Evaluation of RANTES analogue expression in *Nicotiana benthamiana* and *Lycopersicon esculentum* and their topical microbicidal activity

By

Kedibone Gloria Mawela

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Supervisors: Prof JN Eloff

Dr R Chikwamba

Dr E Chakauya

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LIST OF ABBREVIATIONS AND ACRONYMS

apo	:	apoplast
B	:	breaker
bp	:	base pairs
CaMV 35S	:	cauliflower mosaic virus 35S promoter
cDNA	:	complementary DNA
cyt	:	cytosol
DNA	:	deoxyribonucleic acid
dpi	:	day post infiltration
ELISA	:	enzyme-linked immunosorbent assay
ER	:	endoplasmic reticulum
FW	:	fresh weight
g	:	gram
GFP	:	green fluorescent protein
HIV-1	:	human immunodeficiency virus type 1
IC_{50}	:	concentration to inhibit 50%
kDa	:	kilo Dalton
MG	:	mature green
mg	:	milligram
ml	:	millilitre

ng	:	nanogram
P	:	pink
PVDF	:	polyvinylidene difluoride
R	:	ripe
RANTES	:	Regulated upon activation normal T– cell expressed and activated
SD	:	Standard deviation
SDS-PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sLT-B	:	synthetic gene encoding the *Escherichia coli* heat labile enterotoxin B subunit
TCA	:	trichloroacetic acid
T-DNA	:	transfer-DNA
TSP	:	total soluble protein
µg	:	microgram
VSV-G	:	vesicular stomatitis virus glycoprotein
-ve	:	negative

ABSTRACT

The HIV/AIDS pandemic has dramatically altered patterns of morbidity and mortality in sub-Saharan Africa during the last two decades. In the absence of HIV vaccine, microbicides may offer viable option for protection against HIV infection. Microbicides are products that are applied topically inside the vagina or rectum that act to impede transmission of HIV and other sexually transmitted diseases. Small human chemokines such as RANTES (regulated upon activation, normal T cell expressed and secreted) are currently been investigated as microbicides candidates.

A number of N-terminally modified RANTES analogues such as 5P12 and 6P4 with a much higher antiviral potency have been developed and they have strong potential for use as microbicides. Since plants offer an alternative option for cost effective production of protein therapeutics, we evaluated the feasibility of expressing 5P12 and 6P4 in *Nicotiana benthamiana* species. 5P12 is considered the most promising candidate for use in the microbicide pipeline because it inhibits HIV infection through cellular receptor antagonism. Hence its feasibility of expression was also evaluated in *Lycopersicon esculentum* (tomato). The two analogues were transiently expressed in the selected plant species via agrobacterium-mediated transfection.

For expression in *N. benthamiana*, two different vectors (pTRA and MagnICON) were used to deliver the two analogues for transient expression. About 6-8 weeks-old *N. benthamiana* plants were agroinfiltrated via needle injection and vacuum infiltration methods and targeted to four subcellular compartments viz: apoplast, chloroplast, cytosol and endoplasmic reticulum (ER). The agroinfiltrated leaves were replanted, grown in a tissue culture laboratory and harvested after different periods. For expression in *L. esculentum*, the MagnICON constructs were used to deliver the 5P12 gene into four different developmental stages of tomato fruits viz: mature green (MG), breaker (B), pink (P) and ripe (R) via needle injection. The agroinjected tomato fruits were incubated in a dark cupboard and harvested after different periods.

Proteins were extracted from the harvested material and evaluated for 5P12 and 6P4 expression. ELISA results showed expression of 5P12 and 6P4 in *N. benthamiana* leaves which was detectable at 3-9 days post infiltration (dpi). Similar results were obtained for 5P12 and 6P4, consequently only results for 5P12 are reported. The vacuum infiltrated leaves of both pTRA and MagnICON constructs led to higher yields than the needle injected leaves. The highest yields were obtained with the MagnICON constructs. The highest 5P12 expression level of 603 µg/kg fresh weight leaf tissues (~0.024% TSP) was obtained in the apoplast at 9 dpi. The pTRA constructs had the highest expression levels of 0.63µg/kg FW in the cytosol at 3 dpi.

5P12 was also detectable at 3-9 dpi in *L. esculentum*, based on ELISA results. The highest 5P12 expression of 23.56 µg/kg FW and pH 4.75 tissues was obtained at the MG stage in the apoplast at 9 dpi. Western blot analysis confirmed the size of plant-made 5P12. Moreover, the plant extracts had anti-viral activity and were not toxic to TZM-bl cells.

Our results show that the RANTES can be made in both *N. benthamiana* and *L. esculentum* and that the levels are not different from other systems reported previously. Furthermore, this is the first report that a chemokine has been expressed in plants. The quantities expressed were low making the commercial development of a microbicide from these species impractical. However, production of bulky leaf material may enhance the quantities.

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Background on HIV/AIDS

It is over 30 years since the Centres for Disease Control and prevention (CDC) first reported acquired immunodeficiency syndrome (AIDS) in 1981 (Fortson, 2011). Since then, UNAIDS (2007a) reported a global total of 2.7 million new infections in 2008. Out of approximately 60 million infections, more than 25 million persons have died of AIDS and currently, more than 33 million persons are HIV positive or living with AIDS (Dieffenbach and Fauci, 2011). Sub-Saharan Africa constitutes 3% of the global population and has an alarming 68% of the world's individuals living with HIV/AIDS (Lurie and Rosenthal, 2010). According to UNAIDS (2007b), there is no other region with HIV prevalence greater than 1%, yet 5% of adults have been reported to be infected with HIV in sub-Saharan Africa.

Heterosexual intercourse is the predominant mode of HIV type 1 (HIV-1) transmission across the globe which accounts for more than 90% of HIV-1 infections (Kish-Catalone *et al*., 2006). The World Health Organisation (2009) reported that AIDS is the main cause of death among women of reproductive age across the globe, thus women increasingly bear a disproportionate burden of the pandemic. UNAIDS / WHO (2004) reported that females in the sub-Saharan Africa account just about 57% of the total infected population. These should not raise so many questions because women have become and continue to be victims of sexual violence due to personal, economic, social and cultural issues (Ramjee *et al*., 2006a). Thus the high HIV infection rates have prompted the UN Joint Programme on HIV/AIDS to call for extra efforts in HIV/AIDS interventions (Sidibe, 2010).

1.2 Microbicides as the most effective means to prevent HIV-1 transmission

1.2.1 HIV life cycle

Figure 1 is a brief summary of the life cycle of HIV starting from its attachment to the host cell membrane to the budding off from the cell. The mechanism of actions of each crucial step was discussed in succession in the following context.

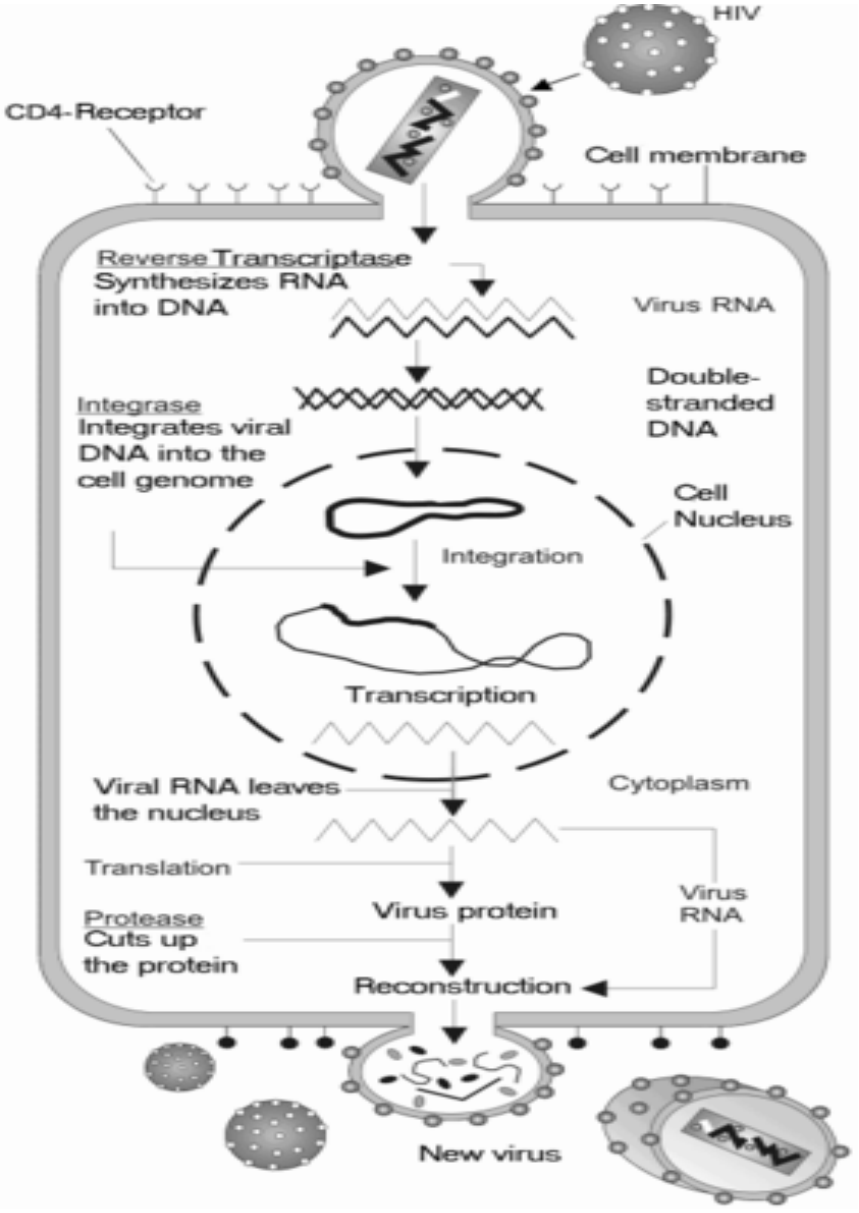

Figure 1 Life cycle of HIV starting from the receptor attachment, fusion, entry into the host cell and ending with the reconstruction that leads to the budding off of the new virus **(http://www.en.wikipedia.org/wiki/HIV)**

1.2.2 Entry and fusion of the HIV to the host cell

HIV particles have a diameter of 100 nm and are surrounded by viral envelope (or membrane) which is made up of a lipid bilayer (Figure 2) (Fanales-Belasio *et al*., 2010). Three gp120 envelope glycoproteins that are noncovalently associated to three gp41 transmembrane molecules project from the viral envelope as viral spikes (Pancera *et al*., 2010). Inside the viral envelope, there is a layer of matrix protein (p17). The capsid (viral core) surrounds two copies of the viral ssRNA, reverse transcriptase, integrase and protease enzymes that are required for HIV replication (reviewed in Gelderblom *et al*., 1989; Luciw, 1996). A capsid is made up of polymers of protein p24 (Fanales-Belasio *et al*., 2010).

The virus infects target cells (not only in the genital tract) including dendritic cells, CD4+ lymphocytes and macrophages (Olinger *et al*., 2000; Clapham and McKnight, 2001; Saphire *et al*., 2001; Buckheit *et al*., 2010). Figure 2 also illustrates the structure of HIV subsequent to post-translational modification processes including glycosylation, oligomerisation and cleavage resulting into two subunits, gp120 and gp41 (Zhang *et al*., 2001).

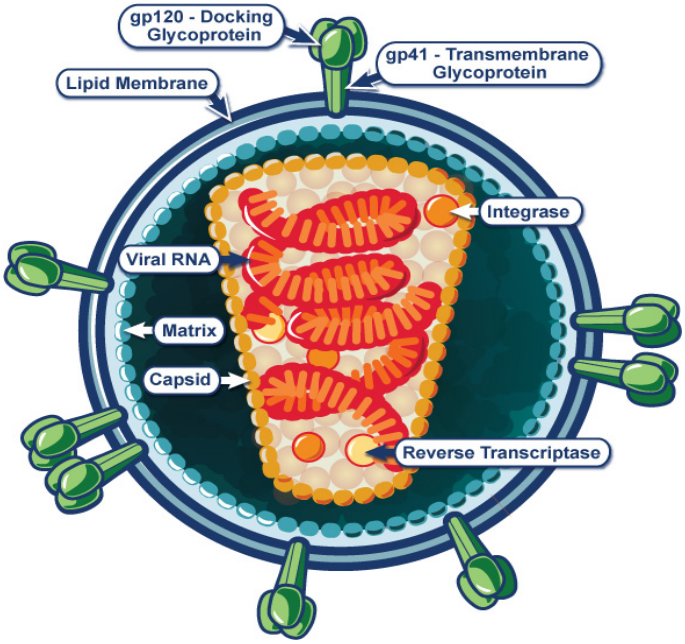

Figure 2 Structure of human immunodeficiency virus showing viral envelope with gp120 and gp41 projecting as spikes. The viral envelope covers the viral core

(capsid) that surrounds the two copies of ssRNA, reverse transcriptase and integrase **(http://www.niaid.nih.gov)**

HIV infection is established by attachment of gp120 to the cellular lectins (DC-Sign and related C-type lectins) found on the surface of CD4+ lymphocytes, macrophages and dendritic cells (Figure 3) (Dhawan and Mayer, 2006). Three steps are involved in the entry of the virus thus, (1) attachment to the CD4 receptor; (2) binding to the co-receptor; and (3) fusion process. All three steps are mediated by the viral envelope (env) proteins, gp120 and gp41 projecting from the membrane of the virus (Tilton and Doms, 2010). During infection, the gp120 subunits first contact CD4 (Anastassopoulou *et al*., 2009). The CD4-gp120 complex must also bind to HIV co-receptors, which are 7-transmembrane G-protein-coupled receptors (GPCRs) for chemokines, normally expressed on the cell membrane of various cells including T cells for infection to occur (Jin *et al*., 2010). The principal HIV co-receptors are CCR5 and CXCR4 (Choi and An, 2011). Binding to the HIV co-receptors causes conformational rearrangement within the trimer that drives gp41 fusion peptide (FP) region to be inserted into the host cell membrane resulting in membrane fusion (Nuttall *et al*., 2007; Anastassopoulou *et al*., 2009). FP is situated at the N-terminus of gp41 which is adjacent to two heptad repeats (HR1 and HR2). During fusion, the two heptad repeats interacts together and thereby forming a 6-helix bundle that approximates the HIV envelope and host cell membrane together to form a fusion pore which allows transmission of the viral capsid to the target cell (Matos *et al*., 2010).

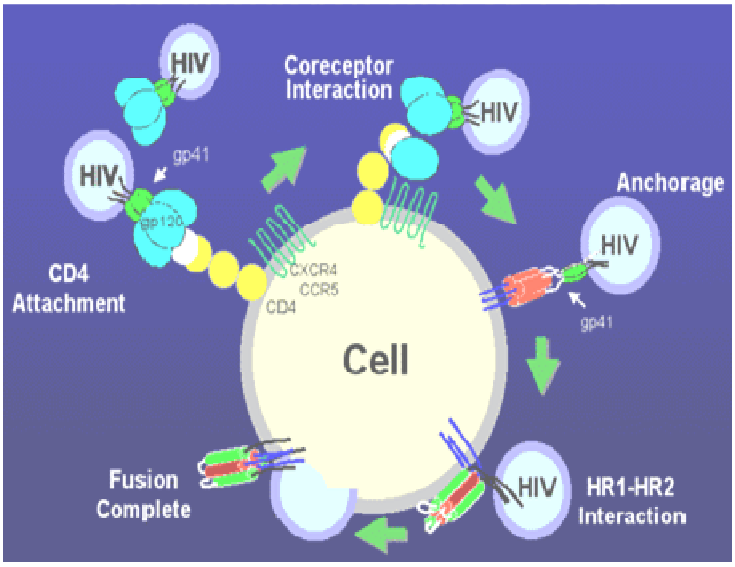

Figure 3 The HIV entry process starting from CD4 receptor attachment, binding of gp120 to CCR5/CXCR4 receptors, gp120-gp41 interaction, HR1- HR2 interaction to begin fusion, formation of fusion pore and entry of the capsid to the host cell **(www.medscape.com)**

1.2.3 Reverse transcription

Subsequent to fusion, the virus releases its viral RNA genome, reverse transcriptase, integrase and virion regulatory proteins into the host cell for further replication of itself (Wang *et al*., 2007). The reverse transcriptase is able to convert ssRNA into dsDNA so that the viral integrase inserts it into the cellular DNA (Safadi *et al*., 2007).

1.2.4 Integration

During HIV replication, a proviral DNA becomes integrated into the host genome of the infected cells (Engelman *et al*., 1991; Lewinski *et al*., 2006; Di Primio *et al*., 2013; Shimura *et al*., 2008). The reverse transcription generates the double-stranded viral DNA for the integrase to bind and mediate integration in the genome so as to produce a functional provirus (McColl and Chen, 2010). There are two catalytic

processes involved during integration; 3´ processing (cleavage of nucleotides from the 3´ ends of both strands of the proviral DNA) and strand transfer (insertion of the proviral DNA into the host DNA) (Asante-Appiah and Skalka, 1997; Lewinski *et al*., 2006).

1.2.5 Transcription, viral assembly and budding-off

The proviral DNA uses the host enzyme called RNA polymerase upon receiving such signal to replicate its genomic material and transcribes early and late viral mRNA. The information contained in the mRNA is translated into long chains of HIV proteins (Greene, 2004). The HIV protease slices the long chains into smaller individual proteins. These individual proteins form the new HIV particles containing each of the proteins and enzymes required to repeat the HIV life cycle (Werner, 2006). The new virus particle buds from the host cell carrying part of the cell's outer envelope to infect other cells (Sarwar, 2007).

1.3 HIV prevention

After almost three decades of research since the Institute Pasteur in France discovered that HIV is linked to AIDS back in 1983, the cure is still elusive (Greene, 2007). In the absence of a cure, a successful protection measure against the sexual transmission of HIV-1 is needed to combat the disease. Vaccines have long been used to prevent the spread of the viral infection hence they are among the most effective public health interventions (Johnston and Fauci, 2008). A limited number of preventative options are available including abstinence, barrier methods (e.g., condoms and diaphragms), antiretroviral drugs and topical microbicides.

HIV prevention tool such as abstinence is not always a feasible option for married couples and those who are victims of sexual violence due to personal, socio-economic, and cultural issues (Ramjee *et al*., 2006b). Condoms are highly effective in preventing HIV transmission (Stadler *et al*., 2008). They have been in use for more than two decades and in 1987 the latex condoms provided a proof-of-concept that

they can be used to prevent HIV infection through sexual intercourse (Centre for Disease Control, 1987). Though the condoms continued to be used effectively, HIV transmission remains high especially in sub-Saharan Africa (Van Damme *et al*., 2008). This could be that most women are unwilling to use them as they believe that they are unreliable, cause sicknesses and physical discomfort, reduce sexual pleasure and engender mistrust in intimate relationships (Stadler and Saethre, 2011). Other women are unable to initiate the use of condoms due to gender-inequity, economic and religious beliefs (Padian *et al*., 2008). Hence other physical barrier methods have been explored.

Female-initiated methods such as the diaphragms have been considered as other means to provide protection against HIV and sexually transmitted infections (STI) (Matthews and Harrison, 2006). Most women found the diaphragms as easy and comfortable to use (Sahin-Hodoglugil *et al*., 2011). Moreover there is an evidence suggesting that physical barriers that cover the cervix offer safe and effective protection against HIV and STIs (Padian *et al*., 2007). Thus cervical barriers have been studied as a means to prevent pregnancy, diseases and as reusable microbicide delivery mechanism (Van der Straten *et al*., 2009). Delany-Moretlwe (2006) examined the safety of vaginal diaphragms such as silicone diaphragm when used with anti-HIV gels such as BufferGel, Acidform and KY Jelly (placebo gel) and Ortho All flex diaphragm when used in combination with 6% cellulose sulphate or KY Jelly (placebo). Due to their safety, both diaphragms and their combinations were recommended for evaluation in Phase III trials. However, another investigation was done on Ortho-All-Flex diaphragm when used in combination with Replens lubricant gel for the prevention of HIV and other STIs such as Chlamydia and gonorrhea. Unfortunately the study showed no effect of the intervention (Sahin-Hodoglugil *et al*., 2011). Therefore, the diaphragm is not recommended in the context of HIV/AIDS because it covers mainly the cervix leaving most of the vaginal epithelium uncovered and exposed to semen (Agboghoroma, 2011). In the absence of more reliable methods that women can initiate, more innovative strategies are required to add on the HIV intervention toolbox.

Whilst still searching for the right preventive measure for HIV/AIDS, the best way to combat HIV-1 infections is via preventive strategies such as vaccines. Vaccine candidates such as HIV recombinant canarypox have been investigated and shown to stimulate CD8 and CD4 Th1 cell response to HIV in healthy human volunteers (Autran *et al*., 2008). Furthermore, Fauci *et al*. (2008) reviewed an investigation of the recombinant form of HIV-1 gp120 as a vaccine candidate hoping to neutralise a broad array of primary isolates of HIV. In addition, the immunogenicity and safety of the MRK adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine was also assessed in a Phase I trial. The vaccine *per se* induced an immune response against HIV-1 and was well tolerated in most healthy individuals; unfortunately it was not efficacious (Priddy *et al*., 2008).

HIV vaccine has thus far been elusive and the search disappointing and frustrating (Johnston and Fauci, 2008). However, HIV vaccine (RV144 Phase IIb) efficacy trial which was conducted in Thailand gave the first encouraging results. Ross *et al*., (2010) also reviewed RV144 Phase IIb trial and showed a reduction of HIV acquisition to 31.2%. On the other hand, the Step Study resulted in higher HIV-1 incidence in vaccine-treated men than in placebo-control men (McElrath *et al*., 2008). Whilst still waiting for the discovery of the reliable HIV vaccine, antiretroviral drugs have been used effectively to diminish HIV transmission.

In 1987, the United States of America Food and Drug and Administration (USFDA) approved the first nucleoside reverse transcriptase inhibitor, zidovudine (AZT) for the fight against AIDS (www.fda.gov). Other reverse transcriptase inhibitors were introduced in the period of 1991-1993 and were designed to suppress the reverse transcription of viral RNA in healthy cells (Mamo *et al*., 2010). This led to the approval of about 25 antiretroviral drugs to date (Mamo *et al*., 2010). In 1995-96 the protease inhibitors were approved and the triple-drug therapy emerged that revolutionised the HIV/AIDS treatment (Mamo *et al*., 2010). The triple-drug or highly active antiretroviral therapy (HAART) combines three or more different antiretroviral drugs according to the particular stage of infection and administered simultaneously. The application of HAART regimen became a success in improving health, patients' quality of lives and in reducing the likelihood of developing drug-resistant HIV (Lee

and Harrigan, 2010). Unfortunately, HAART regimens and the potency of the drugs themselves cannot remove the HIV from the body and suffer from toxicity, complicated treatment regimens, insufficient pharmacology and emergence of multi-drug resistant virus strain (Buckheit *et al*., 2010).

1.3.1 Classes of microbicides and their mode of action

Nuttall *et al.* (2007) described microbicides as antiviral agents that can be applied topically to genital mucosa prior to sexual intercourse that would act to impede HIV transmission or other STIs. They are designed to provide women with the means to protect themselves against sexually transmitted infections, particularly in settings where their partners are unwilling to use condoms, (McGowan, 2011).

Microbicides are topical pre-exposure prophylaxis (PrEP) products such as gels, capsules, tablets, films and intravaginal rings (Shattock and Rosenberg, 2011). They may be applied either around the time of coitus (capsules and tablets) and used on daily basis (gels and films) or to deliver the product over a prolonged period (intravaginal rings) (Shattock and Rosenberg, 2011). Their role would be to; “1), enhance the natural defences of the body against infection, 2), damage the surface membranes of pathogens rendering them ineffective (surfactant), 3), bind to either a pathogen or healthy cell to prevent attachment by an infectious agent (entry and fusion inhibitor) and 4), incorporate antiretrovirals to prevent viruses from replicating in a cell (replication inhibitors)” (Matthews and Harrison, 2006). They can be used alone or in combination with other physical barrier methods to enhance protection or as a backup in case a barrier fails (Kilmarx *et al*., 2006).

1.3.2 Surfactants / membrane disruptors

The first candidate microbicide to be subjected to trial was surfactant nonoxynol-9 (N-9) because it has demonstrated *in vitro* antiviral activity (Stone, 2009; Zalenskaya *et al*., 2011). N-9 had been previously demonstrated to disrupt the cell membranes and to possess an antibacterial membrane and anti-HIV-1 envelope *in vitro* activities (Dhawan and Mayer, 2006). Its first clinical trial demonstrated that it failed to protect

against HIV infection since it showed ulceration and genital irritation which increased the risk of HIV-1 transmission (Zalenskaya *et al*., 2011). Other surfactant-based microbicides such as savvy/C31G have been evaluated for safety both in animals and human but failed in Phase III clinical trials owing to lack of efficacy (Misaki, 2007).

1.3.3 Vaginal defense enhancers

Lactobacilli found in the vagina maintain the normal pH between 3.0 and 4.2 (Ariën *et al*., 2011). They produce chemical agents such as hydrogen peroxides to aid in protection against HIV infection (Lederman *et al*., 2006). For this reason, topical microbicides that enhance *lactobacilli* and buffer the vaginal pH are being evaluated. BufferGel, *Lactobacillus crispatus* and Acidform have been evaluated for acid-buffering properties (Cutler and Justman, 2008). The BufferGel was unable to prevent HIV-1 acquisition (Pirrone *et al*., 2011). Acidform in combination with the diaphragm was safe but no statistically significant differences were achieved (Von Mollendorf *et al*., 2010).

1.3.4 Entry / fusion inhibitors

Entry inhibitors or negatively charged agents such as polyanionic substances block the HIV entry by electrostatic interaction with gp120 (Pirrone *et al*., 2011). These entry inhibitors include Carraguard (sulfated polyanion), PRO 2000 and Cellulose sulfate (sulfonated polyanion) (Cutler and Justman, 2008). All three polyanionic compounds were evaluated in microbicide clinical trials for safety and efficacy (Pirrone *et al*., 2011). A seaweed extract (Carraguard) was tested in a large efficacy trial at three centres in South Africa and a statistical significance on the acquisition of HIV was not demonstrated (Mehendale, 2008; Ramjee *et al*., 2010). Cellulose sulphate and PRO 2000 were also safe but no evidence was provided for their efficacy against heterosexual HIV-1 transmission (Pirrone *et al*., 2011). On the other hand, RANTES may be different from these microbicides in terms of safety and efficacy. Safety is guaranteed because it is a human chemokine and it has also been

shown to be efficacious in its initial assessment as a candidate microbicide (Hartley *et al*., 2004; Gaertner *et al*., 2008b; Veazy *et al*., 2009; Jin *et al*., 2010).

1.3.5 Antiretroviral-based microbicides

Reverse transcription inhibitors have also been considered in clinical trials for development as potential microbicides. Tenofovir (PMPA; 9-[2-(phosphonomethoxy)propyl]adenine) has been evaluated as a topical microbicide (Doncel, 2008). CAPRISA 004 trial showed that antiretroviral-based microbicide (1% tenofovir) could block HIV transmission (i.e., 39% reduction in HIV incidence) when formulated for vaginal application (Karim *et al*., 2010). Nevirapine has been used for prevention of mother to child transmission (PMTCT) whereas other antiretroviral drugs such as dapirivine, UC781, MIV-150 and MC1220 have undergone preclinical evaluation as microbicides (Shattock and Rosenberg, 2011). Dapivirine showed high activity, hence it has a potential to be active in preventing HIV transmission when delivered as a microbicide (Shattock and Rosenberg, 2011). Most of the antiretroviral drugs that are currently in use may be used in future conjunction with tenofovir.

1.3.6 Anti-HIV proteins

Since gp120 has a binding affinity to the CCR5 and CXCR4 receptors of the targeted $CD4^+$ cell, a number of antiretroviral drugs have been developed that act directly upon this site (Nisius *et al*., 2008). Most of these antiretroviral drugs are the by-products of the conventional and biological systems. Maraviroc which is an end product of medicinal chemistry is thought to bind to the transmembrane domains, thereby inhibiting the binding of gp120 to CCR5 receptors (Dorr *et al*., 2005; Shattock and Rosenberg, 2011). In 2008, the CCR5 antagonistic effect of maraviroc prompted The International Partnership for Microbicides to license it for development as a topical microbicides (www.ipmglobal.org/). On the other hand, scytovirin (SVN) is among anti-HIV proteins that originate from the biological system. SVN is an aqueous extract of cyanobacterium *Scytonema varium* which has been shown to

neutralize both laboratory-adapted strains and primary isolates of HIV-1 by interacting with mannose-rich glycans on the viral envelope (Alexandre *et al*., 2010). Although it has not been tested in human clinical trials, it can be developed as a topical microbicide based on its non-toxicity to human cells (Alexandre *et al*., 2010).

From a number of anti-HIV proteins that have been reported, small chemokines such as RANTES have been investigated. The biological activity of RANTES has also been reported to block or down-modulate the CCR5 *in vitro*, which suppresses HIV-1 infections (Shimizu *et al*., 2006). In-depth discussion of RANTES is presented in the following context. This is based on the grounds that advanced research interventions upon targeting the CCR5 receptors are required since epidemiological evidence shows that the virus using these receptors is responsible for 90% of infections across the globe (Shattock and Rosenberg, 2011).

1.3.7 Chemokines in HIV management

1.3.7.1 Chemokines and their classification

Chemokines belong to a family of proinflammatory cytokines that are involved in the regulation of the immune system and inflammation (Mennicken *et al*., 1999).These are small, highly basic proteins, with molecular weights between 8 and 12 kDa (Flanagan and Kaufman, 2006).They are classified into four subfamilies based on the conserved and number of the cysteine residues present within their amino (N)-termini, thus; CC, CXC, CX3C and C (Choi and An, 2011). All chemokines have a 3-dimensional structure which is stabilised by two disulfide bonds joining the first cysteine to the third and the second cysteine to the fourth (Huang and Geng, 2010). The CC chemokine is the largest subfamily which is characterised by the adjacent positions of the first two of four cysteine residues (Gao and Ji, 2010). The CXC chemokines have a single amino acid residue in between the two cysteine residues (Oo and Adams, 2010). They are subdivided into two groups; the ELR positive (with a specific glutamic acid–leucine-arginine motif before the first cysteine) and the ELR

negative (without a specific motif) (Huang and Geng, 2010). The CX3C subfamily has three intervening amino acids in between the first two cysteine residues whereas the C subfamily has only two cysteine residues (Gao and Ji, 2010).

1.3.7.2 Role of chemokines in disease management

Chemokines play a central role in many biological events such as embryonic development, wound healing, angiogenesis, tissue homeostasis, lymphatic organ development, inflammatory diseases and coordinated immune responses during viral infection (Hosking and Lane, 2010; Huang and Geng, 2010). They are divided into two functional groups; 'homeostatic or constitutive' and 'inflammatory'. The homeostatic or constitutive chemokines are constitutively expressed and play an essential role in homeostatic immune regulation. They are made in individual microenvironments within the bone marrow, thymus, secondary lymphoid organs, mucosa and skin tissues and are involved in immune surveillance, antigen sampling in secondary lymphoid tissue and physiological trafficking and positioning of cells (Oo and Adams, 2010). In contrast, inflammatory chemokines are induced by inflammation (Oo and Adams, 2010). They are expressed in the resident and infiltrating cells following stimuli by pro-inflammatory cytokines or contact with the pathogens (Oo and Adams, 2010).

Chemokine receptors are similarly grouped as chemokines as described in section 1.3.7.1, hence chemokines bind to correspondingly grouped and often to multiple chemokine receptors (Zernecke and Weber, 2010). Chemokine receptors are a group of transmembrane proteins belonging to the superfamily of guanine-protein-coupled receptors (GPCRs) (Choi and An, 2011). Amongst the GPCRs, CCR5 is a CC chemokine receptor for CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β) which was later described as the main co-receptor for macrophage tropic HIV (Haworth *et al*., 2007; Kroetz and Deepe, 2010). Thus binding of chemokines to receptors generates a regulatory network characterised by specificity and robustness and involved in regulating the diversity of leukocyte recruitment (Fabene *et al*., 2010). However, CC chemokines such as CCL3, CCL4, CCL5, CCL7, CCL8 and

CCL13 activate the receptor by binding to the CCR5 with different abilities and affinities (Haworth *et al*., 2007). CCR5 and chemokine interaction leads to signaling processes such as Ca^{2+} mobilisation which is followed by receptor phosphorylation (Figure 4) (Haworth *et al*., 2007). However, signaling may lead to detrimental effects such as mucosal inflammation which is known to boost HIV infection (Gaertner *et al*., 2008a). But to circumvent prolonged activation of the receptors, the GPCR internalises through clathrin-mediated endocytosis followed by either degradation in lysosomes (down-modulation) or termination of the activated state and recycling to the cell surface (resensitisation) (Borroni *et al*., 2010; Calebiro *et al*., 2010). Moreover, the chemokine-induced internalisation of CCR5 and CXCR4 could inhibit HIV-1 entry because the cells become indistinguishable for the virus envelope interaction (Alkhatib, 2009).

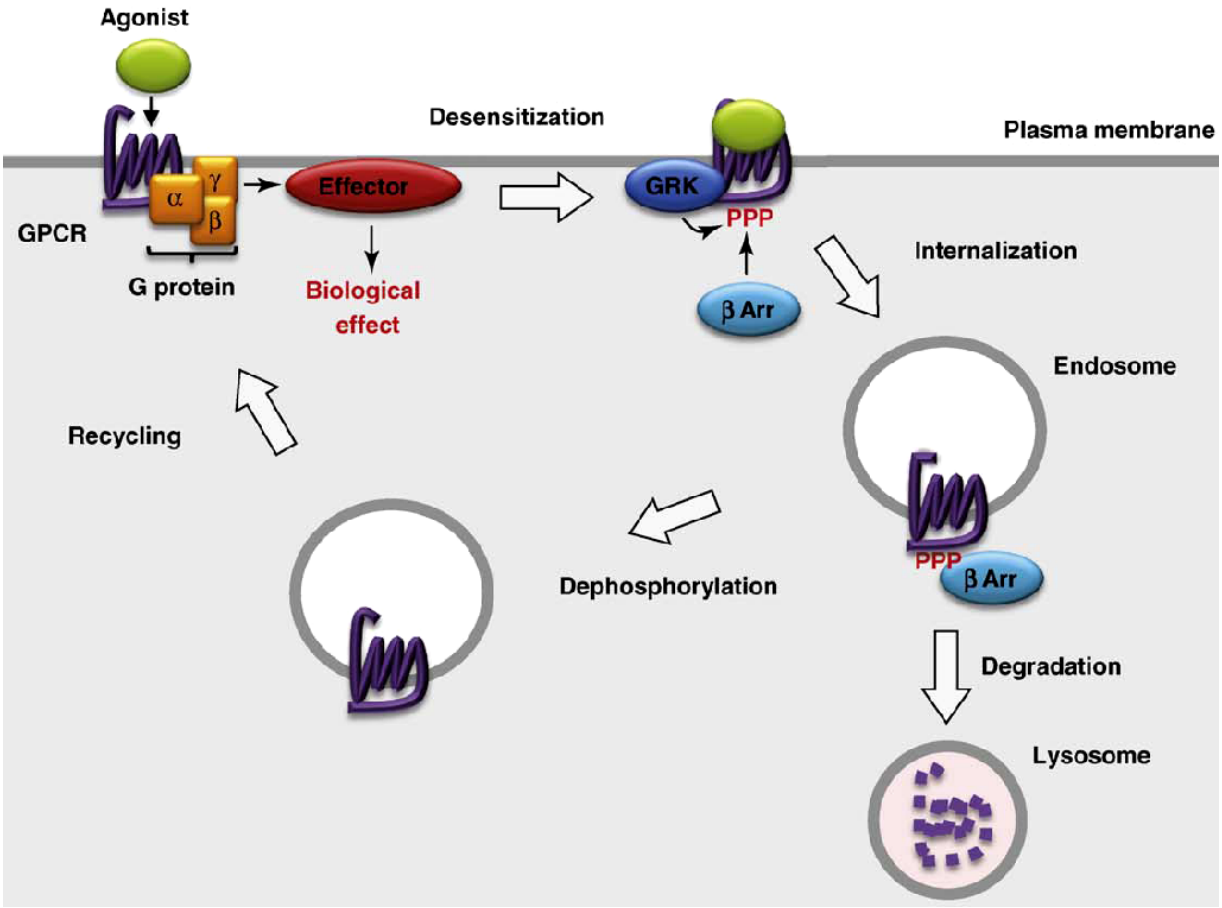

Figure 4 GPCR signaling and trafficking. Binding of agonist to GPCR activates and releases the heterotrimeric G-proteins (α, β and γ subunits). The free subunits stimulate or inhibit effector enzymes such as ion channels and adenylyl cyclases. The activation of downstream signaling cascades ultimately produces biological effects. Receptor desensitisation and internalisation terminate G-protein signaling. Desensitisation is initiated by phosphorylation of activated GPCR by GPCR kinases (GRKs). This phosphorylation recruits β-arrestin (βArr) proteins to the membrane so as to bind to the receptors and promote internalisation. Internalised GPCRs are either targeted to lysosomes for degradation or dephosphorylated and recycled back to the cell surface to sustain a new cycle of activation (Calebiro *et al*., 2010).

Gα is one of the heterotrimeric G-proteins released after HIV-1 has bound to GPCR. It has two subunit isoforms with different properties namely, Gαi and Gαq. Binding of gp120 to CCR5 and CXCR4 induces Gαq for viral entry and activates multiple intracellular signaling cascades that differ from those induced by natural chemokine ligands, perhaps due to differing ligand binding sites or affinities (Figure 5) (Juno and

Fowke, 2010). However, activation of cellular signaling pathway by gp120 that leads to expression of cytokine and chemokine genes could enable viral replication (Herbein *et al*., 2010).

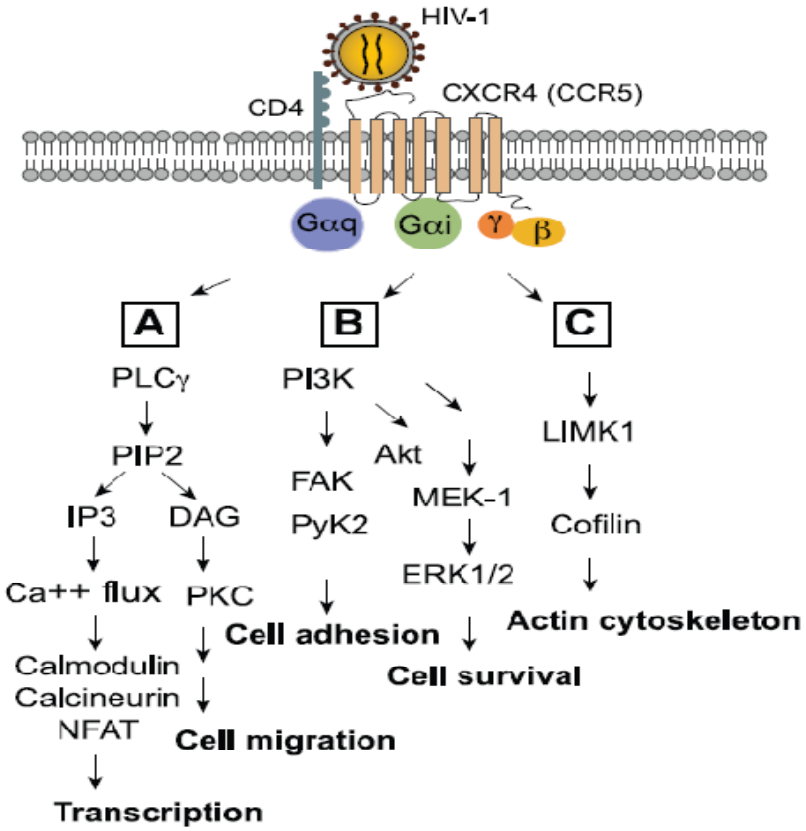

Figure 5 HIV-1 envelope co-receptor signaling pathways. Binding of HIV-1 gp120 to CXCR4 or CCR5 has shown to activate the following heterotrimeric G-proteins as signaling molecules that mediates transcriptional activation, cell migration, cell adhesion, cell survival and actin cytoskeleton rearrangement. (A) Gαq activates a phospholipase C-γ (PLC-γ) that hydrolyses phosphatidylinositol-4,5-biphosphate (PIP2) to generate inositol triphosphate (IP3) and diacylglyerol (DAG) which stimulate calcium influx and activation of protein kinase C (PKC). (B) Gαi activates phosphoinositide 3-kinases (PI3K) which stimulates downstream targets such as protein kinase B (PKB/Akt), NF-κB, mitogen/extracellular signal-regulated kinase (MEK-1), and extracellular signal-regulated kinase (ERK1/2). PI3K also triggers activation of proline-rich tyrosine kinase (Pyk2). (C) GTP-bound Gβγ stimulates guanine nucleotide exchange factors (GEFs) specific for the Rho family GTPases (Rac/CDC42/RhoA). These GTPases are regulators of the actin cytoskeleton rearrangements hence they activate the following pathways: Rac activates p21-

activated kinase (PAK), which then activates LIM kinase (LIMK) resulting in phosphorylation of cofilin and actin polymerization. CDC42 stimulates actin nucleation through its binding to neutral Wiskott-Aldrich Syndrome protein (WASP) that leads to recruitment of actin-related protein 2/3 (Arp2/3). RhoA activates Rho kinase (ROCK), leading to phosphorylation of myosin light-chain (MLC) and microtubule rearrangement (Wu and Yoder, 2009).

The gp120 signaling is known to induce apoptosis in T-cells, a process which involves CD4 signaling but which can be enhanced by involvement of the co-receptors (Juno and Fowke, 2010). Wan and Chen (2010) reported that lower concentrations of up to 500 ng/ml of soluble gp120 were sufficient to mediate significant T-cell death. Moreover, Juno and Fowke (2010) reported that binding of gp120 to CCR5 resulted in activation of ERK1/2 and phospholipase D which promotes transactivation of tat and HIV long terminal repeat (LTR) (perhaps through NF-κB) *in vitro.* Hence reverse transcription is dependent on Gαi-mediated ERK1/2 induction since the tat protein plays a role in transcription and replication of the virus (Herbein *et al*., 2010). HIV-1 tat could promote HIV-1 infection as it is able to induce expression of the viral cognate receptors in monocytes/macrophages (Herbein *et al*., 2010). It can also trigger Ca^{2+} mobilisation in macrophages through CCR2 and CCR3 receptors (Herbein *et al*., 2010). The HIV-1 virus strains that use CCR5 receptors for entry are called R5 viruses whereas those that use CXCR4 receptors are called X4 viruses. R5 gp120 has been reported to elicit higher Ca^{2+} peaks than X4 gp120 (Herbein *et al*., 2010). Additionally, Ca^{2+} mobilisation is also reported to occur during endocytosis-mediated entry of several enveloped viruses (Scherbik and Brinton, 2010). The role of Ca^{2+} is to regulate rapid cell processes for instance, cytoskeleton remodelling and the release of vesicles contents and slower ones including transcription, proliferation, exocytosis, apoptosis and contraction (Scherbik and Brinton, 2010). Moreover, previous studies showed that addition of Ca^{2+} to cells expressing gag (the structural precursor polyprotein) enhanced virus particle production (Ehrlich *et al*., 2010).

The gp120 interaction may lead to chemotaxis, rearrangement of actin cytoskeleton and activation of cofilin, to increase the cortical actin dynamics in resting CD4 T cells (Wu and Yoder, 2009). Also, it has been shown to trigger Pyk2 and CD4/CXCR4-dependent NFAT (nuclear factor of activated T cells) nuclear translocation (Wu and Yoder, 2009). Furthermore, pulsing of CD4 cells with gp120 can induce phosphorylation of ERK1/2 to activate PI3K and to lead to membrane recruitment and phosphorylation of Akt (Juno and Fowke, 2010).

1.3.7.3 Chemokine inhibitors

Chemokine response is associated with a number of detrimental effects not only in HIV transmission, for instance, Zernecke and Weber, (2010) reviewed vascular inflammation that is promoted by numerous chemokines including CXCL1, CXCL8 and CXCR2. It has also been stated that progression of chronic inflammatory diseases such as atherosclerosis is determined by disturbed equilibrium of immune responses. Furthermore, studies show that CXCL12-CXCR4 axis may be related to tumor metastasis of distant organs whereas CCL12-CCR7 axis may be associated with lymph node metastasis in tumours such as prostrate, colon and lung cancers (Huang and Geng, 2010). Moreover, chemokines and their cognate receptors provide a platform of leukocyte influx to the vascular wall (Zernecke and Weber, 2010). Therefore, several strategies to block the unfavourable effects of chemokines are now being developed, including receptor-blocking antibodies or receptor-blocking molecules or downstream signalling inhibitors.

Compounds with chemokine-inhibitory properties are classified into two broad classes: (a) receptor antagonist that inhibits the action of one or small number of chemokine receptors and (b) broad-spectrum chemokine inhibitors that block leukocyte recruitment in response to many chemokines (Grainger *et al*., 2005).The broad-spectrum chemokine inhibitors may be developed as anti-inflammatory therapies (Grainger *et al*., 2005), to alleviate major set-backs as a results of signaling pathways. Moreover, Naidu *et al*., (2003) showed that the broad-spectrum chemokine inhibitors may potentially be used as therapeutic tools in slowing the

progression of obliterative bronchitis (OB) which affects more than half of all long-term survivors after lung transplantation. Furthermore, Granger and Reckless, (2003) developed a broad-spectrum inhibitor (BSCI) NR58-3.14.3 as a powerful anti-inflammatory agent which suppresses the migration of leukocytes in response to chemokines such as CCL2, CXCL8 (IL8), CCL3 and CCL5. In contrast, the receptor antagonists offer the prospect of delicate control of the recruitment of specific leukocytes subsets under particular conditions; however, the redundant system means that the effect of specific receptor blockade may be subtle (Schroff *et al*., 2005).

The receptor antagonists are most likely to become new antiviral agents that may be used to fight against HIV transmission. Since HIV uses CCR5 and CXCR4 receptors for entry into the host cell, small CCR5 and CXCR4 antagonistic compounds with potent antiviral activity have been described. The bicyclam derivatives which consistently block X4 viral replication have been described as the best CXCR antagonists (Princen and Schols, 2005). A cyclic peptide CVX15 and a small molecule IT1t have been reported to be CXCR4 antagonists (Wu *et al*., 2010). On the other hand, there are CCR5 antagonists such as maraviroc (UK-427,857) (Dorr *et al*., 2005), and 4-{[4-({(3R)-1-Butyl-3-[(R)-cyclohexyl(hydroxy)methyl]-2,5dioxo-1,4,9-triazaspiro[5.5]undec-9-yl}methyl)phenyl]oxy}benzoic acid hydrochloride (873140) that have demonstrated potent antiviral activity againsts CCR5-tropic HIV-1. But the latter have been shown to be ineffectual antagonist as it was able to block binding of 125I-MIP-1α) and not RANTES (Watson *et al*., 2005). Otherwise, a lot of receptor inhibitors have been developed that may be used as effective tools to combat HIV transmission.

1.3.8 RANTES analogues as microbicide candidates

RANTES/CCL5 is a CC chemokine which has been reported to bind to CCR1, CCR3, CCR4 and CCR5 receptors (Dhawan and Mayer, 2006; Sun *et al*., 2008). It has become the cornerstone of research because it is the most potent HIV-suppressive chemokine (Duma *et al*., 2007). RANTES/CCL5 may be secreted by

various cells such as T cells, platelets, epithelial cells, eosinophils, fibroblasts, endothelial, endometrial and macrophages (Levy, 2009). Subsequent to its secretion, it binds to one of its cognate chemokine (e.g., CCR5) receptors (Choi and An, 2011). RANTES obstructs interaction of CCR5 with the viral gp120 to hinder HIV infection (Nisius *et al*., 2008). Furthermore, the extracellular N-terminus of CCR5 contributes significantly to interaction with both RANTES and viral gp120. There are several RANTES analogues that have been developed to improve the binding affinity to the CCR5 receptors (Ham *et al*., 2009).

1.3.8.1 PSC-RANTES

PSC-RANTES which stands for N^{α}–(n-nonanoyl)-*des*-Ser^{1}-[L-thioproline^{2}, L-α-cyclohexyl-glycine^{3}]RANTES, is a human chemokine analogue that has the same chemical structure as the native RANTES except that the first three N-terminal amino acids i.e., serine, proline and tyrosine of the native RANTES were replaced with the non-natural and non-coded structures namely; nonanoyl group, thioproline and cyclohexylglycine of PSC-RANTES (Figure 6) (Lederman *et al*., 2004; Gaertner *et al*., 2008b). PSC-RANTES acts as a CCR5 agonist and has the capacity to inhibit the recycling of internalised CCR5 as a result of prolonged co-receptor sequestration. It can prevent vaginal HIV-1 transmission in a monkey model (Hartley *et al*., 2004; Ham *et al*., 2009). Hence it is a potent HIV entry inhibitor which showed promising efficacy in its initial assessment as a candidate microbicide (Gaertner *et al*., 2008b; Jin *et al*., 2010).

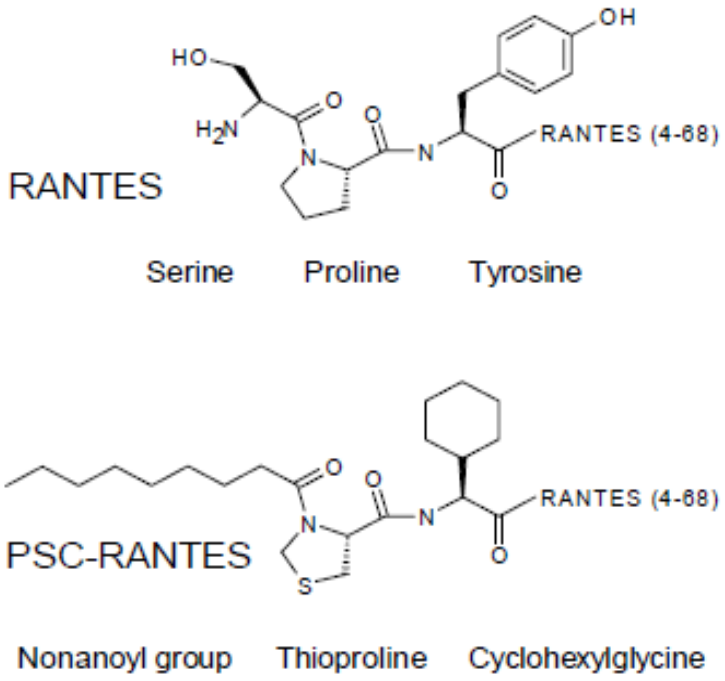

Figure 6 Structure of native RANTES and PSC-RANTES

1.3.8.2 5P12-RANTES and 6P4-RANTES

As a results of the non-natural and non-coded structures within the N-terminal pharmacophore of PSC-RANTES which required some semisynthetic strategies (Gaertner *et al*., 2008b), its fully recombinant chemokine analogues with potent anti-HIV activity were generated using phage-display strategy (Gaertner *et al*., 2008a). Thus hydrophobic amino acids which were similar to the first hydrophobic chain of PSC-RANTES at the N-terminal were selected and ultimately the Gln^{0}-Gly^{1}-Pro^{2} motif was identified and favoured for the N-terminal tripeptide (Gaertner *et al*., 2008a). Potent anti-HIV molecules such as 5P12-RANTES (Q^{0}-[G^{1}-P^{2}-P^{3}-L^{4}-M^{5}-A^{6}-T^{7}-Q^{8}-S^{9}]RANTES/CCL5) and 6P4-RANTES (Q^{0}-[G^{1}-P^{2}-P^{3}-G^{4}-D^{5}-I^{6}-V^{7}-L^{8}-A^{9}]RANTES/CCL5) were isolated following selection of two next-generation libraries (Gaertner *et al*., 2008a). 5P12-RANTES is a CCR5 antagonist that does not have a G protein-linked signaling activity and cannot induce intracellular sequestration (Gaertner *et al*., 2008a; Veazy *et al*., 2009; Choi and An, 2011). On the other hand, 6P4-RANTES is a strong agonist like PSC-RANTES that allows signaling and

induces prolonged intracellular sequestration (Cerini *et al*., 2008; Gaertner *et al*., 2008a).

1.3.9 Approaches in production of RANTES analogues

1.3.9.1 Chemical synthesis

A range of N-terminally chemically engineered derivatives of RANTES, including AOP-RANTES, NNY-RANTES and PSC-RANTES were widely reported as potent inhibitors of HIV-1 transmission (Hartley *et al*., 2004; Veazy *et al*., 2009). Since PSC-RANTES is composed of non-natural and non-coded structures located within an N-terminal pharmacophore, it requires semisynthetic strategies. These expensive synthetic strategies made its use unaffordable in the developing world (Veazy *et al*., 2009). Hence it has led to the production of the two PSC-RANTES derivatives (5P12-RANTES and 6P4-RANTES) with antiviral potency (Gaertner *et al*., 2008a). Both 5P12- and 6P4-RANTES analogues were prepared by total chemical synthesis (Hartley *et al*., 2004).

1.3.9.2 Microbial fermentation

Potent 5P12-RANTES and 6P4-RANTES may also be produced using a biotechnology approach (Gaertner *et al*., 2008a; Whaley *et al*., 2010). Not only N-terminally modified RANTES molecules were explored, Vangelista *et al*. (2010) investigated *Lactobacillus jensenii* that secreted anti-HIV RANTES and C1C5 RANTES. Both RANTES molecules showed potent anti-HIV activities. Superior protein folding capacity of C1C5-RANTES was reported in *L. jensenii* than in mammalian Chinese hamster ovary (CHO) cell lines (Secchi *et al*., 2009). CHO cells secreted low levels of C1C5-RANTES which were mostly dimers whereas *L. jensenii* secreted proteins in monomeric forms (Secchi *et al*., 2009). Generally, chemokines were reported to form inclusion bodies in *Escherichia coli* as an expression host resulting in low expression yields (Cho *et al*., 2008). Different types of RANTES which were fused to either glutathione-S-transferase or maltose binding protein were

cloned and expressed in *E. coli.* The type of fusion protein influenced the yield of soluble proteins hence greater yields were obtained when maltose binding protein was used (Cho *et al*., 2008).

1.3.9.3 Challenges in production of chemokines

Chemokines have high-affinity receptor and low-affinity glycosaminoglycans (GAGs) interactions (Charni *et al*., 2009). GAGs help sequester and retain chemokines on the endothelium and extracellular matrix (Campanella *et al*., 2006), and are also involved in multiple cancer-related processes (Sutton *et al*., 2007). Several chemokines form higher order oligomers in solution by themselves or upon binding to glycosaminoglycans (GAGs) (Salanga and Handel, 2011). Thus both chemokine receptors and their cognate chemokines can form stable dimers (Nisius *et al*., 2008). Depending on the pH and concentration of the medium, chemokines such as MIP-1α, MIP-1β and RANTES occur in monomeric and dimeric forms (Nisius *et al*., 2008). However, chemokines are biologically active as monomers (Campanella *et al*., 2006). On the other hand, chemokine oligomers including CXCL12 seem to induce alternative signaling responses such as cellular activation or signals to halt migration (Wu *et al*., 2010). In contrast, Wang *et al.* (2011) reported that chemokine monomers can cause cell migration *in vitro* whereas previous studies have shown that induction of migration *in vivo* depends on oligomerisation.

Heterologous expression of proteins in biological system can provide access to large quantities of protein and allow for the exchange of 1 of 20 common amino acid residues for another. But aggregation is the major setback as it limits the yield of properly folded proteins (Nilsson *et al*., 2005). Hence chemical synthesis and semi-synthesis of proteins harbour the potential to overcome many of the limitations of production in the biological system. Although the natural L-amino acids are incorporated in the nascent peptide chains *in vivo*, chemical synthesis offers the opportunity to incorporate also the D-amino acids and chemically modified amino acids resulting in increased stability of peptides as a result of resistance to cleavage by proteases (Huther and Dietrich, 2007). Even though semi-synthetic routes are

considered facile, efficient and scalable they are associated with potential side effects (Cai *et al*., 2012). Moreover, synthetic routes are generally too costly as they require expensive chemicals and reagents. For instance, PSC-RANTES showed antiviral potency but could not be used as a microbicide due to its high costs (Hartley *et al*., 2004; Gaertner *et al*., 2008b). Therefore, advanced expression systems are required since the application of recombinant proteins as pharmaceutical products is steadily increasing.

1.4 Plants as alternative production systems

Genetic engineering of bacteria, yeast and animal cells has extensively been used to deliver biopharmaceutical products (Faye and Gormod, 2010). The cost of equipment needed to produce protein-based microbicides deter the progress of research, hence scientists tried modifying plants to express proteins in an affordable manner (Gilbert, 2009). Transgenic plants comprise a feasible option that has extensively demonstrated great potential when it comes to complex polypeptides such as recombinant proteins that must be synthesized by living organisms (Santi and Mason, 2006). Moreover, plants are considered the largest and most economical generators of biomass, they possess eukaryotic post-translational modification machinery and they do not harbour human pathogens (Shoji *et al*., 2009).

Since glycosylation is an essential post-translational modification in which sugars are added onto proteins to aid in their activity, folding and guarding against denaturation (Coku, 2007), plant and mammalian systems have a different way of adding final sugars onto their glycoproteins. Thus plants have xylose and/or α (1,3)-fucose added onto the *N*-glycan. On the other hand, galactose residues with sialic acids are added onto the *N*-glycan on mammalian glycoproteins (Coku, 2007). To avoid expressing pharmaceutical products with the presence of xylose and fucose that might result in allergies, strategies were followed to knockout the genes responsible for the two sugars, thus the genes for 1,3-fucosyltransferase (Fuc-T) and 1,2-xylosyltransferase (Xyl-T) were disrupted and ultimately both xylose and fucose were not added on the final products (De Muynck *et al*., 2010). However, chemokines like RANTES do not

have the sites for *N*-linked glycosylation, therefore they cannot be glycosylated (Schall *et al*., 2009).

1.4.1 An ideal host for plant protein expression

Many efforts have been made in plant molecular farming since the first production of an antibody in a plant expression system about two decades ago (Faye and Gormod, 2010). The question is; which plant can be used as an ideal host for recombinant protein expression?

A diverse number of plants have been used ranging from food/feed and non-food plants (Breyer *et al.,* 2009). They all have their own advantages and disadvantages as expression systems. Most advantages have been discussed in Section 1.4 however, one common setback of plant-made recombinant proteins includes inadequate accumulation levels and purification cost as there is no efficient purification method in place (Joensuu *et al*., 2010). However, high costs may be reduced by selecting watery tissues such as tomato to express the recombinant protein rather than dry tissues such as wheat which ultimately bring about a challenging protein extraction (Coku, 2007).

Factors such as bio-safety, protein expression level, a means for product delivery or quality of the end product must be considered when selecting the plant species to be used as an expression host (Breyer *et al.,* 2009). However, the recombinant proteins should have structural integrity and be functionally active (Chikwamba *et al*., 2002). Hence the subcellular location of the recombinant proteins is also important for its accumulation, folding and assembly, and, depending on their use, may influence their functionality (Chikwamba *et al*., 2003). The most spread plant production systems have been arranged into three groups, thus 1) cereals; 2) fruits and vegetables; 3) tobacco production system (Kamenarova *et al*., 2005).

1.4.1.1. Cereals

Wheat (*Triticum aestivum*), maize (*Zea mays*) and rice (*Oryza sativa*) are the world's most commonly cultivated cereals (Coudert *et al.,* 2010). Cereals are considered as the main source of dietary protein for humans (Shewry, 2007). Recombinant proteins in cereal seeds permit best storage at ambient temperatures. The dryness of seeds ensures that stored proteins are not exposed to protease degradation and non-enzymatic hydrolysis (Kamenarova *et al.,* 2005). The absence of phenolic substances in cereal seeds enhances the efficiency of downstream processing (Kamenarova et *al*., 2005).

A lot has been done in using cereals as production systems. Maize has been used for the production of pharmaceutical products such as laccase, trypsin and aprotinin (Kamenarova *et al.,* 2005). Moreover, Barros and Nelson (2010) used the conventional breeding to produce human monoclonal antibody 2G12 in maize, of which the antibody *per se* is known to prevent HIV transmission. Transgenic rice was used to produce a synthetic fusion gene comprising *E. coli* LT-B and an epitope of porcine epidermic diarrhea virus; chickens were given an oral vaccination with transgenic rice seed containing infectious bursal disease virus (IBDV) VP2 protein which resulted in protection from virulent IBDV challenge (Rybicki, 2010). Genetically modified rice and wheat were used to produce recombinant antibodies against a single antigen (Kamenarova *et al.,* 2005). Moreover, carotenoids were produced in transgenic rice endosperm when two bicistronic systems were used (synthetic 2A and IRES sequences) (Ha *et al*., 2010).

Due to the possibility of the escape of transgenic material through pollen dispersal and volunteer plants, cereals such as maize are difficult to contain (Chakauya *et al*., 2006). However, most of the biopharmaceutical products that are currently on the market are produced in maize (Breyer *et al.,* 2009).

1.4.1.2 Fruits and vegetables

Many biopharmaceutical products have been delivered using fruits and vegetables as production systems. For instance, transgenic tomatoes have been used to produce variety of antigens such as a hepatitis E virus surface protein, rabies virus glycoprotein, Norwalk virus capsid antigen, respiratory syncytial virus F glycoprotein and hepatitis B virus surface antigen (HbsAg) (Rybicki, 2010). Potatoes have also been engineered to express *Escherichia coli* heat-labile enterotoxin (LT-B), Norwalk virus coat protein, rabbit haemorrhagic disease virus (RHDV) VP60, HBsAg, a combination cholera / *E. coli* / rotavirus vaccine, human papillomavirus E7 and L1 proteins (Rybicki, 2010).

There has been a lot of controversy about the use of food crops as production systems for pharmaceutical products because there is a high risk of genetically modified plants interfering with the feed or food chain and the risk of gene transfer to related crop species (Breyer *et al.,* 2009). However, this study followed a small-scale research in the use of food crops as production system.

1.4.1.3 Tobacco production system

Cultivated tobacco and other high-bulk plant varieties are increasingly being seen as the production vehicle of the future (Rybicki, 2010). A lot of pharmaceutical products have been successfully made in transgenic tobacco. Because it is easy to transform tobacco and regulation of its transgene expression is well-understood (Chakauya *et al*., 2006). *Nicotiana tabacum* and *Nicotiana benthamiana* have been used as model species for the plant sciences for more than two decades because two plant species are able to modulate gene expression rapidly and efficiently via plant genetic engineering (Clemente, 2006). Furthermore, *N. benthamiana* is a hyper-susceptible host to many viral vectors and many diseases (Gleba *et al.,* 2004).

The first plant-derived pharmaceutical protein from transgenic tobacco and potato was human serum albumin (Ma *et al*., 2005a). Several antibodies and vaccines were

later produced and have reached the preclinical testing stage and clinical trials (Miao *et al.,* 2008). Serum albumin, a tetanus toxin fragment, cholera toxin B subunit and human growth hormone have been produced at high levels in tobacco chloroplasts and were found to be active after screening for biological activity (Kamenarova *et al.,* 2005). A transgenic tobacco (*N. tabacum* L.) successfully produced a mouse hybridoma-derived monoclonal antibody (mAb) (Hiatt *et al*., 1989). Candidate microbicides such as Griffithsin (GRFT), which is a protein produced by red algae, is effective against HIV and was successfully expressed in tobacco plants (Gilbert, 2009; www.nature.com). Cyanovirin-N (CV-N) which is also a candidate microbicide that inactivates a wide range of HIV strains by binding to gp120, has also been expressed in transgenic tobacco plants (Sexton *et al.,* 2005).

Following the successful history of the plant production systems, the plant-derived pharmaceuticals are currently in clinical stages of development or on the market (Obembe *et al*., 2011).

1.4.2 Protein expression systems

1.4.2.1 Transient expression of proteins

In the transient expression system, the transgene, which may either be a foreign DNA, viral or bacterial vector, is not integrated into the plant genome but is still transcribed within the host cell (Gómez *et al*., 2010). The system allows for the quick, high expression level and evaluation of protein expression within weeks without a selection procedure required to identify transformed cells as cells are being used for temporary basis (Baur *et al*., 2005). There are three major approaches available to introduce DNA for transient expression; delivery of “naked” DNA by particle bombardment; infection with modified viral vectors; and infiltration of plant tissue with recombinant *Agrobacterium tumefaciens* species (Negrouk *et al*., 2005).

1.4.2.2 Modified viral vectors

Viral vectors are preferred as vehicles for recombinant protein expression and have been extensively studied and developed (Bråve *et al*., 2007). Plant viral vectors offer an efficient alternative for heterologous expression of proteins because they do not integrate into the plant genome, they do not form a heritable trait and the recombinant proteins are produced rapidly and in high yield (Gleba *et al*., 2007). Hence replicating plant viruses have been used to insert a gene of interest among their viral replicating elements, episomically amplified and translated in the plant cytosol (Lico *et al*., 2008).

Various plant viruses have been explored to produce pharmaceutical products (Brave *et al*., 2007). This include potex viruses such as potato virus X (PVX) and bamboo mosaic virus (BaMV), tobacco mosaic virus (TMV), papaya mosaic virus (PapMV), cowpea mosaic comovirus (CPMV), alfalfa mosaic virus (AIMV), bean yellow dwarf mastrevirus (BeYDV), cucumber mosaic viruses (CMV) and tomato bushy stunt tombusvirus (Rybicki, 2010). Tobacco mosaic virus (TMV) vectors express the highest levels of recombinant protein when compared with many modified plant viruses that function as expression vectors (Lindbo, 2007).

In this study, we followed the 'deconstructed virus' strategy that uses different *Agrobacteria* to deliver various modules of the viral vector and of the gene of interest (MagnICON). In this strategy, the virus has been deconstructed, i.e., some of its limiting or undesirable functions such as species-specificity has been eliminated and the process was rebuilt by either replacing the missing functions with analogous functions that are not necessarily of the viral origin or delegating the missing functions (Gleba *et al*., 2005). The functions of the bacteria are primary infection and systemic movement whereas the viral vector is responsible for cell-to-cell spread, amplification and high level expression (Gleba *et al.,* 2005). The site-specific recombinase helps the modules to assemble inside a plant cell (Marillonnet *et al*., 2004). The resulting DNA is transcribed and the undesired elements such as recombination sites are excised to generate a fully functional RNA replicon (Marillonnet *et al.,* 2004). The constitutive *Arabidopsis thaliana* Act2 promoter drives

the transgene on the tobacco mosaic virus (TMV)-based system (MagnICON). The deconstructed MagnICON system also allows the scalable production of a desired protein with high expression level and yield, low up- and downstream costs, reduced time and reduced biosafety concerns (Gómez *et al*., 2010). It has also been extensively engineered to express the highest levels of proteins in leaves with speed in production and scalability (Santi *et al*., 2006). MagnICON can result in the expression of up to 5 g recombinant protein per kg of fresh leaf biomass depending on the specific gene of interest (Gleba *et al*., 2007).

1.4.2.3 Stable expression of proteins

Agrobacterium-mediated transformation and particle bombardment methods are used quite often for stable transformation (Lico *et al*., 2008). The gene of interest between the right border and the left border marking the T-DNA becomes integrated into the plant genome. The integration process *per se* is lengthy for instance, production of stable transgenic plants may require 3-4 months for *Arabidopsis*, 9-12 months for *Rosa* and 2-3 months for *Nicotiana* (Yasmin and Debener, 2010).

Although most of plant biotechnology research is based on nuclear transformation for expression, both the nuclear and plastids (chloroplasts and mitochondria) genomes as well as endoplasmic reticulum have been used to express the recombinant proteins in transgenic plants (Rasala *et al*., 2010). Transformed chloroplast expression has been carried out and was demonstrated with the production of viable *E. coli* LT-B and *Cholera vibrio* CI-B antigens in tobacco. The resulting high-level recombinant protein expression does not seem to influence plant phenotypes as much as nuclear-mediated expression (Rybicki, 2010).

Additionally, stably transformed plants usually result in low yield of the recombinant protein because they are transformed with weak promoters. Plant viral vectors transiently produce recombinant proteins rapidly with high yields (Gleba *et al*., 2007). Unfortunately, viral vectors may not be used for stable transformation because they do not integrate into the plant genome.

To circumvent the lengthy period of protein expression and low yields of stable transgenic plants, this study opted to follow two transient expression routes i.e., (1), the standard integrative plant expression system, and (2), the 'deconstructed' virus strategy.

1.5 Aims of study

The overall aim of the study is to assess the feasibility of expressing 5P12-RANTES analogue in plants. It builds on the work done in the Combine Highly Active Antiretroviral Treatment (CHAARM) Consortium on finding an efficacious anti-HIV microbicide for HIV control. The following lines of investigation were followed:

a. Construction of vectors for expressing 5P12-RANTES gene in *Nicotiana benthamiana* and *Lycopersicon esculentum*
b. Assess the effect of transgenes on leaf morphology
c. Assess methods to extract and purify the protein from plant matrix and biochemically characterise the plant-made 5P12-RANTES for integrity
d. Screen the plant-made 5P12-RANTES protein for biological activity against HIV

For the purpose of presentation, the results will be presented in two sections as evaluation of 5P12–RANTES analogue in *Nicotiana benthamiana* (Chapter 3) and evaluation of 5P12-RANTES analogue expression in *Lycopersicon esculentum* (Chapter 4).

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Sources of reagents and materials

2.1.1 Chemical and non-chemical reagents

All reagents used in the study were analytical grade and were obtained from Sigma-Aldrich (South Africa) unless otherwise stated. Restriction endonucleases and *Taq* DNA polymerase were obtained from Inqababiotec (Pretoria, South Africa). PVDF membrane was obtained from Amersham Pharmacia Biotechnology. Chemically synthesised 5P12-RANTES standard was kindly provided by Prof O. Hartley (University of Geneva, Switzerland).

Unless stated otherwise, all general DNA manipulations and basic molecular biology were as described by Sambrook and Russell (2000). *Escherichia coli* DH10B was used as a general host for cloning and plasmid propagation. Plasmids encoding the 5P12-RANTES analogue were synthesised by GENEART (www.GeneART.com). The sequence of the analogue was plant codon-optimised and 6xhis tag was included at the C-terminal end and an extra nine nucleotide sequence encoding methionine, valine and serine in succession was also included at the N-terminal end to facilitate protein translation. The codon-optimised 5P12 gene was fused to an *E. coli* heat labile enterotoxin B subunit (LT-B) as a signal peptide (Mason *et al*., 1998) that cleaves *in vivo*. Hence the new sequence has to start with a glutamine instead of a methionine (Figure 7).

```
1   M   V   K   V   K   C   Y   V   L   F   T   A   L   L   S   S   L   C   A   Y
1   ATG GTG AAG GTG AAG TGC TAT GTG CTC TTC ACT GCT CTC CTC AGC TCT CTT TGT GCT TAT

21  G   V   S   Q   G   P   P   L   M   A   T   Q   S   C   C   F   A   Y   I   A
61  GGA GTC TCT CAA GGA CCA CCA CTT ATG GCT ACT CAG TCT TGC TGC TTC GCT TAT ATT GCT

41  R   P   L   P   R   A   H   I   K   E   Y   F   Y   T   S   G   K   C   S   N
121 AGG CCA CTT CCA AGG GCT CAC ATT AAG GAA TAC TTC TAC ACT TCT GGA AAG TGC TCT AAC

61  P   A   V   V   F   V   T   R   K   N   R   Q   V   C   A   N   P   E   K   K
181 CCA GCT GTT GTT TTC GTG ACT AGG AAG AAC AGG CAA GTT TGC GCT AAT CCA GAG AAG AAG

81  W   V   R   E   Y   I   N   S   L   E   M   S   H   H   H   H   H   H   -   -
241 TGG GTT AGG GAG TAC ATT AAC TCA CTT GAG ATG TCT CAT CAC CAT CAC CAC CAC TGA TGA

101 E   L   E   F
301 GAG CTC GAA TTC TT
```

Figure 7 Protein (top row) and nucleotide (bottom row) sequences of 5P12 showing the signal peptide, gene and 6xhis tag. Freeware DNA translation tool was used to translate the nucleotide sequences (www.vivo.colostate.edu). Colour shaded sequences: blue – signal peptide; black – 5P12 gene; green – 6xhis tag; red – stop codons; purple – the rest of the vector.

2.1.2 Kits

Plasmid DNA was prepared from *E. coli* by using Zymogen Zyppy Plasmid Miniprep kit (20 µg capacity as described by the manufacturer Zymogen, CA, USA). TOPO® cloning kits were obtained from Invitrogen (Life Technologies, South Africa) whilst T4 DNA ligase and DNA molecular markers were supplied by New England Biolabs Inc (Hitchin, UK).

2.1.3 Plasmids

Table 1 Description of plasmids that were used in this study

Name	Description	Reference
pCs5P12	*E. coli* 5P12-RANTES cloned into the *Nco*I/*Xho*I sites of pTRAc	This study
pCs6P4	*E. coli* 6P4-RANTES cloned into the *Nco*I/*Xho*I sites of pTRAc	This study
pcTPs5P12	*E. coli* 5P12-RANTES cloned into the *Eco*RI/*Xho*I sites of pTRAkc-rbcs1-cTP	This study
pcTPs6P4	*E. coli* 6P4-RANTES cloned into the *Mlu*I/*Not*I sites of pTRAkc-rbcs1-cTP	This study
pERs5P12	*E. coli* 5P12-RANTES cloned into the *Nco*I/*Not*I sites of pTRAkc-ERH	This study
pERs6P4	*E. coli* 6P4-RANTES cloned into the *Nco*I/*Not*I sites of pTRAkc-ERH	This study
pGEM-T Easy vector	3.015 kb vector with multiple restriction sites for cloning PCR products.	www.promega.com/

pICH11599	Tobacco mosaic virus (TMV)-based provector (3´ module cloning vector)	ICON Genetics, Halle, Germany; Giritch *et al*., (2006).
pICH14011	TMV-based provector (integrase module)	ICON Genetics, Halle, Germany; Giritch *et al*., (2006).
pICH17388	TMV-based provector (5´ cytosol targeting module)	ICON Genetics, Halle, Germany; Giritch *et al*., (2006).
pICH17620	TMV-based provector (5´ apoplast targeting module)	ICON Genetics, Halle, Germany
pICH7410	TMV-based provector (3´ module for expression of GFP.	ICON Genetics, Halle, Germany; Giritch *et al*., (2006).
pICHs5P12	*E. coli* 5P12-RANTES cloned into the *Nco*I/*Xho*I sites of pICH11599	This study
pICHs5P12apo	Integration of pICHs5P12 and pICH17620	This study
pICHs5P12cyt	Integration of pICHs5P12 and pICH17388	This study
pICHs6P4	*E. coli* 6P4-RANTES cloned into the *Nco*I/*Not*I sites of pICH11599	This study
pICHs6P4apo	Integration of pICHs6P4 and pICH17620	This study
pICHs6P4cyt	Integration of pICHs6P4 and pICH17388	This study
pTRAc	6.15 kb *Agrobacterium* vector which consists of CaMV 35S promoter, chalcone synthase 59 untranslated region, two copies of scaffold attachment region flanking expression cassette,	Maclean *et al*., (2007)

	left and right borders for T-DNA integration and *bla* gene for antibiotic selection.	
pTRAkc-ERH	7.701 kb *Agrobacterium* vector. A derivative of pTRAc vector with a plant codon-optimised signal-peptide sequence from the murine mAb24 heavy chain gene and the his6 and endoplasmic reticulum retention (SEKDEL) sequences. Has *nptII* gene for kanamycin resistance.	Maclean *et al*., (2007)
pTRAkc-rbcs1-cTP	7.743 kb *Agrobacterium* vector. A derivative of pTRAc vector with the chloroplast-transit peptide sequence of the potato *rbcS*1 gene. Has *nptII* gene for kanamycin resistance.	Maclean *et al*., (2007)

2.1.4 Computer analysis

Primers for PCR were designed using published cDNA sequences and Primer3 software. Usually the primers were between 20 and 87 base pairs with annealing temperatures of 50 to 72.3^{o}C and a GC content of about 50%. Primers were also checked for hairpins and dimerisation. BioEDIT (Hall, 1999) and VectorNTI programs were used for sequence alignments and comparisons while BLAST (Altschul *et al*., 1990) was used for database searches.

2.1.5 Primers

Table 2 List of vectors, primers and the designed constructs used in this study

Vector	Insert	Primers (5´-3´ sequences)	Restriction enzymes	Construct
pTRAc	5P12	**CF1 :** CATGCCATGGTGAAGGTGAAGTGCTA TGTGCTCTTCACTGCTCTCCTCAGCTCTCTT TGTGCTTATG GACAAGGACCACCACTTATG	*Nco*I	pCs5P12
		CR1 : TCCGGACTCGAGATTAAGA ATTCGA GCTCTCATCAG	*Xho*I	
	6P4	**CF2:** CATGCCATGGTGAAGGTGAAGTGCTAT GTGCTCTTCACTGCTCTCCTCAGCTCTCTTT GTGCTTATGGACAAGGACCACCTGGAGAT	*Nco*I	pCs6P4
		CR2: TCCGGACTCGAGATTAAGAATTCGAGC TCTCATCAG	*Xho*I	
pTRAkc-ERH	5P12	**ERF1 :** tccATGgTGAAGGTGAAGTGCTATGT GCTC	*Nco*I	pERs5P12
		ERR1 : CTCATCACCATCACCACCACCGCGG CCGC*TC*	*Not*I	
	6P4	**ERF2: :** tccATGgTGAAGGTGAAGTGCTATG TGCTC	*Nco*I	pERs6P4
		ERR2 : CTCATCACCATCACCACCACCGCGG CCGC*TC*	*Not*I	
pTRAkc-rbcs1-	5P12	**CTPF1 :** gtggacgcgttaggtgATGGTGAAGGTG	*Mlu*I	pcTPs5P12

cTP		AAGTGCTATGTGC TC		
		CTPF1 : CTCATCACCATCACCACCACc GCG GCCGC*TC*	*Not*I	
	6P4	**CTPF2 :** gtggacgcgttaggtgATGGTGAAGGTGA AGTGCTATGTGC TC	*Mlu*I	pcTPs6P4
		CTPF2 : CTCATCACCATCACCACCACc GCG GCCGC*TC*	*Not*I	
pICH11599	5P12	**IconF1 :** CATGCCATGGTGAAGGTGAAGTGCT ATGTGCTCTTCACTGCTCTCCTCAGCTCTCT TTGTGCTTATGGACAAG GACCACCACTTATG	*Nco*I	pICHs5P12
		IconR1: TCCGGACTCGAGATTAAGAATTCGA GCTCTCATCAG	*Xho*I	
	6P4	**IconF2:** CATGCCATGGTGAAGGTGAAGTGCT ATGTGCTCTTCACTGCTCTCCTCAGCTCTCT TTG TGCTTATGGACAAGGACCACCTGGAGAT	*Nco*I	pICHs6P4
		IconR2: TCCGGACTCGAGATTA AGAATTCGAGCTCTCATCAG	*Xho*I	

Custom oligonucleotides were synthesised by Inqaba Biotech (Pretoria, SA). Nucleotide and sequences are given with a brief description.

2.2. Microbiological techniques

2.2.1 Bacterial strains

Table 3 Bacterial strains used in the study

Name	Genotype	Full genotype and reference
DH10B	*E. coli*	F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-
GV3101::pMP90	*Agrobacterium tumefaciens*	MP90(pTiC58ΔT-DNA) (Koncz and Schell, 1986)
LBA4404	*A. tumefaciens*	pAL4404 (Hoekema *et al*., 1983)

2.2.2 Transformation of *E. coli*

The pTRA and MagnICON constructs were used for transformation of *E. coli* DH10B. About 50 µl of electrocompetent *A. tumefaciens* cells and 2 µl of the constructs were transferred into the chilled cuvettes. The cells were electroporated by BioRad Gene Pulser with a set voltage of 1.60 kV. About 450 µl of Luria Bertani (LB) medium was added and incubated in an Innova^{TM} 4000 Incubator Shaker at 37^{o}C for one hour. Luria agar (LA) medium containing carbenicilin (100 µg/ml) was prepared, poured in Petri dishes and allowed to set. About 100 µl of the cells were spread on media plates and incubated overnight in a Memmert incubator set at 28^{o}C. A colony PCR or miniprep was conducted to confirm the presence of the RANTES genes.

2.2.3 Transformation of *A. tumefaciens*

Approximately 50 to 100 ng of recombinant plasmid was electroporated into host *A. tumefaciens* GV3101::pMP90RK cells using BioRad Gene Pulser with a set voltage

of 1.44 kV. Transformed cells were resuscitated on LB medium and incubated in an Innova™ 4000 Incubator Shaker at 28°C for one hour. Only 100 µl of cells were spread on LA medium plates containing kanamycin (30 µg/ml), rifampicin (50 µg/ml) and carbenicillin (50 µg/ml). The plates were incubated overnight in a Memmert incubator set at 28°C. A colony PCR or miniprep was conducted to confirm the presence of the RANTES genes.

2.3 DNA preparation and analysis

2.3.1 Nucleic acid manipulation

Unless stated otherwise, all nucleic acid manipulations and cloning were as described by Sambrook *et al*. (1989). These include small-scale DNA plasmid preparations, restriction enzyme digestion and ligations.

2.3.2 Preparation of plasmid DNA

Unless stated otherwise a Plasmid Miniprep kit (Qiagen) was used for preparation of plasmid DNA according to manufacturer's protocol.

2.3.3 Polymerase Chain Reaction (PCR)

The PCR reactions were performed in 50 µl volumes containing: 38.5 µl of sterile DI water, 5 µl of 10x NH4 reaction buffer (Bioline), 1.5 µl of MgCl2 (50 mM), 1 µl of forward primer (100 mM), 1 µl of reverse primer (100 mM), 2 µl of dNTPs (10 µM), 1-5 µl of template and 2.5 units *Taq polymerase* (Bioline, USA). The standard PCR programme had 35 cycles and temperature cycling was performed on a 2720 Thermal cycler machine (Applied Biosystems, Life Technologies, South Africa) in a total volume of 50 µl. The PCR amplification consisted of 5 cycles of 95ºC for 5 s, 94°C for 30s, 35ºC for 30 s, 72ºC for 1 s; then 30 cycles at 48°C annealing temperature, and 72°C elongation time. The final PCR cycle was identical to the

above, except that the extension time at 72 °C was increased to 4 minutes. PCR optimisation was carried out to the best conditions for a particular primer.

2.3.4 Agarose gel electrophoresis and isolation of DNA fragments

After PCR about 8 µl of amplified product was mixed with 2 µl of 10x loading buffer III (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and then subjected to electrophoresis using 0.8 to 2% agarose gel containing 1 µg/ml ethidium bromide. Gels were ran in 1x TAE (tris acetate EDTA) buffer and photographed on a UV light transilluminator. Band sizes were estimated with 100 bp, 1 kb ladder or λ-HindIII marker. DNA for further cloning was examined under long-wave UV (254 nm), minimising the exposure time to prevent nicking. Gels with the desired fragment were excised and extracted from the agarose using the Zymogen Gel Extraction Kit supplied by Zymogen Research (CA, USA).

2.3.5 Cloning into expression vectors

The MagnICON is a de-constructed tobacco mosaic virus (TMV) system (from ICON Genetics, Halle, Germany) comprising of three components: a 5´ module for protein targeting to different subcellular compartments (cytosol or apoplast); a 3´ module which contains the gene-of-interest; and *Streptomyces* phage PhiC31 integrase module which mediates integration of the provectors. To generate the MagnICON vector construct, the 5P12 gene was amplified using the forward primer (IconF1) and the reverse primer (IconR1). The PCR product was cloned into pGEM-T Easy vector and subcloned into the corresponding *Nco*I and *Sal*I restriction enzymes of pICH11599 vector (Figure 8) to obtain pICHs5P12 and pICH5P12 constructs. In this study, we reported protein expression using pICHs5P12 construct. In addition to restriction enzyme digestion, all cloned genes were sequenced to verify their identity.

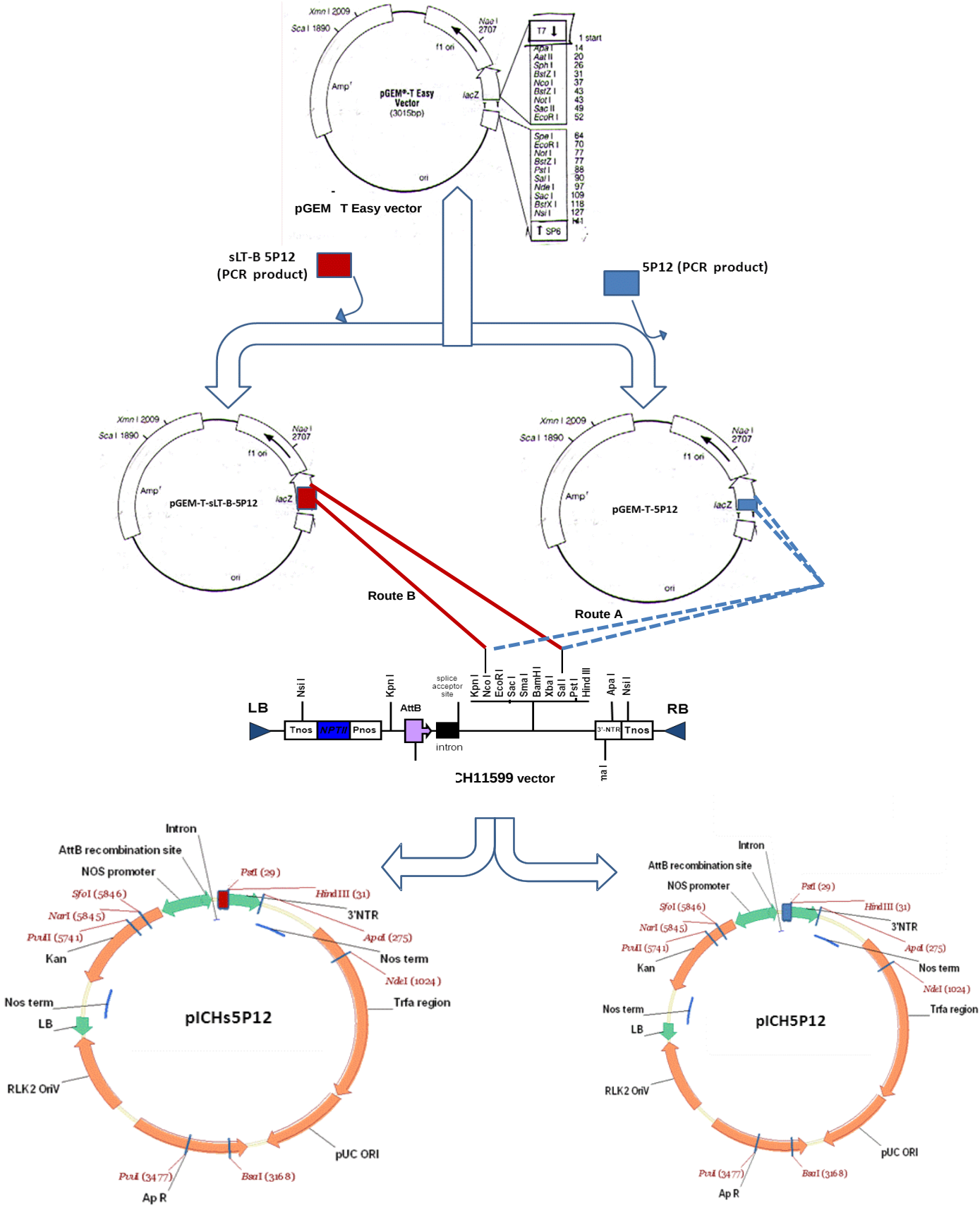

Figure 8 The cloning strategy into the MagnICON vector. PCR products were cloned into the pGEM-T Easy vector as a cloning vector. The 5P12 were sub-cloned from

pGEMT-Easy vector into the pICH11599 vector for expression. The resulting constructs were pICH5P12 and pICHs5P12.

The three pTRA vectors (pTRAc, pTRAkc-ERH and pTRAkc-rbcs1-cTP) were provided by Dr Rainer Fischer (Fraunhofer Institut, Aachem, Germany) (Figure 9). The vector modules target the protein to different subcellular locations viz: cytosol, ER and chloroplast. To generate a cytosolic targeting construct, the 5P12 gene was amplified using the forward primer (CF1) and the reverse primer (CR1). The PCR product was cloned into pGEM-T Easy vector and then subcloned into the *Afl*III/*Xho*I restriction sites of pTRAc vector. The resulting construct was named pCs5P12. Similarly, the ER targeting construct was generated by amplifying the 5P12 gene with forward primer (ERF1) and the reverse prime (ERR1). The PCR product was cloned into pGEM-T Easy vector and subcloned into the *Nco*I/*Not*I restriction sites of pTRAkc-ERH vector, resulting in pERs5P12 construct with a KDEL endoplasmic reticulum retention signal peptide. To generate the chloroplast targeting construct, 5P12 gene was amplified by PCR using the forward primer (CTPF1) and the reverse primer (CTPR1). The PCR product was cloned into pGEM-T Easy vector and then subcloned into the *Mlu*I/*Not*I restriction sites of pTRAkc-rbcs1-cTP vector to afford pcTPs5P12 construct. The sequences of all constructs were also verified by sequencing.

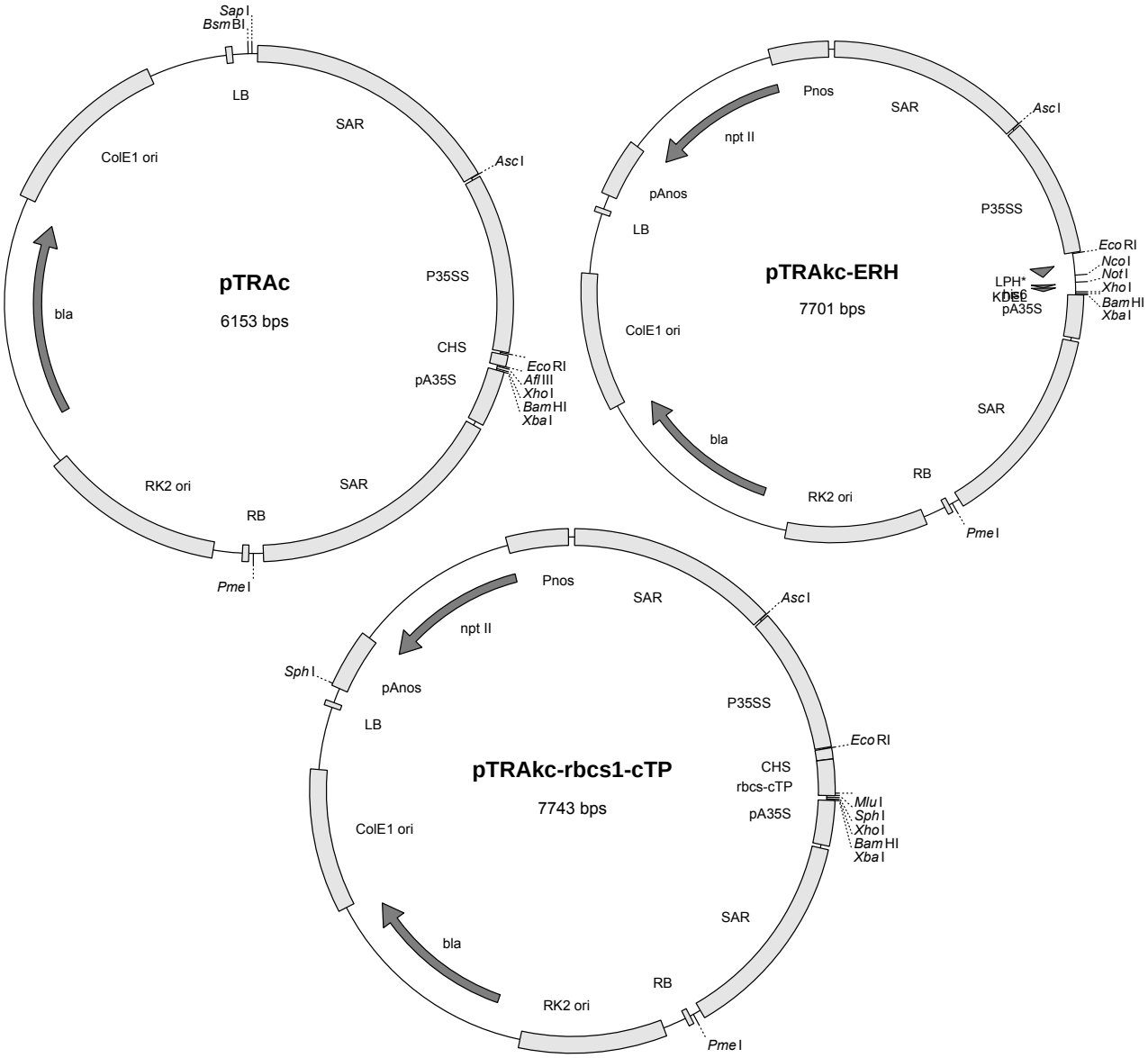

Figure 9 Plasmid representations of pTRA vectors

2.4 Preparation of plant biomass

2.4.1 General plant husbandry (*Nicotiana benthamiana)*

Nicotiana benthamiana (Family: *Solanaceae*; subfamily: *Nicotianoideae*) seeds were obtained from University of Pretoria (Forestry and Agricultural Biotechnology Institute, South Africa) and sterilised using 70% ethanol. The ethanol was discarded and seeds were soaked for few seconds in bleach, a solution containing 3-8%

sodium hypochlorite and 0.01-0.05% sodium hydroxide. The bleach was discarded and seeds were rinsed thrice with distilled water. Sterile seeds were transferred into sterile bottles containing ½ MS (Murashige and Skoog (MS) macronutrients, MS micronutrients (5 ml/l), kanamycin (30 µg/ml), sucrose (30 g/l), agar (8 g/l) and pH 5.8) medium. The seeds were allowed to grow in the tissue culture laboratory under a 16-h/8-hour photoperiod and a temperature regime of 25°C day/20°C night. Three weeks after seeding, individual plantlets were picked out, transplanted in pots containing soil and left to grow in the tissue culture laboratory for 3-5 additional weeks under the same environmental conditions.

2.4.2 Agroinfiltration of *N. benthamiana* with MagnICON constructs

Agrobacterium strain GV3101 containing the MagnICON constructs were cultured in a 250 ml conical flask containing 100 ml of LB supplemented with rifampicin (50 µg/ml) and carbenicillin (50 µg/ml) in an Innova^{TM} 4000 Incubator Shaker at 28^{o}C. About 50 ml of the cells from overnight cultures were transferred into 50 ml falcon tubes, sedimented by centrifugation at 4000 rpm for 10 minutes using Eppendorf centrifuge 5810R and resuspended in infiltration buffer (10mM 2-(*N*-morpholino)ethanesulphonic acid (MES), 10mM $MgSO_4$, pH 5.5). A final OD_{600} of 0.4 was measured with a DU® 800 spectrophotometer (Oak Ridge National Laboratory, United States). Equal volumes of *A. tumefaciens* suspension containing the three viral modules (5´-module, integrase or 3´-module) were mixed into 50 ml falcon tubes and infiltrated into the abaxial leaf surface of 6-8 week old *N. benthamiana* plants as described previously (Webster *et al*., 2009). Agroinfiltration was done by injection using a 5 ml syringe on the abaxial surface of the leaves or by vacuum (25 mbar for 1 minute) into a whole, uprooted plant (Figure 10). Infiltrated plants were replanted, grown at 22^{o}C under a 16 h/8 h light/dark cycle and subsequently harvested on a time course as of 3 dpi.

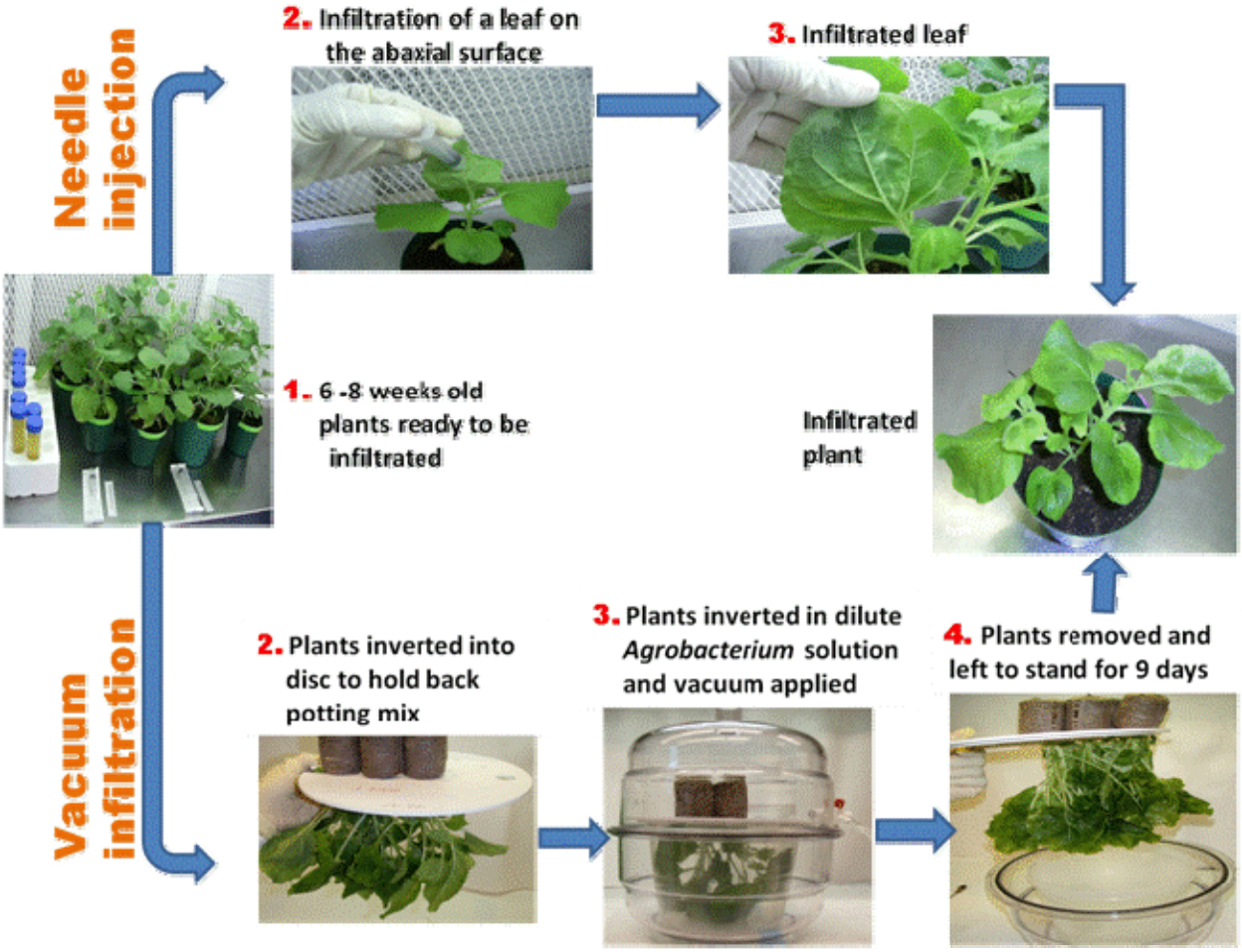

Figure 10 Agroinfiltration of *N. benthamiana* via needle injection and vacuum infiltration.

2.4.3 Agroinfiltration of *N. benthamiana* with pTRA constructs

The method was adapted from Meyers *et al*. (2008). Recombinant strains were cultured overnight in a 250 ml conical flask with an induction medium containing LB and 10 mM MES (pH5.6) supplemented with 20 µM acetosyringone, kanamycin (30 µg/ml), rifampicin (50 µg/ml) and carbenicillin (50 µg/ml) in an Innova^{TM} 4000 Incubator Shaker (New Brunswick Scientific, UK) at 27^{o}C. The culture were transferred into a 50 ml Falcon tubes, cells were pelleted at 4000 rpm using Eppendorf centrifuge 5810R and resuspended in infiltration medium containing 10 mM MES and 1 M $MgCl_2$ (pH 5.6) supplemented with 200 µM acetosyringone. Cells

were diluted in infiltration medium to an OD_{600} of 0.25. After incubation in an Innova^{TM} 4000 Incubator Shaker at 22^{o}C for 2 hours, cells were infiltrated by injection (10 ml syringe) into whole, uprooted *N. benthamiana* plants. Plants were co-infiltrated with *A. tumefaciens* LBA4404 (diluted in infiltration medium to an OD_{600} of 0.25) containing a silencing suppressor pBIN-NSs (provided by Marcel Prins, Laboratory of Virology, Wageningen, The Netherlands) which enhances transient protein expression by suppressing post transcriptional gene silencing. This strain was similarly grown up overnight at 27^{o}C with agitation in induction medium containing LB and 10 mM MES (pH 5.6) supplemented with 20 µM acetosyringone, kanamycin (30 µg/ml), rifampicin (50 µg/ml) and 2 mM MgSO4 to prevent the cells from clumping. Infiltrated plants were replanted, grown at 22^{o}C under a 16 h/8 h light/dark cycle and subsequently harvested on a time course as of 3 dpi.

2.4.4 Agroinfection of *Lycopersicon esculentum* with MagnICON constructs

Agrobacterium strain GV3101 containing the MagnICON constructs were cultured in a 250 ml conical flask containing 100 ml of LB supplemented with rifampicin (50 µg/ml) and carbenicillin (50 µg/ml) in an Innova^{TM} 4000 Incubator Shaker at 28^{o}C. The overnight cultures were transferred into 50 ml falcon tubes, centrifuged at 4000 rpm for 10 minutes using Eppendorf centrifuge 5810R and resuspended in infiltration buffer (10mM 2-(*N*-morpholino)ethanesulphonic acid (MES), 10 mM $MgSO_4$, pH 5.5). A final OD_{600} of 0.4 was measured with a DU® 800 spectrophotometer. Equal volumes (15 ml each) of *A. tumefaciens* suspension containing the three viral modules (5´-module, integrase and 3´-module) were mixed and infiltrated into tomato fruits at different stages of development (Giritch *et al*., 2006; Webster *et al.,* 2009). A volume of 1.5 ml was infiltrated into mature tomato fruits using a 5 ml syringe with 23G Neoject needle (Neomedic, UK). The needle was introduced 3 to 4 mm in depth into the fruit tissue through the stylar apex. Only completely infiltrated fruits were used in the experiment (Orzaez *et al*., 2006). Tomato fruits were left in a cupboard at room temperature and the protein was extracted between 3 and 9 days post infiltration.

2.5 Protein analysis

2.5.1 Crude protein extraction from *N. benthamiana* leaves

This protocol was adapted from various methods-(Schröder, 2000; Chakauya *et al*., 2008). Approximately 100 mg of harvested leaves were weighed and ground in liquid nitrogen to a fine powder with a pestle and mortar and homogenised in 300 µl buffer (phosphate buffered saline (PBS) containing 0.02% Tween20, pH 7.5) for 60 minutes in an ice water mixture. The extract was transferred into 1.5 ml Eppendorf tubes and the cell debris was removed by two rounds of centrifugation at 10 000 rpm and 4^{o}C for 30 minutes using Eppendorf centrifuge 5417R. The supernatant was used for expression analyses and protein purification by gel filtration.

2.5.2 Crude protein extraction from *L. esculentum* fruits

The infiltrated tomato fruits were frozen at -86^{o}C, ground to a powder with a pestle and mortar and lyophilised. Lyophilised material was homogenised in PBS containing 0.02% Tween20, pH 7.5) or citric acid (0.1M aqueous citric acid containing 50% (v/v) ethanol) buffers (Rudack *et al*., 2004). About 5 ml of the extraction buffer was added into a 50 ml beaker containing 1.5 g of tomato fruit lyophilised material. The cell debris was removed by two rounds of centrifugation at 10 000 rpm and 4^{o}C for 30 minutes using Eppendorf centrifuge 5417R.

2.5.3 Protein determination

Crude protein was assayed as described by Bradford (1976). The Bio-Rad protein reagent was used to determine the protein concentration, using the micro-protein assay in accordance with the manufacturer's instructions. Bovine serum albumin was used as a standard. The crude extract and purified protein were determined by measuring absorbance at 595 nm.

2.5.4 Measuring pH of the lyophilised tomato fruit material

About 1.5 g of the lyophilised material at 9 dpi was added into a 50 ml beaker containing 5 ml of distilled water. The solution was stirred with a magnetic stirrer for 5 minutes. The pH of a solution was measured using Mettle s20 pH meter (Mettle Toledo Inlab® Expert Pro) per sample.

2.5.5 Size-exclusion chromatography

Gel filtration was conducted using the Macrosep centrifugal devices (PALL Life Sciences, USA). According to the manufacturer's instructions, about 5-15 ml of sample was poured into the non-membrane side of the sample reservoir of Macrosep device (lot. 10550261) with a 10K molecular weight cutoff. The reservoir was closed with a cap and centrifuged at 5000 rpm for 90 minutes or more to achieve desired concentrate volume using Eppendorf centrifuge 5810R. The filtrate was then collected and transferred into the Macrosep device (lot. 11550202) with a 3K molecular weight cutoff. Both the retentate and filtrate were collected and used for further analysis as it contained the RANTES protein.

2.5.6 Protein purification by Protino Ni-IDA

We followed the protocol from the manufacturer's manual. The Protino Ni-IDA (iminodiacetic acid) packed columns (MACHEREY-NAGEL, cat. no. 745 150.50) were equilibrated with 320 µl LEW buffer. The columns were allowed to drain by gravity. The prepared crude samples were added to the pre-equilibrated columns and the columns were allowed to drain by gravity. The columns were washed with 320 µl LEW buffer. The washing step was repeated. The polyhistidine-tagged proteins were eluted in a collecting tube by adding 240 µl 1x Elution buffer.

2.5.7 Protein concentration by trichloroacetic acid (TCA) method

Protein concentration was determined using a modified method adapted from Wang *et al*., (2006). A buffer containing 50% TCA was added into a 1.5 ml Eppendorf tube

containing the protein solution to yield a final TCA percentage of 20%. The solution was subsequently centrifuged at 15000 rpm at 4°C for 10 minutes using Eppendorf centrifuge 5417R. The supernatant was discarded and the precipitate was washed with 150 µl of 10% TCA once and then 200 µl acetone once and finally dissolved in 20 µl of SDS-PAGE loading buffer.

2.5.8 SDS-PAGE for Coomassie staining and western blot

The gels were run in Mini Protein II™ gel electrophoresis apparatus (Bio-Rad, Herts, UK). SDS-PAGE was performed according to Laemmli (1970). A 15% resolving gel was prepared. Water was laid on the surface of the gel to ensure a smooth interface between the stacking and resolving gels. It was discarded when the resolving gel had set just before the 4.5% stacking gel was added. Protein samples were incubated in 2x SDS Buffer (2% (w/v) SDS, 20% (v/v) glycerol, 20 mM Tris.HCl pH 8.0; 2 mM (w/v) EDTA and 5% (v/v) β-mercaptoethanol and 0.006% (w/v) bromophenol blue) for 3 minutes at 100°C prior to loading. This was followed by a 1 minute spin in a microfuge at maximum speed and the supernatant was loaded. Electrophoresis was performed in running buffer at 40 mA per gel for 1 hour or until the bromophenol blue dye had reached the bottom of the gel. Molecular markers were run on each gel.

2.5.9 High resolution by Tris-tricine gel

High resolution-broad range gel was carried out according to the method of Schägger and von Jagow (1987), using a Bio-Rad Mini Protean II gel apparatus. The protocols for sample preparation, electrophoresis and protein visualisation were as described in the Sigma Technical Bulletin ‘SDS Molecular Weight Markers’ (Bulletin No. MWM-100, September 1990).

2.5.10 Western blot analysis

After separation of proteins by SDS-PAGE the gel was fixed in transfer buffer for 20 minutes. Transfer of proteins onto nitrocellulose was carried out using a semi-dry

blotter (Atto Corp., Horizoblot, Japan) at 13V, 300 mA for 40 minutes using a transfer buffer containing 20% methanol (v/v), 0.2 M glycine, 25 mM Tris and 0.02% (w/v) SDS. Using prestained markers (Rainbow Markers, Norvex) enabled quick verification of a successful transfer as these are visible on membrane once the blot is complete. After blotting, the proteins were visualised on the nitrocellulose with Ponceau STM, which was rinsed off with 1x TBS after the molecular weight standards were marked with pencil.

For immunodetection, proteins were transferred onto a PVDF membrane, blocked with 5% (w/v) solution of skimmed milk powder in TBST (50 mM Tris/100 mM NaCl/0.05% Tween-20, pH 7.4), probed with biotinylated anti-RANTES antibodies (BAF278 R&D Systems) and diluted in a 2.5% (w/v) solution of skimmed milk powder in TBS (1:2000). This was followed by a secondary antibody (peroxidase-conjugated goat anti-rabbit Ig: Streptavidin-HRP, R&D Systems DY998) diluted 1:5000 in TBS and subsequently detected by an enhanced chemiluminiscence kit (ECL Plus, GE Healthcare).

2.5.11 Enzyme-linked immunosorbent assay (ELISA)

This method was adapted from the standard operating procedures from Oliver Hartley's Laboratory at the University of Geneva, Switzerland. A concentration of 5 µg/ml of the capturing primary antibodies (monoclonal anti-human CCL5/RANTES MAB678, R&D Systems) was prepared into 10 ml of PBS, 100 µl was distributed into the 96 well microtiter plate (NUNC, A 62219, AEC Amersham) with a multichannel pipette and incubated for 16 hours at 4^{o}C. The plate was washed 4 times with 200 µl of washing solution (0.05% Tween20 in PBS). 300 µl of the coating solution (PBS / 1% BSA / 5% sucrose / 0.05% NaN_3 pH 7.0) was distributed into each well of the plate and incubated for 2 hours at 37^{o}C. The plate was washed 4 times with 200 µl of washing solution. Serial dilutions of the protein standard (5P12-RANTES) were prepared in a diluent solution (20 mM Tris-HCl / 150 mM NaCl / 0.1% BSA / 0.05% Tween20, pH 7.3) ranging from 10-0.1 ng/ml and about 100 µl were distributed per well into the plate. The plate was incubated for 2 hours at ambient temperature by gently shaking in a thermostar (BMG LABTECH). The plate was washed 4 times with

200 µl of washing solution. A concentration of 250 ng/ml of the secondary antibody (biotinylated anti-human CCL5/RANTES BAF278, R&D systems) was prepared into 10 ml diluent solution, 100 µl was distributed into each well and incubated for 2 hours in a thermostar. The plate was washed 4 times with 200 µl of washing solution. A concentration of 125 ng/ml of the Streptavidin-HRP (peroxidase-conjugated goat anti-rabbit Ig: Streptavidin-HRP, R&D Systems DY998) was prepared into 10ml diluent solution, 100 µl was distributed into each well and incubated for 30 minutes. The plate was washed 4 times with 200 µl of washing solution.

Detection was made with distribution of 100 µl of 3, 3, 5, 5-tetramethylbenzidine (TMB) Liquid substrate (Sigma 040M1646) into each well and the plate was left for some few minutes on a bench to observe the colour change to blue. The reaction was stopped by adding 50 µl of 1M H_2SO_4. The optical density was measured at the range of 450 nm vs 650 nm by KC4 Data Analysis Software (Analytical & Diagnostic Products, South Africa).

2.6 Assays

2.6.1 Cytotoxicity assay

Cell viability was determined using a colorimetric cell metabolic assay (CellTiter 96®AQ_{ueous} Non-Radioactive Cell proliferation Assay kit, Promega, Madison, Wisconsin, USA) based on the MTS tetrazolium compound, {3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium}. The dehydrogenase enzymes found in metabolically active cells convert MTS into the aqueous soluble formazan product. The quantity of formazan product which is directly proportional to the number of living cells in culture is normally measured at the absorbance of 490nm. Etoposide (an anticancer drug - promotes apoptosis of cancer cells) was used as positive control. About 60 µl of Dulbecco's Modified Eagle Medium ($DMEM^5$) was added to all wells except bottom row wells of RANTES extract lanes. 75 µl RANTES extract was added to bottom row wells in duplicate. RANTES sample was serially diluted to upper wells in 5-fold by transferring 15 µl media to upper wells and 15 µl of the top row wells was discarded. 25 µl $DMEM^5$ was added to all wells to

replace for virus volume. 50 µl TZM-bl cells ($2x10^5$ cells/ml) were added to all wells, except the Blank wells. This was incubated for 48 hours at 37°C and 5% CO_2. 28.5 µl PMS/MTS mix was added to all wells and incubated for 45 minutes at 37°C and 5% CO_2. Absorbance of formazan product was read at 492nm with a Luminometer. Results were analysed in Microsoft excel.

2.6.2 HIV-1 pseudovirus neutralisation assay

The HIV-1 pseudovirus neutralization assay was conducted at the Biosciences/Aptamer laboratory (CSIR, South Africa) according to their standard operating procedures. The assay is to measure the capacity of RANTES proteins to neutralise HIV-1 pseudovirions *in vitro* in TZM-bl cells (cells were provided by Lynn Morris, NICD, HIV/AIDS unit, SA). The molecularly cloned HIV-1 pseudovirions were generated by co-transfection of envelope (*env*)-expressing plasmids with backbone plasmid DNA in 293T cells. HIV-1 pseudovirions used in this study include; HXB2 (HIV-1 subtype B, CXCR4 virus); ZM53 (HIV-1 subtype C, CCR5 virus) and Vesicular Stomatitis Virus glycoprotein (VSV-G) which is a HIV backbone genome but contains a VSV-G envelope, used to test specificity of anti-HIV entry inhibitors. TZM-bl cells are CXCR4-positive HeLa cell clones that have been engineered to express CD4 and CCR5. They were also engineered to contain integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under the control of an HIV-1 long terminal repeat (LTR) promoter (Wei *et al*., 2002). After infection, the viral tat-protein induces the expression of the luciferase reporter gene. The luciferase activity is proportional to the infectious viral particles that have entered the cell and is detected and quantified by luminescence. Hence efficacy is calculated as a function of reductions in tat-regulated luciferase reporter gene expression after a single round of infection in TZM-bl cells (Unknown, 2008: http://www.hiv.lanl.gov/).

About 60 µl of media (DMEM) (Gibco, BRL, UK) was added to all wells except bottom row wells of RANTES lanes, an extra 25 µl was added to cell control wells. Assay controls used include T20 (entry/fusion inhibitor) and tenofovir (reverse transcriptase inhibitor). 75 µl RANTES extract was added to bottom row wells in

duplicate. RANTES extract was serially diluted to upper wells in 5-fold by transferring 15 µl media to upper wells. 15 µl of the top row wells was discarded. 25 µl virus (diluted according to titred relative luminescence units (RLU) reading to give a luminescence reading of approximately 20 000 RLU) was added to all wells, except control wells. 50 µl TZM-bl cells ($2x10^5$ cells/ml) were added to all wells. The plate was incubated for 48 hours at 37°C and 5% CO_2. The plate was removed and 75 µl was discarded from all wells. About 50 µl Bright Glo luciferase substrate (Promega, Madison, Wisconsin, USA) was added to all wells and then incubated for 2 minutes. 50 µl of media was transferred from all wells to a black, flat bottom NUNC plate. Luminescence was read with a Luminometer (Tecan i-control, 1.5.14.0, infinite F500, Männedorf, Switzerland) and results were analysed in Microsoft Excel.

CHAPTER 3

3. EVALUATION OF RANTES ANALOGUES EXPRESSION IN *NICOTIANA BENTHAMIANA*

3.1 Motivation

University of Geneva discovered the fully recombinant analogues of PSC-RANTES, i.e., 5P12-RANTES and 6P4-RANTES. Therefore, it is a good idea for the resource-poor countries especially, in the sub-Saharan Africa where HIV infection is alarming, to make efficient use of the discovery so as to alleviate the spread of the disease. Hence both analogues were explored in this study.

5P12-RANTES and 6P4-RANTES analogues were cloned, transiently expressed in *N. benthamiana* leaves, evaluated for the recombinant chemokine expression and screened for their biological activities. The two analogues were treated the same starting from cloning to transient expression. Moreover, the results obtained including yields, cytotoxicity and efficacy were similar. Although we opted to report 5P12-RANTES as the most promising candidate microbicide to avoid massive duplication, 6P4-RANTES may appear when it is absolutely necessary.

3.2 Results and discussion

3.2.1 Sequence alignment, vector design and construction

The amino acids of 5P12-RANTES and 6P4-RANTES analogues were aligned using Clustal 2.0.10 multiple sequence alignment tool (Figure 11). These were compared with the commercially available *E. coli* RANTES (278RN) as it has continuously been used in this study. The three RANTES molecules show common amino acid sequences from the first adjacent cysteine to the last serine.

```
CLUSTAL 2.0.10 multiple sequence alignment

5P12-RANTES       MVSQGPPLMATQSCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKK 60
6P4-RANTES        MVSQGPPGDIVLACCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKK 60
278RN-RANTES      ----SPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKK 56
                      .*    . .************************************************

5P12-RANTES       WVREYINSLEMSHHHHHH 78
6P4-RANTES        WVREYINSLEMSHHHHHH 78
278RN-RANTES      WVREYINSLEMS------ 68
                  ************
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Figure 11 Amino acids alignment of the 5P12, 6P4 and 278RN-RANTES showing their differences.

In order to express 5P12 in *Nicotiana benthamiana*, two vector modules harbouring the gene of interest fused to a signal peptide were successfully generated, that is, MagnICON and the pTRA constructs. The 5P12 gene was tagged with six histidine amino acids (6xHis) on the C-terminus to facilitate protein purification while extra nine nucleotide sequences were inserted on the N-terminus to facilitate protein translation. On the other hand, Moeller *et al*., (2009) provided evidence that sLT-B signal peptide is able to target proteins to the plant secretory pathway and it was used in this study to cleave along with some of the amino acids on the N-terminus as predicted by TargetP software tool (see Appendix A). Cleavage position was designed such that the final 5P12 molecule begins with glutamine for activity purposes (Figure 12). This was based on the observation that recombinant proteins of the N-terminally modified RANTES analogues lose activity when an additional amino acid was added at the N-terminus.

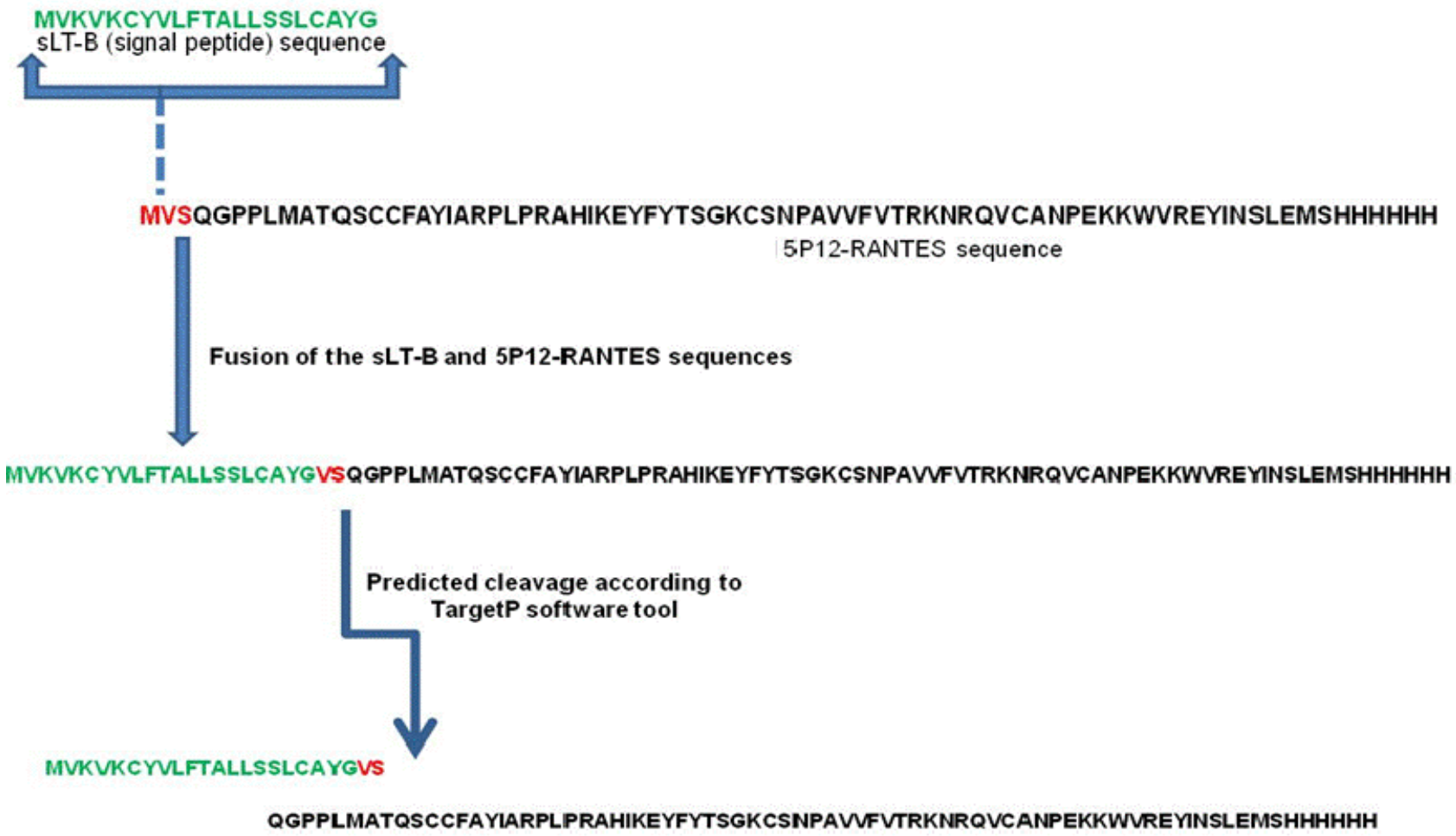

Figure 12 A schematic representations of sLT-B and 5P12 fusion and their predicted cleavage. Firstly, the C-terminal end of the sLT-B sequence fuses with the N-terminal end of 5P12 sequence. Predicted cleavage of the sLT-B signal peptide carries along the first two amino acids of 5P12 sequences.

The MagnICON vector system *per se* is divided into two major complementary DNA (cDNA) modules, a 5´ module which contains the viral RNA dependent RNA polymerase gene whereas a 3´ module harbours the gene of interest as well as the integrase module. A 5´ module carries organelle targeting signal, which fuses in frame with the transgene after recombination and nuclear processing (Marillonnet *et al.,* 2004). In this study, we used the 3´ modules; pICHs5P12 module carrying the plant codon-optimised 5P12 gene fused to sLT-B signal peptide; pICH7410 module carrying green fluorescent protein (GFP) as a reporter to confirm the efficiency of infiltration; pICH11599 module as a negative control and the 5´ modules targeting the cytosol (pICH17388) and apoplast (pICH17620) for transient expression (Figure 13). A strategy for expression of proteins in plants using the MagnICON vector system targeting the apoplast is illustrated in Appendix B. Route A resulted in low yields whereas better yields were obtained when route B was followed. Hence the expression levels reported in this study were based on route B.

The designed pTRA constructs used in this study are depicted and described in Figure 14. Also, a strategy for protein expression using pTRA vector system is demonstrated in Appendix C. Again, low yields were obtained when route A was followed and therefore, expression yields based on route B were reported.

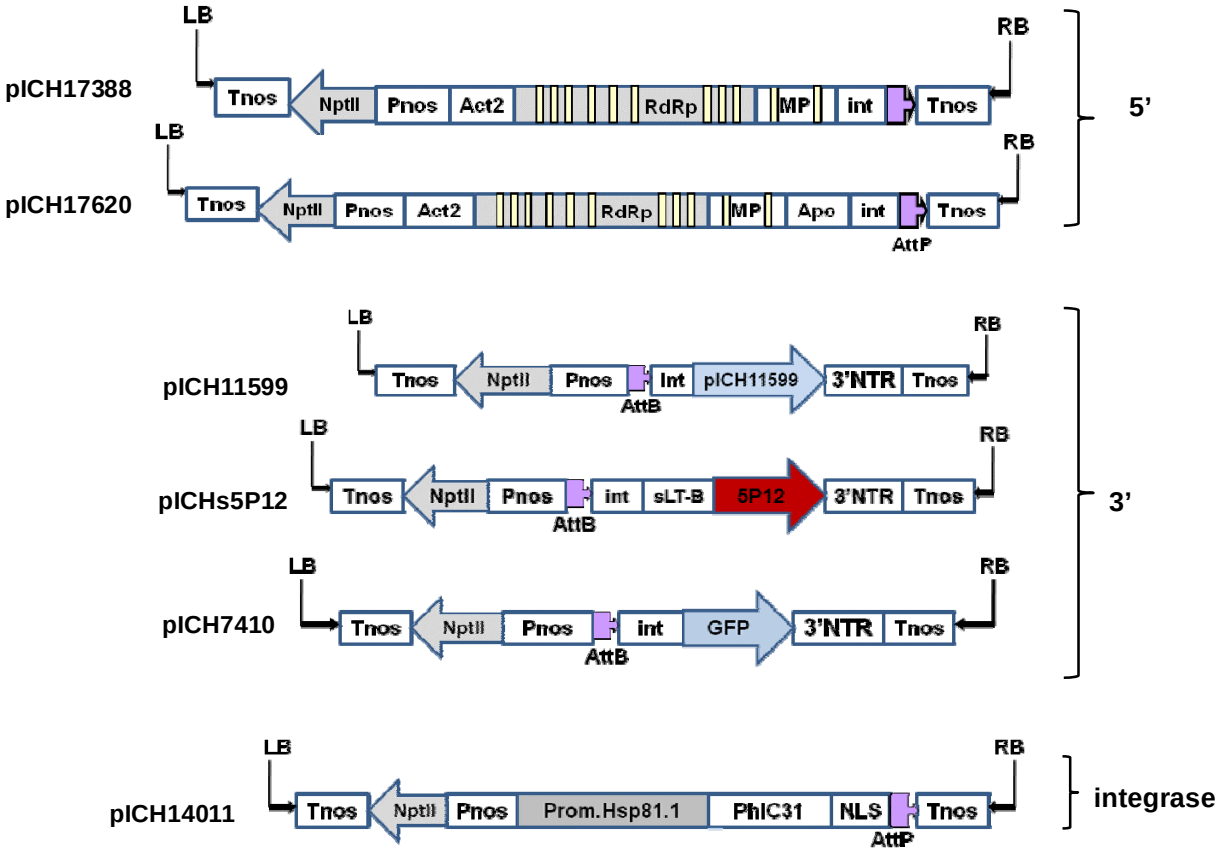

Figure 13 A schematic representations of the T-DNA regions of the MagnICON system used and generated in this study.There are three TMV-based provector modules thus; 1, 5´ modules - pICH17388 (cytosol targeting) and pICH17620 (apoplast targeting); 2, 3´ modules – pICHs5P12 (cloned vector), pICH7410 (for expression of green fluorescent protein (GFP)) and pICH11599; 3, pICH14011 (Streptomyces phage PhiC31 integrase); LB, binary left border of the T-DNA region; RB, binary right border; RdRP, RNA-dependent RNA Polymerase to catalyse replication of RNA from a RNA template; Prom.Hsp81.1, heat shock protein 81.1 promoter from Arabidopsis; Tnos, nopaline synthase terminator; Pnos, nopaline synthase promoter; MP, movement protein; Act2, actin 2 promoter from Arabidopsis; AttB and AttP, sites for PhiC31 integrase recombination; int, intron; Apo, Calreticulin apoplast targeting signal; NLS, nuclear localization signal; NptII, neomycin phosphotransferase II gene; 3´-NTR, 3´ non-translated region; sLT-B signal peptide (plant-optimised synthetic gene encoding the *E. coli* heat labile enterotoxin B subunit (LT-B)) (Mason *et al*., 1998; Giritch *et al*. , 2006).

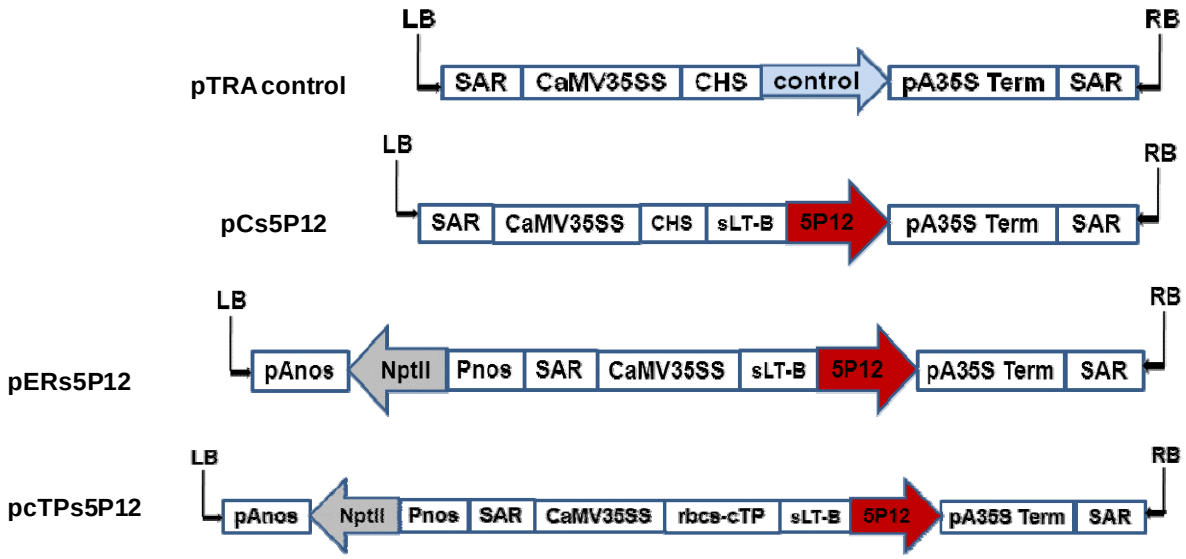

Figure 14 Schematic diagrams of the pTRA constructs generated and used in this study. The pCs5P12 targets the cytosol; pERs5P12 targets the ER; pcTPs5P12 targets the chloroplast. CaMV35SS, cauliflower mosaic virus promoter (P35SS); CHS, chalcone synthase 5´ untranslated region; pA35S, CaMV 35 polyadenylation signal for transgene expression; SAR, tobacco Rb7 scaffold attachment region; LB, left border for T-DNA integration; RB, right border for T-DNA integration; NptII, neomycin phosphotransferase II gene; Pnos, nopaline synthase promoter; pAnos, polyadenylation signal of the nopaline synthase gene; sLT-B, synthetic signal peptide encoding the *E. coli* heat labile enterotoxin B subunit (LT-B) (Mason *et al*., 1998; Maclean *et al*., 2007).

3.2.2 Effect of subcellular targeting of 5P12 on accumulation and cell viability

3.2.2.1 Phenotypes of infiltrated leaves

To test the effect of 5P12 gene in *N. benthamiana* on plant metabolism and phenotype, plant leaves were transfected with the five constructs either by leaf injection or vacuum infiltration and then visualised for obvious phenotypic differences. For the MagnICON vector module, the GFP control treatment showed no expression at 0 dpi, but expression was observed as from 3 dpi and increased till 9 dpi (Figure 15). The green fluorescence peaked at 9 dpi as expected with the

MagnICON vectors where the virus usually has an inherent gene silencing suppressor. Chlorosis which appeared as white circles was observed on the needle injected leaves from 0-9 dpi. This was as a result of a syringe contact with the leaves during agroinfiltration. Interestingly, the vacuum infiltrated leaves showed more GFP expression per day post infiltration than needle injected leaves suggesting that the vacuum managed to infuse more cells and better coverage of the *Agrobacteria* to plant cells. No chlorosis was observed for these leaves over the 9 day observation period.

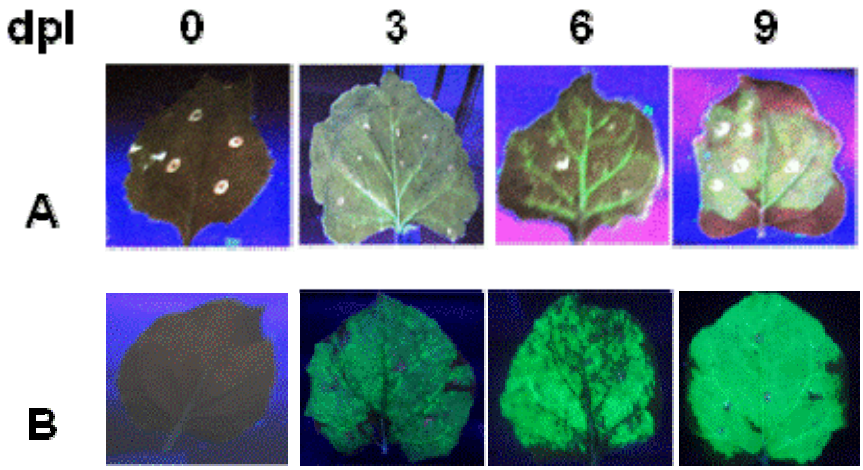

Figure 15 Time course visualisation of green fluorescent protein expression in *N.benthamiana*. A, needle injected leaves; B, vacuum infiltrated leaves

When the MagnICON vectors carrying 5P12 were visualised, all treatment leaves including the negative control appeared normal at 0 dpi. However, the treatment leaves showed increasing severity of stress and chlorosis from 3-9 dpi (Figure 16). A similar observation was made for the pTRA constructs (Figure 17). When put together, the results suggest that agroinfiltration of *N. benthamiana* leaves with the vector carrying 5P12 affects the physiology of the plant and possibly tissue viability.

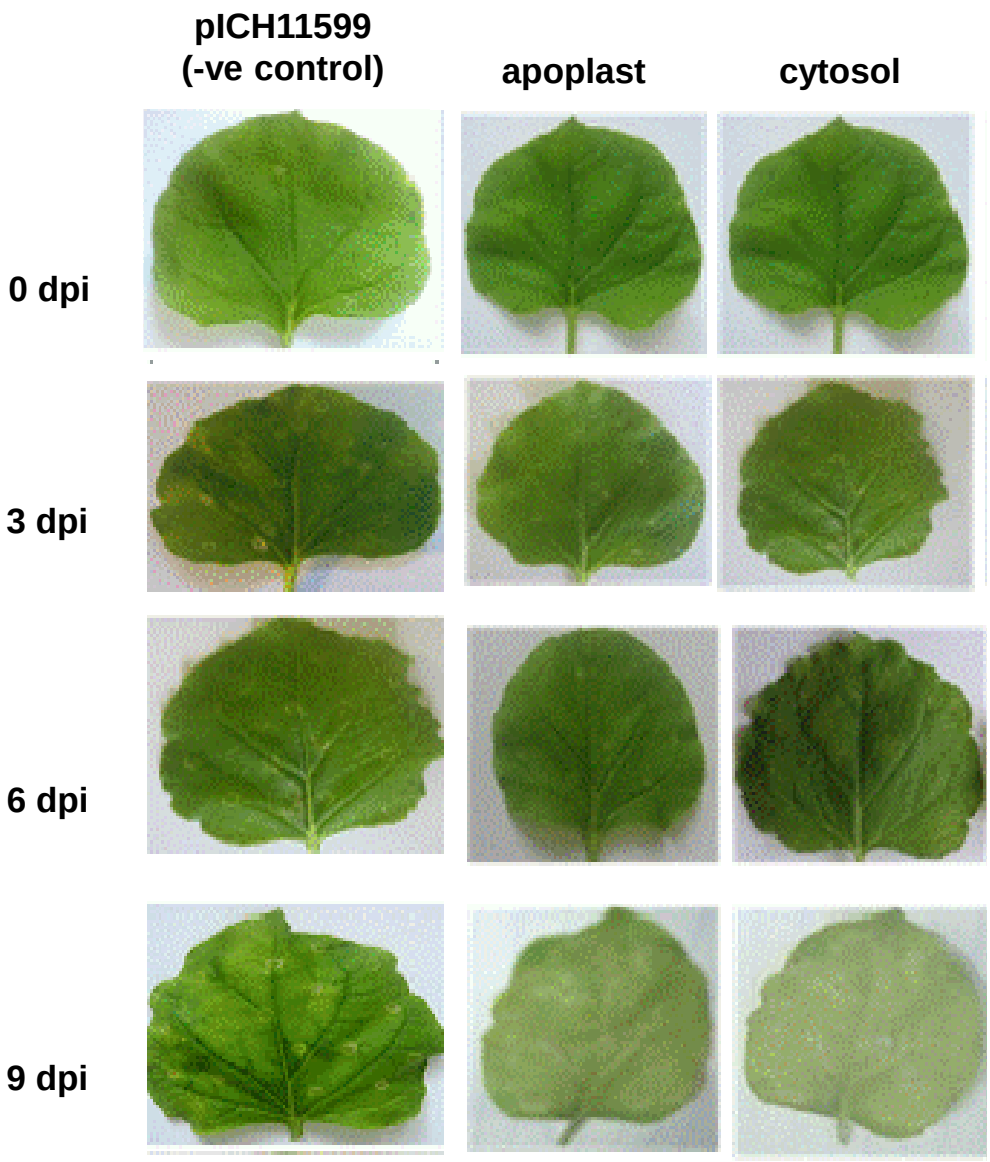

Figure 16 Phenotypes of *N. benthamiana* leaves agroinfiltrated with MagnICON constructs. The three MagnICON constructs used directed targeting to the apoplast, cytosol and the negative control.

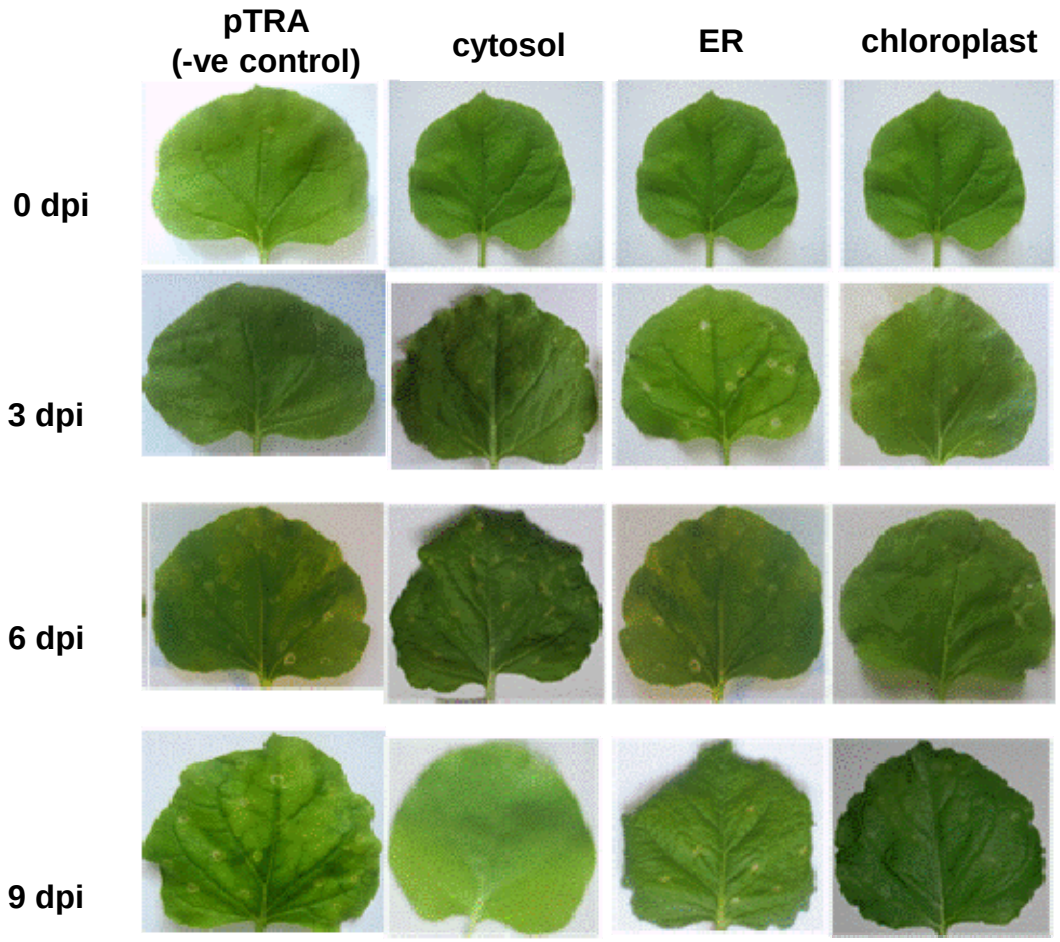

Figure 17 Phenotypes of *N. benthamiana* leaves agroinfiltrated with pTRA constructs. The four pTRA constructs used directed targeting to the chloroplast, cytosol, ER and the negative control.

3.2.2.2 Effect of infiltration method on protein yield

It was considered that the protein yield depends on several factors including the construct, density of *Agrobacterium* strain, method of delivery and the physiology of the plant (age and plant husbandry).

Although needle injection and vacuum infiltration were the two methods explored in this study for transient expression of 5P12 in various subcellular compartments, anti-RANTES ELISA was used to quantify the expression levels using chemically synthesized 5P12-RANTES as a positive control. The samples were measured in

triplicate and the values at each concentration were averaged. The standard curve for the ELISA is shown in Figure 18. A good correlation between the mean absorbance and mean concentration was determined to be y = 0.0354x + 0.1548 where R^2= 0.9555 showed good reproducibility of the assay. The linear range of the ELISA was 0.078-10 ng/ml. Having established the standard curve for the ELISA, the next objective was to determine the optimal harvest time for the leaf samples.

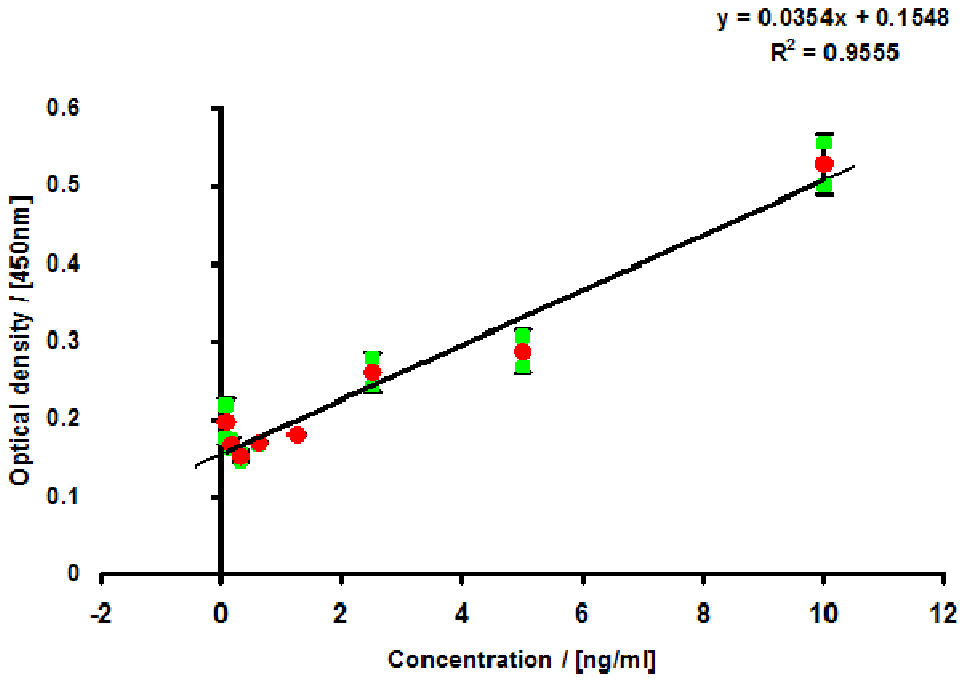

Figure 18 A typical standard curve drawn by the plate reader and linear fit values for the ELISA using chemically synthesised 5P12-RANTES as a standard.

3.2.2.3 Needle injection

The *N. benthamiana* leaves were successfully infiltrated by needle injection. Biomass was harvested on a time-course of 3, 6 and 9 dpi and subsequently, 5P12 levels were measured by ELISA at each time point. Various densities of *Agrobacterium* cells were explored using the MagnICON constructs as depicted in Figure 19. This was done to optimise the expression levels of 5P12. However, based on different expression levels observed (Figures 15A and B), the optical density of

0.4 resulted in the optimum expression level of 5P12 in *N. benthamiana* leaves. Hence it was used for subsequent experiments.

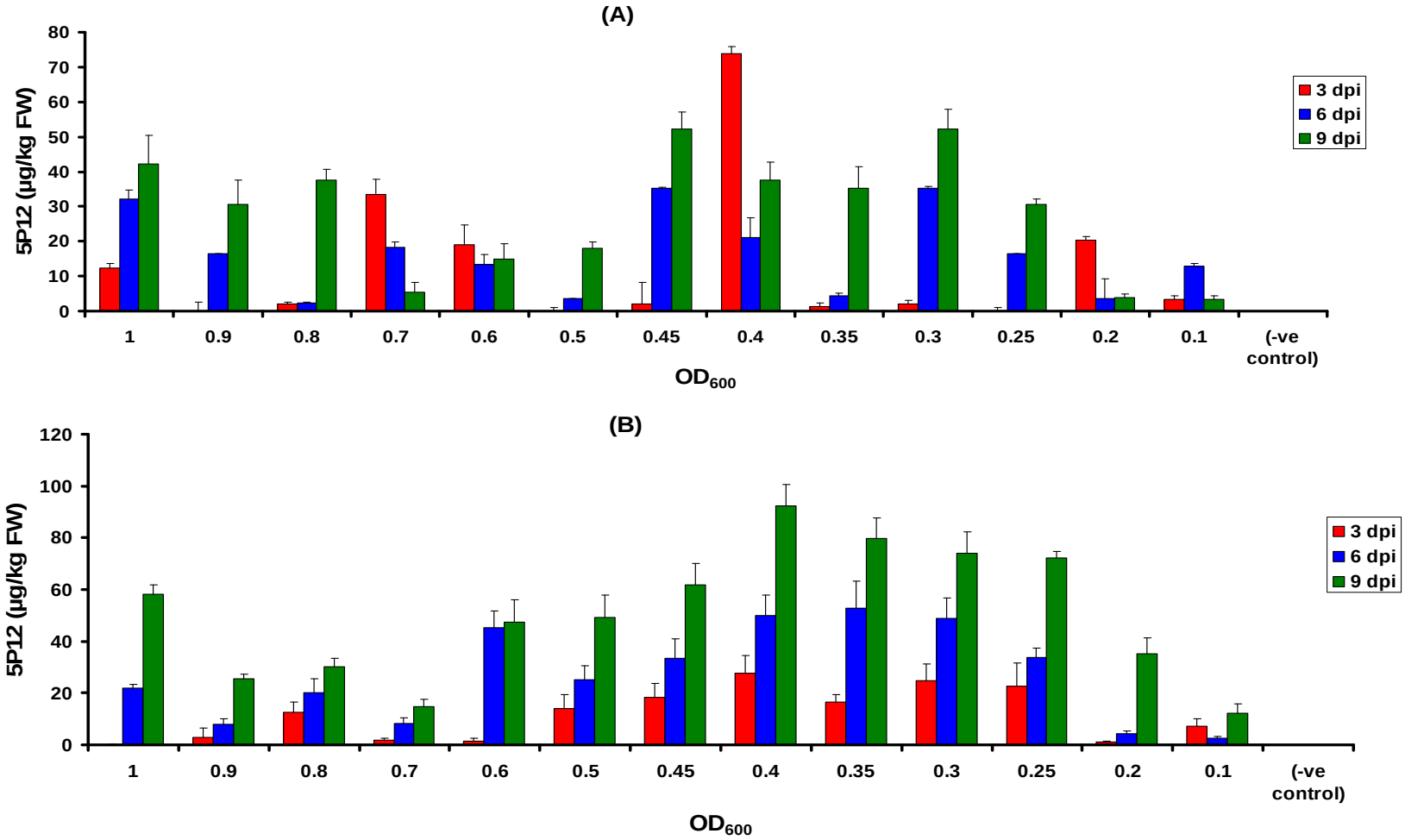

Figure 19 Determination of the optimum culture OD for transient expression. A, apoplast-targeted leaf samples; B, cytosol-targeted leaf samples. The error bars represents the standard deviations of three replicates.

For the pTRA constructs, the pattern of expression over the 9 day observation period was also variable (Figure 20A). There was no 5P12 detected in the negative control as expected. All compartments showed a decrease in protein level over time from 0.147-0.045 µg/kg FW due to protein-limiting factors such as gene silencing and proteases. This was expected in the cytosol as it is known for the incorrect synthesis and misfolding of proteins (Desai *et al*., 2010). In contrast, this was not expected in the ER and chloroplast because both have little protease activities (Bally *et al*., 2009; Avesani *et al*., 2010; Obembe *et al*., 2011). Moreover, the highest expression level of 0.147 µg/kg FW was obtained in the chloroplast as expected because it was reported that the chloroplast is able to synthesise and accumulate high levels of foreign proteins such as 5P12 that do not require post translational modification (Bally *et al*., 2009).

With the MagnICON constructs, 5P12 was also undetectable in the negative control (Figure 20B). The yields in the cytosol showed a gradual increase from 3-9 dpi resulting in the highest level of 92.5 µg/kg FW to confirm stability of 5P12 in that environment. Low yields were obtained in the apoplastic compartment. This was not expected due to less hydrolytic activities by proteases in the apoplast than in the cytosol.

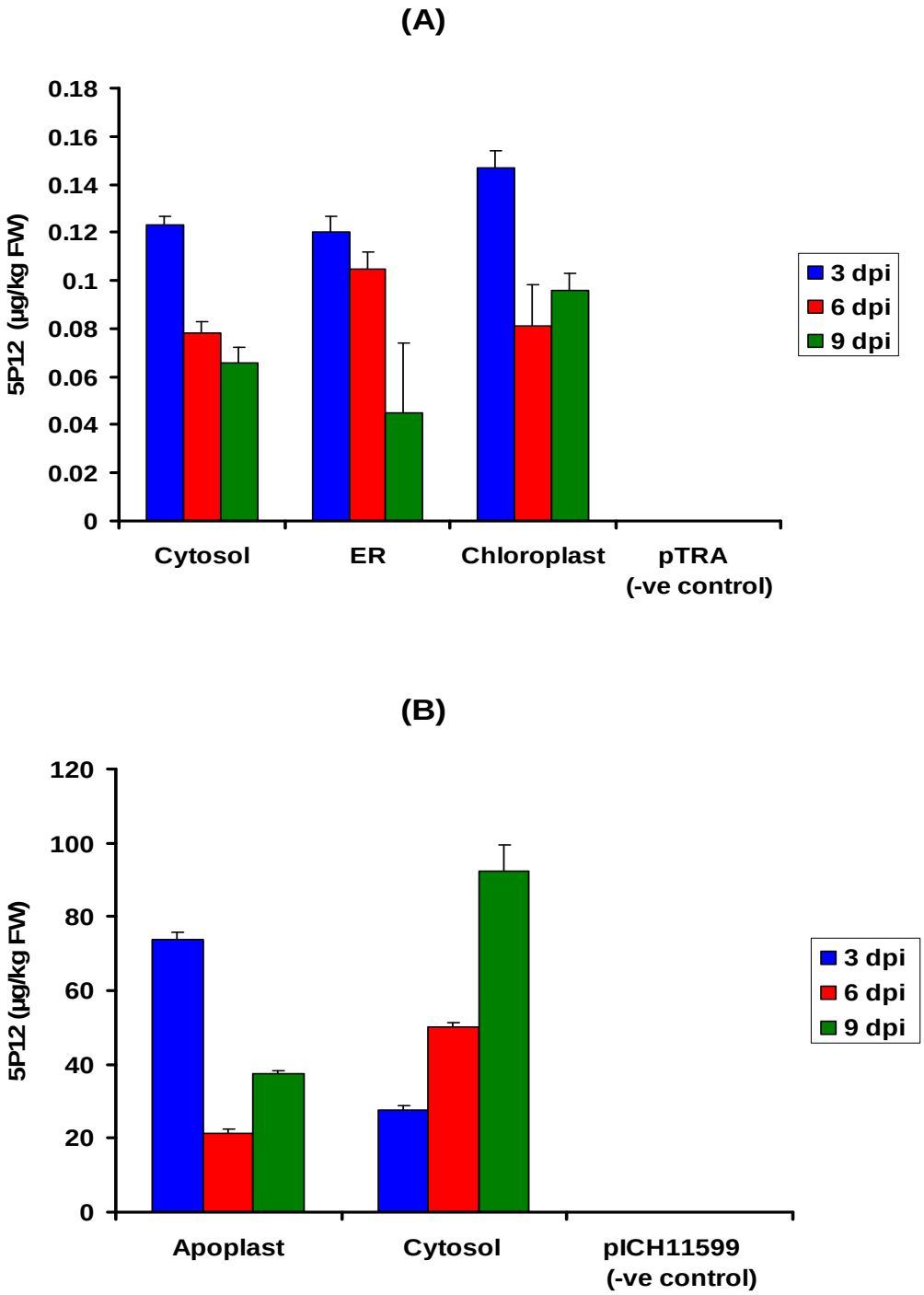

Figure 20 ELISA analysis of the protein extracts of the needle injected *N. benthamiana* leaves at different days post infiltration (dpi). Samples were extracted with PBS-T buffer and quantified by ELISA. A, pTRA-based leaf samples; B, MagnICON-based leaf samples. The error bars represent the standard deviations of three replicates.

3.2.2.4 Vacuum infiltration

With the pTRA constructs, the pattern of expression over 9 day observation period was variable (Figure 21A). 5P12 was also not detected in the negative control as expected. However, the cytosol and ER compartments showed a decreasing trend in 5P12 level from 0.63 to 0.17µg/kg FW. Conversely, the chloroplast compartment showed a gradual increase from 3-9 dpi resulting in the highest yield of 0.49 µg/kg FW. Although the yield was less than that obtained in the cytosol compartment, the observed trend from 3-9 dpi was expected because the chloroplast has an extraordinary capacity to synthesize and accumulate high levels of foreign proteins and has also been used to target proteins that do not require posttranslational modification (Bally *et al*., 2009; Obembe *et al*., 2011).

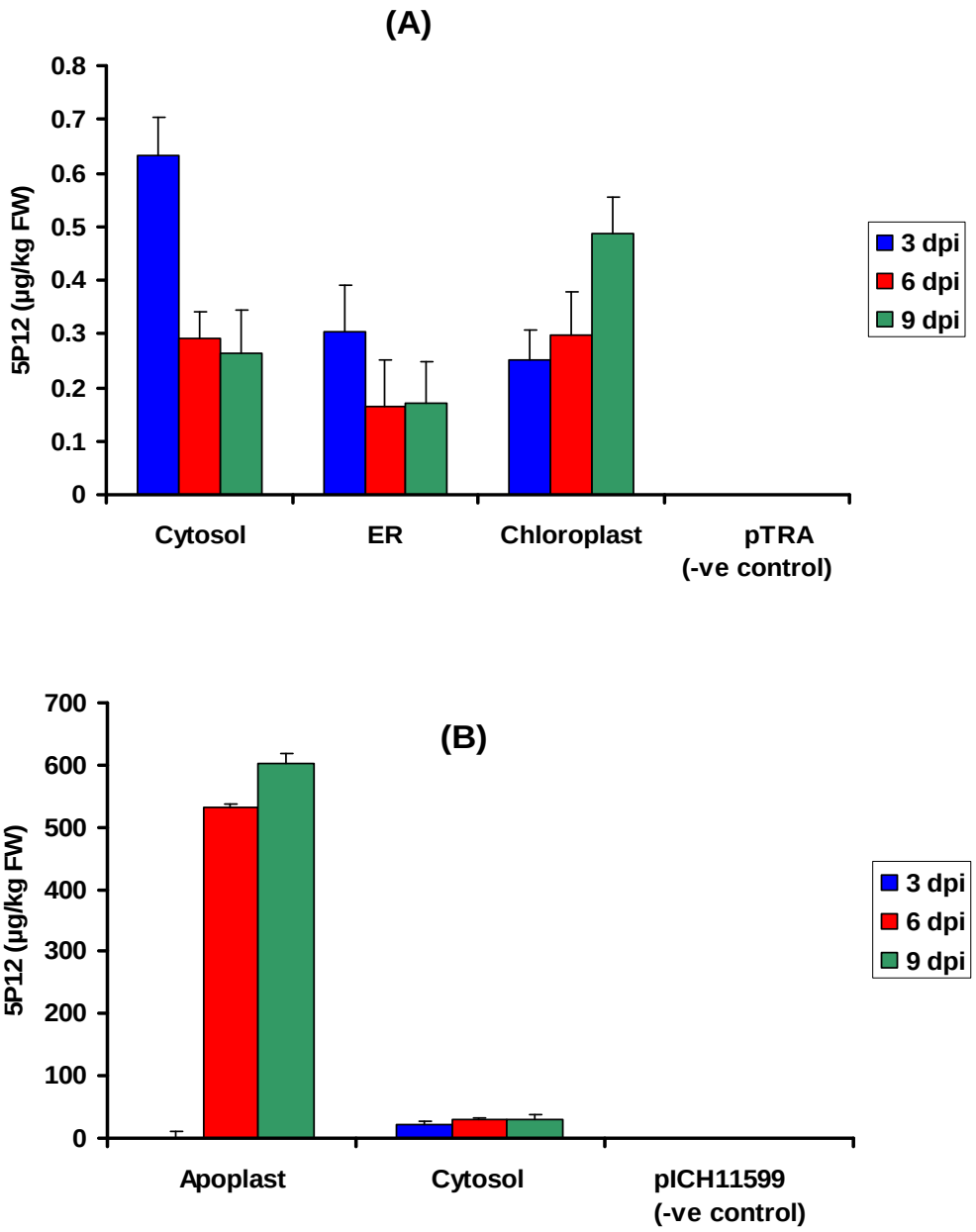

Figure 21 ELISA analysis of the protein extracts of the vacuum infiltrated *N. benthamiana* leaves at different days post infiltration. Samples were extracted using PBS-T buffer. A, pTRA-based leaf samples; B, MagnICON-based leaf samples. The error bars represent the standard deviations of three replicates.

There are several possible reasons for the variable expression levels between the ER, cytosol and the chloroplast. One can speculate that the trend observed with the cytosol and ER may be explained by the gene silencing effect or normal protease degradation. The post-transcriptional gene silencing where sequence specific

degradation of RNA transcripts occurs due to the presence of homologous interfering double stranded RNA (dsRNA) is known to reduce protein level in ectopic expression of specific proteins (Desai *et al*., 2010).

Nonetheless, proteins may be degraded *in planta* immediately after biosynthesis or *ex planta* during extraction (Goulet *et al*., 2010). Proteases degrade the heterologous proteins that are incorrectly synthesized or not properly folded in plants (Desai *et al*., 2010). Furthermore, stability of the recombinant proteins in the foreign cellular environment depends upon the number of protease-susceptible sites for peptide bond hydrolysis by endogenous proteases (Goulet *et al*., 2010). It is also possible that the proteins were degraded in the cytosol due to incorrect/incomplete posttranslational modification which triggers protein degradation (Kamenarova *et al*., 2005). This is contrary to the literature which suggests ER as the best compartment for recombinant protein expression due to little peptide hydrolytic activity by endogenous proteases (Avesani *et al*., 2010). Moreover, it seemed that the best time to harvest was 3 dpi for the cytosol and ER constructs.

In view of the fact that *N*-glycosylation is an important posttranslational modification in which sugars are added onto proteins for protein folding, activity and protection against denaturation, plant systems add xylose and 1,3-fucose whereas mammalian systems add galactose residues with sialic acids (Coku, 2007). Fortunately, RANTES does not have sites for *N*-linked glycosylation (Schall *et al*., 2009). In spite of the absence of *N*-linked glycosylation, folding was considered appropriate in the chloroplast as accumulation and stability of 5P12 was observed over the 9 days period. Moreover the stroma, which is the area inside the chloroplast where reactions take place and starches (sugars) are created, has been reported to allow correct formation of disulfide bonds (Bally *et al*., 2008).

When the MagnICON system was considered, the 5P12 targeted to the apoplast was barely detectable at 3 dpi (Figure 21B). However, there was a dramatic peak at 6 dpi (533 µg/kg FW) which increased at 9 dpi (603 µg/kg FW (~0.024% total soluble protein (TSP)). The maximum yield of 5P12 in the cytosol was about 50 µg/kg FW at 3 dpi and remained at that level over the observation period. When comparing the protein accumulation at 9 dpi of the two subcellular compartments, a 20-fold protein

expression was measured in the apoplast. This was expected because most plant-derived recombinant proteins have been reported to show expression levels in the cytosol of up to 100-fold less than in the apoplast (Obembe *et al*., 2011). Moreover, low expression levels in the cytosol are associated with the presence of proteases (Avesani *et al*., 2010).

3.2.3 SDS-PAGE and immunoblot analysis of plant-made 5P12

Having established the expression levels of 5P12 in leaf tissues, we used SDS-PAGE and immunoblot analysis to determine the expression and molecular weight of the recombinant 5P12. The concentration of transiently expressed 5P12 was too low to be clearly seen on Coomassie stained SDS-PAGE, hence protein samples were concentrated 20-50 folds using the TCA method prior to loading and about 80-100 µg of crude samples were loaded. After separation under reducing conditions, protein profiling of the recombinant 5P12 extract vs the 5P12-RANTES standard was conducted (Figure 22). Interestingly, the 5P12-RANTES standard migrated at approximately 11 kDa (lane 1) instead of 7.9 kDa relative to the used protein marker (lane M). The reason for this anomalous migration could perhaps be the high degree of polymerisation of the self-prepared 15% gel used for SDS-PAGE. Otherwise when using the commercial precast 12.5% and Tris-tricine gels, 5P12-RANTES migration was usually around 7.9 kDa. Although the crude extracts were concentrated, 5P12 still remained undetectable. This is not surprising because the detection limit of the 5P12-RANTES standard is 4 µg/lane in Coomassie stained SDS-PAGE. For the negative control, no bands were expected because it does not carry the 5P12 gene.

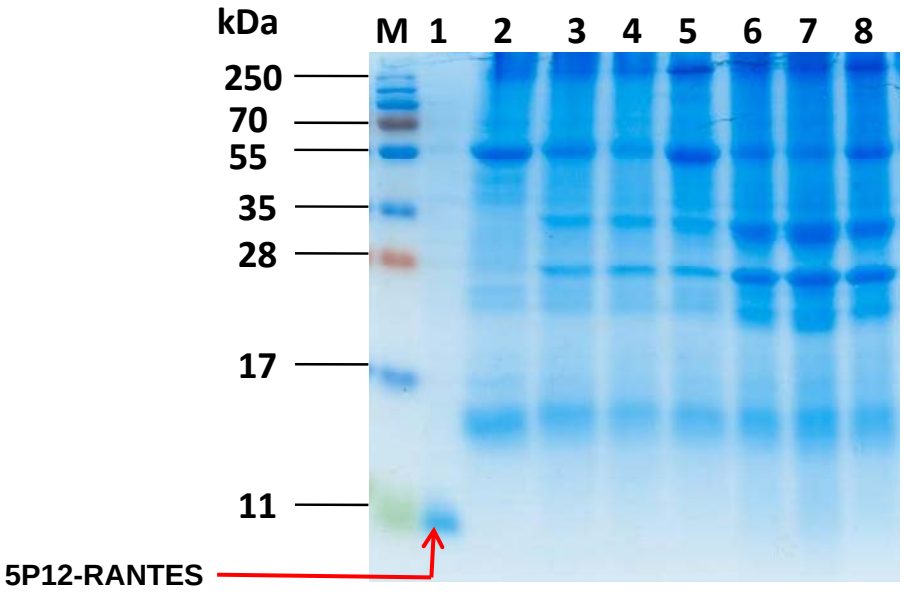

Figure 22 Crude protein extract profiles obtained by 15% SDS-PAGE analysis. M, Molecular marker (SM1811, Fermentas); 1, positive control (5P12-RANTES); 2, pICH11599 crude sample (negative control); 3, apoplastic proteins at 3 dpi; 4, apoplastic proteins at 6 dpi; 5, apoplastic proteins at 9 dpi; 6, cytosolic proteins at 3 dpi; 7, cytosolic proteins at 6 dpi; 8, cytosolic proteins at 9 dpi.

Unfortunately, 5P12 in the crude plant extract was also undetectable by western blot analysis. The detection limit for RANTES in western blot is approximately 1 ng/lane under non-reducing and reducing conditions (www.RnDSystems.com). We then purified the crude extract through Protino Nickel affinity columns and only managed to obtain a single band of approximately 11 kDa (Figure 23) after TCA precipitation. This confirmed that 5P12 was successfully expressed in *N. benthamiana* leaves.

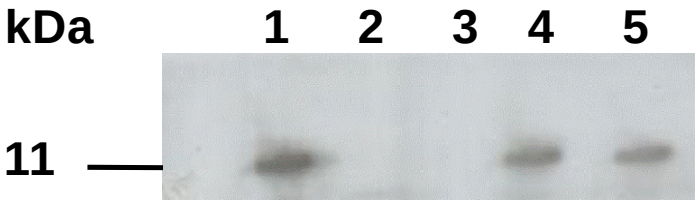

Figure 23 Western blot analysis of purified recombinant 5P12. 1, 10 ng of positive control (5P12-RANTES); 2, 60-80 ng of negative control crude sample; 3, 60-80 ng of pooled wash buffer (apoplastic proteins); 4, 50 ng of elute (apoplastic proteins); 5, 50 ng of elute (cytosolic proteins).

3.2.4 *In vitro* cytotoxicity effects of plant-made 5P12

To test the toxicity of the plant-made 5P12, we conducted the cytotoxicity assay against HIV-1 pseudovirions. TCA precipitated samples could not be used for efficacy studies because precipitated proteins lose activity. We then used partially purified samples by size exclusion chromatography with two cut-off molecular sizes: large proteins (>10 kDa) and small proteins (<10 kDa). Considering the expected potency of 5P12, the hypothesis was that there might be enough protein to show efficiency. For cytotoxicity, MTS–based assay was conducted and the cell viability was measured with etoposide as positive control.

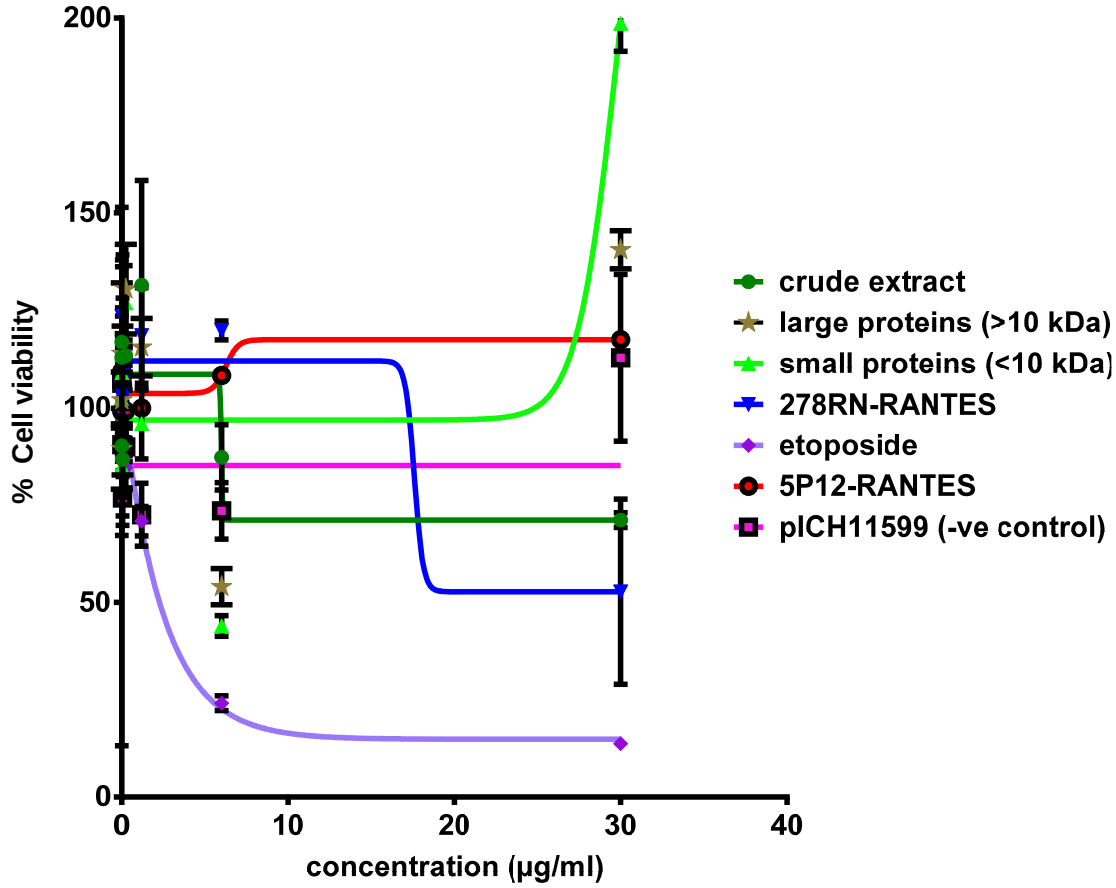

Figure 24 MTS-based cell viability of plant protein extracts (crude extract, large and small proteins), 5P12-RANTES, *E. coli*-based 278RN-RANTES, etoposide as a positive control, pICH11599 as a negative control. TZM-bl cells were used for analysis. Different concentrations were recorded and the error bars represents the standard deviations of three replicates.

Figure 24 shows the cytotoxicity assay based on the MTS reduction into formazan product. Since formazan product is directly proportional to the number of living cells, cell viability decreased with an increase in etoposide concentration. This is because etoposide kills the cells by stabilising a cleavage complex which is a transient intermediate during catalysis by topoisomerase II (Baldwin and Osheroff, 2005). Topoisomerase II is known to regulate DNA under and overwinding and removes knots and tangles from the genetic material by generating transient double-stranded breaks in the sugar-phosphate backbone of the double helix (McClendon and Osheroff, 2007). Moreover, accumulation of cleavage complexes leads to the generation of permanent DNA strand breaks, and if these breaks overwhelm the cell, they initiate death pathway or apoptosis in cancer cells (Baldwin and Osheroff, 2005). However, all the plant extracts were not cytotoxic towards TZM-bl cells when tested on concentrations up to 30 µg/ml. The partially purified small proteins showed the highest cell viability at the highest concentration tested (small proteins > large proteins > 5P12-RANTES standard > negative control > crude extract > *E. coli*-made 278RN-RANTES). Although it may be practically not possible, we expected to see all tested samples excluding etoposide and 278RN-RANTES, to show 100% cell viability. It was obviously expected that the negative control may show higher cell viability than 278RN-RANTES because generally, the *E. coli*-made products usually result in incorrect folding that may interfere with cell viability.

3.2.5 *In vitro* efficacy effects of plant-made 5P12

We then evaluated the activity of the plant extracts to HIV-1 entry using a pseudovirion assay based on a Luciferase Reporter Gene in TZM-bl cells. We wanted to find out as to whether the plant extracts have an effect in blocking the CCR5 and CXCR4 receptors that the HIV-1 uses to enter the cells. However, *N. benthamiana* leaves have lectins which are involved in various biological recognition phenomena (Vandenborre *et al*., 2008). They are able to recognise certain carbohydrates and some have been reported to be expressed after well-defined stress conditions such as drought, salt stress or plant hormone treatment (Lannoo *et al*., 2007). For instance, *Nicotiana tabacum* lectin (nictaba) is expressed in tobacco leaves after being induced with jasmonates and also upon insect herbivory

(Vandenborre *et al*., 2008). Therefore, to circumvent false activities as a results of the presence of some naturally occurring broad-specificity components in *N. benthamiana* leaves, IC_{50} values >0.50 µg/ml were considered inactive in the assay. The positive control samples, T20 (enfuvirtide) and chemically synthesised 5P12-RANTES showed neutralisation activity against ZM53 pseudovirions with IC_{50} of 0.081 µg/ml and <0.0006 µg/ml respectively (Table 4). This was expected because T20 is a 36-amino acid peptide that has been synthesised based on the sequence of the heptad repeat 2 (HR-2) of gp41 (Copeland, 2006; Matos *et al*., 2010). Thus T20 competes for binding to the hydrophobic grooves of HR-1 to inhibit HIV-1 fusion to the cell membrane (Ray *et al*., 2009). On the other hand, 5P12-RANTES is a potent CCR5 blocking agent that inhibits HIV-1 entry into the cells (Nedellec *et al*., 2011).

Table 4 Summary of IC_{50} values of various samples tested against the ZM53 and HXB pseudovirions

Sample identification	**ZM53 (CCR5 virus) IC_{50} (µg/ml)**	**HXB2 (CXCR4 virus) IC_{50} (µg/ml)**
Crude extract	0.59	2.05
Large proteins (>10 kDa)	0.09	0.02
Small proteins (<10 kDa)	0.06	0.02
5P12-RANTES standard	<0.0006	NT
278RN-RANTES	20-100	>100
T20 (entry/fusion inhibitor)	0.081	0.04459
pICH11599 (-ve control)	1.66	0.56

Note: NT denotes not tested

Conversely, the commercial *E. coli* made RANTES (278RN-RANTES) was unable to neutralise all pseudovirions. This may be due to the possible misfolding of eukaryotic proteins upon production in bacteria (Siller *et al*., 2010). Or its N-terminal end was

not modified the same as 5P12-RANTES to give much higher antiviral potency (Hartley *et al*., 2004). Nevertheless, the negative control and the crude extract were unable to neutralise ZM53 and HXB2 pseudovirions, thus the negative control was about 12-20 folds less potent whereas the crude extract was 7-45 folds less potent when compared to T20. This may be an indication that the material expressed in plants was either (i), correctly cleaved and folded, but at too low a concentration to provide a detectable activity, and/or (ii) that the material was incorrectly cleaved/folded.

In contrast, the purified fractions showed enriched antiviral potency. The enrichment increased the concentration of other plant-derived anti-HIV compound(s) present in the extract (e.g., lectins) that act on both CCR5-tropic and CXCR4-tropic viruses.

3.2.6 Specificity of plant-made 5P12

We also evaluated the specificity of the antiviral activity by testing neutralisation against VSV-G pseudovirions which have an HIV-1 backbone genome and VSV-G envelope. This was done based on the fact that viruses have evolved a variety of mechanisms for entry into the host cells, including endocytosis by which components are taken up by invagination of the plasma membrane to form vesicles that enclose these materials (Oh *et al*., 2010). In this case, vesicular stomatitis virus is able to enter cells via clathrin-mediated endocytosis and be subjected to the acidic endosomal pH, resulting in fusion of the viral and endosomal membranes and release of the viral genome into the cytosol (Wang *et al*., 2008). In this assay, two controls were used i.e., tenofovir (reverse transcriptase inhibitor) should show activity against VSV-G, while T20 (entry inhibitor) should not show any viral activity at all. The 5P12-RANTES protein is also an entry inhibitor and expected to behave the same as the T20. Figure 25 show the neutralisation activity of VSV-G by the different samples.

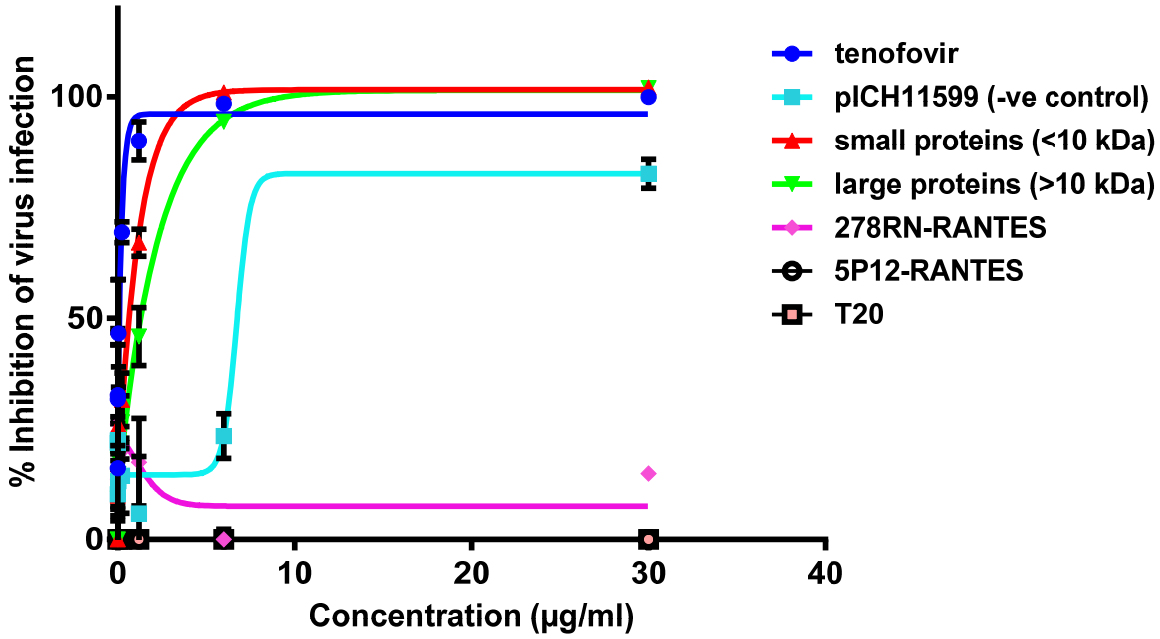

Figure 25 HIV-1 inhibition of the plant protein extracts (large and small proteins), *E. coli*-based 278RN-RANTES, tenofovir (positive control) and three negative controls (5P12-RANTES, T20 and pICH11599) tested against VSV-G pseudovirion. The error bars represents the standard deviations of three replicates

The assay results showed that tenofovir (IC_{50} = 0.463 µg/ml) neutralised VSV-G pseudovirions as expected since it acts downstream of viral entry. 5P12-RANTES standard and T20 showed no inhibition of the VSV-G pseudovirions as expected. This is because both do not act downstream of viral entry, thus T20 is an HIV-1 fusion inhibitor whereas 5P12-RANTES blocks entry by binding to the CCR5 receptors. The 278RN-RANTES was unable to neutralise the VSV-G pseudovirions because it is inactive and has been typically used as a reagent. The negative control (pICH11599), small and large proteins were able to neutralise the pseudovirions. This may be due to the presence of other plant-derived anti-HIV compounds such as lectins that act on both CCR5-tropic and CXCR4-tropic viruses.

3.3 Summary and conclusion

It is imperative that a cost effective and scalable production system is used for producing proteins of high public health importance such as microbicides. This is the only way of making sure that the products can make a difference in managing the HIV pandemic especially in areas where the disease is a burden. In the current study, *N. benthamiana*, a model plant species was investigated as a potential host for ectopic expression of recombinant 5P12, a promising candidate for a microbicide. Plant-based production systems are known to be comparatively low cost and scalable for producing proteins compared to yeast, bacterial and mammalian cells (Chen and Liu, 2011). Moreover, heterologous expression of chemokines is relatively complicated. With this in mind, the feasibility of expressing 5P12 in plants was tested by investigating the effect of the transgenes on plant phenotype, influence of the agroinfiltration method and subcellular targeting on the accumulation of the protein. Subsequently, the plant-made 5P12 protein was partially purified and tested for biological activity as an HIV entry inhibitor using HIV-1 pseudovirus neutralisation assay.

The designed constructs were made to target the 5P12 to four subcellular locations i.e., apoplast, cytosol, chloroplast and ER to assess accumulation and stability. The constructs and infiltration methods explored also influenced the yields, thus the vacuum infiltrated leaves resulted in 7-fold more 5P12 than the needle injected leaves when the MagnICON constructs were considered. Furthermore, the MagnICON constructs resulted in 1000-fold more 5P12 when compared to the pTRA-based vector module. In addition, the apoplast localised proteins gave the highest yields hence it was the most suitable compartment for better accumulation, stability and perhaps proper folding of 5P12.

Unfortunately, no plant-made 5P12 was detectable by Coomassie staining, possibly due to a low concentration. These results can be explained by the low expression level and the difficulty of separating the chemokine from the plant matrix. To overcome these drawbacks, we tried bulking up the plant material and purified using Fast Performance Liquid Chromatography (FPLC). This did not make any difference because eventually, we had to concentrate the dilute solution.

However, the chemokine was detectable by western blot and thus the identity and molecular weight (~11 kDa) was confirmed. The partially purified plant-made 5P12 was non-toxic to TZM-bl cells. Furthermore, the chemokine showed an antiviral activity against CCR5 and CXCR4 pseudovirions. Activity was also compared to the *E. coli*-made commercial sample (278RN-RANTES) which showed no activity as expected due to incorrect folding in the host. When put together, the results demonstrated that *N. benthamiana* can be used as a host for expression of therapeutic proteins including 5P12. This was based on the observation that plant-derived extracts showed no cytotoxicity in TZM-bl cells (Figure 24). Although the anti-HIV compounds found present in *N. benthamiana* boosted antiviral potency, 5P12 was expressed in the leaves and this was confirmed by western blot (Figure 23). Furthermore, the results also validated that N-terminal modification of the RANTES protein is essential in the antiviral potency. In conclusion, although *N. benthamiana* has been used to express various types of eukaryotic proteins giving better yields, it needs further optimisation to get economic yields for 5P12-RANTES.

CHAPTER 4

4. EVALUATION OF 5P12-RANTES ANALOGUE EXPRESSION IN *LYCOPERSICON ESCULENTUM*

4.1 Motivation

Heterologous expression of 5P12 in *N. benthamiana* leaves resulted in low yields hence we were prompted to explore other plant species considering the diverse agroinfiltration efficiency which has been observed in different host species. This diversity may perhaps be influenced by various factors such as the compactness of the tissue and bacteria-host compatibility (Orzaez *et al.,* 2006). Although *N. benthamiana* is the most popular host to carry out transient expression through agroinfiltration (Leckie and Stewart Jr, 2011), other non-food and food/feed plant species such as *Arabidopsis thaliana*, *Medicago sativum* and *Lycopersicon esculentum* have been demonstrated to be effective in delivering the novel recombinant proteins. Therefore, for the purpose of this study, *L. esculentum* was selected as a host for transient expression of 5P12 because its fruits have been proposed as biofactories for the production of oral vaccines and other immunotherapeutic proteins (Orzaez *et al*., 2006). In addition, Fischer *et al.* (2009) reported that tomato fruits can be grown in greenhouses for containment of the transgene.

Expression of the recombinant chemokines is generally a challenge, chemokines such as RANTES tend to occur in monomeric and dimeric forms of which the monomers are biologically active (Campanella *et al*., 2006; Nisius *et al*., 2008). Previous studies showed better expression yields of RANTES monomers in *Lactobacillus* species that is known to cause acidic environment for the recombinant chemokines (Secchi *et al*., 2009; Vangelista *et al*., 2010). Most chemokines would favour low pH to circumvent aggregation and tomato fruits could become good hosts due to their citric and malic acid contents (Benton-Jones, 2011). Perhaps tomato fruits may confirm the suspected 5P12 dimers contained in the large protein (>10 kDa) following size-exclusion chromatography as reported in the previous chapter.

We then used the MagnICON constructs for transient expression of 5P12 via the needle injection method which is referred to here as agroinjection. This was done on the grounds that MagnICON constructs resulted in 1000-fold more 5P12 than the pTRA-based constructs (refer to Figure 21, Chapter 3).

4.2 Results and discussion

Since tomato fruits have already been proposed as good hosts for expressing novel proteins (Jani *et al*., 2002), we then explored the use of the ripening tomato fruits to express 5P12. Four groups of tomato fruits of the same Floradade cultivar at different stages of ripening were selected and grouped according to the chlorophyll contents, thus; 100% for mature green (MG) stage; 80% for breaker (B) stage; 50% for pink (P) stage; 0% for ripe (R) stage. Initially, we investigated if the MagnICON constructs can be used effectively to express novel proteins. To establish this, tomato fruits were agroinjected with a GFP construct and observed for 9 days post infiltration. For transient expression of 5P12, the tomato fruits material were also harvested on a time course for a period of 9 days and analysed for expression by ELISA and SDS-PAGE (Coomassie stained and western blot) techniques.

4.2.1 Phenotypes of tomato fruits agroinjected with a GFP construct

All agroinjected tomato fruits at different developmental stages were harvested and analysed for GFP expression. A better view of GFP expression was observed in the MG tomato fruits hence they were sliced and visualised on a time course (Figure 26). The non-agroinjected tomato fruits were used as negative controls.

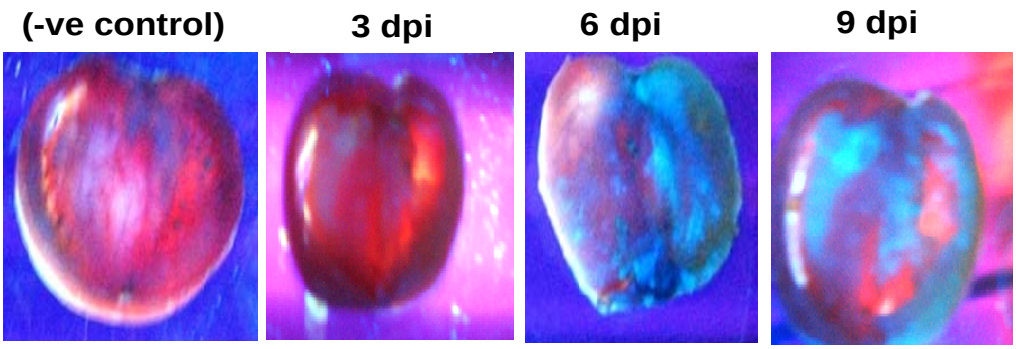

Figure 26 Time course UV visualisation of green fluorescent protein expression in *L. esculentum.* The green fluorescence around the placenta and pericarp at 6 and 9 dpi indicates expression of the GFP.

The negative control and the tomato fruit harvested at 3 dpi showed no fluorescence of the GFP. This was expected only in the negative control. However, any green fluorescence observed anywhere in the agroinjected tomato fruits signifies GFP expression. Green fluorescence was faint around the placenta and pericarp at 6 and 9 dpi. It was difficult to observe the GFP as a result of poor resolution, similar results have been reported in the literature. Orzaez *et al*. (2006) reported that GFP can only be visualised in the central lamella, placenta, pericarp and not in the locular tissues as illustrated at 9 dpi. Furthermore, intense GFP expression was observed in the placenta and less in the pericarp. In summary, better expression of therapeutic proteins may be expected at 9 dpi.

4.2.2 Phenotypes of whole tomato fruits agroinjected with 5P12 construct

The tomato fruits were also harvested over a period of 9 day time course. The crude proteins were extracted and analysed for 5P12 expression. Figure 27 shows phenotypes and the ripening process of all agroinjected tomato fruits. No remarkable change was observed from 3-9 dpi, except for the expected ripening process of the tomato.

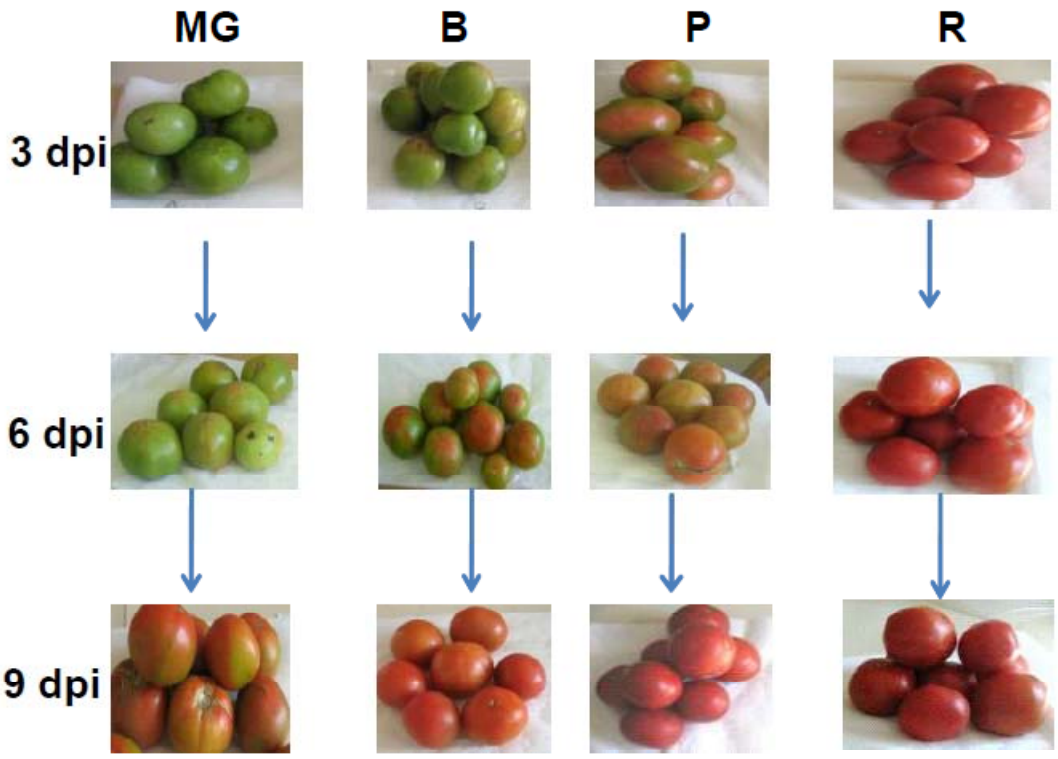

Figure 27 Time course visualisation to compare tomato fruits agroinjected with 5P12 construct at 3-9 days post infiltration. The acronyms used: MG, mature green tomato fruits; B, breaker tomato fruits; P, pink tomato fruits; R, ripe tomato fruits. MG, mature green tomato fruits; B, breaker tomato fruits; P, pink tomato fruits; R, ripe tomato fruits.

4.2.3 Total soluble protein (TSP) in tomato fruits

The Bradford assay (Bradford, 1976) was used to measure total soluble protein of the negative control and agroinjected tomato fruits. Protein samples were measured in triplicate and the values at each concentration were averaged. The relationship between the optical density and the averaged concentrations were calculated.

TSP levels of the negative controls and agroinjected tomato fruits were measured on a fresh weight (FW) basis. The negative controls showed no change in TSP from 3-9 dpi (Table 5). Moreover, there was no clear trend of protein accumulation from 3-9 dpi in the apoplast compartment. However, increasing protein levels were observed

at the R stage from 3-9 dpi. The B stage showed a drop in protein levels from 3-9 dpi. Regardless of the actual TSP levels, a similar trend was observed at the MG and P stages.

Table 5 Total soluble protein levels of agroinjected tomato fruits and negative control

Tomato fruits developmental stage	Day post infiltration (dpi)	Total soluble protein (TSP) (mg total protein /g FW) ± SD	
		Apoplast	**Cytosol**
MG	3	1.42±0.012	2.55±0.063
	6	3.24±0.015	5.48±0.050
	9	1.41±0.003	1.48±0.019
	3	4.45±0.036	4.20±0.010
B	6	2.70±0.010	4.62±0.032
	9	2.71±0.016	2.08±0.004
P	3	1.38±0.005	1.88±0.001
	6	4.50±0.004	2.68±0.010
	9	4.26±0.005	2.56±0.013
R	3	2.73±0.001	5.09±0.000
	6	3.10±0.001	4.89±0.002
	9	3.33±0.014	5.53±0.001
Negative control	3	4.84±0.013	4.84±0.013
	6	4.84±0.052	4.84±0.052
	9	4.84±0.018	4.84±0.018

Despite the actual TSP levels obtained in the cytosol compartment, the MG, B and P stages showed a similar trend, thus protein levels increased from 3-6 dpi and ultimately dropped from 6-9 dpi. This trend is true for the R stage cytosol, but not for the apoplast, with protein levels dropping from 3-6 dpi and then increasing from 6-9 dpi.

In summary, the decrease in TSP levels could be due to the presence of hydrolytic activities by proteases (Kamenarova *et al*., 2005; Avesani *et al*., 2010; Goulet *et al*., 2010). Although the TSP levels were low, the apoplast compartment of the R stage showed stability and better accumulation of proteins.

4.2.4 Total 5P12 expression in *L. esculentum*

5P12 expression yields were determined for each targeted subcellular compartment viz: apoplast and the cytosol, to check for stability and accumulation. The pH values of the lyophilised material were also measured per day post infiltration. The trends of 5P12 expression levels and pH value of all tomato fruits with cytosolic targeting is illustrated in Figure 28. There was no expression in the negative control as expected. However, the pH of all tomato fruits except the R stage decreased from 3-9 dpi. The expression levels except the R stage peaked at 6 dpi and almost the same levels were obtained at 3 and 9 dpi. Moreover, the best maturity stage for agroinjection was the B stage (21.24 µg/kg FW, pH 4.52) which accumulated almost double the levels of the other maturity stages (MG, P and R). In summary, an inverse correlation between the expression levels and the pH was observed except at 9 dpi of the MG, B and R stages.

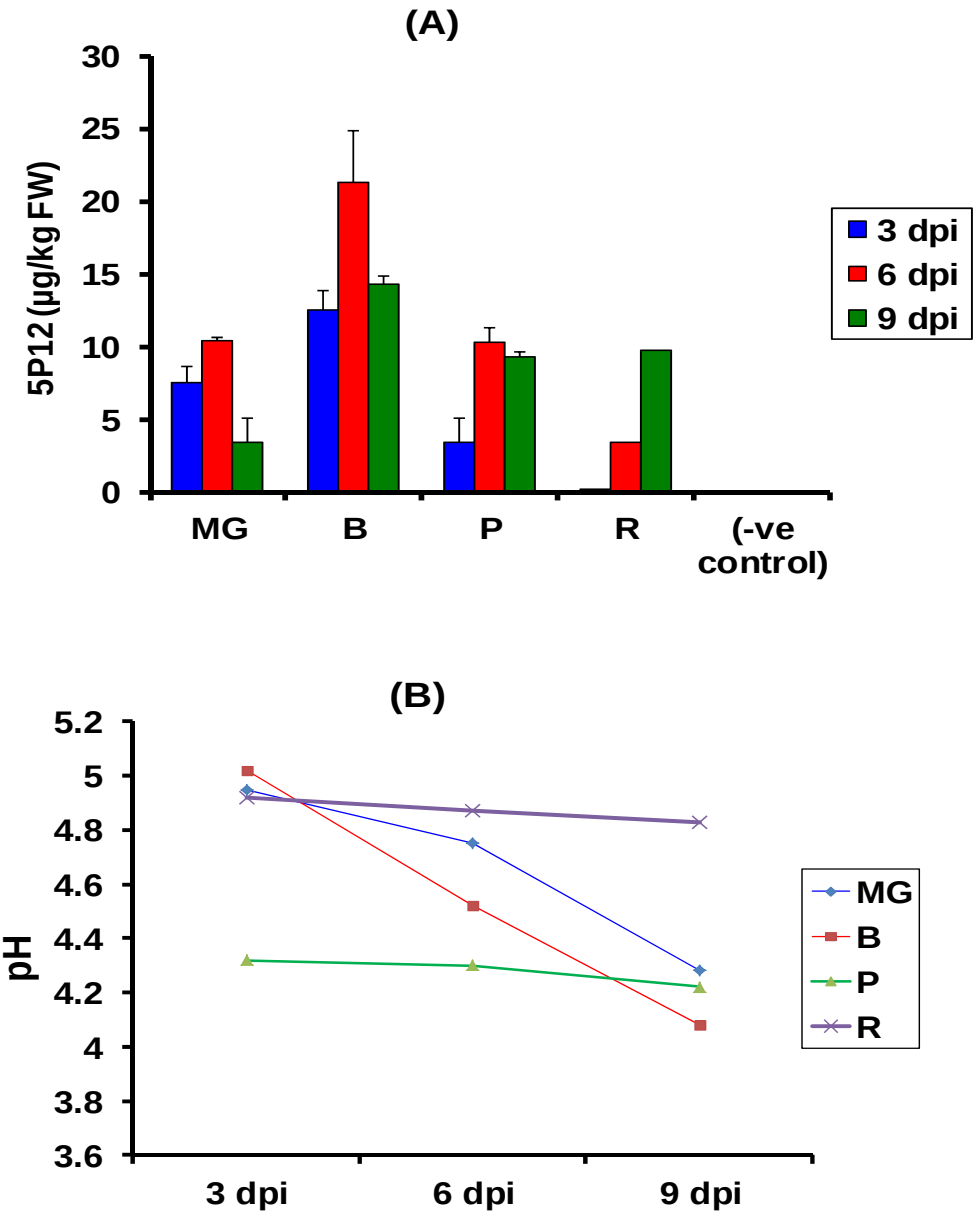

Figure 28 Expression level and pH correlation analyses of tomato fruits targeted to the cytosol. A, ELISA assay; B, pH analysis. The error bars represents the standard deviations of three replicates.

The pH values in the apoplastic compartment showed similar trend as in the cytosol. Nevertheless, a different pattern was observed for 5P12 expression (Figure 29). Thus a gradual increase from 3-9 dpi was observed for all maturity stages except for the P stage. MG was the best maturity stage for agroinjection as it resulted in the highest expression level of 23.56 µg/kg FW and pH 4.75. Although a similar pattern was observed in the MG, B and R stages, the P stage showed a slightly different pattern where expression levels peaked at 6 dpi (17.31 µg/kg FW) and then

decreased at 9 dpi (11.6 µg/kg FW). When put together, an inverse correlation between the expression levels and the pH was also observed except at 9 dpi of the P stage.

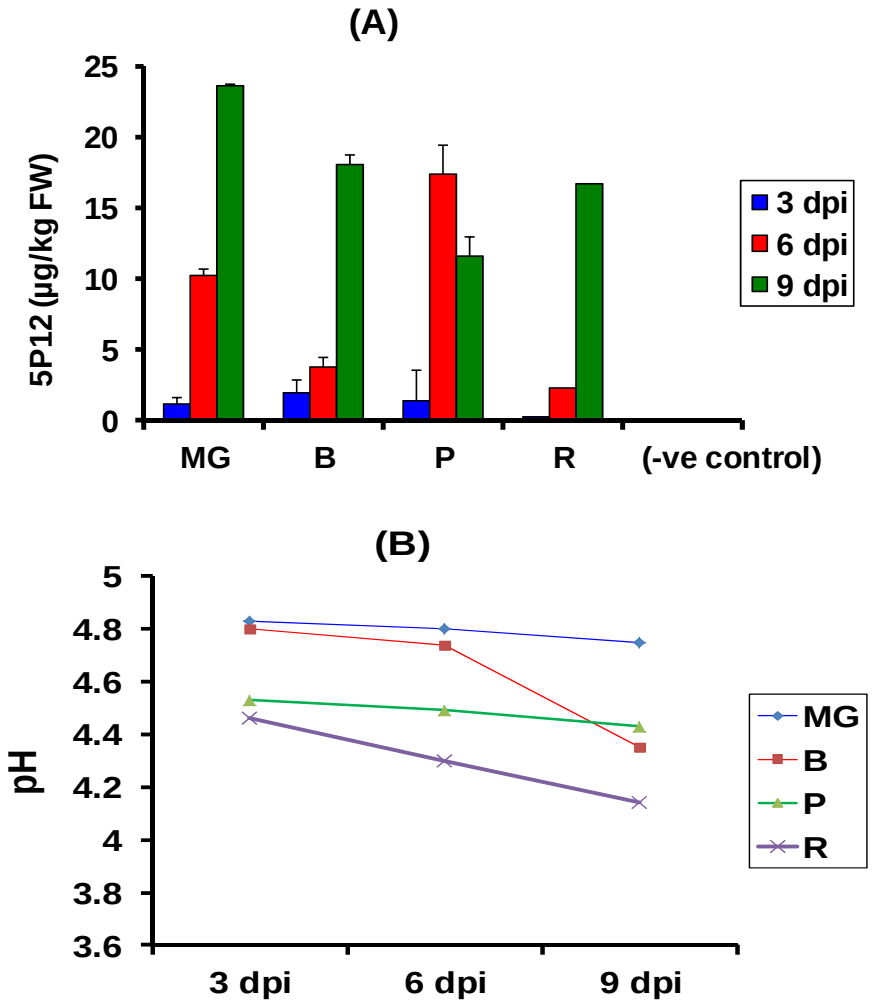

Figure 29 Expression levels and pH correlation analyses of tomato fruits targeted to the apoplast at different day post infiltrations (dpi). A, ELISA; B, pH values. The error bars represents the standard deviations.

In conclusion, the yields obtained in this study were comparable with the previous studies. Thus, we obtained the highest expression levels at the MG stage and the lowest level at the R stage. Giovinazzo *et al*. (2005) also reported the highest expression levels of the hFIX protein at the MG stage and the lowest level at the B stage.

4.2.5 Coomassie stained SDS-PAGE analysis

Having detected 5P12 by ELISA, the samples were further analysed by SDS-PAGE to establish the molecular weight of the recombinant chemokine. Based on expression levels in Figure 29, samples that showed higher expression levels in each group were selected for SDS-PAGE analysis. Unfortunately, 5P12 was not detectable even after the crude extracts were concentrated 50-100 fold (Figure 30). However, 5P12-RANTES standard (lane 1) showed a single band that migrated at approximately 11 kDa relative to the protein marker (lane M).

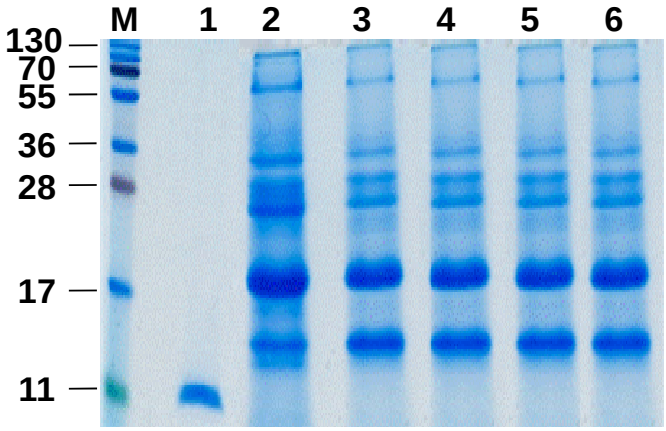

Figure 30 Apoplastic crude extract protein profiles obtained by 15% SDS-PAGE analysis. 60-80 µg of sample was loaded per well. M, Molecular marker (SM1811, Fermentas); 1, 5P12-RANTES standard; 2, negative control; 3, MG stage at 3 dpi; 4, B stage at 9 dpi; 5, P stage at 6 dpi; 6, R stage at 9 dpi.

4.2.6 Western blot analysis

We proceeded to analyse the samples by western blot to confirm the size of the recombinant 5P12. Figure 31 shows recognition and cross-reaction of proteins blotted on PVDF membrane with anti-RANTES antibodies. The 5P12-RANTES standard (lane 1) gave a clear protein band which migrated at the size of 11 kDa. The negative control (lane 2) showed nonspecific reaction with the anti-RANTES antibodies, but none of the bands corresponded in size with the 5P12-RANTES standard. All tomato fruit stages cross-reacted the same way as the negative control.

However, they all showed bands corresponding in size with the 5P12-RANTES. The distinct bands above the 11 kDa band could possibly be a dimer because theymigrated at approximately 16 kDa. Otherwise, the results show that tomato fruits expressed 5P12 molecules at levels detectable by a western. Regardless of the low yields, a proof of principle was obtained that tomato fruits can express 5P12.

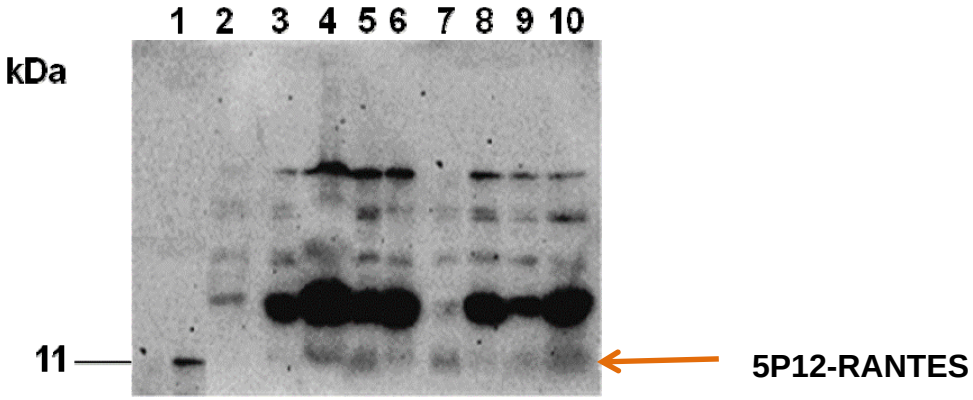

Figure 31 Western blot analysis of 5P12 crude extracts at different days post infiltration. 1, 5P12-RANTES standard; 2, negative control; 3, B stage targeting apoplast at 3 dpi; 4, MG stage targeting apoplast at 6 dpi; 5, P stage targeting apoplast at 3 dpi; 6, R stage targeting apoplast at 9 dpi; 7, MG stage targeting cytosol at 9 dpi; 8, B stage targeting cytosol at 9 dpi; 9, P stage targeting cytosol at 9 dpi; 10, R stage targeting cytosol at 9 dpi.

4.3 Conclusion

5P12-RANTES is a promising candidate for a topical HIV microbicide. For the microbicide to play a role in HIV prevention in resource-poor nations in the sub-Saharan Africa where HIV is a menace, it has to be produced in enough quantities to meet the demands and at a relatively low cost of less than $1 per dose. Shattock and Moore (2003) estimated that for CV-N, it would need 5 tonnes of the microbicide to supply 10 million women two doses per week. They also postulated that such demands can only be achieved using plants as a production system. Tomatoes have been proposed as candidates for producing pharmaceutical products owing to their high biomass yields even approaching the yields possible in tobacco species (Fischer *et al*., 2009; Orzaez *et al*., 2006). Another advantage of tomato fruits as host

is that they could be easy and cheaper storage for recombinant novel proteins as lyophilised material before they can be extracted. Proteins may be easier to purify from lyophilised tomatoes since they contain less bio burden such as fibres and alkaloids. Moreover, the tomatoes can be purchased and used whenever necessary or grown specifically for this purpose. Having said that, the current expression levels obtained in this study is not within the reported range (0.0001-0.3% TSP) of the recombinant proteins in tomato (Zhang *et al*., 2007). In conclusion, our results show for the first time that plant hosts and tomato fruits in particular, are potential candidates for producing 5P12-RANTES as a promising microbicide.

CHAPTER 5

5. DISCUSSION AND CONCLUSION

The primary goal of this study was to economically express RANTES analogues as promising topical microbicide candidates. This was done based on the steadily increasing proportion of biologics among new drugs and microbicides in development. Moreover, production of these biologics are placing an increasing burden on the health care systems of the developed and developing countries as they are currently produced at great expense and in small quantities (Boothe *et al.,* 2010). Plant-based production systems are highly accessible and offer an opportunity for safe, effective, economic and large-scale production (Coku, 2007). Hence we investigated the production of N-terminally modified RANTES analogues (5P12 and 6P4) in plants owing to their antiviral potency. The two analogues have been shown *in vivo* to prevent vaginal HIV-1 transmission (Gaertner *et al*., 2008a). Furthermore plants are poised to become the next major commercial development in biotechnology to deliver pharmaceuticals (Ma *et al*., 2005b).

We decided to investigate *Nicotiana benthamiana* as expression host since it has been used in research for many years due to its attractive features such as high biomass and large-scale production possibility (Zahmanova *et al*., 2005). Moreover, it is a hyper-susceptible host to many viral vectors and many diseases and it has the capacity to modulate gene expression rapidly and efficiently via plant genetic engineering (Clemente, 2006; Gleba *et al*., 2004). On the other hand, *Lycopersicon esculentum* (tomato) has been selected because of its biomass yields which approach the yields in tobacco species (Fischer *et al*., 2009; Orzaez *et al*., 2006). Furthermore, these species have not been investigated for the expression of N-terminally modified RANTES yet.

The RANTES analogues were successfully cloned into pTRA (bacterial) and MagnICON (deconstructed viral) as expression vectors targeting various subcellular compartments viz; apoplast, chloroplast, cytosol and ER. The motive was to compare the two expression vector systems in terms of their yields and protein stability. In order to establish this, *Agrobacterium tumefaciens* was successfully

transformed with the RANTES (5P12 and 6P4) constructs which were used to transfect the cells of *N. benthamiana* and *L. esculentum* species for transient expression. Thus the recombinant T DNA of the *A. tumefaciens* was transferred for non-integration into the genomes of both selected plant species. Hence the recombinant 5P12 and 6P4 were successfully expressed and targeted to various subcellular compartments for better accumulation, stability and probably proper folding (Avesani *et al*., 2010).

5.1 Agroinfiltration methods

For *N. benthamiana*, two agroinfiltration methods were explored, i.e., needle injection and vacuum infiltration. The needle injected leaves resulted in low GFP and 5P12 expression levels when compared to vacuum infiltrated leaves. This was expected as vacuum infiltration, which is used in large-scale production, requires more pressure to be applied to cover the whole surface area of the leaves so that all cells participate in protein expression (Rybicki, 2009; Huang *et al,* 2010). Conversely, only the agroinjection method was applied in *L. esculentum* as too much pressure would have ruined the fruit.

5.2 MagnICON constructs

Accumulation of 5P12 in two different subcellular compartments was determined. The vacuum infiltrated leaves resulted in the highest yield in the apoplast at 9 dpi which showed more 5P12 stability. This may be because the apoplastic space of the cell wall provided a good environment with less protease activities to accumulate up to 100-fold more total protein than the cytosol (Kamenarova *et al*., 2005; Avesani *et al*., 2010; Goulet *et al*., 2010; Obembe *et al*., 2011). The highest yield of the needle injected leaves was however, obtained in the cytosol at 9 dpi.

Similarly, the highest yield in *L. esculentum* was obtained in the apoplast of the MG (mature green) stage at 9 dpi. This was in line with the previous studies that reported highest protein expression at the MG stage (Giovinazzo *et al*., 2005). When comparing the highest yields obtained in the apoplast of *L. esculentum* (at 9 dpi) and

N. benthamiana (at 3 dpi) using the same method i.e., agroinfiltration and needle injection, *N. benthamiana* resulted in 3-fold greater 5P12 level. The difference in the yields could possibly be influenced by factors such as protein content, water content and presence of proteases and protease inhibitors found in both plant species (Streatfield, 2007; Scotti *et al*., 2009). *N. benthamiana* leaves were therefore considered to be a better host for 5P12 expression than tomato fruits since the highest expression levels of 73.7 and 92.5 µg/kg FW were obtained in the apoplast and cytosol, respectively. On the other hand, the highest expression levels obtained in tomato fruits were 23.56 and 21.24 µg/kg FW in the apoplast and cytosol, respectively.

5.3 pTRA constructs

pTRA constructs produced the highest 5P12 expression levels in the cytosol at 3 dpi. However, the MagnICON system produced 1000-fold more than the pTRA system, making the MagnICON vectors more reliable for *in planta* production. The yields were even lower than those obtained in *L. esculentum*. The reason could be that viral vectors can produce protein products more rapidly and in abundance than the bacterial vectors (Gleba *et al.,* 2007; Werner *et al*., 2011). Viral vectors contain a movement protein (MP) which is responsible for cell-to-cell movement leading to whole plant infection (Werner *et al*., 2011). Nonetheless, the current findings are in line with the previous findings that showed *Arabidopsis thaliana* Act2 promoter that was used in MagnICON vectors to be significantly more active than the CaMV 35S promoter which was used in pTRA vectors (An and Meagher, 2010). Although 5P12 expression in pTRA was confirmed by dot blot analysis to provide a proof of concept, low yields are one of the major setbacks associated with plant-based production systems (Scotti *et al.,* 2009).

5.4 Efficacy testing and specificity of plant-made 5P12-RANTES

In vitro efficacy testing was conducted and the plant extracts neutralised pseudovirions targeting the CCR5 and CXCR4 receptors. If the analogue had been expressed sufficiently and completely purified, it would not have inhibited the VSV-G

pseudovirions, but the CCR5-tropic viruses. Therefore, the plant protein extracts showed antiviral activities and were not specific to the HIV entry.

5.5 Conclusion

In summary, we obtained a proof of concept that the N-terminally modified RANTES analogues can be expressed in *N. benthamiana* and *L. esculentum* species. However, the expression yields were not good when compared with the reports on expressing other proteins. For instance, 5 mg/g of the recombinant proteins including GFP were previously reported (Santi *et al*., 2006; Gleba *et al*., 2007). Therefore, more alternative hosts are required for the cost –effective production of these analogues because they are promising candidates in the development of microbicides. Otherwise, genetic engineering of the best compartments and further optimising the conditions for better expression of these analogues may be topics for future research. Success could lead to the production of safe, cheap and efficient products to help women especially, in the sub-Saharan Africa, to protect themselves against HIV-1 infection.

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APPENDIX A: Results of cleavage site prediction by TargetP v1.1 software tool

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TargetP 1.1 Server - prediction results
Technical University of Denmark

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### targetp v1.1 prediction results
####################################
Number of query sequences:  1
Cleavage site predictions included.
Using PLANT networks.
```
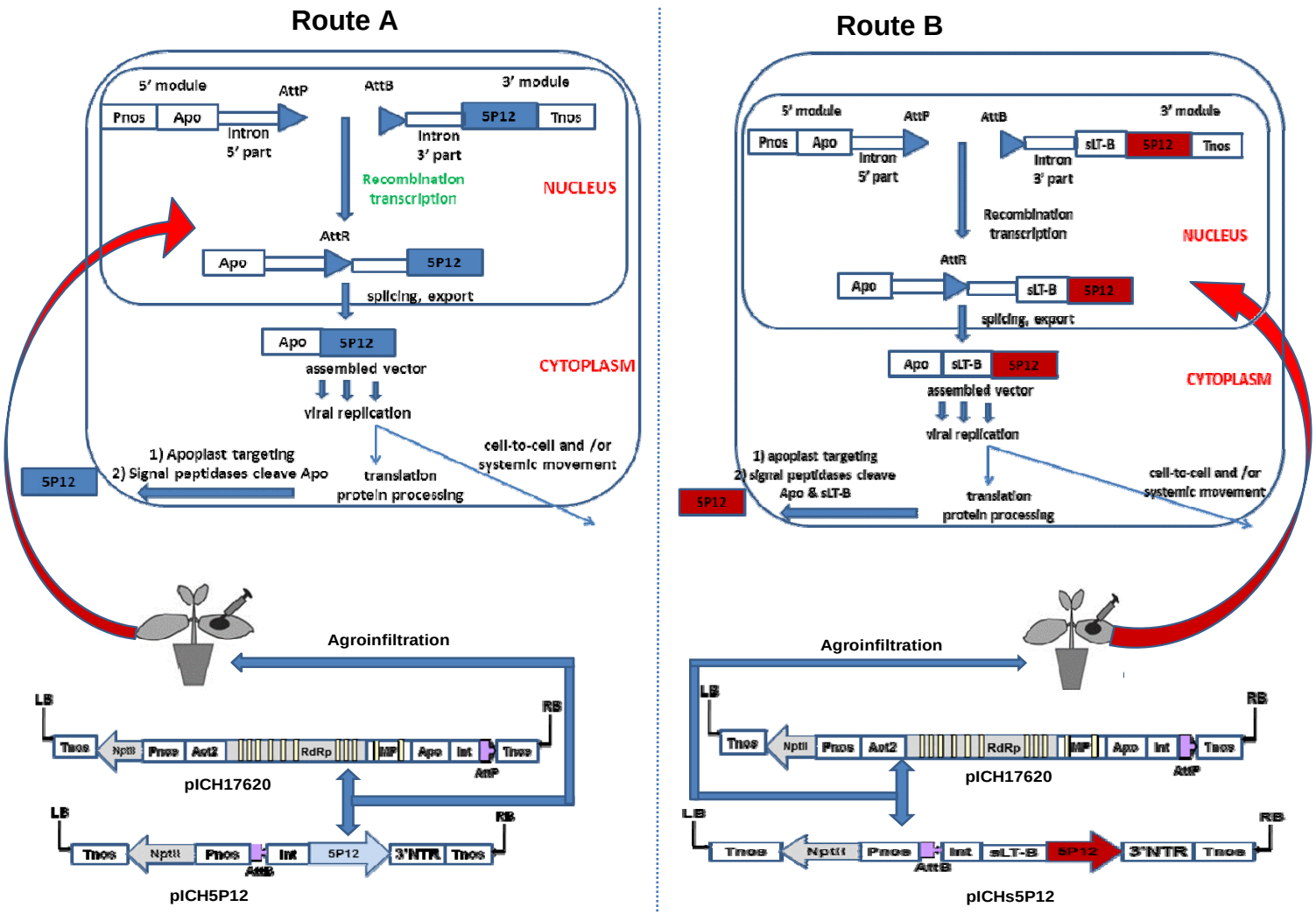

Name	Len	cTP	mTP	SP	other	Loc	RC	TPlen
Sequence	98	0.011	0.192	0.893	0.013	S	2	23
cutoff		0.000	0.000	0.000	0.000			

DESCRIPTION

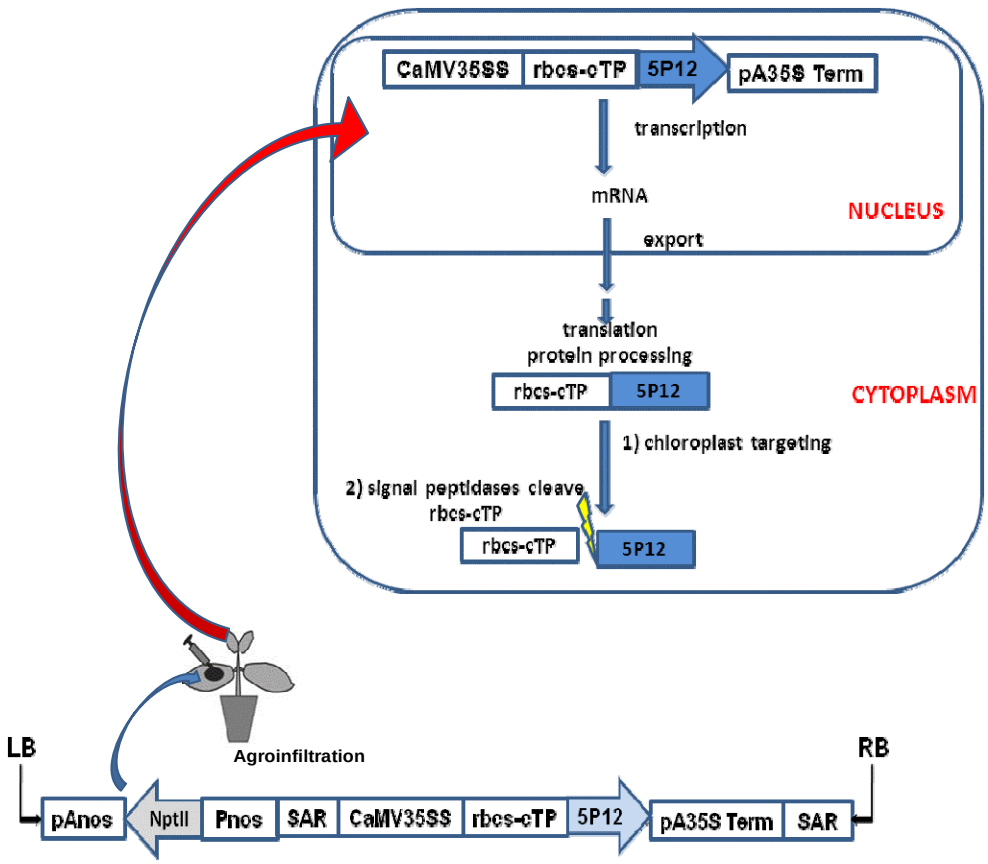
Name	Sequence name truncated to 20 characters
Len	Sequence length
cTP, mTP, SP, other	Final NN scores on which the final prediction is based (Loc, see below). Note that the scores are not really probabilities, and they do not necessarily add to one. However, the location with the highest score is the most likely according to TargetP, and the relationship between the scores (the reliability class, see below) may be an indication of how certain the prediction is.
Loc	Prediction of localization, based on the scores above; the possible values are: **C** Chloroplast, i.e. the sequence contains **cTP**, a chloroplast transit peptide; **M** Mitochondrion, i.e. the sequence contains **mTP**, a mitochondrial targeting peptide; **S** Secretory pathway, i.e. the sequence contains **SP**, a signal peptide; _ Any other location; * "don't know"; indicates that cutoff restrictions were set (see instructions) and the winning network output score was below the requested cutoff for that category.
RC	Reliability class, from 1 to 5, where 1 indicates the strongest prediction. RC is a measure of the size of the difference ('diff') between the highest (winning) and the second highest output scores. There are 5 reliability classes, defined as follows: 1: diff> 0.800 2: 0.800 > diff > 0.600 3: 0.600 > diff > 0.400 4: 0.400 > diff > 0.200 5: 0.200 > diff Thus, the lower the value of RC the safer the prediction.
TPlen	Predicted presequence length; it appears only when TargetP was asked to perform cleavage site predictions (see instructions).

APPENDIX B: Strategy for expression of proteins in plants using MagnICON vector system



APPENDIX C: Strategy for expression of proteins in plants using pTRA vector system

Route A



Route B