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Rice endosperm as a production platform for third-generation HIV microbicides

Evangelia Vamvaka

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School of Agricultural and Forestry Engineering

Department of Plant Production and Forest Science

University of Lleida

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Evangelia Vamvaka

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Supervisor: **Prof. Paul Christou**

Department of Plant Production and Forest Science

School of Agricultural and Forestry Engineering

University of Lleida

_____ _____

Signature Date

Co-supervisor: **Prof. Teresa Capell**

Department of Plant Production and Forest Science

School of Agricultural and Forestry Engineering

University of Lleida

_____ _____

Signature Date

"Eureka!"

Archimedes

DEDICATION

To my parents

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ABSTRACT

My dissertation focuses on the endosperm-specific production of three different HIV-neutralizing proteins in transgenic rice plants, alone and in combination, followed by tests to determine their yield, integrity and ability to bind and inactivate HIV. The three components were the monoclonal antibody 2G12 and the lectins griffithsin (GRFT) and cyanovirin-N (CV-N). The overall aim was to evaluate rice as a production platform and determine the best overall production strategies for the preparation of an efficacious microbicide cocktail *in planta*. Initially I tested the expression of 2G12 not only to determine whether rice endosperm was viable as a production platform but also to gain insight into factors that affect the production and functional efficacy of neutralizing antibodies in plants and the impact they have has on endogenous gene expression. The antibody was expressed successfully in rice endosperm, but unlike all previous reports of antibodies expressed in plants, the most prevalent form was the aglycosylated version with the remaining forms featuring oligomannose-type glycans, complex vacuolar-type glycans or single GlcNAc residues. These results suggest that a large proportion of the heavy chain is co-translationally imported into a region of the endoplasmic reticulum (ER) that lacks glycosyltransferases and accumulates in protein bodies without further modification. The remaining proportion appears to follow the canonical pathway, undergoing initial glycosylation in the ER lumen followed by either complex modification in the Golgi body and/or trimming back to a single GlcNAc residue. The aglycosylated form of the antibody produced in rice showed greater HIV-neutralizing activity than the glycosylated version produced in tobacco seeds, although neither was as potent as the original antibody produced in CHO cells. Proteomic analysis suggested that yields of recombinant proteins could be improved in seed endosperm by regulating protein/carbohydrate metabolism and by protecting the endosperm from abiotic stress. Having established that rice is suitable for the expression of 2G12, I expressed GRFT in the endosperm to determine whether rice is also a suitable platform for the expression of HIV-neutralizing lectins. GRFT was expressed successfully in rice, with yields comparable to or even higher than other antibodies expressed in rice and GRFT produced by transient expression in tobacco leaves. GRFT produced in rice showed similar potency in HIV-inhibition assays to the control GRFT produced in *Escherichia coli*. CV-N was also expressed successfully in rice endosperm but the yield was low and the potency in HIV-inhibition assays was lower than CV-N produced in other platforms, suggesting rice is not the ideal expression system for this protein.

Having shown that all three proteins could be produced individually in rice endosperm, I tested them in all possible pairwise combinations. The aim of these experiments was to determine whether the proteins could be coexpressed in rice endosperm and whether the extracts containing multiple proteins were more potent against HIV. The CV-N/GRFT combination was tested using a whole-cell assay against HIV and showed good activity. In addition, all the combinations showed enhanced activity against HIV in an infectivity assay. The 2G12 crude extract showed concentration-dependent activity. This is the first time that more than one HIV-neutralizing protein has been stably expressed in rice endosperm, opening the way for more combinatorial experiments to determine the best microbicide cocktails for the prevention of HIV.

RESUMEN

Mi tesis doctoral está enfocada en la producción en el endospermo de arroz, de tres proteínas diferentes con capacidad para neutralizar el virus del SIDA, solas o en combinación y en la detección de su producción, integridad y capacidad para unirse e inactivar al HIV. Las tres proteínas son el anticuerpo monoclonal 2G12, la lecitina grifithsin (GRFT) y la lecitina cianovirina (CV-N). El objetivo general era evaluar si se podía utilizar arroz como una plataforma de producción y determinar la mejor estrategia de producción en planta de un coctel microbicida eficaz. En la primera parte de mi tesis investigué si podía producir 2G12 en el endospermo de arroz y cuáles eran los factores que afectaban a su producción y su eficacia funcional. Exprese con éxito el anticuerpo monoclonal Ab2G12 humano en el endospermo de arroz, el cual se acumuló en una forma aglicosilada, además de con alguna forma con glicanos del tipo oligomanosa y otros del tipo de complejos vacuolares o residuos de GlcNAc sencillos. Estos resultados sugieren que una proporción muy elevada de la cadena pesada del anticuerpo era importada, al mismo tiempo que transcrita, a una región del retículo endoplasmatico que carece de glucosiltransferasa y acumulándose en los cuerpos proteicos sin modificaciones posteriores. La proporción restante parece que sigue la ruta canónica, produciéndose una glucosilacion inicial en el lumen del retículo endoplasmatico seguida de complejas modificaciones en el cuerpo de Golgui y/o recortando a residuos de GlcNAc sencillos. La forma aglicosilada del anticuerpo producida en arroz demostró un mayor actividad neutralizante del virus HIV que la forma glicosilada, producida con anterioridad en semillas de tabaco; pero ninguna de las dos era tan potente como la molécula producida en células CHO. Los análisis del proteoma sugirieron que los rendimientos obtenidos de proteínas recombinantes podrían ser mejorados en el endospermo de la semilla mediante la regulación del metabolismo de las proteínas/carbohidratos y también protegiendo al endospermo de estreses abióticos. Una vez establecido que en arroz se puede expresar 2G12, pase a introducir la lectina GRFT en el endospermo de arroz para determinar si este podía ser una plataforma de expresión de esta lectina neutralizante del HIV. GRFT se acumuló en arroz, con una producción comparable o incluso más alta que otros anticuerpos producidos en arroz y a GRFT producida de forma transitoria en tabaco. GRFT producida en arroz tuvo una potencia inhibitoria en ensayos de inhibición del virus HIV semejante a la control GRFT producida en E. coli. También exprese la proteína CV-N en endospermo de arroz, pero tanto la concentración como la actividad

neutralizante fueron bajas, y la potencia de inhibición del virus HIV fue más baja que en ensayos donde CV-N fue producida utilizando otras plataformas, indicando que arroz no es el sistema ideal para la acumulación de esta proteína. Una vez demostré que las tres proteínas podían producirse individualmente en el endospermo del arroz, probé si era posible producirlas en combinaciones. El objetivo de estos experimentos era ver si las proteínas podían ser co-expresada en el endospermo de arroz y si los extractos conteniendo múltiples proteínas eran más potentes contra HIV. La combinación CV-N/GRFT fue testada contra el virus HIV y demostró muy buena actividad neutralizante. Además todas las combinaciones mostraron actividad mejorada contra HIV en ensayos de infecciones. El extracto crudo 2G12 demostró que su actividad dependía de la concentración. Esta es la primera vez que más de una proteína que neutraliza el virus HIV es producida de forma estable en el endospermo de un cereal, abriendo un nuevo camino hacia la producción de un cóctel microbicida para evitar la transmisión del virus del sida.

RESUM

La meva tesis doctoral està enfocada en la producció a endosperma d'arròs, de tres proteïnes diferents amb capacitat per a neutralitzar l'HIV, soles o en combinació i en la detecció de la seva producció, integritat i capacitat par a unir-se e inactivar a l'HIV. Les tres proteïnes son l'anticòs monoclonal 2G12, la lecitina griffithsin (GRFT) i la lecitina cianovirina (CV-N). L'objectiu general era avaluar si es podia utilitzar arròs com a una plataforma de producció i determinar la millor estratègia de producció en planta d'un còctel microbicida eficaç. En la primera part de la meva tesis he investigat si es podia produir 2G12 a l'endosperma d'arròs i quins eren els factors que afectaven la seva producció i la seva eficàcia funcional. Vaig expressar amb èxit l'anticòs monoclonal 2G12 humà a l'endosperma d'arròs, el qual es va acumular en una forma aglicosilada, a mes amb alguna forma amb glicans del tipus oligomanosa i altres del tipus de complexes vacuolars o residus de GlcNAc senzills. Aquest resultats suggereixen que una proporció molt elevada de la cadena pesada de l'anticòs era importada, al mateix temps que transcrita, a una regió del reticle endoplasmàtic que no te la glucosiltransferasa i s'acumula als cossos proteics sense modificacions posteriors. La proporció romanent sembla que segueix la ruta canònica, produint-se una glucosilació inicial al lumen del reticle endoplasmàtic seguida de modificacions complexes als cossos de Golgui i/o retallan a residuos de GlcNAc senzills. La forma aglicosilada de l'anticòs produïda en arròs va demostrar tenir una major activitat neutralitzant del virus HIV que la forma glicosilada, produïda amb anterioritat en llavors de tabac; però cap d'elles va ser tant potent como la molècula produïda en cèl•lules CHO. Els anàlisis del proteoma van suggerir que els rendiments obtinguts de proteïnes recombinants podrien esser millorats a l'endosperma de la llavor mitjançant la regulació del metabolisme de les proteïnes/carbohidrats i també protegint l'endosperma d'estressos abiòtics. Un cop establert que a l'arròs es pot expressar 2G12, vaig passar a introduir la lectina GRFT a l'endosperma d'arròs per a determinar si aquest podria ser una plataforma d'expressió d'aquest lectina neutralitzant de l'HIV. GRFT s'acumula en arròs, amb una producció semblant i inclús mes alta que altres anticossos produïts en el mateix sistema i a GRFT produïda de forma transitòria en tabac. GRFT produïda a l'arròs va tenir una potencia inhibitòria en assajos de inhibició del virus HIV semblant a la control GRFT produïda en E. coli. També vaig expressar la proteïna CV-N a l'endosperma d'arròs, però tant la concentració com l'activitat neutralitzant van esser baixes, i la potencia d'inhibició del virus HIV va esser inclús mes baixa que als assajos on CV-N va esser

produïda utilitzant altres plataformes, indicant que l'arròs no es el sistema ideal per a l'acumulació d'aquesta proteïna. Un cop demostrat que les tres proteïnes es podien produir individualment a l'endosperma de l'arros, vaig provar si era possible produir-les en combinacions. L'objectiu d'aquest experiments era veure si les proteïnes podien esser co-expresades a l'endosperma d'arròs i si els extractes contenint múltiples proteïnes eren mes potents contra HIV. La combinació CV-N/GRFT va esser provada contra el HIV i va demostrar molt bona activitat neutralitzant. Ames, totes les combinacions van mostrar activitat millorada contra HIV en assajos d'infeccions. El extracto cru 2G12 va demostrar que la seva activitat depenia de la concentració. Aquest es el primer cop que mes d'una proteïna que neutralitza el virus HIV es produïda de forma estable en l'endosperma d'un cereal, obrint un camí nou cap a la producció d'un còctel microbicida per a evitar la transmissió del virus de la sida.

ABBREVIATIONS

ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
bp	Base Pairs
BiP	Binding Immunoglobulin Protein
BSA	Bovine Serum Albumin
CaMV 35S	Cauliflower Mosaic Virus 35S
CAP	Cellulose Acetate Phthalate
CBAs	Carbohydrate-binding Agents
CCR5	C-C chemokine Receptor type 5
CD4	Cluster of Differentiation 4
CDC	Complement-Dependent Cytotoxicity
CDC	Centers for Disease Control and Prevention
CHO	Chinese Hamster Ovary
CS	Cellulose Sulfate
CV-N	Cyanovirin-N
CXCR4	C-X-C chemokine receptor type 4
DARPA	Defense Advanced Research Projects Agency
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic Acid
DS	Dextrin Sulfate
EC	The half maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELP	Elastin Like Peptide
EMRT	Exact Mass Retention Time
ENGase	Endoglycosidase
Env	Envelope
ER	Endoplasmic Reticulum
EU	European Union
Fab	Fragment Antigen-binding
FDA	US Food and Drug Administration
GlcNAc	N-Acetyl-glucosamine
GLGB	Glycogen Branching Enzyme
GLGS	Glycogen Synthesis Protein
GN	Gene Name
gp120	Glycoprotein 120KDa
gp41	Glycoprotein 41KDa
GRAS	Generally Regarded As Safe
GRFT	Griffithsin
GRXC8	Glutaredoxin-C8
HAART	Highly Active Antiretroviral Therapy
HC	Heavy Chain
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hpt	Hygromycin
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
H1N1	Influenza A
IC	The half maximal Inhibitory Concentration
Ig	Immunoglobulin

kDa	Kilo Dalton
LC	Light Chain
MAb	Monoclonal Antibody
MnP	Manganese peroxidase
MS	Mass Spectrometry
MPER	Membrane Proximal Region
MPDS	MassPREP Digestion Standards
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MWCO	Molecular Weight Cut-off
NCI	National Cancer Institute
ngram AVG	nanogram Average
NMR	Nuclear Magnetic Resonance
NNRTIs	Non- Nucleoside Reverse Transcriptase Inhibitors
Nos	Nopaline Synthase gene
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
N-9	Nonoxynol-9
OD	Optical Density at 540 nm
OS	Oryza Sativa
PB-I	Protein body type one
PB-II	Protein body type two
PBMCs	Peripherial Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBs	Protein Bodies
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PE	Protein Existence
PIC	Pre-integration complex
PrEP	Pre-Exposure Prophylaxis
PRO7	Prolamin
PSVs	Protein Storage Vacuoles
RAmyD3	rice α-amylase signal sequence
RNA	Ribonucleic Acid
RT-PCR	Real Time- Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
SARS-CoV	Severe Acute Respiratory Syndrome-associated coronavirus
SD	Standard Deviation
SE	Standard Error
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SLS	Sodium lauryl sulfate
SV	SequenceVersion
TBS	Tris-Buffered Saline
TEM	Transmission Electron Microscopy
TMB	3,3',5,5'-Tetramethylbenzidine substrate
TMV	5' 5'-leader sequence of Tobacco Mosaic Virus
TSP	Total Soluble Protein
US	United States
USA	United States of America
USDA	United States Department of Agriculture
VMO-1	Vitelline Membrane Outer layer protein 1
WHO	World Health Organisation
XTT	2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt tetrazolium

I. GENERAL INTRODUCTION

I.1 MOLECULAR PHARMING

Molecular pharming describes the use of plants as factories to produce recombinant pharmaceutical proteins such as antibodies, vaccines, blood components, and other therapeutic and diagnostic macromolecules (Ramessar et al., 2008a; Twyman et al., 2003). Plants can be scaled up rapidly and inexpensively, allowing the economical production of pharmaceutical proteins that are required in amounts exceeding 100 kg per year. In addition, plants have many advantages compared with conventional systems such as mammalian cells, *Escherichia coli* and yeast (Table I.1). Plants fold and assemble complex proteins, carry out essential posttranslational modifications and do not contain the endotoxins and pathogens (e.g. prions) that are found in other production platforms (Ma et al., 2003; Stöger et al., 2005).

Table I.1: Production systems for recombinant human pharmaceutical proteins (adapted from Ma et al., 2003). *Compared to production in conventional systems such as mammalian cells, *E. coli* and yeast.

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risk	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Mammalian cell culture	High	Long	Very low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Plant cell culture	Medium	Medium	Medium	High	Minor differences*	Low risk	Moderate
Transgenic plants	Very low	Long	Very high	High	Minor differences*	Low risk	Inexpensive

Molecular pharming has been used to produce many commercial products, including avidin and trypsin (Twyman et al., 2014), collagen, aprotinin, growth hormone and human serum albumin (Ma et al., 2003). Antibodies have also been successfully produced in plants, including single-chain variable fragments (scFv) and full-size IgGs targeting *Herpes simplex virus* (HSV), *Respiratory syncytial virus*, *Rabies virus*, colon cancer antigens and *Hepatitis B virus* (Ko and Koprowski, 2005; Ramessar et al., 2008a; Stöger et al., 2005) as well as IgMs targeting a

neuropeptide hapten (Ma et al., 2003). Antibodies for the prevention of sexually transmitted diseases have been produced in plants, including an IgG against HSV which prevented transmission in mice (Zeitlin et al., 1998) and a diagnostic antibody for *Human immunodeficiency virus* (HIV) produced in barley (Schünmann et al., 2002). Tobacco and maize have been used to produce the full size anti-HIV antibodies 2G12 and 2F5 (Rademacher et al., 2008; Ramessar et al., 2008b; Sack et al., 2007). Cyanovirin-N has been produced transiently in tobacco (*Nicotiana tabacum*) and griffithsin in *N. benthamiana* (O'Keefe et al., 2009; Sexton et al., 2009).

The US Department of Defense Advanced Research Projects Agency (DARPA) has recently challenged plant biotechnology companies to develop new strategies for the large-scale manufacture of influenza vaccines. This resulted in a successful phase I clinical trial of the H1N1 HAC1 subunit, showing that the product was safe and well tolerated at all dose levels (iBio Inc, 2012). Pharma-Planta was an EU Sixth Framework Program integrated project that secured approval for the HIV-neutralizing antibody 2G12 produced in transgenic tobacco (Pharma-Planta, 2011), which completed a first-in-human phase I clinical trial to confirm its safety and tolerability (Fischer et al., 2012).

The pharmaceutical company Synthon (Nijmegen, NL) acquired a molecular pharming platform based on aquatic plants from Biolex Therapeutics (Pittsboro, NC, USA) and is now developing clinical candidates such as Locteron (interferon-α2b) and a human CD30-specific antibody (McCarthy, 2005; Synthon, 2014). Protalix Biotherapeutics recently gained FDA approval for the commercialization of a recombinant form of the enzyme glucocerebrosidase (prGCD/ELELYSO™) produced in carrot cells. This enzyme is also produced in mammalian cells by Genzyme (Cerezyme™) and Shire (VpriV®), but it requires *in vitro* modification before formulation to remove sialic acid groups from the glycans thus exposing terminal mannose residues (Shaaltiel et al., 2007). Protalix targeted this enzyme to the vacuole, producing the oiligomannose version directly, thus avoiding the need for *in vitro* processing (Aviezer et al., 2009). Protalix Biotherapeuics was the first company to gain approval for the marketing of a plant-derived pharmaceutical protein intended for use in humans (Paul et al., 2013).

I.2 SEEDS AS A PRODUCTION PLATFORM FOR MOLECULAR PHARMING

Many different plant-based systems have been described for the production of pharmaceutical proteins, often using leaf biomass, fruits, tubers, or specialized aquatic plants or cultured plant cells (Fischer et al., 2004; Ma et al., 2003; Twyman et al., 2003). Seeds provide further advantages over these systems because they accumulate large amounts of protein in a relatively small volume and provide a stable environment that promotes protein accumulation and inhibits degradation, thus facilitating long-term storage (Ma et al., 2003; Stöger et al., 2005). For example, antibodies accumulate at high levels in seeds and remain stable for several years with no loss of activity when stored at ambient temperatures (Stöger et al., 2000). In practical terms, this means that cereal seeds containing pharmaceutical proteins can be stored and distributed in countries lacking a reliable cold chain.

A relatively high protein concentration is achieved because most seeds are small and compact with a simple proteome, which also reduces the number of competing proteins released during processing. Seeds also tend to lack the metabolites present in leaves that interfere with downstream processing by fouling membranes and chromatography media, e.g. polyphenolic compounds (Moloney et al., 1999). The specialized organelles in seeds provide further advantages such as increased stability and capacity for accumulation, bioencapsulation and containment, and enhanced processing strategies such as the extraction of oil bodies found in oilseeds such as safflower and rapeseed (Moloney et al., 1999).

Seed-based production systems can be scaled up and down rapidly in response to market demand simply by adjusting the amount of land used for a specific pharmaceutical crop. Field plants can be scaled up between 100-fold and 1000-fold in a single season depending on the species (Schillberg et al., 2002) and plant lines can be stored indefinitely and in an easily accessible form simply by storing seeds (Twyman et al., 2003). In addition, edible seeds have GRAS status (generally regarded as safe for human consumption) making them particularly suitable for the development of oral vaccines that can be administered as flakes or flour with minimal purification (Peters and Stöger, 2011).

Cereal seeds naturally accumulate large amounts of protein in the endosperm, which has evolved to store proteins and other macromolecules for consumption during germination. By subverting the natural protein storage mechanisms, cereal endosperm tissue therefore provides an ideal biochemical environment for the accumulation of recombinant proteins, and this is generally achieved by the formation of specialized storage compartments such as protein bodies and protein storage vacuoles, which are derived from the secretory pathway (Ramessar et al., 2008a). Recombinant proteins expressed in seeds have remained stable and active after storage at room temperature for more than six years (Ma et al., 2003; Stöger et al., 2000; Twyman et al., 2003). Commercial development has focused on three staple cereal crops, i.e. maize, rice and barley, with maize as a clear market leader (Ma et al., 2003; Ramessar et al., 2008b; Sabalza et al., 2013; Stöger et al., 2005).

I.3 RICE AS A PRODUCTION PLATFORM FOR MOLECULAR PHARMING

Rice has many advantages, such as the high grain yield, the existence of straightforward *in vitro* cultivation and transformation procedures, and the ability to scale up production rapidly. In addition, rice is a self-pollinating species so in theory there should be a lower risk of gene flow from pharmaceutical to non-pharmaceutical rice crops and wild relatives. However, the protein content of rice seeds is lower than maize, wheat and barley and the cost of growing rice is also much higher than the other cereals (Stöger et al., 2005). The US company Ventria Bioscience Inc. has developed rice as a commercial platform and has produced USDA-approved lines that express high levels of lactoferrin and lysozyme for non-pharmaceutical use (Hennegan et al., 2005; Lau and Sun, 2009; Nandi et al., 2002; Ramessar et al., 2008a; Yang et al., 2003, 2001).

An interesting and apparently unique property of rice endosperm is the diversity of storage compartments. The endosperm cells of most cereals contain specialized storage compartments known as protein bodies, which have evolved specifically for the storage or proteins, and these bud from the endoplasmic reticulum (ER) when sufficient protein has accumulated (Takaiwa et al., 2009). These compartments can also be used to accumulate recombinant proteins expressed

using the appropriate promoters and targeting sequences, increasing protein yields by providing an ideal stable environment (Muntz, 1998; Yamagata and Tanaka, 1986).

Most cereals form a single type of protein body but two distinct compartments are found in the rice endosperm, known as PB-I and PB-II (Takaiwa et al., 2009). Protein bodies are insoluble in low-salt aqueous buffers, so they can be purified by centrifugation (Sabalza et al., 2013). The presence of two different protein bodies in rice potentially allows different proteins to be expressed and targeted to different compartments simultaneously, and then recovered by first separating the compartments by density centrifugation.

I.4 HIV PREVALENCE

In 2012 there were 35.3 million people living with HIV, up from 29.4 million in 2001. This increase reflected general population growth, the appearance of new infections (UNAIDS, 2013) and also the increased longevity of HIV patients because antiretroviral therapy is becoming more effective (Abdool Karim and Baxter, 2012; Lynch et al., 2012).

Although HIV is now a pandemic, some geographical regions are more affected than others. More than 95% of people with HIV live in developing countries, where most new infections occur (UNAIDS, 2013). The global prevalence among people aged 15–49 was 0.8% in 2012 (Figure I.1). Sub-Saharan Africa, which is home to only 11% of the world's population, accounted for 4.7% of all HIV infections in 2013 (Figure I.2) (UNAIDS, 2013).

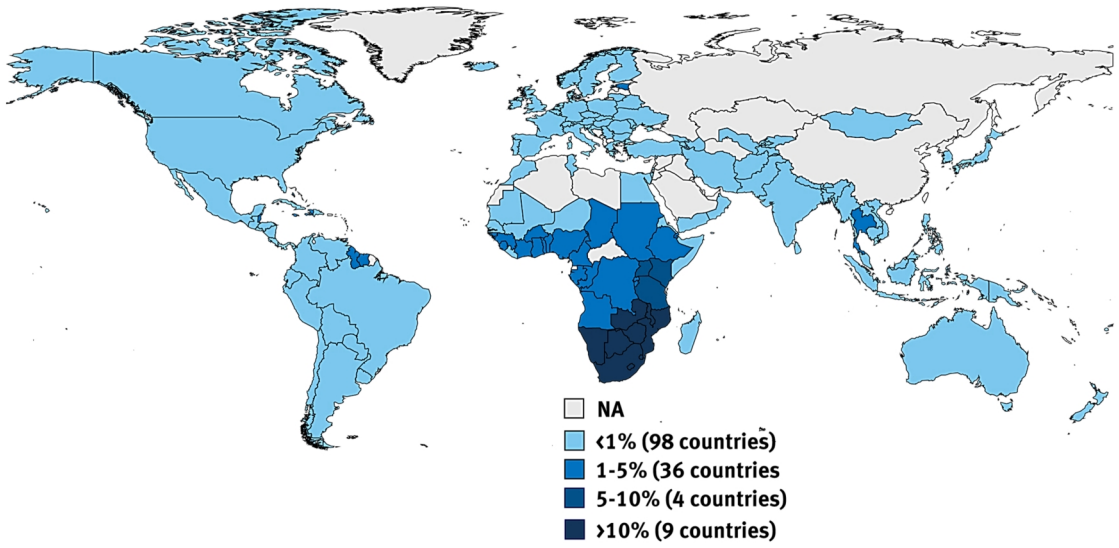

Figure I.1: A global view of HIV infection. HIV prevalence in adults (aged 15–49) in 2012 was ~0.8% although the prevalence differs significantly by region (**www.globalhealthfacts.org**, based on UNAIDS, 2013).

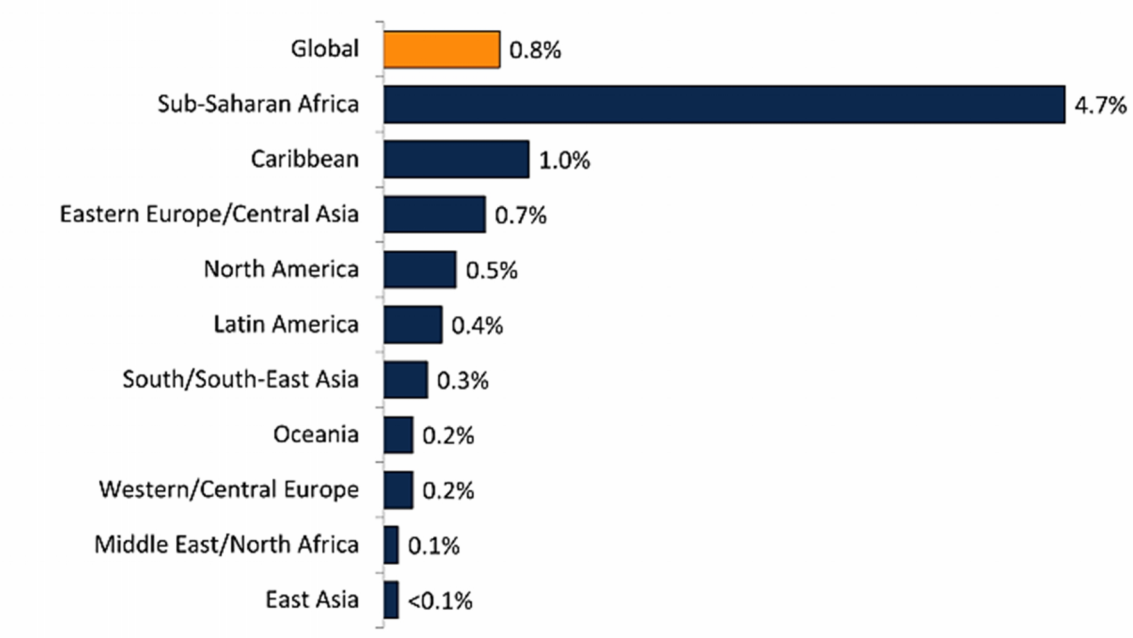

Figure I.2: Adult HIV prevalence by region in 2012 (UNAIDS, 2013).

Currently there is no cure for HIV. Nevertheless, the disease can be managed with antiretroviral drugs that reduce the rate of HIV replication and hence the mortality and morbidity associated with acquired immunodeficiency syndrome (AIDS). The best current treatment is highly active antiretroviral therapy (HAART), which comprises a cocktail of antiretroviral drugs that combat HIV proliferation by inhibiting different components of the virus. However, resistance to HAART is a growing problem and despite measures to increase the distribution of anti-retroviral drugs, the number of new HIV infections is outstripping the number of recipients of HAART by 2:1 (MTN, 2014). For this reason, new strategies are required to prevent the spread of HIV.

I.5 MECHANISMS OF HIV-1 SEXUAL TRANSMISSION

HIV-1 is transmitted by males more often than females, and requires the virus to pass through the mucosal epithelium of the vagina or rectum (Shattock and Moore, 2003; McGowan, 2006). The exact transmission mechanism remains unknown, and several different pathways may be involved (McGowan, 2006; Shattock and Moore, 2003) (Figure I.3). The virus must penetrate multiple layers of stratified squamous epithelium in the vagina and ectocervix of females or the inner foreskin, penile glans and fossa navicularis in males. The effectiveness of this barrier was demonstrated when the epithelial layer of rhesus macaques was thinned by hormonal treatment, substantially reducing 'physical resistance' to the virus (Marx et al., 1996; Smith et al., 2000).

It is broadly accepted that HIV infection requires some breakdown of the epithelial stroma and this happens frequently during intercourse. In addition, the positive effects of male circumcision against HIV might be explained by the fact that the circumcision reduces the possibility of microtrauma to the large surface area of foreskin (Auvert et al., 2005; AVAC, 2014).

Rectal infection is more difficult to prevent because the rectum features a single layer of columnar epithelium instead of stratified epithelial cells. Anal intercourse combined with some kind of injury increases the possibility of HIV-1 infection (McGowan, 2006). Anything that may cause mild epithelial disruption, such as trauma, ulceration or inflammation, makes the epithelium more susceptible to HIV (Mostad et al., 1997; Strathdee et al., 1996).

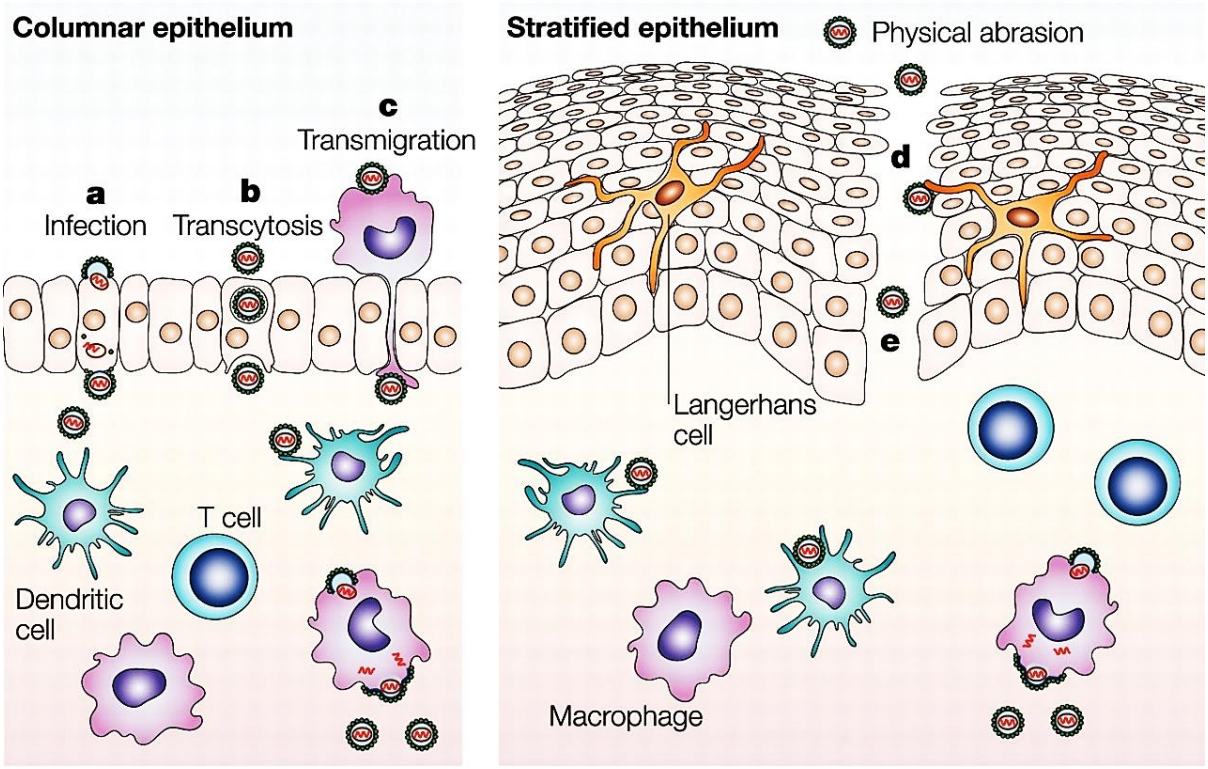

Figure I.3: Potential mechanisms for HIV-1 transmission across mucosal epithelium. a) Direct infection of epithelial cells. b) Transcytosis through epithelial cells and/or specialized microfold (M) cells. c) Epithelial transmigration of infected donor cells. d) Uptake by intra-epithelial Langerhans cells. e) Circumvention of the epithelial barrier through physical breaches. Successful transfer of virus across epithelial barriers would result in HIV-1 uptake by migratory dendritic cells (DC-SIGN or another mannose C-type lectin receptor) and subsequent dissemination to draining lymph nodes, and/or localized mucosal HIV-1 infection leading to recruitment of additional susceptible cells (Shattock and Moore, 2003).

I.6 CONVENTIONAL APPROACHES FOR THE TREATMENT OF HIV

In 2012, 1.6 million people died of AIDS, which was a 30% reduction since 2005. Deaths have declined in part due to antiretroviral therapy. Antiretroviral drugs block or inhibit the replication of retroviruses such as HIV, and HAART in particular has been highly beneficial to many HIV patients since its introduction in 1996, when the protease inhibitor-based HAART initially became available (Palella et al., 1998). Currently, there are 30 antiretroviral drugs approved by the FDA for the treatment of HIV carriers. These drugs are usually combinations or cocktails and

they fall into five major classes: reverse transcriptase (RT) inhibitors, protease inhibitors, entry and fusion inhibitors, integrase inhibitors and multidrug combination products (NAID, 2014). However, HAART neither cures HIV nor eliminates all symptoms, and high virus titers, often HAART-resistant, return if treatment is stopped (Dybul et al., 2002; Martinez-Picado et al., 2000). HAART can suppress the virus, even to undetectable levels, as long as the patient remains under treatment (Blankson et al., 2002). This means that the drugs must be taken for life, and although the number of HAART recipients has increased, universal access remains limited. Almost 26 million people are eligible for HAART based on WHO 2013 guidelines but the treatment gap was 16.2 million in 2012 indicating a requirement for new therapeutic strategies (WHO, 2013).

The prevention of sexual transmission is necessary to curtail the HIV pandemic. Heterosexual sex accounts for approximately two thirds of new infections (AVERT, 2014). In the past, simple preventative methods have been advocated such as abstinence, monogamy, a reduction in the number of sexual partners, and the use of barrier methods such as male and female condoms. However, these methods are inadequate because they depend on compliance from both partners to work effectively. These challenges have encouraged research into HIV prophylaxis and therapeutic vaccines, but there has been little progress and early efficacy trials were disappointing (Dolgin, 2010; Walker and Burton, 2010). Researchers have also focused on prevention strategies, such as pre-exposure prophylaxis, microbicides and voluntary male circumcision (AVAC, 2014). Microbicides are self-administered agents that can be applied to the vaginal or rectal mucosal surfaces with the goal of preventing or reducing the transmission of sexually transmitted infections (Singh et al., 2014).

Truvada (Gilead Sciences, Inc., Foster City, CA) is the first product indicated for pre-exposure prophylaxis (PrEP) in uninfected people who have a high risk of infection, e.g. those engaging in sexual activity with a known HIV-infected partner (FDA, 2012). The FDA originally approved Truvada for use in combination with other antiretroviral agents for the treatment (not prevention) of HIV-infected adults and children 12 years or older (FDA, 2012) but also approved it for PrEP in July 2012. Truvada comprises two antiretroviral drugs, the RT inhibitor emtricitabine and tenofovir disoproxil fumarate. In order to reduce the risk of HIV infection, Truvada must be

taken daily by the at-risk group and individuals must be tested to confirm their HIV– status at least every 3 months (FDA, 2012).

I.7 THE DEVELOPMENT OF MUCOSAL MICROBICIDES AND POTENTIAL MECHANISMS OF ACTION

Based on recent epidemiological data, women are 2–4 times more susceptible than men to new HIV infections (Wang et al., 2013). In addition, women carry a greater burden of HIV risk because of the greater exposed area of mucosal membrane, the biology of the virus, and unknown or high-risk behavior among partners and the inability to enforce compliance with the use of barrier methods. In developing countries these risks are exacerbated by low socioeconomic status, the high prevalence of non-consensual sex, unprotected sexual intercourse and other socio-economic and cultural factors (Ibrahim et al., 2013).

For this reason new technologies must be designed in order to empower women. Topical self-administered microbicides are being developed as a subset of PrEP strategies. These could significantly reduce the rate of HIV infection especially in women and also reduce the risks of HIV infection in women with multiple sexual partners.

Microbicides need to be designed for different uses (vaginal or anal intercourse) in order to meet the needs of every individual. For this reason they are formulated as gels, creams, films, or suppositories. In addition, a contraceptive effect (with or without spermicidal activity) might be useful serving a dual purpose by reducing the risk of pregnancy (Lopez et al., 2013).

Women in Brazil, India, South Africa, Thailand, the USA and Zimbabwe have expressed a positive attitude towards the use of microbicides with or without contraceptives (Coggins et al., 1998; Darroch and Frost, 1999; Han et al., 2009). In South Africa, in a study among HIV^+ men and women, neither sex objected to using the candidate microbicide (Ramjee et al., 2007).

Until recently, the incomplete understanding of the mechanism of HIV transmission hindered the development of microbicides. Most of the initial agents (including surfactants and acidifying agents) acted non-specifically, either by disrupting viral and cellular membranes or creating a more hostile environment for the virus (Nutan and Gupta, 2011). Progress in understanding how

HIV gains entry into the host and establishes lasting infection has allowed the development of compounds that target specific viral–host cell interactions and has facilitated a more tailored approach to microbicide development. Topical microbicides can therefore be classified according to their mechanism of action and their target during HIV transmission (Figure I.4) (McGowan, 2006).

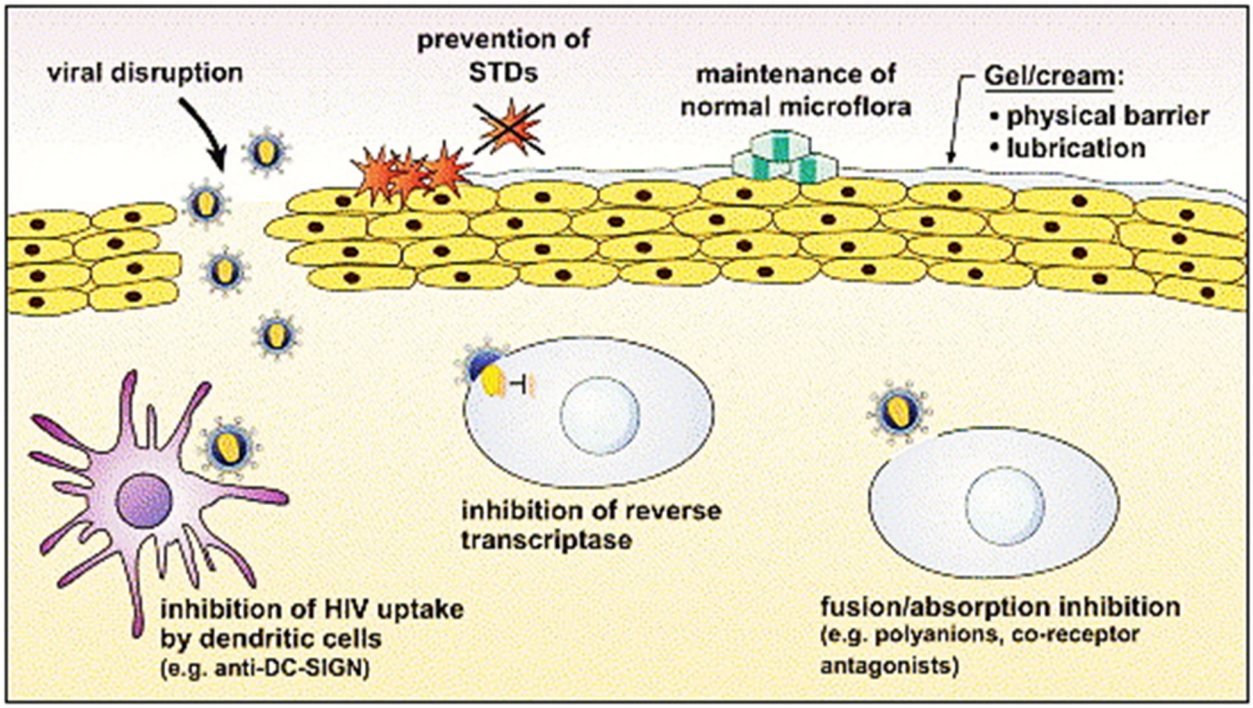

Figure I.4: Potential mechanisms of action of microbicide compounds (McGowan, 2006).

Microbicides that kill the pathogen (surfactants):

Some microbicides are surfactants that break down the membrane or envelope of the target pathogen non-specifically, preventing interactions with vaginal epithelial cells. Three surfactants have been evaluated as topical microbicides: Nonoxynol-9 (nonylphenoxypolyethoxyethanol or N-9), C31G (Savvy^{TM}, Cellegy Pharmaceuticals Inc., Huntingdon Valley, PA, USA) and sodium lauryl sulfate (Invisible Condom, Universite Laval, Quebec, Canada). N-9 was developed as a spermicide in the 1960s but was later tested for its ability to act as a vaginal microbicide against HIV (Bourinbaiar and Fruhstorfer, 1996). The antiviral activity of N-9 was demonstrated *in vitro*

(Hicks et al., 1985) but it was not effective against HIV in phase 2/3 trials, and it was also associated with local vaginal toxicity (Van Damme et al., 2002). C31G (Savvy^{TM}, Cellegy Pharmaceuticals Inc., Huntingdon Valley, PA, USA) is a spermicidal and antimicrobial agent consisting of two surface-active amphoteric agents (cetylbetaine and myristamine oxide). C31G was shown to be non-toxic to human cells *in vitro* along with broad-spectrum activity against *Chlamydia trachomatis*, HSV and HIV(Krebs et al., 2000; Wyrick et al., 1997). However, phase III clinical studies in three countries failed to show any protection against HIV-1, resulting in the termination of C31G as a potential microbicide (Peterson et al., 2007; Feldblum et al., 2008). Sodium lauryl sulfate (SLS, Invisible condom) is a potent surfactant that inhibits both non-enveloped viruses (e.g. papillomavirus, rotavirus and poliovirus) and enveloped viruses such as HSV and HIV (Piret et al., 2002). A randomized, double blind, placebo-controlled Phase II study in Cameroonian women demonstrated that the Invisible condom was well tolerated and acceptable, and further clinical evaluation is underway (Mbopi-Keou et al., 2010).

Microbicides that prevent virus replication (replication inhibitors):

Other microbicides are not designed to prevent infection but to block virus replication soon after initial infection, thus slowing the progression of AIDS before the disease starts to take hold (Balzarini, 2007). These are the same class of antiretroviral drugs used in HAART and are particularly useful for the prevention of mother-to-child HIV transmission during birth. For this reason, interest has grown in the use of RT inhibitors for HIV prevention and there is already a long list of products that have demonstrated efficacy, inexpensive production and the benefits of oral administration. However, there are concerns that the use of such microbicides will result in the emergence in antiretroviral resistance (McGowan, 2006). Tenofovir (Gilead Sciences Inc., Foster City, CA, USA), UC-781 and TMC-120 belong to this group of microbicides. Tenofovir is a nucleoside reverse transcriptase inhibitor (NRTI) and both UC-781 and TMC-120 are non-nucleoside reverse transcriptase inhibitors (NNRTI) (McGowan, 2006; Nutan and Gupta, 2011). Tenofovir has shown both *in vitro* and *in vivo* efficacy and was therefore the first antiretroviral drug to be assessed as a vaginal microbicide in clinical trials (Herrera et al., 2009). Even though NNRTIs have a high therapeutic index and act directly against virus, their low solubility in water makes their formulation as microbicides challenging (Nutan and Gupta, 2011).

Microbicides that enhance the vaginal milieu of protectors (buffers):

The acidity of the vaginal canal inhibits several bacteria and viruses (Croughan and Behbehani, 1988; Sturm and Zanen, 1984; Yasin et al., 2002). Various microbicidal compounds have been developed that protect the acidic vaginal milieu, either by buffering the neutralizing effect of semen or maintaining sufficient lactobacilli in the vaginal canal (Ramessar et al., 2010). Acidform (Amphora, Instead Inc., Dallas, TX, USA) is an acidic gel containing gelling agents, buffer salts, humectants, preservatives and water in a proprietary mixture. A phase I study revealed that this product was safe, but it caused vaginal irritation in combination with N-9 (Amaral et al., 1999). Carbopol 974P (BufferGel ReProtect, Baltmore, MD, USA) is a buffering carbopol polymer with spermicidal activity. Preclinical tests showed that it can prevent pregnancy and the transmission of HIV, HSV, human papilloma virus (HPV) and chlamydia (Zeitlin et al., 2001). Lactobacilli protect the vaginal milieu from pathogens, and the introduction of exogenous lactobacilli such as *Lactobacillus crispatus* achieved a reduction in HIV proliferation (Klebanoff and Coombs, 1991; Martin et al., 1999). Bioengineered lactobacilli (live microbicides) are therefore being tested as an alternative microbicidal approach.

Microbicides that block virus entry into host cells (entry/fusion inhibitors):

Entry inhibitors form another broad class of microbicide agents that block the attachment, fusion or uptake of HIV into host cells. Such agents work either by binding to HIV and disrupting the interaction with its receptors on the host cell membrane, or by saturating available binding sites. Entry inhibitors are divided into three groups: anionic polymers or polyanions, CCR5 inhibitors and fusion inhibitors that bind glycans or their receptors (Ramessar et al., 2010). The anionic polymers include cellulose sulfate (CS), PRO-2000 (Indevus Pharmaceuticals, Lexington, MA, USA), dextrin sulfate (DS), SPL7013, Carraguard, mandelic acid condensation polymer SAMMA (Topical Prevention for Conception and Disease, Rush University, Chicago, IL), and cellulose acetate phthalate (CAP). All have been evaluated in the clinic and have been shown to be safe and well tolerated (El-Sadr et al., 2006; Joshi et al., 2006; McCormack et al., 2005; Patton et al., 2006). The CCR5 inhibitors include PSC-RANTES, which demonstrates picomolar-range IC_{50} values against all HIV-1 clades (Kawamura et al., 2003). Finally, the

fusion inhibitors include cyanovirin-N, an 11 kDa protein extracted from the cyanobacterium *Nostoc ellipsosporum* (Mori and Boyd, 2001). Although CV-N shows efficient neutralization activity *in vitro*, the dose has to be increased to 10^5 times greater than the *in vitro* $ED_{100}$to achieve efficient neutralization *in vivo*, thus making the production of this protein expensive (McGowan, 2006). This protein has been produced in transgenic plants (Sexton et al., 2006) and lactobacilli (Pusch et al., 2005) to reduce production costs accordingly. Microbicides that are currently undergoing preclinical and clinical development are discussed on the AVAC Global Advocacy for HIV Prevention website (AVAC, 2014).

a. Potential viral targets for microbicide components

Once HIV enters its target cells, the infection is irreversible (Shattock and Rosenberg, 2012). Therefore, there is a short window of opportunity between exposure and infection during which PrEP methods must block the uptake of the virus, and experiments in non-human primates suggest that this window is 30–60 minutes in duration (Shattock and Rosenberg, 2012). When the virus interacts with the mucosal tissue, an infection is established in 16–72 hours, forming an initial focus of infection (Haase, 2011). One strategy to prevent transmission is to block the HIV proteins that interact with cellular receptors, i.e. gp41 and/or gp120, or the receptors themselves, i.e. CD4 and/or CCR5. Another is to prevent the production of provirus DNA by using reverse transcriptase inhibitors and/or to prevent the integration of the provirus using integrase inhibitors. The final opportunity is to block virus maturation with protease inhibitors thus preventing the release of progeny viruses with the ability to infect other cells (Figure I.5).

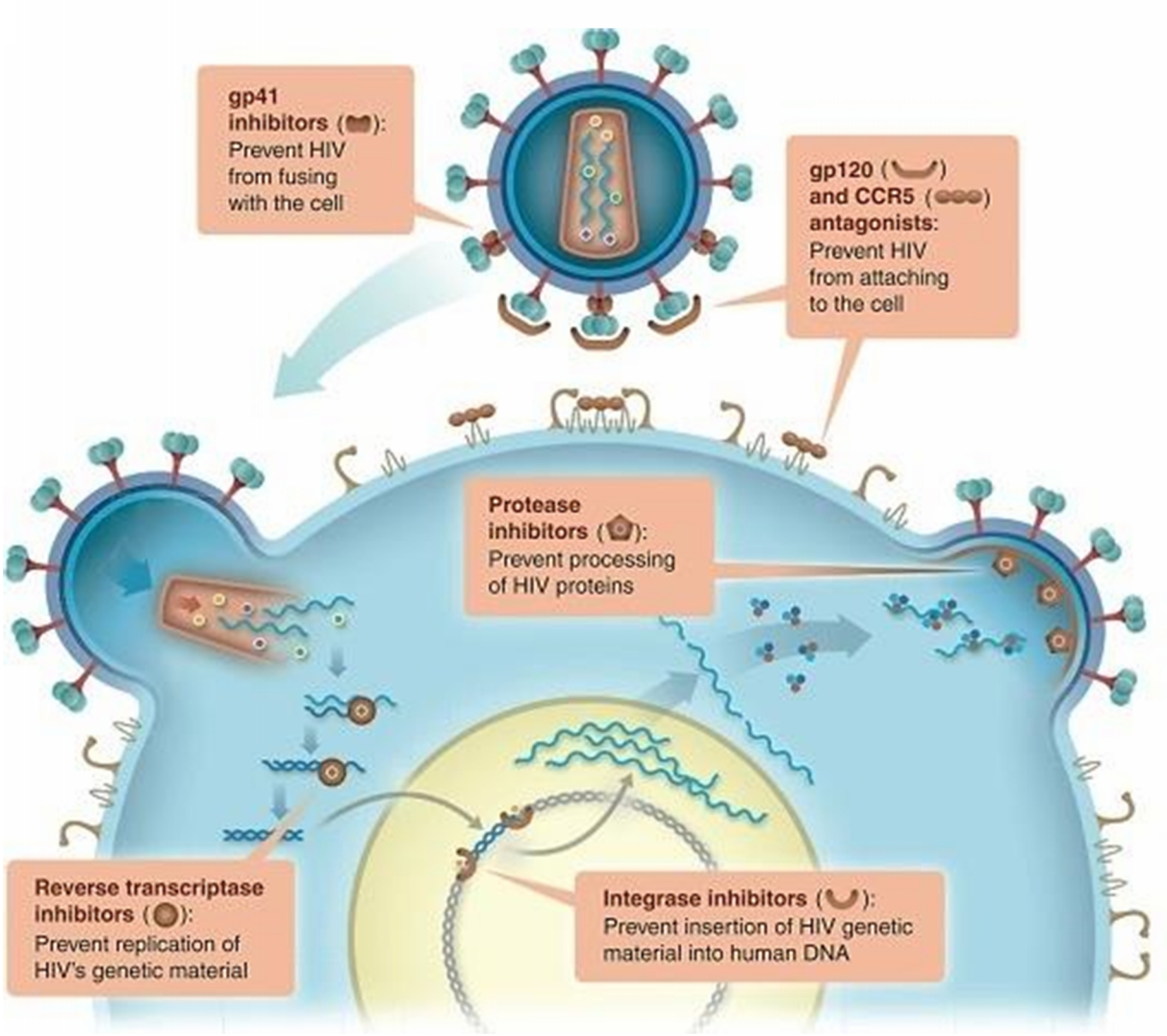

Figure I.5: Drugs targeting different stages of the HIV life cycle. Entry into target cells represents the first point at which microbicides could interrupt initial transmission events either by binding to the viral envelope or blocking the cellular receptor CD4 or its coreceptor. Reverse transcription represents the second point at which a microbicide could potentially intervene through the activity of nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs). Integration, a third intervention point, can be prevented by integrase inhibitors. Finally, protease inhibitors can block viral maturation thus preventing the release of defective non-infectious virions (Shattock and Rosenberg, 2012).

b. Recombinant microbicides including combination strategies

HIV treatment is challenging because the incoming virus may already contain mutations rendering it resistant to certain antiretroviral drugs. Although HIV mutates almost every time a new copy is made, not every mutation causes resistance (The Body, 2006).

HIV has been able to evolve *in vitro* escape mutants that circumvent the effects of polyanionic compounds, which are some of the most promising microbicide candidates (Bobardt et al., 2004; Este et al., 1997). In order to avoid the development of resistant isolates, combinations of two or more compounds that target separate viral structures or functions are used in microbicide formulations. Therefore, the development of effective microbicides will probably require a combination of several components to prevent the rapid evolution of resistance and to provide sufficient cross-clade protection (Sexton et al., 2009).

Combination microbicides are advantageous because they have a wider antiviral spectrum, a higher genetic barrier to resistance, greater potency, and broader cell/tissue coverage than most individual microbicides, and they often provide simultaneous protection against other sexually transmitted infections. Examples include combinations of NNRTIs such as efavirenz (EFV, Sustiva, Stocrin, Efavir) and nevirapine (NVP, Viramune, Boehringer Ingelheim) which are also used in combination antiretroviral therapy, or the lipophilic molecules UC781 and TMC120, which have been formulated as vaginal gels for phase III trials (Herrera et al., 2009). Rather than mixing different components, the microbicidal activities of different polypeptides can be combined by expressing them as fusion proteins, as shown for the antibody b12 and cyanovirin-N expressed in tobacco leaves (Sexton et al., 2009). This protein binds gp120 at two sites: the antibody binds to a conformational amino acid epitope and cyanovirin-N binds to the high-mannose residues on the surface of the protein. Microbicide combinations may also be used to target two diseases simultaneously, not only because some HIV microbicides are active against other viruses but also because diseases caused by one virus may facilitate transmission and pathogenesis of another. For example epidemiological studies show that HSV infection increases the risk of HIV (Freeman et al., 2006). Another example is the HIV–malaria link in which both diseases increase the severity of each other's symptoms and can make both diagnosis and therapy more complex (Vamvaka et al., 2014).

Although microbicides may help to prevent HIV, their success depends on the ability to produce sufficiently large quantities at a low cost. Estimations based on a 5-mg dose administered twice a week suggest that a production capacity of 5000 kg/y is required to supply 10 million women (Shattock and Moore, 2003). Therefore only plants provide the economy of scale required to ensure the availability of these products in the HIV-endemic regions of Africa and Asia (Fischer et al., 2004; Ma et al., 2003; Stöger et al., 2005, 2002).

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II. AIMS AND OBJECTIVES

My research project focused on the expression of multi-component third-generation microbicides in rice endosperm. My specific objectives were to express individually and in combination one human monoclonal antibody (2G12), and two anti-HIV proteins (cyanovirin-N and griffithsin) in order to explore whether rice seeds can be used as a production platform for individual and combination HIV microbicides. I analyzed the expression of these components to identify a lead event for further characterization, and investigated factors affecting the expression and accumulation of the 2G12 heavy and light chains so that their expression and accumulation can be modulated. My ultimate aim was to produce a multicomponent microbicide in rice endosperm tissue and to investigate how this performs against HIV.

CHAPTER 1

A recombinant HIV-neutralizing antibody produced in rice endosperm accumulates predominantly as an aglycosylated derivative with enhanced neutralizing activity

1.1 INTRODUCTION

HIV/AIDS is typically a sexually-transmitted disease, although the virus can also be transmitted via contaminated needles or blood stocks and by mother-to-child transfer during pregnancy, childbirth or lactation (UNAIDS, 2013). The virus is taken up by T-cells, macrophages and dendritic cells expressing the surface receptor CD4, which is recognized by envelope glycoprotein 120 (gp120). Subsequent conformational changes in gp120 initiate virus binding to the co-receptor (CCR5 or CXCR4) and fusion between the cell membrane and the virus, mediated by envelope glycoprotein 41 (gp41) (Clapham and McKnight, 2001). HIV carries two copies of a single-stranded RNA genome, which are reverse transcribed after uncoating to generate a double-stranded DNA provirus that integrates into the host genome. The integrated provirus is transcribed by cellular RNA polymerases to generate full-length progeny genomic RNAs and spliced mRNAs encoding viral proteins (Shors, 2011). After export from the nucleus, the mRNAs are translated and the resulting proteins are processed by a viral protease before combining with the genomic RNAs to form new virions. These bud from the membrane, acquiring lipid envelopes in the process, and become infectious particles (Figure 1.1) (Greene et al., 2008).

Focusing in the clinical course of HIV-1 infection, the initial step is recognized by the high peak of plasma viremia. This occurs within 2 weeks of transmission and declines as $CD4^+$ T lymphocytes accumulate, representing a specific immune response to HIV-1 (Figure 1.2) (Simon and Ho, 2003). During the chronic stage of HIV infection, infected individuals have different viral set-points due to variations in genetics, strain specificity and the cytotoxicity of host and viral factors (Figure 1.2) (Connick et al., 2001). The gradual increase in plasma viremia and loss of $CD4^+$ cells marks the onset of AIDS (Figure 1.2). Two weeks after infection, the virus begins to multiply efficiently. This is followed a week later by the production of anti-HIV antibodies, which inhibit further HIV replication causing the plasma viremia to decline once more (Figure 1.2). The presence of anti-HIV antibodies indicates the end of the first (acute) stage of HIV infection and the beginning of the second, chronic stage (Morrison et al., 2010; Pilcher et al., 2004; Schacker et al., 1998).

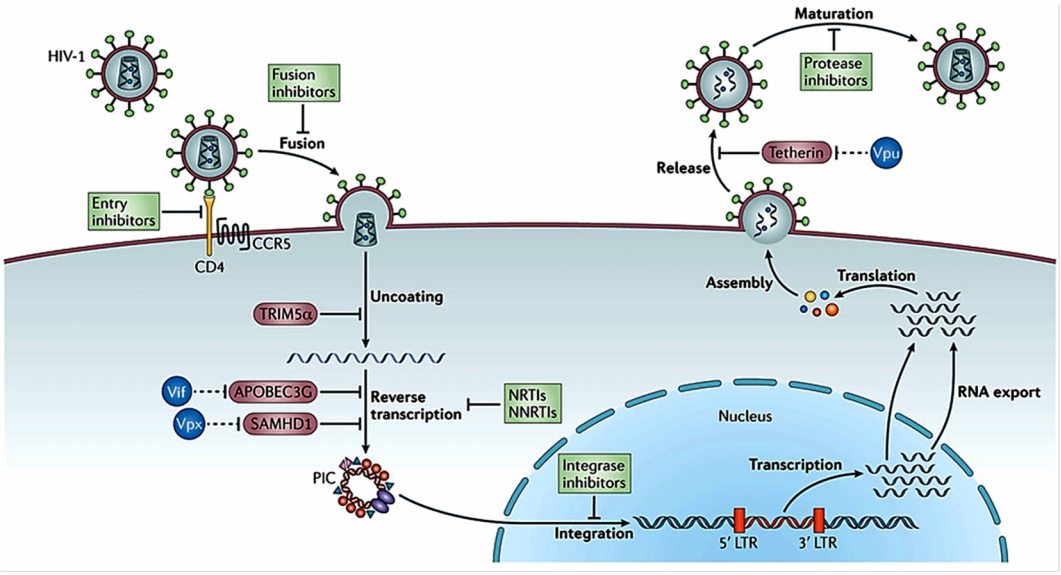


Figure 1.1: Schematic overview of the main steps in the HIV replication cycle: binding to CD4 and co-receptors; fusion with the host cell membrane; uncoating of the viral genome; release of the RNA and proteins into the cytoplasm; reverse transcription of RNA to DNA; formation of the pre-integration complex (PIC); and translocation into the nucleus. Once in the nucleus, the proviral DNA is integrated into the host DNA and subsequently transcribed and translated to form new viral RNA and viral proteins that translocate to the cell surface and assemble into new immature particles. The virions bud off and are released. Finally, during maturation, the protease cleaves the structural polyprotein to form mature Gag proteins, resulting in the production of new infectious virions (Barré-Sinoussi et al., 2013).

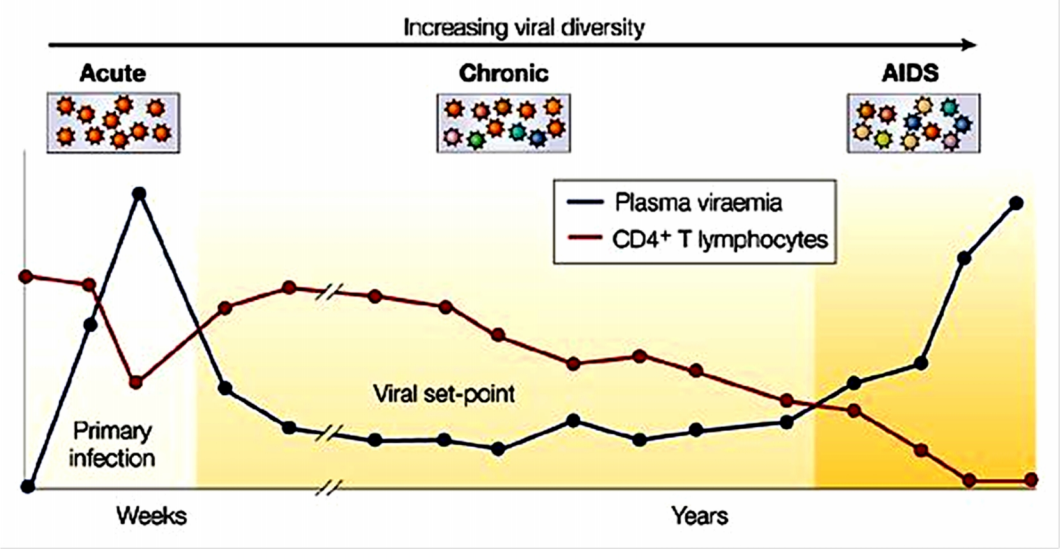


Figure 1.2: The natural course of HIV-1 infection based on the longitudinal evolution of the two key surrogate markers — plasma viremia and CD4+ T-lymphocyte count. The primary infection phase is associated with clinical symptoms of acute retroviral syndrome. After the first months of infection, plasma viremia stabilizes at an

individual-specific level and the CD4+ T-lymphocyte number normalizes. The protracted chronic phase of infection is clinically silent despite the fact that HIV-1 replicates continuously at high levels. AIDS is defined by the occurrence of opportunistic infections or HIV-1-associated malignancies as defined by the Centers for Disease Control and Prevention (CDC) classification of 1993. The risk of opportunistic infections increases significantly when the number of CD4+ T lymphocytes falls to below 200 per mm3. Viral diversity increases over the course of infection in accordance with the continuously high level of viral replication and the error-prone nature of HIV-1 reverse transcription (Simon and Ho, 2003).

Neutralizing antibodies play a major role in the defense against HIV (Albert et al., 1990; Montefiori et al., 2001). However, their precise impact is unclear because this early antibody response specifically targets the autologous circulating virus and is ineffective against heterologous viruses (Wei et al., 2003). Studies of asymptomatic HIV^+ individuals, who have been infected for at least 1 year without clinical signs of AIDS and who have not received antiretroviral drug therapy, revealed that 10–30% of such individuals develop broadly neutralizing antibodies and these tend to accumulate three or more years after infection (Stamatatos et al., 2009). However, only 1% of HIV^+ individuals develop antibodies with outstanding breadth and potency (Simek et al., 2009).

Early studies led to the isolation of only four broadly neutralizing monoclonal antibodies: b12, 2G12, 2F5 and 4E10 (Cardoso et al., 2005). These are not highly potent, but they nevertheless helped to define some HIV vulnerable sites and are also efficacious against SHIV infection in macaques (Baba et al., 2000; Hessell et al., 2010; Mascola et al., 2000). All four antibodies are atypical and are not found in nature: b12 is a phage-derived antibody (Klein et al., 2013), 2G12 has three rather than the normal two combining sites (Calarese et al., 2003; Trkola et al., 1996), and 4E10 and 2F5 are self-reactive (Haynes et al., 2005; Yang et al., 2013). The unsuccessful attempts to stimulate antibody production by vaccination and the large quantities required for transmission blocking in macaques resulted in limited enthusiasm for the development of these antibodies (Mascola and Nabel, 2001).

Two recent developments, i.e. single-cell antibody cloning techniques (Scheid et al., 2009) and the reduced risk of infection achieved by the RV144 human vaccine, have refocused attention on these antibodies and facilitated the discovery of additional and more suitable candidates (Haynes

et al., 2012). New antibodies have been discovered that target two or more different sites on the HIV envelope spike (Bonsignori et al., 2012; Georgiev et al., 2013; Klein et al., 2012; Scheid et al., 2011, 2009). This second generation of broadly neutralizing antibodies target four major vulnerable sites on the HIV envelope spike, i.e. the CD4-binding site, the N160 glycan-dependent site associated with the V1/V2 loops, the N332 glycan-dependent site at the base of V3 loop and the membrane proximal external region (MPER) on gp41 (Figure 1.3).

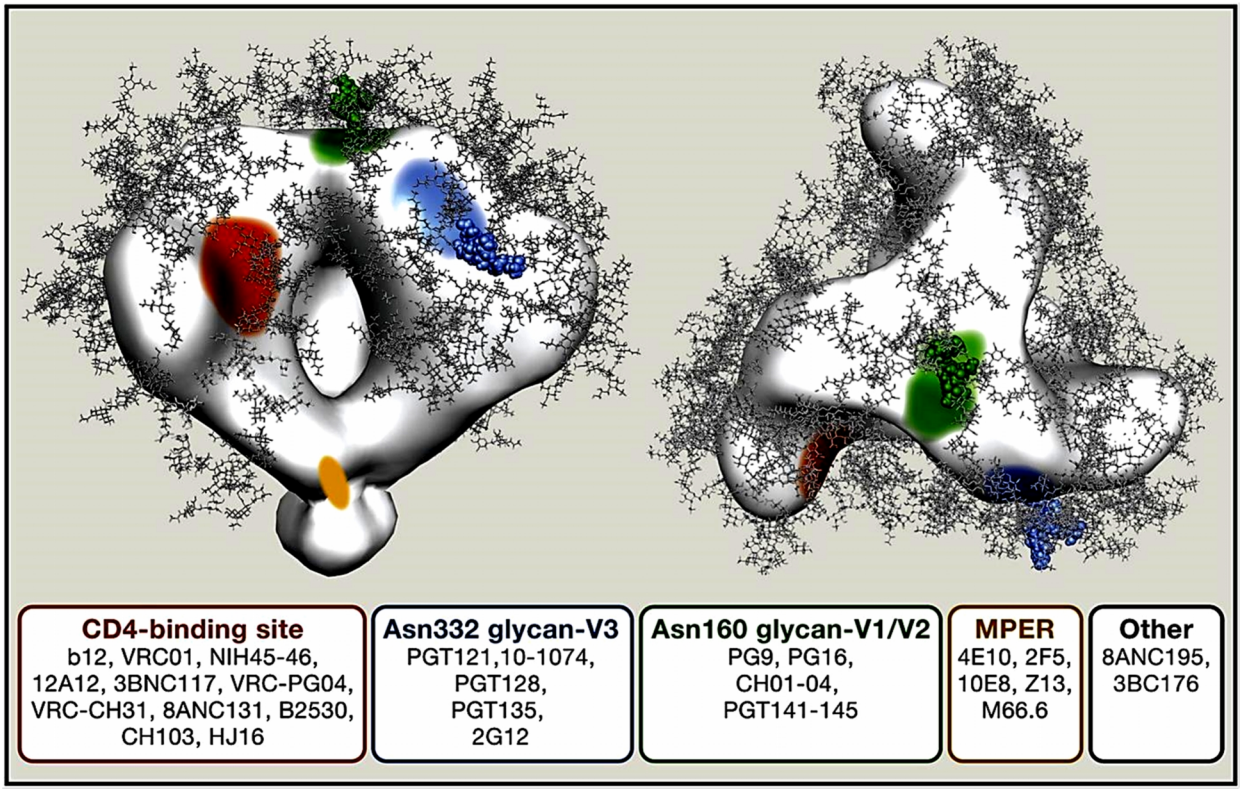


Figure 1.3: Epitopes of broadly neutralizing antibodies on HIV-1 Env. Structure of the Env trimer as determined by electron microscopy (Liu et al., 2008) showing the approximate locations of epitopes on the surface (each epitope is shown once per trimer): CD4-binding site epitope (red), V3 loop/Asn332 epitope (glycan attached to Asn332 as a space-filling model) (blue), V1/V2 loop/Asn160 epitope (glycan attached to Asn160 as a space-filling model) (green), MPER epitope (yellow). N-linked glycans are shown as gray sticks and were added to all potential acceptor sites present in the coordinates for BG505 SOSIP Env (PDB 4NCO) using the GlyProt server (West et al., 2014).

Even though the CD4 binding site is protected, i.e. surrounded by glycans and variable regions (Zhou et al., 2007), this functionally-conserved site remains accessible to CD4 and the corresponding antibodies. Two of the most potent antibodies, NIH45-46 AND 3BNC117, share a

common mode of antigen recognition even though they were isolated from different individuals (Scheid et al., 2011).

PG9, PG16, CH01–CH04 and PGT141–PGT145 target the N160 glycan-V1/V2 site by penetrating the glycan shield with a long anionic third heavy chain complementary-determining region (Julien et al., 2013a; McLellan et al., 2011). The PGT121-like and PGT128-like antibodies target the N332 glycan-V3 loop site. PGT121-like antibodies contact gp120 at the base of the V3 loop and a high-mannose or complex-type N-glycan at N332 (Julien et al., 2013b; Mouquet et al., 2012). In addition, PGT128-like antibodies contact the C-terminus of the V3 loop, mannose Man8/9 glycans and a synthetic Man5 glycan lacking the core N-acetylglucosamine (Man5GlcNAc2) at positions N332 and N301, respectively (Pejchal et al., 2011). Finally, 10E8 targets the MPER in gp41 by recognizing an α-helix near the 2F5 and 4E10 recognition site. However, 10E8 is a more potent antibody, which is neither autoreactive nor phospholipid-reactive (Huang et al., 2012).

A panel of broadly-neutralizing anti-HIV antibodies has been described which differ in terms of the number of HIV strains and clades neutralized and the potency of *in vitro* neutralization (reviewed by McCoy and Weiss, 2013). However, only a few of these have been tested in preclinical studies for their ability to prevent the transmission of HIV or SHIV (Ramessar et al., 2010). Importantly, most HIV-neutralizing antibodies are highly mutated and can also carry insertions and deletions that are uncommon in other antibodies. In addition, these antibodies lose their neutralizing activity and even fail to bind to most HIV-1 envelope proteins when reverted to their germ line sequences (Scheid et al., 2011).

The first-generation antibody 2G12 recognizes a cluster of high-mannose glycans attached to Asn332, Asn295 and Asn339, using a unique domain-swapped architecture that combines both Fab arms into a single, large antigen-binding $(Fab)_2$ unit (Calarese et al., 2003). Domain-swapped 2G12 dimers show higher neutralization potency and breadth (West et al., 2009) through the binding of two $(Fab)_2$ units that are flexibly linked to two Fc regions in the 2G12 dimer (Wu et al., 2013). HIV-neutralizing antibodies 2G12, 2F5 and 4E10 have been produced in Chinese hamster ovary (CHO) cells for prophylactic and therapeutic use (Hofmann-Lehmann et al., 2001; Stiegler et al., 2002).

Several HIV-neutralizing antibodies have been produced in plants. The first was b12, which was expressed in tobacco as an IgG (Sexton et al., 2009) and also as a fusion with the anti-HIV lectin cyanovirin-N (Boyd et al., 1997; Dey et al., 2000). Then 4E10 was produced by secretion from hydroponic tobacco and was shown to assemble and function correctly although the overall yields were low (Drake et al., 2009). Most work has been carried out with 2G12, which has been expressed successfully in tobacco and was the first plant-derived antibody to complete a phase I clinical study in Europe (Fischer et al., 2012). This antibody has also been produced in maize in two separate studies, in one case by secretion to the apoplast and in the other by retention in the endoplasmic reticulum (ER) (Rademacher et al., 2008; Ramessar et al., 2008b). In both cases the yields were high (40–100 mg/kg dry seed weight) and the purified antibody had a neutralization activity that was similar to or greater than the same antibody produced in CHO cells. Furthermore, 2G12 has also been expressed transiently in *Nicotiana benthamiana* with yields of up to 50 mg/kg fresh biomass (Sainsbury and Lomonossoff, 2008; Strasser et al., 2008). Finally, 2F5 has been produced in tobacco plants as a fusion protein with an elastin like peptide (ELP) (Floss et al., 2008). Free 2F5 has also been expressed in tobacco suspension cells, with similar antigen-binding affinity to its CHO counterpart but less potent HIV-1 neutralization activity (Sack et al., 2007).

Broadly-neutralizing human monoclonal antibodies against HIV can be used as microbicides to prevent virus transmission. HIV-1 entry into susceptible cells requires the envelope (Env) protein and a trimer of gp120/gp41 heterodimers, with gp120 acting as the external surface subunit that engages cellular receptors, and gp41 as the transmembrane subunit that mediates membrane fusion (Poignard et al., 2001). The gp120 protein interacts with the T-cell surface glycoprotein CD4 and the co-receptors CCR5 or CXCR4, so antibodies against these proteins can be used to prevent the uptake of HIV into target cells. The high cost and limited capacity of cell culture platforms make it unfeasible to meet the demand for these proteins in developing countries because 100–1000 kg of each antibody would be required annually and each microbicide would require 2–3 antibodies targeting different epitopes to prevent the emergence of escapes (Ramessar et al., 2010).

1.2 AIMS

The aims of the work described in this chapter were to test whether rice endosperm can function as a platform for the production of 2G12 and to gain insight into the factors that affect the production and functional efficacy of neutralizing antibodies in plants and the impact this has on endogenous gene expression.

1.3 MATERIALS AND METHODS

1.3.1 Cloning and construct design

All transformation constructs were based on the binary vector pTRA, a derivative of pPAM (GenBank accession no. AY027531). The vector contains two tobacco *RB7* scaffold attachment regions flanking the expression cassette. The coding regions of the 2G12 heavy and light chains (obtained from Polymun, Vienna, Austria) included N-terminal signal sequences targeting each polypeptide to the secretory pathway (Figure 1.4). The expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the maize ubiquitin-1 first intron, the *Tobacco etch virus* 5' leader, which acts as a translational enhancer, the coding region, and the *Cauliflower mosaic virus* 35S terminator, resulting in final constructs pTRAgtiGH and pTRAgtiGL.

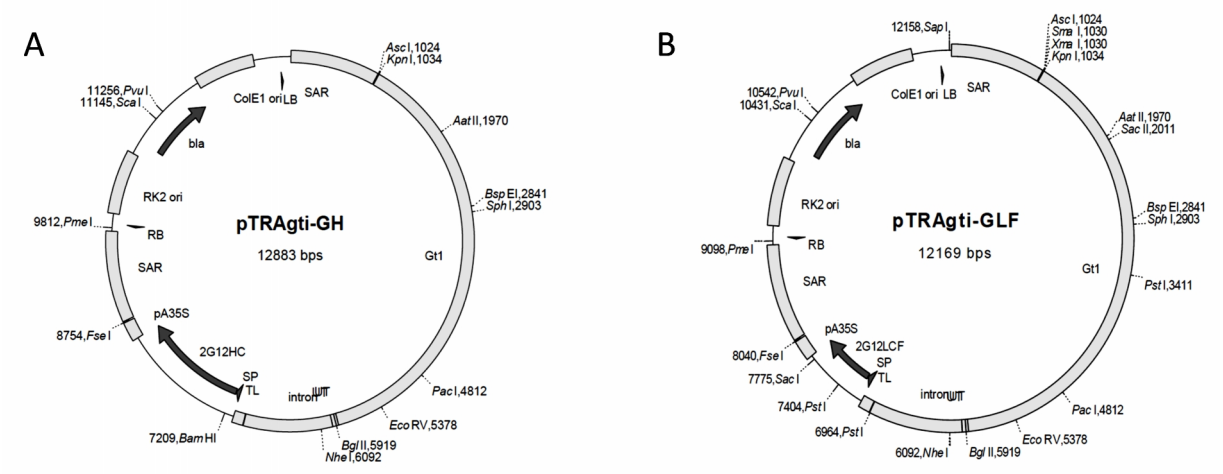


Figure 1.4: Transformation constructs for stable expression in rice endosperm. A) pTRAgti-GH containing the 2G12 heavy chain (HC) gene. B) pTRAgti-GLF containing the 2G12 light chain (LC) gene. In both constructs the

expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the *Tobacco etch virus* 5' leader, the coding region and the CaMV 35S terminator (Ramessar et al., 2008b).

1.3.2 Transformation and regeneration of transgenic plants

Seven-day-old mature rice zygotic embryos (*Oryza sativa* cv. Nipponbarre) were transferred to osmotic medium (4.4 g/l MS powder supplemented with 0.3 g/l casein hydrolysate, 0.5 g/l proline, 72.8 g/l mannitol and 30 g/l sucrose) 4 h before bombardment with 10 mg gold particles coated with the 2G12 heavy and light chain constructs and the selectable marker *hpt* at a 3:3:1 ratio (Sudhakar et al., 1998; Valdez, 1998). The embryos were returned to osmotic medium for 12 h before selection on MS medium (4.4 g/l MS powder, 0.3 g/l casein, 0.5 g/l proline and 30 g/l sucrose) supplemented with 50 mg/l hygromycin and 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark for 2–3 weeks. Transgenic plantlets were regenerated and hardened off in soil. Plants were grown in the greenhouse and growth chamber at 28/20°C day/night temperature with a 10-h photoperiod and 60-90% relative humidity for the first 50 days, followed by maintenance at 21/18°C day/night temperature with a 16-h photoperiod thereafter in a growth chamber.

1.3.3 Characterization of rice plants and transgene expression

Genomic DNA was isolated from rice leaves by phenol extraction (Edwards et al., 1991) and 50-ng aliquots were used for PCR with three primers designed to generate overlapping products to confirm transgene integrity (Figure 1.5). GoTaq DNA polymerase was used with the program recommended by the manufacturer (Promega). The primers are listed in Table 1.1. The positive lines were then screened for transgene expression by dot blot. The total soluble protein (TSP) was extracted in three volumes (v/w) of phosphate buffered saline (PBS). The samples were centrifuged twice (15 min, 4°C, 13,000 x g) and 4 µl of supernatant was spotted onto a nitrocellulose membrane (Amersham, GE Healthcare, Buckinghamshire, UK). The membrane was blocked for 1 h with 5% blocking buffer and probed overnight, shaking at room temperature with alkaline phosphatase-labeled antibodies against the heavy and light chains (goat anti-human IgG Fc chain, Sigma (St. Louis, MO, USA) A9544, and goat anti-human kappa light chain,

Sigma A3813). The blots were washed three times with PBS supplemented with 0.2% Tween-20 (PBST) and the antibody was detected using Sigma FAST^{TM} BCIP/NBT in distilled water.

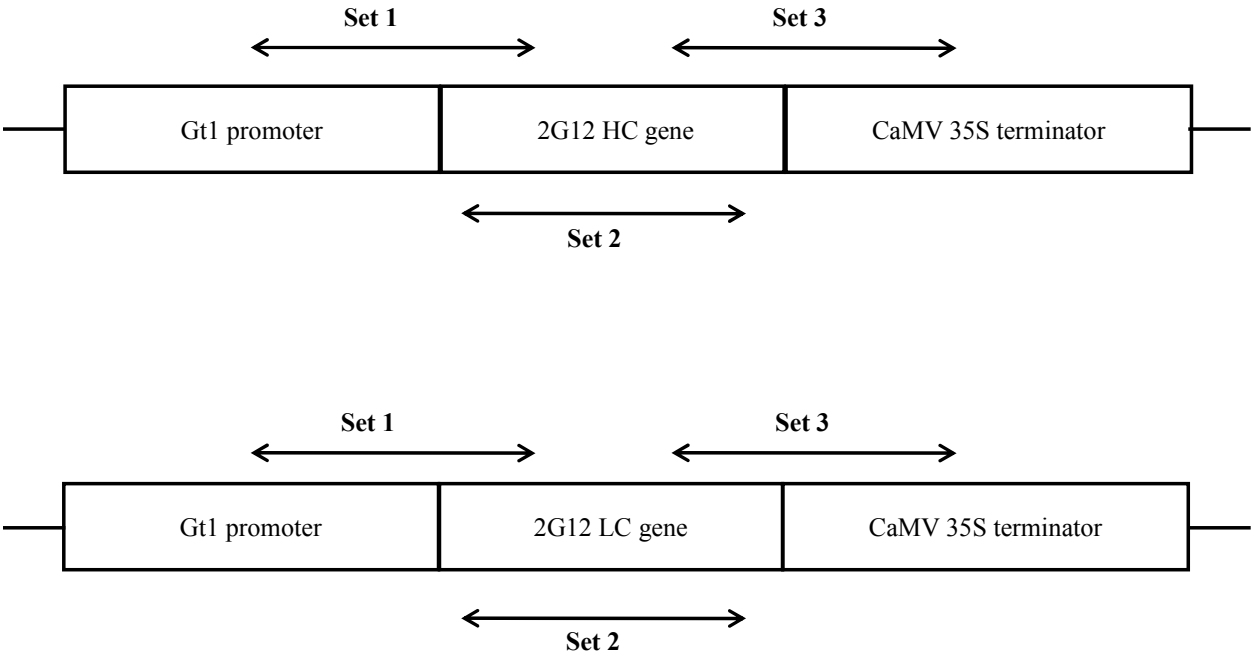


Figure 1.5: Three primers for the PCR-based detection of intact expression cassettes in transgenic plants.

Table 1.1: Primers used to screen putative transgenic plants by PCR.

Set of primers	Gene	Primer Name	Primer sequence
Set 1 2G12	HC	Gt1-F	5'- CAC CTT TCG TGT ACC ACA CTT C -3'
		Gene-R	5'-CAC GGT GAG CTT GCT GTA GA -3'
	LC	Gt1-F	5'- CAC CTT TCG TGT ACC ACA CTT C -3'
		Gene-R	5'- TCC CCT GTT GAA GCT CTT TG -3'
Set 2 2G12	HC	HC-F	5'- GGC TAT TTT AAA AGG TGT CCA GTG TG-3'
		Gene-R	5'-CAC GGT GAG CTT GCT GTA GA -3'
	LC	Gene-F	5'- TCA GTC TCC TTC CAC CCT GT-3'
		Gene-R	5'- TCC CCT GTT GAA GCT CTT TG -3'
Set 3 2G12	HC	Gene-F	5'- AAA GGT GTC CAG TGT GAG GTG-3'
		EV-Camv-R	5'- GGT TTC TTA TAT GCT CAA CA-3'
	LC	LC-F	5'- TAC TCT GGC TCC CAG GTG CCA AAT-3'
		EV-Camv-R	5'- GGT TTC TTA TAT GCT CAA CA-3'

1.3.4 Confirmation of antibody assembly and accumulation (ELISA)

Three seeds representing each event were analyzed by ELISA to quantify 2G12 expression, using goat anti-human kappa light chain (Sigma K3502) or goat anti-human IgG Fc chain (Sigma K2136) as capture antibodies. These were diluted 1:200 and coated directly onto 96-well Nunc-Immuno Maxisorp surface plates (Nalge Nunc) and incubated overnight at 4°C. The plates were blocked with 5% nonfat milk for 2 h and then washed with PBST. Serial dilutions of the samples were added to the wells and incubated for 2–3 h at room temperature. After washing, a horseradish peroxidase (HRP)-conjugated sheep anti-human kappa-chain antiserum (The Binding Site, Birmingham, UK) was added to the plates at a 1:1000 dilution and incubated at room temperature for 2 h before the signal was detected with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). After 4–5 min, the signal was quantified by measuring the absorbance at 450 nm.

1.3.5 Fluorescence microscopy

Fluorescence microscopy was carried out by E Arcalis, Department of Genetics and Cell Biology, BOKU, Vienna. Sections of immature rice endosperm (20 days after pollination) were mounted on glass slides and pre-incubated in 5% (w/v) bovine serum albumin (BSA Fraction V) in phosphate buffer (0.1 M, pH 7.4) and then incubated with polyclonal rabbit anti-HC or anti-LC antiserum diluted 1:100. Sections were then treated with the secondary antibody diluted in phosphate buffer (0.1 M, pH 7.4). An AlexaFluor 594-conjugated goat anti-rabbit IgG (red fluorophore) was used to detect 2G12 and an anti-glutelin antibody (green fluorophore) was used to detect endogenous glutelins.

1.3.6 Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out by E Arcalis, Department of Genetics and Cell Biology, BOKU, Vienna. Developing rice seeds (20 days after pollination) were fixed

and processed as described by Arcalis et al. (2004). Immature seeds from wild-type and transgenic rice plants were cut into small pieces with a razor blade under PBS (pH 7.4) and fixed in 4% (w/v) paraformaldehyde with 0.5% (v/v) glutaraldehyde in PBS overnight at 4°C. After several washes in PBS, the samples were dehydrated through an ethanol series (50%, 70%, 90%, 100%) at 4°C and progressively embedded in LR White resin (25% in ethanol, 50%, 75% and pure resin for 3 h each) followed by an additional step with fresh 100% resin. Blocks were mounted in Beem capsules and polymerized overnight at 60°C. Sections showing silver interference colors were collected on gold grids, pre-incubated in 5% (w/v) BSA in PBS for 15 min at room temperature and then incubated with either polyclonal goat anti-HC or anti-LC (1:100 in PBS) for 2 h at room temperature. The primary LC antibody is not specific and also binds HC, whereas the primary HC antibody is specific. After three washes for 10 min in PBST, sections were incubated with the secondary antibody (donkey anti-goat IgG coupled to 15-nm colloidal gold particles) diluted 1:30 in PBS and incubated for 1 h at room temperature. After two washes for 10 min in PBS and two in distilled water, samples were air dried and the sections were observed using a FEI Tecnai G2 transmission electron microscope.

1.3.7 Protein A affinity chromatography and SDS/PAGE

Rice seeds were ground to a fine powder and extracted overnight at 4°C in five volumes of buffer (1× PBS, 5 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4). Insoluble material was removed by centrifuging twice at 8000 × g for 30 min at 4°C. The supernatant was filtered and the antibody concentration in the crude extract determined by ELISA. The sample was loaded onto a protein A affinity column (ceramic HyperDF) at a flow rate of 2 ml/min. The column was washed with PBST and PBS, and the antibody was eluted in 100 mM glycine (pH 3.6) containing 100 mM fructose, and buffered with 0.1 volumes of 1 M sodium acetate (pH 4.75). Antibody-containing fractions were identified by the droplet Bradford method (Bradford, 1976), and 2G12 concentrations were determined by ELISA. Fractions containing >50 µg/ml were pooled and dialyzed for 2 days against 10 mM sodium acetate (pH 4.75) containing 1 mM EDTA and 0.1 mM 2-mercaptoethanol (omitted in the final dialysis step). The dialyzed antibody was concentrated by ultrafiltration using spin-columns with a 30-kDa molecular weight cut-off

(MWCO). Antibody heavy and light chains were separated by SDS-PAGE (12% polyacrylamide) as previously described (Laemmli, 1970).

1.3.8 Glycan analysis

Glycan analysis was carried out by F Altmann, Department of Chemistry, BOKU, Vienna. Antibody bands from Coomassie-stained polyacrylamide gels were destained, carbamidomethylated, digested with trypsin, and extracted from gel pieces as described (Shukla et al., 2007). The peptides were separated by capillary reversed phase chromatography with a Q-TOF Ultima Global mass spectrometer (Waters) for detection (Van Droogenbroeck et al., 2007). The MS data from the tryptic peptides were analyzed as previously described (Van Droogenbroeck et al., 2007) and compared with datasets generated by *in silico* tryptic digestion of the 2G12 coding region, using the PeptideMass program (www.expasy.org/tools/peptide-mass.html). Based on the tryptic peptide datasets, tryptic glycopeptide datasets were generated by adding glycan mass increments to the masses of the two identified glycopeptides.

1.3.9 *In vitro* binding to gp120 (ELISA)

The specific antigen-binding activity of 2G12 was determined by coating ELISA wells with 100 ng recombinant gp120 from HIV strain IIIB, provided by the MRC Centralized Facility for AIDS reagents, Potters Bar, UK. After washing with PBST and blocking with BSA, serial dilutions of 2G12 were added and the amount of bound antibody determined using a mix of HRP-conjugated anti gamma-chain and HRP-conjugated anti-kappa chain (The Binding Site) diluted 1:1000. The wells were washed with PBS and the signal was developed with TMB substrate followed by reading the absorbance at 450 nm.

1.3.10 Syncytium-inhibition assay for HIV neutralization

The HIV-neutralization assays were carried out by AYH Teh, Molecular Immunology Unit, Division of Clinical Sciences, St. George's Hospital Medical School, University of London.

HIV-1 neutralization was assessed using a syncytium-inhibition assay. Ten two-fold serial dilutions (starting concentration 100 µg/ml) of ^{OS}2G12, ^{CHO}2G12, ^{NT}2G12 and a non-neutralizing control were pre-incubated with HIV-1 Bal at 10 half-maximum tissue culture infectious doses (Terman and Bertram, 1985) per ml for 1 h at 37°C. $CD4^+$ human AA-2 cells were added at a density of 4×10^5 cells/ml and incubated for a further 5 days. We analyzed eight replicates per antibody dilution step. The presence of one or more syncytia per well after 5 days was scored as positive for infection. The half maximal inhibitory concentrations (IC_{50}) were calculated using the concentrations present during the antibody-virus pre-incubation step (Montefiori, 2005; Wei et al., 2003).

1.3.11 Comparative proteomic analysis

Comparative proteomic analysis was carried out by A Murad, Embrapa Genetic Resources and Biotechnology, Laboratory of Synthetic Biology, Parque Estacao Biologica, Distrito Federal, Brasil. Approximately 100 mg of rice seed powder was vortexed with 500 µl buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM KCl, 10 mM DTT, 1 mM PMSF and 0.1% (v/v) SDS) at room temperature for 10 min and centrifuged for 5 min at 10,000 x g. The protein content of the supernatant was measured using a NanoDrop (Thermo Fisher Scientific, USA) and 100 µg of TSP was precipitated using 1:4 cold acetone. After centrifugation as above, the pellet was dried at room temperature for 10 min and dissolved in 50 µl 50 mM NH_4HCO_3 to a final concentration of 2 µg/µl, then mixed with 10 µl 50 mM ammonium bicarbonate and 25 µl 0.2% (v/v) RapiGEST™ at 80°C for 15 min before adding 2.5 µl 100 mM DTT and incubating at 60°C for a further 30 min. After centrifuging as above, samples were mixed with 2.5 µl 300 mM iodoacetamide in the dark at room temperature for 30 min and then with 10 µl 300 mM iodoacetamide at 37°C overnight. RapiGEST was precipitated with 10 µl 5% TFA for 90 min at 37°C and the samples were centrifuged at 10,000 x g at 6°C for 30 min. The supernatants were transferred to Waters Total Recovery vials (Milford, USA), freeze dried and resuspended in 100 µl 200 mM ammonium formate containing 50 fmol/µl Waters protein standards digested with rabbit phosphorylase B. The final protein concentration was 1 µg/µl.

The tryptic peptides were fractionated using a nanoACQUITY™ system (Waters). First-dimension separation was carried out using an XBridge™ reversed phase column (300 µm x 50 mm, 5-µm C18 resin) with 20 mM ammonium formate as mobile phase A and acetonitrile as mobile phase B. Second-dimension separation was carried out using a Symmetry pre-column (300 µm x 50 mm, 5-µm C18 resin) and a HSST3 reversed phase column (75 µm x 150 mm, 1.8-µm C18 resin) with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B.

The samples were injected into the first column at a flow rate of 2 µl/min in 0.1% mobile phase B and were eluted in 10.8 % mobile phase B for 2 min at the same flow rate. After dilution with aqueous 0.1% formic acid in the pre-column at a flow rate of 10 µl/min for 8.5 min, the peptides were separated on the second column using a gradient of 7–35 % mobile phase B for 37 min at a flow rate of 500 µl/min followed by a 5-min rinse with 85 % mobile phase B. The column temperature was maintained at 35 °C. After re-equilibration, peptides for the second, third, fourth and fifth fractions were eluted using 14, 16.7, 20.4 and 50 % of mobile phase B, respectively.

The tryptic peptides were analyzed using a Synapt G2 HDMS™ mass spectrometer (Waters, Manchester, UK) with a hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight (oa-TOF) geometry in positive nanoelectrospray ion mode. Exact mass retention time (EMRT) nanoLC-MSE data (Silva et al., 2006) were collected in alternating low and elevated energy mode at 3 eV and 12–45 eV respectively with a continuum spectra acquisition time of 1.5 s and a 0.1-s interscan delay.

MS data were processed and searched using ProteinLynx Global Server (PLGS) v2.5 (Waters, Manchester, UK). Proteins were identified using the ion accounting algorithm and a search of the UniProt Oryza spp database with MassPREP digestion standards (MPDS). Identifications and quantitative data packaging were generated using dedicated algorithms (Li et al., 2009; Silva et al., 2006) and a search against a species-specific UniProt database. The fixed modification of carbamidomethyl-C was specified, and the variable modifications included were amidation C-terminus, deamidation N, deamidation Q, methyl N-terminus, methyl C-terminus, glycation N-terminus, O-GlcNac ST and oxidation M. Components were typically clustered with a 10 ppm mass precision and a 0.25-min time tolerance against the database-generated theoretical peptide ion masses with a minimum of one matched peptide. One missed cleavage site was allowed. The

protein identification criteria also included the detection of at least three fragment ions per peptide, six fragments per protein, the determination of at least one peptide per protein, and the identification of the protein was allowed with a maximum 4% false positive discovery rate in at least three technical repeatability injections. The intensity measurements were normalized to those representing peptides of the digested internal standard (Murad and Rech, 2012).

1.3.12 Quantitative real-time PCR

RNA was isolated from rice endosperm 25 days after pollination as described by Li and Trick (2005) with corrections and modifications. Extraction buffer I was 100 mM Tris (pH 8.0), 150 mM LiCl, 50 mM EDTA, 1.5% SDS, 1.5% 2-mercaptoethanol and extraction buffer II was 4.2 M guanidine thiocyanate, 1 M sodium acetate, (pH 4.0), 0.5% lauryl sarcosine, 25 mM sodium citrate (pH 7.0). The samples were processed on a Bio-Rad CFX96 system using the Quantitect reverse transcription kit (Qiagen) in 25 µl reactions comprising 10 ng cDNA, 1x iQ SYBR Green Supermix (Bio-Rad) and 0.2 µM forward and reverse primers. To calculate relative expression levels, serial dilutions (60–0.096 ng) were used to produce standard curves for each gene. PCRs were carried out in triplicate using 96-well optical reaction plates, initially heating for 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 50 °C for GLGB, 50.7°C for GRXC8 and PRO7 and 58.3 °C for GLGS for 1 min, and 72 °C for 20 s. Amplification specificity was confirmed by melt curve analysis on the final PCR products in the temperature range 50–90 °C with fluorescence acquired after each 0.5 °C increment. The fluorescence threshold value and gene expression data were calculated using CFX96 system software. Values represent the mean of three replicates ± SD. The forward and reverse primers for each gene are shown in Table1.2.

Table 1.2: Oligonucleotide sequences of primers used for quantitative real-time RT-PCR analysis

Transgenes	Primer sequence
OsACTIN-F	Forward 5'-GACTCTGGTGATGGTGTCAGC-3'
OsACTIN-R	Forward 5'-TCATGTCCCTCACAATTT-3'
OsGRXC8-F	Forward 5'-TCCGTATGTGGTGGAACTGG-3'
OsGRXC8-R	Forward 5'-TTCCACGGTATCATCGCTGCC-3'
OsPRO7-F	Forward 5'-GCAGCAGAGCCATTATCAGGAT-3'
OsPRO7-R	Forward 5'-CCGAGCACCATTACCACCCT-3'
OsGLGS-F	Forward 5'-TAGCGTGATTGGCGAAGGC-3'
OsGLGS-R	Forward 5'-GCAGTTTTTTATCCGCTTCG-3'
OsGLGB-F	Forward 5'-CGAAGGCAACAACTGGAGC-3'
OsGLGB-R	Forward 5'-AATCTGTTTGCTGCTGCTCA-3'

1.4 RESULTS

1.4.1 Generation of transgenic rice plants expressing 2G12

Rice embryos were transformed by particle bombardment with constructs containing the coding sequences of the 2G12 heavy and light chains, under the control of the rice glutelin-1 promoter (Ramessar et al., 2008b) plus a third construct containing the selectable marker *hpt*. Embryo-derived callus was selected on hygromycin-supplemented medium and 20 independent transformants were regenerated and transferred to the greenhouse. Leaves and seeds from these primary transformants were analyzed by PCR to confirm the presence of the transgene (Figure 1.6) and by dot blot to confirm transgene expression (Figure 1.7). For the heavy chain, the amplified products for primer sets 1–3 were 1878, 1279 and 1514 bp, respectively. For the light chain, the amplified products for primer sets 1–3 were 1269, 622 and 808 bp, respectively. The positive control was used to ensure that the amplification reactions proceeded correctly (Figure 1.6). Six independent lines expressing 2G12 at high levels based on ELISA results were self-pollinated to produce T1 and T2 seeds for more detailed analysis.

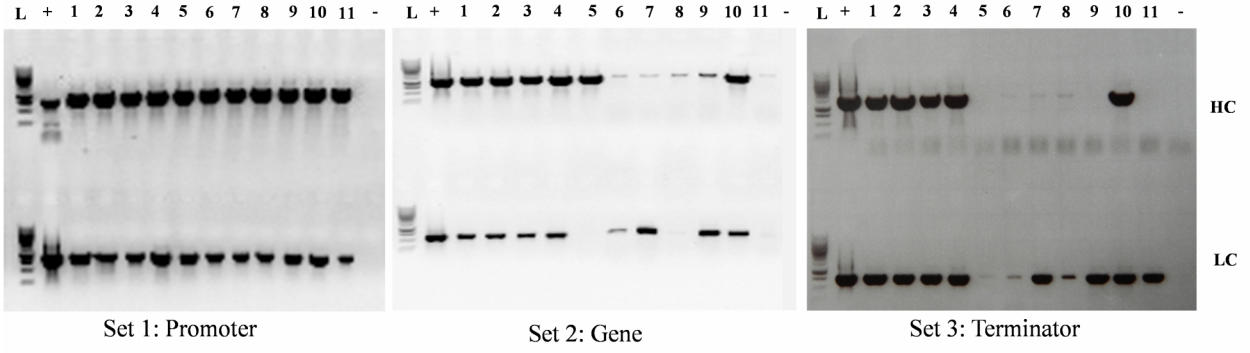


Figure 1.6: PCR amplification of heavy and light chain genes in independent transgenic events. (L) 1-kb DNA ladder from Promega, (+) positive control plasmids pTRAGti-GH and pTRAGti-G, (–) negative control wild-type rice to rule out sample contamination.

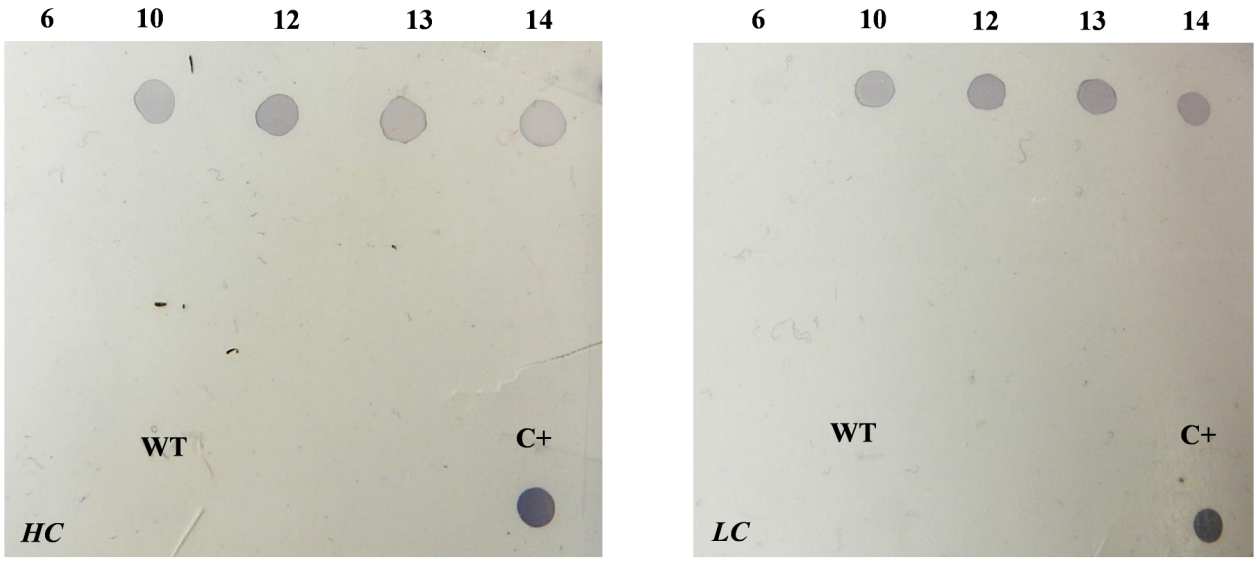


Figure 1.7: Dot blot analysis of proteins extracted from T2 transgenic seeds expressing the 2G12 heavy chain (*HC*) and light chain (*LC*), using 4 µl of total soluble protein extract per spot, probed with anti-human K antibody followed by detection with an alkaline phosphatase-conjugated anti-human IgG antibody.

1.4.2 Confirmation of antibody chain expression and assembly in T1 transgenic endosperm

The presence of correctly-assembled 2G12 in T1 endosperm was confirmed using an immunoglobulin-specific sandwich ELISA. The antibody content of the endosperm was determined by calculating the concentrations from titration curves based on positive controls spiked with known concentrations of the same antibody produced in maize (Figure 1.8). We used non-transformed rice endosperm as a negative control to rule out cross reaction with endogenous plant proteins. Among the six independent lines expressing both antibody chains, we selected the two lines (4 and 14) producing the highest levels of assembled 2G12 for more detailed investigation. The yield of 2G12 in lines 4 and 14 was 2.323 and 3.789 µg/ml, respectively.

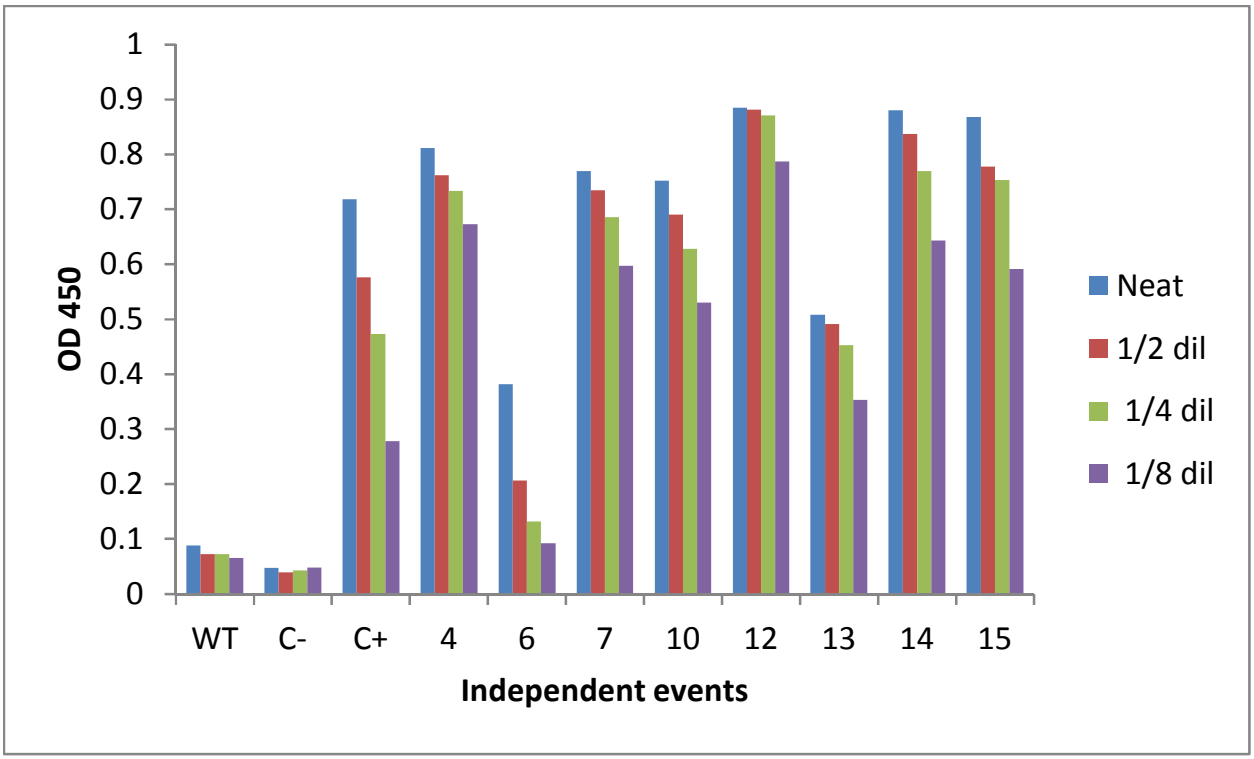


Figure 1.8: The expression of 2G12 in mature rice endosperm. An immunoglobulin-specific sandwich ELISA was used to screen endosperm extracts from independent transgenic lines for 2G12. The plates were coated with an anti-human κ-chain antibody and captured 2G12 was detected with an anti-human IgG antibody labeled with horseradish peroxidase. Four serial dilutions were loaded per sample (neat, 1/2, 1/4 and 1/8). WT = wild type extracts; C– = negative control (PBS); C+ = 2G12 purified from maize (500 ng/ml) as positive control. OD = optical density at 450 nm.

1.4.3 Accumulation of 2G12 in the rice endosperm induces the formation of novel protein bodies

The localization of 2G12 in the endosperm of lines 4 and 14 was investigated by fluorescence microscopy and transmission electron microscopy (TEM). Colocalization with glutelin indicated that most of the antibody had accumulated in the protein storage vacuoles (glutelin bodies, PB-II) but additional labeling was detected outside these organelles (Figure 1.9). Consistent with this result, TEM showed that most of the antibody had accumulated in glutelin bodies and only a small amount in the ER-derived prolamin bodies (also known as type-I protein bodies PB-I). In the transgenic endosperm we also observed novel protein bodies that were studded with

ribosomes but did not contain 2G12 (Figure 1.10). There was no evidence of 2G12 labeling in the untransformed control plants (data not shown).

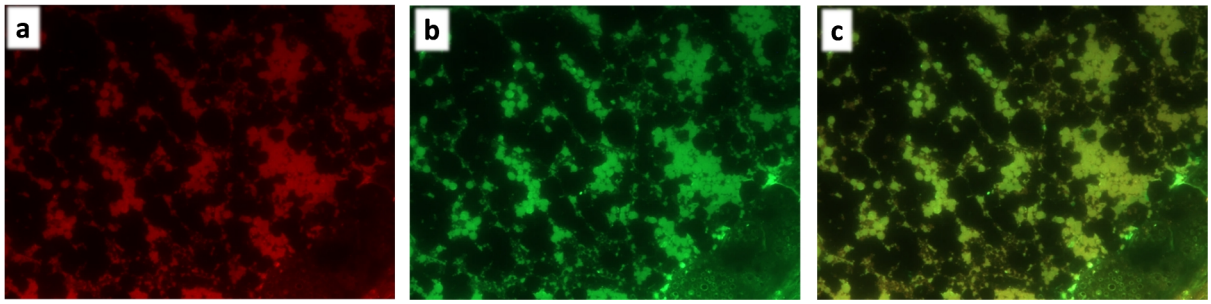


Figure 1.9: Localization of 2G12 and the seed storage protein glutelin in rice endosperm. Immunofluorescence microscopy confirmed that 2G12 accumulated in the PSV. (a) Red fluorescence shows the localization of 2G12; (b) Green fluorescence shows the localization of glutelin; (c) Yellow fluorescence shows the colocalization of 2G12 and glutelin.

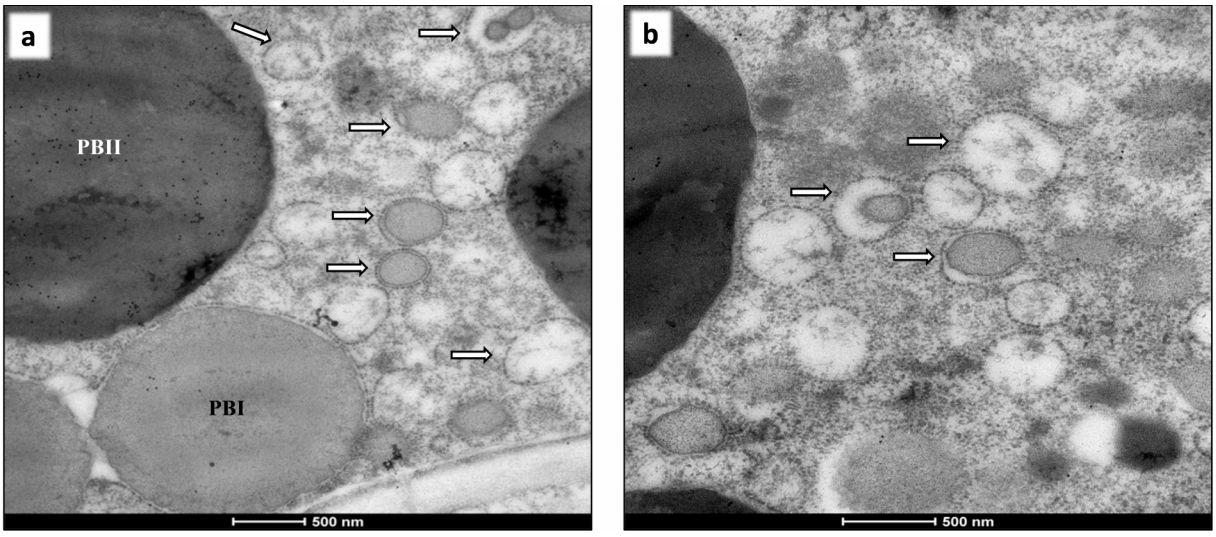


Figure 1.10: Localization of 2G12 in the rice endosperm by electron microscopy. Ultrathin sections of immature rice seeds (line 14) revealed (a) the accumulation of 2G12 mostly in PB-II structures and more sparsely in PB-I structures, as well as (b) novel bodies surrounded by ribosomes (white arrows). Bar = 500 nm, PB-I = protein body I, prolamins; PB-II = protein body II, glutelins and globulins

1.4.4 Glycan analysis reveals that 2G12 produced in rice endosperm is predominantly aglycosylated

Antibody 2G12 was purified from rice endosperm extracts by protein A affinity chromatography. Neither the flow-through nor the wash fractions contained measurable amounts of the 2G12 heavy and light chains indicating that the antibody was folded correctly in planta and retained efficiently on the resin. The final concentration of pure antibody from the T1 generation was 34 μg/g dry seed weight, but after protocol optimization and two rounds of self-pollination to produce the T3 generation of seeds, the antibody concentration increased to 42 μg/g dry seed weight. The presence of the heavy and light chains was demonstrated by reducing SDS-PAGE (50 and 25 kDa bands). No degradation products were present, suggesting the antibody remained stable in the seeds (Figure 1.11).

The purified antibody heavy chain was digested with trypsin and analyzed by MALDI-TOF mass spectrometry. Remarkably, we found that 50% of the EEQYNSTYR peptide, which contains the specific NSx glycan acceptor sequence responsible for heavy chain glycosylation, was completely aglycosylated. Among the remainder, 19% and 14% contained vacuolar-type N-glycans (MUXF and MMXF, respectively), 13% carried a single N-acetylglucosamine (GlcNAc) attached to the asparagine residue, suggesting the presence of ENGase activity in the early part of the secretory pathway, and the remaining 4% carried high-mannose glycans of the Man_7 configuration (Figure 1.12).

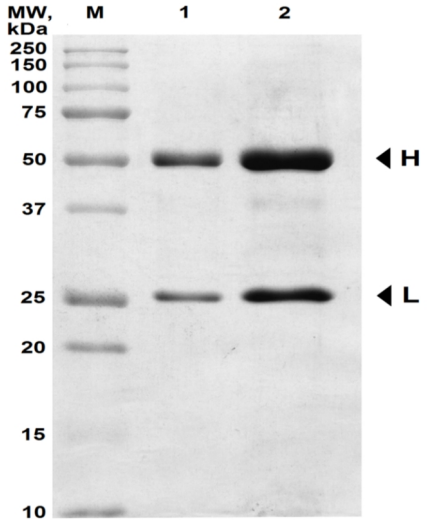


Figure 1.11: SDS-PAGE analysis of 2G12 purified from rice endosperm. Fractions eluting from the protein A column were separated by SDS-PAGE (12% polyacrylamide) under reducing conditions and the gels were stained with Coomassie Brilliant Blue. The heavy (H) and light (L) chains were identified and no degradation products were observed. M = molecular weight markers. Lanes 1 and 2 were loaded with 2 and 5 μg of 2G12, respectively.

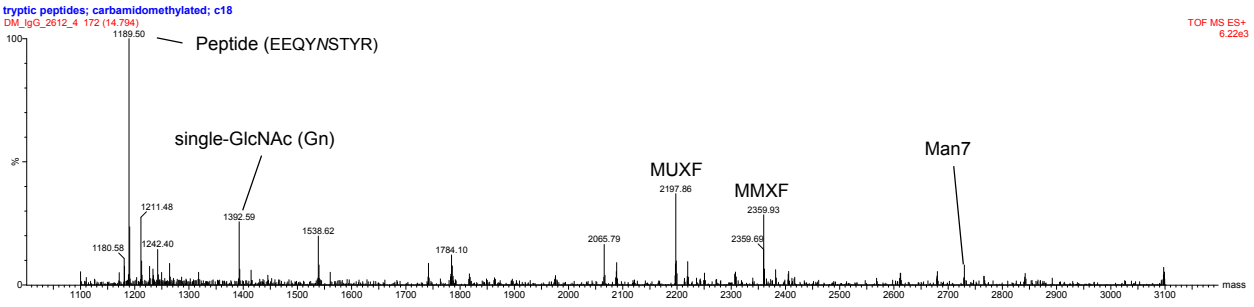


Figure 1.12: Glycosylation profile of 2G12 purified from rice endosperm. De-convoluted mass spectrum (*m/z* = 1100–3100) of the tryptic glycopeptide derived from the 2G12 heavy chain (band H shown in Fig. 4). MMXF = paucimannosidic structures, Man 7 = mannose 7.

1.4.5 The aglycosylated 2G12 shows enhanced *in vitro* binding and HIV-neutralization activity

The ability of the endosperm-derived 2G12 to bind gp120 was tested *in vitro* using an antigen-specific ELISA with both the purified antibody and the crude rice extract. The capture reagent was gp120IIIB and the bound 2G12 was detected using a mixture of anti-human gamma and anti-human kappa antibodies which bind the heavy and light chains, respectively. The transgenic endosperm extracts showed higher gp120IIIB-binding activity than wild-type endosperm extracts as expected, which confirmed that the assembled 2G12 produced in the transgenic endosperm was also functional and did not cross-react with endogenous plant proteins. The crude extracts showed a higher gp120-binding activity than the purified 2G12 produced in rice endosperm and the purified 2G12 with glycans produced in maize endosperm (Figure 1.13).

The HIV-neutralizing activity of rice endosperm-derived 2G12 (^{OS}2G12) was compared to the same antibodies produced in CHO cells (^{CHO}2G12) and tobacco (^{NT}2G12) and to an unrelated antibody recognizing a different antigen (anti-rabies antibody E559). The potency of HIV neutralization was determined using a syncytium inhibition assay with HIV-1 BaL. The IC_{50} was 1.197 µg/ml for ^{OS}2G12, 0.083 µg/ml for ^{CHO}2G12 and 7.889 µg/ml for ^{NT}2G12, demonstrating that ^{CHO}2G12 was 14 times more potent than ^{OS}2G12 but ^{OS}2G12 was approximately seven times more potent than ^{NT}2G12, which was a significant difference at $p < 0.05$ (Figure 1.14).

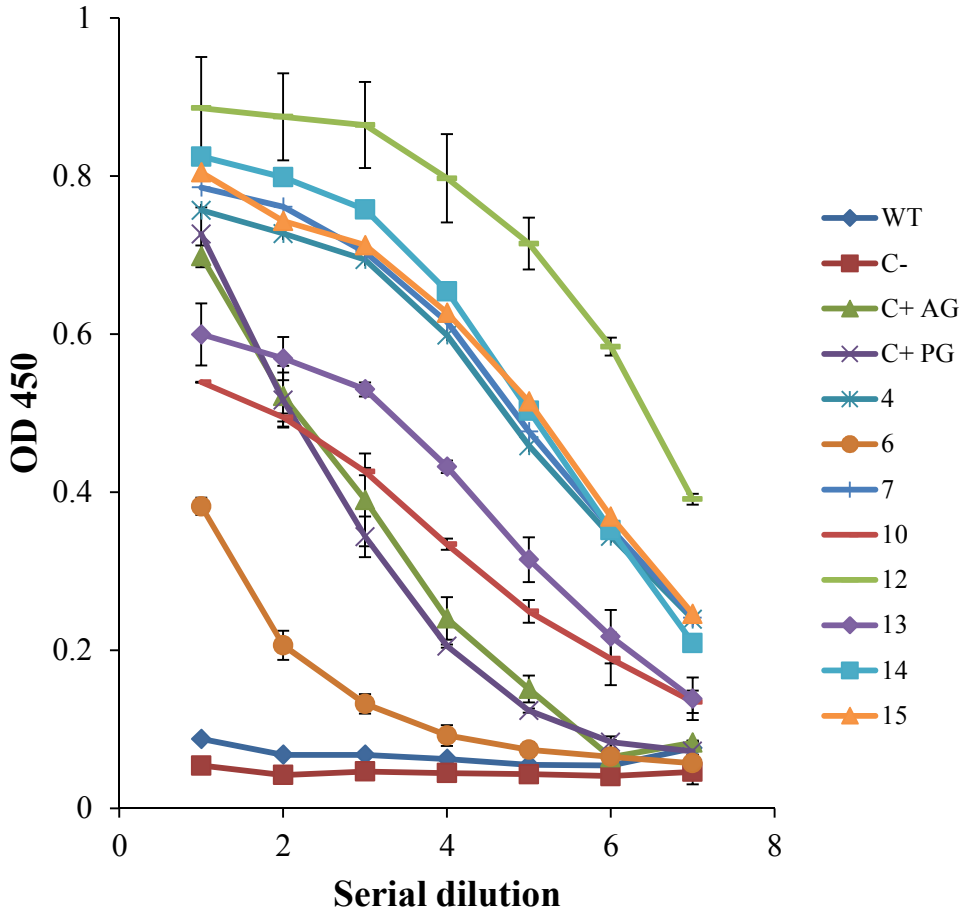
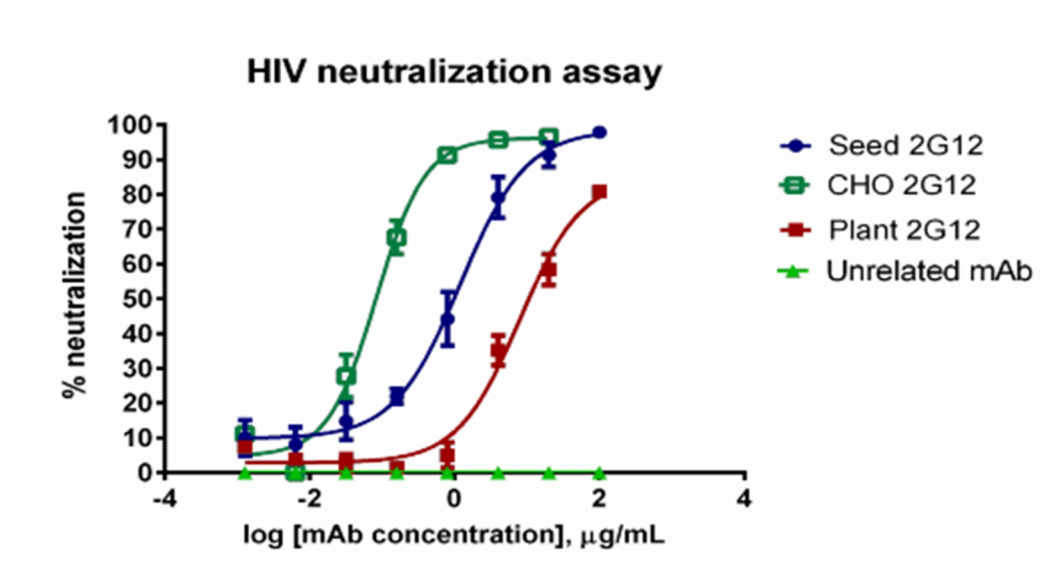


Figure 1.13: Antigen-binding activity of crude extracts from rice endosperm and purified 2G12. ELISA was used to determine binding activity of purified 2G12 and serially-diluted crude extracts of rice endosperm expressing the same protein. Plates were coated with the HIV gp120 protein and bound 2G12 was detected with a mixture of anti-human κ-chain and anti-human IgG antibodies labeled with horseradish peroxidase. Values represent the average of two experiments and bars indicate standard deviations. The binding kinetics showed a concentration-dependent relationship. All the crude extracts showed higher binding kinetics compared to wild-type extracts (negative control) and purified 2G12 (positive control). WT = wild type extracts; C– = negative control (PBS); C+ = 2G12 purified from maize (500 ng/ml) as positive control. OD = optical density at 450 nm.



		IC_{50} titer (µg/ml)		
Virus	**Clade**	**Seed 2G12**	**CHO 2G12**	**Plant 2G12**
HIV-1 BaL	B	1.197	0.083	7.889

Figure 1.14: *In vitro* virus neutralization efficacy of 2G12 from different sources. HIV neutralization was measured using a syncytium inhibition assay (Wei et al., 2003; Montefiori, 2005) with HIV-1 strain BaL. An unrelated plant-derived anti-rabies antibody E559 was used as a negative control.

1.4.6 Proteomic analysis in rice seeds

Proteomic analysis in rice seeds also helped to determine the 2G12 heavy and light chain protein and peptide levels, covering almost 80.1% of the heavy chain and 64.4% of the light chain (Figure 1.15). The different colors represent matches to intact peptides (e.g. LSDNDPFDAWGPGTVVTVSPASTK = LSDNDPFDAWGPGTVVTVSPASTK), partial peptides (e.g. SVFIFPPSDEQLK = TVAAPSVFIFPPSDEQLK), modified peptides (e.g. HKVYACEVTHQGLSSPVTK = HKVYACEVTHQGLSSPVTK, showing carbamidomethyl cysteine modification) or partial modified peptides (e.g. ASVVCLLNNFYPR = SGTASVVCLLNNFYPR, also showing carbamidomethyl cysteine modification).

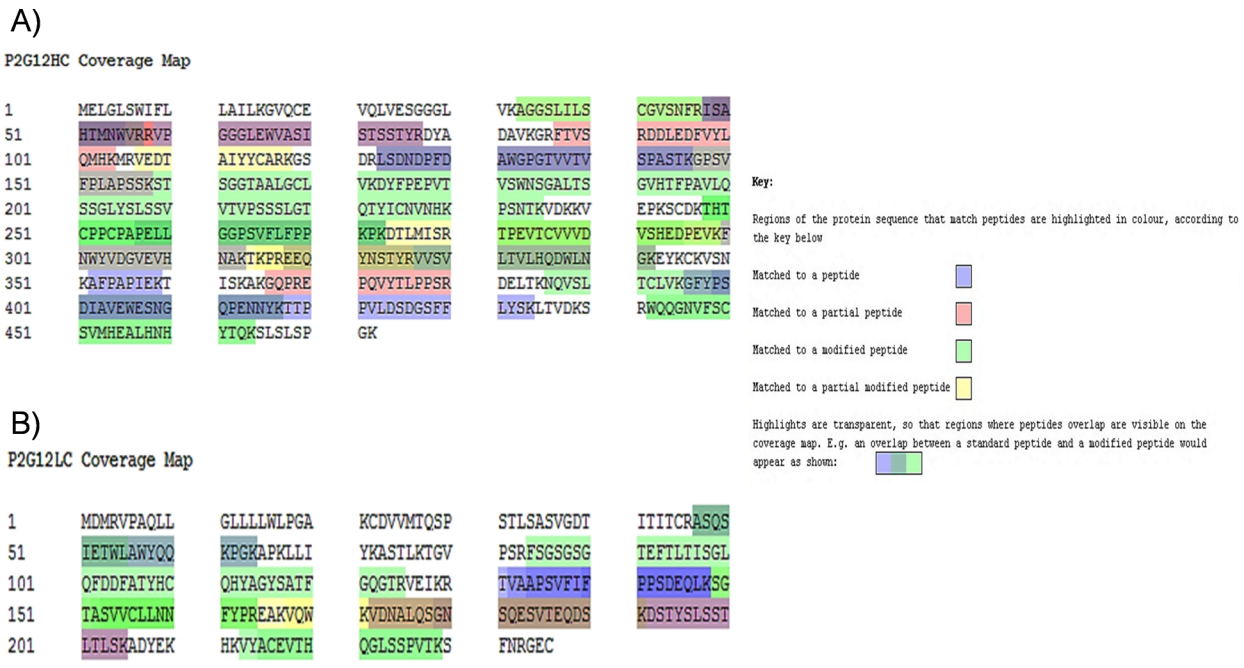


Figure 1.15: Coverage of the 2G12 antibody by tryptic peptides. The coverage map was A) 80.1% for the 2G12 heavy chain and B) 64.4% for the 2G12 light chain. Different colors represent intact and partial peptides with and without modifications.

Because 2G12 is a human monoclonal antibody that does not exist naturally in rice seeds, it was necessary to manually add the sequences for both the heavy and light chains to the rice database downloaded from UniProt in order to test the distribution of peptides and proteins in the rice database, and in the seed proteome by NanoUPLC-MS^{E}.

The 2G12 heavy and light chains were detected at both the protein and peptide levels as expected (Figure 1.16). The data represented by blue squares match entries in the rice database, whereas those shown as red squares correspond to rice seed proteins. We also tested the distribution of seed proteins identified within the rice database in terms of their isoelectric points (Figure 1.17). The data represented by blue squares correspond to the rice peptide database whereas those shown as red squares correspond to rice seed peptides and those shown as green squares represent the peptides of the 2G12 antibody.

In addition, the rice proteins from a transgenic plant (line 15) were identified by mass spectrometry. Table 1.3 shows the proteins that were identified in the rice endosperm and their abundance in terms of %TSP. Among the 69 proteins that were identified, the most abundant were the seed allergenic protein, the α1,4-glycan branching enzyme, glutelin type A1 and the 19 kDa globulin protein. The least abundant proteins were the ubiquitin 60S ribosomal protein, the ubiquitin NEDD8-like protein, a 14-3-3-like protein, a calmodium-like protein and a superoxide dismutase (Table 1.3).

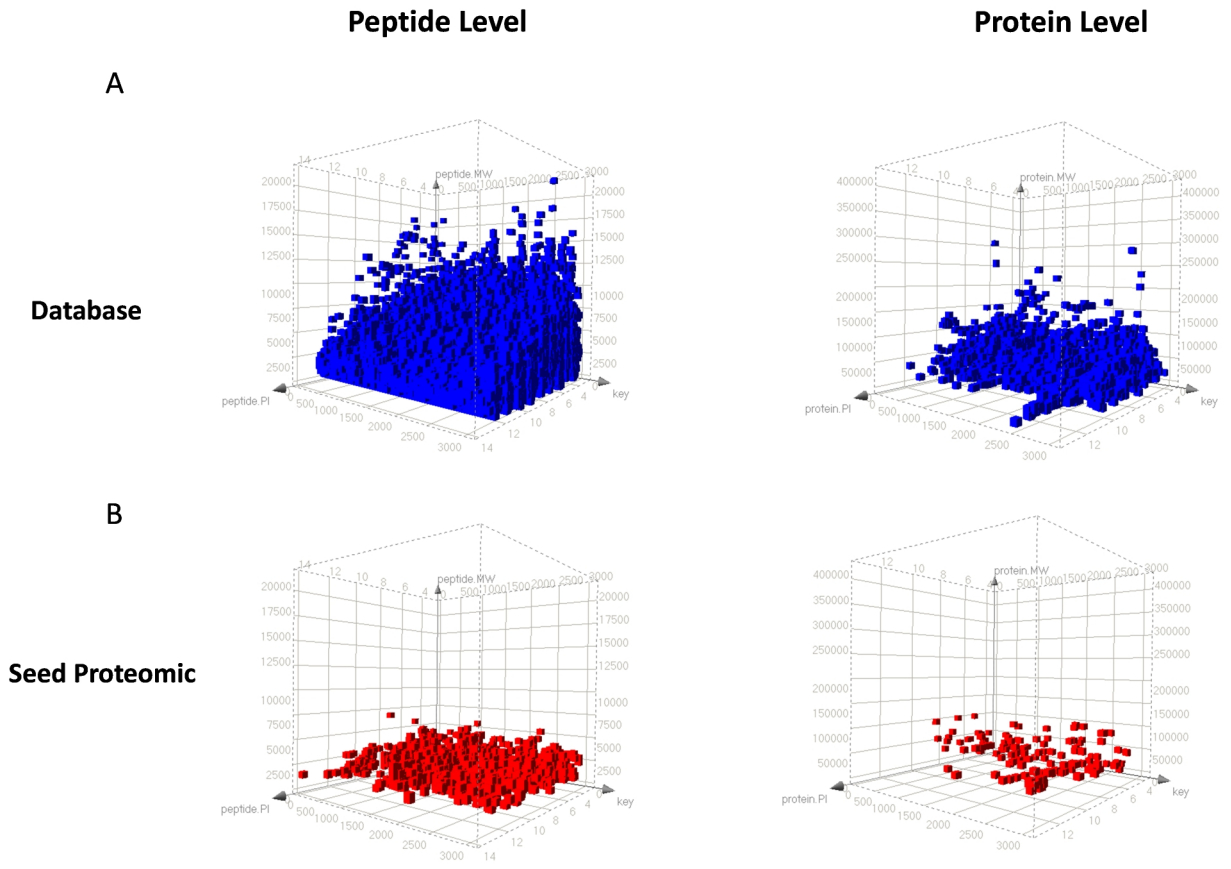


Figure 1.16: Distribution of peptides and proteins in (A) the rice database and (B) the seed proteome as determined by NanoUPLC-MS^{E}.

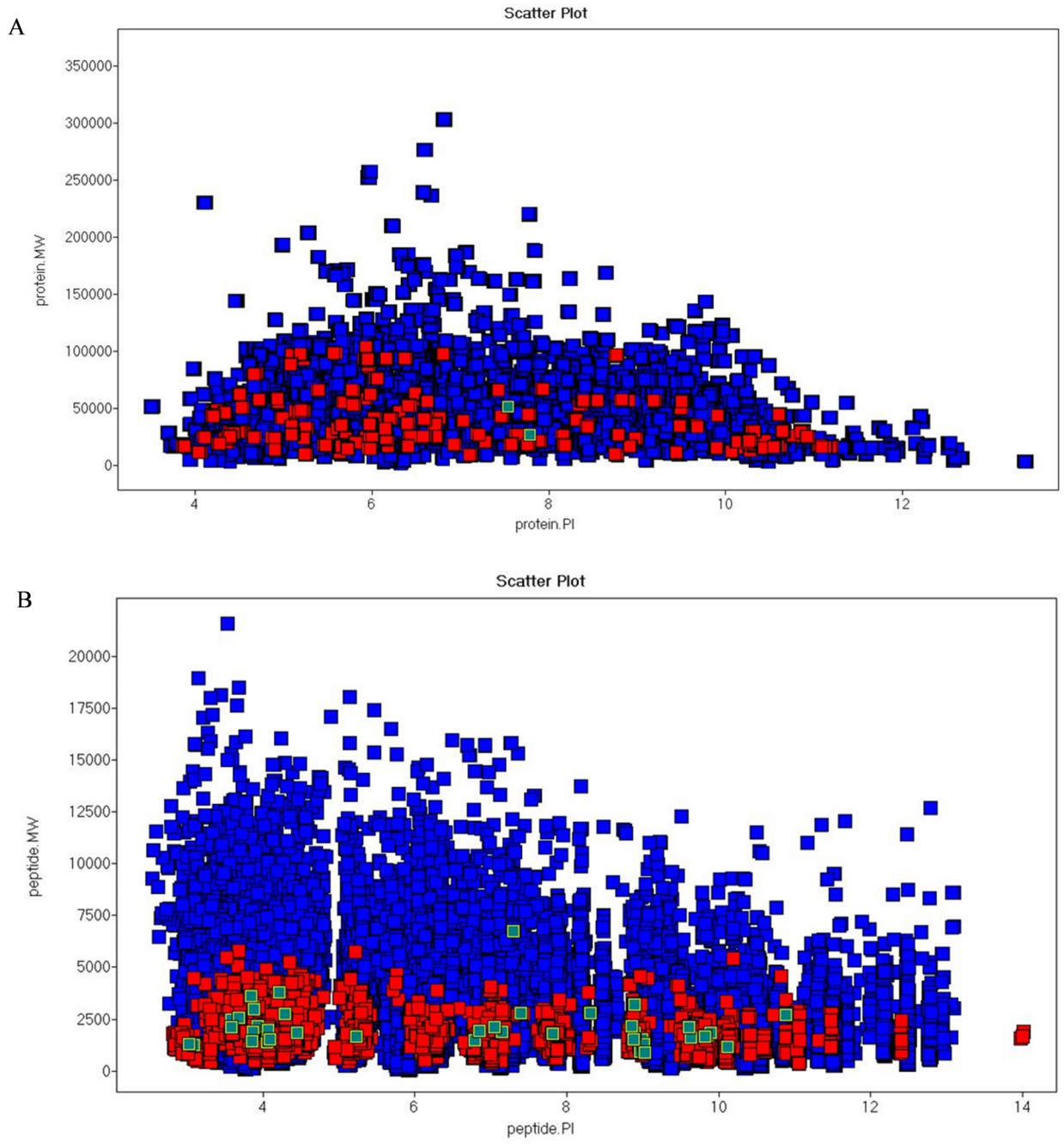


Figure 1.17: Distribution of A) seed proteins identified in the rice database sorted by isoelectric point, and B) the isoelectric points of the identified peptides. Data represented by blue squares correspond to the rice database, whereas red squares correspond to rice seed proteins and green squares represent the 2G12 heavy and light chains.

Table 1.3: Protein identification, including the entry number and description of each protein, the molecular weight (MW) in kDa, ngram average value (ngram AVG) and percent of total soluble protein (% of TSP) in rice seeds.

Entry	Description	MW(kDa)	ngramAVG	%TSP
P2G12HC	2G12HC HEAVY CHAIN	52,376.0438	7.7440667	1.395327333
P2G12LC	2G12LC LC CHAIN	26,088.3006	8.030434	1.446925045
P00489	GLYCOGEN PHOSPHORYLASE MUSCLE FORM EC	97,671.6378	24.4179	4.399621622
Q7XTE8	14 3 3 like protein GF14 B OS Oryza sativa subsp japonica GN GF14B PE 1 SV 2	29,977.7418	1.0308	0.18572973
Q6ZKC0	14 3 3 like protein GF14 C OS Oryza sativa subsp japonica GN GF14C PE 1 SV 1	28,997.6467	1.2241666	0.220570559
Q2R2W2	14 3 3 like protein GF14 D OS Oryza sativa subsp japonica GN GF14D PE 2 SV 1	29,375.7807	0.1882	0.03390991
Q6EUP4	14 3 3 like protein GF14 E OS Oryza sativa subsp japonica GN GF14E PE 2 SV 1	29,863.585	1.5033	0.270864865
P37833	Aspartate aminotransferase cytoplasmic OS Oryza sativa subsp japonica GN Os01g0760600 PE 2 SV 1	44,678.2525	4.1755333	0.752348342
Q10DV7	Actin 1 OS Oryza sativa subsp japonica GN ACT1 PE 2 SV 1	42,041.0675	1.2513666	0.225471459
A3C6D7	Actin 2 OS Oryza sativa subsp japonica GN ACT2 PE 2 SV 1	41,907.9403	0	0
Q10AZ4	Actin 3 OS Oryza sativa subsp japonica GN ACT3 PE 2 SV 1	41,991	0	0
P0C540	Actin 7 OS Oryza sativa subsp japonica GN ACT7 PE 3 SV 1	41,899.8943	0.43563333	0.078492492
Q2R8Z5	Alcohol dehydrogenase 1 OS Oryza sativa subsp japonica GN ADH1 PE 2 SV 2	41,725.7371	4.5635333	0.822258252
P17784	Fructose bisphosphate aldolase cytoplasmic isozyme OS Oryza sativa subsp japonica GN FBA PE 1 SV 2	39,262.656	7.0143666	1.263849838
Q01859	ATP synthase subunit beta mitochondrial OS Oryza sativa subsp japonica GN ATPB PE 1 SV 2	59,048.3303	2.6957	0.485711712
Q0IQB6	Calmodulin like protein 3 OS Oryza sativa subsp japonica GN CML3 PE 2 SV 1	20,365.487	0.27015	0.048675676
P30287	Dehydrin Rab25 OS Oryza sativa subsp japonica GN RAB25 PE 2 SV 2	23,236.4077	2.1459668	0.386660685
O64937	Elongation factor 1 alpha OS Oryza sativa subsp japonica GN REFA1 PE 2 SV 2	49,635.2552	2.8262665	0.509237207
P29545	Elongation factor 1 beta OS Oryza sativa subsp japonica GN Os07g0662500 PE 1 SV 3	23,856.7601	0.85525	0.154099099
P46520	Embryonic abundant protein 1 OS Oryza sativa subsp japonica GN EMP1 PE 2 SV 1	10,165.0399	1.1598667	0.208984991
Q42971	Enolase OS Oryza sativa subsp japonica GN ENO1 PE 1 SV 2	48,314.7712	16.1253	2.905459459
Q0J8A4	Glyceraldehyde 3 phosphate dehydrogenase cytosolic OS Oryza sativa subsp japonica GN GAPC PE 1 SV	36,583.788	8.391999	1.512071892

P49027	Guanine nucleotide binding protein subunit beta like protein A OS Oryza sativa subsp japonica GN RA	36,687.9658	1.8069	0.325567568
P29835	19 kDa globulin OS Oryza sativa subsp japonica GN Os05g0499100 PE 1 SV 2	21,511.0285	39.572765	7.130227928
Q01401	1 4 alpha glucan branching enzyme chloroplastic amyloplastic OS Oryza sativa subsp japonica GN SBE	93,806.8118	51.25363	9.234888288
Q01401-2	Isoform 2 of 1 4 alpha glucan branching enzyme chloroplastic amyloplastic OS Oryza sativa subsp ja	87,148.1652	1.6293334	0.293573586
P15280	Glucose 1 phosphate adenylyltransferase small subunit chloroplastic amyloplastic OS Oryza sativa su	56,503.3604	0	0
P15280-2	Isoform 2 of Glucose 1 phosphate adenylyltransferase small subunit chloroplastic amyloplastic OS Or	53,235.6669	10.042966	1.809543423
P07728	Glutelin type A 1 OS Oryza sativa subsp japonica GN GLUA1 PE 1 SV 2	56,817.2816	39.866932	7.183230991
P07730	Glutelin type A 2 OS Oryza sativa subsp japonica GN GLUA2 PE 1 SV 1	56,876.4798	21.544767	3.88194
Q09151	Glutelin type A 3 OS Oryza sativa subsp japonica GN GLUA3 PE 2 SV 2	56,585.3839	17.446333	3.143483423
P14323	Glutelin type B 1 OS Oryza sativa subsp japonica GN GluB1 A PE 2 SV 3	56,949.8358	24.3509	4.38754955
Q02897	Glutelin type B 2 OS Oryza sativa subsp japonica GN GLUB2 PE 2 SV 2	56,446.1378	8.123934	1.463771892
P14614	Glutelin type B 4 OS Oryza sativa subsp japonica GN GLUB4 PE 1 SV 1	57,274.3697	9.673766	1.743020901
Q6ERU3	Glutelin type B 5 OS Oryza sativa subsp japonica GN GLUB5 PE 2 SV 1	57,264.3312	6.4039664	1.15386782
Q75KH3	Glucose and ribitol dehydrogenase homolog OS Oryza sativa subsp japonica GN Os05g0140800 PE 2 SV 2	32,495.7058	3.3847666	0.609867856
P55142	Glutaredoxin C6 OS Oryza sativa subsp japonica GN GRXC6 PE 1 SV 2	11,945.5888	1.7503667	0.315381387
P27777	16 9 kDa class I heat shock protein 1 OS Oryza sativa subsp japonica GN HSP16 9A PE 1 SV 1	16,939.1789	0.65900004	0.118738746
Q07078	Heat shock protein 81 3 OS Oryza sativa subsp japonica GN HSP81 3 PE 2 SV 2	80,469.2625	1.531	0.275855856
P29421	Alpha amylase subtilisin inhibitor OS Oryza sativa subsp japonica GN RASI PE 1 SV 2	21,702.6362	6.105133	1.100023964
Q0JR25	Bowman Birk type bran trypsin inhibitor OS Oryza sativa subsp japonica GN RBBI3 3 PE 1 SV 2	29,272.5046	5.238933	0.943951892
Q65XK0	Ketol acid reductoisomerase chloroplastic OS Oryza sativa subsp japonica GN Os05g0573700 PE 1 SV 1	62,719.4694	1.7585499	0.316855838
A3AHG5	Late embryogenesis abundant protein 1 OS Oryza sativa subsp japonica GN LEA1 PE 2 SV 1	37,019.3515	3.9307334	0.708240252
P0C5A4	Late embryogenesis abundant protein group 3 OS Oryza sativa subsp japonica GN LEA3 PE 2 SV 1	20,514.1501	2.754	0.496216216

Q948T6	Lactoylglutathione lyase OS Oryza sativa subsp japonica GN GLX I PE 1 SV 2	32,895.6179	5.0275664	0.90586782
Q7XDC8	Malate dehydrogenase cytoplasmic OS Oryza sativa subsp japonica GN Os10g0478200 PE 1 SV 3	35,911.1204	7.535567	1.35775982
Q07661	Nucleoside diphosphate kinase 1 OS Oryza sativa subsp japonica GN NDKR PE 1 SV 1	16,861.3596	2.5668333	0.462492486
Q0IQK9	Non specific lipid transfer protein 1 OS Oryza sativa subsp japonica GN LTP PE 1 SV 1	11,801.4456	1.4014667	0.252516523
Q42980	Oleosin 16 kDa OS Oryza sativa subsp japonica GN OLE16 PE 2 SV 2	15,258.7809	2.08985	0.37654955
Q10EK7	Oleosin 18 kDa OS Oryza sativa subsp japonica GN OLE18 PE 2 SV 1	17,221.7455	6.2076335	1.118492523
Q10MW3	Pyruvate decarboxylase isozyme 2 OS Oryza sativa subsp japonica GN PDC2 PE 2 SV 1	65,802.4291	3.52205	0.634603604
Q53LQ0	Protein disulfide isomerase like 1 1 OS Oryza sativa subsp japonica GN PDIL1 1 PE 2 SV 1	57,082.7688	11.8336	2.13218018
Q6AVA8	Pyruvate phosphate dikinase 1 chloroplastic OS Oryza sativa subsp japonica GN PPDK1 PE 1 SV 1	103,643.5481	7.225567	1.301903964
Q6AVA8-2	Isoform 2 of Pyruvate phosphate dikinase 1 chloroplastic OS Oryza sativa subsp japonica GN PPDK1	96,971.0503	12.148467	2.188912973
Q42465	Prolamin PPROL 14P OS Oryza sativa subsp japonica GN PROLM20 PE 2 SV 1	17,052.4845	1.9576501	0.352729748
Q0DJ45	Prolamin PPROL 14E OS Oryza sativa subsp japonica GN PROLM7 PE 2 SV 1	16,926.3287	2.0298667	0.365741748
Q9ARZ9	Ubiquitin 40S ribosomal protein S27a 1 OS Oryza sativa subsp japonica GN RPS27AA PE 2 SV 1	17,896.6488	0	0
P51431	Ubiquitin 40S ribosomal protein S27a 2 OS Oryza sativa subsp japonica GN RPS27AB PE 2 SV 3	17,910.7191	0	0
Q01881	Seed allergenic protein RA5 OS Oryza sativa subsp japonica GN RA5 PE 1 SV 2	17,863.329	7.5655	1.363153153
Q01883	Seed allergenic protein RAG1 OS Oryza sativa subsp japonica GN RAG1 PE 1 SV 2	18,138.4391	23.414398	4.21881045
Q01882	Seed allergenic protein RAG2 OS Oryza sativa subsp japonica GN RAG2 PE 1 SV 2	18,434.8769	63.272434	11.40043856
P0C5C9	1 Cys peroxiredoxin A OS Oryza sativa subsp japonica GN Os07g0638300 PE 2 SV 1	24,213.5309	15.704501	2.82963982
P0CH34	Ubiquitin 60S ribosomal protein L40 1 OS Oryza sativa subsp japonica GN Ub CEP52 1 PE 2 SV 1	15,012.4505	0.005666667	0.001021021
P0CH35	Ubiquitin 60S ribosomal protein L40 2 OS Oryza sativa subsp japonica GN Ub CEP52 2 PE 3 SV 1	15,012.4505	0	0
P41095	60S acidic ribosomal protein P0 OS Oryza sativa subsp japonica GN Os08g0130500 PE 1 SV 3	34,491.7325	1.3687	0.246612613
Q8LJU5	40S ribosomal protein S7 OS Oryza sativa subsp japonica GN RPS7 PE 2 SV 2	22,364.0004	1.7593999	0.317008991
P0C030	Ubiquitin NEDD8 like protein RUB1 OS Oryza sativa subsp japonica GN RUB1 PE 2 SV 2	17,129.7071	0.030333333	0.005465465

P0C031	Ubiquitin NEDD8 like protein RUB2 OS Oryza sativa subsp japonica GN RUB2 PE 2 SV 2	17,094.6619	0	0
P0C032	Ubiquitin like protein NEDD8 like protein RUB3 OS Oryza sativa subsp japonica GN RUB3 PE 3 SV 2	17,292.8519	0	0
Q0DRV6	Superoxide dismutase Cu Zn 1 OS Oryza sativa subsp japonica GN SODCC1 PE 1 SV 1	15,365.0518	0.35345	0.063684685
P28757	Superoxide dismutase Cu Zn 2 OS Oryza sativa subsp japonica GN SODCC2 PE 1 SV 2	15,194.7565	0.5611667	0.101111117
Q43008	Superoxide dismutase Mn mitochondrial OS Oryza sativa subsp japonica GN SODA PE 2 SV 2	24,997.5114	1.2993335	0.234114144
P30298	Sucrose synthase 2 OS Oryza sativa subsp japonica GN SUS2 PE 1 SV 2	92,642.9095	5.718	1.03027027
P48494	Triosephosphate isomerase cytosolic OS Oryza sativa subsp japonica GN TPI PE 1 SV 3	27,291.0846	4.7694	0.859351351
Q58G87	Polyubiquitin 3 OS Oryza sativa subsp japonica GN UBQ3 PE 2 SV 2	42,725.1482	3.1382	0.565441441
Q53NL5	Xylanase inhibitor protein 2 OS Oryza sativa subsp japonica GN Chib3H h PE 1 SV 1	31,881.8156	7.733667	1.393453514

We also clustered the identified proteins by functionality into groups such as storage, starch biosynthesis, type I hypersensitivity and other functions (Figure 1.18). We found that 32% of the identified proteins were seed storage proteins, 12% were related to starch biosynthesis and other functions and 18% were related to type I hypersensitivity. Other proteins were clustered in smaller groups, such as those corresponding to stress responses (7%), glycolysis (5%), photosynthesis (4%), oxidoreductase activity (3%), plant defense (2%), lipid droplet formation (2%) and late embryogenesis abundant proteins (1%). The 2G12 heavy and light chains each accounted for 1% of the total protein content.

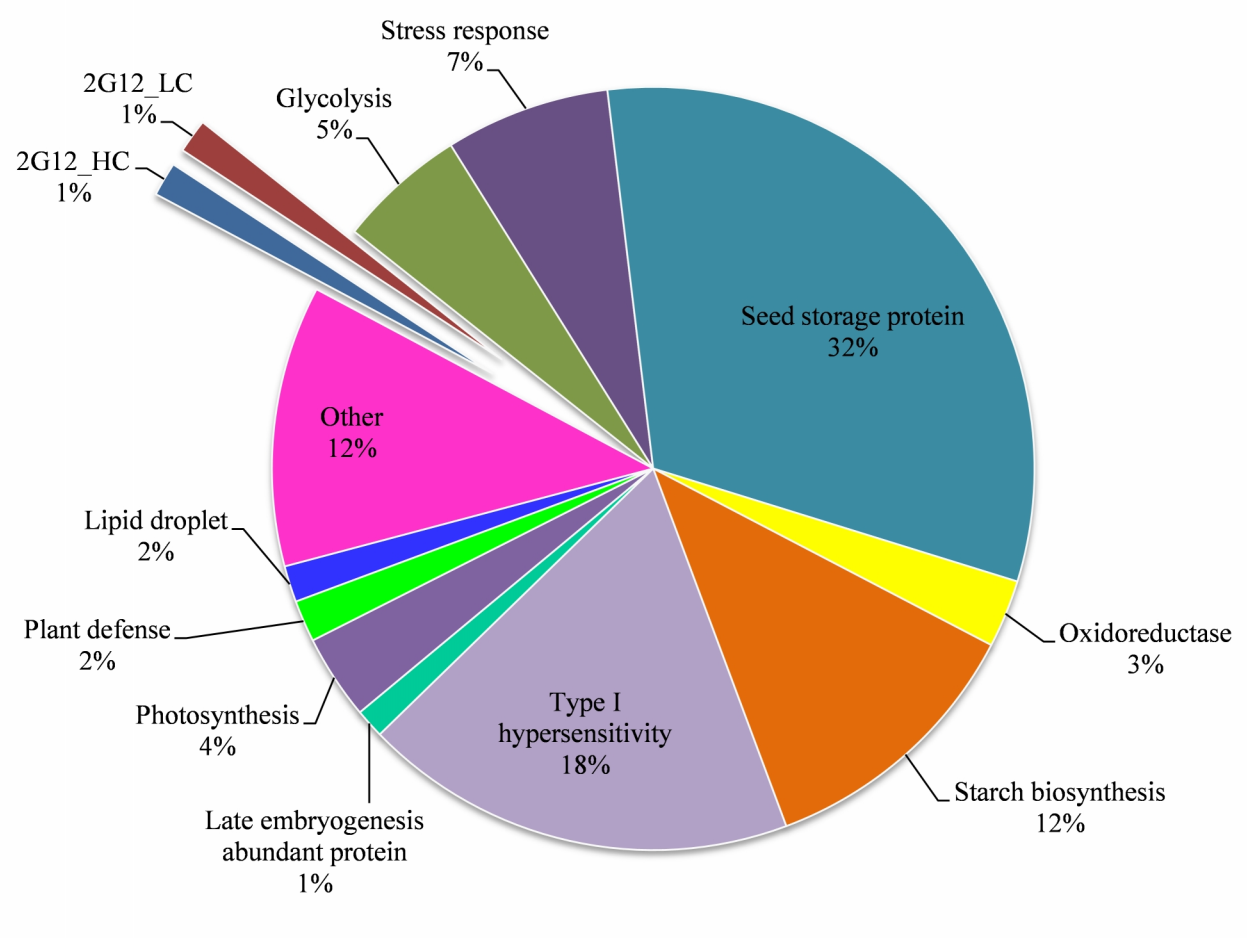


Figure 1.18: Functional clustering of the seed proteome of transgenic rice plants expressing 2G12.

1.4.7 Proteomic analysis and transcript profiling reveal genes modulated by 2G12 expression in rice endosperm

The comparative proteomic analysis of wild-type and transgenic rice endosperm using a threshold value of 1 (Figure 1.19) revealed two major upregulated proteins and two major downregulated proteins in the transgenic plants (Table 1.4). One of the upregulated proteins was the storage protein prolamin and the other was the enzyme glutaredoxin-C8. Both the downregulated proteins were related to glycogen synthesis and storage.

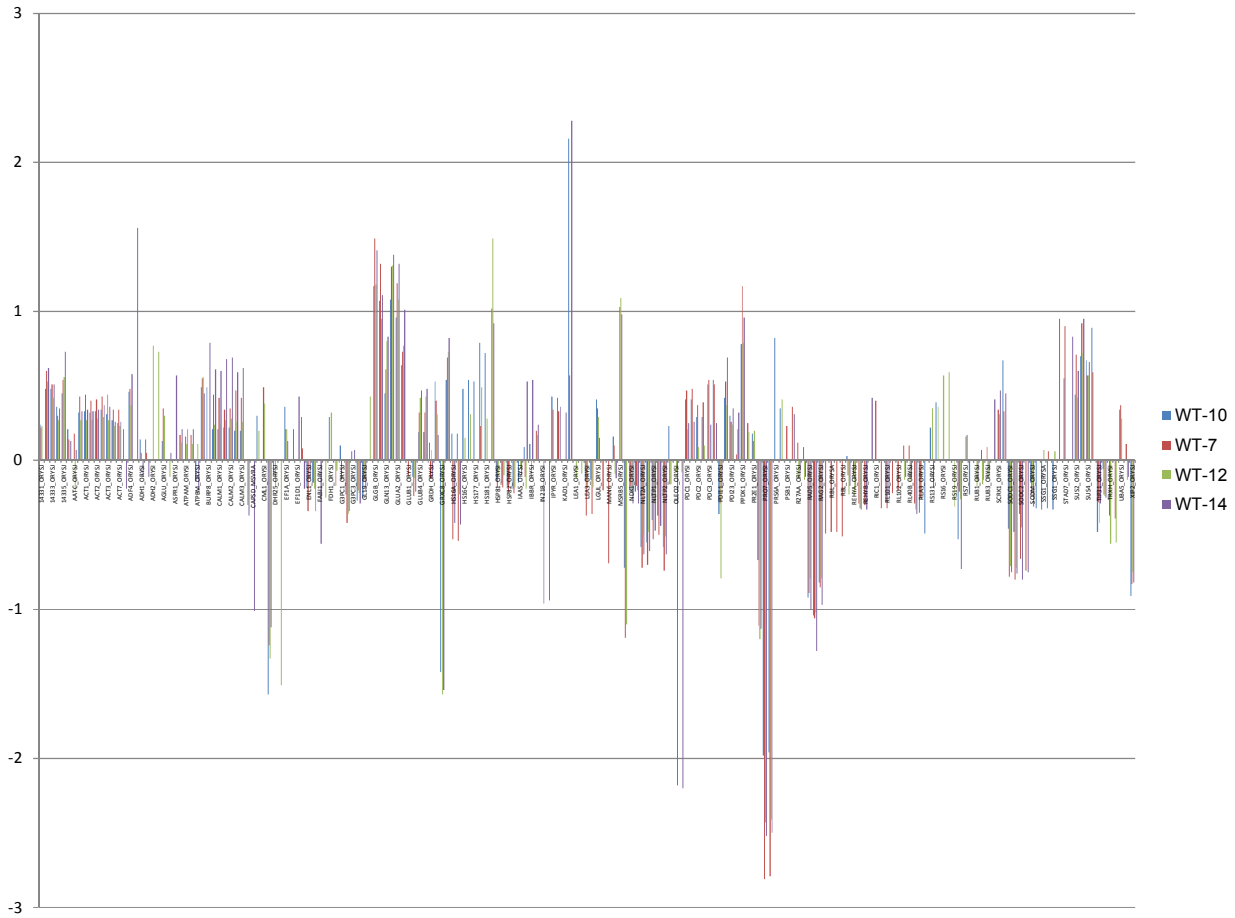


Figure 1.19: Pivot chart representing the proteomic analysis of transgenic rice endosperm. Proteins showing a fold difference (increase or decrease) greater than 1 were chosen for RT-PCR analysis.

Table 1.4: The two most strongly upregulated and downregulated proteins (fold increase/decrease) in transgenic plants compared to wild-type rice plants.

Protein	B110:WT Fold increase	B17:WT Fold increase	C26:WT Fold increase	C29:WT Fold increase
PRO7_ORYSJ	1.96	2.79	2.41	2.5
GRXC8_ORYSJ	1.42	1.08	1.57	1.54

Protein	B110:WT Fold decrease	B17:WT Fold decrease	C26:WT Fold decrease	C29:WT Fold decrease
GLGB_ORYSJ	-1.17	-1.49	-1.18	-1.41
GLGS_ORYSJ	-1.07	-1.32	-0.95	-1.11

We also measured the abundance of the corresponding transcripts by quantitative real-time RT-PCR in order to correlate the mRNA and protein levels of the modulated genes. Four lines (7, 10, 12 and 14) that express 2G12 at the highest levels were chosen for quantitative RT-PCR analysis. Accordingly, we found that the transcript levels of endogenous glycogen branching enzyme (GLGB _ORYSJ) and glycogen synthesis protein (GLGS _ORYSJ) were downregulated in all the transgenic lines compared to wild-type plants (Figure 1.20 a-b) and that the expression levels of endogenous glutaredoxin-C8 (GRXC8 _ORYSJ) and prolamin PPROL 14E (PRO7_ORYSJ) were upregulated in all the transgenic lines compared to wild-type plants (Figure 1.20 c-d).

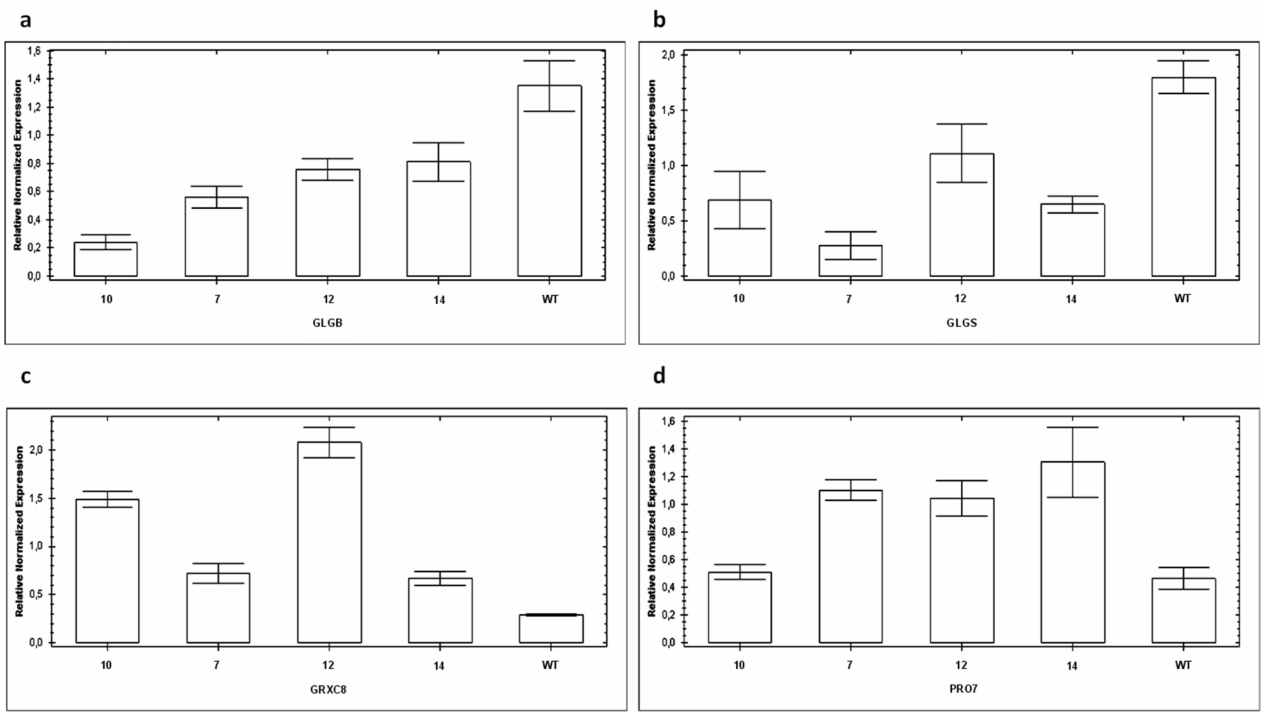


Figure 1.20: Quantitative real-time RT-PCR analysis of modulated genes. Gene expression was monitored in lines 7, 10, 12, 14 and wild-type plants. The relative expression level of each gene was determined by normalizing against the housekeeping gene *actin*. (a) The downregulated gene *GLGB*; (b) The downregulated gene *GLGS*; (c) The upregulated gene *GRXC8*; (d) The upregulated gene *PRO7*. Total RNA was extracted from mature T4 seeds from each line (n = 3). Bars represent means ± SE for three independent experiments.

1.5 DISCUSSION

Protein microbicides against HIV can prevent infection, as demonstrated in a number of preclinical and clinical studies (De Clercq, 2007). However, the production and distribution of therapeutic proteins in key HIV-endemic areas is challenging and expensive because there is neither sufficient production infrastructure nor a reliable cold chain, meaning that the people most likely to benefit from the microbicides are effectively excluded on economic grounds. Many therapeutic proteins have been expressed in plants, including a large number of vaccine candidates and recombinant antibodies, so the principle of pharmaceutical molecular farming is well established (Ramessar et al., 2008a; Twyman et al., 2005). Plants have many advantages over fermenter-based systems (Ma et al., 2003; Paul et al., 2013; Ramessar et al., 2008a) but rice seeds are particularly suitable in the context of developing countries because they maintain the stability of recombinant proteins accumulating in the endosperm so that a cold chain is not necessary for product distribution, and the economy of large-scale production makes the product affordable without detracting from its safety or performance (Fischer et al., 2012; Sabalza et al., 2013).

We expressed the HIV-neutralizing antibody 2G12 in transgenic rice endosperm to evaluate the potential of rice seeds as a vehicle for inexpensive microbicide production. We also investigated the biochemical and cellular processes that are influenced by the accumulation of a heterologous recombinant protein such as 2G12 in the endosperm to gain a better understanding of factors that might control the accumulation of recombinant proteins in plants. We regenerated transgenic rice plants and confirmed the presence and strong expression of the transgenes in six independent lines, ultimately choosing two lines with the highest antibody yields for more detailed analysis. The yields of 2G12 in rice were similar to those reported for 2G12 in other cereal seeds and better than those reported for other antibodies. We achieved a maximum yield of 42 μg/g dry seed weight in T3 seeds, which compares well with the 30 μg/g initially achieved for 2G12 in maize (Rademacher et al., 2008) although this was subsequently improved to 75 μg/g by the selective breeding of high-performance lines and then to 100 μg/g by passing the best-performing line through a dedifferentiation–regeneration cycle (Ramessar et al., 2008b). Our results for 2G12 were far better than those reported for HIV-neutralizing antibody 2F5 in maize seeds, where the maximum accumulation was 0.80 μg/ml (Sabalza et al., 2012). Our data suggest

that the 2G12 yields in rice could also be improved beyond 42 μg/g dry seed weight by selective breeding.

The localization of 2G12 in the transgenic endosperm was investigated by immunohistochemistry and TEM, revealing that most of the antibody accumulated in PB-II compartments and to a lesser extent in PB-I compartments. The subcellular location of a recombinant protein can influence its yield, structure and functionality, but it is difficult to predict the localization of recombinant proteins in the endosperm of cereal seeds because protein trafficking appears to be influenced not only by the species and tissue but also by intrinsic properties of the expressed protein (Drakakaki et al., 2006). When a 2G12 antibody carrying a C-terminal KDEL tag was expressed in maize endosperm, it accumulated within ER-derived zein bodies but no significant labeling was observed in storage vacuoles or any other cell compartment (Rademacher et al., 2008). In contrast, an untagged version of 2G12 accumulated in the storage vacuoles and, to a much lesser extent, also in zein bodies (Ramessar et al., 2008b). The expression of recombinant fungal phytase in rice resulted in the accumulation of phytase in both types of protein bodies (PB-I and PB-II) but no additional labeling was observed in the apoplast or other cell compartments (Drakakaki et al., 2006).

Our rice plants were transformed with the untagged version of 2G12 and the results were similar to those reported in maize (Ramessar et al., 2008b). Immunohistochemistry showed that the signal for 2G12 overlapped with that for glutelin, indicating that most of the antibody co-accumulated with this storage protein in PB-II compartments, also known as glutelin bodies. More detailed analysis by TEM confirmed that most of the 2G12 was localized in PB-II compartments and a smaller amount in PB-I compartments, but we also observed novel protein bodies that were not present in untransformed seeds, suggesting the compartments were formed *de novo* as a consequence of high-level antibody expression even though they did not contain significant amounts of the antibody. The novel protein bodies were unusual in architecture, featuring prominent studs in the membrane caused by the presence of ribosomes. The induction of novel protein bodies in transgenic rice cells has been reported before (Torres et al., 2001, 1999; Yang et al., 2012). In our transgenic lines, the antibody was able to induce novel protein bodies without the benefit of a KDEL tag, suggesting that the protein was abundant enough in the rough ER to induce the budding of protein bodies even without a dedicated retention sequence. Green fluorescent protein fused to rice prolamin has also been shown to induce the

formation of protein body-like structures in transgenic rice endosperm, although the protein ultimately accumulated in PB-I compartments (Saito et al., 2009).

Further information about the intracellular trafficking of recombinant proteins can be gathered by analyzing the glycan profile if the protein contains canonical N-glycan acceptor sites. The heavy chain of IgG antibodies carries an acceptor site at residue N297 of the Fc domain, and the structure of the glycan affects antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which can thus be modulated by choosing an appropriate production platform (Jung et al., 2011). Antibodies expressed in plants typically comprise a mixture of glycoforms, ranging from the absence of any glycans (aglycosylated heavy chain) to high-mannose and complex-type glycans, and antibodies with single oligosaccharides resulting from extensive glycan trimming (Baez et al., 2000; De Wilde et al., 1998; Hristodorov et al., 2013; Khoudi et al., 1999; Ma et al., 1994). The aglycosylated fraction is usually a minor component. For example, 13.3% of 2G12 was aglycosylated when the KDEL-tagged version was expressed in standard maize and only 10.6% in a sweetcorn variety (Rademacher et al., 2008), whereas in the absence of KDEL tag the aglycosylated fraction was 11.5% (Ramessar et al., 2008b). Surprisingly, we found that the aglycosylated fraction of 2G12 in transgenic rice endosperm represented 50% of the total, the remainder comprising vacuolar-type complex N-glycans (19% MUXF and 14% MMXF), 13% individual N-acetylglucosamine (GlcNAc) residues confirming the presence of ENGase activity, and only 4% oligomannose glycans (Man_7) as expected for ER-resident proteins, suggesting that the recombinant protein was directed through distinct trafficking pathways characterized by different glycan signatures. In most previous investigations, the predominant glycoforms were the complex type (if the protein passed through the secretory pathway to the apoplast or the storage vacuole) or the oligomannose type (if the protein was retrieved to the ER lumen). Only one previous study reported a prevalent aglycosylated fraction and that involved the expression of an scFv-Fc antibody in Arabidopsis seeds, where most of the antibodies carried oligomannose glycans but 35–40% were aglycosylated (Van Droogenbroeck et al., 2007). The authors proposed that the glycosylation machinery may have been saturated at the high expression levels they achieved, but we observed no change in the steady-state mRNA levels of BiP and PDI, two proteins that are usually upregulated when the secretory pathway is under heavy load. Indeed we even noted a slight decline in the level of *PDI* mRNA in the some transgenic lines. These results suggest that a

large proportion of the heavy chain is co-translationally imported into a region of the ER that lacks glycosyltransferases and accumulates in protein bodies without further modification. The remaining proportion appears to follow the canonical pathway, undergoing initial glycosylation in the ER lumen followed by either complex modifications in the Golgi body and/or trimming back to a single GlcNAc residue. The glycosylation of 2G12 is not only important from a protein trafficking and accumulation perspective but also because it affects the functionality of the protein once it has been purified from the rice grains. We tested the antigen-binding efficiency of the purified ^{OS}2G12, the crude extracts of different lines expressing 2G12 and the ^{ZM}2G12 with plant glycans produced in maize. We found that ^{OS}2G12 binds to HIV gp120 with a similar efficiency to ^{ZM}2G12, but the ^{OS}2G12 was more potent in HIV neutralization assays, suggesting that the absence of glycans is advantageous for the intended function of the protein. Functional aglycosylated antibodies are advantageous because potentially they can be expressed in any platform, including bacteria (which do not add glycans to recombinant proteins). Fc glycosylation is necessary only for IgG effector functions. Aglycosylated antibodies can be used when these functions are unnecessary or undesirable, such as for neutralizing, agonistic or antagonistic antibodies and the treatment of chronic inflammatory diseases (Hristodorov et al., 2013). Therefore, the glycosylation of the 2G12 Fc domain should not be necessary for its HIV-neutralizing activity but this is the first evidence that the absence of glycans may actually be better than the presence of plant-specific glycans.

The IC_{50} of 2G12 with mammalian glycans (i.e. 2G12 produced in CHO cells) is lower than that of aglycosylated 2G12 purified from rice endosperm, which is in turn lower than that of the glycosylated 2G12 variants produced in other plants. Although the glycans do not appear to be required to establish the domain-exchange structure that binds the mannose cluster on gp210 or for any other aspect of antigen binding (Calarese et al., 2003) there is increasing evidence that aglycosylated antibodies are at least equivalent but in some cases even better in terms of neutralizing activity than their glycosylated counterparts. For example, a plant-derived aglycosylated antibody against *Bacillus anthracis* PA (pp-mAb^{PANG}) was able to neutralize the anthrax LeTx more efficiently *in vitro* and in non-human primates than the glycosylated equivalent (Mett et al., 2011). Several studies have shown the equivalence or near-equivalence of aglycosylated and glycosylated variants of the same antibody produced in CHO cells

(Hristodorov et al., 2013), maize (Baez et al., 2000) and soybean compared with mouse cells (Zeitlin et al., 1998).

We also tested the distribution of peptides and proteins in the rice database and seed proteome by NanoUPLC-MS^{E}. The 2G12 peptide and protein sequences were found as anticipated because the sequences of both chains were added manually to the database. We identified the major proteins in the seed proteome of transgenic rice line 15 and these were clustered into different functional groups including storage proteins, type I hypersensitivity proteins, starch biosynthesis proteins and other functions. The most abundant proteins were the seed allergen protein, the α 1,4 glycan-branching enzyme, glutelin type A1 and 19 kDa globulin.

We also investigated the wider effects of high-level antibody production by carrying out a proteomic analysis of four transgenic lines compared to wild-type endosperm tissue followed by the analysis of transcripts corresponding to the four differentially-expressed proteins we identified. We sampled 24 seeds from line 10, 36 seeds from lines 7, 12 and 14, and 48 wild-type seeds. The two downregulated proteins were both related to starch biosynthesis, i.e. ADP-glucose pyrophosphorylase (GLGS) and starch-branching enzyme (GLGB). GLGS controls a rate-limiting step in starch biosynthesis involving carbon partitioning between starch and sucrose in photosynthetic tissues (Heldt et al., 1977; Preiss et al., 1991). It also regulates starch biosynthesis in non-photosynthetic sink organs such as seeds, and in cereals the major form of this enzyme is located in the cytoplasm (Denyer et al., 1996; Sikka et al., 2001; Thorbjornsen et al., 1996). GLGB is the only enzyme that can introduce α-1,6-glucosidic linkages into α-glucans and it is required for amylopectin biosynthesis. Plants express two classes of this enzyme (BEI and BEII) with different functions (Nakamura et al., 2003). Rice and maize have two isoforms of the BEII enzyme (BEIIa and BEIIb), the latter expressed only in the endosperm (Abe et al., 2014). The downregulation of GLGS and GLGB in the transgenic plants was confirmed at both the mRNA and protein levels suggesting that the accumulation of 2G12 has a significant impact on starch metabolism and results in the accumulation of less starch in the seeds. Accordingly, we found that the weight of the transgenic seeds was ~22% lower than the wild-type seeds, potentially indicating that the expression of recombinant proteins in the rice endosperm can disrupt the balance between protein and carbohydrate storage.

This hypothesis was supported by the greater abundance of PRO7, a seed storage protein, and GRXC8 (glutaredoxin-C8), an enzyme that scavenges reactive oxygen species (Lin et al., 2014). In rice, prolamins accumulate in the PB-I compartments which bud directly from the ER. The greater abundance of prolamin in the transgenic endosperm may be related to the downregulation of carbohydrate metabolism discussed above and/or may reflect the disruption of protein trafficking in the cell due to the accumulation of 2G12 in the ER, resulting in a compensatory mechanism as the cell attempts to sort several abundant proteins into different compartments. The concentration of 2G12 in PB-II compartments and the genesis of novel ER-derived protein bodies may generate feedback at the level of storage protein deposition that results in the upregulation of particular prolamin genes. The surge of proteins through the endomembrane system is likely to cause local stress that induces the production of reactive oxygen species, resulting in the production of glutaredoxin-C8 as a stress response. These data suggest that yields of proteins could be improved in rice seeds by regulating protein/carbohydrate metabolism and protecting the endosperm from abiotic stress.

1.6 CONCLUSIONS

Our data show that a recombinant HIV-neutralizing antibody can be produced in transgenic rice endosperm with yields similar to those achieved in transgenic maize. Unusually, the most prevalent form was the aglycosylated version of the antibody, representing 50% of the total, with the remainder featuring complex vacuolar-type and oligomannose-type glycans as well as single GlcNAc residues, suggesting that the antibody was not trafficked through any specific pathway but was distributed via several different routes featuring different glycan signatures. This was supported by the accumulation of the antibody primarily in PB-II compartments but also in PB-I. In addition novel protein bodies was observed that were not present in wild-type endosperm. The aglycosylated version of the antibody proved to have a greater HIV-neutralizing activity than the glycosylated version produced in tobacco seeds, although neither were as potent as the original form of 2G12 produced in CHO cells. Proteomic analysis allowed us to identify the 2G12 heavy and light chains at the protein and peptide levels and we also identified the major seed proteins in transgenic rice line 15 and clustered them into different functional groups. Proteomics analysis revealed that starch metabolism was suppressed in the transgenic seeds and a prolamin gene was

upregulated, indicating that transgene expression caused some disruption in the balance between carbohydrate and protein storage in the seed. The metabolic disruption and unusual subcellular architecture of the cell appears to cause stress within the protein trafficking networks, perhaps due to the unusually high level of traffic that needs to be processed, resulting in the further induction of genes encoding the radical scavenger glutaredoxin-C8. These data suggest that yields of proteins could be improved in rice seeds by regulating protein/carbohydrate metabolism and protecting the endosperm from abiotic stress.

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CHAPTER 2

Production of the anti-HIV protein griffithsin (GRFT) in rice endosperm

2.1 INTRODUCTION

Lectins are proteins that bind carbohydrates without causing enzymatic chemical modifications (Weis and Drickamer, 1996). Lectins are found in most organisms, including viruses and bacteria, sea corals, algae, fungi, plants and animals (De Mejía and Prisecaru, 2005; Gabius, 1997; Lis and Sharon, 1998). The carbohydrate-binding domains have been studied mainly in the plant kingdom (Sharon, 2007; Vandenborre et al., 2011). Lectins have diverse structures (De Hoff et al., 2009) and are therefore defined functionally, often in terms of the biological processes they mediate, e.g. host–pathogen interactions, cell signaling, induction of apoptosis, cancer metastasis and differentiation (Ziółkowska et al., 2006).

The function of many lectins in plants remains unclear (Etzler, 1986; Goldstein and Poretz, 1986; Kijne et al., 1986; Kumar et al., 2012; Pusztai, 1991). Lectins bind reversibly to soluble carbohydrates and glycan chains on glycoproteins or glycolipids. They bind to the N-linked glycans of glycoproteins predominantly via mannose, glucose, fucose, N-acetylglucosamine, galactose, N-acetylgalactosamine and/or sialic acid residues (Goldstein and Poretz, 1986). Lectins can be used as antiviral agents because the surfaces of many retroviruses including HIV contain glycans (François and Balzarini, 2012). Lectins that bind virus glycans can disrupt interactions between proteins on the viral envelope and their cellular receptors (Balzarini, 2006; Botos and Wlodawer, 2005; Sacchettini et al., 2001; Shenoy et al., 2002).

The most widely studied prokaryotic antiviral lectins are griffithsin (GRFT) and cyanovirin-N (CV-N). Both are entry inhibitor molecules that bind to mannose residues in N-linked glycans displayed on the surface of *Human immunodeficiency virus* (HIV) envelope glycoproteins. GRFT is a potent anti-HIV lectin isolated from the red alga *Griffithsia* spp. (Mori et al., 2005) and it selectively targets mannose-rich glycan arrays on HIV-1 (Balzarini, 2005). As shown in Table 2.1, it has a higher potency (EC_{50} ~43 pM) against HIV-1 than other lectins, such as jacalin, concanavalin A and scytovirin (Charan et al., 2000; Ziółkowska and Wlodawer, 2006). This makes it an attractive candidate for microbicide development (Alexandre et al., 2010; Mori et al., 2005). In addition, GRFT shows little or no toxicity towards human cells and does not activate T cells (Emau et al., 2007; Kouokam et al., 2011). It acts synergistically with HIV-

neutralizing antibodies such as 2G12, and other lectins such as scytovirin (Férir et al., 2012). Only CV-N has been shown to interfere significantly with GRFT binding (Mori et al., 2005).

GRFT has broad-spectrum antiviral activity, inhibiting not only HIV-1 (Mori et al., 2005), but also severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and other coronaviruses (O'Keefe et al., 2010; Ziółkowska et al., 2006), *Hepatitis C virus* (Meuleman et al., 2011), *Japanese encephalitis virus* (Ishag et al., 2013) and *Herpes simplex virus* (Nixon et al., 2013). The antiviral activity of GRFT reflects its ability to bind high-mannose oligosaccharides on viral envelope glycoproteins, specifically terminal mannose residues on Man5–9-GlcNAc2 (Moulaei et al., 2010; Ziółkowska et al., 2007). The inactivation of HIV-1 by GRFT occurs almost immediately after contact with the virus (Emau et al., 2007). Although GRFT has no sequence homology to other lectins and shows protective effects against HIV at lower concentrations than other lectins, the basis of these unique differences remains unknown and needs further investigation (Giomarelli et al., 2006; Mori et al., 2005; O'Keefe, 2001).

GRFT has no mitogenic effect on peripheral blood mononuclear cells (PBMCs), but shows full activity in the presence of macaque vaginal secretions (Emau et al., 2007) and has a good safety profile in the rabbit vaginal irritation model (O'Keefe et al., 2009). The treatment of human cervical explants with GRFT induced minimal alterations in the expression profile of a panel of pro-inflammatory chemokines and cytokines (Kouokam et al., 2011). GRFT strongly inhibited the infection of human cervical explants by HIV-1, and the dissemination of the virus by resident cells to T cells (O'Keefe et al., 2009). GRFT can also inhibit HIV-1 binding to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and prevent DC-SIGN-mediated transfer of the virus to target cells (Alexandre et al., 2010).

Table 2.1: The anti-HIV activity of several lectins (Ziółkowska et al., 2006). The half maximal effective concentration (EC_{50}) is the lectin concentration that induces a response half way between the baseline and maximum after a specified exposure time. The EC_{50} of a graded dose response curve therefore represents the concentration at which 50% of the maximal effect is observed. The half maximal inhibitory concentration (IC_{50}) measures the ability of a compound to inhibit a biological or biochemical function. This quantitative measure indicates how much of a particular lectin is needed to inhibit a given biological process by 50%.

Lectins	***EC_{50} (nM)***
Jacalin	*> 227*
Myrianthus holstii lectin	*150*
Urtica diocia agglutinin	*105*
Concanavalin A	*98*
Narcissus pseudonarcissus lectin	*96*
Psophocarpus tetragonolobus lectin	*52*
Microcystis viridis lectin *(MVL)**	*30*
Scytovirin (SVN)	*0.3*
Cyanovirin-N (CV-N)	*0.1*
Griffithsin (GRFT)	*0.04*
**IC_{50} rather than EC50 reported*	

GRFT has a molecular weight of 12.7 kDa and is 121 amino acids in length, with three strictly conserved repeats of the sequence GGSGG (Figure 2.1) (Mori et al., 2005; Ziółkowska et al., 2006). The residue at position 31 does not appear to correspond to any standard amino acid and its identity and functional role are still unknown (Mori et al., 2005). This residue was replaced by an alanine in both *Escherichia coli* (Giomarelli et al., 2006) and *Nicotiana benthamiana* (Ziółkowska et al., 2006) expression constructs without affecting the ability of GRFT to bind carbohydrates. GRFT exists exclusively as a 25 kDa dimer and has been crystallized as a domain-swapped dimer, yielding several high-resolution structures (Moulaei et al., 2010; Ziółkowska et al., 2006). The GRFT fold is a β-prism-I motif as found in a variety of lectins, vitelline membrane outer layer protein 1 (VMO-1) and δ-endotoxin (Shimizu and Morikawa,

1996). The motif consists of three repeats of an anti-parallel four-stranded β-sheet that form a triangular prism (Figure 2.2).

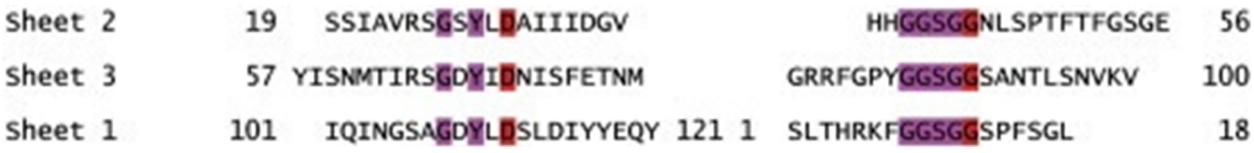

Figure 2.1: Structure-based sequence alignment of the three β sheets (blades) in GRFT. Conserved residues that interact directly with the bound carbohydrates are highlighted in red, whereas conserved residues that do not make such contacts are shown in magenta (Ziółkowska et al., 2006).

Figure 2.2: The tertiary structure of GRFT. The motif of monomer GRFT consists of three repeats of an anti-parallel four-stranded β-sheet that form a triangular prism (Ziółkowska and Wlodawer, 2006).

Unlike other proteins in the same fold family, a single molecule of GRFT contains three almost identical carbohydrate-binding sites, each capable of binding a monosaccharide through multiple epitopes. Therefore, GRFT presents six binding sites for mannose per dimer, and hence shows a significant ability to bind the high-mannose glycans on gp120 (Figure 2.3) (Ziółkowska and Wlodawer, 2006). This is why the reported biological activity of GRFT against HIV is much

more potent than the activities of the monosaccharide-specific lectins listed in Table 2.1 (Charan et al., 2000; Ziółkowska et al., 2006).

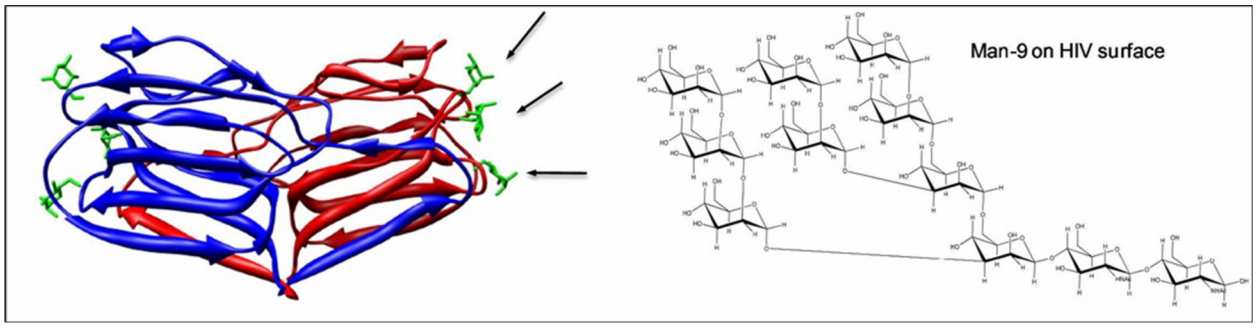

Figure 2.3: Left: The structure of the GRFT dimer in a complex with mannose (Ziółkowska et al., 2006) based on the Chimera package (Pettersen et al., 2004) using PDB structure 2gud. The arrows show the three mannose binding sites on one monomer. Right: The structure of Man-9 (Shenoy et al., 2002), an oligosaccharide on the surface of gp120 that is probably bound by GRFT.

Only small quantities of anti-HIV lectins can be isolated from nature (plants, algae, cyanobacteria and fungi) so the development of lectin-based microbicides requires the expression of recombinant lectins in larger amounts. The production of recombinant lectins in plants was considered challenging because lectins have the potential to interfere with endogenous biological processes (Lotter-Stark et al., 2012). Plants often produce large quantitates of lectins in their seeds but smaller amounts in vegetative tissues, where they may accumulate in the cytoplasm or nucleus (Peumans and Van Damme, 1995; Lannoo and Van Damme, 2010). The production of recombinant lectins could therefore interfere with signaling processes and plant development (Lotter-Stark et al., 2012).

GRFT has been expressed as a recombinant protein in *E. coli* (Giomarelli et al., 2006) and *N. benthamiana* (O'Keefe et al., 2009). The yield of recombinant GRFT in *E. coli* was 819 mg/l but the protein accumulated mostly as inclusion bodies where up to one third was mis-folded and nonfunctional, so it was not possible to produce soluble and biologically active protein (Giomarelli et al., 2006). In contrast, the production of GRFT in *N. benthamiana* was

inexpensive and large quantities of the protein (up to 1 g per kg of fresh leaf weight) were produced using a vector based on *Tobacco mosaic virus* (O'Keefe et al., 2009).

2.2 AIMS OF THE STUDY

The aims of the work described in this chapter were to determine whether rice endosperm can be used as a production platform for recombinant GRFT and to test whether the rice-derived GRFT retains its potent HIV-neutralizing activity.

2.3 MATERIALS AND METHODS

2.3.1 Cloning and construct design

The gene encoding GRFT in vector pET-28a (+) was kindly provided by Dr. Barry O'Keefe (National Cancer Institute, Frederick, MD, USA). Cloning was carried out by Dr M. Sabalza. The *GRFT* gene was amplified by PCR using primers designed to add restriction sites (Table 2.2) that facilitated subcloning. The reagents are listed in Table 2.3 and the thermal cycling conditions in Table 2.4. The PCR products were inserted into the shuttle vector pGEM-T easy (Promega, Madison, Wisconsin, USA), introduced into competent *E. coli* cells and incubated overnight at 37°C under ampicillin selection. The integrity of the plasmid DNA was confirmed by sequencing (Universidad Autónoma de Barcelona, Spain) before digestion with SphI and SacI to release the expression cassette, which was then inserted into vector pgZ63 containing the endosperm-specific maize zein promoter (Naqvi et al., 2009) and the rice α-amylase 3A signal peptide sequence (Figure 2.4).

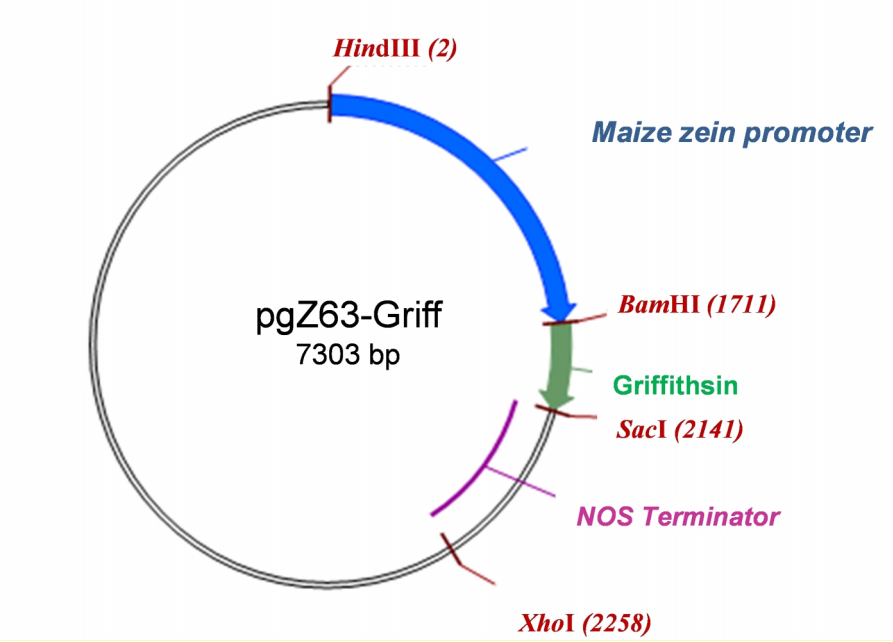

Figure 2.4: Transformation construct pgZ63-GRFT for stable expression in rice endosperm. The expression cassette comprised the endosperm-specific maize zein promoter, the rice α-amylase 3A signal peptide sequence, the coding region, a His_6 tag and the *nos* terminator.

Table 2.2: Primers used to amplify the *GRFT* gene and add restriction sites for subcloning.

GENE	PRIMER	RESTRICTION SITE	PRIMER SEQUENCE
Griffithsin (GRFT)	Forward	BamHI restriction site 5'…G▼GATCC…3' 3'…CCTAG▲G…5'	5'-TGC ATG CAT GGG CAG CAG CCA TCA T-3'
	Reverse	SacI restriction site 5'…G▼AGCTC…3' 3'…CTCGA▲G…5'	5'-GGG GAG CTC TTA GTA CTG TTC ATA GTA G-3'

Table 2.3: PCR reagents used to amplify the *GRFT* gene.

Component	GRFT (in µl)
10x Buffer (GoTaq, Promega, Madison, WI,USA)	10
10 mM dNTP	1
Forward primer (20 µM)	2.5
Reverse primer (20 µM)	2.5
Taq polymerase (Promega, Madison, WI, USA)	0.25
dH_20	41.25
Plasmid containing the gene of interest (250 ng)	2.5
Total PCR volume per reaction	50

Table 2.4: PCR thermal cycling conditions.

Step	Temperature (°C)	Time (min)		
Initial denaturation	94	3		
Denaturation	94	45	⎫	
Annealing	60	45	⎬	35 Cycles
Elongation	72	3	⎭	
Final elongation	72	10		

2.3.2 Transformation and regeneration of transgenic rice plants

This procedure is described in detail in Chapter 1, section 1.1.2.

2.3.3 Confirmation of protein expression

Protein expression was confirmed using an enzyme-linked immunosorbent assay (ELISA). Mature rice seeds were ground in three volumes of phosphate buffered saline (PBS) and centrifuged twice at 13,000 x g for 10 min at 4°C to remove debris. GRFT expression was confirmed by coating the wells of ELISA plates with 100 ng recombinant gp120 from HIV strain

IIIB, provided by the MRC Centralized Facility for AIDS Reagents, Potters Bar, UK. After washing and blocking with 5% nonfat milk, serial dilutions of each seed extract were added. Protein assembly was confirmed using a primary rabbit anti-GRFT polyclonal antiserum and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antiserum (The Binding Site, Birmingham, UK) diluted 1:1000. After washing, HRP was detected using the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

2.3.4 His_6-tag affinity purification

Rice seeds were crushed to a fine powder and extracted overnight at 4°C in five volumes of lysis buffer (50 mM NaH_2PO_4, ≥ 300 mM NaCl, 5 mM imidazole, pH 8). Insoluble material was removed by centrifuging twice at 8000 × g for 30 min at 4°C. The sample was loaded onto a profinity IMAC-resin column at a flow rate of 2 ml/min. The column was washed twice with wash buffer (50 mM NaH_2PO_4, ≥ 300 mM NaCl, 10 mM imidazole, pH 8) and the protein was eluted four times with elution buffer (50 mM NaH_2PO_4, ≥ 300 mM NaCl, 250 mM imidazole, pH 8). Protein-containing fractions were identified by the droplet Bradford method, and GRFT concentrations were determined by ELISA. Fractions containing >50 µg/ml were pooled and concentrated by ultrafiltration using spin-columns with a 3-kDa molecular mass cut-off.

2.3.5 SDS-PAGE analysis and immunoblotting

The efficiency of protein purification was tested by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4–12% pre-cast Bis-Tris Nu-PAGE gels (Invitrogen, Carlsbad, CA, USA) using Precision Plus protein standards (BioRad, Hercules, USA). Each sample comprised 5 µl of rice seed extract and 5 µl SDS loading buffer (0.3 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.125% (w/v) bromophenol blue). For immunoblotting, samples were transferred to nitrocellulose membranes using the Hoefer TE70 semidry transfer system (Amersham Biosciences, Piscataway, NJ) and blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.6). After three washes with TBS

containing 0.1% Tween-20 (TBST), protein expression was confirmed using a primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG antiserum diluted 1:1000. After further washes, the signal was detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, Little Chalfont, UK).

2.3.6 In vitro binding of GRFT to gp120

The specific antigen-binding activity of GRFT was determined by coating the wells of ELISA plates with 100 ng recombinant gp120 from HIV strain IIIB, provided by the MRC Centralized Facility for AIDS reagents, Potters Bar, UK. After washing with PBST and blocking with 5% nonfat milk, serial dilutions of the GRFT protein were added and the amount of bound protein determined using a secondary HRP-conjugated anti-rabbit IgG antiserum diluted 1:1000. After washing, the signal was developed with TMB substrate and the absorbance was read at 450 nm.

2.3.7 Whole-cell anti-HIV bioassays

Whole-cell anti-HIV bioassays were carried out by Dr K. Ramessar at the NIH National Cancer Institute, Rockville, Maryland, USA, using the 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) tetrazolium system. The HIV-neutralizing activity of GRFT produced in *E. coli* was compared to ^{OS}GRFT in HIV-1_{RF}-challenged T-lymphoblastic CEM-SS cells (Gulakowski et al., 1991). XTT was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. CEM-SS cells were maintained in RPMI 1640 medium without phenol red and supplemented with 5% fetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin (all from BioWhittaker). Exponentially growing cells were washed and resuspended in the complete medium, and a 50-µl aliquot containing 5 x 10^3 cells was added to individual wells of a 96-well round-bottomed microtiter plates containing serial dilutions of GRFT produced in *E. coli* or ^{OS}GRFT, in 100 µl of medium. Stock supernatants of HIV-1_{RF} were diluted in complete medium to yield sufficient cytopathicity (80–90% cell death in 6 days), and a

50-µl aliquot was added to the appropriate wells. Plates were incubated for 6 days at 37°C and then stained with XTT to detect viable cells. All experiments were carried out in triplicate.

2.4 RESULTS

2.4.1 Production of *GRFT* transgenic rice plants

Rice (*Oryza sativa* cv. *Nipponbarre*) mature seed-derived embryos were transformed by particle bombardment with a construct containing the GRFT coding sequence under the control of the maize zein promoter, and a second construct containing the selectable marker *hpt*. Embryo-derived callus was selected on hygromycin-supplemented medium and 13 independent transformants were regenerated and transferred to the greenhouse. Endosperm tissue was analyzed by ELISA to confirm transgene expression.

2.4.2 Protein accumulation and assembly in rice endosperm

The presence of correctly-assembled GRFT in the T1 endosperm was confirmed using an immunoglobulin-specific sandwich ELISA. The protein content of the endosperm was determined by calculating the concentrations of different lines expressing GRFT from titration curves based on positive controls spiked with known concentrations of the same protein produced in *E. coli* (Figure 2.5). We used non-transformed rice endosperm as a negative control to rule out cross reaction with endogenous plant proteins. From the 13 independent events, one line (line 6) accumulating GRFT at the highest levels (0.38 µg/ml) was selected for more detailed studies.

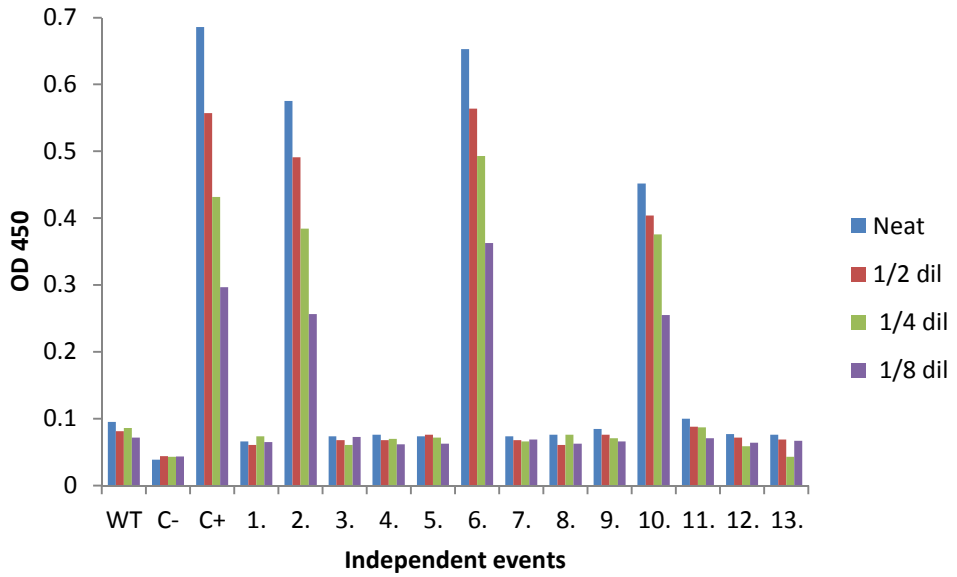

Figure 2.5: GRFT accumulation in mature rice endosperm. An immunoglobulin-specific sandwich ELISA was used to screen all independent events. The ELISA plate was coated with gp120 and GRFT protein assembly was confirmed using a primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG antiserum. Four dilutions per sample are shown. WT = wild-type plants; C^- = PBS buffer used as a negative control; C^+ = GRFT purified from *E. coli* (concentration 500 ng/ml) used as a positive control. OD = optical density at 450 nm.

2.4.3 Purification of GRFT from rice endosperm

GRFT was purified from the endosperm extract of line 6 by IMAC targeting the His_6 tag. Neither the flow-through nor wash fractions contained measurable amounts of GRFT (data not shown) indicating that the protein was folded correctly *in planta* and retained efficiently on the nickel resin. The final concentration of GRFT from the T1 endosperm was ~223 µg/g dry seed weight with a purity of almost 80%. The purified protein was separated by SDS-PAGE under reducing conditions, with 5 µl loaded per lane corresponding to 3.5 and 3.8 µg of protein (Figure 2.6, lanes A and B). A 14.6-kDa band was observed, corresponding to the expected size of GRFT produced in *E. coli*, as well as some degradation products probably caused by the imidazole present in the lysis, wash and elution buffers (Figure 2.6).

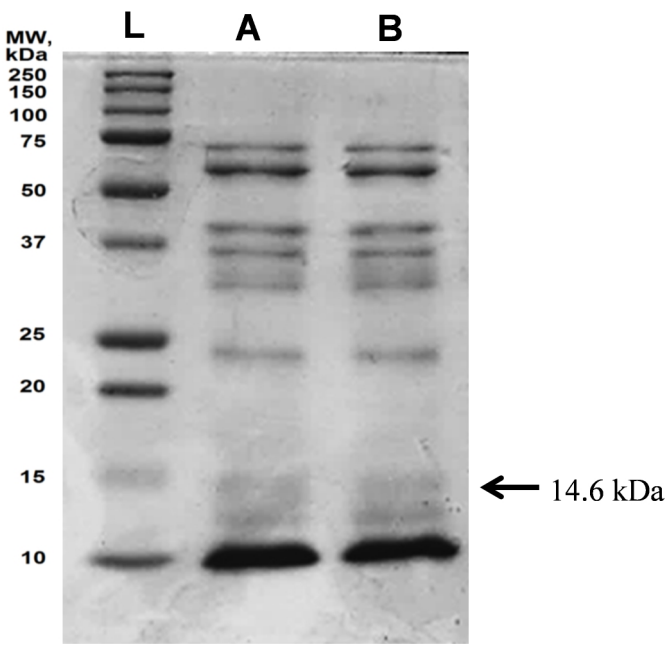

Figure 2.6: Separation of the purified GRFT fraction by SDS-PAGE under reducing conditions. The presence of the GRFT protein was confirmed (arrow) as well as degradation products. L = Precision Plus Protein™ All Blue Standards (Biorad), lane A = 3.5 µg GRFT, and lane B = 3.8µg of GRFT protein.

2.4.4 Immunoblotting analysis of GRFT

The purified GRFT fraction was separated by SDS-PAGE under reducing conditions followed by immunoblotting and detection using a primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. This revealed the anticipated 14.6-kDa band plus an additional band of the same intensity with a size of 16–17 kDa. The larger band could potentially represent a post-translational modification that alters the charge of the protein as well as its molecular mass, but the twin bands could also represent versions of the protein with and without the rice α-amylase (RAmy3D) signal sequence, which is 25 residues in length and has a predicted molecular mass of ~2.6 kDa (Figure 2.7).

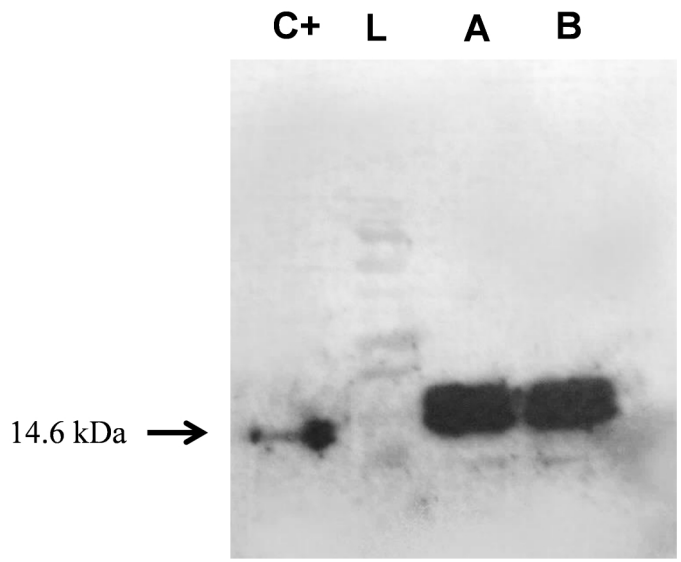

Figure 2.7: Analysis of rice-derived GRFT by SDS-PAGE under reducing conditions and immunoblotting using a primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. C^+ = positive control (50 ng GRFT produced in E.coli), L = protein size marker Precision Plus Protein™ All Blue Standards (Biorad). Lanes A and B represent 1µl of the purified simple of 3.5 µg GRFT and 3.8µg of GRFT protein accordingly. The arrow represents the anticipated size of the GRFT-His_6 protein.

2.4.5 HIV envelope glycoprotein binding by GRFT crude extracts produced in rice endosperm

The ability of the endosperm-derived GRFT to bind gp120 was tested *in vitro* using an antigen-specific ELISA. The capture reagent was gp120IIIB and the bound GRFT was detected using primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. The wild-type extract showed no binding to gp120IIIB, confirming that there was no cross reaction with the endogenous plant proteins. Lines 2, 6 and 10 showed the same gp120IIIB-binding efficiency as the positive control. Line 1 showed no binding confirming the lack of GRFT expression. These results indicate that the assembled GRFT protein produced in rice was functional and comparable to the GRFT produced in *E. coli* (Figure 2.8).

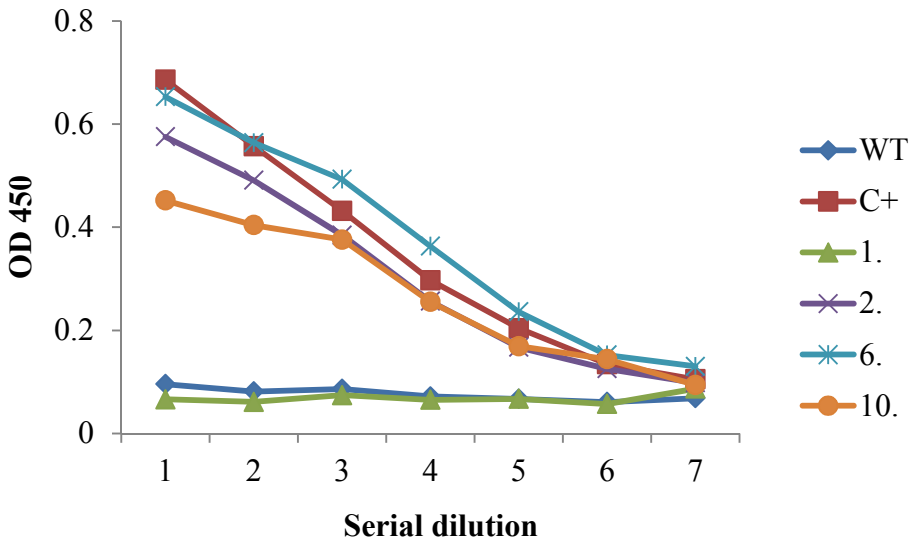

Figure 2.8: Antigen-binding activity of crude rice endosperm extracts containing GRFT and the purified GRFT produced in *E. coli* by ELISA. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. Plates coated with the HIV gp120 were tested with different concentrations of crude extracts or fixed concentrations of purified GRFT and binding activity was measured using a primary rabbit anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased. All the crude extracts showed higher binding kinetics than wild-type extracts and similar kinetics to the purified GRFT. WT = wild type plants, C^+ = GRFT produced in *E. coli* (concentration 500 ng/ml) as a positive control. 2, 6, 10 = independent events expressing GRFT protein. 1= independent event that does not express GRFT protein, OD = optical density at 450 nm.

2.4.6 Whole-cell anti HIV bioassays

Rice-derived GRFT (crude extract) and GRFT produced in *E. coli* were tested for their ability to inhibit the cytopathic effects of HIV-1_{RF} against T-lymphoblastic CEM-SS cells. Wild-type rice was used as a negative control to exclude cross-reaction with endogenous plant proteins. Previously, native GRFT and GRFT produced in *N. benthamiana* showed remarkably potent activity against HIV-1, with EC_{50} values of 0.054 and 0.156 nM, respectively (O'Keefe et al., 2009). In our experiments, GRFT at a concentration of 100 µg/ml showed potent activity against HIV-1, with an EC_{50} value of 0.29 nM, comparable to the 0.28 nM of the positive control. No IC_{50} value was measured, confirming that no toxic constituents had co-purified with the protein.

Wild-type crude extract showed no activity confirming that endogenous plant lectins had no effect on the virus (Figure 2.9).

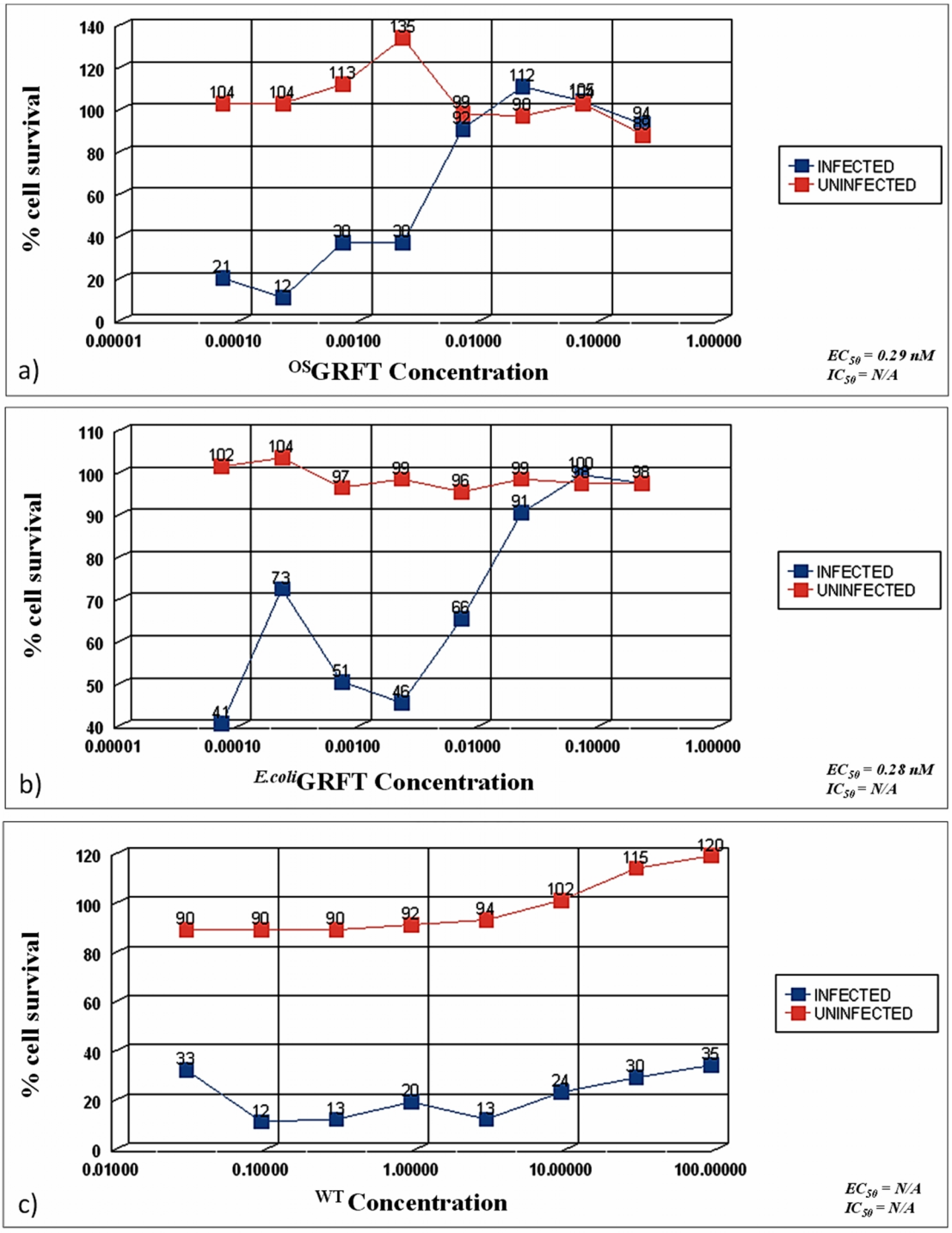

Figure 2.9: Concentration-dependent effects of GRFT on cellular viability. The *in vitro* anti-HIV activity of a) rice-derived GRFT, b) GRFT produced in *E. coli* and c) wild-type rice extracts against CEM-SS cells infected with HIV-

1_{RF} is presented as cell viability relative to uninfected and untreated controls. Cell viability was assessed using the XTT assay. All points are averages (± SD) of triplicate measurements.

2.5 DISCUSSION

Griffithsin (GRFT) is a lectin isolated from the red alga *Griffithsia* spp. and it binds to mannose residues in the N-linked glycans displayed on the surface of many different viruses (Xue et al., 2013), including HIV (Ziółkowska et al., 2007). GRFT is active against HIV clades A, B and C, which are predominant in sub-Saharan Africa, India and the West (Lotter-Stark et al., 2012) and is equally active against T-tropic and M-tropic strains of HIV-1 (Mori et al., 2005). GRFT acts as an HIV entry inhibitor, it blocks virus–cell fusion at sub-nanomolar concentrations and reduces the cytopathic effect of laboratory strains and clinical primary isolates of HIV-1 at picomolar concentrations (Mori et al., 2005). Native GRFT acts as a tight dimer (Xue et al., 2012) with a swapped domain. Each subunit contains three similar mannose-binding sites (Ziółkowska et al., 2006; Xue et al., 2013). Monomeric GRFT has no activity against HIV (Moulaei et al., 2010).

Native GRFT can be purified directly from its source (Mori et al., 2005) but the low yields mean that the production of recombinant GRFT is necessary to meet the demand for this protein as a microbicide component. GRFT was initially produced with a His-tag in *E. coli* (Giomarelli et al., 2006) and the yield was 819 mg/l, of which 66% (542 mg) was in the soluble fraction and 33% (277 mg) was in inclusion bodies. The authors attempted to isolate His-GRFT from the inclusion bodies using different detergents but this did not release significant amounts of active protein. O'Keefe et al. (2009) addressed this challenge by producing GRFT in tobacco leaves using a TMV-based vector, achieving a yield of 1 g/kg leaf tissue.

We investigated the potential use of rice endosperm as an alternative production platform for recombinant His-GRFT (^{OS}His-GRFT). We used particle bombardment to transform mature seed-derived embryos with a construct containing the GRFT coding sequence under the control of the maize zein promoter, plus a second construct containing the selectable marker *hpt*. We regenerated 13 independent transgenic events under hygromycin selection and confirmed the presence and strong expression of the transgene in three independent lines. This is the first time that the His-GRFT protein has been produced in cereal endosperm. Ultimately we chose line 6

for further investigation because this had the highest ^{OS}His-GRFT yield of 223 μg/g dry seed weight, which compares well with yields previously achieved for the HIV-neutralizing antibody 2G12 in maize (30–75 μg/g; Rademacher et al., 2008; Ramessar et al., 2008) and rice (42 μg/g; see Chapter 1) and the HIV-neutralizing antibody 2F5 in maize (0.80 μg/g; Sabalza et al., 2012). GRFT has been produced at higher levels in *N. benthamiana* by transient expression (1 mg/g after 12 days) but after purification this was reduced to 300 mg/g which is only slightly higher than the yields we achieved in rice (Zeitlin et al., 2009). Furthermore, we extracted ^{OS}His-GRFT from T1 generation seeds, which were not homozygous. It is therefore likely that the yield can be improved by breeding to homozygosity and selection in each generation, as previously described for the expression of enzymes in maize by Hood et al. (2012).

In order to determine whether any production system is economically viable there are three major factors: the amount of product that accumulates per unit biomass (productivity, specific yield), the amount of biomass that can be produced per hectare per year, and the quality of the final product in terms of homogeneity and functionality (Sabalza et al., 2013). The production system should also be facile, i.e. plants should be easy to transform and regenerate. The most suitable production system for a particular protein should therefore be determined on a case-by-case basis because no system is perfect for all proteins (Schillberg et al., 2005).

Although tobacco combines most of the above requirements, it also produces toxic compounds such as nicotine that must be removed during downstream processing, thus increasing the final cost of the product (Obembe et al., 2011). Furthermore, recombinant proteins expressed in tobacco leaves and other leafy crops are often unstable (so that harvested material must be frozen if not processed immediately) and can affect plant growth and development (Fischer et al., 2004). Importantly, the yield from *N. benthamiana* was in fresh leaf weight meaning that the infrastructure must be present to maintain the plants and carry out downstream purification rapidly and at the optimum pH to achieve the maximum yield (O'Keefe et al., 2009).

Unlike tobacco, rice does not produce toxic compounds (hence its GRAS status) and recombinant proteins are expressed in the seeds, so they are stable and even toxic proteins do not affect vegetative growth and development. The absence of phenolic compounds and alkaloids in the seeds means that chromatography media do not suffer from fouling and thus retain their capacity for longer (Moloney et al., 1999). Like tobacco, the *in vitro* cultivation and

transformation of rice are straightforward and production can be scaled up rapidly. Field plants can be scaled up between 100-fold and 1000-fold in a single season depending on the species (Schillberg et al., 2002). These advantages are shared by other cereals, but rice (unlike maize and barley) is a self-pollinating crop, so there is a much lower risk of gene flow from pharmaceutical to non-pharmaceutical crops and wild relatives (Sabalza et al., 2013). Rice endosperm also has an interesting and apparently unique property in the diversity of its storage compartments. The endosperm cells of most cereals contain specialized storage compartments known as protein bodies, which have evolved for protein storage and bud from the endoplasmic reticulum when sufficient protein has accumulated (Takaiwa et al., 2009). These compartments can also be used to accumulate recombinant proteins using the appropriate promoters and targeting sequences, increasing protein yields by providing an ideal stable environment (Muntz, 1998; Yamagata and Tanaka, 1986). In practical terms, this means that cereal seeds containing pharmaceutical proteins can be stored and distributed in countries lacking a reliable cold chain. Most cereals form a single type of protein body, whereas rice endosperm forms two distinct types of compartment, known as PB-I and PB-II (Takaiwa et al., 2009). Protein bodies are insoluble in low-salt aqueous buffers, so they can be purified by centrifugation. Therefore the presence of two different protein bodies in rice potentially allows different proteins to be expressed and targeted to different compartments simultaneously, and then recovered by first separating the compartments by density centrifugation (Sabalza et al., 2013).

The yields of recombinant proteins produced in rice and other cereals can often be improved by breeding and selection (Hood et al., 2012), which suggests that our GRFT yields could be improved in the same manner. For example, human transferrin was produced with a yield of 8.8 mg/g (0.88%) in first-generation rice plants, but this increased to 10 mg/g (1%) in the second generation (100-fold more than the commercial threshold) and remained stable at that level in subsequent generations (Zhang et al., 2010). As discussed above, the yield of GRFT in tobacco was 0.3 mg/g or 0.03% TSP and our initial yields were similar at 0.223 mg/g or 0.0223% TSP.

The number and nature of purification steps and the overall cost of processing are key issues that determine the commercial viability of a production platform. Mori et al. (2005) purified the native GRFT from *Griffithsia* spp. by ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, reversed-phase chromatography and size

exclusion chromatography to yield a homogeneous, highly pure and biologically active protein. We found that ammonium sulfate precipitation did not work, not even when combined with ethanol precipitation. However, protein extraction using phosphate buffer combined with IMAC resin purification gave us satisfactory results and >80% protein purity. Several purification methods have been used with the *E. coli* production platform. For example, Giomarelli et al. (2006) purified soluble His-GRFT from *E. coli* using Ni-NTA resin and the dialyzed protein was then applied to a TALON resin before stepwise elution using two different buffers containing different amounts of imidazole (50 mM and 250 mM, respectively). Ishag et al. (2013) used IMAC followed by elution in a buffer containing 250 mM imidazole before further purification by size exclusion chromatography using a Superdex^{TM} 75 column (GE Healthcare). The resulting purified protein contained both the monomeric and dimeric forms of GRFT. Finally, Xue et al. (2013) used a nickel-chelating column (Qiagen) and eluted the His-GRFT protein with 500 mM imidazole, refolding the protein by drop-wise addition to a low-salt refolding buffer before final purification using a C4 reversed-phase chromatography column (Vydac, Hesperia, CA). When GRFT was produced in tobacco using viral vectors, the first purification step involved the removal of the virus coat protein by filtration through a ceramic membrane, followed by ion exchange chromatography over SP-Sepharose resin, ultrafiltration/diafiltration (UF/DF) to concentrate the protein and elution with 100 mM NaCl, achieving >99.8% purity (O'Keefe et al., 2009). In our case, we used non-reducing conditions to prepare the protein extract thus keeping GRFT in its native confirmation to avoid the need for a refolding step. The protein was captured on a Profinity IMAC column and eluted with 250 mM imidazole. There are many commercial kits and protocols for the purification of His-tagged proteins but we used the simplest, one-step method to see if purification costs could be reduced without sacrificing the purity and bioactivity of the protein. Accordingly, we achieved a purity of ~80% and the protein remained active.

Multiple bands were observed when the purified sample was separated by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. A western blot using a primary rabbit anti-GRFT polyclonal antiserum and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antiserum revealed an intense band at 14.6 kDa (the anticipated molecular mass of GRFT) and another at 16–17 kDa. The gel was run under denaturing conditions so the additional band is unlikely to represent a conformational isomer or a non-covalent interaction. It is possible that GRFT undergoes partial post-translational modification and is therefore present in two forms,

but the sequence does not contain a glycan acceptor site and no other modifications would explain the difference in mobility (2–3 kDa) unless the modification also caused a significant change to the charge of the protein (e.g. phosphorylation). A more likely explanation is the incomplete removal of the rice α-amylase (RAmy3D) signal peptide, which is 25 residues in length and has a predicted molecular mass of 2.6 kDa.

GRFT can interfere with the interaction between HIV and CD4 cells by binding to several mannose-rich viral glycoproteins, namely gp120, gp41 and gp160 (Mori et al., 2005). GRFT interacts with a number of viruses, but has particular potency against HIV because it can bind to multiple sites on gp120 (Giomarelli et al., 2006). We therefore compared the biological activity of ^{OS}His-GRFT and GRFT produced in *E. coli* by testing their ability to bind gp120 in a plate-based binding assay, initially comparing the purified *E. coli* GRFT to crude rice extracts from different lines. We found that ^{OS}His-GRFT bound to gp120 in a manner similar to *E. coli* GRFT and that the binding activity was concentration dependent. Line 6 showed slightly greater affinity for gp120 than *E. coli* GRFT, indicating that the ^{OS}His-GRFT possessed essentially the same oligosaccharide-dependent binding properties as the bacterial recombinant protein. These results agree with O'Keefe et al. (2009), who showed that tobacco GRFT had a comparable or even higher affinity for gp120IIIB than *E. coli* GRFT. Giomarelli et al. (2002) also demonstrated that the His-GRFT produced in *E. coli* retained the biological activity and gp120-binding activity of its native counterpart, and endogenous plant lectins did not interfere with binding.

We also tested ^{OS}His-GRFT and *E. coli* GRFT side-by-side in a whole-cell anti-HIV assay in order to define their anti-HIV activity. Giomarelli et al. (2006) showed that the recombinant His-GRFT inhibited HIV-induced cytopathicity with an EC_{50} value of 0.089 nM, similar to the EC_{50} value of native GRFT (0.163 nM) and they concluded that both forms of GRFT were functionally equivalent. However, these data were different from those reported by O'Keefe et al. (2009), who recorded an EC_{50} value of 0.054 nM for native GRFT and 0.156 nM for tobacco GRFT. This discrepancy may have reflected differences in the assay set up or the nature of the samples. As expected from the gp120 binding assay, our comparison of ^{OS}His-GRFT and *E. coli* GRFT confirmed that both displayed nanomolar activity against HIV. The ^{OS}His-GRFT has an EC_{50} value of 0.29 nM compared to *E. coli* GRFT with an EC_{50} value of 0.28 nM, well within the

range previously reported by Mori et al. (2005). The wild-type crude extract showed no activity against HIV, confirming there was no cross reaction with endogenous plant lectins.

2.6 CONCLUSIONS

Our data show that ^{OS}His-GRFT can be expressed in rice endosperm with yields comparable to or even higher than GRFT produced transiently in tobacco. One-step His-tag purification resulted in the isolation of GRFT with almost 80% purity. Furthermore, we obtained moderate yields of ^{OS}His-GRFT even in first-generation plants suggesting that rice seeds could be used as a production platform for GRFT after future optimization. The ability of ^{OS}His-GRFT to bind and inhibit HIV was tested by ELISA against gp120 and a whole-cell anti-HIV assay. In both cases, ^{OS}His-GRFT showed similar behavior to the control GRFT produced in *E. coli*. This is the first time that GRFT has been produced in cereals and this allows the comparison of different plant-based production platforms to see whether they affect the anti-HIV activity of GRFT. We therefore demonstrated that rice endosperm can be used as an economical production platform for GRFT.

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CHAPTER 3

Preliminary investigations for the production of the anti-HIV protein Cyanovirin-N (CV-N) in rice endosperm

3.1 INTRODUCTION

Cyanovirin-N (CV-N) is an 11-kDa monomeric non-glycosylated protein originally isolated from an aqueous extract of the cyanobacterium *Nostoc ellipsosporum* while screening for anti-HIV activity carried out at the US NCI Natural Products Repository in the late 1990s (Boyd et al., 1997). CV-N inhibits all HIV clades *in vitro* by blocking multiple stages of membrane fusion and virus entry (Dey et al., 2000). CV-N binds to the HIV surface envelope glycoprotein gp120 (Boyd et al., 1997; Mori et al., 1997), specifically interacting with N-linked high-mannose residues (Bolmstedt et al., 2001; Botos et al., 2002) blocking the attachment and fusion of the virus particle to target cells, and thereby preventing infection. CV-N also blocks vaginal HIV transmission in non-human primates but relatively high concentrations are needed to demonstrate this positive effect because of the protein has a short half-life and undergoes rapid clearance (Taha et al., 1998). In addition, *in vivo* studies showed that CV-N formulated as a gel had promising efficacy as a microbicide candidate, whereas *ex vivo* studies demonstrated that CV-N has stronger antiviral effect than the nonspecific polyanion microbicide PRO 20O (Fischetti et al., 2009; Huskens et al., 2009). CV-N also inhibits HIV-1 binding to the DC-SIGN receptor and DC-SIGN mediated transfer of the virus to target cells (Alexandre et al., 2012). This is important because in addition to the infection of $CD4^+$ T lymphocytes and macrophages by cell-free virions and donor-infected cells, the DC-SIGN-directed capture of HIV-1 and transmission to $CD4^+$ T lymphocytes is considered an avenue of primary infection in women exposed to HIV-1 through sexual intercourse (Hladik and Hope, 2009).

Conventional biochemical techniques were used to determine the primary structure of CV-N and the pattern of disulfide bridges (Boyd et al., 1997; Gustafson et al., 1997). CV-N has a unique sequence of 101 amino acids (Boyd et al., 1997) comprising two internal repeats of 50 and 51 amino acids, respectively (Figure 3.1) (Bewley et al., 1998; Ziółkowska and Wlodawer, 2006). The 1-50 and 51-101 repeats have 70% similarity. Structural alignment of the repeats showed that 16 residues were identical and 19 were conservative replacements (Figure 3.1) (Bewley et al., 1998).

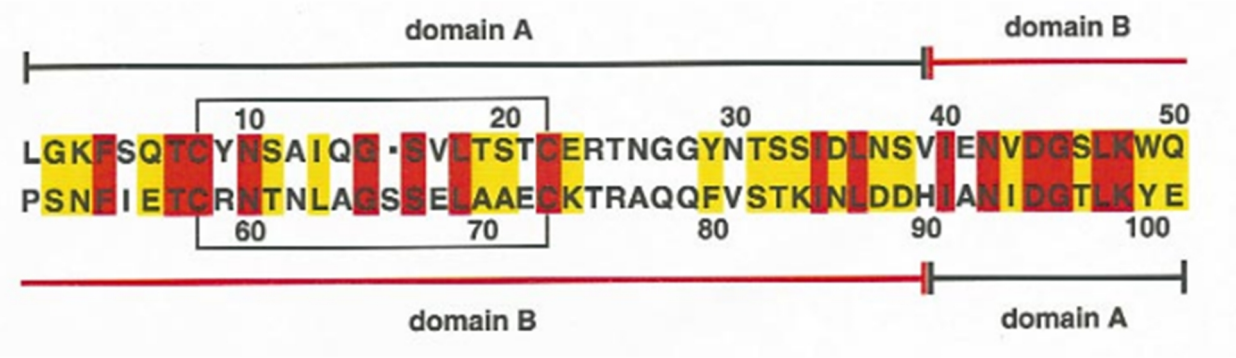

Figure 3.1: Structure alignment of the first and second sequence repeats of the algal lectin CV-N. Conserved residues are highlighted in yellow, whereas identical residues are highlighted in red. Black and red lines demarcate domains A and B, respectively (Bewley et al., 1998).

CV-N contains four cysteine residues that form two intramolecular disulfide bonds. Disruption of these disulfide bonds causes the loss of anti-HIV activity (Bewley et al., 1998; Yang et al., 1999). In addition to HIV, CV-N is also active against rhinoviruses, *Herpes simplex virus* (HSV-1), *Hepatitis C virus* (HCV), *Ebola virus*, *Human herpes virus 6* (HHV-6), measles virus, influenza viruses A and B (Balzarini, 2007; Barrientos et al., 2002; Buffa et al., 2009; Helle et al., 2006; O'Keefe et al., 2003; Smee et al., 2007; Xiong et al., 2010), murine leukemia virus and vesicular stomatitis virus (Shahzad-ul-Hussan et al., 2011).

The Protein Data Bank contains 24 entries for *Nostoc ellipsosporum* CV-N based on x-ray crystallography (17) and nuclear magnetic resonance (NMR) spectroscopy (7) data. The predominant form of CV-N in solution is the monomeric form, but because it contains two carbohydrate recognition sites on symmetrically-opposed domains, it can cross-link branched oligomannosides to form higher-order structures (Bewley and Otero-Quintero, 2001; Bewley, 2001; Bewley et al., 1998; Liu et al., 2009). CV-N specifically recognizes Manα(1–2)Man linked mannose substructures in the D1 and D3 arms of Man9 (Figure 3.2) (Bewley, 2001; Botos et al., 2002). Although the two halves in the monomeric CV-N share a high degree of sequence similarity, there are never more than eight contiguous amino acids or 20% of the total sequence that are related to any other known protein (Yang et al., 1999).

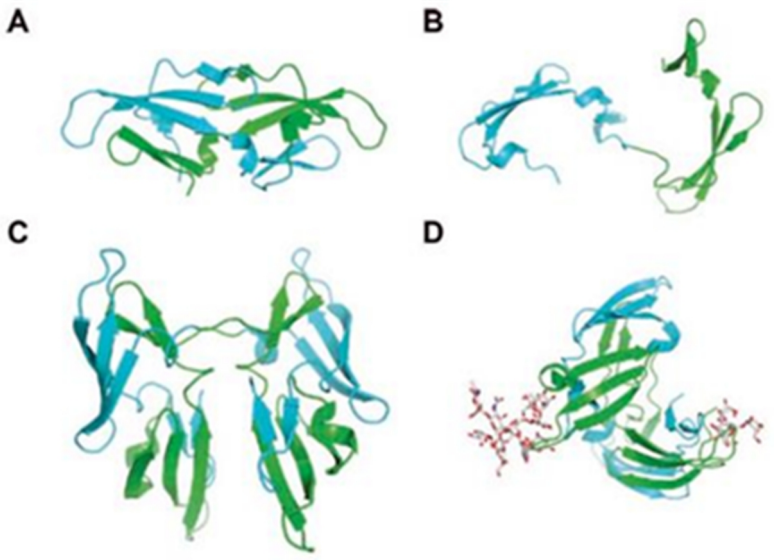

Figure 3.2: Different structures reported for CV-N. Domain A of each monomer is shown in cyan and domain B in green. A) Compact monomer observed by NMR; B) Extended monomer that is found in all crystal structures and some NMR structures as part of a dimer – it does not exist in isolation; C) Domain-swapped dimer comprising two monomers as shown in panel B; D) A complex of CV-N with Man9 (Ziółkowska and Wlodawer, 2006).

A 5-mg dose of CV-N was effective as a microbicide candidate in macaques, but this equates to a demand of 5000 kg per year to supply twice-weekly doses for 10 million women (Shattock and Moore, 2003). This scale of production requires an expression system that could produce recombinant CV-N efficiently at a low cost. CV-N has been expressed in *Escherichia coli*, but the yields were low at 10 mg/l (Boyd et al., 1997). The yield was increased to ~40 mg/l in shaking-flask cultures by targeting CV-N for accumulation in cytosolic inclusion bodies, and this increased again to 140 mg/l in high-density fed-batch cultures, but the final product was heterogeneous (Colleluori et al., 2005). CV-N has also been expressed using a chaperone fusion system in *E. coli*, in which the protein was double-tagged with hexa-histidine and a small ubiquitin-related modifier (SUMO). This achieved CV-N yields of >30% total soluble protein (TSP) (Gao et al., 2010). CV-N was also expressed in commensal *Streptococcus gordonii* either as a secreted protein or attached to the bacterial cell surface. The secreted CV-N was able to bind HIV-1 and the surface-attached version was able to capture HIV-1 (Giomarelli et al., 2002). *Lactobacillus jensenii* has also been shown to secrete recombinant CV-N with potent antiviral activity against laboratory and primary HIV-1 isolates (Liu et al., 2006). This recombinant

bacterium has been shown to deliver CV-N directly to the mucosal surface in macaques, reducing the transmission of a the chimeric virus SHIV by 63% after repeated vaginal challenges (Lagenaur et al., 2011). Finally, CV-N has also been produced in the yeast *Pichia pastoris*, which is widely used for the production of recombinant proteins because it is adapted for highly efficient secretion. However, the recombinant CV-N produced in yeast was inactive due to the high mannose N-glycosylation at position N30 and P51-mediated dimerization of the final product in the hinge region, both of which result in a loss of activity (Mori et al., 2002). The overall yield was less than 10 mg/l.

Transgenic plants provide an alternative and more scalable platform for CV-N expression and tobacco plants (*Nicotiana tabacum*) have been used to produce recombinant CV-N at yields of ~130 μg per gram of fresh leaf tissue, or 0.635 ± 0.298 mg/l in a 24-day rhizosecretion system, with most of the recombinant protein present in the monomeric form demonstrating high stability (Sexton et al., 2006). Under optimized conditions, the yield of the rhizosecretion system was increased to 3.2 mg/l, or 766 μg/g root dry weight per 24 h. This is the highest reported yield for the rhizosecretion of a heterologous protein although further optimization would be required to achieve commercial viability (Colgan et al., 2010; Drake et al., 2009). CV-N has also been produced in the marshmallow plant (*Althaea officinalis* L.) using *Agrobacterium rhizogenes* with yields of 2.4 μg/g fresh weight (Drake et al., 2013).

3.2 AIMS OF THE STUDY

The aims of the experiments described in this chapter were to explore rice endosperm as a production platform for the production of CV-N and to evaluate the efficiency of rice-derived CV-N for the neutralization of HIV.

3.3 MATERIALS AND METHODS

3.3.1 Cloning and construct design

The gene encoding CV-N (N30Q/P51G) in plasmid pET30b, recombinant purified CV-N produced in *E. coli*, and anti-CV-N polyclonal antisera, were all kindly provided by Prof. Julian Ma, St George's Hospital Medical School, University of London, UK, under a material transfer agreement with Dr. Barry R. O'Keefe, National Cancer Institute, Frederick, MD, USA. Cloning was carried out by Dr M. Sabalza. The *CV-N* gene was amplified by PCR using primers designed to append specific restriction sites (Table 3.1) facilitating the construction of transformation vectors using the reagents listed in Table 3.2 and thermal cycling conditions as shown in Table 3.3.

The PCR products were ligated into the shuttle vector pGEM-T Easy (Promega, Madison, Wisconsin, USA) and introduced into competent *E. coli* cells, which were incubated overnight at 37°C under ampicillin selection. Plasmid DNA from positive samples was verified by sequencing (Universidad Autónoma de Barcelona, Spain). Plasmid DNA was digested with restriction enzymes SphI and SacI and the CV-N gene was transferred to the pRP5 vector containing the endosperm-specific rice prolamin promoter (Naqvi et al. 2009a) and the rice α-amylase 3A signal peptide sequence (Figure 3.3).

Table 3.1: PCR primers for the *CV-N* gene and appended restriction sites

GENE	PRIMER	RESTRICTION SITE	PRIMER SEQUENCE
Cyanovirin-N (CV-N)	Forward	BamHI restriction site 5'…GGATCC…3' 3'…CCTAGG…5'	5'-GGG ATC CAT GCT TGG TAA ATT CTC CCA G-3'
	Reverse	EcoRI restriction site 5'…GAATTC…3' 3'…CTTAAG…5'	5'-CCG AAT TCT TAT TCG TAT TTC AGG GTA CCG-3'

Table 3.2: Reagents used to amplify the *CV-N* gene.

Component	CV-N (in µl)
10x Buffer (GoTaq, Promega, Madison, WI,USA)	10
10 mM dNTP	1
Forward primer (20 µM)	2.5
Reverse primer (20 µM)	2.5
Taq polymerase (Promega, Madison, WI, USA)	0.25
dH_20	41.75
Plasmid containing the gene of interest (250 ng)	2
Total PCR volume per reaction	50

Table 3.3: PCR thermal cycling conditions.

Step	Temperature (°C)	Time (min)		
Initial denaturation	94	3		
Denaturation	94	45	⎫	
Annealing	60	45	⎬	35 Cycles
Elongation	72	3	⎭	
Final elongation	72	10		

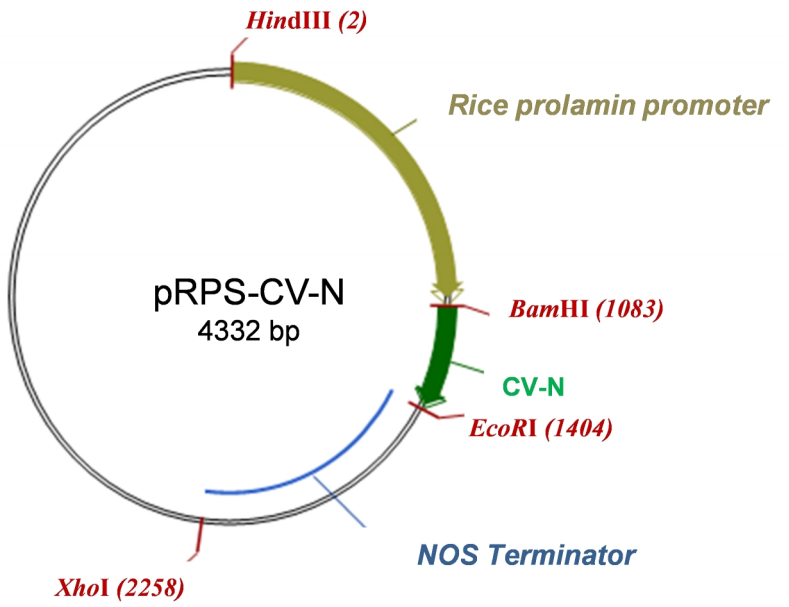

Figure 3.3: Transformation construct pRP5-CV-N for stable expression in rice seeds. The expression cassette comprised the endosperm-specific rice prolamin promoter, the rice α-amylase 3A signal peptide sequence, the coding region, and the *nos* terminator.

3.3.2 Transformation and regeneration of transgenic plants

This method is described in detail in Chapter 1, section 1.3.2.

3.3.3 Crude extract of CV-N from rice endosperm

We crushed 5 g of mature rice seeds into a fine powder using a glass mortar. The total soluble protein (TSP) was extracted in three volumes (v/w) of PBS by vortexing overnight at 4°C. The samples were then centrifuged twice (13,000 x g, 15 min, 4°C) and the supernatant was filtered and transferred into a 50-ml falcon tube.

3.3.4 SDS-PAGE and immunoblotting

Proteins were separated under non-reducing conditions on 4–12 % gradient pre-cast Bis-Tris Nu-PAGE gels (Invitrogen, Carlsbad, CA, USA) using Precision Plus protein standards (BioRad,

Hercules, USA). Each sample comprised 5 µl of rice seed extract plus 5 µl SDS loading buffer (0.3 M Tris HCl pH 6.8, 10 % (w/v) SDS, 50 % (v/v) glycerol, 0.125 % (w/v) bromophenol blue). For immunoblotting, samples were transferred to nitrocellulose membranes using the Hoefer TE70 semidry transfer system (Amersham Biosciences, Piscataway, NJ) and blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.6). After three washes with TBS plus 0.1 % Tween-20 (TBST), protein expression was detected using a primary rabbit anti-CV-N polyclonal antiserum and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antiserum (The Binding Site, Birmingham, UK; 1:1000 dilution). After three further washes, the signal was detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, Little Chalfont, UK).

3.3.5 Confirmation of protein expression (ELISA)

Mature rice seeds were ground in three volumes of PBS and centrifuged twice (13,000 x g, 10 min, 4°C) to remove seed debris, and serial dilutions of the extract were added to ELISA wells previously coated with 100 ng recombinant gp120 from HIV strain IIIB (MRC Centralized Facility for AIDS Reagents, Potters Bar, UK), washed and blocked with 5% nonfat milk. Protein assembly was confirmed using a primary rabbit anti-CV-N polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG antiserum (1:1000 dilution). After washing, HRP was detected by adding 3,3',5,5' tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

3.3.6 In vitro binding of cyanovirin-N to gp120 (ELISA)

The specific antigen-binding activity of CV-N was detected by coating ELISA wells with 100 ng recombinant gp120 from HIV strain IIIB. After washing with PBST and blocking with 5% nonfat milk, serial dilutions of CV-N were added and the amount of bound protein determined using a secondary HRP-conjugated anti-rabbit IgG antiserum (1:1000 dilution) detected as described above.

3.3.7 Whole-cell anti-HIV bioassays

Whole-cell anti-HIV bioassays were carried out by Dr K. Ramessar at the NIH/NCI, Frederic, Maryland, USA. A 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT)-tetrazolium-based assay was used to determine the anti-HIV activity of rice-derived CV-N against HIV-1_{RF} challenged T-lymphoblastic CEM-SS cells, as described previously (Gulakowski et al., 1991). XTT was supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. CEM-SS cells were maintained in RPMI 1640 medium without phenol red and supplemented with 5% fetal bovine serum, 2 mM l-glutamine and 50 µg/ml gentamicin (all BioWhittaker). Exponentially-growing cells were washed and resuspended in this complete medium, and a 50-µl aliquot containing 5 x 10^3 cells was added to the individual wells of a 96-well round-bottomed microtiter plate containing serial dilutions of rice-derived CV-N in 100 µl of medium. Stock supernatants of HIV-1_{RF} were diluted in complete medium to yield sufficient cytopathicity (80–90% cells killed in 6 days), and a 50-µl aliquot was added to the appropriate wells. Plates were incubated for 6 days at 37 °C and then stained for cellular viability using XTT. All experiments were carried out in triplicate.

3.4 RESULTS

3.4.1 Production of CV-N in rice endosperm

Rice (*Oryza sativa* cv. *Nipponbarre*) mature seed-derived embryos were transformed by particle bombardment with a construct containing the CV-N coding sequence, under the control of the rice prolamin promoter, plus a second construct containing the selectable marker *hpt*. Embryo-derived callus was selected on hygromycin-supplemented medium and 10 independent transformants were regenerated and transferred to the greenhouse. Endosperm tissue was analyzed by ELISA to confirm transgene expression.

3.4.2 Confirmation of protein expression and assembly in rice endosperm

The presence of correctly-assembled CV-N in the T1 endosperm was confirmed using an immunoglobulin-specific sandwich ELISA. The protein content of the endosperm was determined by calculating the concentrations of different lines expressing CV-N from titration curves based on positive controls spiked with known concentrations of the same protein produced in *E. coli* (Figure 3.4). We used non-transformed rice endosperm as a negative control to rule out cross-reaction with endogenous plant proteins. Among 10 independent events, two lines were found to express CV-N (lines 2 and 6, with yields of 6 and 0.5 µg/ml, respectively) although it is necessary to take into consideration that a 1/25 dilution was necessary for detection. We selected line 2, with the highest yields of assembled CV-N, for more detailed investigation.

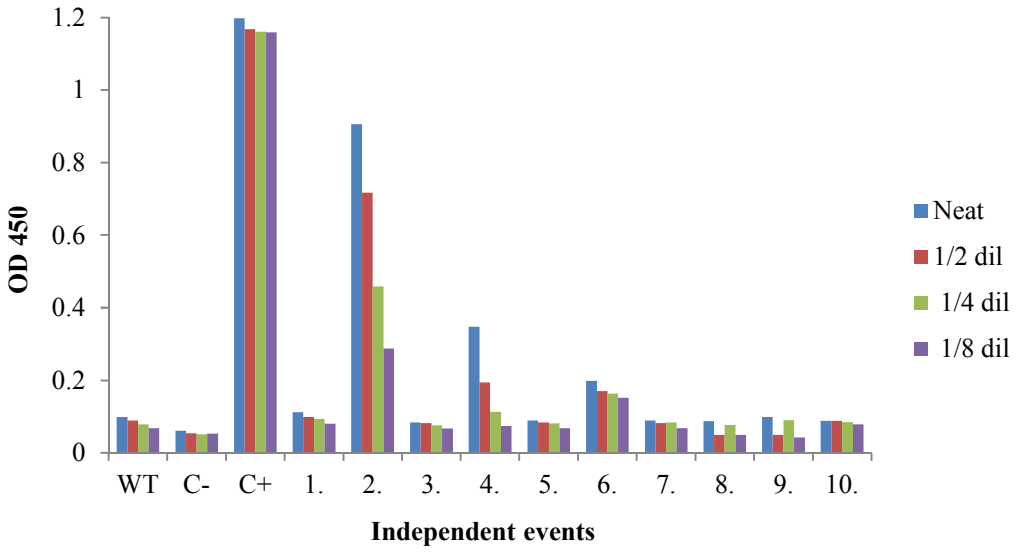

Figure 3.4: CV-N accumulation in mature rice endosperm. An immunoglobulin-specific sandwich ELISA procedure was used to screen all the independent events for CV-N production. The ELISA plate was coated with gp120 IIIB and the bound CV-N was detected using primary rabbit anti-CV-N polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG antiserum (1:1000 dilution). Four dilutions per sample are shown; WT = wild type plants and C^- = PBS buffer used as negative control. C^+ = CV-N produced in *E. coli* (concentration of 500 ng/ml) as a positive control.

3.4.3 Binding activity of the CV-N crude extract from rice endosperm

The ability of the endosperm-derived CV-N to bind gp120 was tested *in vitro* using an antigen-specific ELISA. The capture reagent was gp120 IIIB and the bound CV-N was detected using primary rabbit anti-CV-N polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. The wild-type extract showed no binding to gp120, confirming that there was no cross reaction with the endogenous plant proteins. The positive control showed higher gp120-binding activity than any of the independent events and remained at the same level throughout the range of serial dilutions. Line 2 showed promising gp120-binding activity and both lines 4 and 6 were also positive for gp120 binding but only at the highest concentration (or lowest dilution). These results indicate that the recombinant CV-N protein produced in the transgenic endosperm was functional (Figure 3.5).

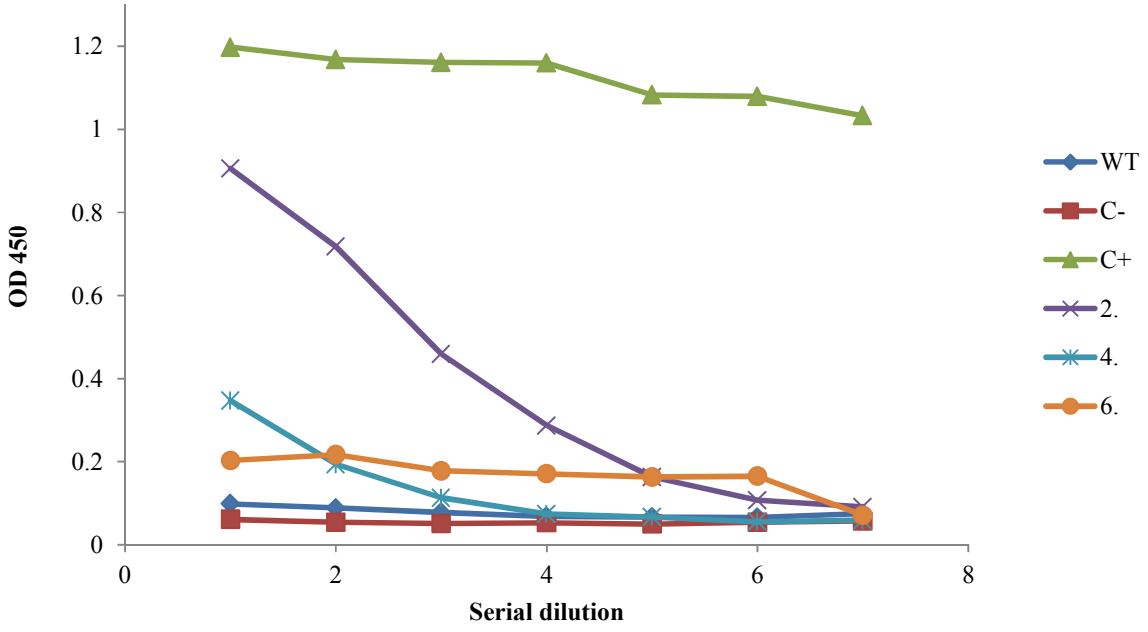

Figure 3.5: Antigen-binding activity of the crude rice endosperm extracts containing CV-N measured by ELISA compared to the purified CV-N produced in *E. coli*. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. Binding activity was measured with a primary rabbit anti-CV-N polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased in all the samples except the positive control. All the crude extracts showed higher binding kinetics than wild-type extracts but lower binding kinetics than

the purified CV-N. WT = wild type plants. C^- = PBS buffer used as negative control. C^+ = CV-N produced in *E. coli* (concentration of 500 ng/ml) as a positive control. Lines 2, 4 and 6 are independent events expressing CV-N.

3.4.4 Higher yields of CV-N in rice endosperm over successive generations

Three lines (1, 2 and 4) were self-pollinated over three generations to produce T4 seeds. The yields of CV-N were compared in the T1 and T4 seeds, revealing a substantial increase in lines 2 and 6 (Figure 3.6). In the T1 generation, the expression of CV-N was lower than the ELISA detection limit and therefore indistinguishable from the wild-type extracts, but a significant upward trend was apparent during through to the T4 generation (Figure 3.6).

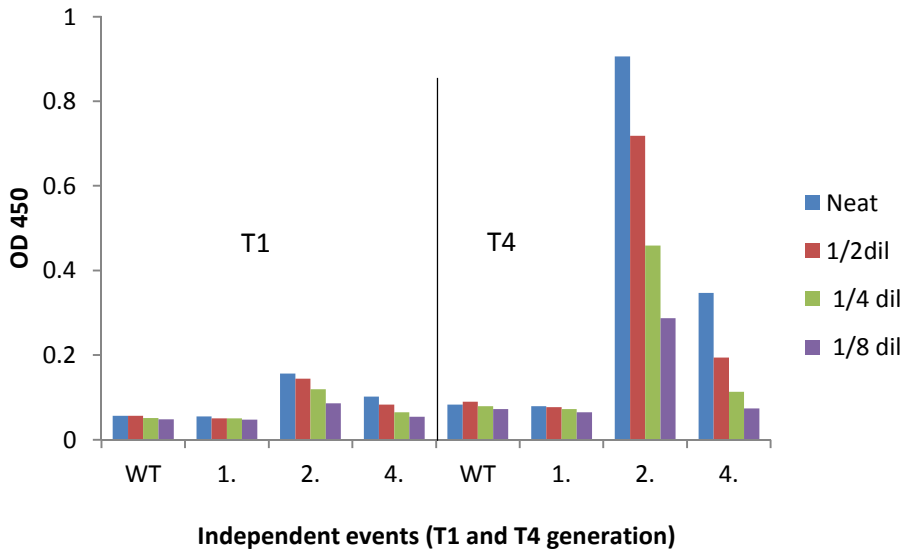

Figure 3.6: Comparison of CV-N yields in generations T1 and T4. Four dilutions per sample are shown; WT = wild-type plants. C+ = CV-N purified from E.coli (concentration 500ng/ml) as a positive control.

3.4.5 HIV-neutralization activity of rice-derived CV-N in whole-cell bioassays

Rice-derived CV-N (crude extract) was tested for its ability to inhibit the cytopathic effects of HIV-1_{RF} against T-lymphoblastic CEM-SS cells. Wild-type rice was used as a negative control to exclude any potential cytotoxicity or antiviral activity caused by endogenous plant proteins.

Rice-derived CV-N at a concentration of 100 µg/ml showed low activity against HIV-1, with an EC_{50} value of 28 µg/ml, much higher than that reported previously (O'Keefe, 2001). This probably was due to the fact that even though a specific concentration of extract was used, the concentration of CV-N inside the final extract was low (Figure 3.7 a). It was not possible to determine an IC_{50} value, confirming that no toxic constituents had co-purified with the protein. Wild-type crude extract showed no activity confirming that there was no toxicity or antiviral activity caused by endogenous plant lectins or other components (Figure 3.7 b).

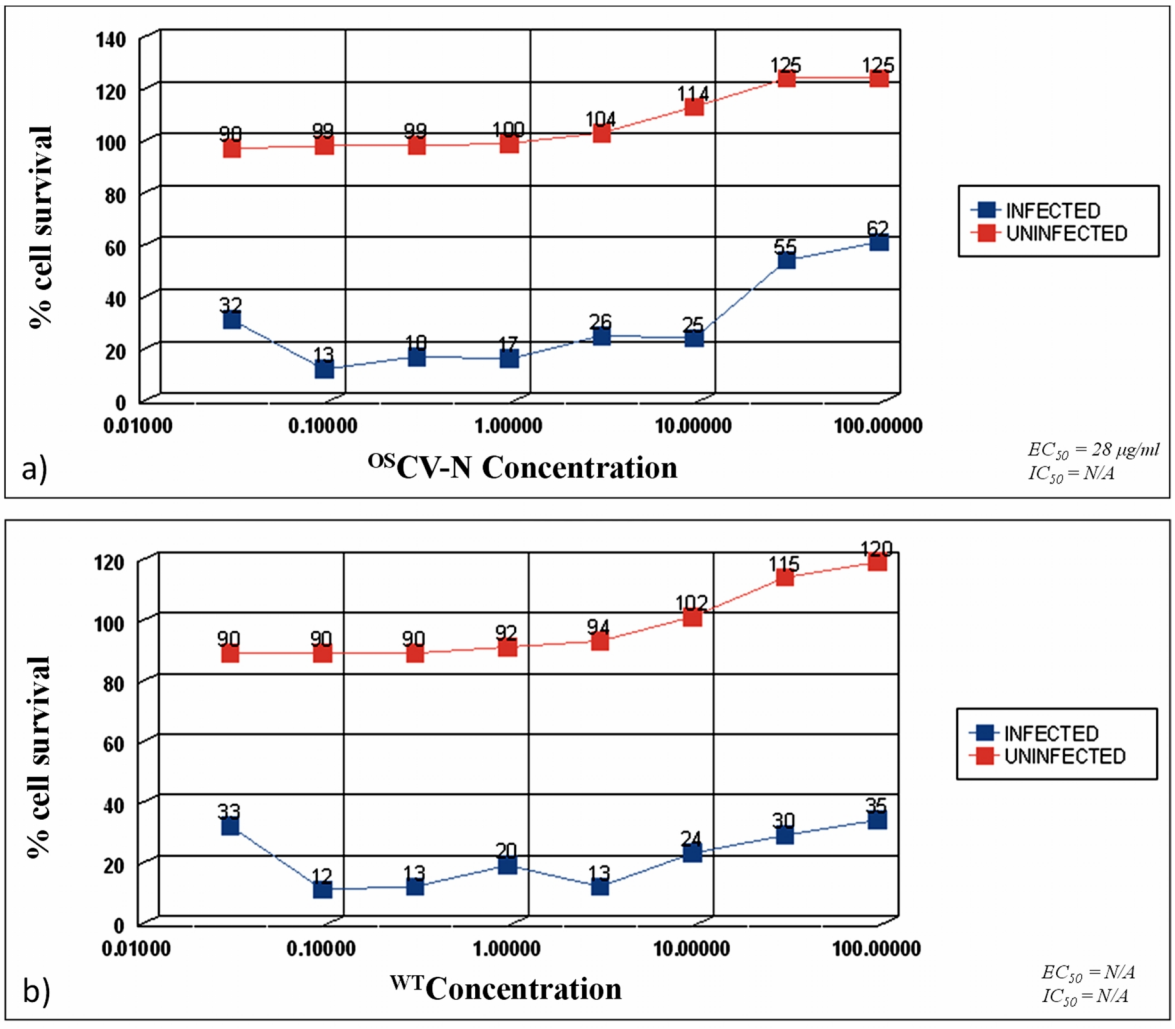

Figure 3.7: *In vitro* anti-HIV activity of rice-derived CV-N. The anti-HIV activity of a) CV-N and b) wild-type rice in CEM-SS cells infected with HIV-1_{RF} revealing concentration-dependent effects of CV-N on cellular viability

relative to the uninfected and untreated controls. Cell viability was assessed using the XTT assay. All points are averages (± SD) of experiments carried out in triplicate.

3.5 DISCUSSION

CV-N is an entry inhibitor originally isolated form the cyanobacterium *Nostoc elipsosporum* which shows promising *in vivo* efficacy against HIV (Boyd et al., 1997; Buffa et al., 2009). CV-N binds to N-linked high-mannose glycans on the surface of HIV envelope glycoproteins, such as gp120 and gp41, inhibiting viral attachment to its target cells (Dey et al., 2000; Shenoy et al., 2001). Furthermore, CV-N is resistant to chemical and thermal denaturation and degradation. It is stable in a low-pH environment and survives multiple freeze-thaw cycles (Boyd et al., 1997). Native CV-N can be purified directly from its source but only low yields can be achieved (Gustafson et al., 1997). Other platforms are therefore required for the large-scale expression of this protein, and recombinant CV-N has accordingly been produced in *E. coli* (Boyd et al., 1997) and other bacteria such as *Streptococcus gordonii* and *Lactobacillus jensenii* (Giomarelli et al., 2002; Liu et al., 2006), the yeast *Pichia pastoris* (Mori et al., 2002) and the plants *Nicotiana tabacum* and *Althaea officinalis* (Drake et al., 2013; Sexton et al., 2006).

We transformed mature seed derived rice embryos by particle bombardment using a construct containing the coding sequence of CV-N under the control of the rice prolamin promoter, plus a second construct containing the selectable marker *hpt*. We successfully expressed CV-N in rice endosperm and tested its HIV-neutralization activity. We regenerated 10 independent events under hygromycin selection and confirmed CV-N expression in three independent events, ultimately choosing one event (line 2) with the highest yield of ^{OS}CV-N (6 mg/l) for more detailed analysis. This yield was comparable to the 10–40 mg/l achieved in *E. coli* (Boyd et al., 1997; Mori et al., 2002, 1998). Boyd et al. (1997) expressed a FLAG-CV-N fusion protein targeted to the periplasmic space, resulting in a yield of less than 10 mg/l. The FLAG tag inhibited the activity of CV-N, and the recombinant protein was a mixture of intact and truncated forms of CV-N. Mori et al. (1998) produced full-length soluble CV-N by directing the protein to the periplasmic space using the *pel*B leader sequence, again achieving a yield of ~10 mg/l. They attempted to improve the yield by adding a C-terminal His_5 tag and expressing Asn30 and/or Pro51 substitution mutants but the maximum yield remained at ~10 mg/l. We did not use any

fusion tags to express recombinant CV-N in rice, although we did use the rice α-amylase 3A signal peptide sequence to direct the protein to the secretory pathway.

Because periplasmic expression in *E. coli* resulted in low yields, others have attempted to express the protein in the cytosol (Colleluori et al., 2005; Gao et al., 2010). Colleluori et al. (2005) expressed CV-N within cytosolic inclusion bodies, increasing the yield to 40 mg/l in shaking flasks and 140 mg/l in high-density fed-batch cultures, which is the highest yield achieved thus far. However, the final product was a mixture of monomeric and dimeric forms including variants with N-terminal deletions. For this reason, Gao et al (2010) used a chaperone-fusion system to express soluble in the *E. coli* cytosol, yielding 3–4 mg CV-N per gram wet cell yielding equivalent to 30% TSP. The purification process was simplified and the product was intact and homogeneous. CV-N production in the yeast *Pichia pastoris* was less successful. A secreted version of the protein with a C-terminal His_5 tag was expressed with a maximum yield of 10 mg/l and N-glycosylation resulted in the formation of a heterogeneous and largely inactive product; however, a P51G mutant showed comparable activity and stability to the wild-type enzyme (Mori et al., 2002). CV-N has also been expressed in tobacco and marshmallow (Sexton et al., 2006; Drake et al., 2013). Sexton et al. (2006) expressed soluble CV-N in tobacco, with a yield of 130 μg/g fresh leaf tissue or 0.85% TSP. The yield achieved by rhizosecretion after 24 days was 0.635 ± 0.298 mg/l and almost all of the CV-N protein was in the monomeric form. In comparison, the yield in *A. officinalis* was 0.02 mg/l/day or 0.5 μg/g root fresh weight/day.

We observed an increase in ^{OS}CV-N yields over successive generations indicating that breeding cycles could be used to improve the yields even further, as previously reported (Clough et al., 2006; Hood et al., 2012; 2007; 2003; Streatfield et al., 2002) demonstrating that germplasms manipulation could lead to increases in recombinant proteins. Clough et al. (2006) backcrossed multiple independent transgenic maize lines with elite inbred lines for several generations demonstrating that the high-level expression of manganese peroxidase (MnP) was maintained. Hood et al. (2003) used a high-oil germplasm to increase germination efficiency and promote the expression of a fungal laccase gene by breeding and selection over several generations. Hood et al. (2012) also used a breeding program to introgress the E1 and CBH I cellulases into elite, high-oil germplasm to enhance protein accumulation in the grain. Streatfield et al. (2002) produced the GM1 receptor binding B subunit of the heat-labile toxin from enterotoxigenic

strains of E. coli in maize and increased the yields again by several generations of breeding and selection. We did not need to carry out backcrossing or selective crossing with elite inbred lines because rice is a self-pollinating crop. Ramessar et al. (2008) by subjecting immature zygotic embryos from one 2G12 highly expressing line to dedifferentiation and subsequent regeneration subjecting immature zygotic embryos from one 2G12 highly expressing line not only boosted protein yields but also eliminated most of the seed-to-seed variation.

Native CV-N has a potent virucidal activity against multiple strains of HIV-1 and HIV-2, with EC_{50} < 10 nM (Xiong et al., 2010). Only two studies have reported a precise anti-HIV activity (EC_{50} or IC_{50}) for CV-N (Gao et al., 2010; Mori et al., 2002). Mori et al. (2002) calculated an EC_{50} value of 0.3 ± 0.09 nM for recombinant CV-N produced in *P. pastoris* and Gao et al. (2010) calculated an IC_{50} value of 31.3 ± 2.15 nM for recombinant CV-N produced in *E. coli*. Other studies have merely stated that the anti-HIV activity of CV-N is concentration dependent, and have emphasized the need to use the correct concentration of recombinant CV-N in the virus neutralization assay. We found that ^{OS}CV-N had a low potency against HIV-1, with an EC_{50} value of 28 μg/ml. Furthermore, ^{OS}CV-N showed little evidence of cytotoxicity. This may reflect the low concentration of ^{OS}CV-N in the crude endosperm extracts.

In addition to these results, three of the above studies showed that gp120 binding was concentration dependent, and only one demonstrated that the affinity of the recombinant CV-N was lower than the native CV-N (Giomarelli et al., 2002). We observed a concentration dependent relationship in gp120-binding efficiency for all the samples except the control, which appeared to be stable through the entire range of serial dilutions. In addition, ^{OS}CV-N showed relatively good binding efficiency compared to the control (CV-N produced in *E.coli*) at the highest concentration (lowest dilution). This might explain the low anti-HIV activity, emphasizing the importance of the correct dilution of the samples. It is therefore important to use an optimized extraction protocol to obtain large quantities of recombinant CV-N in crude extracts. We also tried to purify ^{OS}CV-N using ethanol and ammonium sulfate precipitation but only minimal amounts of the protein were recovered, resulting in no anti-HIV activity in the whole-cell assays. A high-yield crude extraction protocol to separate CV-N from starch and other proteins is therefore necessary.

3.6 CONCLUSIONS

Our data show that CV-N can be produced in rice endosperm albeit at lower yields than has previously been achieved in *E. coli* and *P. pastoris* but at higher yields than previously reported in tobacco and marshmallow. This is the first time that CV-N has been stably expressed in rice endosperm and we succeeded in increasing the expression levels by breeding the transgenic lines over several generations. Crude extracts from the transgenic plants were not cytotoxic towards CEM-SS cells indicating that the production of CV-N in rice seeds is safe. The low neutralization activity and concentration-dependent gp120-binding efficiency probably reflect the low CV-N concentration in the crude extracts. A simple crude extraction method for lectins is therefore essential to increase the recovery and purity of recombinant CV-N.

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CHAPTER 4

Towards the production of microbicide cocktails in rice endosperm

4.1 INTRODUCTION

The number of people living with HIV increased from 29.4 million in 2001 to 35.3 million in 2012 (UNAIDS, 2013). Even though there is no cure for HIV, the disease can be managed with antiretroviral drugs that reduce the rate of viral replication and hence the mortality and morbidity associated with acquired immunodeficiency syndrome (AIDS). However, long-term side effects and particularly the emergence of resistant strains under drug selection pressure is a growing problem that reduces the impact of therapy (Balzarini, 2007; MTN, 2014). For this reason, new strategies are required to prevent the spread of HIV.

HIV is a retrovirus with a high mutation rate, resulting in numerous subtypes and clades (Vigerust and Shepherd, 2007). The glycans on the viral envelope are essential for transmission (Balzarini, 2007). HIV infection is a multi-stage process involving: (i) binding of the viral envelope glycoprotein gp120 to the cellular receptor CD4, (ii) interaction between gp120 and the viral chemokine co-receptor CXCR4 or CCR5, (iii) conversion of gp41 into a fusogenic conformational state, and (iv) transfer of the fusion peptide into the target cell (Figure 4.1) (Permanyer et al., 2010).

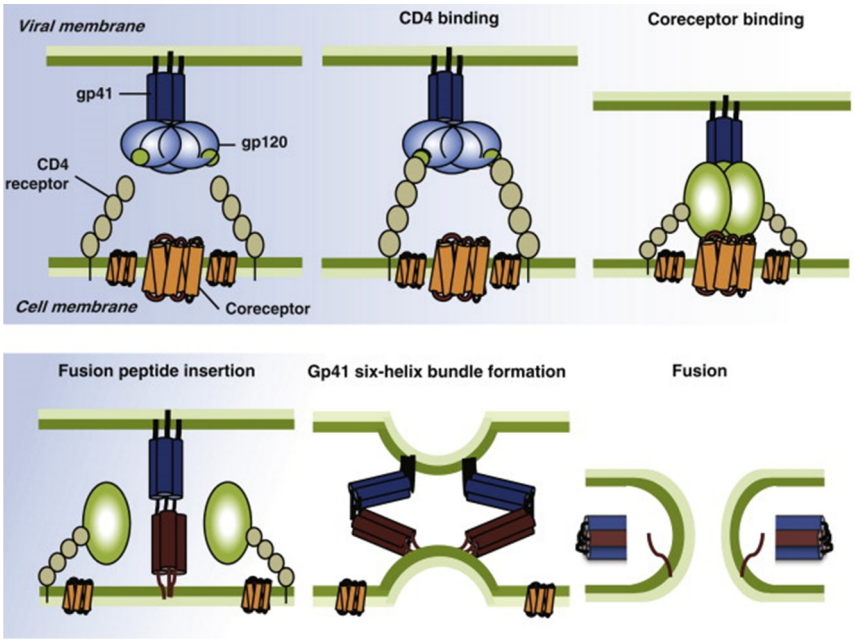

Figure 4.1: The multistep process of HIV infection. The well-defined HIV infection process leading to the delivery of the viral core into the cytoplasm begins when the viral envelope glycoprotein gp120 binds to the cellular receptor CD4 (binding site shown in green). After gp120–CD4 engagement, conformational changes in gp120 trigger its interaction with the viral chemokine co-receptor CXCR4 or CCR5. Thereafter, gp41 is converted into a fusogenic conformational state that triggers the transfer of the fusion peptide into the target cell. Finally, the formation of a six-helix bundle brings the membranes close together and enables fusion (Permanyer et al., 2010).

Carbohydrate-binding agents (CBAs) obstruct viral entry into target cells by interacting with the glycans on the HIV envelope (Huskens and Schols, 2012). The CBAs include proteins that recognize carbohydrate structures (lectins) and non-peptide small agents with affinity for monosaccharides and/or oligosaccharide structures (Balzarini, 2007). HIV gp120 is heavily glycosylated and is presented on the virion surface as trimers of gp120/gp41complexes (Kwong et al., 1998; Leonard et al., 1990; Wyatt et al., 1998). These glycans affect Env conformations and oligomerization, viral entry, infectivity and antibody recognition (Huang et al., 2012; Kumar et al., 2011; McLellan et al., 2011; Scanlan et al., 2002). Specifically, gp120 comprises 30–40% high-mannose glycans (Figure 4.2 a) which are preferably deleted under CBA selection pressure (Figure 4.2 b) (Balzarini, 2007). Thus there is a clear relationship between the number of glycan deletions and resistance to CBAs (Balzarini et al., 2005b). Although HIV mutates almost every time a new copy is made, not every mutation causes resistance. For example, the deletion of individual N-linked glycans would not be sufficient for the virus to escape the antiviral action of lectins, and indeed multiple oligosaccharide deletions are necessary for the virus to evolve resistance (Balzarini et al., 2005a).

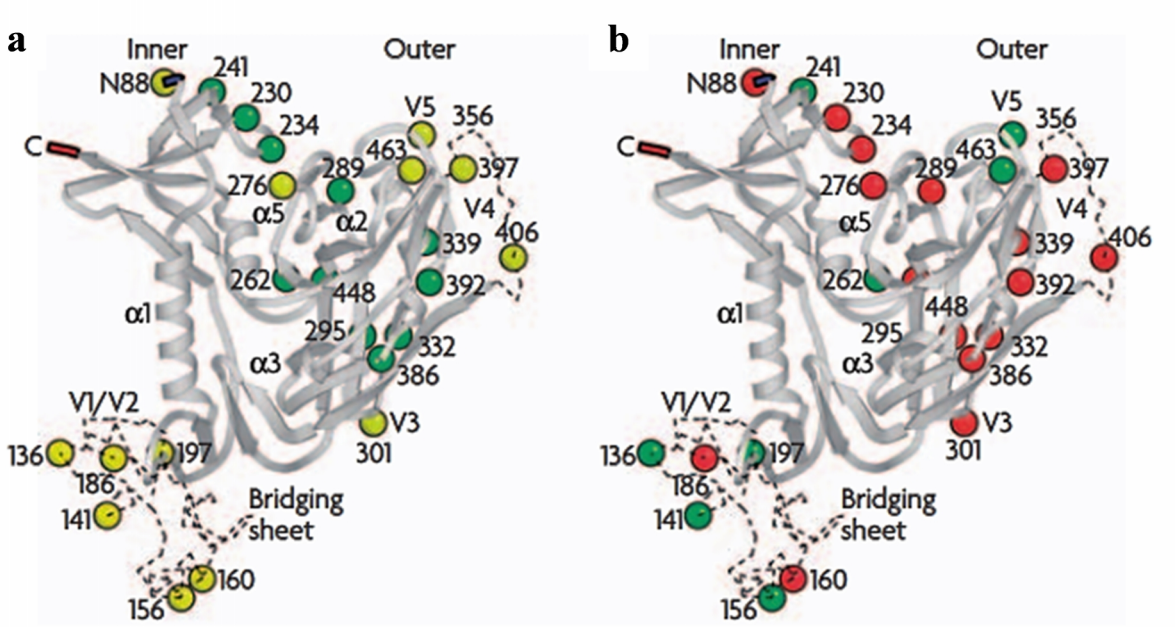

Figure 4.2: The HIV envelope glycoprotein gp120. Ribbon diagrams showing the 24 putative N-glycosylation sites (colored circles) in the HIV-1(III_B) envelope glycoprotein gp120 according to Kwong et al. (1998) and Leonard et al. (1990) a. High-mannose (green) and complex/hybrid-type (yellow) glycans. b. The red circles indicate the deleted N-glycosylation sites that appear under pressure from carbohydrate-binding agents (CBAs) in more than 30 different mutant virus isolates. The green circles represent glycosylation sites that, as yet, have not been deleted under CBA pressure (Balzarini, 2007; Kwong et al., 1998).

One major challenge for the treatment of HIV infections is that the incoming virus may already contain mutations rendering it resistant to antiretroviral drugs. *In vitro* studies have identified HIV-1 escape mutants resistant to polyanionic compounds, which are some of the most promising microbicide candidates (Bobardt et al., 2004; Este et al., 1997). In order to prevent the emergence of resistant HIV-1 isolates, combinations of two or more compounds that target separate viral structures or functions are used in microbicide formulations. Therefore, the development of effective microbicides will likely require a combination of several compounds to prevent the rapid evolution of HIV resistance and to provide sufficient cross-clade protection (Sexton et al., 2009).

Highly active antiretroviral therapy (HAART) involves the administration of combinations of antiviral drugs and is already well-established as a treatment for HIV (Portsmouth and Scott,

2007) because it is much more effective than a monotherapy (Palella et al., 1998). Likewise, combination microbicides are likely to demonstrate greater efficacy than microbicides with only one active component (Garg et al., 2009). The theory behind the combinatorial microbicides is based on three principles. The first is that they increase the range of activity, reducing the likelihood that infections could be initiated by existing resistant viruses or new escape mutants. Secondly, combination microbicides can reduce the amount of each drug needed to achieve the same efficacy if there is synergy between them drugs. Finally, combination microbicides can target not only in the infection process but also in the viral life cycle, expanding the mechanisms of protection. This hypothesis is demonstrated by the prevention of mother-to-child transmission using antiretroviral therapy (Sturt et al., 2010).

There are numerous examples of combination microbicides. Truvada (Gilead Sciences, Inc., Foster City, CA) is a combination of two antiretroviral drugs (the NRTI emtricitabine plus tenofovir disoproxil fumarate) and was approved in 2012 for HIV pre-exposure prophylaxis and is taken daily to reduce the risk of infection (see General Introduction). In addition, the b12-CV-N fusion product combined two HIV-neutralizing components in one protein and accordingly showed a much greater neutralizing efficacy than either protein acting alone (Sexton et al., 2009). The synergistic inhibition of HIV-1 infection by combinations of potential microbicide components has also been demonstrated, e.g. the two leading polyanionic microbicides, dextran sulfate and PRO 2000 (Gantlett et al., 2007). They have been combined with the neutralizing antibody b12 and the cyanobacterial protein CV-N and demonstrated synergistic activity, although the degree of synergy depended on the inhibitor concentration and combination (Pirrone et al., 2011; Ramessar et al., 2010). These examples also include successful combinations of NNRTIs such as efavirenz and nevirapine, which are also used in combination antiretroviral therapy, or the lipophilic molecules UC781 and TMC120, which are being developed as vaginal gels for phase III trials (Herrera et al., 2009). Phase I trial MTN-013/IPM026 is the first clinical trial of a vaginal ring consisting of two antiretroviral drugs: dapivirine and maraviroc (AVAC, 2014).

Vaccine candidates have already been developed in which malaria antigens from different stages of the life cycle of the parasite have been combined into a single fusion protein (Patarroyo et al., 2012) or combined with the cholera toxin B chain as an adjuvant. Indeed, apical membrane

antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) have been expressed as fusions with the cholera toxin B chain in tobacco and lettuce chloroplasts exhibiting dual immunity against cholera and malaria either by oral or injectable delivery (Davoodi-Semiromi et al., 2010). Finally, MAPP66 is a cocktail of several antibodies targeting HIV and *Herpes simplex virus* (HSV), and was produced using the MagnICON (magnifection) system in *N. benthamiana* (Gleba et al., 2005). HIV microbicides work in the first line of defense on the exposed surfaces by blocking the earliest steps of the infection cycle. By using the HIV microbicides, the concentration of the active substance is high but the long-term toxicity in healthy but at-risk individuals is reduced (Nuttall, 2014; Shattock and Rosenberg, 2012).

CBAs such as the lectins CV-N and GRFT, and human HIV-neutralizing monoclonal antibodies such as 2G12, can acts as entry/fusion inhibitors to inhibit the first steps of HIV infection by binding directly to glycans on the envelope proteins or indirectly promoting glycan deletion (Balzarini, 2007). The interactions between several CBAs and gp120 have been investigated, e.g. the prokaryotic lectins CV-N and actinohivin, the plant lectins HHA and UDA, and the monoclonal antibody 2G12. These studies have shown that binding does not prevent the initial contact between HIV and the cell, but blocks the subsequent binding to co-receptors (Balzarini et al., 2007, 1991; Bewley, 2001; Huskens et al., 2007). Although the precise molecular mechanism is unknown, it may reflect the inhibition of gp120 conformational changes that normally occur once the virus has made contact with CD4 on the cell surface (Balzarini et al., 1991).

Several important characteristics need to take into consideration for the development of a combinatorial microbicide. These include: safety, efficacy, cost, acceptability, appropriate drug delivery, long-term effect, potential for resistance and impact on therapy, and prioritization of best-in-class products for clinical trials. For example, phase III clinical trials are not only expensive and time-consuming, but also a large number of participants is necessary in order to demonstrate efficacy (Shattock and Rosenberg, 2012). A production platform combining all the above characteristics with the lowest cost would be highly appropriate for the production of HIV microbicides.

4.2 AIMS OF THE STUDY

The main aim of the work described in this chapter was to produce combination microbicides in rice endosperm tissue by co-expressing the monoclonal antibody 2G12 together with two antiviral lectins (CV-N and GRFT) and testing the cytotoxicity and anti-HIV activity of crude extracts containing these components.

4.3 MATERIALS AND METHODS

4.3.1 Cloning and construct design

Constructs for the expression of 2G12, GRFT and CV-N are described in detail in Chapter 1 section 1.3.1, Chapter 2 section 2.3.1, and Chapter 3 section 3.3.1, respectively.

4.3.2 Transformation and regeneration of transgenic plants

This procedure is described in detail in Chapter 1, section 1.3.2.

4.3.3 Crude extracts from rice endosperm

We crushed 5 g of mature rice seeds into a fine powder using a glass mortar. The total soluble protein (TSP) was extracted in three volumes (v/w) of PBS by vortexing overnight at 4°C. The samples were then centrifuged twice (13,000 x g, 15 min, 4°C) and the supernatant was filtered before transfer to a 50-ml Falcon tube.

4.3.4 Confirmation of protein expression (ELISA)

A pool of mature rice seeds was ground in three volumes of PBS and centrifuged twice (13,000 x g, 10 min, 4°C) to remove seed debris. The expression of 2G12, CV-N and GRFT was confirmed using three separate enzyme-linked immunosorbent assays (ELISAs). Antibody 2G12 was

detected by coating ELISA wells with goat anti-human kappa light chain (Sigma K3502) or goat anti-human IgG Fc chain (Sigma K2136) as capture antibodies. After washing and blocking with 5% nonfat milk, serial dilutions of each seed extract were added and bound 2G12 was detected using a horseradish peroxidase (HRP)-conjugated sheep anti-human kappa-chain antiserum (The Binding Site, Birmingham, UK, 1:1000 dilution). For GRFT and CV-N, the ELISA plates were coated with 100 ng recombinant gp120 from HIV strain IIIB (MRC Centralized Facility for AIDS Reagents, Potters Bar, UK). After washing and blocking with 5% nonfat milk, serial dilutions of each seed extract were added and the lectins were detected primary rabbit anti-GRFT anti-CV-N or polyclonal antisera, followed by the HRP-conjugated anti-rabbit IgG as above. HRP was detected by using 3,3',5,5' tetramethylbenzidine (TMB) as the substrate (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

4.3.5 In vitro binding of crude extracts containing multiple combinations to gp120 (ELISA)

The specific antigen binding activity of 2G12, GRFT and CV-N was confirmed by coating ELISA wells with 100 ng recombinant gp120 from HIV strain IIIB. After washing with PBST and blocking with 5% nonfat milk, serial dilutions of the crude extracts were added and the amount of bound antibody or protein determined using HRP-conjugated sheep anti-human kappa-chain antiserum for 2G12 or HRP-conjugated anti-rabbit IgG (1:1000 dilution) for GRFT and CV-N. The signal was detected using TMB substrate as described above.

4.3.6 Whole-cell anti-HIV bioassays

This procedure is described in detail in Chapter 3, section 3.3.7

4.3.7 Cytotoxicity and antiviral assay

Cytotoxicity and antiviral assays were carried out by Dr. A. Evans at St. Mary's Campus Imperial College, Department of Medicine, London. The cytotoxicity of all samples was

assessed using the MTT colorimetric assay as previously described (Mesquita et al., 2008). Crude extracts of ^{OS}2G12, ^{OS}GRFT, ^{OS}CV-N and combinations of these were titrated in triplicate onto TZM-bl cells (10^5/well), a cervical epithelial cell line expressing CD4, CCR5 and luciferase. Twofold serial dilutions of the extracts were prepared for testing and Nonoxynol-9 was used as a positive control. Results were expressed as the percentage of cellular viability compared to the negative (medium-only) control, plus or minus the standard deviation (SD).

To determine antiviral potency, the same crude extracts were titrated onto TZM-bl cells (10^4/well) in triplicate and cultured for 72 h with HIV-1 BaL using a concentration optimized for infectivity. Twofold serial dilutions of the extracts were prepared for testing. The extent of viral replication was determined by measuring luciferase activity in the cell lysates (Promega, Madison, WI, USA). Results were expressed as the percentage of infection attained relative to the virus-only control, plus or minus the standard error (SE).

4.4 RESULTS

4.4.1 Production of 2G12 + GRFT + CV-N in rice endosperm

Rice (*Oryza sativa* cv. *Nipponbarre*) mature seed-derived embryos were transformed by particle bombardment with a construct containing the coding sequences of the 2G12 heavy and light chains, under the control of the rice glutelin-1 promoter (Ramessar et al., 2008), the GRFT coding sequence under the control of the maize zein promoter, the CV-N coding sequence, under the control of the rice prolamin promoter, plus a fifth construct containing the selectable marker *hpt*. Embryo-derived callus was selected on hygromycin-supplemented medium and multiple independent transformants were regenerated and transferred to the greenhouse. Endosperm tissue was analyzed by ELISA to confirm transgenes expression.

4.4.2 Confirmation of combinatorial expression and assembly in rice endosperm

The presence of correctly-assembled 2G12, GRFT and CV-N in the T1 endosperm was confirmed using an immunoglobulin-specific sandwich ELISA. The concentration of each protein in the endosperm was determined by calculating the concentrations of different lines expressing 2G12, GRFT and CV-N from titration curves based on positive controls spiked with known concentrations of the same proteins produced in rice endosperm or *E. coli* (Figure 4.3). We used non-transformed rice endosperm as a negative control to rule out cross reaction with endogenous plant proteins. Among the nine independent events, lines 1', 2', 3' and 4' were found to co-express CV-N and GRFT. Line 1' expressed CV-N (0.15 µg/ml) at higher levels than GRFT (0.09 µg/ml) and line 2' expressed GRFT (8.73 µg/ml) at higher levels than CV-N (0.14 µg/ml). Lines 5', 6' and 7' co-expressed 2G12 and CV-N, whereas line 8' co-expressed 2G12 (1.43 µg/ml) and GRFT (0.12 µg/ml). Finally, line 9' co-expressed all three proteins: 2G12 (0.61 µg/ml), GRFT (2.28 µg/ml) and CV-N (0.04 µg/ml). These lines were selected for more detailed studies.

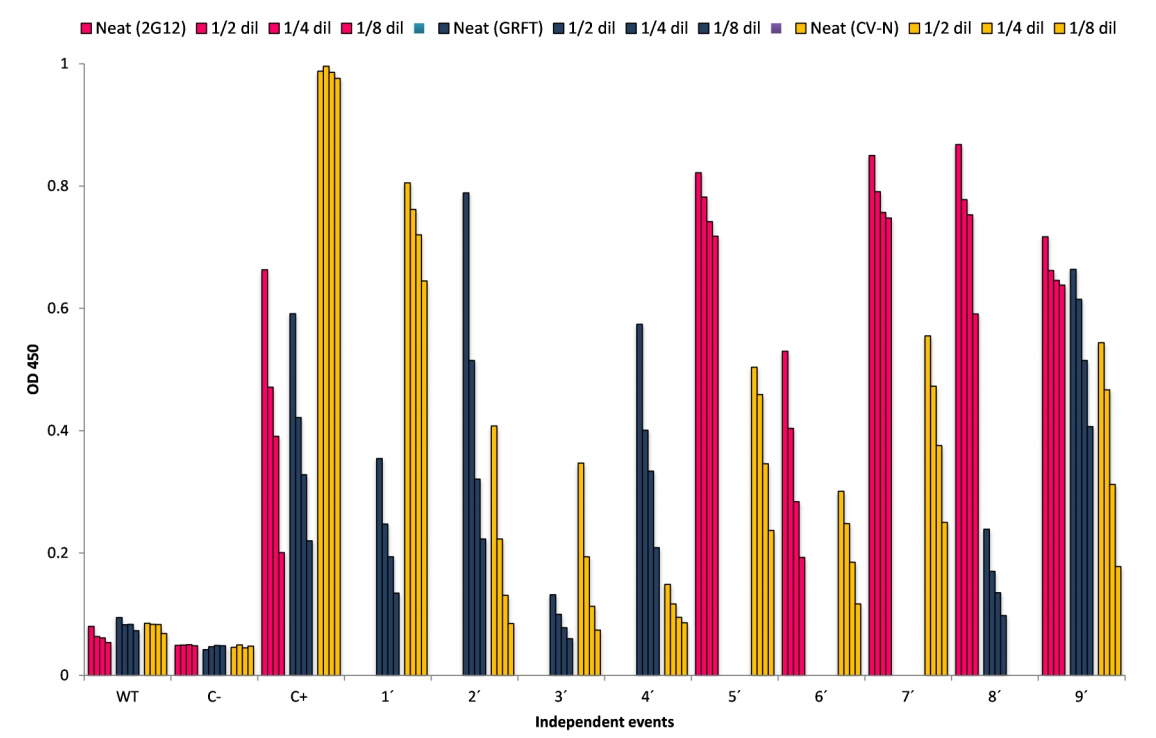

Figure 4.3: The accumulation of 2G12, GRFT and CV-N in mature rice endosperm. An immunoglobulin-specific sandwich ELISA was used to screen all independent events. The ELISA plate was coated with gp120, and 2G12 antibody assembly was confirmed using a primary goat anti-human kappa light chain or goat anti-human IgG Fc chain and a secondary HRP-conjugated sheep anti-human kappa-chain antiserum. CV-N and GRFT protein assembly was confirmed using a primary rabbit anti-CV-N and anti-GRFT polyclonal antiserum and a secondary HRP-conjugated anti-rabbit IgG for both. Four dilutions per sample are shown. Non-expressed transgenes are not shown. WT = wild-type plants; C^- = PBS negative control; C^+ = 2G12 (pink) purified from rice (500 ng/ml), GRFT (blue) and CV-N (yellow) purified from *E. coli* (concentration 500 ng/ml) as positive controls.

4.4.3 HIV-binding activity of the crude extracts

The ability of the endosperm-derived 2G12, GRFT and CV-N in to bind gp120 in combination was tested *in vitro* using an antigen-specific ELISA. The capture reagent was gp120 IIIB and the bound proteins were detected using primary goat anti-human kappa light chain or goat anti-human IgG Fc chain for 2G12, and a primary rabbit anti-GRFT or anti-CV-N polyclonal antiserum for the lectins. A secondary HRP-conjugated sheep anti-human kappa-chain antiserum was used to detect 2G12 and a secondary HRP-conjugated anti-rabbit IgG antiserum was used to detect GRFT and CV-N. In all the combinations, the wild-type extract showed no binding to gp120, confirming the absence of a cross reaction with endogenous plant proteins. We obtained three groups of extracts expressing different combinations of 2G12, GRFT and CV-N.

- *Fist group: Lectin combination*

The first group comprised four different events expressing GRFT and CV-N at different relevant levels. Line 1' showed moderate binding kinetics for GRFT (midway between the positive and negative controls) but the highest binding kinetics for CV-N. Lines 2' and 4' showed similar GRFT binding kinetics as the positive control but low binding activity for CV-N (Figure 4.4). Line 3' showed moderate binding kinetics for CV-N but the lowest binding kinetics for GRFT.

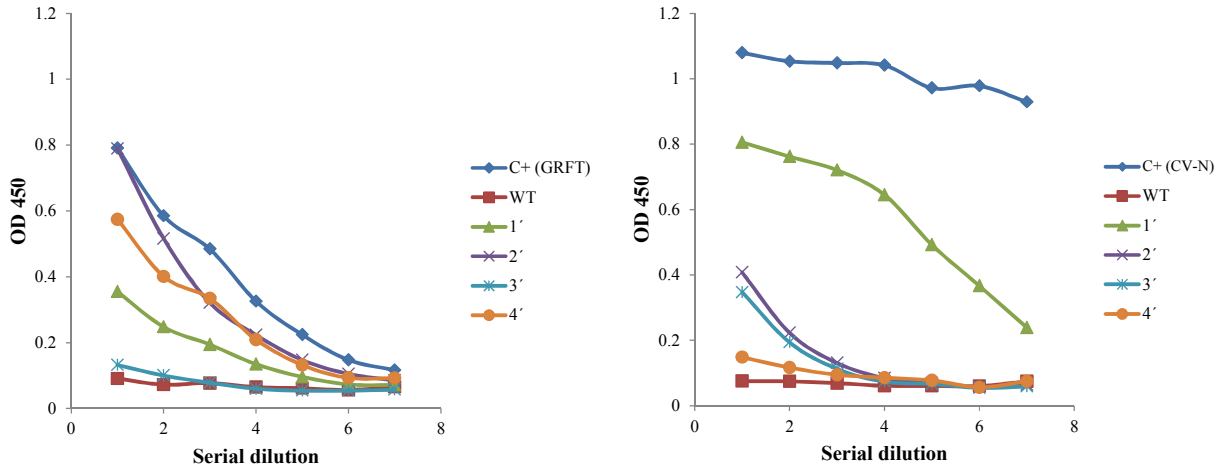

Figure 4.4: Antigen-binding activity (ELISA) of crude rice endosperm extracts containing GRFT and CV-N and the purified GRFT and CV-N produced in *E. coli*. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. ELISA plates were coated with the gp120 and were tested with different concentrations of crude extracts or fixed concentration of purified GRFT and CV-N. Binding activity was measured with primary rabbit anti-GRFT and anti-CV-N polyclonal antisera and a secondary HRP-conjugated anti-rabbit IgG. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased in all the samples except the positive control for CV-N. WT = wild type plants. C^+ = GRFT and CV-N produced in *E. coli* (concentration 500 ng/ml) as positive controls. Lines 1', 2', 3' and 4' are independent events expressing GRFT and CV-N.

- *Second group: Antibody/lectin combinations (2G12 + CV-N or 2G12 + GRFT)*

The second group consisted of two subgroups, combining (i) 2G12 and CV-N, or (ii) 2G12 and GRFT. The first subgroup comprised three independent events. Lines 5 and 7 showed the best binding kinetics in both ELISAs, with higher binding kinetics for 2G12 than the positive control and the highest binding kinetics for CV-N, although still at half the value of the positive control at the highest concentration (Figure 4.5). The second subgroup comprised one independent event (line 8) coexpressing 2G12 and GRFT, with higher binding kinetics than the 2G12 positive control but lower binding kinetics than the GRFT positive control (Figure 4.6).

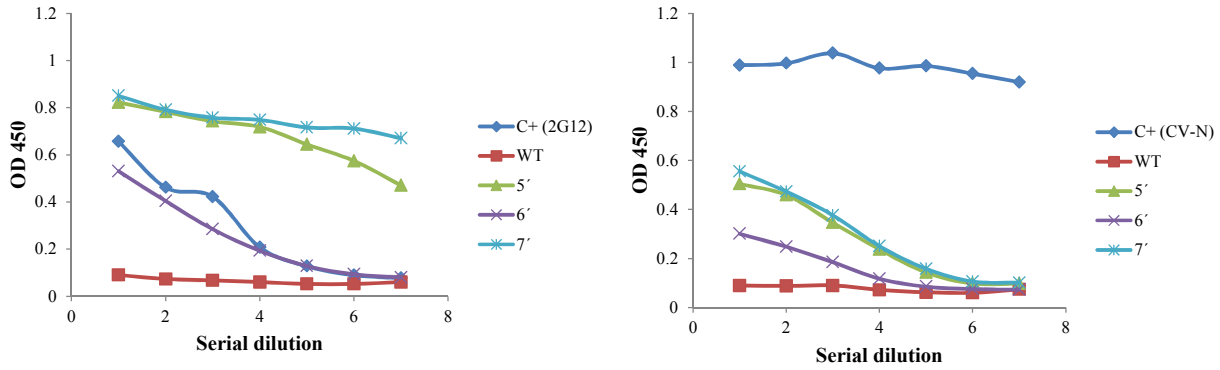

Figure 4.5: Antigen-binding activity (ELISA) of crude rice endosperm extracts containing 2G12 and CV-N and the purified 2G12 produced in maize and CV-N produced in *E.coli*. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. ELISA plates were coated with the gp120 and were tested with different concentrations of crude extracts or fixed concentration of purified 2G12 and CV-N. Binding activity was measured with a primary goat anti-human kappa light chain or goat anti-human IgG Fc chain for 2G12 and a primary rabbit anti-CV-N polyclonal antiserum for CV-N, followed by a secondary HRP-conjugated sheep anti-human kappa-chain antiserum for 2G12 or a secondary HRP-conjugated anti-rabbit IgG for CV-N. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased. WT = wild type plants. C^+ = 2G12 produced in maize and CV-N produced in *E. coli* (concentration of 500 ng/ml) as positive controls. Lines 5', 6' and 7 ' are independent events expressing 2G12 and CV-N.

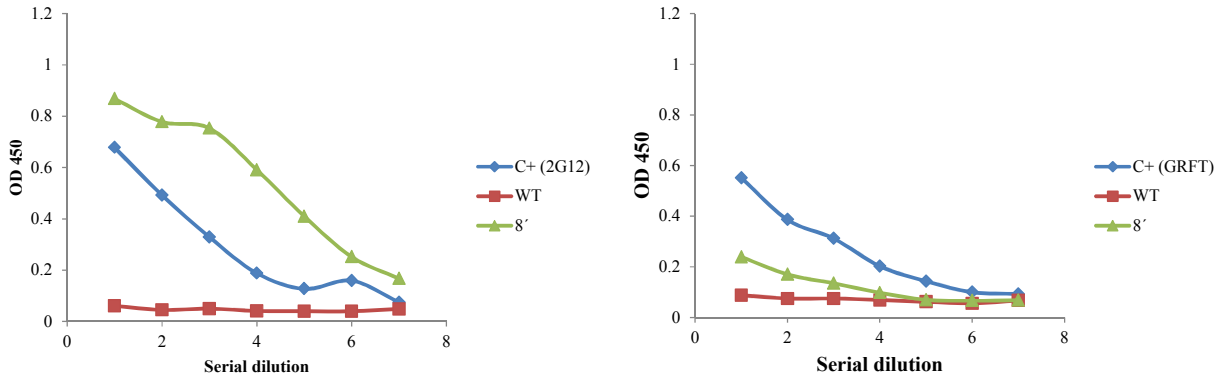

Figure 4.6: Antigen-binding activity (ELISA) of crude rice endosperm extracts containing 2G12 and GRFT and the purified 2G12 produced in maize and GRFT produced in *E. coli*. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. ELISA plates were coated with the gp120 and were tested with different concentrations of crude extracts or fixed concentration of purified 2G12 and GRFT. Binding activity was measured with a primary goat anti-human kappa light chain or goat anti-human IgG Fc chain for 2G12 and a primary rabbit anti-GRFT polyclonal antiserum for GRFT, followed by a secondary HRP-conjugated sheep

anti-human kappa-chain antiserum for 2G12 or a secondary HRP-conjugated anti-rabbit IgG for GRFT. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased. WT = wild type plants. C^+ = 2G12 produced in maize and GRFT produced in *E. coli* (concentration of 500 ng/ml) as positive controls. Line 8' is an independent event expressing 2G12 and GRFT.

- *Third group: 2G12+GRFT+CV-N combination*

The third group comprised one event (line 9') expressing all three recombinant proteins. This showed higher binding kinetics than the 2G12 and GRFT positive controls and relatively low binding kinetics compared to the CV-N positive control (Figure 4.7).

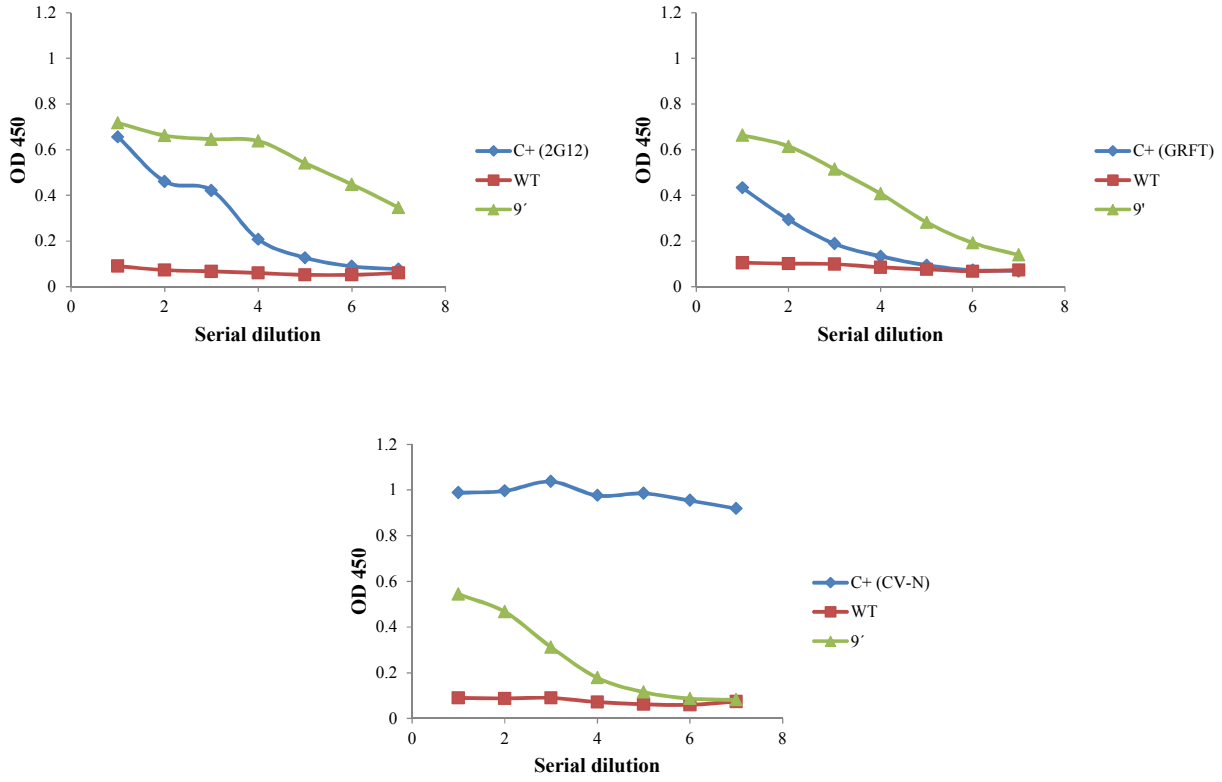

Figure 4.7: Antigen-binding activity (ELISA) of crude rice endosperm extracts containing 2G12, CV-N and GRFT and the purified 2G12 produced in maize and GRFT and CV-N produced in *E. coli*. Ordinate: Optical density at 450 nm. Abscissa: Serial dilutions. Values are the average of two experiments. ELISA plates were coated with the gp120 and were tested with different concentrations of crude extracts or fixed concentration of purified 2G12, CV-N and GRFT. Binding activity was measured with a primary goat anti-human kappa light chain or goat anti-human IgG Fc

chain for 2G12 and primary rabbit anti-GRFT and anti-CV-N polyclonal antisera for GRFT and CV-N, followed by a secondary HRP-conjugated sheep anti-human kappa-chain antiserum for 2G12 or a secondary HRP-conjugated anti-rabbit IgG for GRFT and CV-N. The binding kinetics showed a concentration-dependent relationship, declining as the dilution factor increased. WT = wild type plants. C^+ = 2G12 produced in maize and GRFT/CV-N produced in *E. coli* (concentration of 500 ng/ml) as positive controls. Line 9' is an independent event expressing 2G12, CV-N and GRFT.

4.4.4 Anti-HIV activity bioassays

The crude extract from line 2' expressing CV-N and GRFT was tested for its ability to inhibit the cytopathic effects of HIV-1_{RF} against T-lymphoblastic CEM-SS cells. Wild-type rice was used as a negative control to exclude antiviral effects caused by endogenous plant proteins. Previously, native GRFT and GRFT produced in *N. benthamiana* showed remarkably potent activity against HIV-1, with EC_{50} values of 0.054 and 0.156 nM, respectively (O'Keefe et al., 2009). In addition, CV-N displays potent virucidal activity against diverse primary isolates and laboratory strains of HIV-1 and HIV-2, with EC_{50} values generally in the 1–10 nM range (Boyd et al., 1997). In our experiments, the crude extract at a concentration 50 μg/ml showed potent activity against HIV-1, with an EC_{50} value of 3.15 μg/ml. It was not possible to determine an IC_{50} value, confirming that no toxic constituents had co-purified with the protein. Wild-type crude extract showed no activity confirming that endogenous plant lectins had no effect on the virus (Figure 4.8).

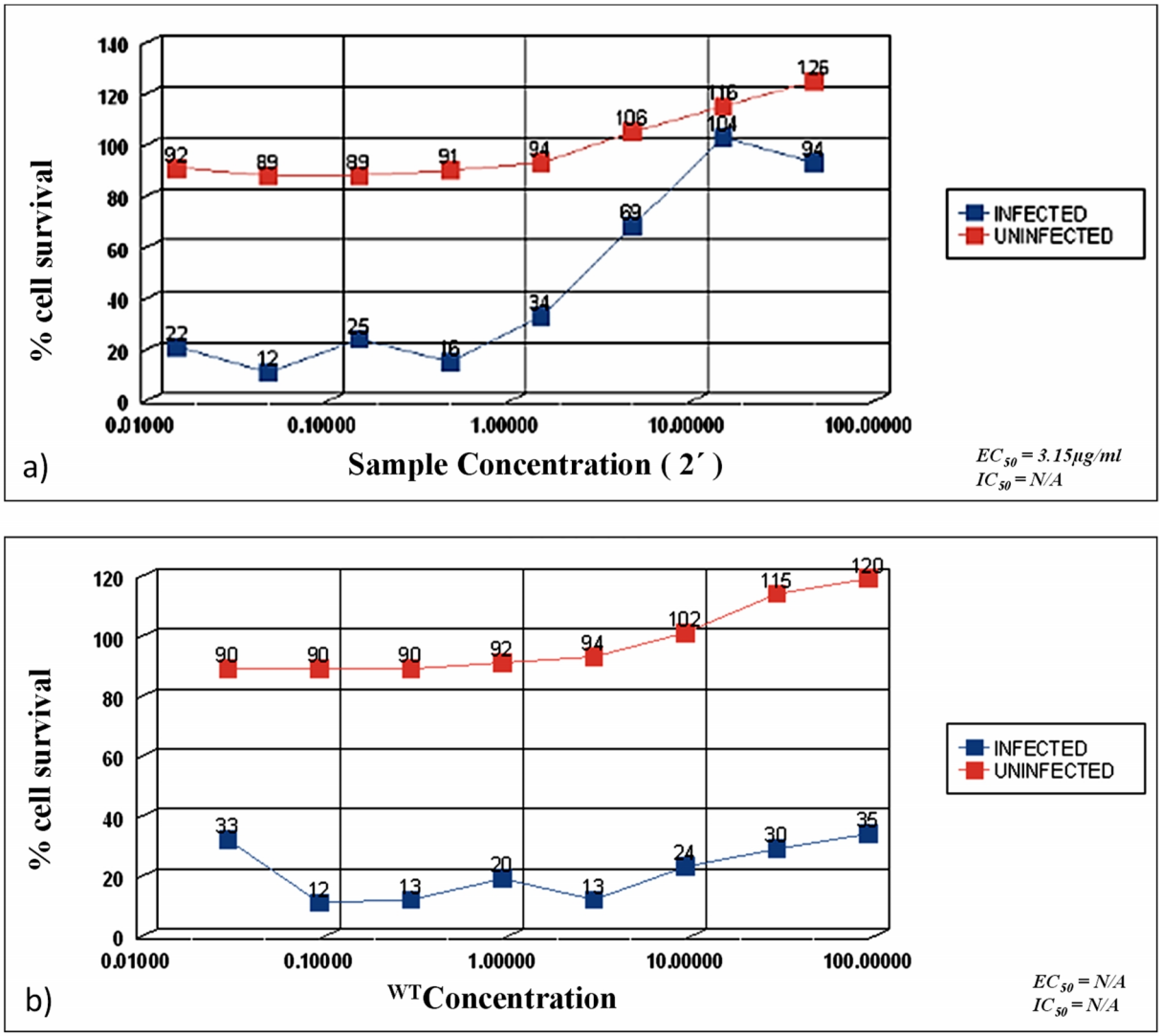

Figure 4.8: *In vitro* anti-HIV activity of crude rice extracts. The anti-HIV activity of (a) line 2' and (b) wild-type rice in CEM-SS cells infected with HIV-1_{RF}. Concentration-dependent effects of CV-N on cellular viability were observed. Cellular viability data are shown relative to uninfected, untreated controls. Cell viability was assessed using the XTT assay. All points are averages (± SD) of experiments carried out in triplicate.

4.4.5 Cytotoxicity and antiviral assay

An MTT assay (Mesquita et al., 2008) was used to determine the potential cytotoxicity of crude extracts from wild-type rice endosperm and lines expressing 2G12, GRFT, CV-N, 2G12 + GRFT, 2G12 + CV-N, and two lines (1' and 2') expressing CV-N + GRFT against TZM-bl cells, which were used to study the effects of these crude extracts on HIV-1 infection. As shown in

Figure 4.9, all the crude extracts at dilutions 1/4 and higher exhibited no significant cytotoxicity against the cell line. The same samples were used for an infectivity assay.

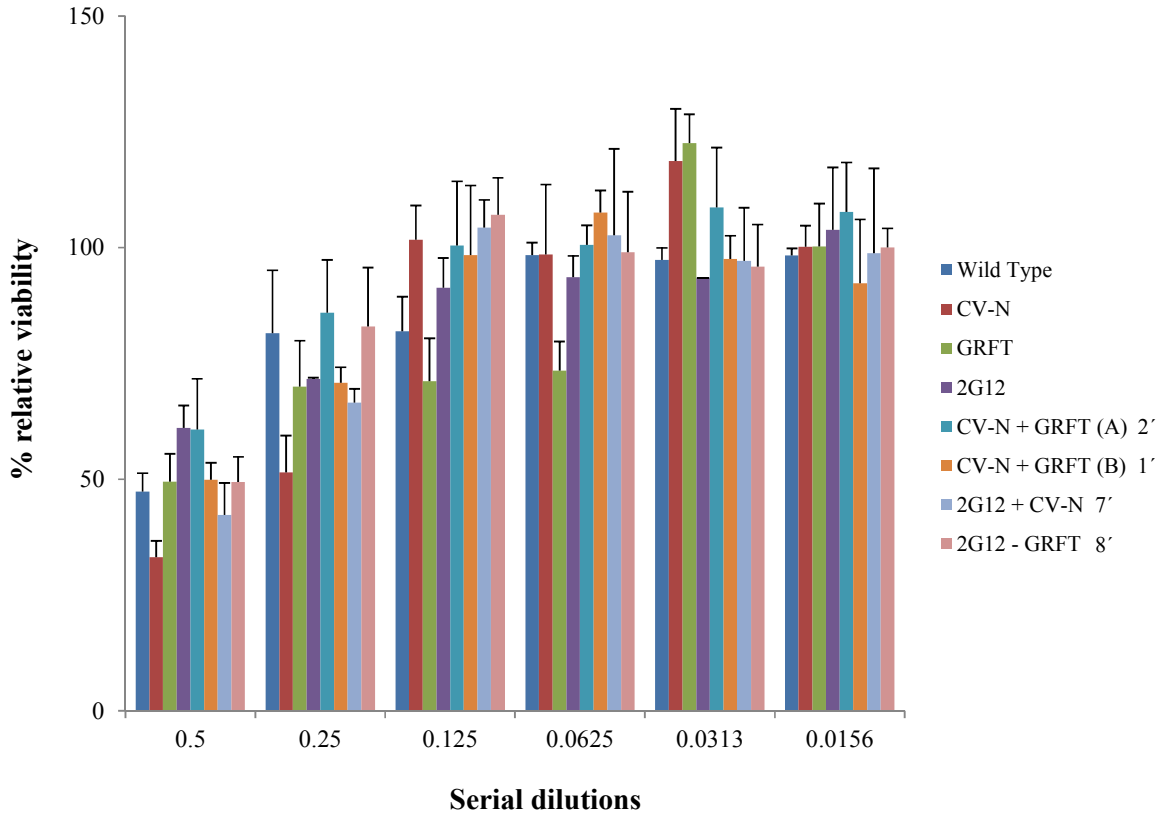

Figure 4.9: Effect of different concentrations of numerous crude extracts on cell viability. Cytotoxicity was determined using an MTT assay and was expressed as the percentage of dead cells (mean ± SD, n = 3). Ordinate: % of relative viability. Abscissa: Serial dilutions. Nonoxynol-9 was used as a cytotoxic positive control.

The same crude extracts were tested for their effect on HIV infectivity. To avoid the cytotoxicity of crude extracts, the neat extract and the half-strength dilution were not tested. Figure 4.10 shows the proportion of infection achieved relative to the virus-only control (no extract). A value of 0% indicates that the extracts have completely inhibited viral replication and 100% means that the extracts have no antiviral effect. We observed potent antiviral activity in the extracts containing GRFT and to a lesser extent CV-N. The extracts containing 2G12 showed a generally low antiviral activity that was only observed at the highest concentration (lowest dilution). The

wild-type extract showed a low level of viral inhibition, maybe by preventing the virus from contacting the cells. The extract containing 2G12+CV-N showed some antiviral activity at the 1/8 and 1/16 dilution (0.0625 and 0.03125). However the 1/4 and 1/32 dilutions showed no antiviral activity. All the extracts containing GRFT alone or in a combination with CV-N or 2G12 showed high antiviral activity, probably reflecting the presence of GRFT (Figure 4.10).

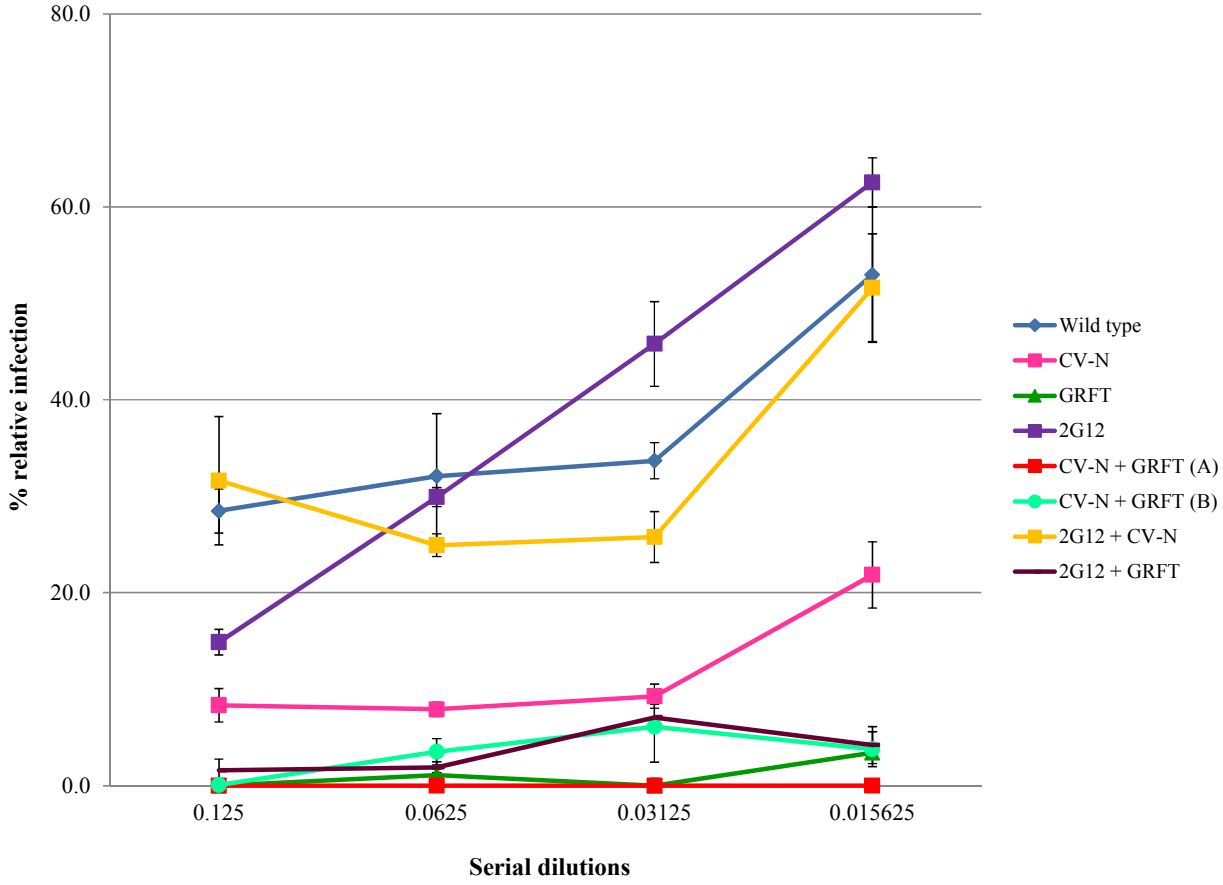

Figure 4.10: HIV-1 infectivity assay. TZM-bl cells were incubated with serial dilutions (in triplicate) and cultured for 72 h with HIV-1BaL using a concentration optimized for infectivity. The extent of viral replication was determined by measuring the luciferase activity of cell lysates. Ordinate: percentage of relative infection. Abscissa: Serial dilutions. Results are shown as the percentage of infection relative to the virus-only control, plus or minus the standard error (SE).

4.5 DISCUSSION

Topical microbicides are considered to be one of the most promising approaches for the prevention of HIV. An effective microbicide should have multiple advantages, such as a potent and preferentially broad-spectrum microbicidal activity, limited to no toxicity on the vaginal or rectal epithelial cell layer, and a beneficial pharmacokinetic/dynamic profile (Doncel and Clark, 2010). For this reason, the development of effective microbicides will likely require a combination of several compounds to prevent the rapid evolution of HIV resistance and to provide sufficient cross-clade protection (Sexton et al., 2009). Therefore the feasibility of production is a significant concern. Because the HIV envelope protein gp120 is highly glycosylated and N-linked glycans account for ~50% of its molecular mass, carbohydrate-binging agents such as human monoclonal antibody 2G12, and the lectins CV-N and GRFT could be used as potent HIV microbicidal agents with a high genetic barrier to resistance (Férir et al., 2012).

The design of a suitable combinatorial microbicide must take into account any potential competition for binding sites on the HIV envelope. Although GRFT and CV-N do not compete for these sites, they both compete with 2G12 (Mori et al., 2005) such that lectin-resistant HIV strains are often susceptible to the antibody. We have previously expressed 2G12, CV-N and GRFT independently in rice endosperm (Chapters 1, 2, and 3) and in this chapter we combined them to identify lead events expressing different combinations of 2G12, GRFT and CV-N, allowing us to compare their HIV-neutralizing activities.

No combination microbicides have yet been produced in plants, but several CV-N fusion proteins have been investigated (McFadden et al., 2007; Sexton et al., 2009). In one case, the antibody b12 was fused with the lectin CV-N, and the fusion protein was more potent than its components (Sexton et al., 2009). In another case, CV-N was fused with the linear peptide 12pi and this also showed enhanced anti-HIV activity (McFadden et al., 2007). We used a mixture of plasmids containing the coding sequences for each protein so that each could be stably expressed in rice endosperm. Both the gp120 binding efficiency and anti-HIV whole-cell assays demonstrated that crude endosperm extracts producing combinations of anti-HIV proteins were promising as combinatorial microbicides. The candidate microbicides need to be tested for efficacy and safety in order to be accepted and finally enter the market, using *in vitro* cellular

models, *ex vivo* mucosal tissue explants, small animal models and non-human primates. There are several cell lines that are widely used for efficacy tests, including PM-1 CDA+T cells (Lusso et al., 1995) and the TZM-bl luciferase reporter cell line (Wei et al., 2002). TZM-bl cells provide a single viral cycle assay model that requires 2 days of culture, comparted to at least 7 days for PM-1 cells, before infectivity is evaluated by the measurement of luciferase activity driven by the expression of the viral trans-activator of transcription (Tat) (Nuttall, 2014). In terms of safety, cellular models are used to measure the potential cytotoxicity of microbicides, often by measuring the cleavage of a tetrazolium salt (MTT) into a blue-colored product (formazan) in viable cells (Slater et al., 1963).

We found that crude rice endosperm extracts at dilutions of 1/4 and higher exhibited no significant cytotoxicity to the cell line, suggesting that the enhanced HIV-1 infection did not result from the cytotoxicity of the rice crude extracts at the concentrations we tested. Thus the lower viability that was measured in this assay in the more concentrated dilutions is possible to be due to the fact that the contents of the extracts are covering the cells so thoroughly that they cannot access the nutrients in the culture media and are not growing properly.

The infectivity assay confirmed the potent activity of the combination of CV-N + GRFT against HIV-1, particularly line 2', where GRFT is more abundant than CV-N. The crude extract containing 2G12 showed satisfactory antiviral activity but only at the highest concentration. The combination of 2G12 + CV-N in the extract from line 7' at 1/4 dilution showed antiviral activity similar to the wild-type extract, but in both consecutive dilutions the activity was more potent. The CV-N crude extract was active against HIV-1 throughout the dilution series. This result combined with the anti-HIV assay described in chapter 3 confirms that CV-N has antiviral activity when expressed in rice. Finally, in the combination of 2G12 + GRFT (line 8'), the antiviral activity was most probably due to the GRFT and not 2G12, even though the concentration of 2G12 was higher. It is possible that these components demonstrate synergy, but the exact concentration of each protein in the crude extracts is unknown so we would need to perform a much wider titration to establish a dose response curve and calculate the IC_{50} values in order to compare the combination extracts with the extract components individually. Even then it would be difficult to say whether we have synergy or additive effects. Nevertheless, we can

conclude that we have achieved our goals and demonstrated that combinatorial microbicides can be produced in rice endosperm.

4.6 CONCLUSIONS

The data from our experiments show that multiple anti-HIV microbicides can be successfully produced in rice endosperm with potent anti-HIV activity. These results open the way for the production of more combinatorial microbicides in rice endosperm, which is a suitable low-cost production platform for molecular pharming applications.

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GENERAL CONCLUSIONS

1. A recombinant HIV-neutralizing antibody (2G12) was produced in transgenic rice endosperm with a maximum yield of 42 μg/g, which is similar to the yields previously achieved in transgenic maize. The most prevalent form of the antibody was aglycosylated with the remainder featuring complex vacuolar-type and oligomannose-type glycans as well as single GlcNAc residues, suggesting that the protein was translated in a region of the ER where the addition of the N-glycan does not occur.
2. The aglycosylated antibody exhibited a greater HIV-neutralizing activity than the glycosylated version produced in tobacco seeds, although neither were as potent as 2G12 produced in CHO cells.
3. Proteomics analysis revealed that starch metabolism was suppressed in the transgenic seeds and a prolamin gene (*PRO7*) was upregulated, indicating that transgene expression caused some disruption in the balance between carbohydrate and protein storage in the seed. The metabolic disruption and unusual subcellular architecture of the cell appears to cause stress within the protein trafficking networks, perhaps due to the unusually high level of traffic that needs to be processed, resulting in the further induction of genes encoding the radical scavenger glutaredoxin-C8. These data suggest that yields of proteins could be improved in rice seeds by regulating protein/carbohydrate metabolism and protecting the endosperm from stress.
4. GRFT was expressed in rice endosperm with yields comparable to or even higher than GRFT produced transiently in tobacco and other recombinant proteins produced in rice. Our yield of GRFT (223 μg/g) was the highest among the three recombinant proteins under investigation. A one-step purification protocol using the His_6 tag achieved ~80% purity. Recombinant GRFT produced in rice showed potent anti-HIV activity in a cell-based neutralization assay.
5. CV-N was produced in rice endosperm at lower yields than previously reported in *E. coli* and *P. pastoris* but with higher yields than tobacco and marshmallow. Our yield of CV-N (6 μg/g) was the lowest among the three recombinant proteins under investigation. Recombinant CV-N showed moderate anti-HIV activity in a cell-based neutralization assay.
6. The binding activity of each protein (2G12, GRFT and CV-N) was concentration dependent.

7. Whole-cell neutralization assays confirmed that the combination of CV-N and GRFT produced in the same plant showed potent anti-HIV activity and may therefore constitute a promising combination microbicide cocktail.
8. Overall, rice endosperm was shown to be an effective production platform for combination microbicides.

ANNEX