

Expression of anti-HIV peptides in Tobacco Cell Culture Systems

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REFERENCE DECLARATION IN RESPECT OF MASTER'S DISSERTATION

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DEDICATION

I dedicate this work to my parents, **Mr and Mrs Moodley**, for their unconditional love, support and motivation.

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ABSTRACT

Nearly half of all individuals living with HIV worldwide at present are woman and the best current strategy to prevent sexually transmitted HIV is antiretrovirals (ARVs). Microbicides are ARV's which directly target viral entry and avert infection at mucosal surfaces. However, most promising ARV entry inhibitors are biologicals which are costly to manufacture and deliver to resource-poor areas. Microbicides formulated as simple gels, which are currently not commonly used in ARV therapy, show immense potential for use in prevention and treatment of multidrug-resistant viral infections in developing countries.

Among the most potent HIV entry inhibitory molecules are lectins, which target the high mannose N-linked glycans which are displayed on the surface of HIV envelope glycoproteins. Of the microbicides, the red algal protein griffithsin (GRFT) has potent anti-HIV inhibitory activity and is active by targeting the terminal mannose residues on high mannose oligosaccharides. It has a total of 6 carbohydrate binding sites per homodimer, which likely accounts for its unparalleled potency. The antiviral potency of GRFT, coupled with its lack of cellular toxicity and exceptional environmental stability make it an ideal active ingredient of a topical HIV microbicide.

Scytovirin (SVN) is an equally potent anti-HIV protein, isolated from aqueous extracts of the cyanobacterium, *Scytonema varium*. Low, nanomolar concentrations of SVN have been reported to inactivate laboratory strains and primary isolates of HIV-1. The inhibition of HIV by SVN involves interactions between the protein and HIV-1 envelope glycoproteins gp120, gp160 and gp41.

Current recombinant production methods for GRFT and SVN molecules are unfortunately hampered by inadequate production capacities. This project therefore aimed to determine if these molecules can be produced in plant cell culture systems. The transgenic tobacco cell culture system was evaluated to determine if it can be an alternative, cost effective production system for these molecules.

Results of the study show that the microbicide genes can be cloned into plant transformation vectors, used to successfully transform SR1 tobacco cell lines and adequately produce 3.38ng and 10.5ng of GRFT and SVN protein respectively, per gram of SR1 tobacco callus fresh weight.

The promising results attained in this study form the basis for further work in optimising plant cell based production systems for producing valuable anti-HIV microbicides, a possible means to curbing the elevated HIV infection rates worldwide.

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List of Abbreviations and symbols

AIDS	Acquired Immunodeficiency Syndrome
APA	Allium porum agglutinin
bp	Base pairs
BY2	Bright Yellow 2
CA	Cybidium agglutinin
CaMV 35S	Cauliflower Mosaic Virus 35S
CV-N	Cyanovirin –N
Da	Daltons
DNA	Deoxyribonucleic Acid
ESA-2	Euchema serra agglutinin 2
g	grams
GlcNac	N-acetyl-glucosamine
GMP	Good Manufacturing Practice
GNA	Galanthus nivalis agglutinin
GRFT	Griffithsin
HHA	Hippeastrum hybrid agglutinin
HIV	Human Immunodeficiency Virus
Hr	Hour
KOH	Potassium hydroxide
kV	Kilovolts
L	Litre
LOA	Listera ovata agglutinin

IgA	Immunoglobulin A
MBHA	Myxococcus Xanthus hemagglutinin
MES	2-(N-morpholino)ethanesulfonic acid
Mg/l	Milligram per litre
Min	Minutes
ml	Millilitres
mM	Millimolar
MS	Murshige and Skoog
MVI	Microcystis viridis lectin
NaOCl	Sodium hypochlorite
ng	Nanogram
NPA	Narcissus pseudomonas agglutinin
NT1	<i>Nicotiana tabacum</i> 1
OAA	Oscillatoria agardhii agglutinin
PCR	Polymerase Chain Reaction
pm	Pico Molar
RNase	Ribonuclease
r.p.m	revolutions per minute
SARS-CoV	Severe Acute Respiratory Syndrome-Coronavirus
SD2	Scytovirin domain 2
SIV	Simian Immunodeficiency Virus
SR1	<i>Nicotiana tabacum</i> cv Petit Havana
SVN	Scytovirin
T-DNA	Transfer DNA

Ti	Tumor inducing
UDA	Urtica dioica agglutinin
UN	United Nations
v/v	volume/volum
w/v	weight/volume
WT	Wild type
2,4D	2,4- dichlorophenoxyacetic acid
2-Ip	2-Isopentyl adenine

Symbols

α	alpha
β	beta
%	percent
°C	degree celcius
μ l	microlitre

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THE AIMS AND OBJECTIVES

Aim

The primary aim of this project is to determine if anti-HIV molecules, Scytovirin and Griffithsin, can be expressed stably in transgenic tobacco cells.

Objectives

The broader objective of this project is to curb HIV infection in the Southern African sub-region. Several microbicides are currently under trial in Southern Africa. Should any of these molecules prove to be effective in curbing HIV, their affordability remains to be seen – cost is a major limitation to adoption of some measures to control infectious disease. It is therefore essential to develop technologies that will allow local production of these molecules at a scale that is relevant to regional demand and a cost that is affordable to regional governments. This project will explore plant based expression systems for microbicidal molecules that are low tech, cheaper and locally developed.

The objectives of the research of this project are:

- To establish methodology for the development of *Nicotiana tabacum* cell lines
- To clone the Scytovirin and Griffithson genes into plant transformation vectors and to thereafter transform tobacco plant cells
- To determine cytosolic expression of novel molecules in cell culture

Thesis Design

The thesis is in the format of five chapters and each chapter can be broken down as follows:

Chapter One: The literature review discusses the need for microbicides, plants and plant cell culture systems for pharmaceutical drug production, an introduction to lectins and more specifically, the Griffthsin and Scytovirin lectins.

Chapter Two: This chapter discusses the development of a robust *N.tabacum* SR1 cell line.

Chapter Three: Chapter three focuses on the cloning of the transformation vectors for SVN and GRFT, as well as the EHA101 agrobacterium transformation of the SR1 cell line previously developed (Chapter Two). This chapter includes the observation of callus development after transformation and the confirmation of the putative transformants via polymerase chain reaction (PCR).

Chapter Four: This chapter completes the study by determining if plant cells are indeed capable of microbicide expression and quantification, confirmed by protein analysis via western blot and gp120 binding ELISA assays.

Chapter Five: This chapter summarizes the findings in all of the above chapters and briefly discusses the future perspectives.

Chapter One

Introduction

Human Immunodeficiency Virus (HIV) continues to be a major global health problem, and HIV infection is rising more rapidly among women than men in many parts of the world. Half of all adults living with the virus that causes AIDS are female, according to the U.N, and 28% of the Sub-Saharan African population adds to the 33 million people infected globally (UNAIDS, 2008), with most new infections acquired through heterosexual intercourse. In this region, cultures limit women's autonomy and can leave them vulnerable to a spouse's unsafe sexual practices. Currently available prevention options such as condoms and mutual monogamy are not feasible for millions of people, especially women, who do not have the social or economic power necessary to insist on condom use and fidelity. Thus a massively expanded prevention program that specifically targets women is required to curb global HIV infection. The highly mutable nature of HIV and the daunting complexities of developing a broadly effective vaccine for multiple clades of HIV are increasingly apparent. Therefore, there is a pressing need to develop anti-HIV microbicides to prevent the sexual transmission of HIV. (Mori *et al*, 2005).

Microbicides are products that prevent the sexual transmission of HIV and other sexually transmitted infections (STIs) when applied topically to the vagina or rectum. In the absence of an effective vaccine against HIV, microbicides are a powerful, viable tool in the fight against HIV transmission. Microbicides could be used covertly without a partner's cooperation and would particularly empower women in AIDS prevention. The two microbicides selected for this study, Scytovirin and Griffithsin, isolated from

Cyanobacterium and Red Alga respectively, have both been shown to bind to viral coat glycoproteins. These molecules are currently produced in *E. coli*, where production capacity is limited. Cost, stability and ease of manufacturing are critical for the effectiveness and accessibility of a microbicide by the poor.

Literature Review

1.1. HIV infection and current control strategies

South Africa is currently experiencing one of the most severe AIDS epidemics in the world. At the end of 2007, there were approximately 5.7 million people living with HIV in South Africa, whilst Sub-Saharan Africa has the highest HIV infection prevalence in the world (Fig. 1.1). Almost 1,000 AIDS deaths occur every day in South Africa (UN report on global AIDS epidemic, 2008) and it is unfortunate that at present, a staggering 71% of all deaths of people between the ages of 15 and 49 are caused by AIDS (South African Medical Research Council, 2006). Although HIV prevention campaigns usually encourage people to use condoms and have fewer sexual partners, women and girls in South Africa are often unable to negotiate safer sex and are frequently involved with men who have several sexual partners. They are also particularly vulnerable to sexual abuse and rape, and are economically and socially subordinate to men. Women often face more severe discrimination than men if they are known to be HIV-positive. This can lead to physical abuse and the loss of economic stability if their partners leave them. Since antenatal testing gives them a greater chance of being identified as HIV-positive, women are sometimes branded as ‘spreaders’ of infection. Fig.1.2 shows Sub-Saharan Africa having the highest percentage of females living with HIV, as compared to other countries across the world (UN AIDS Report, 2008).

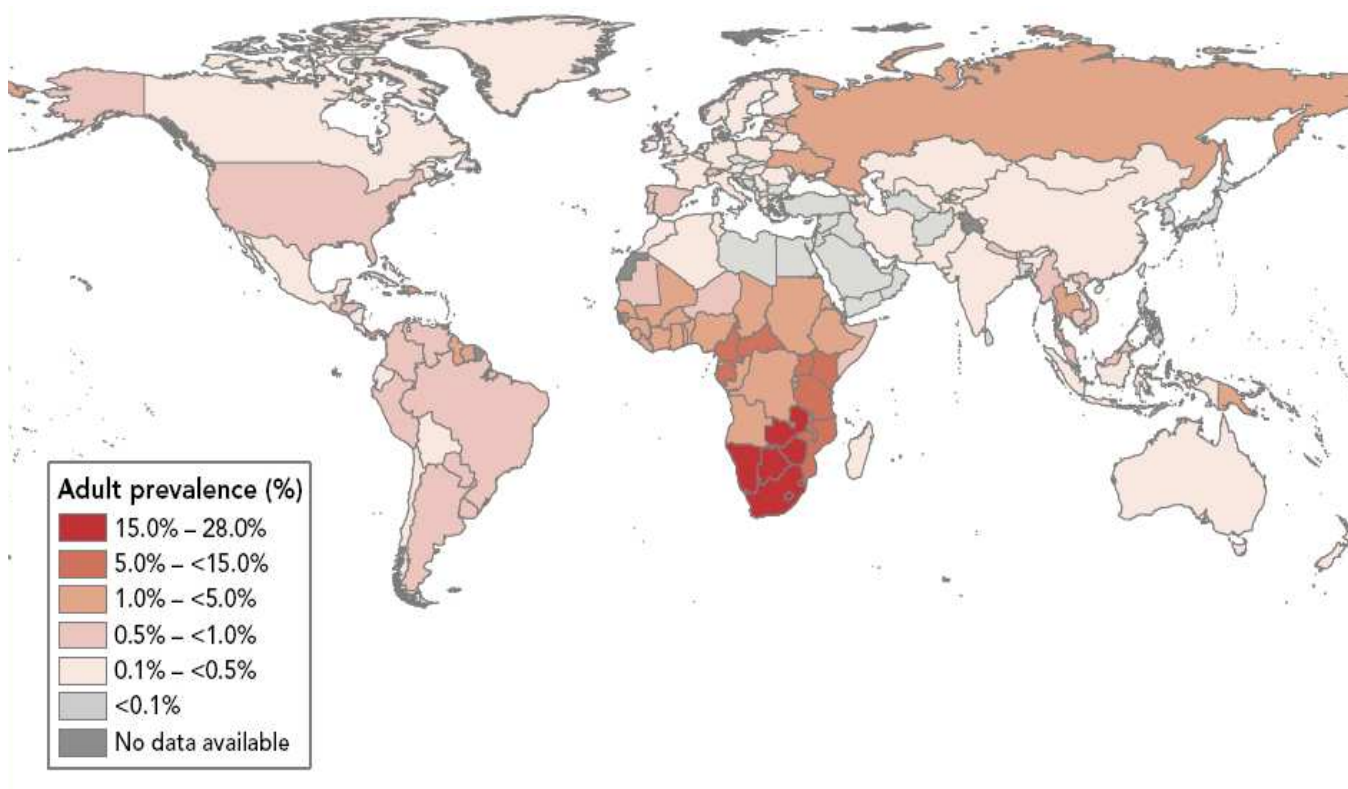


Fig.1.1 A global view of HIV prevalence across the world in 2007. Thirty three million people living with HIV (UNAIDS report, 2008)

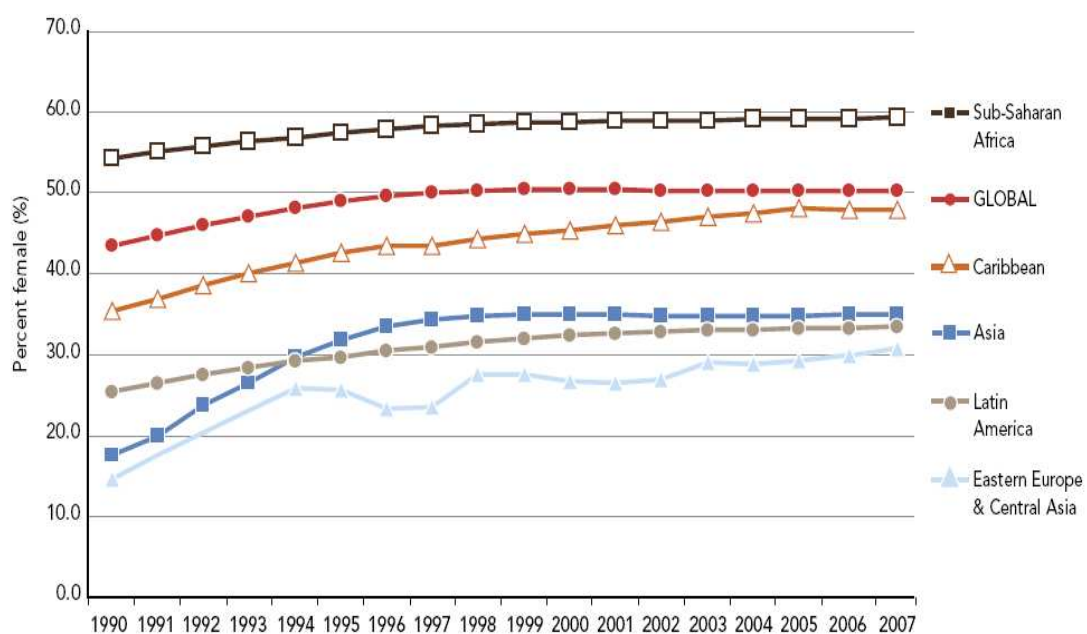


Fig. 1.2 Percentage of adults living with HIV, who are female 1990-2007 (UN AIDS report, 2008)

Emerging and re-emerging diseases such as HIV, the increasing age and size of the world's population and the threat of biological warfare are challenging the research, development and production arenas of the pharmaceutical industry. Current methods to curb HIV infection rates are not successful and the numbers of infected people continue to grow. Apart from methods such as insistent condom usage during sexual practice, monogamy, or complete abstinence all together, the discovery of anti-retroviral recombinant proteins may possibly be the answer to minimizing or stopping the transmission of HIV altogether.

Microbicides, which inhibit viral entry, may be available in gel or cream form and can be used in the same way as available spermicides i.e. by topical application. This would be a way to empower woman and more importantly, curb infection rates.

Current avenues for the production of recombinant pharmaceutical proteins include prokaryotes and eukaryotes (yeasts and mammalian cell cultures, insect cells); however, the main challenge lies in achieving cost-effective production on a large scale (Walmsely and Arnten, 2003). Altogether, the biochemical, technical and economic limitations on existing prokaryotic and eukaryotic expression systems, as well as the growing clinical demand for complex therapeutic proteins, have created substantial interest in developing new expression systems for the production of therapeutic proteins. To that end, plants have emerged in the past decade as suitable alternative to the current production systems (Faye *et al*, 2004).

1.2. Plants as recombinant protein production systems

Plant based systems for the production of recombinant proteins are advantageous as they are economical, use low cost inputs such as light, water and minerals, they are easily adaptable to large scale operations, they pose minimal risk of contamination with bacteria or mammal-borne pathogens, the products may not require rigorous purification and the pharmaceuticals produced this way can be given mucosally, hence simplifying delivery and decreasing overall costs (Walmsely and Arntzen, 2003). One of the major advantages of using transgenic plants over other production systems available for large scale production, such as yeasts or *E.coli*, is the ability of plants cells to perform most post-translational modifications required for protein activity and suitable pharmacokinetics properties. This is illustrated by their capacity to produce functional forms of complex mammalian proteins, including for instance collagens, hemoglobin and antibodies, including secretory IgA (Faye *et al*, 2004).

A range of different plant and vector systems have been used for the expression of antigens. Current plant derived proteins produced in genetically modified plants and those that are closest to commercialization are summarized in Table 1.1

Table 1.1 Plant derived pharmaceutical proteins that are closest to commercialization for the treatment of human diseases

Product	Class	Indications	Company/Organisation	Crop	Status
Various single chain antibody fragments	Antibody	Non-Hodgkins lymphoma	Large Scale Biology Corp	Viral vectors in tobacco	Phase I
CaroRx	Antibody	Dental caries	Planet biotechnology	Transgenic tobacco	Phase II
<i>E.coli</i> heat labile toxin	Vaccine	Diarrhoea	Prodigene Inc Arntzen group (Tacket <i>et al</i> , 1998)	Transgenic maize Transgenic potato	
Gastric Lipase	Therapeutic enzyme	Cystic fibrosis, pancreatitis	Meristem therapeutics	Transgenic maize	Phase II
Hepatitis B virus surface antigen	Vaccine	Hepatitis B	Arntzen group (Richter <i>et al</i> , 2000). Thomas Jefferson University/ Polish Academy of Sciences	Transgenic potato Transgenic Lettuce	Phase I Phase I
Human Intrinsic Factor	Dietary	Vitamin B12 deficiency	Cobento Biotech AS	Transgenic <i>Arabidopsis</i>	Phase II
Lactoferrin	Dietary	Gastrointestinal infections	Meristem therapeutics	Transgenic maize	Phase I
Norwalk Virus Capsid Protein	Vaccine	Norwalk Virus infection	Arntzen Group (Tacket <i>et al</i> , 2000)	Transgenic potato	Phase I
Rabies glycoprotein	Vaccine	Rabies	Yusibov <i>et al</i> , 2002	Viral vectors in spinach	Phase 1

(The European Union Framework 6 Pharma-Planta Consortium, EMBO Reports, 2005)

To date many different plant species have been used for vaccine production. Early studies used tobacco and potato, but now tomato, banana, corn, lupine, lettuce and others are being used for this purpose. The choice of the plant species (and tissue in which the protein accumulates) is important and is usually determined through how the vaccine or other therapeutic is to be applied in the future e.g. an edible, palatable plant is necessary if the vaccine is planned for oral administration. This limitation is overcome in non-edible plants by antigen extraction and purification, as performed when using tobacco cells (Sala *et al*, 2003).

Plant cell culture is a technique designed for the growth and multiplication of cells using nutrient solutions in an aseptic and controlled environment. This technology explores conditions that promote cell division and genetic reprogramming under *in vitro* conditions. Mainly developed in the 1960's, plant tissue culture has turned into a standard procedure for modern biotechnology and today one can recognize at least five different areas where *in vitro* cultures are currently applied: large scale propagation of elite materials; generation of genetically modified fertile strains; as a model system for fundamental plant physiology aspects; preservation of endangered species and metabolic engineering of fine chemicals (Methods in Molecular Biology, Plant Cell Protocols, 2006)

Plant cell suspension cultures also have advantages over whole plant production systems. The requirements of plant cell systems necessitate the containment and control of laboratory conditions, thereby avoiding the concerns of environmental contamination and the gene-transfer potential with the field production of plants. Rapid growth, simple inexpensive media, and amenability of plant cell cultures to large scale production in bioreactors also represent a distinct advantage (Mayo *et al*, 2006).

1.3. The use of *Nicotiana tabacum* cell cultures for the production of recombinant proteins

The stable transformation of *Nicotiana tabacum* cells to produce transgenic cell lines is a robust method for obtaining rapid cell growth in a highly contained and stringently controlled environment with the potential for abundant protein production. Thus, the transgenic tobacco cells can facilitate protein production in conditions of ‘good manufacturing practices’ and avoids the complications of alternative in planta production methods which raise concerns regarding germplasm escape.

The transgenic tobacco cell system has been used as a production platform for erythropoietin (Matsumoto *et al*, 1995), hepatitis B surface antigen (Kumar *et al*, 2003), *Escherichia coli* intimin (Judge *et al*, 2004), gum Arabic protein (Zhang and Mason, 2006) and Norwalk virus capsid protein (Mason *et al*, 1996). However, factors that could limit the use of this protocol include toxicity effects of intended products, improper protein folding and conformation, and potential extraction difficulties if the foreign protein is membrane bound.

Plant cell cultures can be easily established under aseptic conditions. Friable callus cultures are attainable in two and a half months and they are thereafter inoculated into liquid culture. After three subsequent transfers (± 3 weeks), transformation protocol can be carried out using the cells in liquid culture. The transformation of tobacco cell suspension cultures may yield transformants on solid media within about five weeks and that with strong growth can be sampled, analyzed and transferred to liquid within an additional two- three months. Whole plant systems, especially combined with fruit specific expression can require a year or more to complete. In addition, whole plant culture requires a separate facility for field or greenhouse production to support the growth, maintenance and processing of the plants (Mayo *et al*, 2006). By including a secretory signal peptide to the novel protein, secretion of

the product into the medium will be enhanced or the protein could be localized in various cell destinations.

1.4. Plant transformation methods

Higher plants require the combination of many molecular tools to manipulate their metabolic processes owing to complex organization and metabolic compartmentation. In order for a transformation system to be successful a suitable gene and promoter sequence for cell and tissue specific expression and effective targeting signal to direct protein to its final destination within the cell are necessary (Stitt and Sonnewald, 1995)

Direct transfer methods for plant transformation rely entirely on physical or chemical principles to deliver DNA into the plant cell. Several different DNA transfer methods have been described, including particle bombardment (Klein *et al*, 1987 and Christou *et al*, 1991), microinjection (Crossway *et al*, 1986), transformation of protoplasts mediated by polyethylene glycol or calcium phosphate (Negrutiu *et al*, 1987 and Fromm *et al*, 1986) and transformation using silicon carbide whiskers (Framme *et al*, 1994). Among these methods, particle bombardment has been used the most widely for generating commercial transgenic crops, and the delivery of transgenes into embryogenic tissues by particle bombardment remains the principle direct DNA transfer technique in plant biotechnology (James, 2003). Particle bombardment facilitates a wide range of transformation strategies. Genetic transformation occurs in two stages: DNA transfer into the cell followed by DNA integration into the genome. The integration stage is much less efficient than the DNA transfer stage, with the result that only a small proportion of the cells that initially receive DNA actually become stably transformed. In the remaining cells the DNA enters the cell and may be expressed for a short time (transient expression), but it is never integrated and is eventually degraded by nucleases. Transient expression occurs almost immediately after gene transfer, it

does not require regeneration of whole plants, and it occurs at a much higher frequency than stable integration (Alpeter *et al*, 2005). Where the aim is to extract recombinant proteins from transgenic plants, transient expression following particle bombardment may also be used to produce small amounts of protein rapidly for testing (Twyman *et al*, 2003). Stable transformation via particle bombardment allows for the transformation of plastids and mitochondria and is also useful in transformation strategies involving viruses (Johnson *et al*, 1988 and Hoffman *et al*, 2001).

Several investigators have recently suggested that particle bombardment will inevitably be supplanted by transformation methods using soil bacterium *Agrobacterium tumefaciens* and its relatives, at least for the production of commercially enhanced crops (Gelvin, 2003, Tzfira and Citovsky, 2003 and Valentine 2003). Many regard *Agrobacterium* spp. as natural conduits for gene transfer between bacteria and plants and to therefore be more “natural” than direct transfer methods (Verhoog, 2003). A limitation to the use of *A. tumefaciens* thus far is the bacterium’s inadequacy in transforming monocots. This is a result of most cereal crops missing a wound response, where cells adjacent to wounded cells do not dedifferentiate but instead accumulate phenolics and die (Stitt and Sonnewald, 1995)

Agrobacterium is a soil-borne bacterium that, in the presence of a wounded plant, moves toward it, attaches itself to the wound site and proceeds to transform the cell. The sugars and phenolic compounds exuded by the plant not only signal the pathogenic opportunity to the bacterium but also induce transcription of the virulence genes. These virulence genes are located on a specific plasmid known as the tumor inducing (Ti) plasmid, which also contains the transferred DNA (T-DNA). Virulence proteins have roles ranging from transcriptional activation to T-DNA processing and export, with certain proteins also having a function in the host. *Agrobacterium* has evolved to transfer the T-DNA, which codes for (a) plant

hormone producing enzymes that stimulate growth of a tumor, and (b) metabolic enzymes responsible for producing opines (can be used as a source of carbon and/or nitrogen), metabolizable only by *Agrobacterium*. The resultant crown gall is a microcosm where the bacteria can thrive. In addition, the T-DNA contains genes for the synthesis of a unique class of compounds called opines, which *A. tumefaciens* can utilize as a carbon and energy source. The site of gene integration into the genome also influences epitope and transgene integration in plants. *A. tumefaciens* infection is most frequently used to achieve permanent integration into the nuclear DNA, where integration occurs (Sala *et al*, 2003). Figure 1.3 below outlines the ten step process required for *agrobacterium*-mediated genetic transformation (Tzavira and Citovsky, 2006)

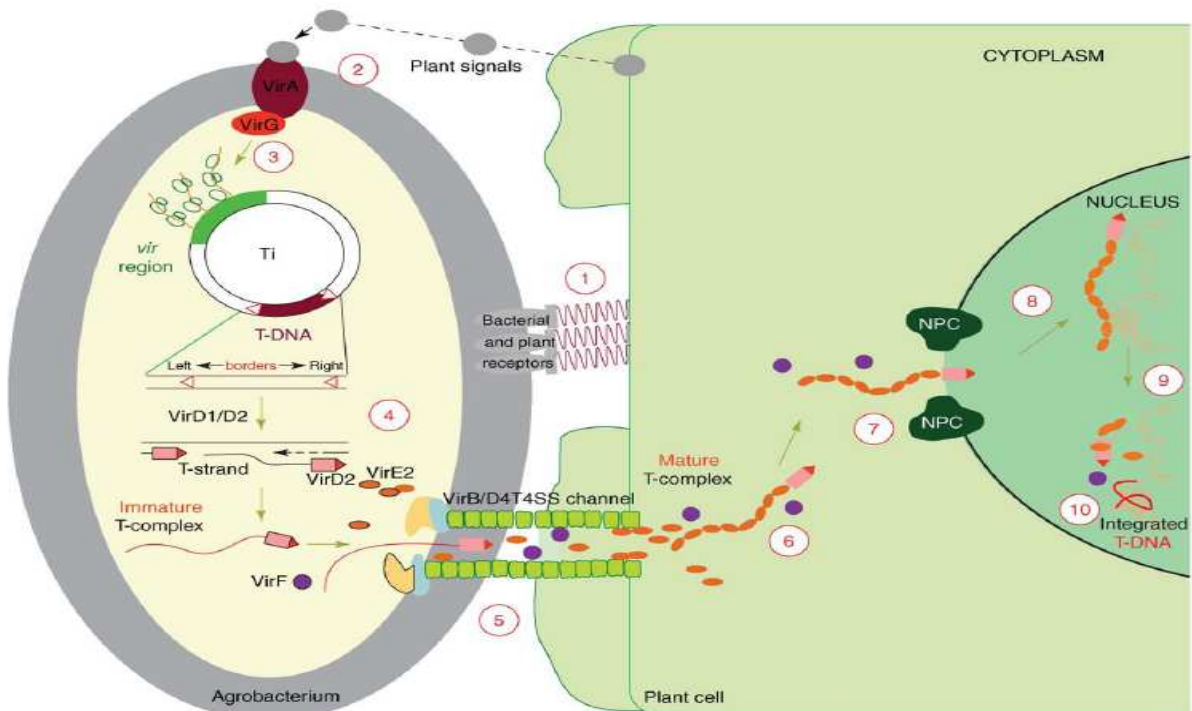


Fig.1.3

A model for the Agrobacterium-mediated genetic transformation. The transformation process begins with the recognition and attachment of the Agrobacterium to the host cells (1) and the sensing on specific plant signals by the Agrobacterium VirA/VirG two component signal-transduction systems (2). Following activation of the vir gene (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a Vir-D2 DNA complex (immature T-complex), together with several other Vir proteins, into the host cell cytoplasm (5). Following the association of VirE2-DNA with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10) (Tzfira and Citovsky, 2006)

1.5 Lectins, their occurrence and properties

Lectins were first discovered in 1888 by Stillmark as a proteinaceous agglutinating factor in castor bean seeds and owing to this early discovery; it has developed a far longer scientific history than most other plant proteins. Lectins were for a long time referred to as agglutinins as they are capable of clumping erythrocytes, but it is nowadays greatly emphasized that agglutination is no longer used as a criterion to consider a protein as a lectin (Van Damme *et al*, 2004).

A more accepted definition of a lectin is a plant protein which possesses at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharides. (Peumans *et al*, 1995).

Van Damme *et al* (1998) have reported that over five hundred plant lectins have been characterised with regards to the biochemical properties and carbohydrate binding specificity, and the corresponding genes for approximately 200 of these lectins have been cloned.

There is still little insight into why and when plants produce these lectins with defined sugar specificity and biological activity. Lectins can, at present, be divided into two categories i.e. “classical plant lectins”, with an involvement in protein storage and/or plant defence and “novel plant lectins”, which are involved in specific carbohydrate interactions within the plant cell, essential for normal growth, development and functioning of the plant (Van Damme *et al*, 1998).

Plant lectins do not represent a single superfamily of proteins but definitely exhibit a marked structural diversity. Based on structural, biochemical and molecular analysis, evidence suggests that virtually all plant lectins can be classified into seven families of structurally and evolutionary related proteins, namely, the amaranthins, chitin binding lectins comprising hevein domains, the Curcubitaceae phloem lectins, the jacalin-related lectins, legume lectins, the monoct-mannose-binding lectins and the type-2 inactivating proteins. Table 1.2

gives an overview of occurrence and cellular localization of the above mentioned plant lectins (Van Damme *et al*, 2004).

Table 1.2 Overview of the occurrence and cellular localisation of plant lectins

Lectin family	Occurrence	Subcellular location
<i>Amaranthins</i>	Documented only in the genus <i>Amaranthus</i> of the Amaranthaceae	Presumably cytoplasmic proteins
<i>Chitin binding lectins comprising hevein domains</i>	Probably ubiquitous in plants	Some proteins are located in the vacuole, others are secreted into the extracellular space
<i>Curcubitaceae phloem lectins</i>		
Classical Curcubitaceae phloem lectins	Curcubitaceae family only	Cytoplasm
Nictaba orthologs	Documented in Solanaceae	Cytoplasm and Nucleus
<i>Jacalin related lectins</i>		
Galactose-specific subgroup	Moraceae family only	Vacuole
Mannose-specific subgroup	Widespread in all major taxonomic groups	Cytoplasm (and nucleus)
Legume lectins	Documented in Fabaceae and Lamiaceae	Vacuole
Monocot mannose-binding lectins	Documented in 8 families of monocots, 3 families of dicots and one livewort	Vacuole
Type 2 ribosome-inactivating proteins	Documented in more than 10 families from all major taxonomic groups	Vacuole

(Van Damme *et al*, 2004)

Initial lectin studies were widely conducted on leguminosae lectins. Common leguminosae lectins include Concanavalin A, isolated from *Canavalia ensiformis*, with mannose and glucose binding properties and Soybean agglutinin isolated from Soybean with N-acetylgalactosamine and galactose binding capabilities (Rudiger, 1984)

Griffithsin, one of the compounds of interest in this study, is an algal lectin with the ability to target the heavily glycosylated gp120 envelope of HIV. Many recombinant proteins such as microbicides, function as HIV fusion inhibitors, preventing virus-cell fusion and infection of target cells. Figure 1.4 shows the life cycle of HIV. Briefly, HIV-1 attaches to the surface of a CD4⁺ lymphoid cell via an interaction between the viral gp120 glycoprotein and the CD4 receptor molecule of the cell. This event is then followed by fusion of the viral envelope with the cell membrane, a process facilitated by gp 41, a cleavage product, along with gp120, of the viral glycoprotein gp160, involving also secondary host cell chemokine receptors. At this initial infection phase, two targets can be exploited for the screening of antiviral agents. Specifically, the antiviral agents can act at the cell surface to inhibit the interaction of viral and cellular receptors, or they can prevent cell fusion by blocking the viral transmembrane gp 41. Blocking of cell fusion also blocks cell-cell transmission of HIV virus (Yang *et al*, 2001)

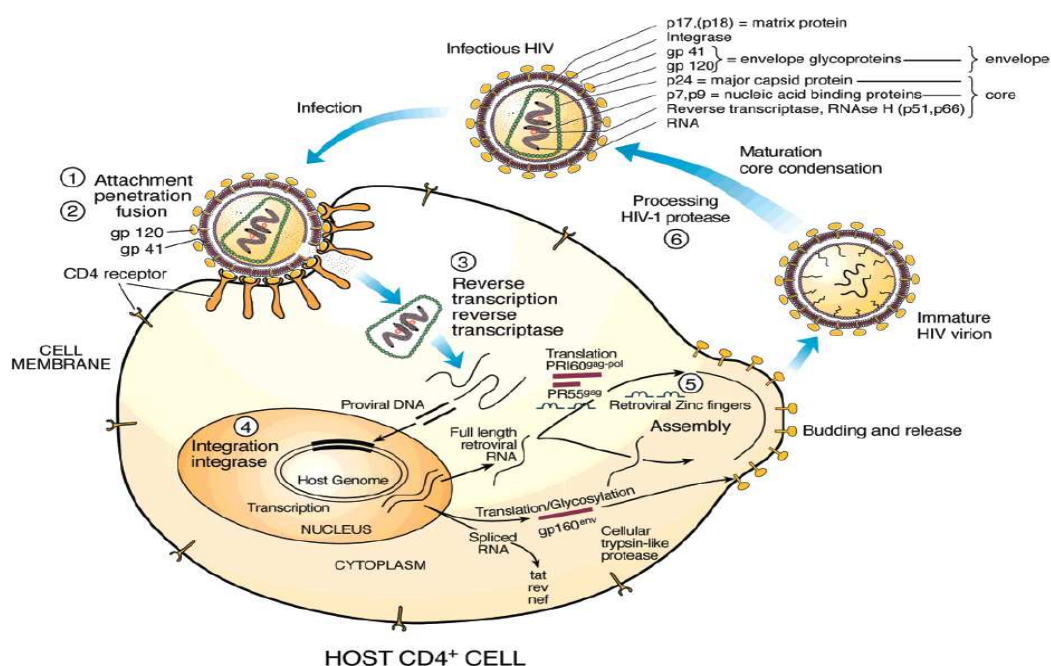


Fig.1.4 The HIV life cycle (Yang *et al*, 2001)

Another macroalgal lectin, ESA-2 isolated from *Euchema serra*, also shows specificity for high mannose type *N-glycans*. The sequence of ESA-2 is highly homologous with myxobacterium, *Myxococcus xanthus* hemagglutinin (MBHA). The structural similarity between lectins of prokaryotic bacterium and eukaryotic macro algae eventually led to the focus on lectins of cyanobacterium origin, as cyanobacteria are classified between bacteria and macro algae (Sato *et al*, 2007). Scytovirin (SVN), also a novel anti-HIV lectin isolated from the cyanobacterium *Scytonema varium* and is therefore also a compound selected for use in this study. Other cyanobacteria isolated lectins include Cyanovirin-N (CV-N), isolated from *Nostoc ellipsosporum* and has potent HIV inactivating activity (Li *et al* 2008 and Balzarini *et al*, 2005) and Sato *et al.* (2007) have recently purified and partially characterised a novel lectin from freshwater cyanobacterium, *Oscillatoria agardhii*. The compound, *Oscillatoria agardhii* agglutinin (OAA), interestingly shows high N-terminal sequence similarity to ESA-2 and MBHA. *Microcystis viridis* lectin (MVL) is a 13kDa protein isolated from the cyanobacterium *Microcystis viridis*. It is also mannose binding and inhibits HIV-1 fusion at nanomolar concentrations (Li *et al*, 2008). Balzarini *et al* (1990, 2004) have conducted studies on *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum hybrid* agglutinin (HHA). In their 1990 study, they found that the above mentioned D- mannose specific plant lectins as well as *Narcissus pseudonarcissus* agglutinin (NPA) and *Listera ovata* agglutinin (LOA) were able to inhibit MT4 cell infection by HIV1, HIV2 and Simian Immunodeficiency Virus (SIV). They were also able to conclude that these are also inhibitory to Cytomegalovirus infection. The lectins interact with either CD4 or gp120 to block virus adsorption, virus mediated cell fusion, infection and cytopathogenicity. In the 2004 study, their findings showed that HHA and GNA did decrease in sensitivity towards HIV-1 strains after 20-40 subcultures; however the nature of the mutations caused to the gp120 glycosylation sites by the plant lectins are entirely different to the mutations known to appear

under the pressure of other viral inhibitors. This is confirmed by the susceptibility of the plant lectin-resistant viruses to any other HIV inhibitor, including the mannose-specific CVN. Balzarini *et al* (2005) also showed that the stinging nettle *Urtica dioica* produces an agglutinin (UDA) that is an N-acetylglucosamine (GlcNac) recognising protein, which inhibits HIV in cell culture. Their findings indicated GlcNac-binding compounds are a novel and unique class of antivirals endowed with a highly specific and targeted resistance profile. They propose that UDA be used in combined therapy with neutralising antibodies as UDA binds and deletes the glycosylation sites, exposing underlying amino acid epitopes that are crucial for the virus to maintain its *in vivo* virulence (Balzarini *et al*, 2005). Both the mannose binding plant lectins, HHA, GNA and the Cybidium agglutinin (CA), as well as UDA inhibit entry and replication of Hepatitis C virus as well (Bertaux *et al*, 2007). Keyearts *et al* (2007) showed that many plant lectins with different binding capabilities, were potent inhibitors of coronaviruses, with the mannose specific lectin from *Allium porum* (Leek) (APA) exhibiting the best anti-coronavirus activity. The GlcNac specific lectins UDA and tobacco lectin Nictaba were also markedly active against SAR-CoV. N-acetylgalactosamine-specific lectins, *Helix aspera* agglutinin and *Helix pomatia* agglutinin (HPA), are used to detect abnormal glycosylation patterns of IgA1 in patients with IgA nephropathy (Moore *et al*, 2006).

Owing to the above studies exhibiting promising results with regards to lectins acting as viral, and specifically HIV inhibitors, two HIV binding lectins, Griffithsin and Scytovirin, were chosen for this study as viable candidates for heterologous expression in plant cell culture. The need for expression of these proteins in such a system is justified by the difficulty encountered in producing these valuable molecules in bacteria.

1.5.1. Syctovirin

Scytovirin (SVN), a potent anti- HIV protein was originally isolated from extracts of the cyanobacteria, *Scytonema varium*. SVN has a molecular mass of 9713Da, contains five intrachain disulfide bonds, and binds to HIV proteins gp120, gp160 and gp41 but not to the cellular receptor CD4. Low nanomolar concentrations of SVN inactivate laboratory strains and primary isolates of HIV-1. The inhibition has been shown to involve selective interactions between SVN and high mannose oligosaccharide-bearing proteins. Specifically, SVN interacts with oligosaccharides containing α 1-2, α 1-6 trisaccharides units. (Xiong *et al*, 2005). Figures 1.5 and 1.6 below illustrate the resolved and solution structures of SVN.

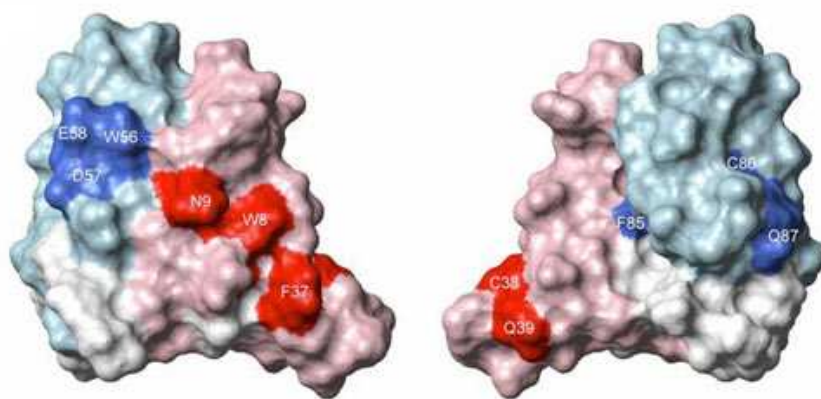


Fig.1.5 High resolution structure of Scytovirin (Xiong *et al*, 2005)

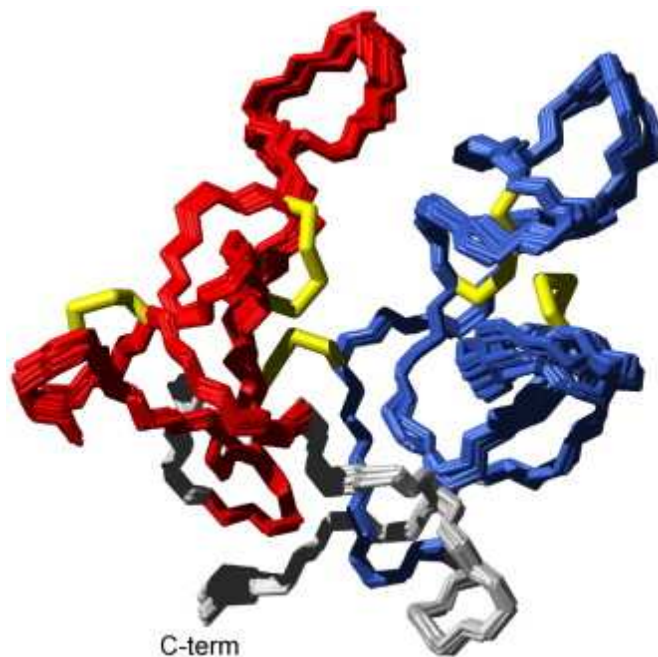


Fig.1.6 Solution structure of Scytovirin calculated calculated from refinement against $H^{\alpha}C^{\alpha}$ and NH RDCs. SD1 is colored red, SD2 blue, disulfide bonds in yellow, and the linker and termini gray. (McFeeters *et al*, 2007)

The solution structure of the potent 95 residue anti-HIV protein scytovirin has been determined and two carbohydrate-binding sites have been identified i.e. SD1 and SD2, as seen if figure 1.5 (Mcfeeters *et al*, 2007). This unique protein, containing five structurally important disulfide bonds, demonstrates a novel fold with no elements of extended regular secondary structure. Scytovirin contains two -39 residue sequence repeats, differing in only three amino acid residues, and each repeat has primary sequence similarity to chitin binding proteins. Both sequence repeats form similarly structured domains, with the exception of one region. The result is two carbohydrate-binding sites with substantially different affinities. The

unusual fold clusters aromatic residues in sites, suggesting a binding mechanism similar to other known hevein-like carbohydrate-binding proteins but differing in carbohydrate specificity (McFeeters *et al*, 2007).

Syctovirin (SVN) has been recombinantly produced in *E. coli* cells. However, this method is not adequate as the disulfide bonds of the protein require specific partners to generate correctly folded protein, and recombinant *E. coli* production of SVN produced significant amounts of misfolded protein (Xiong *et al*, 2005).

1.5.2 Griffithsin

Collected from the waters off New Zealand, Griffithsia is best known for producing the fluorescent proteins of the phycoerythrin class, which are used as labels in a variety of biochemical and cell biology methods. In addition, the isolation of certain matrix polysaccharides and photosynthetic pigments, as well as the identification of proteins of common evolutionary interest such as cyclophilins have been published (Mori *et al*, 2005). Griffithsin (GRFT) is the most recently isolated anti-viral lectin, isolated from red alga, *Griffithsia* sp. and it has been shown to inhibit cytopathic effects of different isolates of HIV-1 at concentrations as low as 43pM, and its binding to viral envelope glycoproteins was shown to be directly dependant on their glycosylation sites. These properties make Griffithsin a promising potential candidate for development as a future pharmaceutical agent. Careful analysis of Griffithsin structure and sequence (Fig.1.7), indicates that this protein belongs to a diverse family of β -prisms-I or Jacalin related lectins and shares structural characteristic with proteins such as Jacalin, artocapin and heltuba. However sequence identity between Griffithsin and any of these proteins is less than 30% (Ziolkowska *et al*, 2006).

In a study conducted by Mori *et al* (2005), both recombinant and native GRFT showed potent antiviral activity against laboratory strains and primary isolates of T- and M- trophic HIV-1 with EC₅₀ values ranging from 0.043-0.63nm. High concentrations of GRFT were not lethal to any tested host cell type.

Even though GRFT can be produced in large quantities in recombinant *E. coli* cells, the shortfall of this method for its production is that there is improper protein folding which directly affects the molecules efficiency.

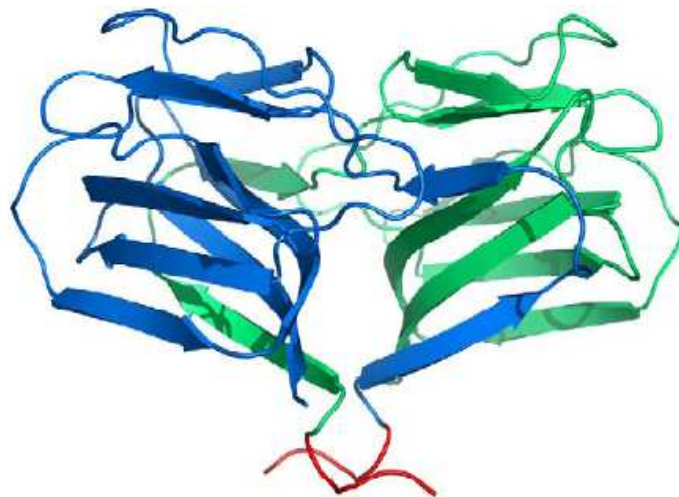


Fig.1.7 A ribbon α Trace of a Griffithsin Dimer, based on the co-ordinates of the His-Tagged version of the protein. Molecule A is colored blue, and molecule B green, with the N-terminal extension that resulted from the cloning procedure colored red (Ziolowska *et al*, 2006)

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Chapter Two

Establishment of Plant Cell Lines

2.1 Introduction to the Chapter

Plant cell suspensions have been used in several recent studies as a means of producing a variety of foreign proteins, with researchers stating that plant cells allow for expression levels similar to those of animal cells, have lower cost implications for culture maintenance, allow for simple purification and low possibility for product contamination (Lee *et al* 2004 and Streatfield, 2007). Even though rice and tomato cell cultures have also been tested for protein synthesis by Terashima *et al*, (1999) and Kwon *et al*, (2003), tobacco remains the most commonly used host species in accordance with literature (Doran, 2000), with focus being placed on the well characterised cell lines of the tobacco cultivars Bright Yellow 2 (BY2) and *Nicotiana tabacum* 1 (NT1) (Hellwig *et al*, 2004). The establishment of stable cell lines is an integral part to any plant cell transformation study and various parameters for cell growth need be optimised before transformation protocols can be employed. We describe in this chapter, the establishment of a proprietary tobacco cell suspension culture called SR1. This cell line was derived by callus induction of *Nicotiana tabacum* L. cv Petit Havana (SR1) leaf discs, which was cultured on Murashige and Skoog (MS) based callus medium supplemented with 2.21 mg/l 2,4 -dichlorophenoxyacetic acid (2,4-D). Friable callus development within 3 weeks of initiation resulted in the initiation of homogenous SR1 cell suspensions which enabled growth curve and cell morphology comparisons to be made between SR1 and BY-2 cell lines. The end result of the work in this chapter was the development of stable *Nicotiana tabacum* L. cv Petit Havana (SR1) cell lines for transformation experiments to follow.

2.2 Plant Cell Propagation

Plant cell propagation methods were developed in the 1950's, when it was realised that plant cells possessed the potential to synthesize a variety of useful, low molecular weight molecules and is safe, flexible and an efficient production platform metabolite production. It outweighs whole plant production systems by by-passing the long development time's, variations in product yield and quality and it eliminates contamination with fertilizers and pesticides.

2.2.1 *Some species used for cell suspension cultures*

Many plant species, both indigenous medicinal and cereal crops, have been used for metabolite production *in vitro*. At present, two plant secondary metabolites, Paclitaxel (Taxol) and Shikonin, are produced on a commercial scale using plant cell culture systems. Paclitaxel is extracted from cell cultures of *Taxus brevifolia* and is currently one of the most successful drugs used as cancer chemotherapy treatment (Hellwig *et al*, 2004). Shikonin has been shown to demonstrate antimicrobial, anti-inflammatory, wound healing and anti-tumor activity. Shikonin, extracted from root induced callus of *Lithospermum erythrorhizon*, is not only the first metabolite to be produced via industrial scale plant cell culture systems but is also the model system for secondary metabolism in plant cell basic research (Yamamoto *et al*, 2000). Other plant cell suspension cultures include those of *Arabidopsis thaliana*, *Taxus cuspidate*, *Catharanthus roseus* and domestic crops such as alfalfa, rice, tomato and soybean (Hellwig *et al*, 2004). In recent studies the authors were able to produce a sorghum cell suspension culture system for proteomics studies (Ngara *et al*, 2008) and Ritala *et al* (2008) showed that they were able to use barley cell culture systems for recombinant protein

production. Tobacco plant cell lines are extensively characterised, with the cultivars Bright Yellow 2 (BY2) and *Nicotiana tabacum* 1 (NT1) being the most popular species used (Hellwig *et al*, 2004).

2.2.2 Cell suspension culture development

In vitro plant tissue culture technique enables for the production of callus cultures i.e. the undifferentiated tissue that develops on or around an injured or cut plant surface or in tissue culture. It is these undifferentiated cell masses, in friable form, that are integral for the production of homogenous cell suspension cultures. A typical callus culture generally has three stages of development in both liquid medium and agar, namely a) the induction of cell division (b) a period of active cell division during which differentiated cells lose any specialised features that may have acquired and become dedifferentiated and (c) a period when cell division slows down and when, within the callus, there is increasing cellular differentiation. These stages of growth, when monitored according to various parameters such as cell number, cell dry weight and total DNA obtained, can be used to plot a growth curve. The growth curve is made of four distinct phase's which follow each other i.e. a lag phase, a period of exponential growth, a period of decline in growth, which is followed by a stationary phase when growth comes to a halt. Figure 2.1 below shows the phases of growth of a batch suspension culture (George *et al*, 2008).

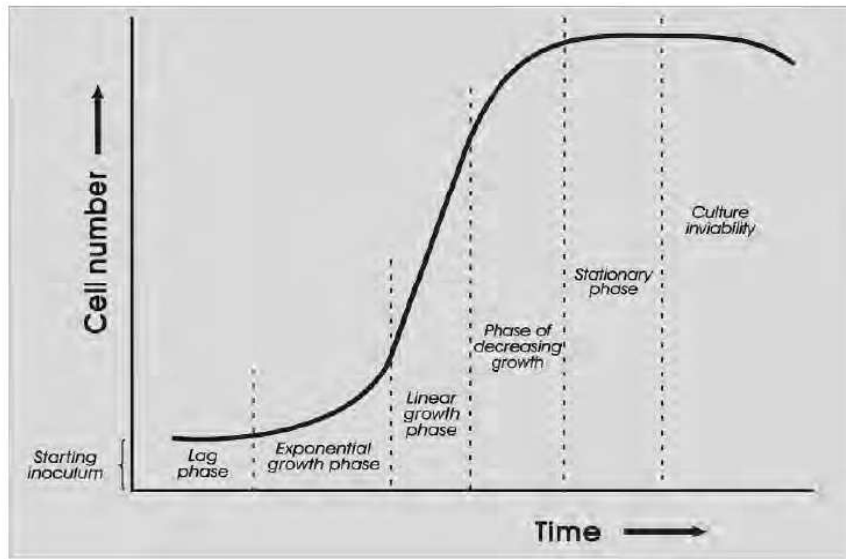


Fig.2.1. Phases of growth in a batch suspension culture (George *et al*, 2008)

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cells have to be met by the culture vessel, the growth medium and the external environment (light, temperature, etc.).

The growth medium usually consists of a solution of salts supplying the major and minor elements necessary for plant cell growth; together with an optional variety of vitamins and amino acids, an energy source and plant growth regulators to stimulate cell division and morphogenesis (George *et al*, 2008). Table 2.1 summarizes the function of some of the elements that play an integral part in plant physiology. (Slater *et al*, 2003).

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS), for tobacco tissue culture (Murashige and Skoog, 1962). The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia.

Table 2.1 Elements that are important for plant nutrition and physiological function (Slater *et al*, 2003)

Element	Function
Nitrogen	Component of proteins, nucleic acids and some co-enzymes. Required in greatest amount
Potassium	Regulates osmotic potential, principal inorganic cation
Calcium	Cell wall synthesis, membrane function, cell signalling
Magnesium	Enzyme cofactor, component of chlorophyll
Phosphorus	Component of nucleic acids, energy transfer, component of intermediates in respiration and photosynthesis
Sulphur	Component of some amino acids (methionine, cysteine) and some cofactors
Chlorine	Required for photosynthesis
Iron	Electron transfer and component of cytochromes
Manganese	Enzyme cofactor
Cobalt	Component of some vitamins
Zinc	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum	Enzyme cofactor, component of nitrate reductase
Copper	Enzyme cofactor, electron transfer reactions

As plant cell cultures are not photosynthetic, they also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). Sucrose is cheap, easily available, readily assimilated and is relatively stable. Other carbohydrates such as glucose, maltose, galactose and sorbitol can also be used. A gelling agent such as agar, produced from seaweed is most commonly used, but a range of purer agents such as Gelrite is often preferred. One other vital component that must also be supplied is water, the principal biological solvent.

Plant tissue culture media is supplemented with plant growth regulators, which are critical for determining the developmental pathway of the plant cells. There are seven classes of plant growth hormones, as shown in table 2.2. The most commonly used of these classes are auxins (Table 2.3) and cytokinins (Table 2.4). Auxins stimulate both cell division and cell growth. The most important naturally occurring auxin is Indole-3-acetic acid (IAA), however its use in plant cell culture media is limited because it is unstable to both heat and light. 2, 4-Dichlorophenxyacetic acid (2,4D) is a chemical analogue of IAA and is the most commonly used auxin as it is extremely effective in most circumstances (Slater, 2003). Cytokinins are a large group of structurally related, purine derivative compounds. Naturally occurring cytokinins such as Zeatin and 2-isopentyl adenine (2-iP) do not have widespread use as they are costly and relatively unstable. Often, cytokinins and auxins are used together, and the ratio in which they are used depicts the type of culture established. A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

Table 2.2 Plant growth regulators, chemical structures and major functions
(Sigma-Aldrich, Tissue Culture Protocols)

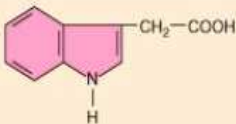

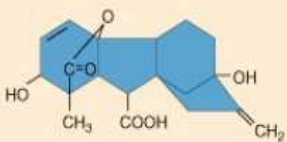
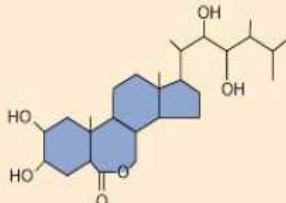
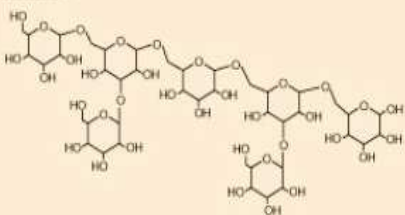

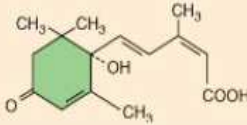
Hormone		Major Functions	Where Produced or Found in Plant
Auxins		Promotion of stem elongation and growth; formation of adventitious roots; inhibition of leaf abscission; promotion of cell division (with cytokinins); inducement of ethylene production; promotion of lateral bud dormancy	Apical meristems; other immature parts of plants
Cytokinins		Stimulation of cell division, but only in the presence of auxin; promotion of chloroplast development; delay of leaf aging; promotion of bud formation	Root apical meristems; immature fruits
Gibberellins		Promotion of stem elongation; stimulation of enzyme production in germinating seeds	Roots and shoot tips; young leaves; seeds
Brassinosteroids		Overlapping functions with auxins and gibberellins	Pollen, immature seeds, shoots, leaves
Oligosaccharins		Pathogen defense, possibly reproductive development	Cell walls
Ethylene		Control of leaf, flower, and fruit abscission; promotion of fruit ripening	Roots, shoot apical meristems; leaf nodes; aging flowers; ripening fruits
Absciscic acid		Inhibition of bud growth; control of stomatal closure; some control of seed dormancy; inhibition of effects of other hormones	Leaves, fruits, root caps, seeds

Table 2.3 Table of auxins (Slater *et al*, 2003)

Abbreviation/Name	Chemical Name
2,4 D	2,4-dichlorophenoxyacetic acid
2,4,5 -T	2,4,5-trichlorophenoxyacetic acid
Dicamba	2-methoxy-3,6-dichlorobenzioc acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MCPA	2-methyl-4-chlorophenoxyacetic acid
NAA	1-naphthylacetic acid
NOA	2- naphthoxyacetic acid
Picloram	4-amino-2,5,6-trichloropicolinic acid

Table 2.4 Table of cytokinins (Slater *et al*, 2003)

Abbreviation/Name	Chemical Name
BAP ^a	6-benzylaminopurine
2iP (IPA) ^b	[N ⁶ -(2-isopentyl)adenine]
Kinetin ^a	6-furfurylaminopurine
thidiazuron ^c	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea
Zeatin ^b	4-hydroxy-3-methyl-trans-2-butyrylaminopurine

^a Synthetic analogues^b Naturally occurring cytokinins^c A substituted phenylurea-type cytokinin

“Explant” is the term used to describe sterile pieces of whole plant used to initiate cultures and it may consist of organs such as leaves, roots or specific cell types such as pollen or endosperm. Feature of the explant are known to affect the efficiency of the culture and younger, more rapidly growing tissue is most effective for callus development. (Slater *et al*, 2003)

As *in vitro* cultures are normally axenic, it is necessary to eliminate possible contaminants from the surface of the explants. Such contaminants include fungal, bacterial and algal spores. Various sterilants are used to achieve this (as shown in table 2.5), the most commonly used compound being sodium hypochlorite (NaOCl), either as a chemical-grade reagent or as a commercial household bleach formulation. Other sterilants used include calcium hypochlorite, mercuric chloride and hydrogen peroxide. A wetting agent such as Tween 20 is often included to improve contact between the sterilant and the plant tissue

Table 2.5 Commonly used Disinfectants for Plant tissue Culture

(Sigma Aldrich Tissue Culture Protocols)

Disinfectant	Concentration (%)	Exposure Time (minutes)
Calcium Hypochlorite	9-10	5-30
Sodium Hypochlorite*	0.5-5	5-30
Hydrogen peroxide	3-12	5-15
Ethyl alcohol	70-95	0.1-5.0
Silver Nitrate	1	5-30
Mercuric chloride	0.1-1.0	2-10
Benzalkonium chloride	0.01-0.1	5-20

* Commercial bleach contains about 5% sodium hypochlorite, and thus may be used at a concentration of 10-20%, which is equivalent to 0.5-1.0% sodium hypochlorite

Callus culture is often performed in the dark as this avoids the cells photosynthetic ability which is a drawback for callus development as light can encourage differentiation of the callus. Callus cultures are usually composed of unspecialised parenchyma cells.

Friable callus provides ideal inoculum for cell suspensions. When friable callus is placed in liquid medium and then agitated, single cells or small clumps are released

into the medium. Under correct conditions these cells continue to grow and divide and subsequent subcultures builds biomass. Cells should be transferred at each stationary phase and it is therefore important for growth cycles to be determined.

(Slater *et al*, 2003)

Cell suspension cultures and its proliferative growth must be attained prior to cell transformation. This chapter describes the work which focused on the development of *N.tabacum* L. cv Petit Havana (SR1) cell lines.

2.3 Materials

2.3.1 Plant Material

Nicotiana tabacum cv Petit Havana (SR1) seeds were obtained from CSIR Biosciences, Plant Biotechnology seed collection from Dr. Bridget Crampton.

N.tabacum cv BY2 callus culture (on NT1 agar plates) was a gift from Ms. Kristin Mayo (Arizona State University). The BY2 cell line was used for comparison with the SR1 cell line developed in this study.

2.3.2 Media

Half Strength MS Media (1L) - 2.15g Murashige and Skoog salts was dissolved in 400ml of distilled water, to which 30g/l sucrose was added and the pH adjusted to 5.8. The final volume was made up to 1L, to which 3 g of gelrite was added prior to autoclaving.

NT1 liquid medium - Dissolve 30g of sucrose, 4.3g of Murashige and Skoog salts was dissolved in 400ml of distilled water, to which 30 grams of sucrose was added, followed by 50 ml MES(20x), 10 ml B1 inositol stock (100x), 3ml Millers 1 stock and 2.21 mg/l of 2,4D. The pH was adjusted to 5.7. The medium was then made up to 1 litre prior to autoclaving.

NT1 Solid Media (1L) – Same as above, with the addition of 3 grams of gelrite was added prior to autoclaving.

2.4 Methodology

2.4.1 Seed sterilisation and germination

SR1 seeds (± 40) were placed in a 1.5 ml eppendorf tubes and sterilised as follows. One ml of ethanol was added to each tube and mixed by inverting the tube a few times. The ethanol was thereafter carefully removed using a Gilson pipette. 1 ml of 50% commercial bleach (Jik) was subsequently added to the tube and the contents were mixed by inversion occasionally during the five minute sterilisation period. The seeds were then washed three times with 1 ml of sterile water which was once again carefully removed with a Gilson pipette to avoid losing too many seeds. Thereafter, 1 ml of 0.1% sterile, cooled agarose was used to re-suspend the seeds and the suspension was then pipetted onto half strength MS plates. Seeds were spread across the plate and the plates were wrapped and incubated in the plant growth room ($39-90 \mu\text{E}/\text{m}^2/\text{s}$) under controlled conditions of 16h light followed by 8 hr dark photoperiod at a temperature at $26 \pm 1^\circ\text{C}$.

After 2 weeks, the regenerated seedlings were transferred to half strength MS media in tubs to allow for shoot elongation and further root development. These plantlets were maintained on this media by transferring elongated shoot cuttings to fresh medium every 2 weeks, until they were used for callus culture initiation.

BY2 callus was maintained on NT1 callus medium throughout the above experiment.

2.4.2 Callus initiation and Maintenance

Callus initiation entailed using the already sterile leaves from previously regenerated SR1 plantlets, which were excised using sterile scalpels and forceps in a laminar

bench. The edges of the leaves were trimmed and each leaf was then cut into leaf discs. Discs were placed on NT1 solid media (Mayo *et al*, 2006) for callus regeneration. The plates were wrapped and incubated under dark culture conditions until callus developed. Callus was transferred every 3 weeks until a friable cell line was attained, which was used for initiation of cell suspension culture.

2.4.3 Initiation of cell suspensions

Three to four grams of friable callus of SR1 and BY2 cultures, was separately added to 40 ml of NT1 liquid medium and placed on a shaker in the dark culture room at 130 rpm. The first transfer of proliferating callus culture was two weeks after initiation, where 2 mls of cell suspension was added to 40 mls of NT1 liquid medium. Thereafter weekly transfers of uniformly growing cell suspension cultures were performed; once again 2 mls of cell culture was transferred to 40 mls of NT1 liquid.

2.4.4 Maintenance of cell cultures

Cell cultures maintenance entailed weekly transfer of 2 mls of the cell suspension cultures initiated in 2.4.3 above to 40 mls NT1 liquid media. This was thereafter cultured on a shaker in the dark at 130 rpm for a period of 3-4 days prior to transformation. Subsequent transfers also followed (over 6 weeks) to ensure stable cell lines and these were used to generate a growth response over a 7 day period.

2.4.5 Growth curve experiment for SR1 cell line

Cell suspension cultures were initiated by inoculating 80 mls of NT1 medium with 2 mls of 7 day old SR1 culture. Three -5 ml samples were removed daily from each flask for a 7 day duration and the fresh weight was measured by allowing the cells to

settle for a 20 min duration and removing the supernatant. The wet weight was measured and an average of the 3 samples per day was used to plot the curve.

2.4.6 Cell line characterisation

A comparison of cellular characteristics of both the *N.tabacum* L. cv Bright Yellow 2 and *N.tabacum* L. cv SR1 was carried out using the Olympus (112794) light inversion microscope. At day 6 of culture, both cell lines were diluted 1:10 in sterile distilled water prior to being viewed with the 10X optical zoom on the light inversion microscope.

2.5 Results

2.5.1 Germination, callus initiation and cell suspension cultures

Sterilized seedlings germinated and produced rooted plantlets within a month in culture (Fig.2.2) and the leaves of these plantlets were used to initiate callus cultures (Fig.2.3). Callus growth appeared within 3 weeks of culture and was allowed to proliferate. Subsequent transfers of callus resulted in friable SR1 callus cultures ideal for cell suspension initiation. (Fig 2.4). Cell suspension initiations with the friable callus dissociated easily in the liquid medium at 130 rpm and over 3 subcultures a homogenous line was developed (Fig. 2.5).



Fig.2.2 Germinated *SR1* seedling

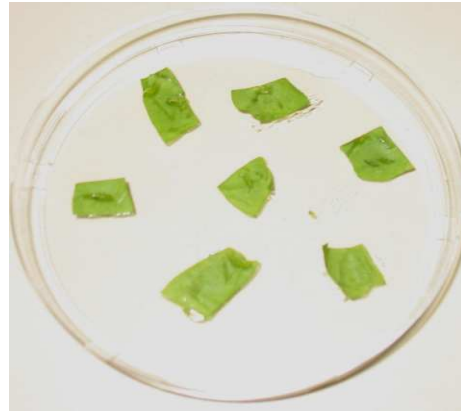
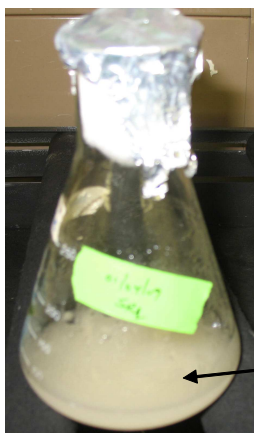


Fig.2.3 Sterile *SR1* leaf discs on NT1 callus
Medium prior to callus development



Fig.2.4 Friable *SR1* callus cultures



Callus disperses easily into liquid NT1, forms homogenous cell suspension

Fig.2.5 Homogenous, clump free *SR1* cell suspension culture

2.5.2 Growth curve of the SR1 cell line

The fresh weight measurement of the SR1 cell line over a 7 day period showed that the callus is in a lag phase for 4 days, and enters the exponential phase of growth until day 6, after which growth declines, as shown in Fig 2.6.

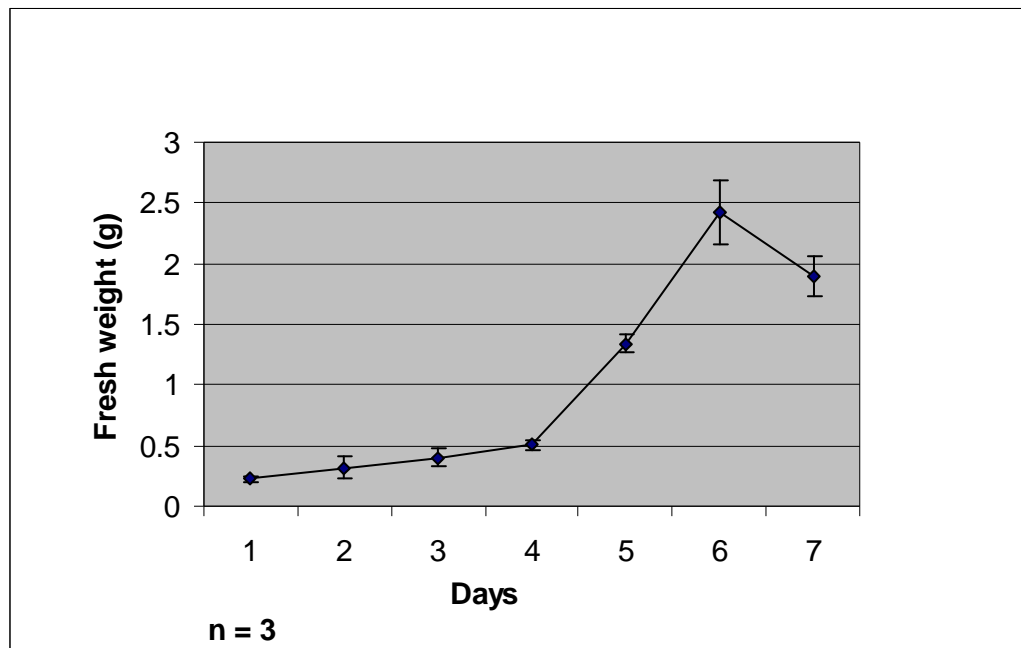


Fig. 2.6 Growth curve of SR1 over cells suspension culture over a 7 day period. The exponential phase of growth is clearly shown between day 4 and day 6, which is confirmed as the optimal transformation time for NT1 cells by Mayo *et al*, 2006.

2.5.3 Comparison of SR1 and BY2

Light microscope images of SR1 showed that the cell suspension is denser, building more biomass than BY2 cell suspensions on day 6 of culture (Fig.2.7). At 10X magnification the SR1 cells appear in elongated chain formation, indicating that they are stable enough for transformation (Fig. 2.8).

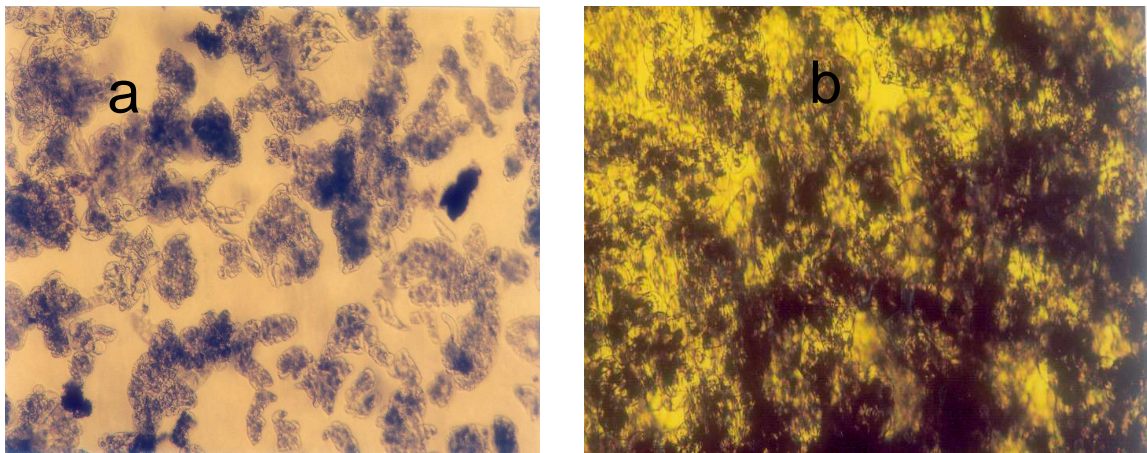


Fig.2.7 Light microscopic [Magnification (10X)] of 6 day old BY-2 (a) and SR1 cell suspensions (b).

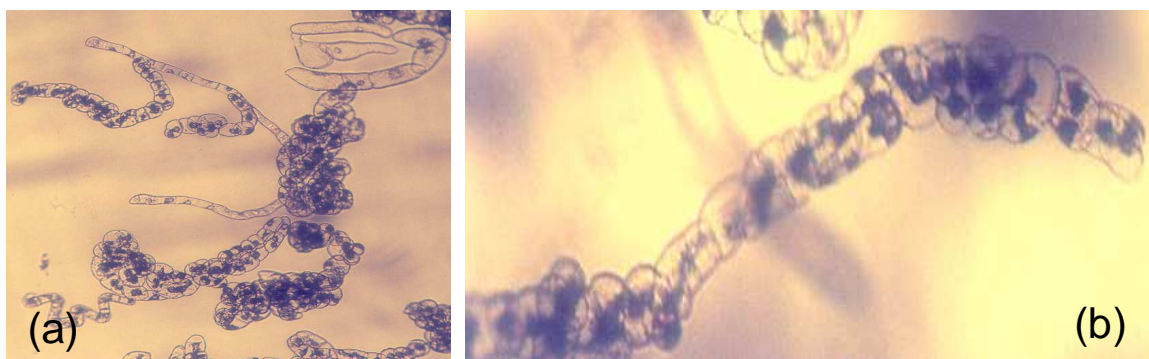


Fig .2.8 Light microscope image of SR1 (a) is the 10X magnification and (b) shows the 40X magnification, both showing distinct chain formation

2.6 Discussion

In an effort to develop *Nicotiana tabacum* cv Petit Havana (SR1) cell suspension cultures for transformation experiments, SR1 seeds were germinated and used to initiated callus cultures. Callus induction resulted in white, friable *N.tabacum* callus similar to callus cultures of BY2 in the studies conducted by Mayo *et al*, (2006). Once cell suspension cultures were initiated, inversion light microscopy was used to determine cellular morphology and make comparisons of the BY2 and SR1 cultivars. SR1 cells suspensions were considerably denser at the sixth day after subculture than BY2 cell suspensions, indicating that SR1 builds greater biomass than BY2. Based on the above observation, as well as the fact that BY2 cells are non-regenerable (Mayo *et al*, 2006), it was decided that the SR1 cultivar would be used for transformation studies. The cellular morphology of SR1 showed several chains of elongated cells, which is a characteristic of untransformed cells. Wild type (WT) cell lines form chains of cells and transformed cell lines form clustered circular cell phenotypes (Suchmelova-Maskova *et al*, 2008). Kim *et al* (1994) confirmed this in their study on cell morphology of *Catharanthus roseus* cell suspensions, and was further backed up by Suzuki *et al* (1990), showing the stability of morphological characteristics of spherical and cylindrical cells for a period of seven years. The SR1 cell line showed no sign of programmed cell death or necrosis at but instead exhibited fully nucleated, non- vacuolated cells. These results can be compared to morphology of tobacco cells exhibiting cell death morphology, as shown by Burbridge *et al*, (2006).

The SR1 cell growth curve showed the typical sigmoid shaped graph, depicting the exponential growth phase of the SR1 cell cultures between day 4 and day 6. It as decided that the cell suspension cultures would be transformed at day 4 as actively

growing cultures are required for transformation and selection (Forreiter *et al* 1997 and An 1985). In a study conducted by James *et al* (2002), they were able to demonstrate that in a bioreactor system, *N.tabacum* cell suspensions show acceptable growth by passive aeration through a foil covering. Therefore, the SR1 cell suspension cultures were grown in 250 ml Erlenmeyer flasks, with a cotton wool bung and covered with foil so as to maintain sterility and allow for passive aeration. These cell suspensions were maintained by transfer every 7 days to fresh NT1 medium, for all subsequent experiments.

2.7 Conclusion

The above study enabled the production of a stable, uniform SR1 cell line, with optimized growth conditions, which will be used in the following chapter for tobacco transformation experiments. By understanding the growth characteristics and cell morphology of untransformed SR1 cell cultures, we are now able to conduct further studies on the cell line. The cell morphology depicted healthy, untransformed cell phenotypes, a comparable point of reference during transformation procedures that follow. A growth curve experiment was essential to determine optimum time for transformation i.e. in the exponential phase, which was between days four and six for the SR1 cell line. This chapter successfully describes the development of a *Nicotiana tabacum* cv. SR1 cell line into suspension cultures that can be used for transformation.

2.8 References

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Chapter Three

Plant Cell Line Transformation

3.1. Introduction

Plant cell line transformation for the production of recombinant peptides of high value protein therapeutics is a fairly undeveloped area in comparison to whole plant transformations, with advantages outweighing whole plant transformation systems. The advantages include economically feasible growth and maintenance, with growth not being hampered by factors that affect field grown transgenic crops, and product isolation and purification being simpler as extraction procedures are not be hampered by products such as fibres, waxes oils, pigments etc. The main advantage of using plant cells for transformation is that the entire procedure is controlled in a bioreactor, meaning that current Good Manufacturing (cGMP) practices can be easily implemented.

Based on the above, as well as on the fact that no literature points to the anti-HIV lectins, Griffithsin and Scytovirin, being produced in cell culture systems, this chapter was focused on the construction of agrobacterium transformation vectors for the expression of GRFT and SVN genes, and to use this to transform SR1 tobacco cell lines. The SVN and GRFT genes were successfully cloned in front of the CaMV35S promoter, and the nopaline synthase (NOS) terminator cloned at it 3' end. Agrobacterium mediated transformation was applied to transform SR1 tobacco cell lines. Plant cell line transformation was confirmed by PCR, and protein expression confirmed by ELISA.

Foreign protein production in plant cells

Current methods for foreign protein production include using microbial cell culture, animal cell culture, plant tissue culture, transgenic animals and transgenic plants (Doran P.M, 2000). Over the recent years, whole plants for the synthesis of recombinant proteins has been given a lot of attention because of economical advantages, scalability and safety compared to microbial and mammalian production systems (Hellwig *et al*, 2004). However, potential issues of concern for plant protein production include containment of genetically modified plants in the environment, allergic reactions to plant protein glycans and other plant antigens, product contamination by mycotoxins, pesticides, herbicides and endogenous plant secondary metabolites, variability in cultivation conditions due to local differences in soil quality and microclimate and regulatory uncertainty for proteins requiring approval for human drug use (Doran P.M, 2000 and Hellwig *et al*,2004).

An alternative, but less well developed technology for producing recombinant proteins is plant cells, which avoids the abovementioned problems whilst retaining advantages such as inexpensive growth and maintenance, the ability to carry out post-translational modifications and freedom from mammalian pathogens and bacterial endotoxins. Plant cell performance is independent of climate, soil quality, season, day length and weather. Cell cultures eliminate the need for herbicides and pesticides, with fewer by-products such as waxes oils phenolics etc. The greatest advantage of a plant cell system over whole plants is that the procedure for product isolation and purification is simpler, making all good manufacturing procedures implement- able throughout the pipeline.

Transformation of plant cell lines

Methods for attaining transformed cell lines include the use of biolistics (particle bombardment) with DNA encoding the gene of interest or with transformed agrobacterium. In a study conducted by Ramussen *et al* (1993), the authors coupled biolistics with agro –mediated transformation, where they used transformed bacterial cells as microprojectiles to infect maize and tobacco cell suspension cultures. Another integrated bombardment and agrobacterium method exists, where micro wounds are inflicted on callus via particle bombardment and the callus is thereafter co-cultivated with *agrobacterium* for transformation (Droste *et al*, 2000). Alternatively, already confirmed transgenic whole plants can be used as a source of starting material to initiate callus cultures for cell suspension culture initiation (Girard *et al*, 2006 and Gao *et al*, 2004), leaf discs may be transformed using agrobacterium and thereafter used to regenerate whole plantlets, and then initiate callus cultures (Hong *et al*, 2001). For the purpose of this study, agrobacterium mediated transformation was performed on homogenous tobacco cell suspension cultures for the intended production of anti-HIV microbicides, using similar agrobacterium transformation methods for cell suspension cultures employed previously by Gao *et al* (2004) for the production of human coagulation factor XIII A-domain and the hepatitis B surface antigen (Sunil Kumar *et al*, 2003, Sunil Kumar *et al* 2005, Rodriguez *et al*, 2003 and Mayo *et al*, 2006).

Another imperative factor affecting product yield is the design of the construct used to express the recombinant protein. In order to attain efficient heterologous protein production, high levels of transcription of the introduced cDNA must be obtained. For this purpose, highly active promoters are required. Very few plant endogenous constitutive promoters have been identified to date, and some of these include

promoters driving the expression of ubiquitin (Callis *et al*, 1990), actin (Zhang *et al*, 1991), translational elongation factors (Axelos *et al*, 1989) and a cryptic promoter identified by random screening using T-DNA tagging (Foster *et al*, 1999). The search for strongly active promoters eventually led to plant viral sequences and to *Agrobacterium tumefaciens* Ti plasmid promoters, where the former ensures efficient viral replication and the latter is responsible for the synthesis of large quantities of opines during plant infection and colonization. Thus, the most widely used constitutive promoters in transgenic plant research are the promoter from the Cauliflower Mosaic Virus 35S transcript and the promoters of the nopaline and octopine synthase genes from *Agrobacterium*. (Rance *et al*, 2002 and An *et al*, 1986). In a study conducted by Zheng *et al* (2007), the authors found that the CaMV 35S promoter, when coupled with tissue and organ specific gene promoters in the same T-plasmid, would increase the activity of the tissue and organ specific gene promoter and enable it active in all tissue types. Thus, showing the powerful nature of the ubiquitous constitutive promoter the CaMV 35S.

A terminator is frequently utilized in plant genetic engineering to terminate expression of the inserted gene i.e. to halt synthesis of desired protein in the plant, after the protein synthesis has occurred. Terminators are further described as sequences that stop RNA polymerases and release both RNA and enzyme from the DNA (Watson *et al*, 1987). Terminators used in plant systems include the CaMV Poly A terminator (Lindh *et al*, 2009), applied in the authors study to halt expression of the p24 capsid protein from HIV-1 in both *Arabidopsis thaliana* and *Daucus carota* expression systems. Bouaziz *et al* (2009) employed the use of the 3' Nopaline synthase (nos) gene terminator in their study to determine if single domain antibody fragments, directed against Potato Virus Y, can be stably expressed in transgenic

potato plants. Hefferon and Fan (2004) showed promising expression of a vaccine protein in a NT1 plant cell line, using a geminivirus –based replicon system, using the Vegetative Storage Protein Terminator (Tvsp) sequence.

Plasmids also contain a selectable marker gene for the selection of transgenic plants or cells and they most often code for antibiotic or herbicide resistance (Xiao-Yi *et al*, 2006). The development of such markers, e.g. for kanamycin resistance, allows for direct selection of transformants without the induction of tumorigenic growth and suppression of cellular totipotency. (An, 1985)

Once cell cultures are transformed and proliferate on selection, it is essential that genomic DNA is extracted from cell cultures and PCR conducted with primers designed specifically for the inserted genes of interest to confirm correct DNA integration in the genome. Gene integration can be further confirmed by Southern analyses. Cell lines with simple gene integration patterns are perceived as potentially stable and are thus selected for further development.

The focus of this chapter was to construct agrobacterium transformation vectors harboring the GRFT and SVN genes, driven by the CaMV 35S promoter, with Kanamycin as the selectable marker gene and to terminate transcription with the VSP terminator (Tvsp). The resultant vectors will be used to transform SR1 tobacco cell lines established in the previous chapter and the correct integration of DNA in transformants will be confirmed by PCR analysis using the GRFT and SVN screening primers.

3.3. Materials

3.3.1 Lauria broth

10g bacto-tryptone

5g NaCl

5g Yeast extract

Make up to 1L at a pH of 7.0

3.3.2 GYT medium

10% (v/v) glycerol

0.125% (w/v) yeast extract

0.25% (w/v) tryptone

3.3.3 SOC medium (For 100ml)

0.5% yeast extract 0.5g

2% tryptone 2g

10Mm NaCl 0.058g

25mM KCl 0.018g

10Mm $\text{MgCl}_{2.6}\text{H}_2\text{O}$ 0.023g

20 Mm $\text{MgSO}_4.7\text{H}_2\text{O}$ 0.0493g

20Mm glucose 0.360g

3.3.4 YEP medium

10g Yeast extract

10g Bacto peptone

5g NaCl

pH7.0 and make up to a final volume of 1L . For solid media, add 8g of bactoagar per litre before autoclaving.

3.3.5 NTC washing medium

Dissolve 30g of sucrose, 4.3g of Murashige and Skoog salts (Sigma cat.no M5524), 50ml MES(20x), 10ml B1 inositol stock (100x), 3ml Millers 1 stock and 2.21ml (1mg/ml) 2,4D stock in deionised water and adjust pH to 5.7 with KOH, make up to 1L and autoclave. Once cool, add 300mg of Cefotaxime antibiotic per litre of media.

3.3.6 NTCK medium 1

Dissolve 30g of sucrose, 4.3g of Murashige and Skoog salts (Sigma cat.no M5524), 50ml MES(20x), 10ml B1 inositol stock (100x), 3ml Millers 1 stock and 2.21ml (1mg/ml) 2,4D stock in deionised water and adjust pH to 5.7 with KOH. After autoclaving, cool media before adding 300mg/l Cefotaxime and 300mg/l Kanamycin.

3.3.7 NTCK medium 2

Same as NTCK medium 1, but with reduced antibiotic concentrations. After autoclaving, cool media before adding 50mg/l Cefotaxime and 50mg/l Kanamycin.

3.3.8 DNA extraction buffer for genomic plant DNA

	Stock solutions	final solution 20 ml
1% Laurylsarkosyl	30% m/v	660 μ l
100 mM Tris-HCl	1 M pH 8.5	2 ml
100 mM NaCl	1 M	2 ml
10 mM EDTA	0.5 M pH 8.0	400 μ l
		14.94 ml H ₂ O to make up 20ml

3.3.9 Hepes (1mM pH 7.4)

Hepes (Sigma Cat.No. H3375)	0.283g
Double distilled Water	1000ml

Adjust to pH 7.5 with potassium hydroxide (KOH) and store at 4°C.

3.3.10 DH10B *E.coli*

A vial DH10B *E.coli* cells was obtained from the CSIR Biosciences culture collection. Competent cells of this *E.coli* strain was prepared for use in the cloning process.

3.3.11 EHA101 agrobacterium

EHA101 agrobacterium strain was obtained from the CSIR Bioscience culture collection. Competent cells were prepared and used in the transformation procedure.

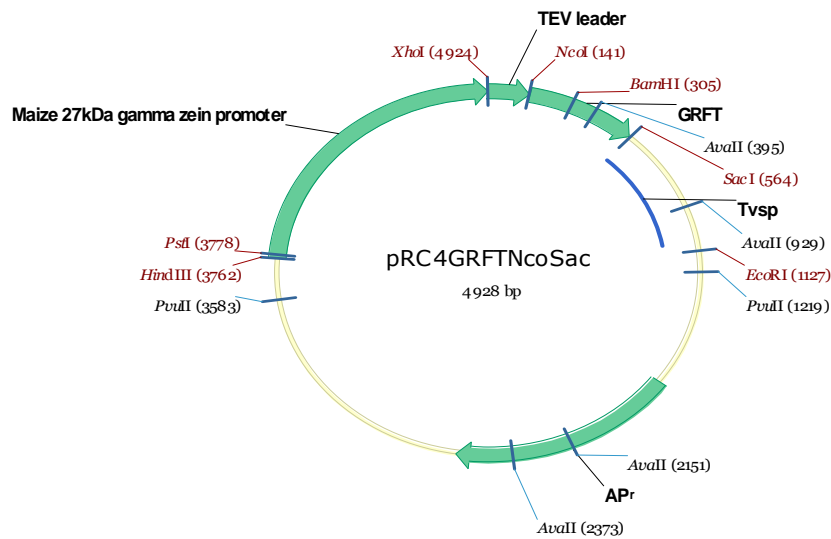
3.3.12 DNA Ladder

Fermentas O'GeneRuler 1kb DNA Ladder (SM1163)

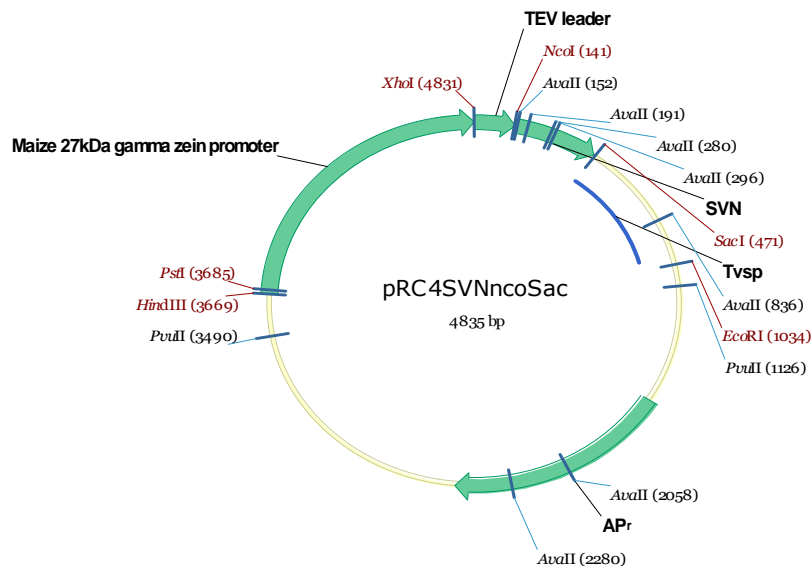
3.4 Methods

3.4.1 Source vectors

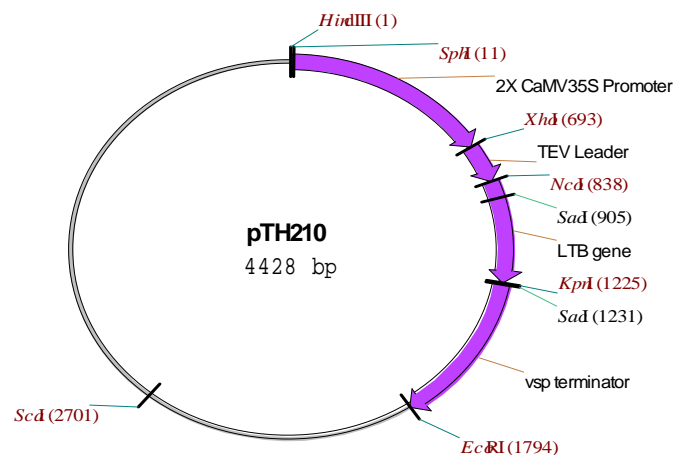
The relevant genes and promoter sequences were obtained from the following source vectors:



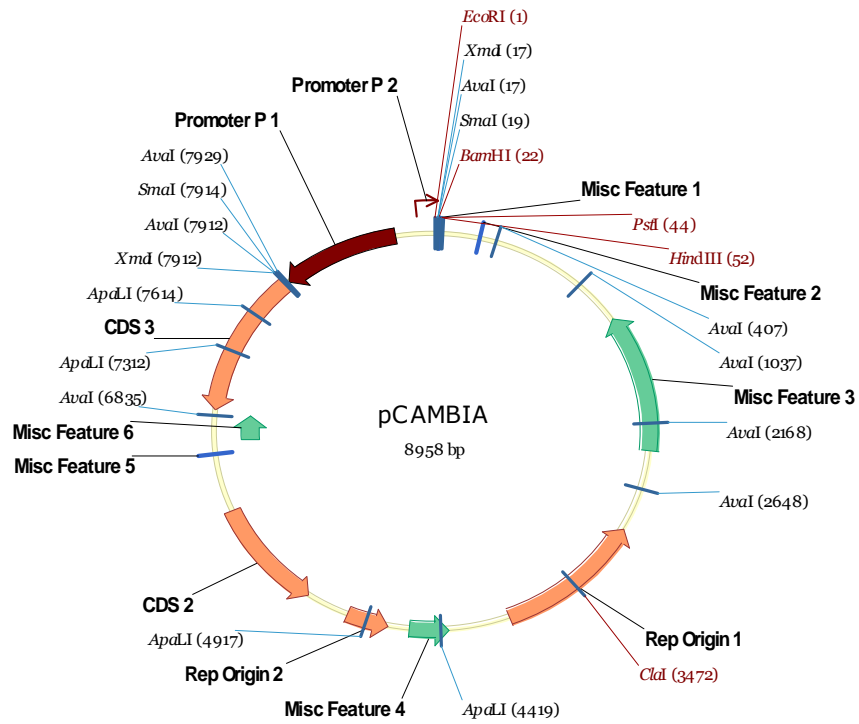
- 3.1.) Prc4GRFTNcoSac vector containing the relevant GRFT insert incorporated in the Nco/Sac restriction site. This GRFT insert is necessary for its incorporation into the pTH210 vector.



- 3.2) Prc4SVNncoSac vector containing the relevant SVN insert incorporated in the Nco/Sac restriction site. This SVN insert is necessary for it incorporation into the pTH210 vector



- 3.3) The pTH210 is an intermediary vector and is necessary for incorporation of the GRFT and SVN genes into its Nco1/Sac1 restriction site, which will then allow for these genes to be moved via restriction of the EcoR1/Hind111 sites and ligated to the pCambia vector. This also enables the incorporation of the vsp terminator and the CaMV35S promoter into the final destination vector.



- 3.4) The pCambia vector is the final destination of GRFT and SVN genes for expression and the Tvsp terminator, all of which will be incorporated into the EcoRI/HindIII restriction sites. These genes will be exported from the pTH210 vector.

3.4.2 Cloning of vectors for transformation of Agrobacterium

In summary, cloning entailed the restriction digest of the pRC4GRFTNcoSac (**Fig 3.1.**) and Prc4sSVNncoSAC (**Fig 3.2**) vectors with NcoI and SacI enzymes to retrieve the SVN and GRFT inserts. These were then inserted into pTH210 vector (**Fig 3.3**), which had to also be restricted with NcoI and SacI to retrieve the vector backbone. The inserts were thereafter ligated to the pTH210 vector. Thereafter, the pTH210 vector containing the inserts of choice were further restricted with EcoRI and HindIII enzymes in order for the inserts to be ligated to the pCambia vector (**Fig 3.4**) as this vector is driven by the CaMV35s promoter and the T-left and T-right borders necessary for agrobacterium initiated cell culture transformations, as well as the kanamycin resistance genes for selection purposes. **Figures 3.5- fig. 3.8** in the results section show the resultant constructs of the cloning process, to be used for agrobacterium transformation of the SR1 cell lines.

NcoI/SacI Digests were set up as follows:

The NcoI/SacI digests for retrieval of the GRFT and SVN inserts and the NcoI/SacI digest of the p TH210 vector to retrieve the vector backbone was conducted as follows: The reaction mix consisted of 500ng of plasmid DNA, 1µl of enzyme i.e. NcoI, SacI, (10 Units concentration each/µl), 2µl of buffer i.e. Buffer SacI and deionised water to make up reactions to 20µl. Reactions were incubated at 37°C for 1 hour. The restriction digests were run on a 1% gel at 90V to retrieve the necessary inserts and vector backbone.; The bands were cut out from the gels and purified using the ZymoClean Gel DNA Recovery Kit (Zymo Research Corp, Catalog no. D4001). The pure DNA from this step was then used in ligation reactions.

Ligation reactions were set up as follows:

Ligation reactions contained 50ng of the vector backbone DNA ligated to either 50ng or 150ng of GRFT/SVN insert DNA. Reactions also contained 2µl of 10x ligation buffer (Fementas Life Sciences) and 1µl of T4 DNA ligase (Fermentas Life Sciences). All reactions were made up to 20µl volume with sterile, deionised water. Ligation reactions were carried out at 16°C overnite. 4µl's of the ligation reaction was used for electroporation into *E.coli* competent cells. Competent cell were first prepared as below:

Preparation of electro-competent *E.coli* and electroporation

An overnight culture of DH10B *E.coli* was cultured in 100ml of Lauria broth (LB) medium with 1ml of *E.coli* and incubated at 37°C in a shaker incubator with vigorous shaking to an OD₆₀₀ of 0.7. The culture was then chilled for 30 min on ice, and then cells were harvested by centrifuging for 15min at 4000g, 4°C. The pellet was washed twice with 50 ml ice cold 10% glycerol, and then re-suspended in ice cold GYT medium to a final volume of 0.2ml. Aliquots of 100µl of the competent cells was made and stored at 80°C.

For electroporation pre-chilled cuvettes were used. 4 µl of ligated plasmid was added to 80 µl of previously made competent cells which was then left to stand for 20 minutes on ice. This was then transferred to the cuvette and electroporated using the Gene Pulser Xcell Electroporation System from (BioRad), at 1.8kV. It was transferred to ice immediately. After 2 minutes 150µl of SOC medium was added. The cells and SOC medium, in a 2 ml eppendorf, was then incubated at 37°C for 1 hour. Thereafter,

the electroporated cells were plated on LA plates with Ampicillin antibiotic for selection.

After overnight culture of plates on ampicillin selection, single colonies were grown in LB (1 colony to 5ml of LB medium). Plasmid extraction followed with a mini-prep kit (GeneJet Plasmid Miniprep Kit #K0502 – Fermentas Life Sciences). Restriction digests were carried out again with the Nco1/Sac1 enzymes and the product was run on a gel to confirm the correct vector/insert plasmid insertion via band size in gel electrophoresis.

After confirmation of the above, the plasmid was thereafter restricted with the EcoR1/HindIII enzymes to retrieve the insert for ligation to the pCambia vector. Restriction digest of the pCambia vector to retrieve the vector backbone and digestion of the pTH210 vector for the inserts were conducted as follows:

The reaction mix consisted of 500ng of plasmid DNA, 1µl of enzyme i.e. EcoR1 and HindIII, (10 Units concentration each/µl), 2µl. Buffer R and deionised water to make up reactions to 20µl. Reactions were incubated at 37°C for 1 hour. The restriction digests were run on a 1% gel at 90V to retrieve the necessary inserts and vector backbone.; The bands were cut out from the gels and purified using the ZymoClean Gel DNA Recovery Kit (Zymo Research Corp, Catalog no. D4001). The pure DNA from this step was then used in ligation reactions, as above. After ligating the pCambia backbone to the EcoR1/Hindiii digested inserts, the resulting plasmid was electroporated into competent *E.coli* cell (as per above), with the selection here being kanamycin antibiotic (100mg/ml). Plasmid extraction and re-digestion to confirm correct gene integration followed (**Fig 3.9- 3.10**) and the plasmid was thereafter electroporated into competent EHA101 agrobacterium.

Preparation of electroporation competent *Agrobacterium* cells

500ml of YEP medium was inoculated with the following antibiotics: Rifampicin (34mg/ml), Kanamycin (100mg/l), Chloramphenicol (34mg/ml) and Spectinomycin (10mg/ml). 400µl of a fresh overnight culture of the *Agrobacterium* (EHA101) cells was then added to the antibiotic/YEP medium and the culture was then grown overnight at 28° to an O.D of 0.5, read using the WPA Lightwave II (Labotec) at 600nm. Cells were harvested by chilling the flask on ice for 15-30 min., transferring to centrifuge bottles and centrifuging for 15 min at 4000g at 4°C. All steps thereafter are conducted on ice.

Supernatant was removed and cells were resuspended in 500 ml of 1mM Hepes pH7.4. Centrifugation followed once again as above. Cells were then resuspended in 250 ml 1mM Hepes. Centrifugation again followed for 15min at 4°C at 4000 g. Cells were then resuspended in 10 ml 1mM Hepes, transferred to tubes and centrifuged at 4°C for 15 min at 4000 g.

Finally, the cells were resuspended in 2ml ice-cold 10% glycerol, and aliquots were made at 45µl of cells per 1 ml eppendorf and were stored at -70°C.

Agrobacterium Cell transformation

For electroporation, 2µl of plasmid and 45ul of competent *agrobacterium* cells were added to a pre-chilled cuvette and electroporated at 1.44V. To confirm plasmid integration into EHA101 cells, plasmid extraction was conducted using the QIAprep Spin Miniprep kit (Qiagen) and the plasmid was run on a 1% agarose gel to confirm that it was indeed the correct inserted plasmid. **(Figure 3.11)**. Thereafter, glycerol stocks of the positive EHA101 cells were made for later use in transformation

experiments. This entailed the 1:1 addition of bacterial culture (O.D 0.5-0.6) and 65% glycerol in a 2ml eppendorf, followed by quick freezing in liquid nitrogen and storage at -80°C.

3.4.3 Transformation of SR1 cell suspensions (Co-cultivation)

a) Transformation of cell suspension cultures were carried out in accordance with the *Mayo et al*, (2006) protocol, with changes as described below.

Three to four days prior to transformation, *A.tumefaciens* strain EHA101, containing the plasmid of interest (i.e. pCambia/SVN, pCambia/GRFT,) from a glycerol stock was plated onto YEP plates with rifampicin (34mg/ml), Chloramphenicol (34mg/ml), Spectinomycin (10mg/ml) and Kanamycin (100mg/ml) for selection. Plates were incubated at 30°C for 24-48 hrs. Two days before transformation, a single colony was picked of the above mentioned YEP plates and cultured in 5 ml of YEP liquid medium with the appropriate antibiotics and incubated overnight at 30° C shaking at 175 rpm. The O.D₆₀₀ of the agrobacterium culture was measured at 600nm and should ideally be between 0.5-0.6

For transformation 40 µl of 20 mM acetosyringone was added to the flask which contained 40ml's of 3-4 day old SR1 cell culture i.e. 1 µl for every millilitre of cell culture. The cells were then abraded by repeatedly pipetting with a 5 ml sterile pipet (± 20 times). 4 ml of cells were removed and sealed in a petri dish (for negative control). The remaining 36 ml of culture was transformed in the 250 ml Erlenmeyer flask, where 200µl of agrobacterium culture was added to 36 mls of abraded cells. Thereafter flasks and the petri dishes were covered in foil and incubated on the shaker at 130 rpm for 24 hrs.

b) Selection

After the 24 hrs of co-cultivation period, cells were transferred to 50 ml conical centrifuge tube and made up to 50ml using NTC medium. Thereafter, the rigorous wash step with the NTC (NT1 medium + the cefotaxime antibiotic) followed. This was done with the intention of killing of or diminishing the agrobacterium in the cell suspension.

Tubes were inverted to mix and then centrifuged for 10mins at 210g using a swinging-bucket rotor. Thereafter, the supernatant was pipetted off and discarded and the wash step was repeated twice more. After the third rinse and removal of supernatant, NTC media was added to a volume of 20ml.

2ml of culture was plated per plate of selection media i.e. NTCK (NT1 medium +300mg/l Cefotaxime, to kill off the agrobacterium in the plant cells + 300mg/l Kanamycin to maintain the selection parameter for transformants). For a positive control, 2ml of transformed cells were plated on non- selection media i.e. NTC plates (NT1 medium + Cefotaxime). For a negative control, the untransformed cells were plated on NTCK media.

Plates were swirled for even distribution of cells and left open with lid ajar on laminar bench for 20-30mins to allow excess liquid to evaporate. Plates were wrapped in parafilm and cultured in the dark.

3.4.4 Bulking up of transgenic cell lines

Within a month of cells being plated on selection media, distinct single colonies began to develop against a lawn of “dead” cells (**Fig. 3.12**). These single colonies were selected and cultured on fresh NTCK selection media (**Fig. 3.13**). Transfers to

fresh media were conducted every three week and the cultures were bulked up in this manner.

3.4.5 Molecular characterisation – PCR analysis genomic DNA from transformed cell lines

Genomic DNA extraction protocol was kindly provided by Dr. Miriam Girgi, University of Hamburg (Germany), and conducted as described below.

DNA extraction from genomic plant DNA

Extraction method:

± Three grams of cell cultures (biomass attained per plate) was ground in liquid nitrogen to fine powder and added to a 2 ml eppendorf. Thereafter, 0.8 ml extraction buffer was added immediately. After gently mixing well, 0.8 ml of phenol/chloroform/isoamylalcohol (25:24:1) was added and the mixture was vortexed (phenol at pH 7.8). This was followed by centrifugation at 5000 rpm for 10 min. The supernatant was then removed and washed with one volume chloroform/isoamylalcohol (24:1). After washing, centrifugation followed again for 10 min at 5000 rpm and the supernatant was kept. DNA precipitation followed with 1/10 volume 3 M NaAc pH 4.8 and 1 volume of isopropanol and left at -20°C for 1-2 hours. The precipitation was then centrifuged at 13000 rpm for 30 minutes. The pellet was then air-dried and the DNA was dissolved in 100 µl TE. 2 µl of a 10 mg/ml stock solution of RNase was added to each eppendorf. DNA concentration was determined using the Nanodrop and DNA quality was confirmed by running 2 µl of DNA on a 1.2% agarose gel (**Fig. 3.17**)

PCR Primers

Sequences for primer development were attained with the GRFT and SVN molecules via Material Transfer Agreement between the National Cancer Institute and the CSIR.

The primers used to amplify the GRFT and SVN inserted genes (423bp and 330bp respectively) were;

1. For *Griffithsin*: GrftScr F 5'- CTG ACC CAT CGC AAG TTC GG-3' (Integrated DNA technologies)

GrftScr R 5'- GAT GTC CAG GCT ATC CAG-3' (Integrated DNA Technologies)

2. For *Scytovirin*: ScSDScr F 5' CTGGTCCGA CCTACTGC-3' (Inqaba Biotechnical Industries (Pty. Ltd)

ScScr R 5' GCCCTGGCAGAAACC-3' (Inqaba Biotechnical Industries Pty. Ltd)

All PCR's were carried out in a 50µl reaction mixture containing the specific Screen forward (Scr F) and Screen reverse (Scr R) primers (100ng or 1..25µl each), 12.5µl of PCR mix (KAPATaq readymix #KK1006), 300ng of template DNA and were made up to 25µl volume with Nuclease free water (Fermentas). PCR conditions were: initial denaturation for 5min at 95°C, followed by 30 cycles of amplification, each consisting of the following steps: 95°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 min, with a final extension at 72°C for 7 min.

PCR products were thereafter run on 1% agarose gels to determine if the GRFT and SVN genes were indeed present in the extracted genomic DNA and were indeed amplified. (Fig. 3.18 – Fig. 3.20)

3.4.6 Bulking up of transgenic cell lines in liquid culture

20 ml transformation experiments on selection media showed distinct areas of callus growth. However, it must be noted that the initial concentration of Kanamycin antibiotic and Cefotaxime in the medium had to be reduced. **Fig. 3.13** in the results section depicts clearly the effect of reducing the two had on calli development.

These raised areas were used for genomic DNA extractions and PCR analysis. Once the transgenic nature of the cells was confirmed, some of the callus was transferred to solid NTCK medium for bulking up whilst the rest was transferred to liquid NTCK medium. 0.4g of transformed calli was added to 40ml of NTCK in a 250ml Erlenmeyer flask. Flasks were placed on a shaker at 130 rpm in the dark and transfers were carried out every 2 weeks, where 10% inoculum was added to 40ml NTCK. After 3 passages, the cell lines were viewed under an inversion light microscope and the transformed cell morphology noted.

3.5 Results

Constructs as a results of cloning process:

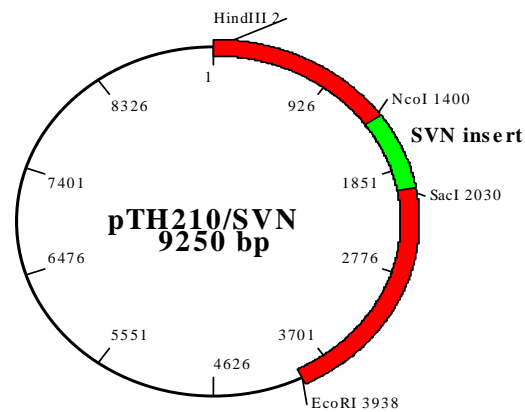


Fig.3.5 The ligation product of the SVN insert (isolated from Prc4SVNncoSAC) and the pTH210 backbone resulted in the formation of pTH210/SVN construct. This now allows for the restriction at the EcoR1/HindIII site for ligation to the pCambia vector

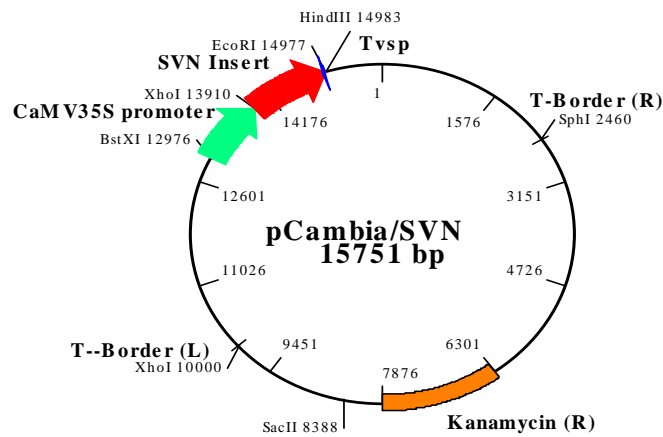


Fig.3.6 The EcoR1/HindIII digest of the pCambia vector resulted in the pCambia backbone, which was then ligated to the EcoR1/HindIII digested insert from p TH210. The final construct, pCambia/SVN was used to transform agrobacterium.

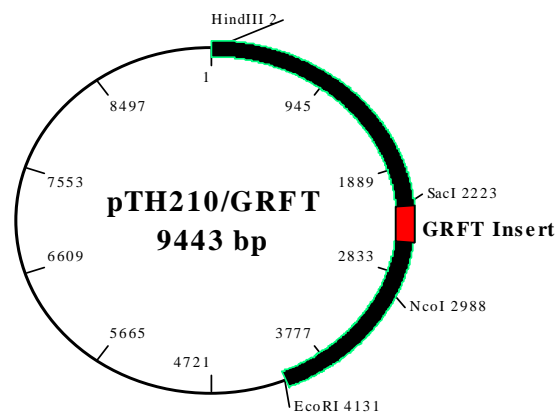


Fig.3.7 The ligation product of the GRFT insert (isolated from Prc4GRFTNcoSac) and the pTH210 backbone resulted in the formation of pTH210/GRFT construct. This now allows for the restriction at the EcoR1/HindIII site for ligation to the pCambia vector

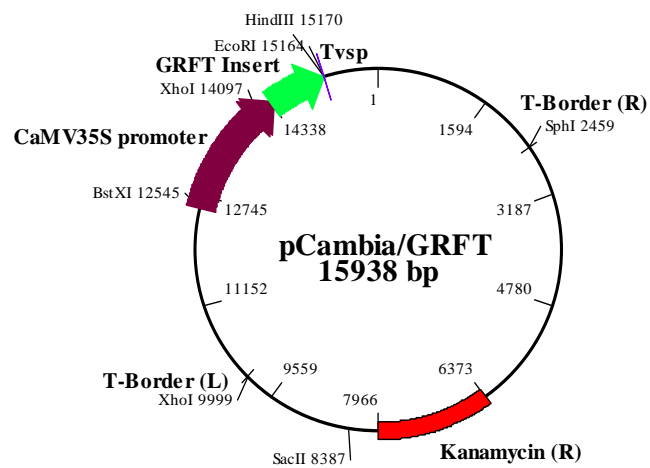


Fig.3.8 The EcoRI/HindIII digest of the pCambia vector resulted in the pCambia backbone, which was then ligated to the EcoRI/HindIII digested insert from pTH210. The final construct, pCambia/SVN was used to transform agrobacterium.

After cloning the GRFT and SVN inserts into the pTH210 vector, ECOR1/HIND111 restriction digests were carried out to obtain the correct sticky ends for ligation to the pCambia transformation vector. Once the sticky ends were ligated to those of the pCambia vector backbone and electroporated into *E.coli*, plasmid extraction and restriction digest with the ECOR1/HIND111 enzymes confirmed the correct insertion of GRFT and SVN inserts, as seen on agarose gels showing the 1819 bp and 1726 inserted bands respectively (Fig. 3.9 and 3.10).

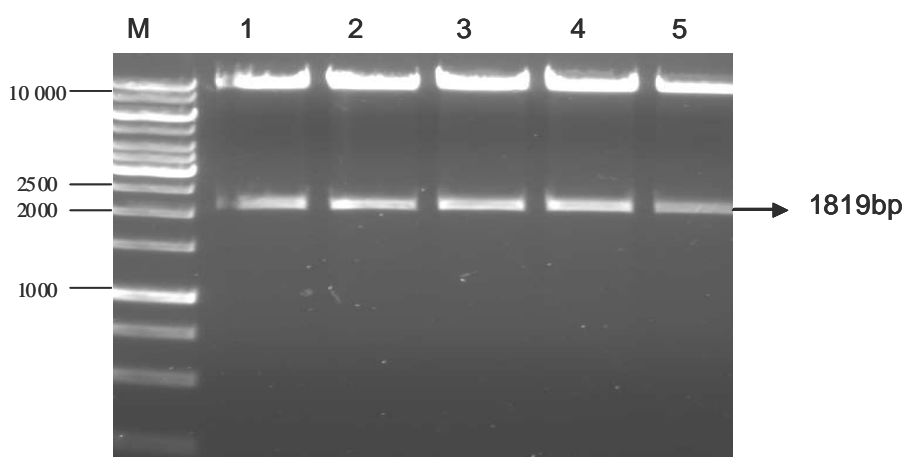


Fig.3.9 Agarose gel showing ECOR1/HIND111 restriction digest, confirming correct GRFT insertion via band size. Lanes 1-5 refer to plasmids isolated from 5 *E.coli* colonies and digested.

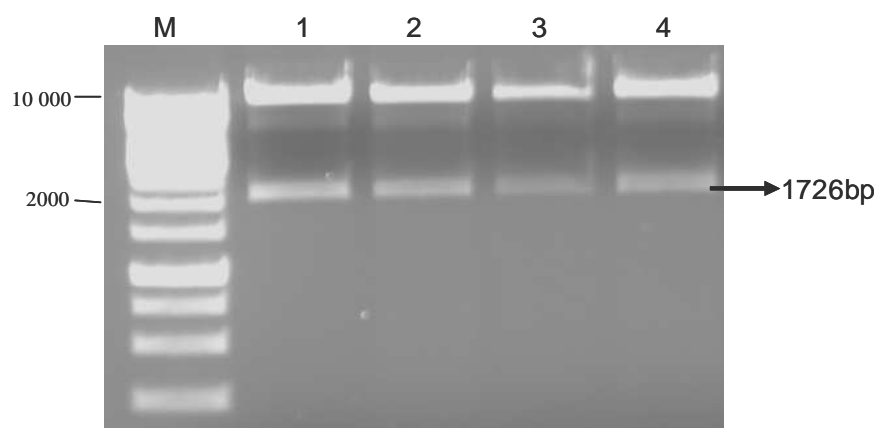


Fig.3.10 Agarose gel showing ECOR1/HIND111 restriction digest, confirming correct SVN insertion via band size (1726bp). Lanes 1-4 indicate 4 different colonies from which plasmids were isolated and digested.

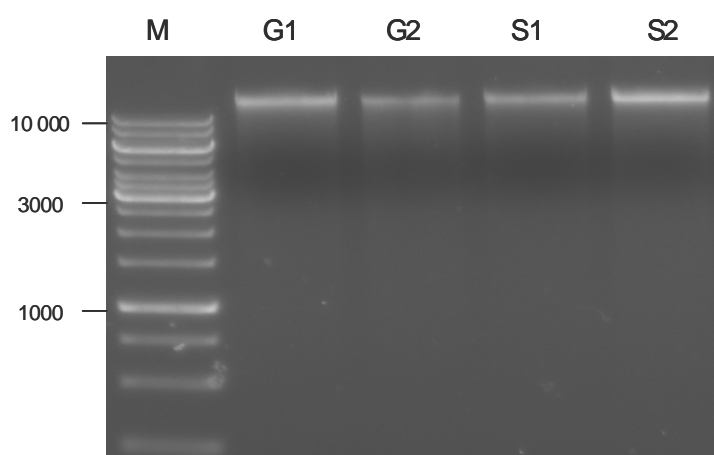


Fig.3.11 Plasmid isolation from EHA101 agrobacterium using QIAprep kit showing correct transformed plasmid size (10 500 -11 500 bp). Plasmids were isolated from two GRFT transformed agrobacterium colonies (G1 and G2) and two SVN transformed agrobacterium coloinies (S1 and S2).

Transformation experiments showed distinct single colonies which developed against a lawn of non-growing cells (Fig. 3.12). It is also worth noting that single colonies only developed on NTCK medium 2, which has a reduced kanamycin and cefotaxime concentration (50mg/l of each) (Fig. 3.13). These colonies were selected after an initial one month of growth and transferred to further selection in NTCK media. Colonies were initially plated on the basis of size i.e. all small colonies were plated together. Thereafter, fortnightly transfers were conducted to bulk up the initial colonies (Fig. 3.14). Not all colonies proliferated well, as depicted in Figure 3.15. Each prolific colony was numbered as a potentially transgenic callus line (Fig. 3.16). Genomic DNA extraction was carried out on the abovementioned lines and PCR analysis conducted, whilst callus was also maintained on selection for both protein analysis and cell suspension culture initiation. For PCR analysis, GRFT colonies 1-20 were screened. DNA extraction was conducted and DNA quality assessed on a 1.2% agarose gel (Fig. 3.17). GRFT PCR showed all colonies, except GRFT colony no. 2 as positive for GRFT gene integration (Figs. 3.18 – 3.19). Seven SVN colonies were screened, all of which were PCR positive (Fig. 3.20), confirming SVN gene integration in the SR1 cell lines screened. As an initial liquid medium experiment with, PCR positive GRFT colonies were used to initiate liquid culture experiments and cell morphology between transformed and non transformed SR1 cells was noted. Transformed cells were pinpoint and in differentiated clusters whereas non transformed cells formed elongated chains (Fig. 3.21)

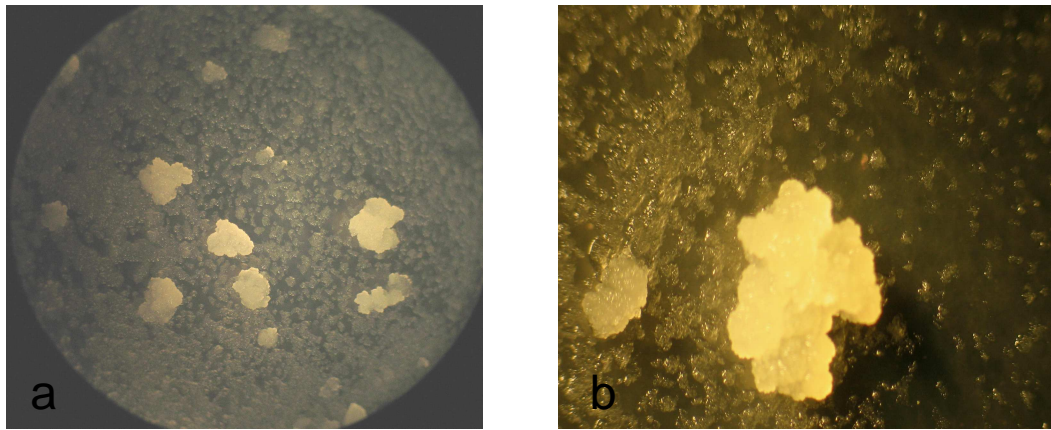


Fig.3.12 (a) Single colonies develop against a lawn of dead cells one month after transformation procedure. (b) 4x optical zoom of a single colony using the Olympus DF plan SZH microscope.

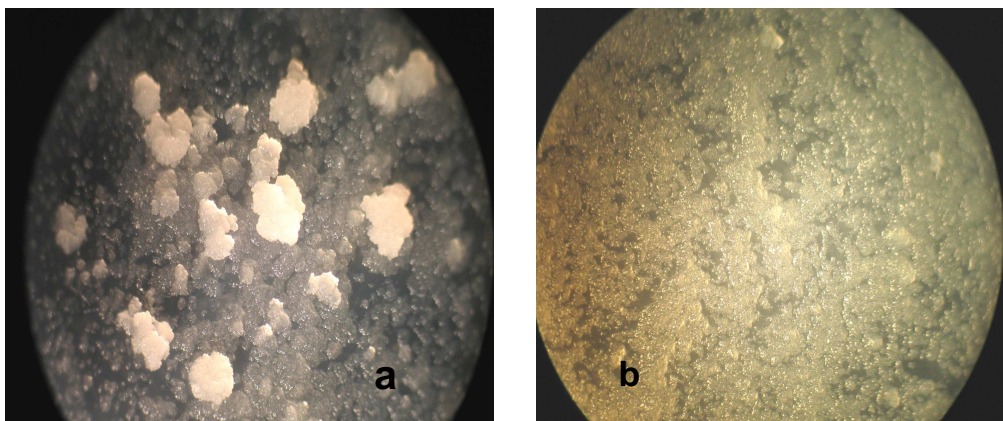


Fig.3.13 (a) Single colonies develop nicely on selection medium with 50mg/l of Cefotaxime and 50mg/l Kanamycin after 6 weeks of plating cells. (b) Cells on selection medium with 300mg/l Cefotaxime and 300mg/l Kanamycin after 6 weeks of plating cells

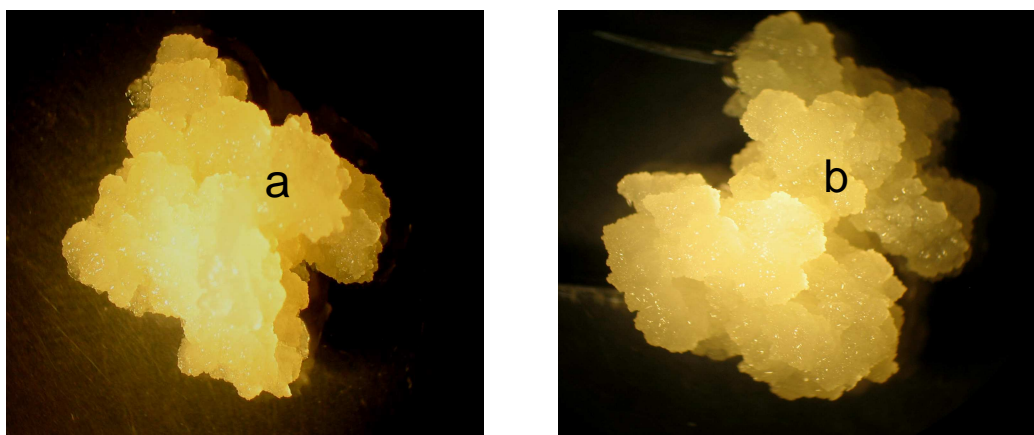


Fig.3.14 (a) and (b) are single colonies which continue to proliferate on selection medium after 2 transfers

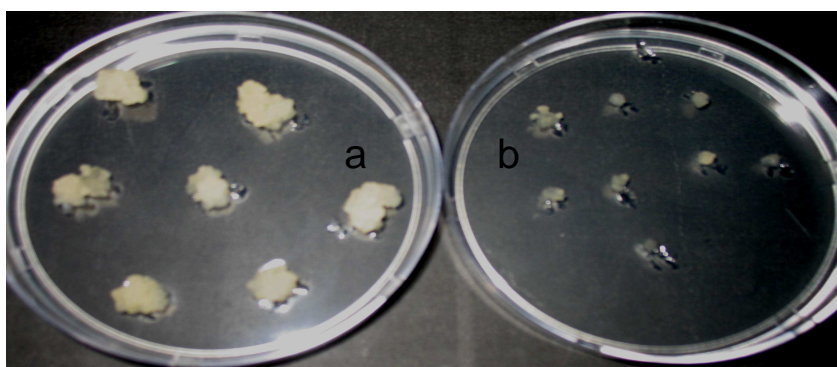


Fig. 3.15 (a) Colonies was initially separated on the basis of size. Plate (a), after the fourth transfer on selection medium proliferates whilst plate (b) does not, showing efficiency of the kanamycin selection

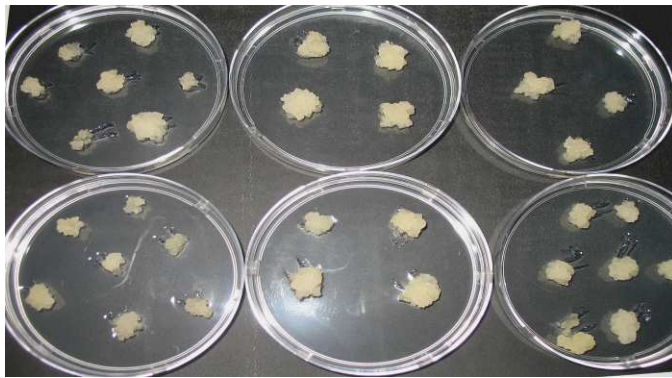


Fig.3.16 Each of the individual colonies on the above plates represents a potential Griffithsin producing callus line

Determination of DNA quality

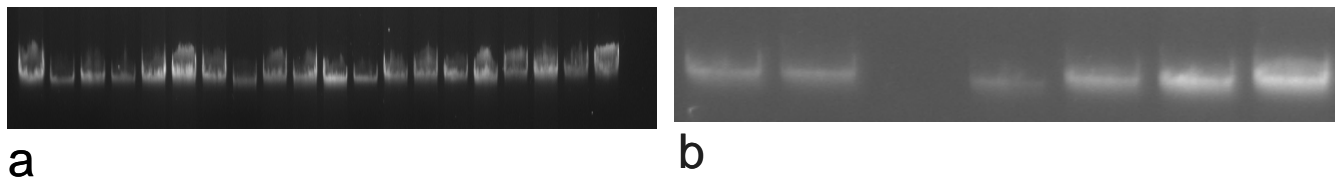


Fig.3.17 a) Genomic DNA extracted from GRFT transformed callus
b) Genomic DNA extracted from SVN transformed callus

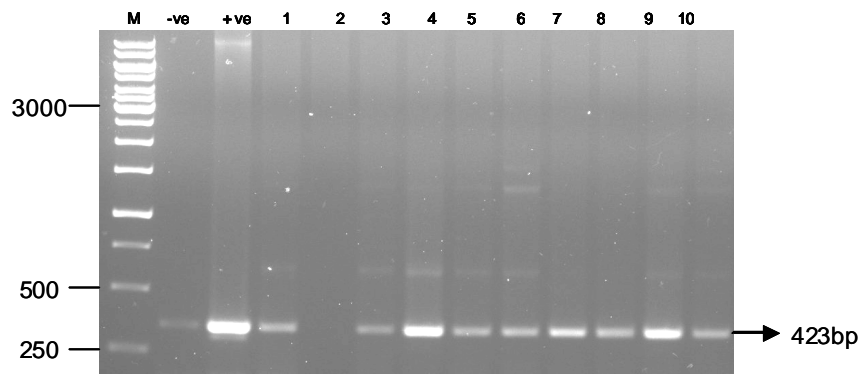


Fig.3.18 GRFT callus lines no. 1-10 were PCR positive, with the exception of callus line no. 2.

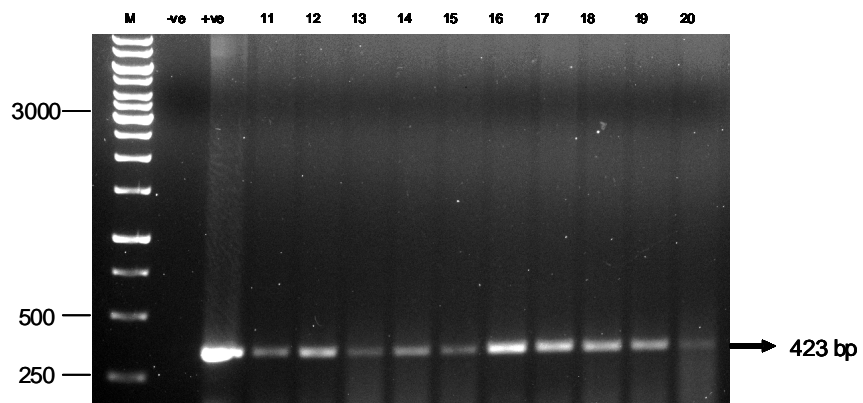


Fig.3.19 GRFT callus lines numbered 11-20 were PCR positive

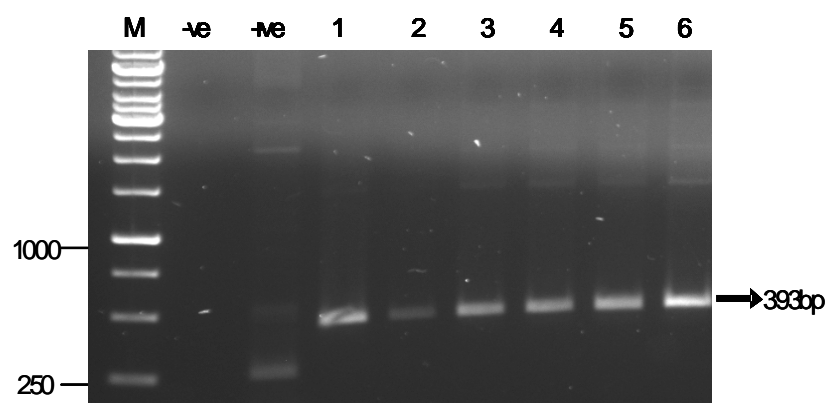


Fig.3.20 SVN callus lines 1-6 were positive positive

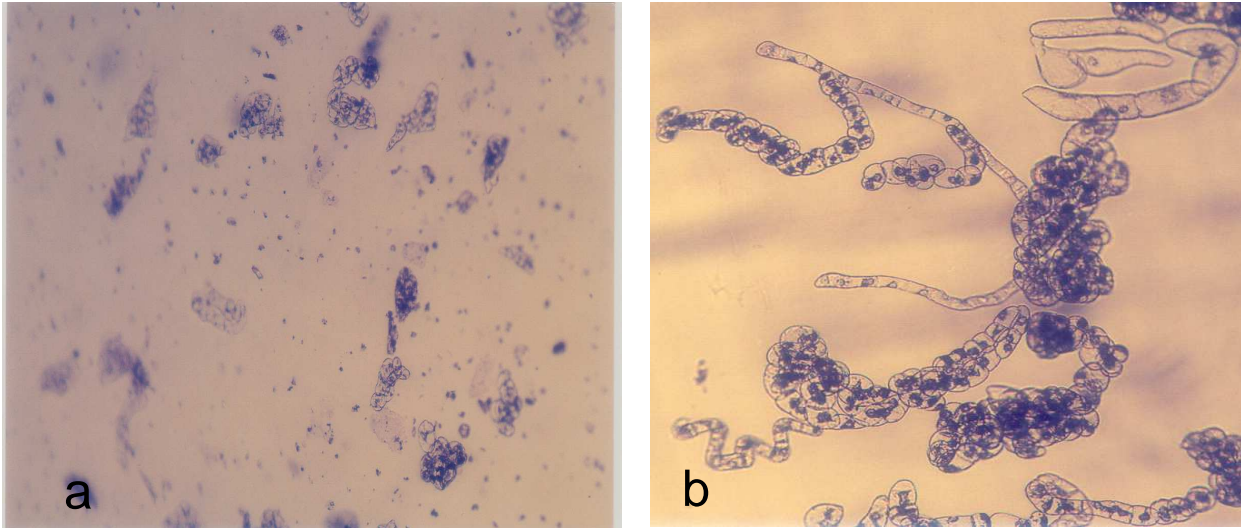


Fig. 3.21 (a) Cells transformed for GRFT expression. After 3 passages, cell suspension viewed under a microscope showed different cell clusters and pin point cells.

(b) Elongated chain formations of wild type SR1 cells

3.6 Discussion

Once the cloning procedure was completed, cell line transformation experiments were conducted. Within a month of culture on selection medium, single colonies developed against a lawn of dead cells. Genomic DNA isolations were performed on the distinct colonies and PCR analysis confirmed the transgenic nature of the cell lines, as the 423bp GRFT insert and the 330bp SVN insert were successfully amplified with the designed primers (Figure's 3.5.14 – 3.5.16). Once the cell lines were confirmed transgenic and transferred to further selection, it was noted that growth appeared at a slower rate than that of the untransformed cells. All initial experiments were carried out with 300mg/l of both Cefotaxime and Kanamycin in the selection media, a selection parameter also maintained by Mayo *et al* (2006) in their NT1 cell suspension transformation for the production of the Hepatitis B Surface Antigen (HBsAg). In their study, the authors maintained transformed cell lines on selection media with antibiotic for 3-4 transfers only, until it appeared free of agrobacterium growth and thereafter continued to bulk up on selection medium without antibiotic. After no transformants were derived from initial experiments, the selection parameter was drastically reduced to 50mg/l cefotaxime and 50mg/l kanamycin. Thereafter colonies of putative transformants developed within a month of plating (Fig. 3.5.9). The cefotaxime could possibly have a negative impact on growth as it was noted that the experimental negative controls (untransformed cells that underwent the abrasion and washing procedures before being plated on selection) also exhibited slower growth rates. Hellwig *et al* (2004) also noted in their review paper that transformed cell lines of *C.roseus* suspension cultures showed slower growth rates in comparison to wild type cells. Further experiments will aim at the eventual exclusion of cefotaxime in an attempt to speed up the bulking up of transformed callus. A few PCR

positive colonies were introduced into liquid culture as a preliminary experiment and after 3 passages in liquid medium; the transformed cells showed cell clusters of various sizes and many pin-point cells (Fig. 3.5.17). This kind of growth morphology was also observed by Suchomelove-Maskova *et al* (2008) and Orchard *et al*, 2005, where the tobacco BY-2 cultivar produced clusters of various sizes when transformed and appeared to not be uniform. These results confirm the vast differences in morphological characteristics of wild type SR1 and transformed SR1 cell lines.

3.7 Conclusion

This chapter aimed at transforming SR1 cell suspension cultures for the eventual production of Griffithsin and Scytovirin. These aims were adequately met as transformation vectors were designed and constructed and transformation experiments were successfully carried out, with the end result of PCR confirming transgenic cell lines of SR1 tobacco. The work conducted here is reproducible and comparable to cell suspension transformation methodology used for other cultivars of *Nicotiana tabacum*. Transgenic lines continue to bulk up on solid medium with the intention of initiating liquid cultures for the production of Griffithsin and Scytovirin and the monitoring of its growth condition thereof.

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Chapter Four

Confirmation of Expression of Griffithsin and Scytovirin Molecules

4.1 Introduction to Chapter Four

To determine if a method of recombinant protein production is comparable and economically feasible, expression needs to be confirmed and quantified. Methods for plant protein extraction and analysis exist, but need to be modified for plant cell produced proteins. This chapter deals with the extraction of plant made Griffithsin (GRFT) and Scytovirin (SVN), protein detection via Western Blot and the functionality and quantification of expression levels with gp120 binding Enzyme Linked Immunosorbant Assay (ELISA).

Quick screening via dot blot analysis confirmed the production of GRFT and SVN in SR1 tobacco cell lines. Further western blotting procedures conducted with total soluble protein extract confirmed that the recombinant SR1 tobacco callus cultures express GRFT protein in monomeric form and dimeric SVN. ELISA results indicate gp120 binding efficacy of the plant made product, however untransformed SR1 callus protein extracts do present some interference and bind concomitantly to the glycosylated gp120.

Expression of recombinant GRFT and SVN

In order to capitalise on the advantages of plant cell produced recombinant proteins in upstream production, it is vital that the downstream procedure be economically feasible with regards to levels of expression, protein stability and protein purification, which needs to be optimised and compared to other production systems.

To date, there are no reports of Griffithsin and Scytovirin being produced in plant cell culture systems, but expression has been noted in recombinant *E.coli* cells. Xiong *et al* (2005) showed that recombinant Scytovirin, produced in *E.coli* via SVN fusion protein, yielded approximately 5mg of HPLC purified scytovirin per litre of cell culture medium and Giomarelli *et al* (2006) showed expression levels of soluble His-tagged Griffithsin at 12mg/l at shake flask level. However, protein extraction and purification procedures make production of these two lectins in *E.coli* systems cumbersome as difficulties such as protein retention within inclusion bodies persists, and is unable to be solved by co-expression with a chaperone. Giomarelli *et al* (2006) have investigated ways of increasing the levels of Griffithsin produced in *E.coli* systems. They achieved this by using complex media containing yeast extract and tryptone, but still faced problems with regards to maintaining repression within cells, yields were still lower than fed-batch systems and problems related to protein solubility also presented.

Methodology for protein extraction from plant and *E.coli* cells differs, with the latter appearing more complex as it requires many more lengthy steps. Plant protein extraction is however faced with problems which need overcoming to obtain maximum total soluble protein as well. Some of the problems include the disruption of the rigid plant cell walls, which is hampered by the cellulose fibres often embedded

in a matrix of pectins, carrageenans (linear sulphated polysaccharides), agarose and other complex molecules. In addition, plant cells contain plastids and vacuoles, with vacuoles containing secondary plant products which can affect protein stability. Phenols and anionic polysaccharides cause problems during chromatographic purification as they bind strongly to the matrices. (Platis and Labrou, 2006). It is therefore necessary to select an extraction buffer which efficiently extracts protein, whilst not affecting protein stability and further purification steps. Agents such as DMSO have been shown to denature proteins and often makes interpretation of results tricky (<http://www.azonano.com>). PBS is most often selected as the extraction buffer for plant material.

Once protein is extracted, it can be analysed via Western Blotting. Western Blotting is a powerful and commonly used tool to identify and quantify a specific protein in a complex mixture. In a quick Dot Blot assay, samples are immobilized on a nitrocellulose or Polyvinylidene Difluoride (PVDF) membrane, whereas in a conventional Western Blot, protein samples are first resolved by size via SDS-PAGE and then electrophoretically transferred to the membrane. The Western Blot technique has been used successfully to identify plant made heterologous proteins in various studies to detect the human anti-rabies virus monoclonal antibody (Girard *et al*, 2006), the hepatitis B surface antigen (Rodriguez *et al*, 2003) (Sunil Kumar *et al*, 2003) and functional human coagulation factor XIII A-domain by Gao *et al*, 2004.

Enzyme Linked Immunosorbent Assay (ELISA) is a biochemical technique used to detect the presence of an antibody or antigen in a sample. *E.coli* derived SVN and GRFT were tested via ELISA to determine its binding efficiency to gp120. Giomarelli *et al* (2005) found that recombinant *E.coli* produced GRFT showed binding to gp120 in a concentration dependant manner, comparable to that of native GRFT.

Recombinant *E.coli* produced SVN binds to gp120 and gp41.

This chapter therefore aims to determine if GRFT and SVN are produced in the tobacco cell culture systems and to biochemically quantitate the levels of expression.

4.2 Materials

4.2.1 Cell cultures

The cell cultures used in this chapter were those previously transformed with the pCambia-GRFT and pCambia-SVN constructs (without signal peptide), and allowed time on selection medium, NTCK

4.2.2 10 x Phosphate Buffered Saline (PBS)

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ - 17.8 g

KH_2PO_4 - 2.4 g

NaCl - 80 g

KCl - 2 g

Weigh out the above and dissolve in 900ml of distilled water. Whilst stirring, adjust the pH to 7.5, then make up to 1L and autoclave.

4.2.3 Tris Buffered Saline (TBS)

20 mM Tris-HCl

150 mM NaCl

pH 7.5

4.2.4 Tris-buffered saline + Tween (TBS-T)

0.05% Tween20 in TBS

4.2.5 Bovine Serum Albumin/Trisbuffered Saline + Tween (BSA/TBS-T)

0.1% BSA in TBS-T

4.2.6 TBST-3% Non-fat dry milk solution

20mM Tris pH 7.5, 150mM 0.05% v/v Tween 20, 3% non fat dry milk

4.2.7 1 x Running Buffer

25mM Tris-HCL

200mM Glycine

0.1% w/v SDS

1 x Running gel (12% acrylamide in a 30ml volume)

H ₂ O	13.2 ml
1.5 M Tris-HCL, pH 8.8	7.5 ml
20% w/v SDS	0.15 ml
Acrylamide/Bis-acrylamide (40%)	9 ml
10% w/v ammonium persulfate (APS)	0.15 ml
TEMED (added last)	0.02 ml

The ingredients (recipe in materials) were gently mixed in the order above, ensuring that no air bubbles formed. It was then poured carefully into the glass assembly plate.

4.2.8 1 x Stacking gel (4% acrylamide in a 10ml volume)

H ₂ O	6.49 ml
0.5M Tris-HCL, pH 8.8	2.5 ml
20% w/v SDS	0.05 ml
Acrylamide/Bis-acrylamide (40%)	1.04 ml
10% w/v ammonium persulfate (APS)	0.05 ml
TEMED (added last)	0.01 ml

The stacking gel was made up and poured onto the top of the set running gel.

4.2.9 The protein sample loading buffer

50mM Tris-HCL pH 6.8

2% SDS

10% glycerol

1% β -mercaptoethanol

12.5 mM EDTA

0.02% bromophenol blue

4.2.10 Transfer buffer

Towbin Transfer Buffer

25mM Trizma-base pH 8.3, 192mM glycine, 20% v/v methanol

4.2.11 Membranes

Dot Blot – Nitrocellulose Membrane (BIORAD)

Western Blot – Polyvinylidene fluoride (PVDF) Membrane

4.2.12 Antibodies

Primary antibody – Anti-GRFT Rabbit polyclonal antibody

Anti-SVN Rabbit polyclonal antibody

The above antibodies were kindly provided by Dr. Barry O' Keefe (NCI, Frederick),
NIH –US.

Secondary antibody- Goat Anti-Rabbit secondary antibody (Sigma)

4.3 Methodology

4.3.1 Protein Extraction

Protein extraction was carried out from cells that were confirmed positive for SVN and GRFT gene integration via PCR.

Total protein was extracted per plate of transformed cells (\pm 3g/plate) as well as from an untransformed negative control. A 1:1 extraction was conducted i.e. 3g of callus: 3ml of PBS.

A miniature pestle was used to disrupt the cells via grinding. The cells in extraction buffer were shaken overnight at 4°C. The cells in buffer was then centrifuged at 12000 rpm for 30 mins at 4°C. The supernatant was removed and used for Western Blot analysis and ELISA analysis.

4.3.2 Quick screen (Dot Blot Analysis)

Dot blot analysis was conducted to rapidly determine if there was GRFT and SVN protein expression indicated by the binding capabilities of the respective antibody to the cell culture protein extract.

The nitrocellulose membrane was cut to the relevant size and a grid was drawn on it. The membrane was soaked in 96%-100% ethanol and allowed to semi-dry. Then, using a narrow mouth pipette, 0.5ul-4ul of protein extract and positive control was slowly spotted on grids on the membrane. The membrane was allowed to dry. Thereafter, non-specific sites were blocked by soaking in 5% BSA in TBS-T for 1hr, on a horizontal shaker. Incubation with the primary antibody followed (1:1000), dissolved in BSA/TBS-T for 30mins, shaking on a belly dancer. This was followed by three 5 minute washes with TBS-T. The membrane was then incubated with secondary antibody GRFT/SVN- anti-rabbit 1:5000, dissolved in BSA/TBS-T for

30mins. Three 15 minute TBS-T washes followed. Blots were then developed in the dark by incubating with SIGMAFAST™ BCIP®/NBT tablets (Product no. B5655, Sigma Aldrich) dissolved in deionised water.

4.3.3 Western Blot Analysis

Western blot analysis was performed by separating 40 ul of total soluble protein extracted from cell cultures (an average of 20 000ng protein per 40 ul sample of GRFT transformed culture extracts and 10 000ng protein per 40ul sample of SVN transformed culture extracts) via Sodium Dodecyl Sulphate (SDS) Gel Electrophoresis, followed by transfer to PVDF membrane, and incubation with the relevant antibodies as described for the dot blot. After transfer to the Hyperfilm™ membrane (Amersham), detection of the proteins was carried out using ECL™ Chemiluminescent detection reagents (GE Healthcare – Amersham) which enabled the determination of kDa size of proteins expressed.

SDS Gel Electrophoresis

After pouring the stacking gel onto the top of the resolving gel, the comb was inserted and the gel was allowed to set. When the gel was set (\pm 30 mins), the comb was removed, the tank was assembled. The main chamber was then filled with 1x Tris-Glycine Running buffer to the “fill line” and the central chamber was filled to cover the gel wells only.

Protein extractions were conducted as mentioned in 4.1.1 and 40 ul of the TSP (20 000ng and 10 000ng for GRFT and SVN samples respectively) was loaded into gel wells, together with the protein marker and positive controls. The samples and positive control were heated together with loading dye at 100°C for 5 minutes prior to

loading into gel wells. The gel was run between 80-90 V for 4 hours. The protein marker used was the Pageruler Prestained Protein Ladder (Fermentas, SMO671).

Transfer of proteins to PVDF membrane

After running the gel, the gel cassettes were removed and rinsed with deionised water. The semi-dry transfer unit was then set up. The blot paper was cut to the same size of the gel and soaked in Towbin buffer. The PVDF membrane was then cut to the same size as the blot paper, equilibrated by soaking in methanol for 5 minutes and then soaked in Towbin buffer for 5 minutes. The gel was then removed from the plastic plates and incubated in Towbin buffer for 15 minutes. The blotting “sandwich” was assembled on the transfer unit in the following order up from the bottom up:

1) Blot paper, 2) PVDF membrane, 3) Gel and 4) Blot paper again. The unit was then connected to the power supply and run at constant current (5V) overnight or 15V for 1 hour.

Thereafter, the membranes were transferred to a vessel followed by a quick rinse in TBST. The further blocking, antibody binding and wash steps continued in the same manner as for the dot blots.

Detection of Western Blots

Blots were detected using the ECL (Chemiluminescence) kit (Amersham: RPN2124)

Detection Solution A and Detection Solution B were mixed in a 2000ul:50ul ration. The excess TBST was drained from the previously washed membranes and they were then placed protein side up on a sheet of Saran wrap. The detection solution was then pipetted onto the membrane and it was left to incubate at room temperature for 1

minute. The excess detection solution was drained of by holding the membrane gently with forceps and touching the edge against tissue paper. The blots were then placed protein side down onto a fresh piece of Saran wrap and the blots were then wrapped in it. The wrapped blots were then placed protein side up in an x-ray film cassette (Amersham: 8"x10" Hypercassette, RPN 1649) and secured with tape. The cassettes were moved to the dark room where a sheet of autoradiography film (Amersham Hyperfilm ECL, 18X24cm, code 28-9068-36) was placed on top of the membrane. The cassette was closed and exposed for 2 hours. The film was then developed by shaking in Developer solution (Manual x-ray developer, Film process/x-ray process), then followed by a rinse in 4% acetic acid, followed by shaking the film in the Fixer solution.

4.3.4 ELISA

To investigate binding of cell culture produced GRFT and SVN to glycosylated gp120, an ELISA assay was performed. The protocol was described by Mori *et al*, 2005, with slight modifications and was conducted in sequence as follows:

Capture antibody coating:

Each well of 96-well protein-adsorbing plates (Maxisorp Immunoplate, Nalge Nunc International) was coated with 100 ng of native HIV-1IIIB gp120 (Advanced Biotechnologies) in PBS at 37°C for 2 hr or overnight at 4°C. Plates were thereafter washed twice with PBS + 0.1% Tween 20 (PBST) and were then blocked with 5 % fat free milk in PBS buffer for a 2 hrs at 37° C or overnight at 4° C. A further two PBST wash steps followed.

Sample loading

Two-fold serial dilutions of recombinant GRFT and SVN (positive controls) and samples (cell culture protein extracts) were made in duplicate and were added to the gp120-treated wells. Cell line controls (untransformed) and BSA control wells were included and the plate was incubated for 2hr at 37°C or overnight at 4°C. Plates were then washed three times with PBST.

Detection antibody coating

Bound GRFT was detected by firstly incubating with a solution of anti-GRFT rabbit polyclonal antibodies. A 1:1000 (antibody: PBS) dilution of the anti-GRFT rabbit polyclonal antibody preparation was used and the incubation period was 37°C for 2 hours whilst shaking the plate. 4 x PBST washes followed, prior to incubation with the secondary antibody, goat anti-rabbit antibody conjugated to HRP (1:2500 dilution in PBS). Plates were washed for a further 4 times before detection.

Bound SVN was determined by incubation with anti-SVN rabbit polyclonal antibodies (1:1000 dilution in PBS) at 37°C for 2 hours whilst shaking, followed by 4 times PBST washes. Incubation with goat anti-rabbit antibody conjugated to HRP (1:2500 dilution in PBS) at 37 °C for 2 hours followed and plates were thereafter stringently washed four times with PBST.

Washing

200 ul of PBST was added into each well with a multi-channel pipette. The buffer was then toppled out of the plate which was then bashed against paper towel on the bench top to remove any residual buffer.

Detection and reading

Following addition of 50ul 3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate (Sigma cat.no 127k0778)) to each of the wells, reactions were stopped by adding 50ul of 1M sulphuric acid. Absorbencies were measured at 405 nm using the Bio-Tek EL808 plate reader and the Bio-Tek KC4 computer programme.

4.4 Results

Rapid dot blot screening of cultures

GRFT and SVN protein expression was noted as darker purple dots against the light purple background.

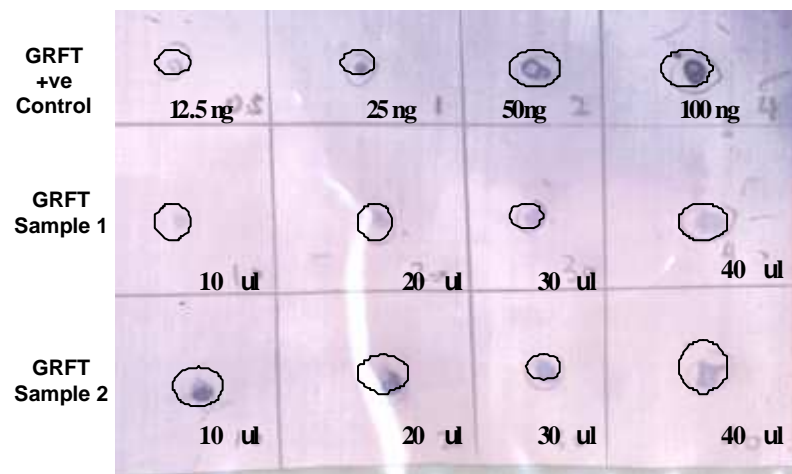


Fig.4.1 Dot Blot screen of GRFT transformed cells was conducted on concentration dependant manner at 10ul to 40ul. Protein was extracted from two callus lines proliferating on NTCK selection (labelled as GRFT Sample1 and GRFT Sample 2).

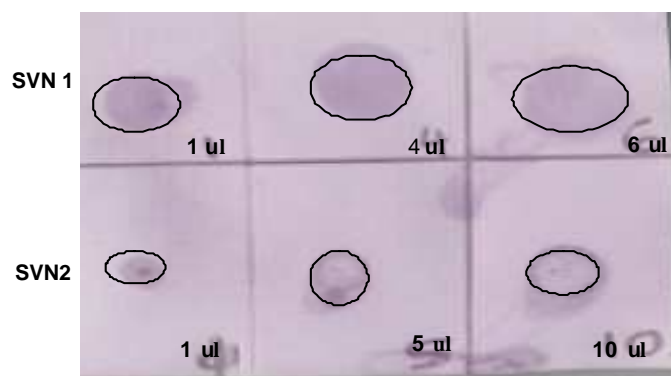


Fig.4.2 Dot Blot screen was conducted on a concentration dependant manner at a range of 10ul to 40ul. Protein was extracted from two callus lines proliferating on NTCK selection (labeled as SVN Sample1 and SVN Sample 2). Results confirm antibody recognition of samples.

Western Blot Analysis

Cell lines that proliferate on NTCK selection medium were screened for GRFT (Fig. 4.3) and SVN (Fig.4.4) below.

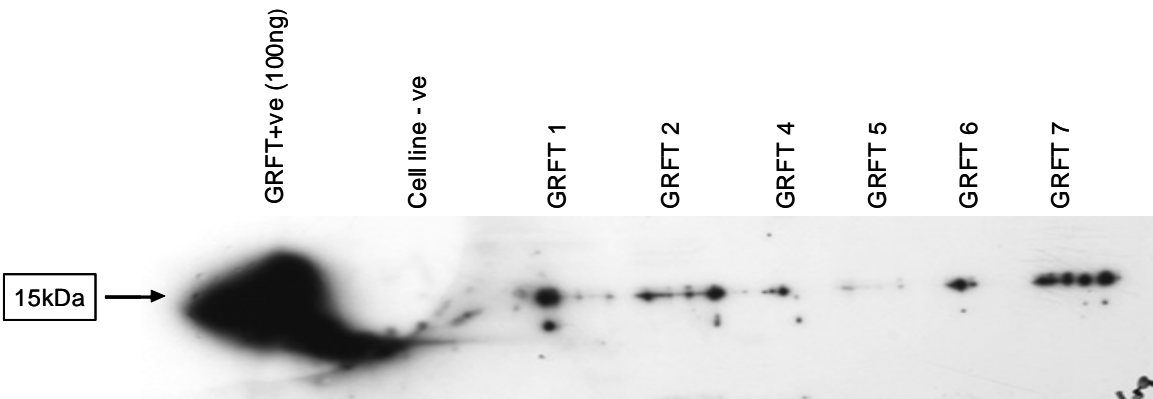


Fig.4. 3 40ul of PBS extracted protein was loaded per well. GRFT monomers at 14.5kDa were present in all samples.

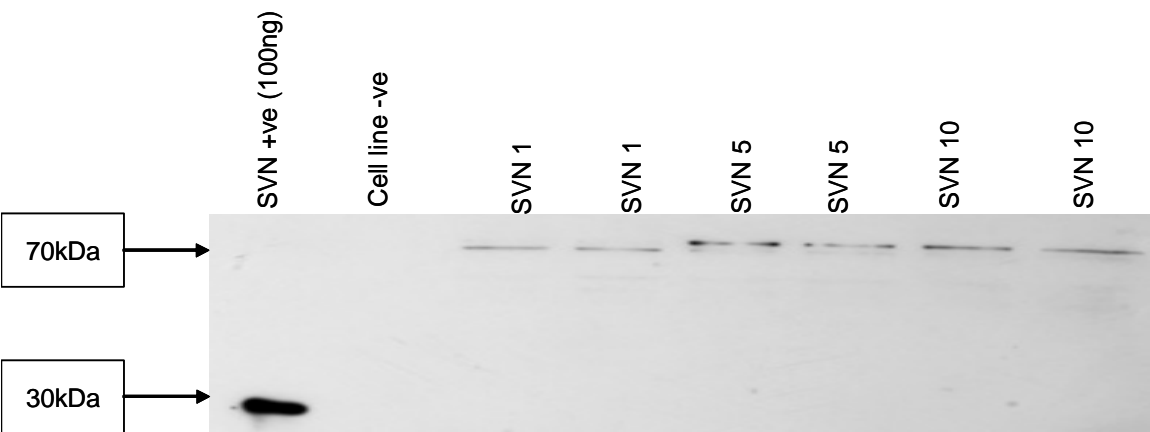


Fig.4.4 The SVN positive control (100ng) loaded on gel appears to be a trimer in solution. All samples contain a 70kDa complex of the SVN protein

Various cell lines which initially proliferated on NTCK selection medium did eventually exhibit cell death characteristics after transfers over an 8 month period.

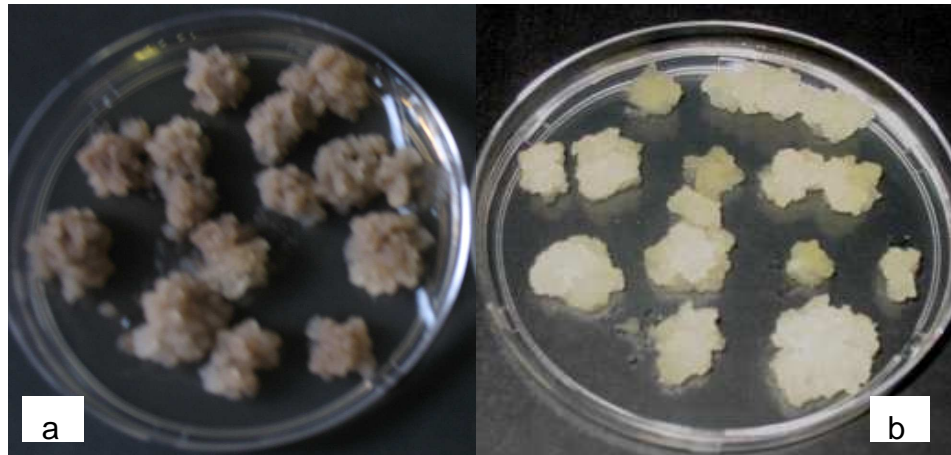


Fig.4.5 (a) GRFT transformed cells that grey-brown and die on selection after 8 months of transfers to fresh medium. Lines show lectin expression as after 1 month on selection and continue to do so as they begin to brown, (b) GRFT transformed cells which proliferate on NTCK selection

Protein was extracted from cells as they began to discolour and it was screened via Western Blot analysis to determine if there was GRFT/SVN protein expression prior to cell death. (Fig 4.6 & Fig. 4.7)



Fig.4.6 All “greying” cell lines produced GRFT (14.5kDa monomer) and a smaller product prior to cell death.

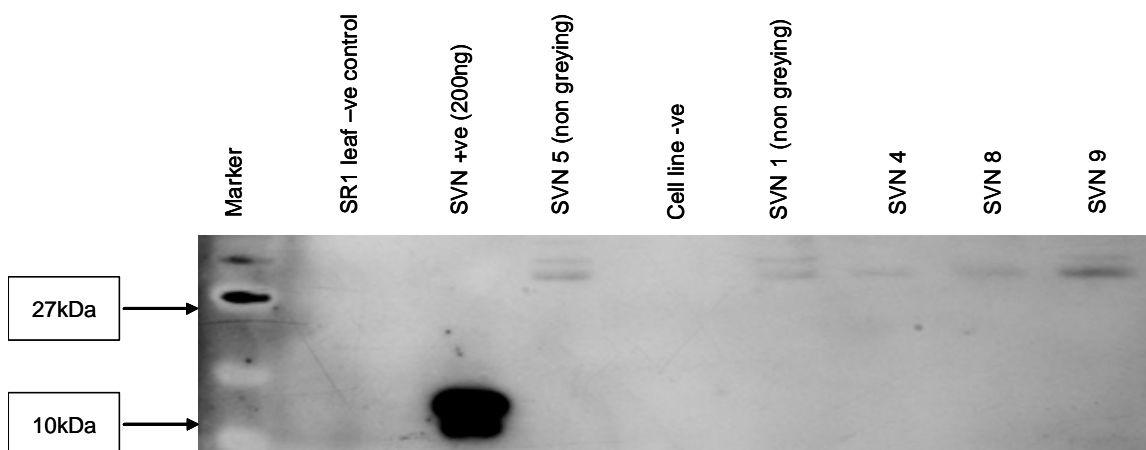


Fig.4.7 SVN cell lines 1 and 5, both non greying lines on NTCK selection, produced the same 29kDa SVN band as the greying lines, indicative of an SVN trimer being produced in the cell lines. Expression of SVN was confirmed for cell lines 1 month after proliferation on selection medium and cell death resulted only after \pm 8 months of transfers to fresh medium.

ELISA's

Two western blot positive cell lines were selected for ELISA analysis i.e. GRFT 7 and SVN 10. Samples were diluted two-fold and loaded in duplicate wells. Results are recorded as the nanogram of GRFT and SVN protein produced per gram of callus that bind gp120. GRFT cell line 7 and its dilutions were ELISA tested. GRFT 7 cell line produced a maximum of 3.38ng of Griffithsin protein per gram of callus fresh weight and was found to bind gp120 in a concentration dependant manner. However, it must be noted that the untransformed SR1 control produces a protein that strongly binds gp120 and this protein is expressed at 10.8 ng/g of callus. Scytovirin protein was produced at a concentration of 10.5ng per gram of callus. The untransformed callus control again binds gp120 and the unknown protein is produced at a concentration of 8.1ng/g of SR1 callus

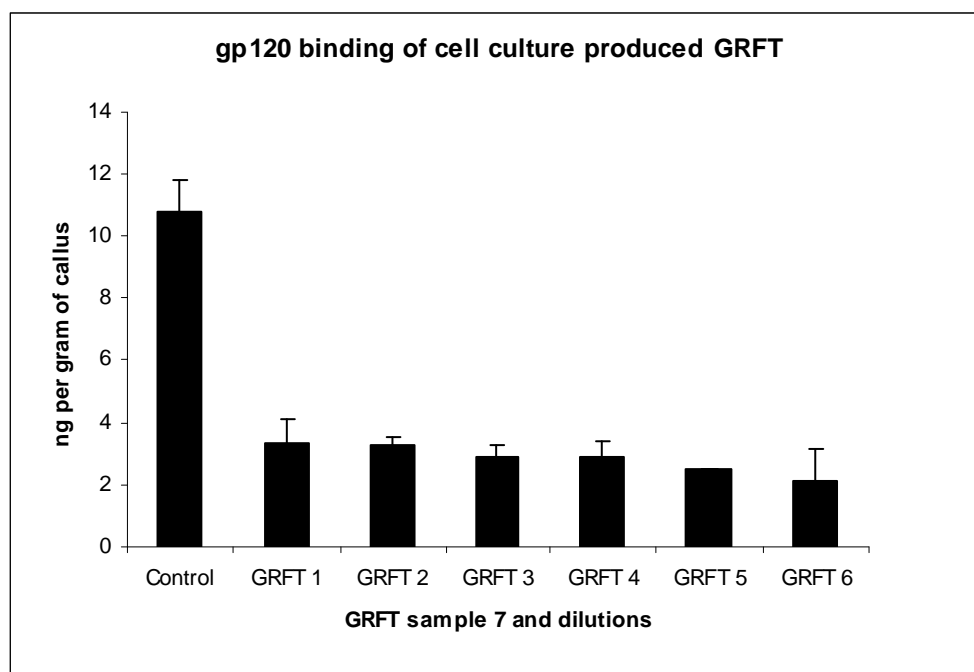


Fig.4.8 ELISA results for the binding GRFT protein (sample cell line 7) to gp120

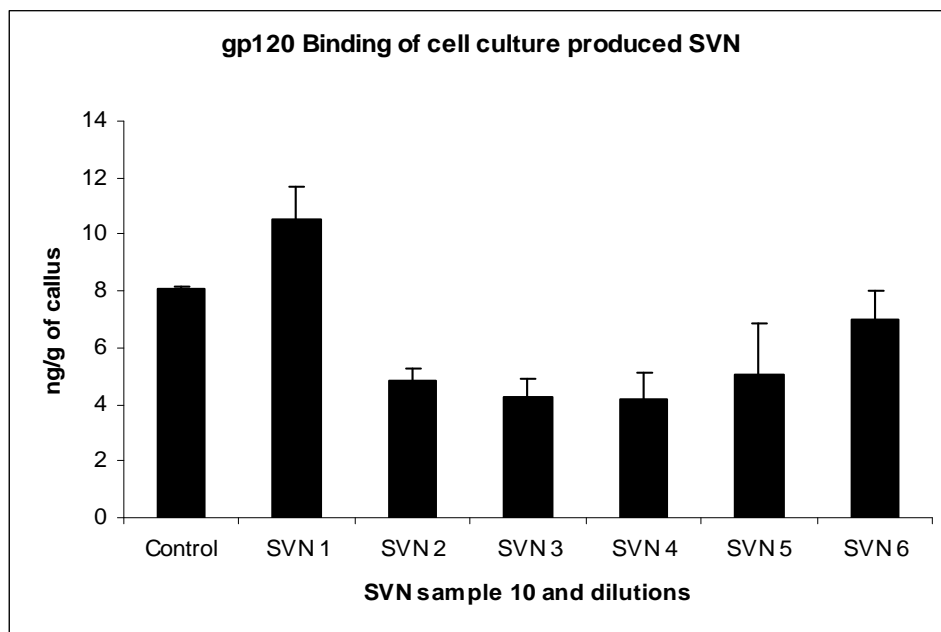


Fig.4.9 SVN cell line 10 and its dilutions (SVN 2-6) ELISA results assessing gp120 binding.

Owing to the binding capacity of the untransformed cell line to gp120, the ELISA method of protein quantification proved unsuccessful in quantifying GRFT and SVN protein expression in SR1 cell lines.

4.5 Discussion

Western Blot analysis of proliferating cell lines

Rapid dot blot analysis facilitated preliminary screening of pcr positive callus cultures and only dot blot positive lines were screened via western blot analysis. The pCambia-GRFT transformed callus lines successfully expressed the 14.5kDa Griffithsin monomer. This is a significant achievement in view of the fact that previous studies on expression of recombinant GRFT in *Escherichia coli* (*E.coli*) often resulted in insoluble protein accumulation in the inclusion bodies, and attempts to extract the GRFT using different surfactant agents and cycloamylose for refolding did not lead to significant recovery (Giomarelli *et al*, 2006). It was only after manipulation of culture conditions via thermoregulation etc. and auto-induction using fermenter culture, where the authors were successful at bulking up cell density and thereby retrieving about two thirds of the soluble GRFT. The results of this study, showing preliminary protein extraction and western blotting, confirms expression of soluble GRFT at basic plant cell culture conditions, forming the monomeric GRFT protein without degradation products, as seen in the immunoblot analysis via the recognition of the recombinant cell culture produced lectins by its respective antibodies. This achievement is a further feat, because several studies using plant systems often result in antibody fragments of molecular mass lower than that of the intact standard on western blots (Doran (2006); Sharp and Doran (2001), when exhibiting possible degradation or proteolysis of the target protein in plant systems, whereas in this study it was not evident.

The pCambia-SVN transformed callus cultures produced a 70kDa complex of the Scytovirin protein detected by the antibody. This is congruent with the 2006 finding

of Xiong *et al*, were the authors expressed SVN in *E. coli* with a thioredoxin fusion for increased solubility. They demonstrate that upon SDS-PAGE and Western blotting, a fraction of the protein existed in dimeric form. The results of this study is the first of its kind, confirming the expression of recombinant SVN in plant systems.

Western Blot analysis of cell lines exhibiting cell death characteristics

Callus cultures exhibiting cell death characteristics also express the lectin prior to death. SVN expressing lines show a 29kDa product in immunoblot analysis (fig. 4.7), which indicates a SVN trimer (exactly 29.1 kDa), recognised by the antibodies.

The GRFT cell lines express a 14.5 kDa singular molecule of the compound prior to or at the commencement of cell death (on average \pm at the 7th month of culture or after 21 subcultures to fresh selection media); however, a smaller degradation product is noted on the western blots. There are several potential sites and mechanisms of foreign protein degradation in plants and plant tissue cultures. Intracellular and apoplastic proteases may be responsible and the formation of foreign protein fragments has been found to occur in many plant hosts including *Nicotiana tabacum* (De Neve *et al*, 1993; Schiermeyer A *et al*, 2005). Sharp and Doran (2001) investigated degradation of IgG1 antibody in tobacco hairy roots, shooty teratomas and suspended cell cultures, and found that the degradation product of their protein was a result of extracellular protease activity in the root and shoot apoplast and that the degradation product also formed in the late-culture suspended cell biomass. This is of particular interest as it was noted that the transformed SR1 cell cultures in this study also exhibited cell death at the later stage of culture, approximately 3.5-4 weeks after the last transfer to fresh culture medium. This indicates that the SR1 cell cultures need transfers to fresh medium every 3 weeks of their growth cycle as protein

degradation occurs at the onset of the later stage of culture. Protein degradation in the SR1 cells may be Ubiquitin mediated. Ubiquitin, being a highly conserved regulatory protein in eukaryotes, responds to biotic and abiotic plant stress, often reacting by ubiquinating foreign proteins in cells and targeting them to the 26S proteasome for degradation. Keeping in mind that the SR1 cell lines were agrobacterium transformed for the expression of the foreign lectins, it is suggestive that the ubiquitin/26S proteasome system may be actively performing its normal housekeeping functions by removing the foreign expressed lectins or initially responding to possible agrobacteria proliferation as Cefotaxime antibiotics diminish in media toward the end of the transfer cycle. The Ubiquitin/26S proteasome pathway begins with the activation of the ubiquitin molecule in an ATP-dependant manner. The activated ubiquitin is then transferred to the active site of the ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin-ligase binds E2 and catalyzes the formation of an isopeptide between the activated ubiquitin and the Lysine residue of the substrate protein. After a chain of multiple ubiquitins are attached to the target protein, it is usually destined to the 26S proteasome, where the target protein is degraded and the ubiquitin monomers are reclaimed by the action of de-ubiquitination enzymes (Zeng *et al*, 2006).

There are various methods which may be employed to diminish or altogether halt protein degradation *in vitro*. The addition of a protease inhibitor to the culture medium may prove affective in small scale production scenarios, assuming the uptake of the inhibitor into the cells (Doran, 2006). Examples of such inhibitors include Leupeptin, Pepstatin, Betastatin, 3, 4-DCI and EDTA, each targeting serine, cystein, aspartic, thiol or metalloproteases (Benchabane *et al*, 2008). However, it has been reported that the protease inhibitor Phenyl-methylsulfonyl Flouride (PMSF), which is known to be responsible for inhibiting both thiol-and serine-type proteases in plants,

has been associated with the inhibition of fresh mass accumulation and is only active in suppressing certain PMSF sensitive proteases (Ananieva and Ananieva, 2001).

At the liquid medium culture stage, protein stabilizing agents such as polyvinylpyrrolidone (PVP), dimethyl sulphoxide (DMSO), albumin, mannitol and salt may be a useful addition to the media to prevent the loss of novel protein. (Doran, 2006 and Soderquist and Lee, 2005).

An alternate strategy to the physical addition of protease inhibitors and protein stabilizers may be to ectopically express a protease inhibitor that is active against specific endogenous proteases (Faye *et al*, 2005). This is more applicable to large scale operations as it simplifies the extraction and downstream processing of large biomass samples. A convincing demonstration supporting this approach was the successful stabilisation of recombinant antibodies secreted by roots of transgenic tobacco plants also expressing and co-secreting a Bownam-Birk trypsin inhibitor from Soybean (Komarnytsky *et al*, 2006).

It is also possible that protein degradation occurred during protein extraction. Some protein loss is expected during the extraction procedure and upon cell disruption; there is systematic release of protein denaturing agents including phenolics and proteases. The smaller molecular weight band in Fig. 4.6 may be a result of the callus containing a mix of metabolically active and inactive cells, as already “greying” tissue was screened (Benchabane *et al*, 2008)

ELISA's

ELISA results could not effectively be concluded owing to the strong gp120 binding capacity of the untransformed SR1 cell line. The interaction of the untransformed *N.tabacum* protein extract and gp120 can be attributed to the presence of a lectin in the culture which recognises gp120. Lectins represent a very diverse and heterogeneous group of proteins of non-immune origin that have the common ability to specifically recognize certain carbohydrates. *Nicotiana tabacum* does express a lectin, most often after treatment with a jasmonate, and this lectin is aptly named *Nicotiana tabacum* agglutinin (Nictaba). Nictaba is therefore a stress response derived lectin which in its native state exists as a homodimer of two identical unglycosylated subunits of approximately 19 kDa (Lannoo et al, 2007). Cell suspension and callus cultures are in a naturally stressed state, often adapting to thermal, oxidative, salt, light and hormonal stresses. It is therefore possible for the physically stressed cell cultures to produce a lectin that would behave in an identical or similar manner to the Nictaba lectin i.e. exhibits a strong affinity for high-mannose and complex N-glycans (Lannoo et al, 2006). In the gp120 envelope of HIV-1 (III_B), all 24 potential N-linked glycosylation sites are utilized as follows: 13 sites containing complex-type oligosaccharides and 11 sites containing hybrid and/or high mannose type structures. All N-linked glycoprotein carbohydrates in gp120 share a common pentasaccharide Man₃GlcNAc₂ linked to the amide nitrogen of asparagine through the reducing hydroxyl group of GlcNAc. 33% of oligosaccharides are of the high mannose type, 4% are of the hybrid type, 63% are of the complex type, making HIV gp120 predominantly fucosylated or sialylated (Balzarini et al, 2005). All of the above make the gp120 envelope an ideal interaction site for various high mannose binding proteins. Balzarini et al (1991) reported inhibitory effects of a number of mannose

specific plant lectins against HIV and Cytomegalovirus infection, however, they also show that the viruses develop decreased sensitivity towards the lectins over a period of time (Balzarini *et al*, 2004).

Further work is necessary with regards to isolating and identifying the unknown lectin or protein complex from the *N.tabacum* callus culture.

4.6 Conclusion

This chapter was aimed at determining if GRFT and SVN can be expressed in plant cell lines. Total soluble protein extractions and western blot analysis confirmed the presence of a 14.5kDa GRFT monomer and a 70kDa SVN complex in cell lines screened. Cell lines that displayed cell death characteristics were screened and were also positive for lectin expression prior to cell death, with the 14,5kDa GRFT product and the 30kDa trimer of the SVN recombinant lectin expressed.

ELISA results show that the protein extracts which were western blot positive expressed GRFT protein at a maximum concentration of 3.38ng/g and SVN at 8.1ng/g of callus fresh weight. These results did however pose questions of concern when the cell line negative (untransformed) also bound to the gp120 captured on ELISA plates. Therefore, even though this chapter confirms positive expression of recombinant GRFT and SVN microbicides in plant cell lines, quantitative western blots and direct binding ELISA assays still need optimisation before expression levels can be further quantitated. Western blot positive cell lines have been introduced into cell suspension culture and they continue to be monitored.

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Chapter Five

General Conclusions and Future Perspective

This study began with a thorough literature review into HIV and its current control strategies, recombinant protein production systems, plants and plant cells as recombinant protein production systems and current Griffithsin and Scytovirin production platforms. The current HIV epidemic calls for the development of cheap and viable control strategies to curb the infection rate. Microbicides appear to be a viable option to curtail the spread of the disease. To date, there are no written reports on the production of recombinant microbicides in plant cell culture systems, which in accordance to the literature review appeared as a possibly feasible production system. All of the above led to the primary aim of this study: To determine if the anti-HIV molecules, Griffithsin and Scytovirin, could be produced in plant cell culture systems. The work conducted in chapter two of this study enabled the development of a stable, uniform SR1 cell line, with optimized growth conditions, for use in transformation experiments. Cell morphology experiments showed healthy, untransformed cell phenotypes, which later allowed for morphological comparisons with transformed cell lines. Another valuable addition to the work covered in this chapter was the growth curve study, which illustrated the optimum time for cell line transformation experiments to be conducted.

Chapter three formed the backbone of this study in totality, as all the transformation vectors were designed and constructed in this chapter, and thereafter used to transform the SR1 cell line developed in chapter two. The end result of the work in this chapter was PCR positive cell lines confirming the GRFT and SVN gene integration in transformed SR1 cultures. Transformed cell lines exhibited pinpoint

clustered cell morphology, differing from the elongated, chain formations of wild type SR1 cell lines. Transgenic lines were also successfully bulked up for protein characterisation in chapter four.

The western blot analysis results of chapter four confirmed the presence of a 14.5kDa GRFT monomer and a 70kDa SVN complex in all cell lines screened. ELISA results show that the protein extracts which were western blot positive expressed GRFT protein at a maximum concentration of 3.38ng/g and SVN at 8.1ng/g of callus fresh weight.

The aim of this study was adequately met, confirming the ability to successfully produced recombinant GRFT and SVN molecules in plant cell culture systems.

Transgenic GRFT and SVN expressing cell lines continue to thrive at shake flask level in cell suspension. Future work would entail optimising the system in order to attain maximum product yield in order to further validate the production method. The addition of a secretory peptide to the cloned constructs may minimise protein extraction costs and increase product yield. The feasibility of a batch fermentation culture method would also be a necessary.

The promising results attained in this study forms the basis of work on an unexplored production method for anti-HIV microbicides, showing that the plant cell production system for recombinant Griffithsin and Scytovirin may in the future be a possible means to curbing the HIV infection rate.