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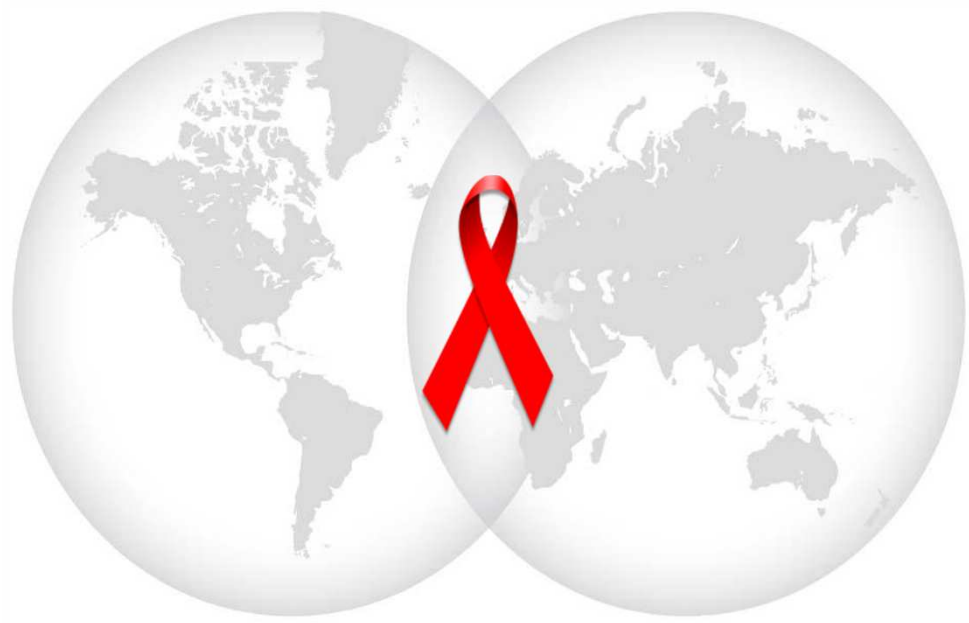
Doctoral dissertation

January 2013



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by

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Applied Plant Biotechnology

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January 2013

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DEDICATION

To my parents,
without their support and efforts this
work would not have been possible

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SUMMARY

HIV remains one of the world's most serious health and development challenges. Although the annual number of new HIV infections declined slightly since the early 2000s, the total number of people living with HIV continues to rise because of the combined effect of new HIV infections and the impact of antiretroviral therapy. Preventing HIV infection is one of the strategies that could slow down the spread of the virus. Microbicides are a new class of products that could address this need. They are low-cost formulations of anti-HIV agents that can be applied topically inside the vagina or rectum to prevent infection with HIV and other sexually transmitted infections. Many potential microbicide candidates are currently being tested. The most advanced clinical trials involve microbicides that include antiretroviral drugs. In addition, protein based microbicides such as human neutralizing monoclonal antibodies and anti-viral lectins are also being considered. However, the feasibility of manufacturing, scalability, and cost are some of the challenges that can undermine the development of cost effective microbicides. Many production platforms are under evaluation for microbicide compounds. One of the most promising such systems is plants because of cost benefits and biological safety. Production of medically important recombinant proteins in plants is known as molecular pharming. Amongst various plant production systems, maize seeds are likely to be an attractive platform for deployment, particularly in developing countries. The focus of this thesis is the production of microbicide components individually or in combination in maize seeds in order to develop a novel and inexpensive strategy for the prevention of HIV infections in the developing world. One such microbicide component is the human neutralizing monoclonal antibody, 2F5. I investigated the production of this antibody in maize seeds. Results showed that the maize-derived antibody was correctly assembled and functional. However, the accumulation levels were lower than previously achieved with another HIV-neutralizing mAb (2G12), suggesting that a combination of factors may limit the accumulation of some antibodies on a case-by-case basis.

Combination microbicides offer more advantages than using only one compound. Therefore a second HIV neutralizing antibody (4E10) in combination with two anti-viral lectins (cyanovirin-N and griffithsin) was contemplated. Due to technical problems during plant transformation and *in vitro* regeneration, no transgenic plants were

regenerated from these particular experiments, consequently new transformation experiments need to be performed.

Recombinant protein synthesis, transport, and storage in plants are critical factors that affect recombinant protein quality in terms of protein folding and posttranslational modification and accumulation levels. Therefore, a lead event accumulating high levels of the HIV neutralizing antibody 2G12, was used to investigate deposition and storage in maize seeds. Results demonstrate that the antibody accumulates in protein bodies as expected. Surprisingly the antibody was found to interact with the protein bodies resulting in amorphous zein bodies. Further experiments will be initiated to determine whether these interactions influence the expression levels and functionality of the antibody.

Regulatory issues relating to genetically engineered crops constitute bottlenecks to their rapid development and commercialization globally. I discuss non-technical barriers to the adoption of GE crops in the European Union and the serious consequences such barriers have not only in industrialized countries but also in the developing world.

RESUM

La infecció pel virus de la immunodeficiència humana (VIH), és un dels problemes sanitaris i de desenvolupament més greus del món actual. Encara que el nombre de noves infeccions ha disminuït lleugerament des de principis de 2000, el nombre total de persones que viuen amb VIH continua augmentant com a conseqüència d'un efecte combinat de noves infeccions i del allargement de la vida dels pacients degut a les teràpies anti-retrovirals. Evitar la infecció és una de les estratègies que podria ajudar a reduir l'expansió del virus. Els microbicides són una nova classe de fàrmacs que podrien ajudar a realitzar-ho. Tenen activitat anti-VIH, es poden produir a baix cost, i es poden incloure en varies formulacions per una aplicació tòpica dins la vagina o al recte. Actualment, molts candidats a microbicides es troben en diverses etapes d'investigació. Els assajos clínics més avançats són de microbicides antiretrovirals. A més a més, també estan sent considerats com a microbicides aquells que estan basats en proteïnes com anticossos monoclonals humans neutralitzant o lectines que presenten activitat antiviral. Alguns dels reptes a superar que podrien frenar el desenvolupament de microbicides efectius son: que les molècules resultants siguin efectives, que la producció es pugui ampliar ràpidament i que els costos de producció puguin ser reduïts. Estan sent avaluades moltes plataformes per la producció de molècules microbicides. Un dels sistemes de producció més prometedors son les plantes, al ser un sistema econòmic i segur. La producció de proteïnes recombinants farmacèutiques en plantes s'anomena "molecular pharming" y les llavors de cereals són probablement les més adequades per estendre'n la producció particularment a països en vies de desenvolupament. El principal objectiu d'aquesta tesis és la producció, individual o combinada, de molècules microbicides en llavors de blat de moro per tal de desenvolupar una nova estratègia de producció mes econòmica aplicable als països en vies de desenvolupament. L'anticòs monoclonal humà neutralitzant, 2F5, és una d'aquestes molècules microbicides que he investigat per la seva producció en llavors de blat de moro. Els resultats han demostrar que l'anticòs 2F5, produït en llavors de blat de moro, es funcional gracies a la correcta unió de les seves unitats en aquest sistema. No obstant això, els seus nivells d'acumulació en les llavors de blat de moro van esser més baixos que els aconseguits anteriorment amb un altre anticòs monoclonal humà neutralitzant (2G12). Aquest resultat ens suggerir que factors aliens al proces, podien influenciar l'acumulació de certs anticossos en l'endosperma de la llavor.

La combinació de diferents microbicides ofereix més avantatges que la utilització d'un de sol. Per tant, la producció d'un segon anticòs monoclonal humà neutralitzant (4E10) en combinació amb dues lectines que presenten activitat antiviral (cyanovirin-N y griffithsin) ha estat un altre objectiu d'aquesta tesis. A causa de problemes tècnics, durant la transformació i la regeneració de plantes *in vitro*, cap planta de blat de moro transgènica va ser regenerada, per tant, aquests experiments han de ser repetits.

La síntesi de proteïnes, el transport i l'emmagatzematge són factors crítics que afecten la qualitat de la proteïna recombinant produïda en plantes. Poden influenciar-ne el plegament, les modificacions postraduccionals i els nivells d'acumulació. Una línea transgènica estable que acumulà elevats nivells d'un anticòs monoclonal humà neutralitzant, 2G12, va ser utilitzada com a model per investigar la producció i l'emmagatzematge de anticòs en les llavors de blat de moro. Els resultats van demostrar que l'anticòs s'acumulà en els cossos proteics de les llavors de blat de moro (zeïnes) com era d'esperar. Sorprenentment també es va trobar l'anticòs interactuant amb les zeïnes i com a conseqüència es produir una deformació dels cossos proteics. Pròximament s'iniciaran experiments addicionals per determinar si aquestes interaccions entre l'anticòs i els cossos proteics influeixen en els nivells d'expressió i en la funcionalitat de l'anticòs.

La bioseguretat i els temes relacionats amb la legislació relativa a cultius millorats genèticament també són una part important d'aquesta tesi, ja que frenen el desenvolupament i la comercialització global d'aquest tipus de cultius. L'estudi normatiu i bibliogràfic que he fet durant el transcurs d'aquesta tesi ha proporcionat una visió àmplia de les barreres no tècniques que s'oposen a l'adopció de cultius millorats genèticament a Europa i de les seves greus conseqüències tant en països desenvolupats com en vies de desenvolupament i que he plasmat en l'últim capítol.

RESUMEN

El virus de la inmunodeficiencia humana (VIH) es en la actualidad uno de los problemas de salud pública más graves en el mundo. A pesar de que la incidencia de nuevas infecciones por VIH ha disminuido ligeramente desde principios del año 2000, el número total de personas que viven con el virus continúa aumentando como resultado de la combinación de las nuevas infecciones y el impacto positivo de la terapia antirretroviral en la que se basa el tratamiento. La aplicación de medidas preventivas para evitar la infección por VIH es la principal vía para reducir la expansión del virus. Los microbicidas son una nueva clase de productos antivirales de bajo coste que podrían ayudar a conseguirlo. Éstos pueden ser diseñados en diversas formulaciones para aplicarlos por vía tópica dentro de la vagina o del recto con el objetivo de prevenir la infección por VIH y otras enfermedades de transmisión sexual. Actualmente, muchos candidatos a microbicidas se encuentran en diferentes etapas de investigación. Los ensayos clínicos más avanzados corresponden a microbicidas basados en antirretrovirales. Además, también se están investigando proteínas microbicidas como anticuerpos monoclonales humanos neutralizantes o lectinas que presentan actividad antiviral. Algunos de los obstáculos que pueden frenar el desarrollo de microbicidas son la viabilidad, el aumento de área de cultivo y los altos costes de producción. Se están evaluando muchas plataformas para la producción de microbicidas. Una de las más prometedoras son las plantas, ya que constituyen un sistema económico y seguro. A la producción de proteínas recombinantes farmacéuticas en plantas se le denomina "molecular pharming". De todos los posibles cultivos que son de interés en molecular pharming, las semillas de cereales son probablemente el vehiculo más adecuado para su distribución, particularmente en países en vía de desarrollo.

El principal objetivo de esta tesis es el estudio de la producción de moléculas microbicidas tanto a nivel individual (producción de una sola molécula) como combinada (producción simultanea de varias moléculas) en semillas de maíz con el fin de desarrollar una nueva estrategia mas económica capaz de evitar la infección por VIH en países en vías de desarrollo.

El anticuerpo monoclonal humano neutralizante, 2F5, es una de estas moléculas microbicidas que he investigado para su producción en semillas de maíz. Los resultados muestran que este anticuerpo ha sido formado correctamente en las semillas de maíz y

es funcional. Sin embargo, sus niveles de acumulación fueron más bajos que los logrados anteriormente con otro anticuerpo monoclonal humano neutralizante (2G12) en semillas de maíz, sugiriendo que una combinación de factores puede limitar la acumulación de ciertos anticuerpos en un determinado caso.

La combinación de moléculas microbicidas ofrece más ventajas que la utilización de sólo una molécula. Por lo tanto, he investigado en esta tesis la producción de un segundo anticuerpo monoclonal humano neutralizante (4E10) en combinación con dos lectinas que presentan actividad antiviral (cyanovirin-N y griffithsin). Debido a problemas técnicos durante la transformación y la regeneración *in vitro* de plantas, no pude regenerar ninguna planta transgénica, por consiguiente estos experimentos deben ser repetidos.

La síntesis de proteínas, su transporte y almacenamiento en plantas son factores críticos que pueden afectar la calidad de la proteína recombinante a nivel de plegamiento, modificaciones postraduccionales y capacidad de acumulación. En base a esta suposición, seleccioné un evento que acumula elevados niveles del anticuerpo 2G12 para investigar su deposición y almacenamiento en semillas de maíz. Los resultados demostraron que el anticuerpo se acumula en los cuerpos proteicos de las semillas de maíz (zeinas). Sorprendentemente también se encontró anticuerpo interactuando con las zeinas y como consecuencia se produjo una deformación de los cuerpos proteicos. Próximamente se iniciarán experimentos adicionales para determinar si estas interacciones entre el anticuerpo y los cuerpos proteicos influyen en los niveles de expresión y en la funcionalidad del anticuerpo.

La bioseguridad y los temas relacionados con la legislación relativa a cultivos mejorados genéticamente también son una parte importante de esta tesis, ya que limitan el desarrollo y la comercialización global de este tipo de cultivos. El estudio bibliográfico y normativo que he desarrollado durante esta tesis proporciona una visión amplia de las barreras no técnicas a la adopción de cultivos mejorados genéticamente en la Unión Europa y de sus graves consecuencias económicas tanto en países desarrollados como los que están en vías de desarrollo.

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ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
AMCV	Artichoke Mottled Crinkle Virus
ART	Antiretroviral Therapy
ARV	Antiretroviral
Bar	Bialaphos resistance gene
BHK	Baby Hamster Kidney
bp	Base Pairs
BSA	Bovine Serum Albumin
Bt	*Bacillus thuringiensis*
CaMV 35S	Cauliflower Mosaic Virus 35S
CAP	Common Agricultural Policy
CCR5	C-C chemokine Receptor type 5
CD4	Cluster of Differentiation 4
CDC	Complement-Dependent Cytotoxicity
CDR	Complementarity Determining Region
CHAARM	Combined Highly Active Antiretroviral Microbicide
CHO	Chinese Hamster Ovary cells
CSPD	Chloro-5-substituted adamantyl-1,2-dioxetane phosphate
CV-N	Cyanovirin-N
CXCR4	C-X-C chemokine receptor type 4
DAP	Days After Pollination
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DIG	Digoxigenin-Labeled nucleic acids
DPI	Days Post Infiltration
EC	European Commission
EC_{50}	The half maximal Effective Concentration
ECC	European Economic Community
EFSA	European Food Safety Authority

ELISA	Enzyme-Linked Immunosorbent Assay
ELP	Elastin Like Peptide
ENGase	Endoglycanase
ER	Endoplasmic Reticulum
EU	European Union
FAO	Food and Agriculture Organization of United Nations
FDA	US Food and Drug Administration
GE	Genetically Engineered
GlcNAc	*N*-Acetylglucosamine
GMP	Good Manufacturing Practice
gp120	Glycoprotein 120KDa
gp41	Glycoprotein 41KDa
GRAS	Generally Regarded As Safe
GRFT	Griffithsin
HAART	Highly Active Antiretroviral Therapy
HC	Heavy Chain
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HMC	Hydroxymethyl Cellulose
HMT	High-Mannose Type
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
IC_{50}	The half maximal Inhibitory Concentration
Ig	Immunoglobulin
ISAAA	Internation Service for the Acquisition of Agri-biotech Applications
ITC	Inverse Transition Cycling
KBBE	Knowledge-Based Bioeconomy
KDa	Kilo Dalton
LC	Light Chain
mAbs	Monoclonal Antibodies
Man	Mannose

MDG	Millennium Development Goal
MPER	Membrane Proximal Region
NCA	National Competent Authorities
NMR	Nuclear Magnetic Resonance
NNRTIs	Non- Nucleoside Reverse Transcriptase Inhibitors
Nos	Nopaline Synthase gene
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
OD_{600}	Optical Density at 600nm
PAT	Phosphinothricin Acetyltransferase
PBMCs	Peripherial Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBs	Protein Bodies
PCR	Polymerase Chain Reaction
PPT	DL-Phosphinothricin
PrEP	Pre-Exposure Prophylaxis
PSVs	Protein Storage Vacuoles
PTGS	Post-Transcriptional Gene Silencing
R&D	Research and Developmenet
SARS	Severe Acute Respiratory Syndrome
SCoFCAH	Committee on the Food Chain and Animal Health
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SMEs	Small and Medium Enterprises
SSC	Saline-Sodium Citrate
TBS	Tris-Buffered Saline
TMB	3,3',5,5'-Tetramethylbenzidine substrate
TMV	5' 5'-leader sequence of Tobacco Mosaic Virus
TSP	Total Soluble Protein
USDA	United States Department of Agriculture

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

1.1 HIV infection statistics

Human immunodeficiency virus (HIV) is now a pandemic with more than 60 million people infected and 25 million deaths since it was first recognized in December 1981 (WHO/UNICEF/UNAIDS, 2011). It was estimated that 34 million people were living with HIV globally by the end of 2010 (Figure 1.1) and there were 2.7 million new HIV infections (UNAIDS, 2010; WHO/UNICEF/UNAIDS, 2011). The number of AIDS-related deaths is declining (WHO/UNICEF/UNAIDS, 2011) but the number of infections is increasing (Shattock et al. 2011). The importance of the HIV/AIDS epidemic is so great that the disease was made the specific target of Millennium Development Goal 6 (MDG6) which aims to halt the spread of HIV/AIDS by 2015 and provide wider access to HIV drugs (UN, 2010; Yuan et al. 2011).

There is a significant regional variation in the incidence of HIV/AIDS (WHO/UNICEF/UNAIDS, 2011). More than 95% of infected people live in developing countries (Figure 1.1) and this is also where most new infections occur (UNAIDS, 2010). For example, 68% of all people infected with HIV in 2010 lived in sub-Saharan Africa, which accounts for only 12% of the global population. Sub-Saharan Africa also accounted for 70% of new HIV infections in 2010. Although there was a notable decline in the regional rate of new infections, the epidemic continues to be most severe in southern Africa with more people living with HIV (an estimated 5.6 million) than in any other part of the world (WHO/UNICEF/UNAIDS, 2011).

There is hope that the HIV epidemic can be overcome because antiretroviral therapy (ART) is becoming more effective (Abdool Karim and Baxter, 2012; Lynch et al. 2012). The greatest challenge is diagnosing HIV-infected people and helping them take full advantage of existing treatments, which prevent AIDS and make HIV-carriers less infectious (Abdool Karim and Baxter, 2012; Lynch et al. 2012; Roberts, 2012). However, despite recent major international commitments to provide access to ART for 15 million people by 2015 (UNAIDS, 2012), there are large funding gaps that limit the

potential to capitalize on the latest scientific progress. Even with the most innovative strategies and tools, confronting and reversing the HIV epidemic will fail without more funding and reduced drug prices (Lynch et al. 2012). Therefore a political will is also required to deliver affordable, quality care for more people.

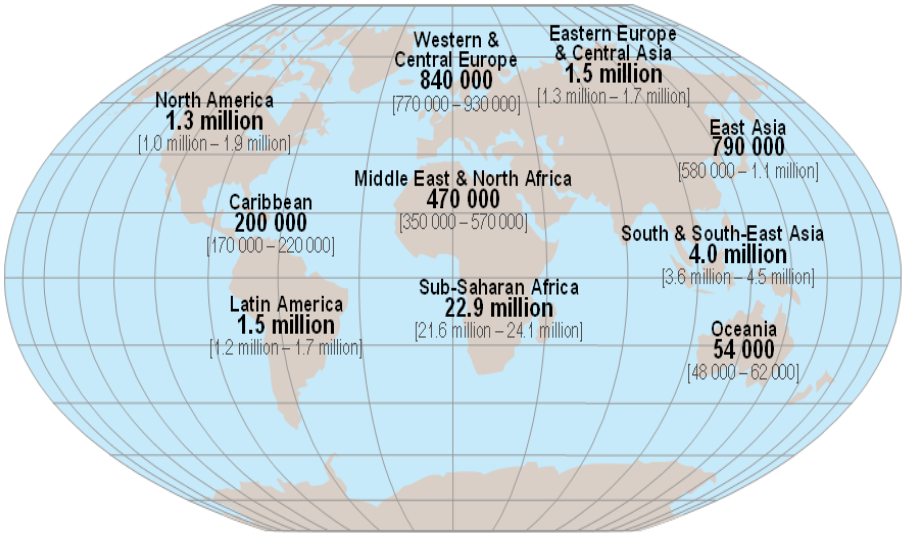

Figure 1.1: Adults and children living with HIV in 2011 (WHO, 2011). Numbers in square brackets represent minimum and maximum estimates and the average is shown in bold.

1.2 Current HIV therapies

There is currently no cure for HIV, but ART is an efficient approach to control and slow down the progression of the disease by reducing the rate of virus replication. 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. The drugs must be taken for life, and although the number of HAART recipients has increased, universal access still appears to be a long way off mainly because the drugs are too expensive (WHO/UNICEF/UNAIDS, 2011). The

number of new HIV infections outstrips the number of HAART recipients by 2:1 (MTN, 2012). Globally, more than 50% of the people eligible for treatment do not have access to HAART and the overwhelming majority that have access to therapy live in industrialized countries (WHO/UNICEF/UNAIDS, 2011). The challenges of drug resistance and systemic toxicity also mean that new strategies for the treatment of HIV/AIDS are necessary (Asahchop et al. 2012).

The prevention of sexual transmission is essential to curtail HIV, and simple preventive methods that have been advocated include abstinence, monogamy, fewer 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.

Currently there is active research to develop HIV prophylactic and therapeutic vaccines but there has been little progress in this area and disappointing results from the first efficacy trials (Nelson, 2007; Fauci et al. 2008; Dolgin, 2010; Walker and Burton, 2010). Researchers have also focused on prevention strategies including pre-exposure prophylaxis, microbicides and voluntary male circumcision (AVAC, 2012a; Global Campaign for Microbicides, 2012).

Truvada (Gilead Sciences, Inc., Foster City, CA) is the first product approved to reduce the risk of HIV infection (Pre-Exposure Prophylaxis (PrEP)) in uninfected individuals at high risk of infection, e.g. those engaging in sexual activity with an HIV-infected partner (FDA, 2012). The US Food and Durg Administration (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, 2004) but in July 2012 approved it for pre-exposure prophylaxis. Truvada comprises two antiretroviral drugs (the nucleoside reverse transcriptase inhibitor emtricitabine and tenofovir disoproxil fumarate) and needs to be taken daily by uninfected subjects to reduce the risk of infection. Truvada is also approved for use as part of a comprehensive HIV prevention strategy that includes other prevention methods, such as safe sex, risk reduction counseling and regular HIV testing to reduce the risk of sexually-acquired HIV infection in high-risk adults.

The approval of Truvada as a prophylactic was controversial, and was based on a non-unanimous FDA decision by the Antiviral Drugs Advisory Committee (ADAC). There was concern that pre-exposure prophylaxis might cause more harm than good, by providing a false sense of security and causing people to abandon proven methods such as condom use (Cohen, 2012). Furthermore, although Truvada works well by itself as a preventive strategy, as a treatment it must be used with other drugs to avoid the emergence of resistant strains. Therefore, the off-label sole use of Truvada by infected individuals needs to be discouraged to avoid the emergence of resistant HIV strains which would render it useless for therapy. The FDA is therefore alerting healthcare professionals and end users that Truvada must only be used by individuals who are confirmed HIV-negative prior to prescription and tested to confirm this status at least every three months, and not by infected individuals unless as part of a combination therapy (FDA, 2012). As a condition of approval, Gilead Sciences must collect viral isolates from individuals who acquire HIV while taking Truvada and evaluate these isolates for the presence of resistance. The company is also required to collect data on pregnancy outcomes for women who become pregnant while taking Truvada prophylactically and to conduct a trial to evaluate drug adherence and its relationship to adverse events, the risk of seroconversion, and the development of resistance in seroconverters (FDA, 2012).

1.3 New preventive strategies: microbicides

Microbicides are low-cost, self-administered topical formulations designed to curb mucosal and perinatal HIV transmission (UNAIDS, 1996; D'Cruz and Uckun, 2004). Microbicides are designed to be applied in the vagina and/or rectum, at the site of potential infection. The virus can enter the body through the bloodstream or by passing through delicate mucous membranes, such as inside the vagina, rectum or urethra (Figure 1.2). Microbicides can be designed to be applied approximately at the time of coitus, on a daily basis or to deliver the product over an extended period. Therefore, they are formulated as gels, creams, suppositories, films, lubricants or a sponge or vaginal ring that slowly releases the active ingredient. Depending on the compounds

present in the formulation, they can also provide a barrier against other sexually-transmitted diseases and pregnancy (Boonstra, 2000; Ramessar et al. 2010; Abdool Karim and Baxter, 2012; Shattock and Rosenberg, 2012). HIV microbicides work by inhibiting or blocking the earliest steps of infection through the topical delivery of the active ingredient to exposed surfaces, producing high topical concentrations of the active substance without significant systematic exposure thus reducing long-term toxicity in healthy but at-risk individuals (Shattock and Rosenberg, 2012).

Women represent half (50%) of all adults living with HIV/AIDS, a proportion that has shifted very little in the last 15 years. HIV/AIDS remains the leading cause of death among women of reproductive age globally, but particularly in sub-Saharan Africa where 59% of the adults living with HIV in 2010 were women (WHO/UNICEF/UNAIDS, 2011). Only a single isolate (a single virus) in 80% of women is needed to establish HIV infection by the vaginal route (Keele et al. 2008; Salazar-Gonzalez et al. 2009; Keele, 2010). The balance between exposure and successful or non-successful infection is therefore likely to be relatively small. Many biological factors make women more vulnerable to HIV than men (Downs and De Vincenzi, 1996). In addition cultural, economic and domestic factors combine to make it very difficult if at all possible for women to ascertain the HIV and treatment status of their male partners (Abdool Karim and Baxter, 2012; Shattock and Rosenberg, 2012). Even with an effective scale-up of HIV testing, treatment and prevention, there would be a high incidence of HIV in African women because of gender inequality. Therefore there is an urgent need to develop a preventive strategy that women can control, and microbicides are currently the only solution which places the power to control the risk of infection in the hands of women (WHO/UNICEF/UNAIDS, 2011). Preventive strategies such as microbicides are therefore needed to achieve an AIDS-free generation in Africa (Abdool Karim and Baxter, 2012).

Some end users may also consider microbicides to be a more flexible prevention option than a vaccine because microbicides are a temporary rather than a systemic barrier to HIV (Tolley and Severy, 2006). Furthermore, the epidemiological modeling of data

from 73 low-income countries indicates that a microbicide with only 60% efficacy could prevent 2.5 million infections in women, men and children (mother-child transmission) over a 3-year period (Watts and Vickerman, 2001; Watts et al. 2002).

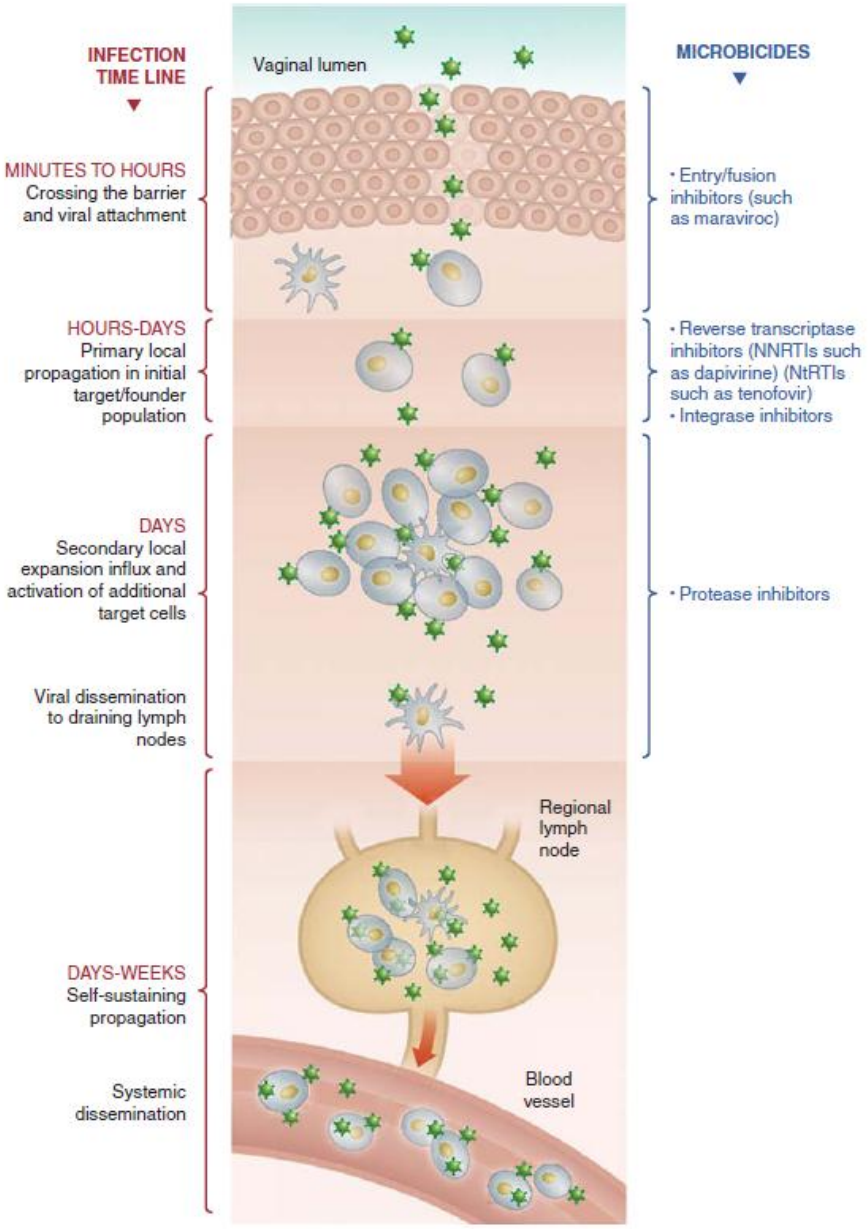

Figure 1.2: Initial events in mucosal infection and the potential points for microbicide intervention (Shattock and Rosenber, 2012).

1.3.1 Types of microbicides and their mechanisms of action

Topical microbicides are classified according to their mechanism(s) of action and their target during HIV transmission (Figures 1.2 and 1.3). Candidate HIV microbicides use one or more of the following mechanisms of action to combat infection:

- *Microbicides that kill the pathogen* (surfactants): These microbicides break down the membrane or envelope of the target pathogen, preventing interactions with vaginal epithelial cells.
- *Microbicides that prevent virus replication* (replication inhibitors): These microbicides are not designed to prevent infection, but they block virus replication soon after initial infection, thus slowing the progression of AIDS before the disease starts to take hold (Balzarini and Van Damme, 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.
- *Microbicides that enhance the vaginal milieu protectors* (buffers): The acidity of the vaginal canal inhibits several bacteria and viruses (Sturm and Zanen, 1984; Croughan and Behbehani, 1988; 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; Abdool Karim and Baxter, 2012).
- *Microbicides that block virus entry into host cells* (inhibitors): Entry inhibitors form another broad class of microbicidal agents that block the attachment, fusion or uptake of HIV into host cells. Such agents work either by binding to HIV and disrupting its interaction with receptors on the host cell membrane, or by saturating available binding sites. Entry inhibitors are divided into three groups: anionic polymers, CCR5 inhibitors and fusion inhibitors (Shattock and Moore, 2003; Ramessar et al. 2010).

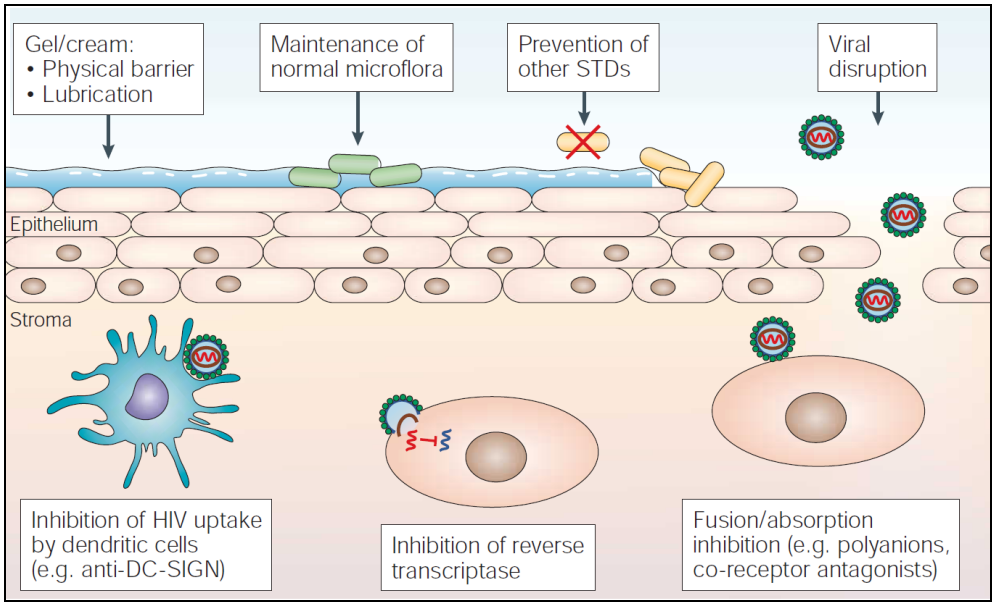

Figure 1.3: Potential mechanisms of action of microbicide compounds (Shattock and Moore, 2003).

1.3.2 Current microbicide candidates

Since the first HIV prevention strategy targeted at women was proposed more than two decades ago (Stein, 1990), several candidate microbicides have entered clinical trials to determine their potential to prevent HIV infection. However, no topical microbicide has yet been proven effective.

Surfactants (e.g. nonoxynol-9 and SAVVY), polyanions (e.g. cellulose sulfate, Carraguard and PRO 2000) and vaginal milieu protectors e.g. (BufferGel) constituted the first generation candidate microbicides (Cutler and Justman, 2008; Abdool Karim et al. 2010a). These were broad acting compounds but they failed to inhibit HIV effectively, even enhancing infection in some instances (Van Damme et al. 2002).

These disappointing results prompted the clinical development of microbicides that contain antiretroviral drugs (Abdool Karim and Baxter, 2012; Shattock and Rosenberg, 2012). The most advanced clinical trials involve microbicides that include antiretroviral

drugs (AVAC, 2012b) (Table 1.1). The most promising results were obtained in 2010 in the CAPRISA 004 tenofovir gel trial. This provided the first evidence that a microbicide gel containing 1% tenofovir, a reverse transcriptase inhibitor, is safe and can reduce HIV and herpes virus (HSV) infections (Abdool Karim et al. 2010b). However, one year later the Microbicide Trial Network VOICE study (NIAID, 2011), which examined the safety and effectiveness of 1% tenofovir gel and two oral antiretroviral agents taken daily (tenofovir and emtricitabine), concluded that tenofovir was no better than placebo in preventing HIV in women (MTN, 2011a,b). One of the key factors may have been the need for daily dosing. A more detailed examination of the VOICE study data is required to understand the reasons for these results and is planned by the end of 2012 (Abdool Karim and Baxter, 2012). In the meantime, another placebo-controlled study, the Follow-on African Consortium for Tenofovir Studies 001 (FACTS 001), continues to confirm and extend the findings of the original CAPRISA 004 trial (Abdool Karim et al. 2010b).

If FACTS 001 can confirm the positive results from CAPRISA, these combined effectiveness data, along with the VOICE safety data for tenofovir gel, may enable the licensing of the first microbicide thus providing a new tool for women to protect themselves from HIV and genital herpes. CAPRISA is also planning to carry out an implementation study (CAPRISA 008) in the communities where the CAPRISA 004 trial took place. CAPRISA 008 aims to address critical implementation questions, i.e. how the tenofovir gel could be incorporated into current health systems and made accessible to women who would benefit most from this product while also providing a mechanism for ongoing post-trial access to the tenofovir gel in these communities (Abdool Karim and Baxter, 2012).

In addition to the clinical trials of tenofovir, two phase III trials are planned with another antiretroviral drug, dapivirine (TMC-120) (Abdool Karim and Baxter, 2012; AVAC, 2012b).

Other than the microbicides described above, new candidates are being developed against novel targets, such as microbicides targeting different components of the HIV infection cycle simultaneously, or individual microbicides that target more than one pathogen (other viruses or bacteria) which may be more effective than single-target strategies (Ramessar et al. 2010). These include HIV-neutralizing human monoclonal antibodies that target conserved but distinct epitopes on the HIV-1 envelope glycoproteins gp120 and gp41, which are responsible for HIV uptake (Ramessar et al. 2010; Lotter-Stark et al. 2012), or the anti-HIV lectins cyanovirin-N (CV-N) and griffithsin (GRFT) (Ramessar et al. 2010; Lotter-Stark et al. 2012). Chapters 3, 4 and 5 describe these alternative microbicide components for the prevention of HIV infection.

Table 1.1: Ongoing and planned clinical trials of microbicide candidates, adapted from the global advocacy for HIV prevention tables on ongoing and planned clinical trials (AVAC, 2012b).

Study Name	Phase	Mechanism of action	Intervention arms	Status/Expected Completion
CHOICE (MTN 018)	Phase IIIb/ Open label	Replication inhibitor	Daily oral TDF/FTC (Tenofovir disoproxil fumarate)	Pending results of VOICE / Q4 2015
CAPRISA 008	Open label	Replication inhibitor	1% tenofovir gel up to 12 hours before vaginal intercourse and within 12 hours following intercourse	Under review by the South African Medicines Control Council (MCC)
ASPIRE (MTN 020)	Phase III	Replication inhibitor	4-week vaginal dapivirine ring	Pending / Q4 2014 - Q1 2015
The Ring Study (IPM 027)		Replication inhibitor	4-week vaginal dapivirine ring	Planned / 2015
FACTS 001		Replication inhibitor	1% tenofovir gel up to 12 hours before vaginal intercourse and within 12 hours following intercourse	Enrolling / Q1-Q2 2014
MTN 017	Phase II	Replication inhibitor	Reformulated (reduced glycerin) 1% tenofovir gel used rectally	In development / Q3-Q4 2014
VOICE (MTN 003)	Phase IIb	Replication inhibitor	Daily oral TDF/FTC. Daily oral TDF and daily 1% tenofovir gel arms dropped for futility based on data from DSMB reviews.	Fully enrolled / Q1 2013
FACTS 002	Phase II	Replication inhibitor	1% tenofovir gel used daily	In development

Open label trial: where all participants are offered the product being tested and there is no placebo

Despite the promise of some HIV microbicides, their success depends on the ability to produce sufficiently large quantities to deal with global demand. Estimations based on a 5 mg protein microbicide dose administered twice a week indicate that a production capacity of 5000 kg/yr is required to supply just 10 million women (Shattock and Moore, 2003). Furthermore, the largest market segment for HIV prophylaxis is in resource limited countries that can ill afford the development costs.

It is therefore crucial that microbicides are produced with minimum upfront capital costs to facilitate development and testing and ultimately to promote availability to end users. Current production systems for recombinant proteins include microbes (yeast and bacteria), mammalian expression systems such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells and myeloma cell lines, baculovirus expression systems in insect cells, transgenic animals producing recombinant proteins in their milk or eggs, and transgenic plants (Figure 1.4) (Ferrer-Miralles et al. 2009). Plants provide significant advantages in terms of economy and safety, making them ideal for the production of microbicides for HIV-endemic regions in Africa and Asia (Table 1.2) (Stoger et al. 2002a, 2005; Ma et al. 2003; Twyman et al. 2003; Fischer et al. 2004). The production of pharmaceutical proteins in plants is also known as molecular pharming.

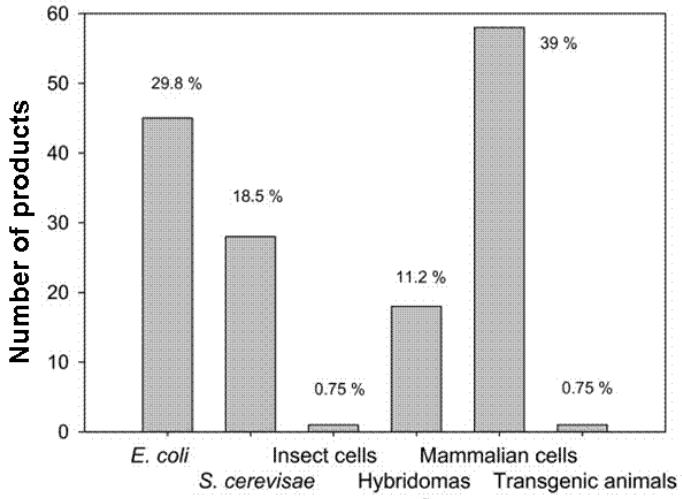

Figure 1.4: Number (percentage) of recombinant proteins approved as biopharmaceuticals in different production systems (Ferrer-Miralles et al. 2009). In 2012 one recombinant pharmaceutical protein from plants (carrot cells) was approved to be on the market (Shaaltiel et al. 2007; Morrow, 2012).

Table 1.2: Production systems for recombinant human pharmaceutical proteins (adapted from Ma et al. 2003; Kunka et al. 2005).

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risk	Storage costs
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

*Compared to production in conventional systems such as mammalian cell cultures

1.4 Molecular pharming

Molecular pharming is the use of plants to produce recombinant pharmaceutical proteins (Twyman et al. 2003, 2005; Paul and Ma, 2011). Examples include human and veterinary vaccines (Streatfield, 2005, 2007), hormones, signaling proteins, blood products, enzymes (Stoger et al. 2002a, 2005; Ma et al. 2003; Claparols et al. 2004; Twyman et al. 2005) protein polymers and structural proteins (Hood et al. 1997, 1999; Fischer et al. 2003). Antibodies of various formats have also been successfully produced in plants, including full length IgGs, scFvs and their fusions (Ko and Koprowski, 2005; Stoger et al. 2005; Ramessar et al. 2008a) and IgMs (Ma et al. 2003).

Plants have significant advantages over microbial or mammalian production systems in that they provide a safe, reliable and cost-effective method for production on an agricultural scale (Table 1.2) depending only upon the land area used for cultivation (Stoger et al. 2002b; Ma et al. 2003; Twyman et al. 2003; Fischer et al. 2004; Kunka et al. 2005). The demand for pharmaceutical proteins is rapidly increasing and requires large production volumes, placing constraints on current production methods which are limited by high production costs and long lead times before production facilities are operational. Therefore, molecular pharming has emerged as a suitable alternative platform to address the drawbacks of established platforms, remaining economically viable even at relatively low expression levels of therapeutic proteins (1% total soluble protein (TSP)).

Plants can fold and assemble complex proteins accurately and are able to carry out many of the essential post-translational modifications required for the optimal biological activity of recombinant proteins. Plants do not support human-tropic pathogens and lack the bacterial toxins that can contaminate recombinant proteins produced using other platforms (Ma et al. 2003; Stoger et al. 2005). Furthermore, compared to mammalian and microbial cells, many plant-based systems are suitable for rapid short-term scale-up or longer agricultural scale-up, overcoming the commercial constraints that prevent the production of pharmaceutical proteins at the 10-100 kg scale. Plants can be grown in soil using only water, fertilizer and light, which is much

less expensive than the complex media requirements of microbial and mammalian cells (Knäblein, 2005).

Early bottlenecks in the developing of molecular pharming have now been largely overcome: yields are now more competitive, the issue of non-human glycan structures can be tackled in a variety of ways and the experience gained now allows plants to be used to produce tailored glycoforms that are not achievable using mammalian cells. The regulators are also beginning to take notice of plants, with several plant-based production processes approved for pharmaceutical production according to good manufacturing practice (GMP) (Peters and Stoger, 2011).

Downstream purification costs are currently a bottleneck not only for plants but also for conventional production systems. However the burden can be alleviated by maximizing production yields and utilizing innovative purification strategies to improve product recovery (Paul and Ma, 2011). In the case of plant production systems, the upfront investment required for infrastructure is lower, potentially reducing the barriers to achieve cost-effective production for pharmaceutical proteins. Several strategies have been developed to reduce protein purification costs in plants, such us the oleosin platform that allows recombinant proteins to be isolated from the lipid fraction of seeds followed by endoprotease cleavage (Boothe et al. 2010; Nykiforuk et al. 2011). A similar concept is exploited when proteins are expressed as fusions with an integral membrane-spanning domain derived from the human T-cell receptor, allowing protein extraction in a small volume using appropriate buffers and detergents (Schillberg et al. 2000). Other separation methods have been devised based on cross-flow filtration, which rely neither on fusions nor on chromatographic separations (Aspelund and Glatz, 2010).

Plants have been used to produce antibodies for the prevention of sexually transmitted infections, including an IgG against Herpes simplex virus (HSV) which prevented transmission in mice (Zeitlin et al. 1998) and an HIV diagnostic antibody produced in barley (Schunmann, 2002). Protein based microbicides, such as neutralizing antibodies

and peptide lectins, have been also successfully expressed in plants (Sack et al. 2007; Rademacher et al. 2008; Ramessar et al. 2008b; O'Keefe et al. 2009; Sexton et al. 2009; De Muynck et al. 2010; Matoba et al. 2010) but these have not yet reached the market. Currently there is one recombinant pharmaceutical protein from plants on the market, an orally delivered recombinant form of human glucocerebrosidase for the treatment of Gaucher's disease produced in carrot cells by Protalix BioTherapeutics (Shaaltiel et al. 2007; Morrow, 2012). A number of other companies have plant-derived pharmaceutical products in late-stage clinical trials (e.g. Ventria Biosciences) and others are producing potential pharmaceutical products which are currently marketed for non-pharmaceutical uses, such as human-like growth factors approved for laboratory use and as cosmetic ingredients produced in barley seeds by ORF Genetics (www.orfgenetic.com). These recombinant proteins produced in plants demonstrate that plant based systems can achieve GMP-like batch-to-batch reproducibility and quality (Faye and Gomord, 2010).

The many advantages provided by molecular pharming will not be realized unless the general barriers to the adoption of genetically engineered crops are overcome, and these are political rather than technical (Farre et al. 2011). Genetically engineered crops are subjected to much stricter and more arbitrary regulations than conventional crops, and these regulations differ from country to country, particularly within the European Union (Ramessar et al. 2008c, 2010b; Sabalza et al. 2011). The benefits of molecular pharming would be promoted by the adoption of a rational, science-based and globally harmonious regulatory framework that removes trade barriers and embraces risk/benefit analysis rather than the current precautionary approach focusing on the elimination of all risks (Twyman et al. 2009). This topic is discussed in more detail in Chapter 6.

1.5 Plant expression systems for the production of pharmaceutical proteins

There are currently many alternative plant production systems for recombinant pharmaceutical proteins (Ma et al. 2003; Twyman et al. 2003; Fischer 2004; Ramessar et al. 2008a). These include leafy crops, fruits and vegetables, cereals, specialized aquatic plants, systems based on plant viruses (transient expression) and plant cell

suspension cultures. The choice of crop used for molecular pharming must take into account a number of factors including overall yield, *in situ* protein stability, ease of purification and the cost of regulatory oversight (Ramessar et al. 2008a).

Cereal seeds are an ideal vehicle for the stable accumulation of recombinant proteins (Ma et al. 2003; Twyman et al. 2003; Ramessar et al. 2008d). The expression of proteins in seeds is advantageous because they are natural protein storage organs with an appropriate biochemical environment to achieve stable protein accumulation with no substantial loss of activity. Seeds also accumulate large amounts of protein in a relatively small volume. The endosperm tissue provides a specialized environment for protein accumulation by promoting the formation of storage compartments such as protein bodies and protein storage vacuoles that are derived from the secretory pathway. Cereal seeds lack phenolic compounds and alkaloids that are present in tobacco leaves, which add to downstream processing costs. Antibodies expressed in cereal seeds remain stable for several years at room temperature with no detectable loss of activity (Stoger et al. 2000). Because of this high stability, cereal seeds containing recombinant proteins can be stored and distributed in countries where a cold chain is unreliable or unavailable; thus cereal seeds are likely to be the most suitable production system for deployment in developing countries (Twyman et al. 2005). Edible seeds also 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 (Twyman et al. 2005; Peters and Stoger, 2011).

1.6 Molecular pharming in maize seeds

Maize (*Zea mays*) has many advantages over other cereal seeds which make it ideal for the production of recombinant pharmaceutical proteins (Ramessar et al. 2008d). Maize is the most widely grown cereal crop in the world. Compared to other cereals, maize has a larger grain size and a higher proportion of endosperm, which can occupy up to 82% of the seed (Watson, 1987). Maize plants have undergone selective breeding for both food and feed production, and have been optimized for high seed yield. Therefore maize has a high biomass yield per hectare and lower production costs than other major cereals

(Giddings et al. 2000). Unlike other cereals, the C4 photosynthetic pathway in maize increases the efficiency of biomass production. Maize also benefits from relatively straightforward and efficient *in vitro* culture, transformation and regeneration protocols as well as a suite of seed-specific promoter systems that can be used to drive transgene expression individually or as part of multigene expression strategies. Studies have shown that proteins in dry maize seed remain stable for more than 6 years at room temperature and for months in cracked and flaked maize seeds stored at up to 10ºC (Hood et al. 1997). Maize was therefore used to produce the first commercial plant-derived recombinant proteins (avidin and β-glucuronidase) marketed by ProdiGene Inc. (Hood et al. 1997, 1999; Kusnadi et al. 1998; Witcher et al. 1998). Prodigene Inc. developed several further products such as laccase and trypsin, and was focusing on medically-relevant proteins before a breach of environmental regulations resulted in fines and cleanup costs levied by the USDA which caused the company to cease trading (Naqvi et al. 2011). The company was punished for its failure to comply with regulations, not because there was actual evidence of risk. It is notable that the USDA has since abandoned its zero tolerance policy, demonstrating clearly that the regulations are still evolving (Ramessar et al. 2008d).

These initial commercial products of molecular pharming demonstrated that plants can be economically viable even when the natural source of a protein is abundant (e.g. egg whites for avidin and *Escherichia coli* for β-glucuronidase) and where a market is already established (Fisher et al. 2012). Maize seeds can also be used as edible vaccines, as demonstrated by the expression of a Newcastle disease virus fusion protein in transgenic maize seeds which, when fed to chickens, induced the production of antibodies conferring protection against viral challenge (Guerrero-Andrade et al. 2006)

One potential drawback of maize is that, unlike other cereals (rice, barley and wheat) is cross-pollinating thus its uncontained cultivation for molecular pharming applications can lead to gene flow into non-pharmaceutical crops (Commandeur et al. 2003). Even so, this can be addressed by appropriate cultivation management practices that mitigate gene flow, and which are already widely established to prevent gene flow between

genetically engineered (GE) and conventional food crops. None of the other cereal crops (rice, barley and wheat) can presently match the yields of recombinant protein achieved in maize (Stoger et al. 2005).

1.7 References

Abdool Karim SS, Richardson B, Ramjee G, Hoffman IF, Chirenje ZM, Taha T, Kapina M, Maslankowski L, Coletti A, Profy A, Moench TR, Piwowar-Manning E, Mâsse B, Hillier SL and Soto-Torres L on behalf of the HIV Prevention Trials Network (HPTN) 035 Study Team (2010a) Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. *AIDS* 25:957-966

Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany ABM, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L and Taylor D on behalf of the CAPRISA 004 Trial Group (2010b) Effectiveness and safety of Tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329:1168-1174

Abdool Karim SS (2012) An AIDS-free generation? *Science* 377:133

Abdool Karim SS and Baxter C (2012) Overview of microbicides for the prevention of human immunodeficiency virus. *Best Practice and Research Clinical Obstetrics and Gynaecology* 26:427-439

Asahchop EL, Wainberg MA, Sloan RD and Tremblay CL (2012) Antiviral drug resistance and the need for development of new HIV-1 reverse transcriptase inhibitors. *Antimicrobial Agents and Chemotherapy* 56:5000-5008

Aspelund MT and Glatz CE (2010) Purification of recombinant plant-made proteins from corn extracts by ultrafiltration. *Journal of Membrane Science* 353:103-110

AVAC, Global advocacy for HIV prevention (2012a), accessed online 3 August 2012, http://www.avac.org/ht/d/sp/i/176/pid/176

AVAC, Global advocacy for HIV prevention (2012b) Ongoing and planned clinical trials of microbicide candidates (April 2012), accessed online 13 August 2012, http://www.avac.org/ht/a/GetDocumentAction/i/3109

Balzarini J and Van Damme L (2007) Microbicide drug candidates to prevent HIV infection. *Lancet* 369:787-797

Boonstra H (2000) Campaign to accelerate microbicide development for STD prevention gets under way. The Guttmacher Report on Public Policy, February. 3:3-5

Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D and Moloney MM (2010) Seed-based expression systems for plant molecular farming. *Plant Biotechnology Journal* 8:588-606

Claparols MI, Bassie L, Miro B, Del Duca S, Rodriguez-Montesinos J, Christou P, Serafini-Fracassini D and Capell T (2004) Transgenic rice as a vehicle for the production of the industrial enzyme transglutaminase. *Transgenic Research* 13:195-199

Cohen J (2012) FDA panel recommends anti-HIV drug for prevention. *Science* 336:792

Commandeur U, Twyman RM and Fischer R (2003) The biosafety of molecular farming in plants. *AgBiotechNet* 5:ABN110

Croughan WS and Behbehani AM (1988) Comparative study of inactivation of herpes simplex virus types 1 and 2 by commonly used antiseptic agents. *Journal of Clinical Microbiology* 6:213-215

Cutler B and Justman J (2008) Vaginal microbicides and the prevention of HIV transmission. *Lancet Infection Diseases* 8:685-697

D'Cruz OJ and Uckun FM (2004) Clinical development of microbicides for the prevention of HIV infection. *Current Pharmaceutical Design* 10:315-316

De Muynck B, Navarre C and Boutry M (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnology Journal* 8:529-563

Dolgin E (2010) Lack of uptake threatens promise of HIV vaccines. *Nature Medicine* 16:937

Downs AM and De Vincenzi I (1996) Probability of heterosexual transmission of HIV: relationship to the number of unprotected sexual contacts. European study group in heterosexual transmission of HIV. *Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology* 11:388-395

Farre G, Twyman RM, Zhu C, Capell T and Christou P (2011) Nutritionally enhanced crops and food security: scientific achievements versus political expediency. *Current Opinion in Biotechnology* 22:245-251

Fauci AS, Johnston MI, Dieffenbach CW, Burton DR, Hammer SM, Hoxie JA, Martin M, Overbaugh J, Watkins DI, Mahmoud A and Greene WC (2008) HIV vaccine research: the way forward. *Science* 321:530-532

Faye L and Gomord V (2010) Success stories in molecular farming-a brief overview. *Plant Biotechnology Journal* 8:525-528

FDA (U.S. Food and Drug Administration) (2004) FDA approves two fixed-dose combination drug products for the treatment of HIV-1 infection, accessed online 1 September 2012, http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2004/ucm108335.htm

FDA (U.S. Food and Drug Administration) (2012) Truvada approved to reduce the risk of sexually transmitted HIV in people who are not infected with the virus, accessed online 30 August 2012, http://www.fda.gov/forconsumers/byaudience/forpatientadvocates/hivandaidsactivities/ucm312264.htm

Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E and Villaverde A (2009) Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories* 8:17

Fischer R, Emans NJ, Twyman RM and Schillberg S (2003) Molecular farming of industrial proteins in plants. *In*: Fingerman M, Nagabhusmanam R (eds) Recent advances in marine biotechnology, vol 9 (biomaterials and bioprocessing). Science publishers Inc., Enfield NH, pp. 279-313

Fischer R, Stoger E, Schillberg S, Christou P and Twyman RM (2004) Plant-based production of biophramaceuticals. *Current Opinion in Plant Biotechnology* 7:152-158

Fischer R, Schillberg S, Hellwig S, Twyman RM and Drossard J (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnology Advances* 30:434-439

Giddings G, Allison G, Brooks D and Carter A (2000) Transgenic plants as factories for biopharmaceuticals. *Nature Biotechnology* 18:1151-1155

Global campaign for microbicides (2012) More prevention options, accessed online 3 August 2012, http://www.global-campaign.org/prevention_options.htm

Guerrero-Andrade O, Loza-Rubio E, Olivera-Flores T, Fehérvári-Bone T and Gómez-Lim MA (2006) Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. *Transgenic Research* 5:455-463

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Hernan R, Kappel WK, Ritland D, Ping Li C and Howard JA (1997) Commercial production of avidin from transgenic maize: characterization of

transformant, production, processing, extraction and purification. *Molecular Breeding* 3:291-306

Hood EE, Kusnadi A, Nikolov Z and Howar JD (1999) Molecular farming of industrial proteins from transgenic maize. *Chemicals via Higher Plant Bioengineer*ing, *Kluwer/Plenum* 464:127-148

Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH and Shaw GM (2008) Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proceedings of the National Academy of Sciences USA* 105:7552-7557

Keele BF (2010) Identifying and characterizing recently transmitted viruses. *Current Opinion in HIV and AIDS* 5:327-334

Knäblein J (2005) Plant-based expression of biopharmaceuticals. *In*: Meyers RA (editor) Encyclopedia of molecular cell biology and molecular medicine, vol 10. Publisher: Wiley and Sons, 2nd ed., pp:385-410

Ko K and Koprowski H (2005) Plant biopharming of monoclonal antibodies. *Virus Research* 111:93-95

Kunka K, Nabil A, Kostadin G and Atanas A (2005) Molecular farming in plants: An approach of agricultural biotechnology. *Journal of Cell and Molecular Biology* 4:77-86

Kusnadi AR, Hood EE, Witcher DR, Howard JA and Nikolov ZL (1998) Production and purification of two recombinant proteins from transgenic corn. *Biotechnology Progress* 14:149-155

Lotter-Stark HCT, Rybicki EP and Chikwamba RK (2012) Plant made anti-HIV microbicides- A field of opportunity. *Biotechnology Advances* 30:1614-1626

Lynch S, Ford N, Van Cutsem G, Bygrave H, Janssens B, Decroo T, Andrieux-Meyer I, Roberts T, Balkan S, Casas E, Ferreyra C, Bemelmans M, Cohn J, Kahn P and Goemaere E (2012) Public health. Getting HIV treatment to the most people. *Science* 337:298-300

Ma JKC, Drake PMW and Christou P (2003) The production of recombinant pharmaceutical proteins in plants. *Nature Review Genetics* 4:794-805

Matoba N, Husk AS, Barnett BW, Pickel MM, Arntzen CJ, Montefiori DC, Takahashi A, Tanno K, Omura S, Cao H, Mooney JP, Hanson CV and Tanaka H (2010) HIV-1 neutralization profile and plant-based recombinant expression of actinohivin, an Env glycan-specific lectin devoid of T-Cell mitogenic activity. *PLoS One* 5:e11143

Morrow T (2012) Gaucher's disease treatment option rides on carrot cells' biologic power. *Managed Care* 21:45-46

MTN (Microbicide Trials Network) (2011a) Microbicide trials network statement on decision to discontinue use of oral Tenofovir tablets in VOICE, a major HIV prevention study in women 2011, accessed online 15 August 2012, http://www.mtnstopshiv.org/node/

MTN (Microbicide Trials Network) (2011b) MTN Microbicide trials network statement on decision to discontinue use of oral Tenofovir gel in VOICE, in VOICE, a major HIV prevention study in women 2011, accessed online 15 August 2012, http://www.mtnstopshiv.org/node/3909

MTN (Microbicide Trials Network) (2012) Microbicide trials network: HIV/AIDS: a global crisis, accessed online 3 August 2012, http://www.mtnstopshiv.org/about/hiv_aids

Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C and Christou P (2011) High-value products from transgenic maize. *Biotechnology Advances* 29:40-53

Nelson R (2007) Female-initiated prevention strategies key to tackling HIV. *Lancet Infectious Diseases* 7:637

NIAID (National Institute of Allergy and Infectious Diseases) (2011) Safety and effectiveness of Tenofovir 1% gel, Tenofovir disproxil fumarate, and Emtricitabine/Tenofovir disoproxil fumarate tablets in preventing HIV in women 2011, accessed online 15 August 2012, http://clinicaltrials.gov/ct2/show/NCT00705679

Nykiforuk CL, Shen Y, Murray EW, Boothe JG, Busseuil D, Rhéaume E, Tardif JC, Reid A and Moloney MM (2011) Expression and recovery of biologically active recombinant apolipoprotein AI (Milano) from transgenic safflower (*Carthamus tinctorius*) seeds. *Plant Biotechnology Journal* 9:250-263

O'Keefe BR, Vojdani F, Buffa V, Shattock RJ, Montefiori DC, Bakke J, Mirsalis J, D'Andrea A, Hume SD, Bratcher B, Saucedo CJ, McMahon JB, Pogue GP and Palmer KE (2009) Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. *Proceedings of the National Academy of Sciences USA* 106:6099-6104

Paul M and Ma JKC (2011) Plant-made pharmaceuticals: Leading products and production platforms. *Biotechnology and Applied Biochemistry* 58:58-67

Peters J and Stoger E (2011) Transgenic crops for the production of recombinant vaccines and anti-microbial antibodies. *Human Vaccine* 7:367-374

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

Ramessar K, Capell T and Christou P (2008a) Molecular pharming in cereal crops. *Phytochemistry Reviews* 7:579-592

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma JKC, Stoger E, Capell T and Christou P (2008b) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proceedings of the National Academy of Sciences USA* 105:3727-3732

Ramessar K, Capell T, Twyman RM, Quemada H and Christou P (2008c) Trace and traceability-a call for regulatory harmony. *Nature Biotechnology* 26:975-978

Ramessar K, Sabalza M, Capell T and Christou P (2008d) Maize plants: An ideal production platform for effective and safe molecular pharming. *Plant Science*174:409-419

Ramessar K, Sabalza M, Miralpeix B, Capell T and Christou P (2010) Can microbicides turn the tide against HIV? *Current Pharmatheutical Design* 16:468-485

Roberts L (2012) HIV/AIDS in America. *Science* 377:167

Sabalza M, Miralpeix B, Twyman RM, Capell T and Christou P (2011) EU legitimizes GM crop exclusion zones. *Nature Biotechnology* 29:315-317

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoger E and Rademacher T (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB Journal* 21:1655-1664

Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH and Shaw GM (2009) Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *The Journal of Experimental Medicine* 206:1273-1289

Schillberg S, Zimmermann S, Findlay K and Fischer R (2000) Plasma membrane display of anti-viral single chain Fv fragments confers resistance to tobacco mosaic virus. *Molecular Breeding* 6:317-326

Schunmann PHD, Coia G and Waterhouse PM (2002) Biopharming the Simpli-RED™ HIV diagnostic reagent in barley, potato and tobacco. *Molecular Breeding* 9:113-121

Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock RJ and Ma JKC (2009) Transgenic plant production of cyanovirin-N, an HIV microbicide. *FASEB Journal* 20:356-358

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH and Aviezer D (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnology journal* 5:579-590

Shattock RJ and Moore JP (2003) Inhibiting sexual transmission of HIV-1 infection. *Nature Reviews Microbiology* 1:25-34

Shattock RJ, Warren M, McCormack S and Hankins CA (2011) Turning the tide against HIV. *Science* 333:42-43

Shattock RJ and Rosenberg Z (2012) Microbicides: Topical prevention against HIV. *Cold Spring Harbor perspectives in Medicine* 2:1-17

Stein ZA (1990) HIV prevention: the need for methods women can use. *American Journal of Public Health* 80:460-462

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P and Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Molecular Biology* 42:583-590

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P and Fischer R (2002a) Practical considerations for pharmaceutical antibody production in different crop systems. *Molecular Breeding* 9:149-152

Stoger E, Sack M, Fischer R and Christou P (2002b) Plantibodies. Applications, advantages and bottlenecks. *Current Opinion in Biotechnology* 13:161-166

Stoger E, Ma JKC, Fischer R and Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Current Opinion in Biotechnology* 16:167-173

Streatfield SJ (2005) Plant-based vaccines for animal health. *Scientific and Technical Review of the Office International des Epizooties* 24:189-199

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnology Journal* 5:2-15

Sturm AW and Zanen HC (1984) Characteristics of *Haemophilus ducreyi* in culture. *Journal of Clinical Microbiology* 19:672-674

Tolley EE and Severy LJ (2006) Integrating behavioral and social science research into microbicide clinical trials: challenges and opportunities. *American Journal of Public Health* 96:79-83

Twyman RM, Stoger E, Schillberg S, Christou P and Fischer R (2003) Molecular farming in plants: host systems and expresion technology. *Trends in Biotechnology* 21:570-578

Twyman RM, Schillberg S and Fischer R (2005) Transgenic plants in the biopharmaceutical market. *Expert Opinion on Emerging Drugs* 10:185-218

Twyman RM, Ramessar K, Quemada H, Capell T and Christou P (2009) Plant biotechnology: the importance of being accurate. *Trends in Biotechnology* 27:609-612

UNAIDS (1996) International working group on vaginal microbicide. Recommendations for the development of microbicides. *AIDS* 10:1-6

UNAIDS (2010) (The Joint United Nations Programme on HIV/AIDS) Report on the global AIDS epidemic 2010, accessed online 30 July 2012,

http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf

UNAIDS (2012) (The Joint United Nations Programme on HIV/AIDS) United to end AIDS: Achieving the targets of the 2011 Political Declaration; Report of the Secretary-General, United Nations General Assembly 66th Session, 2 April 2012.

UN (United Nations) (2010) The Millennium Development Goals Report 2010. United Nations Department of Economic and Social Affairs (DESA), NY.

Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, Rees H, Sirivongrangson P, Mukenge-Tshibaka L, Ettiègne-Traoré V, Uaheowitchai C, Karim SS, Mâsse B, Perriëns J and Laga M (2002) Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomized controlled trial. *Lancet* 360:971-977

Walker LM and Burton DR (2010) Rational antibody-based HIV-1 vaccine design: current approaches and future directions. *Current Opinion in Immunology* 22:358-366

Watson SA (1987) Structure and composition. *In*: Corn: Chemistry and Technology. Watson SA, Ramstad PT (Eds.) American Association of Cereal Chemists, St. Paul, MN. pp:53-82

Watts C and Vickerman P (2001) The impact of microbicides on HIV and STD transmission: model projections. *AIDS* 15:43-44

Watts C, Kumaranayake L, Vickerman P and Terris-Prestholt F (2002) New hope for HIV prevention: Projections of the impacts of microbicides 2002, Antwerp, Belgium. 12-15 May 2002

WHO (World Health Organization) (2011) HIV/AIDS data and statistics, 2011, accessed online 31 July 2012, http://www.who.int/hiv/data/en/

WHO/UNICEF/UNAIDS (2011) Global HIV/AIDS Response: Epidemic update and health sector progress towards Universal Access. Progress Report, 2011, accessed online 30 July 2012, http://whqlibdoc.who.int/publications/2011/9789241502986_eng.pdf

Witcher DR, Hood EE, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C and Mehigh R (1998) Commercial production of β-glucuronidase (GUS): a model system for the production of proteins in plants. *Molecular Breeding* 4:301-412

Yasin B, Pang M, Wagar EA and Lehrer RI (2002) Examination of *Chlamydia trachomatis* infection in environments mimicking normal and abnormal vaginal pH. *Sexually Transmitted Disease* 29:514-519

Yuan D, Bassie L, Sabalza M, Miralpeix B, Dashevskaya S, Farre G, Rivera SM, Banakar R, Bai C, Sanahuja G, Arjó G, Avilla E, Zorrilla-López U, Ugidos-Damboriena N, López A, Almacellas D, Zhu C, Capell T, Hahne G, Twyman RM and Christou P (2011) The potential impact of plant biotechnology on the Millennium Development Goals. *Plant Cell Report* 30:249-265

Zeitlin L, Castle P, Whaley K, Moench T and Cone R (1998) Comparison of an anti-HSV-2 monoclonal IgG and its IgA switch variant for topical immunoprotection of the mouse vagina. *Journal of Reproductive Immunology* 40:93-101

CHAPTER 2

AIMS AND OBJECTIVES

CHAPTER 2: AIMS AND OBJECTIVES

The aim of my research program was to produce microbicide components in maize seeds in order to develop a novel and inexpensive approach for the prevention of HIV infections in the developing world. A further aim was to analyse the expression of these components in transgenic plants to identify lead events for further characterization and development. In conjunction with the above applications, I also sought to develop a greater fundamental scientific understanding of factors controlling the accumulation and stability of these components in plants, individually or in combination. In that spirit, I investigated the benefits of maize as a production platform for next-generation combination microbicides encompassing HIV-neutralizing human monoclonal antibodies and lectins. Furthermore, I sought to investigate the political aspects of plant biotechnology that hamper its adoption in Europe, impeding the benefits of genetically engineered crops with serious consequences not only in Europe but also in developing countries and emerging economies.

CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF THE RECOMBINANT HIV-NEUTRALIZING MONOCLONAL ANTIBODY 2F5 PRODUCED IN MAIZE SEEDS

CHAPTER 3: FUNCTIONAL CHARACTERIZATION OF THE RECOMBINANT HIV-NEUTRALIZING MONOCLONAL ANTIBODY 2F5 PRODUCED IN MAIZE SEEDS

3.1 Abstract

Monoclonal antibodies (mAbs) that neutralize the human immunodeficiency virus (HIV) can be used as microbicides to help prevent the spread of HIV in human populations. As an industry standard, HIV-neutralizing mAbs are produced as recombinant proteins in mammalian cells, but the high manufacturing costs and limited capacity reduce the ability of target populations in developing countries to gain access to these potentially life-saving medicines. Plants offer a more cost-effective and deployable production platform because they can be grown inexpensively and on a large scale in the regions where the products are required. In this chapter we demonstrate that the maize-derived HIV-neutralizing mAb 2F5 is assembled correctly *in planta* and binds to its antigen with the same affinity as 2F5 produced in mammalian cells. Although 2F5 has been produced at high levels in non-plant platforms such as *Pichia pastoris*, hybridoma lines and CHO cells, the yield in maize seeds is lower than previously achieved with another HIV-neutralizing mAb, 2G12. This suggests that the intrinsic properties of the antibody and the environment provided by the production host may combine to limit the accumulation of some antibodies in plants on a case-by-case basis.

3.2 Introduction

Many potential microbicide candidates are undergoing trials but those containing HIV-neutralizing monoclonal antibodies (mAbs) are particularly advantageous because they target conserved but distinct epitopes on the HIV-1 envelope glycoproteins gp120 and gp41 (Figure 3.1), thus inhibiting the uptake of HIV into human cells (Shattock and Moore, 2003; Cardoso et al. 2005; Ramessar et al. 2010). Such mAbs as passive immune therapy may inhibit virus replication not only by preventing uptake but also by inducing the clearance of virus particles and infected cells by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Armbruster et al. 2004).

Neutralizing antibodies against the viral envelope are generated within weeks after infection, but this early antibody response specifically targets the autologous virus that circulates within each person and is ineffective against heterologous viruses (Wei et al. 2003). However, cross-reactive antibodies capable of neutralizing heterologous primary viral isolates can develop during the course of HIV-1 infection (Stamatatos et al. 2009).

Studies on asymptomatic HIV infected individuals (infected by the virus for at least one year, without clinical signs of AIDS and not on antiretroviral drug therapy) have revealed that between 10% and 30% of asymptomatic individuals develop broadly neutralizing antibodies usually taking three or more years post infection for much antibodies to develop (Stamatatos et al. 2009). However, only 1% of HIV infected individuals develop antibodies with outstanding breadth and potency (Simek et al. 2009). Despite intensive study over two decades, only a small number of broadly neutralizing monoclonal antibodies have been identified. Prior to 2009, only four such antibodies (b12, 2G12, 4E10, and 2F5) were isolated (Cardoso et al. 2005). One year later four new antibodies, PG9, PG16, VRC01 and PGV04 were described (Burton and Weiss, 2010) and in 2011 17 new mAbs have been identified that neutralize broadly across clades and are almost tenfold more potent than the first isolated antibodies (Walker et al. 2011) (Figure 3.2 and Table 3.1).

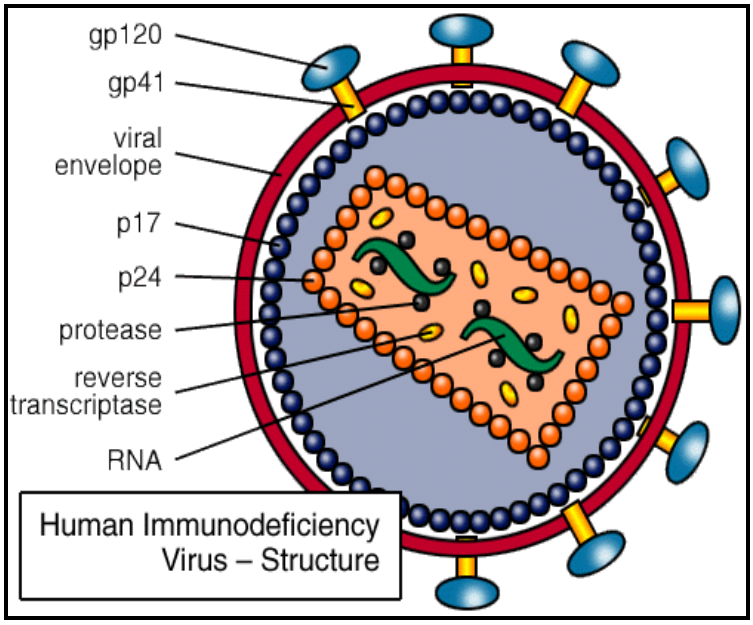

Figure 3.1: Structure of HIV (AVERT, 2012).

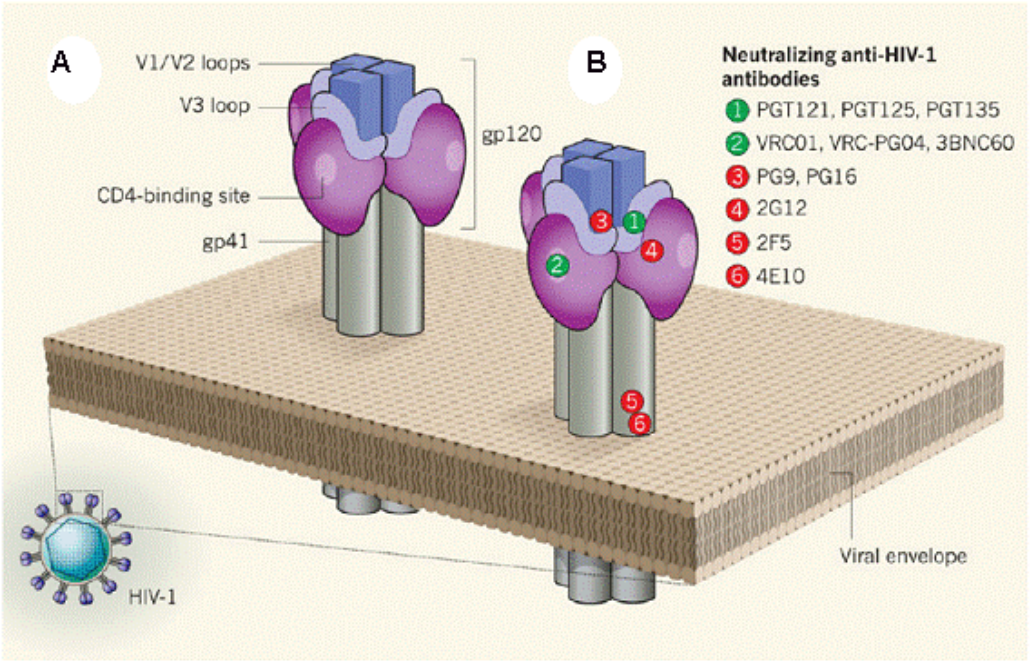

Figure 3.2: The envelope of HIV-1 carries spikes (A) Each spike is made of three molecules of the surface glycoprotein gp120 and three molecules of the transmembrane glycoprotein gp41. Glycoprotein gp120 contains variable V1/V2 and V3 loops, as well as the binding site for CD4. (B) The binding sites of broadly acting and potent HIV-1-specific neutralizing antibodies are shown as colored circles (Koff, 2012).

Table 3.1: Neutralization activity of human HIV monoclonal antibodies. A) Median neutralization potency against viruses neutralized with an IC_{50} < 50 µg/ml. B) Neutralization breadth at different IC_{50} cut-offs (Walker et al. 2011). The half maximal inhibitory concentration, IC_{50}, is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) in this case an antibody, is needed to inhibit a given biological process by half.

A

	Median IC_{50}
PGT121	0.03
PGT122	0.05
PGT123	0.03
PGT125	0.04
PGT126	0.04
PGT127	0.08
PGT128	0.02
PGT130	0.16
PGT131	0.52
PGT135	0.17
PGT136	7.81
PGT137	3.46
PGT141	0.35
PGT142	0.21
PGT143	0.31
PGT144	2.06
PGT145	0.29
PG9	0.23
VRC01	0.32
PGV04	0.20
b12	2.82
2G12	2.38
4E10	3.41

Key (µg/ml):	
	< 0.2
	0.2 - 2
	2 - 20

B

	Viruses neutralized		
	IC_{50} < 50 µg/ml	IC_{50} < 1 µg/ml	IC_{50} < 0.1 µg/ml
PGT121	70	57	44
PGT122	65	48	36
PGT123	67	54	40
PGT125	52	40	32
PGT126	60	50	40
PGT127	50	37	27
PGT128	72	60	50
PGT130	52	35	23
PGT131	40	23	13
PGT135	33	23	13
PGT136	16	6	3
PGT137	22	8	4
PGT141	56	36	15
PGT142	57	40	23
PGT143	56	37	17
PGT144	38	16	3
PGT145	78	52	27
PG9	77	54	29
VRC01	93	74	20
PGV04	88	65	25
b12	34	10	2
2G12	32	11	1
4E10	96	13	1

Key (%):	
	> 90
	60 - 90
	30 - 60
	1 - 30

Thus far only 2G12, 4E10 and 2F5 have undergone clinical evaluation (Armbruster 2002, 2004; Mehandru 2004; Joos, 2006), including a triple microbicide formulation developed as part of the European Microbicides Project [EMPRO; Dr. Charles Kelly-Hull York Medical School (HYMS) York, United Kingdom; and Dr. Charles Lacey-King's College London (London, United Kingdom), personal communication].

Monoclonal antibody 2F5 demonstrates broad neutralizing activity against different HIV-1 strains *in vitro* and in animal models (Huang et al. 2002; Cardoso et al. 2005) because it binds to the highly-conserved linear epitope ELDKWA found on the C-

terminus of the gp41 heptad (Muster et al. 1993; Binley et al. 2008). Binding inhibits the gp41 conformational changes required for HIV to interact with human cells (Parren and Burton, 2001; Zwick et al. 2001). Furthermore, in combination with 2G12 and/or 4E10, 2F5 has been shown to confer protection against intravenous, intrarectal, intravaginal and oral challenge with simian-human immunodeficiency virus (SHIV) in Rhesus macaques (Mascola et al. 1999, 2000; Baba et al. 2000; Hessell et al. 2010; Shen et al. 2010). Phase I clinical trials using passively administered 2F5 individually or in combination with other antibodies produced in Chinese hamster ovary (CHO) cells showed no evidence of toxicity or immunogenicity in human subjects (Armbruster et al. 2002, 2004; Mehandru et al. 2004). Phase II clinical studies with the three antibodies (2G12, 4E10 and 2F5) also passively administered indicate that 2F5 is effective as part of a monoclonal antibody cocktail to prevent the evolution of virus escapes (Joos et al. 2006; Manrique et al. 2007).

The inclusion of 2F5 in a broadly-accessible microbicide entails the use of a large-scale production platform (Shattock and Moore, 2003). Plants offer a more cost-effective and scalable manufacturing platform that could be used to produce mAbs locally (Ma et al. 2003; Ramessar et al. 2008a). Maize is particularly suitable in developing country settings that lack a cold chain and distribution network because recombinant proteins remain stable for months or years at ambient temperatures when produced and stored in seeds (Stoger et al. 2005; Ramessar et al. 2008a,b,c).

Several HIV-neutralizing mAbs have been produced in plants. The first was b12, which was expressed in tobacco as an IgG and also as a fusion (Sexton et al. 2009) with the anti-HIV lectin cyanovirin-N (Boyd et al. 1997; Dey et al. 2000). 4E10 was produced by secretion from hydroponic cultivated tobacco and was shown to assemble and function correctly although the overall yields were low (Drake et al. 2009). 2G12 has been expressed successfully in tobacco and was the first plant-derived mAb to complete a phase I clinical study in Europe (Fischer et al. 2012). This mAb 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 (Ramessar et al. 2008b; Rademacher et al. 2008).

In both cases the yields were high (40-100 mg/kg dry seed weight) and the purified antibody demonstrated HIV-1 neutralization activity that was similar to or greater than the same antibody produced in CHO cells. 2G12 was also expressed transiently in *Nicotiana benthamiana* with yields of up to 50 mg/kg fresh biomass (Sainsbury and Lomonossoff, 2008; Strasser et al. 2008). 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, where it was reported to have similar antigen-binding affinity to its CHO counterpart but less efficient HIV-1 neutralization (Sack et al. 2007).

3.3 Aim

We wished to test the hypothesis that the intrinsic properties of a particular macromolecule determine the level of its accumulation in a given production platform, rather the production platform itself. To that effect we expressed mAb 2F5 in maize seeds to determine whether it was possible to match the high yields and neutralization activity previously achieved with 2G12.

3.4 Materials and Methods

3.4.1 Transformation vectors

The 2F5 heavy (HC) and light chain (LC) genes (obtained from Polymun Scientific Immunobiologische Forschung, Vienna, Austria) were amplified by PCR using primers containing (i) restriction sites to facilitate cloning and (ii) a murine immunoglobulin chain signal peptide sequence to target the secretory pathway (Schillberg et al. 1999) and achieve appropriate post-translational modification. Two sets of transformation vectors were used, i.e. test constructs for constitutive transient expression in *N. benthamiana*, and constructs for stable endosperm-specific expression in transgenic maize plants.

The transient expression constructs were based on vector pL32 and contained the constitutive *Cauliflower mosaic virus* 35S (CaMV 35S) promoter, the murine immunoglobulin signal peptide sequence, the coding region of the HC/LC genes and the nopaline synthase (*nos*) gene terminator (Figure 3.3). Matching constructs were generated with the 2G12 HC and LC genes for comparison (Figure 3.4). A binary vector (pBI) containing the *Artichoke mottled crinkle virus* (AMCV) p19 silencing suppressor gene was used to avoid any potential post-transcriptional gene silencing in the infiltrated plants (Johansen and Carrington, 2001) (Figure 3.5). Post-transcriptional gene silencing (PTGS) is a general plant response that limits the efficiency of *Agrobacterium*-mediated transient expression. It has been reported that transient co-expression of viral suppressors of PTGS alleviates the host silencing response in wild-type *Nicotiana benthamiana*. The p19 protein is one of the most effective suppressor (Voinnet et al. 2003).

The stable transformation constructs pHor-2F5HC and pHor-2F5LC were generated by transferring the 2F5 HC and LC genes to the pHor vector, containing the endosperm-specific barley D-hordein promoter (Naqvi et al. 2009), the mouse immunoglobulin signal peptide and the rice *ADPGPP* gene terminator (Figure 3.6). A third construct, pTRAuxbar (obtained from Drs E. Stoger-BOKU, Vienna, Austria and T. Rademacher-Fraunhofer IME, Aachen, Germany) comprising the constitutive maize ubiquitin-1 promoter, the maize ubiquitin-1 first intron, the *bar* selectable marker gene and the CaMV 35S terminator was used to select transgenic plants on the basis of phosphinothricin resistance (Figure 3.7) (Zhu et al. 2008). Maize seeds accumulating 2G12 were generated previously and are described in Ramessar et al. 2008b (Figure 3.8).

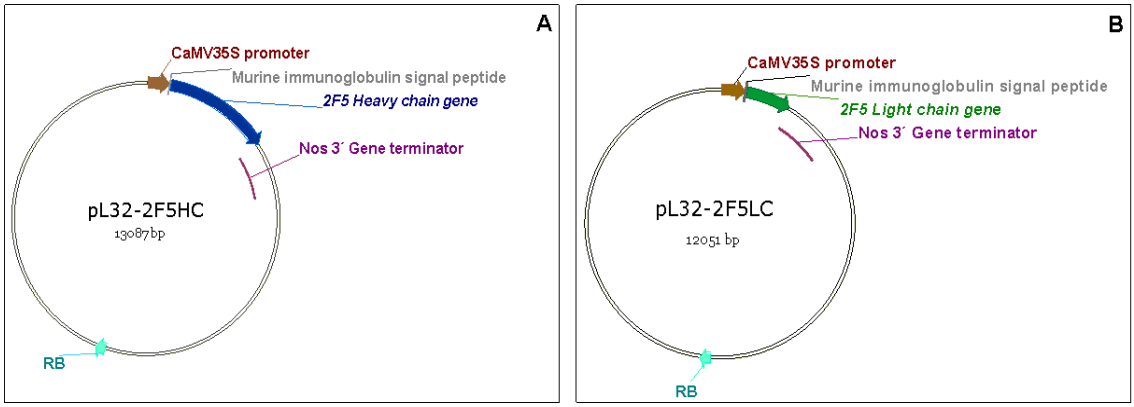

Figure 3.3: Transformation constructs for transient expression in *Nicotiana benthamiana* leaves A) pL32-2F5HC containing the 2F5 heavy chain (HC) gene; B) pL32-2F5LC containing the 2F5 light chain (LC) gene (adapted from Rogers et al. 1987).

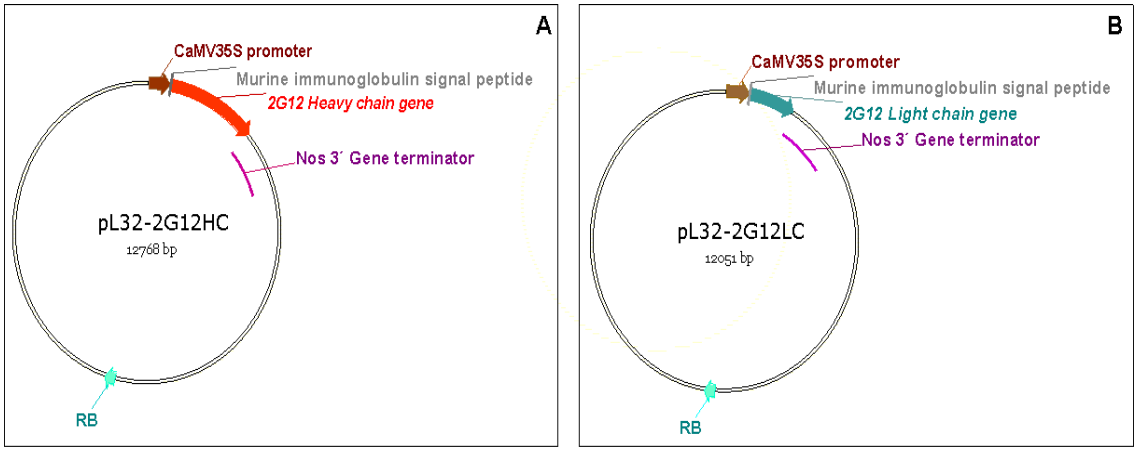

Figure 3.4: Transformation constructs for transient expression in *Nicotiana benthamiana* leaves A) pL32-2G12HC containing the 2G12 heavy chain (HC) gene; B) pL32-2G12LC containing the 2G12 light chain (LC) gene (adapted from Rogers et al. 1987).

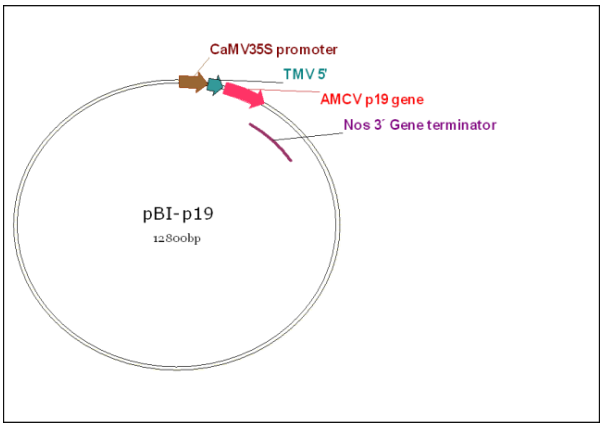

Figure 3.5: p35:AMCV-p19 construct for transient expression containing AMCV-p19 gene silencing suppressor (Lombardi et al. 2010).

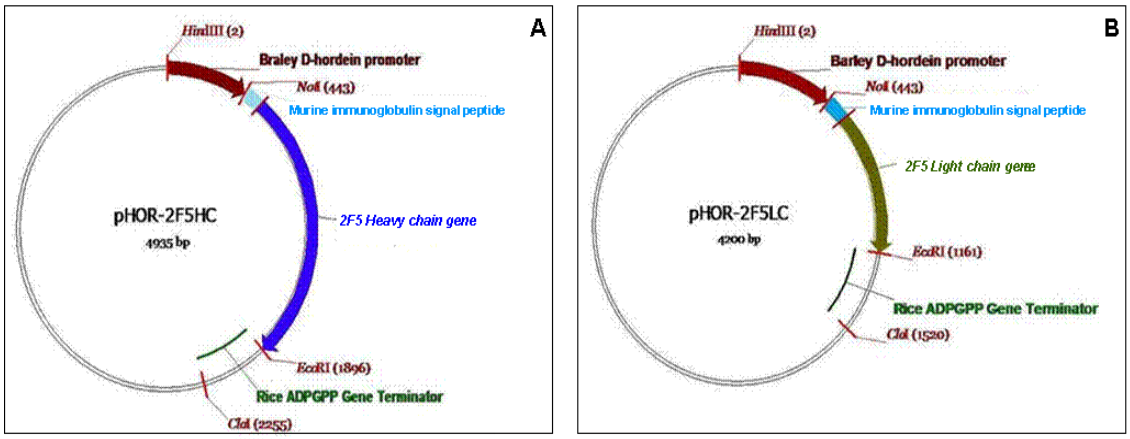

Figure 3.6: Transformation constructs for stable expression in maize seeds A) pHOR-2F5HC containing the 2F5 heavy chain (HC) gene; B) pHOR-2F5LC containing the 2F5 light chain (LC) gene (Naqvi et al. 2009).

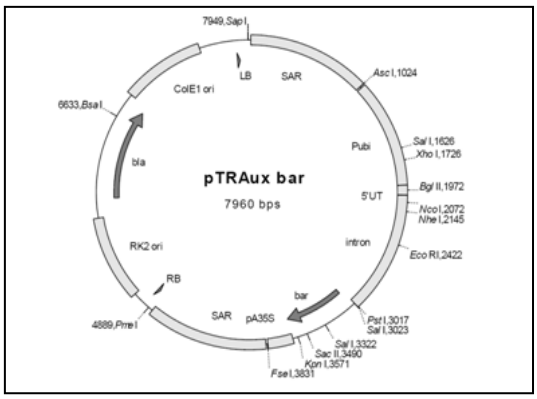

Figure 3.7: Transformation construct pTRAux-bar containing the bar selectable marker gene (Zhu et al. 2008).

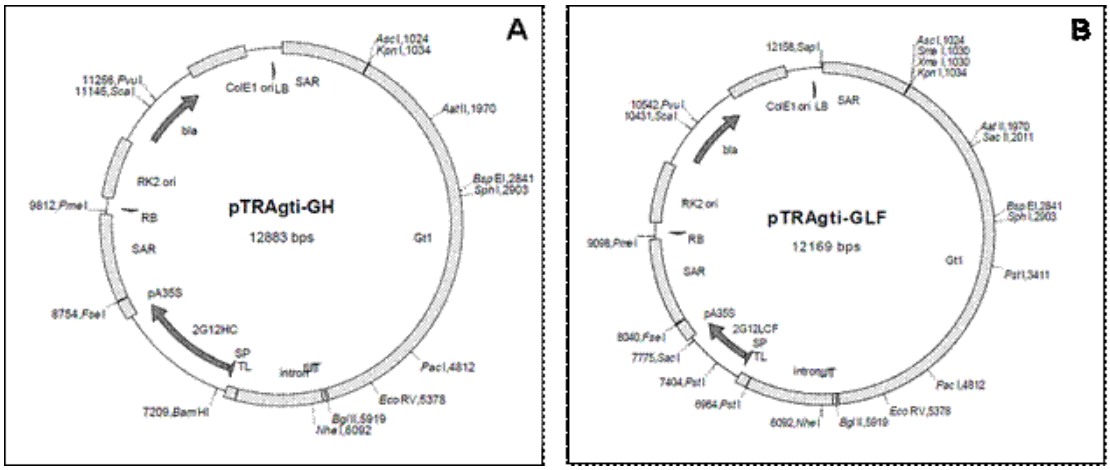

Figure 3.8: Transformation constructs for stable expression in maize seeds A) pTRAgti-GH containing the 2G12 heavy chain (HC) gene; B) pTRAgti-GLF contains the 2G12 light chain (LC) gene; In both constructs the expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the Tobacco Etch Virus 5'leader, the coding region, and the CaMV 35S terminator (Ramessar et al. 2008b).

3.4.2 Transient expression of mAb 2F5 in *N. Benthamiana* leaves

Individual bacterial clones were maintained in liquid culture until the OD_{600} reached 0.6, before mixing into one of two bacterial mixtures: the 2F5 mix (2F5HC, 2F5LC and p19) and the 2G12 mix (2G12HC, 2G12LC and p19). *N. benthamiana* leaves were infiltrated as previously described (Lombardi et al. 2010) with one of two bacterial mixtures described above. Samples were taken for analysis 6 days post infiltration (dpi), when transient accumulation levels are typically highest (Villani et al. 2008).

3.4.3 Stable expression of mAb 2F5 in transgenic maize seeds

Immature zygotic embryos of the South African elite white maize (*Zea mays*) inbred M37W were transformed by particle bombardment 10-14 days after pollination as described by Drakakaki et al. (2005) and modified by Naqvi et al. (2009) using 3:1 (by molecular weight) DNA ratio for the vector containing the gene of interest: *bar* selectable marker. Bombarded embryos were incubated in the dark and transferred every 2 weeks to fresh N6-based medium containing 3 mg/L phosphinothricin (PPT). After 4-6 weeks, pieces of PPT-resistant embryogenic type I callus were transferred to regeneration medium containing 3 mg/L PPT for 2-4 weeks with a 16-h photoperiod. All experiments were performed at 25°C. Developing plantlets with well-formed shoots and roots were hardened off and transferred to soil.

3.4.4 DNA blot analysis

Genomic DNA was isolated from maize leaves by phenol extraction (Edwards et al. 1991) and 20µg aliquots were digested overnight with restriction enzymes recognizing either a single site in the transformation vector (HindIII) or two sites flanking the 2F5 HC and LC transgenes (NotI/EcoRI). The DNA was fractionated by 0.8% agarose gel electrophoresis (Sambrook et al. 1989), transferred to a positively-charged nylon membrane (Roche, UK) and fixed by UV cross-linking. DIG-labeled probes were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and denatured at 90ºC for 10 min prior to hybridization overnight at 42ºC. Membranes were washed at

high stringency (twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 25 min in 0.5x SSC, 0.1% SDS at 68ºC, once for 15 min in 0.2x SSC, 0.1% SDS at 68ºC, and once for 10 min in 0.1x SSC, 0.1% SDS at 68ºC) prior to chemiluminescent detection using the DIG Luminescent Detection Kit (Roche, UK) according to the manufacturer's instructions. After washing, the membranes were incubated with CSPD chemiluminescent substrate (Roche, UK) and exposed on BioMax light film (Kodak, Rochester, NY, USA) at 37ºC.

3.4.5 Enzyme-linked immunosorbent assay (ELISA)

Agroinfiltrated *N. benthamiana* leaves (harvested 6 dpi) and transgenic maize endosperm tissue (embryo removed) were ground in two volumes of (phosphate buffered saline) PBS and centrifuged twice at 20,000 x *g* for 10 min at 4°C to remove plant debris.

The presence of assembled mAb was determined by coating Nunc Maxisorp 96-well Immunoplates (Nunc, Roskilde, Denmark) with anti-human γ-chain antiserum (The Binding Site, Birmingham, UK) and blocking with 2.5% (w/v) bovine serum albumin (BSA). Serial dilutions of the supernatant from plant tissue samples were then added, and captured 2F5 mAb was detected after a brief wash step by adding horseradish peroxidase (HRP)-conjugated sheep anti-human κ-chain antiserum (The Binding Site, Birmingham, UK; 1:1000 dilution), washing, developing the signal with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

The specific antigen binding activities of 2F5 and 2G12 were detected by coating ELISA wells with either 100 ng recombinant gp41 from HIV strain HXB2 (2F5) or 100 ng recombinant gp120 from HIV strain IIIB (2G12), both provided by the MRC Centralized Facility for AIDS reagents, Potters Bar, UK. After washing and blocking with BSA as above, serial dilutions of the 2F5 and 2G12 mAbs were added and the amount of bound antibody determined using HRP-conjugated anti γ-chain and HRP-

conjugated anti κ-chain antiserum as appropriate (The Binding Site, Birmingham, UK; 1:1000 dilution) with signal detection as above.

3.4.6 Western blot analysis

Proteins were separated under non-reducing conditions on a gradient 4-12% pre-cast Bis-Tris Nu- PAGE gels (Invitrogen, Carlsbad, CA, USA) using Precision Plus protein standards (BioRad, Hercules, USA). Each sample comprised 15 µl of maize 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). Separated proteins were blotted onto 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), mAbs were detected with HRP-conjugated IgG γ-chain or HRP-conjugated IgG κ-chain antiserum (The Binding Site, Birmingham, UK; 1:1000 dilution). After further washes, signals were detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, Little Chalfont, UK).

3.5 Results

3.5.1 Functional 2F5 antibody transiently expressed in *N. benthamiana* leaves

We produced 2F5 and 2G12 in *N. benthamiana* leaves by transient expression to test the expression constructs and to confirm the assembly of functional antibodies *in planta*. In separate experiments, leaves were infiltrated with two bacterial preparations, namely the 2F5 mix (2F5HC, 2F5LC and p19) and the 2G12 mix (2G12HC, 2G12LC and p19). In both cases, the presence of correctly assembled antibody molecules was confirmed using an immunoglobulin-specific sandwich ELISA in which the plates were coated with a HC-specific capture antibody, and bound 2F5 and 2G12 were detected with a LC-specific antibody conjugated to HRP. This ensured that only correctly assembled antibodies were detected and free HC and LC molecules were excluded. The concentrations of antibodies were calculated using titration curves compared with the

positive controls at known concentrations and no substantial difference was observed between 2F5 and 2G12 transiently expressed in *N.bentahmiana* leaves (Figure 3.9A). We used corresponding mAbs produced in CHO cells as positive controls and non-infiltrated tobacco leaves as a negative control to rule out cross-reaction with endogenous plant proteins.

Having confirmed mAb expression and assembly, we used an antigen-specific ELISA to investigate mAb binding activity. In this assay, the capture reagent was $gp41_{HXB2}$ for 2F5 and $gp120_{IIIB}$ for 2G12, and a mixture of anti-human gamma and anti-human kappa antibodies (which bind HC and LC, respectively) was used for detection (Figure 3.9B, C). This experiment showed that the plant-derived antibodies bound to their specific antigens as expected.

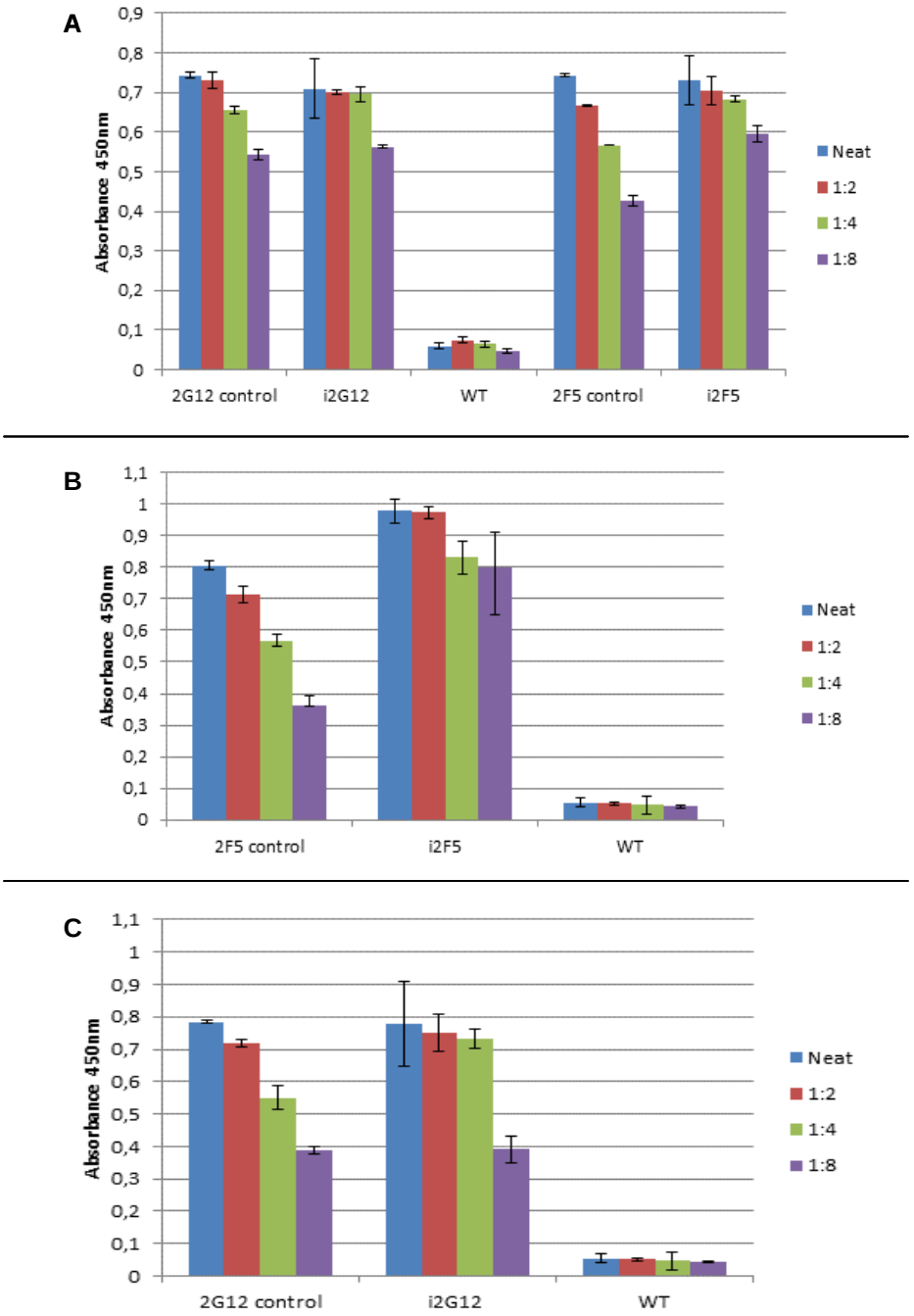

Figure 3.9 Structural and functional analysis of mAbs 2F5 and 2G12 produced by transient expression in *N. benthamiana* leaves (i2F5 and i2G12). A) Assembled 2F5 and 2G12 in leaf extracts were detected using a sandwich ELISA, comprising a heavy chain-specific anti-human IgG γ-chain capture antibody and a light chain-specific HRP-conjugated anti-human IgG κ-chain detection antibody (absorbance measured at 450 nm). Data represent neat extracts and dilutions as well as 1 µg/ml CHO-derived reference antibodies and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings. B) Antigen-specific ELISA using gp41 as capture reagent for 2F5 and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies. Data represent neat extracts and dilutions as well as 1 µg/ml CHO-derived reference antibody and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings. C) Antigen-specific ELISA using gp120 as capture reagent for 2G12 and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies. Data represent neat extracts and dilutions as well as 1 µg/ml CHO-derived reference antibody and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings.

3.5.2 Integration of input genes in transgenic maize plants

Having established that the HC and LC constructs were suitable for expression in plants, we bombarded maize embryos with the vectors pHor-2F5HC, pHor-2F5LC and pTRAuxbar, and regenerated transgenic plants from the resulting callus. These plants were either selfed or crossed with wild-type M37W plants depending on pollen availability to generate 20 independent transgenic lines.

DNA blot analysis confirmed the presence of the antibody transgenes in most of the lines, and the unique integration patterns revealed by cleaving genomic DNA with HindIII (which has a single restriction site in each transgene) confirmed that each line represented an independent transformation event (representative results for the 2F5 HC are shown in Figure 3.10A, results for the 2F5 LC are not shown but are similar to the results for the HC). We anticipated linkage of the three input transgenes based on the tendency of separate transformation constructs to integrate at the same locus (Nicholson et al. 2005; Drakakaki et al. 2005; Altpeter et al. 2005; Kohli et al. 2006) and this was confirmed in the T1 generation by the Mendelian segregation of the transgenes. Digestion with NotI and EcoRI released the 1453-bp 2F5HC cassette (Figure 3.10B) and the 713-bp 2F5LC cassette in all 5 maize lines confirming their integrity (representative results for the 2F5 HC are shown in Figure 3.10B, results for the 2F5 LC are not shown).

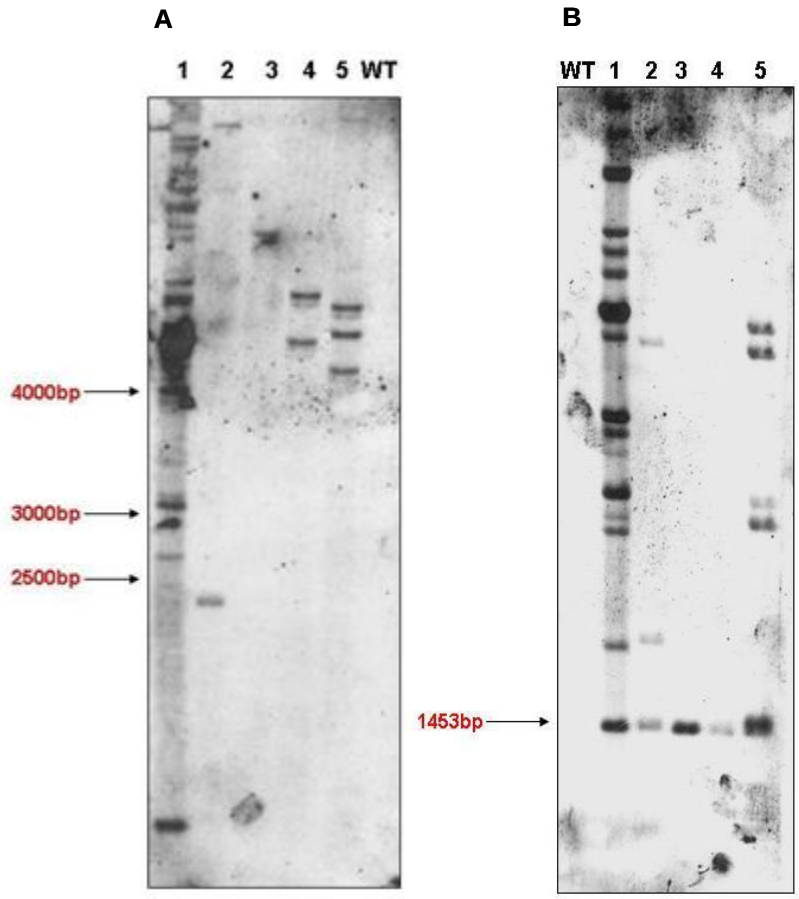

Figure 3.10 DNA blot analysis to verify the independent origin of five transgenic maize lines (lanes 1-5) and the integrity of the integrated transgene cassette. A) Digestion of genomic DNA with HindIII, which cuts once within the transgene cassette, and detection with a heavy chain-specific probe. The different sized fragments in each transgenic line confirm their independent origin. B) Digestion of genomic DNA with NotI and EcoRI, which flank the heavy chain cassette, and detection with a heavy chain-specific probe. The released fragment is consistent with the expected size of 1453 bp. Wild type maize genomic DNA was used as a control. Similar results were obtained with a light chain-specific probe (not shown).

3.5.3 Immunoglobulin-specific ELISA and native western blot demonstrate that 2F5 is correctly assembled in maize endosperm

Six T1 generation seeds each from five representative 2F5 independent transgenic lines (as described above) and five T3 generation seeds from a previously-described transgenic line expressing 2G12 (Ramessar et al. 2008b) were separated into embryo and endosperm. We tested endosperm extracts by ELISA and native western blot to investigate antibody expression, folding and assembly. Embryos from seeds with the highest 2F5 accumulation in the endosperm were germinated, and the best-performing

T1 lines were selected and grown to maturity. The resulting plants were either selfed or crossed with wild-type M37W depending on pollen availability to identify T2 homozygous lines.

The endosperm extracts were tested using the immunoglobulin-specific ELISA procedure discussed above to confirm the presence of assembled antibody. Representative results from 8 seeds from 3 different lines are shown in Figure 3.11A, with corresponding antibodies produced in CHO cells as positive controls and non-transgenic endosperm extract as a negative control to rule out cross-reactivity. These experiments confirmed the presence of assembled 2F5 (Figure 3.11A) and 2G12 (Figure 3.11B). Overall we found that 2F5 accumulated to a consistently lower level (0.80± 0.33 µg/ml) than 2G12 and the best 2F5 seeds produced three-fold lower amounts of antibody compared to the best 2G12 seeds (3.98 ± 0.83 µg/ml).

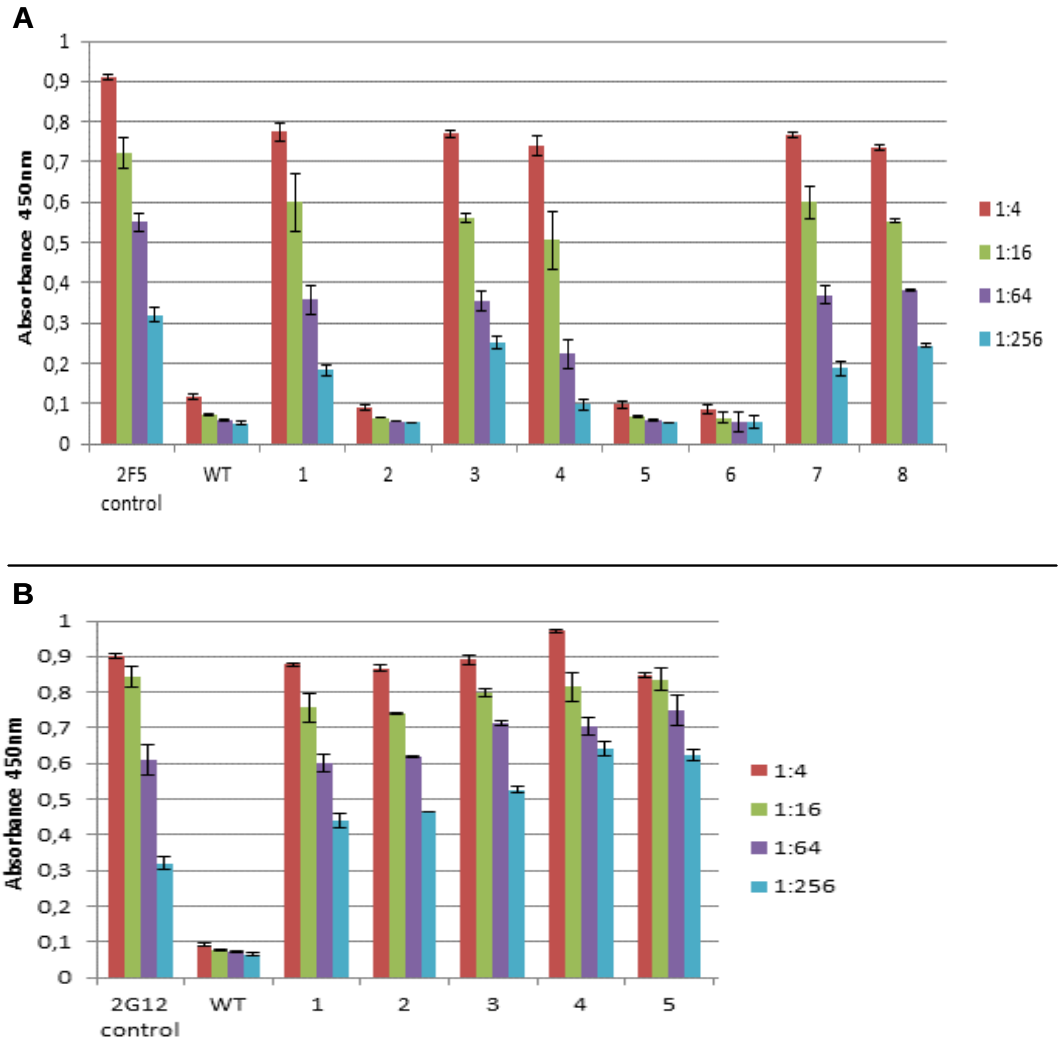

Figure 3.11 Structural analysis of mAbs 2F5 and 2G12 produced by stable expression in maize. Assembled A) 2F5 and B) 2G12 in endosperm extracts were detected using a sandwich ELISA, comprising a heavy chain-specific anti-human IgG γ-chain capture antibody and a light chain-specific HRP-conjugated anti-human IgG κ-chain detection antibody (absorbance measured at 450 nm). In A) lanes 1-8 represent three independent transgenic lines expressing 2F5 (three seeds from one event in lanes 1-3, three seeds from a second event in lanes 4-6 and two seeds from a third event in lanes 7-8); B) lanes 1-5 represent five seeds from a single previously-described transgenic maize line expressing 2G12. Data represent extract dilutions as well as 1 µg/ml CHO-derived reference antibodies and wild-type endosperm extract as a negative control (mean ± SD of triplicate readings).

Non-reducing western blots of seed extracts from the three best-performing 2F5 transgenic lines revealed the presence of a 150-kDa product in all lines. This is the expected size of a tetrameric mAb comprising two 50-kDa HCs and two 25-kDa LCs and is identical to the size of the assembled CHO-derived 2F5 antibody which was used as a control (Figure 3.12). The 150-kDa assembled antibody is also the dominant

product in the 2G12 seeds (Figure 3.13). In both cases, there is also a ~25-kDa product, which corresponds to excess LC, and additional bands (indicated by red arrows in Figure 3.12 that are likely to be degradation products. Excess HC molecules are present in the CHO 2F5 control sample whereas the LC product is absent (Figure 3.12).

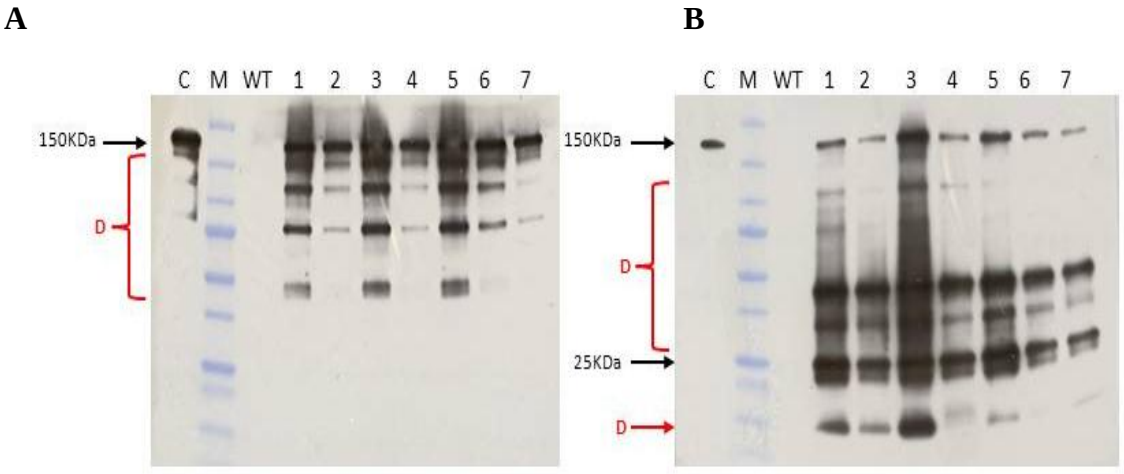

Figure 3.12 Western blot analysis of maize-derived 2F5 under non-reducing conditions using A) heavy chain-specific anti-human IgG γ-chain and B) light chain-specific anti-human IgG κ-chain antibodies. C = positive control (50 ng 2F5 produced in CHO cells), M = protein size markers, WT = wild type seed extract as a negative control. Lanes 1-7 represent three independent transgenic lines expressing 2F5 (three seeds from one event in lanes 1-3, three seeds from a second event in lanes 4-6 and two seed a from a third event in lane 7).

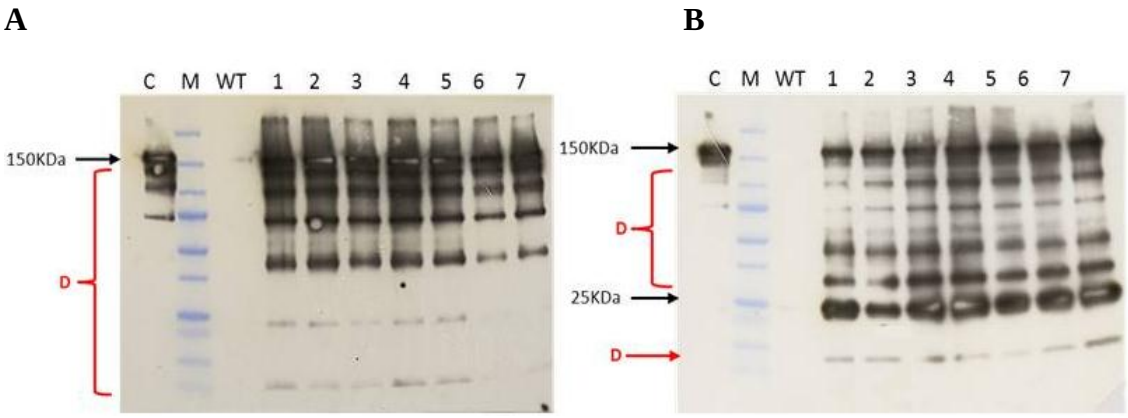

Figure 3.13 Western blot analysis of maize-derived 2G12 under non-reducing conditions using A) heavy chain-specific anti-human IgG γ-chain and B) light chain-specific anti-human IgG κ-chain antibodies. C = positive control (50 ng 2G12 produced in CHO cells), M = protein size markers, WT = wild type seed extract as a negative control. Lanes 1-7 represent one independent transgenic line expressing high levels of 2G12. Lanes 1-7 represent 7 seeds from a single transgenic line maize seed.

3.5.4 2F5 expression in maize endosperm

Having established that 2F5 is correctly assembled in maize endosperm, we used the antigen-specific ELISA procedure described above with crude extracts to estimate the binding activity of plant-derived mAb against gp41 (Figure 3.14). All extracts from transgenic seeds generated a signal greater than the wild type extract background, indicating that all the seeds produced a functional 2F5 antibody. There was no cross-reactivity between 2F5 and endogenous plant proteins showing that the plant-derived antibody was specific. The binding activity between plant-derived 2F5 and gp41 was similar to that between the control 2F5 antibody produced in CHO cells and gp41 and to that between plant-derived 2G12 and gp120 (Figure 3.15). However, these experiments confirmed that the abundance of functional antibody was lower than previously achieved by expressing 2G12 using the same platform. The highest concentration of functional 2F5 antibody in the seed extract was 0.61 ± 0.28 µg/ml, whereas for 2G12 the equivalent value was 3.68 ± 0.56 µg/ml.

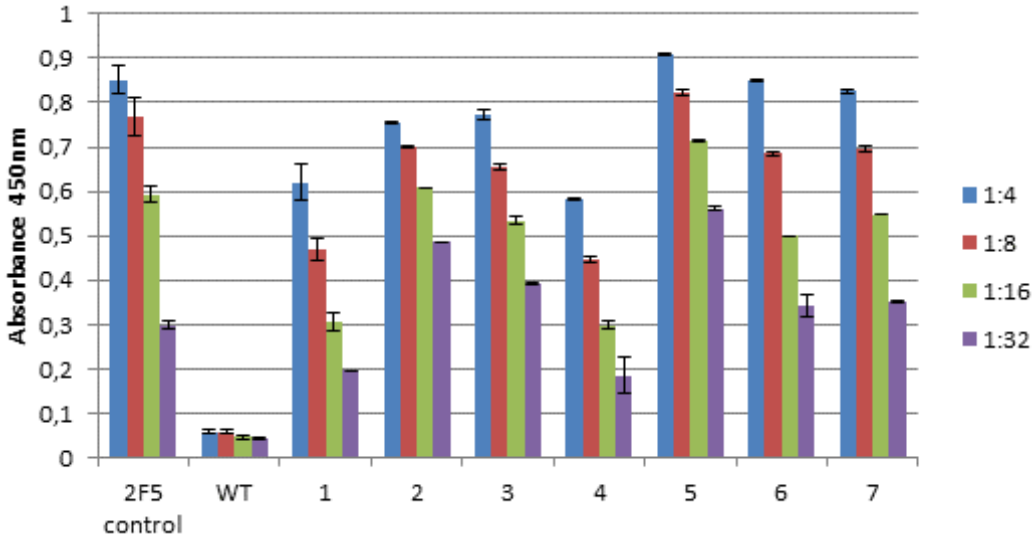

Figure 3.14 Functional analysis of 2F5 produced in transgenic maize seeds using gp41 as the capture reagent and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies (absorbance measured at 450 nm). Data represent crude extract dilutions as well as 1 µg/ml CHO-derived reference 2F5 and wild-type seed extract as a negative control, (mean ± SD of triplicate readings). Three independent events are shown: 1-3 three seeds from one event; 4-5 two seeds from a second event; and 6-7 two seeds from a third event.

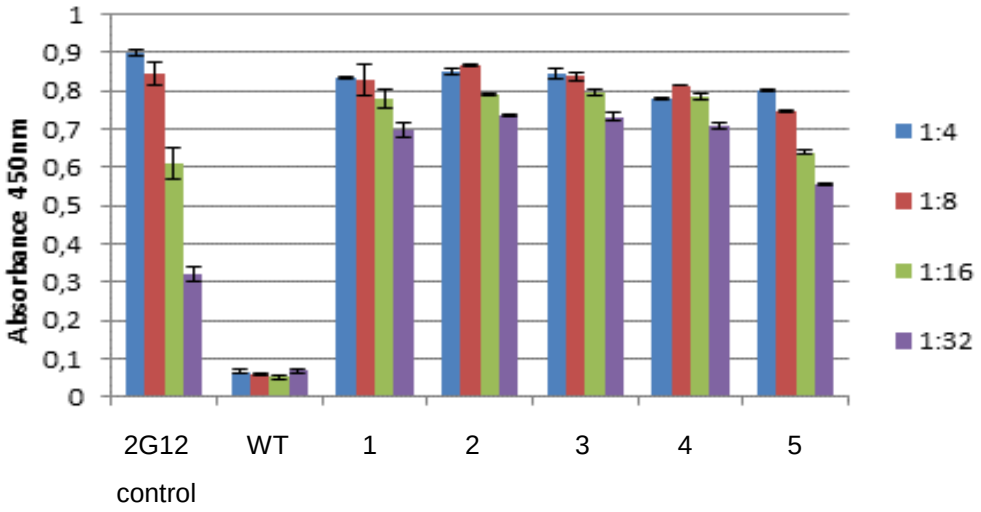

Figure 3.15 Functional analysis of 2G12 produced in transgenic maize seeds using gp120 as the capture reagent and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies (absorbance measured at 450 nm); 1-5 five seeds from a single previously-described transgenic maize line expressing 2G12. Data represent crude extract dilutions as well as 1 µg/ml CHO-derived reference 2G12 and wild-type seed extract as a negative control, (mean ± SD of triplicate readings).

3.6 Discussion

HIV/AIDS is an ongoing pandemic that has caused more than 30 million deaths since the virus was first recognized in 1981 (UNAIDS, 2010). There is no cure for HIV/AIDS and efficacious vaccines against HIV are currently unavailable (Walker and Burton, 2010). Microbicides that block the transmission of HIV are considered one of the most promising strategies to slow the HIV/AIDS pandemic in developing countries (where 20-30% of the population is infected) because it allows women to play a proactive role in sexual health rather than relying on men to cooperate in the use of condoms.

Human antibodies are preferred over humanized or murine antibodies because they tend not to induce an immune response against the microbicide itself even after repeated applications (Radomsky et al. 1992). HIV-neutralizing mAbs are also persistent, and therefore not only reduce male-to-female HIV-1 transmission at the time of intercourse, but also neutralize virus particles in vaginal secretions and thus also reduce HIV-1 transmission from women to men (Veazey et al. 2003). The advantageous properties of HIV-neutralizing mAbs are offset by the costs of manufacture. The current industry standard is to produce antibodies by fermentation in mammalian cells, which is suitable

for products required in relatively small amounts (e.g. for the treatment of cancer), but far too expensive for the manufacture of 'commodity' antibodies that are required on a much larger scale. Plants have emerged as a suitable alternative to mammalian cells where cost-effective large-scale production is necessary (Stoger et al. 2002; Ma et al. 2003; Fischer et al. 2004). 2G12 was the first HIV-neutralizing mAb to be manufactured in plants under good manufacturing practice (GMP) guidelines and recently completed a phase I clinical trial as a microbicide (Fischer et al. 2012).

The clinical-grade 2G12 was produced in transgenic tobacco plants, which grow rapidly and produce large amounts of biomass but require a conventional processing infrastructure including a cold chain. In developing country settings where such facilities are generally not available, seed-based production systems maintain the economy of scale enjoyed by tobacco but also allow the crude bulk drug to be stockpiled at ambient temperatures without product degradation (Stoger et al. 2005). Maize is advantageous because it is widely grown in developing countries and has already been developed as a commercial platform for recombinant protein production (Naqvi et al. 2011).

We had previously expressed 2G12 in maize seeds and found that the antibody was expressed at high levels, was easy to purify from ground endosperm and demonstrated similar or even better HIV-neutralization activity than 2G12 derived from CHO cells (Ramessar et al. 2008b). This established the principle of efficient functional antibody expression in maize. However, in order to reduce the likelihood of viral escape, microbicides must contain a cocktail of mAbs recognizing different epitopes of the HIV virus. Therefore it is necessary to establish whether the same platform can be used to produce additional HIV-neutralizing mAbs.

The production of 2F5 in plants had previously been achieved using two different tobacco-based systems, in one case suspension cells expressing the free antibody (Sack et al. 2007) and in the other transgenic plants expressing a fusion protein comprising the antibody joined to an elastin like peptide (ELP) to increase its stability and facilitate

extraction (Floss et al. 2008). The suspension cells produced low yields of 2F5 and although the antibody demonstrated a similar affinity to its antigen as a CHO-derived counterpart, its HIV-neutralization activity was reduced (Sack et al. 2007). Two fusion constructs were produced in transgenic plants, one with the fusion tag on the heavy chain and one with the tag on the light chain, both strategies achieving higher yields than the untagged protein, which accumulated to only low levels (Floss et al. 2008). A comparison between the untagged 2F5 expressed in transgenic tobacco and the similarly untagged 2G12 expressed in the same platform (Fischer et al. 2012) revealed significant differences in yield which could reflect differences in the strategy for product development (i.e. the breeding program to create the production line) or intrinsic properties of the antibody.

As a first step towards developing a generic maize-based platform for the production of different HIV-neutralizing antibodies for a microbicidal cocktail, we therefore attempted to express 2F5 in maize using precisely the same strategy as previously adopted for 2G12. First it was necessary to confirm that the cDNA constructs containing the antibody HC and LC sequences were suitable for expression in plants, and this was achieved by transient expression in *N. benthamiana* leaves to demonstrate transgene expression and functional antibody assembly. All steps were carried out in parallel for both 2F5 and 2G12, the latter as a control. These experiments demonstrated conclusively that all transgenes were expressed, the antibodies assembled correctly and were able to bind their antigens and there was no cross-reaction to endogenous plant proteins.

The transient expression data suggested that the constructs were suitable for the production of transgenic maize plants and the transgenes were duly transferred to expression vectors promoting endosperm-specific expression in maize. We recovered 20 independent transgenic lines as verified by unique transgene integration patterns in DNA blots and selected a representative number for expression analysis. The 2F5 transgenic maize lines were tested in parallel with our previously-reported elite transgenic line expressing 2G12 to compare their performance. We repeated the

experiments to confirm antibody expression, assembly and functionality, but found that whereas the transient expression data reliably predicted the structural and functional integrity of 2F5, there was no relationship between the yields achieved in the two sets of experiments. Indeed, we found that the yield of 2F5 in the best-performing plants was consistently lower than the best 2G12 lines.

The differences between 2F5 and 2G12 levels achieved in transgenic tobacco plants could reflect differences in experimental conditions, breeding and/or the intrinsic properties of the antibody. Yields of the same recombinant antibody can vary substantially in different plant systems, particularly when comparing transient and stable expression, whole plants and suspension cultures, or transgenic and transplastomic plants (Daniell et al. 2001; Hellwig et al. 2004; Strasser et al. 2008). For example, 2G12 accumulates to different levels when transiently expressed in *N. benthamiana* and stably expressed in tobacco plants and suspension cells (Platis et al. 2008; Dr. Stefan Schillberg, Fraunhofer IME, Aachen, Germany, personal communication).

The plant production species may also affect yields, partly because common regulatory elements may differ in efficiency in different genetic backgrounds, partly because the efficiency of protein synthesis may reflect species-dependent codon preferences, and partly because the intracellular environment may affect protein stability in a species-dependent context (Torres et al 1999; Stoger et al. 2000; Desai et al. 2010; Kahn et al. 2012). Yields in the same production platform can vary when different promoters are used to drive transgene expression (Peremarti et al. 2010) or when different targeting strategies are employed (Kahn et al. 2012). For example, 2G12 accumulated to 75 mg/kg dry seed weight in maize when secreted to the apoplast, but to only 40 mg/kg dry seed weight when retained in the endoplasmic reticulum (Ramessar et al. 2008b; Rademacher et al. 2008).

The track record for 2F5 shows that, in three different transgenic plant systems, the yields are lower than 2G12. Our results in transgenic maize are consistent with

transgenic tobacco (Floss et al. 2008) and tobacco suspension cells (Sack et al. 2007). Our transgenic maize lines expressing 2F5 and 2G12 were prepared using the same techniques and selection procedures, allowing head-to-head comparison between the antibodies with other factors taken into account. The discordant transient and stable expression data in tobacco suggest that the 2F5 and 2G12 molecules probably do not differ significantly in terms of intrinsic stability and that the difference in yields in transgenic systems may reflect a combination of host-dependent interactions, such as the abundance of different proteases and the potential for unique sequences within the transgene to induce silencing. The intrinsic stability of 2F5 is also supported by the high yields achieved in other platforms such as *Pichia pastoris* (Gasser et al. 2006), hybridoma lines (Kunert et al. 1998) and CHO cells (Kunert et al. 2000) and also the sequence homology between 2F5 and 2G12 in the constant region domains of the antibodies. It therefore seems likely that the variable region of the 2F5 antibody may render it more susceptible than 2G12 to the particular spectrum of proteases found in maize seeds (comparing Figure 3.12B with Figure 3.13B shows more degradation in the 2F5 lane, supporting this hypothesis) and/or that certain unique aspects of the 2F5 cDNA sequence render it more susceptible to silencing in maize. These hypotheses are not mutually exclusive - indeed the accumulation of any recombinant protein is always dependent on the combined influences affecting transgene expression (product synthesis) and protein stability (product degradation).

3.7 Conclusions

We have achieved the production of the HIV-neutralizing mAb 2F5 in transgenic maize seeds. Thus maize could be used to produce multiple antibodies as components of a microbicidal cocktail to prevent the spread of HIV. The maize-derived antibody was assembled efficiently *in planta* and bound its corresponding antigen in the same way as a control 2F5 preparation produced in CHO cells. Although the antibody appeared stable and the degradation was not significantly greater than 2G12 produced using the same platform, the overall yield of the antibody was consistently more than threefold lower than 2G12 produced in an identical background, suggesting 2F5 is more sensitive

than 2G12 to platform-specific factors such as maize proteases or transgene silencing. We found that 2F5 accumulated to similar levels as 2G12 by transient expression in *N. benthamiana* leaves suggesting that 2F5 is not intrinsically less stable than 2G12 and that the cDNA does not contain codons that reduce the efficiency of protein synthesis. The low levels of 2F5 expression achieved in other transgenic plant systems including tobacco suggest that the bottleneck may involve a combination of factors including sensitivity to specific proteases, the influence of transgene silencing or some form of incompatibility between the 2F5 transgene and the adjacent regulatory elements in the construct.

3.8 References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N and Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding* 15:305-327

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Michael NL, Vetter N and Katinger HW (2002) A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. *AIDS* 16:227-233

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Köller U, Jilch R, Ammann CG, Pruenster M, Stoiber H and Katinger HWD (2004) Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. *Journal of Antimicrobial Chemotherapy* 54:915-920

AVERT (2012) HIV structure, accessed online 22 October 2012, http://www.avert.org/hiv-virus.htm

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu WD, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu YC, Wright JE, Chou TC and Ruprecht RM (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nature Medicine* 6:200-206

Binley JM, Lybarger EA, Crooks ET, Seaman MS, Gray E, Davis KL, Decker JM, Wycuff D, Harris L, Hawkins N, Wood B, Nathe C, Richman D, Tomaras GD, Bibollet-Ruche F, Robinson JE, Morris L, Shaw GM, Montefiori DC and Mascola JR (2008) Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. *Journal of Virology* 82:11651-11668

Boyd M, Gustafson K, McMahon J, Shoemaker R, O'Keefe B, Mori T, Gulakowski R, Wu L, Rivera M, Laurencot C, Currens M, Cardellina J, Buckheit R Jr, Nara P Jr, Pannell L, Sowder R Jr and Henderson L (1997) Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. *Antimicrobial Agents of Chemotherapy* 41:1521-1530

Burton DR and Weiss RA (2010) A boost for HIV vaccine design. *Science* 329:770-773

Cardoso RMF, Zwick MB, Stanfield RL, Kunert R, Binley JM, Katinger H, Burton DR and Wilson IA (2005) Broadly neutralizing anti-HIV antibody, 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* 22:163-173

Daniell H, Streatfield SJ and Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Science* 6:219-226

Desai P, Shrivastava N and Padh H (2010) Production of heterologous protein in plants: strategies for optimal expression. *Biotechnology Advances* 28:427-435

Dey B, Lerner DL, Lusso P, Boyd MR, Elder J and Berger EA (2000) Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. *Journal of Virology* 74:4562-4569

Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P and Stoger E (2005) Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. *Plant Molecular Biology* 59:869-880

Drake PMW, Barbi T, Sexton A, McGowan E, Stadlmann J, Navarre C, Paul MJ and Ma JKC (2009) Development of rhizosecretion as a production system for recombinant proteins from hydroponic cultivated tobacco. *FASEB Journal* 23:3581-3589

Edwards K, Johnstone C and Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19:1349

Fischer R, Stoger E, Schillberg S, Christou P and Twyman RM (2004) Plant-based production of biophramaceuticals. *Current Opinion in Plant Biotechnology* 7:152-158

Fischer R, Schillberg S, Hellwig S, Twyman RM and Drossard J (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnology Advances* 30:434-439

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R and Conrad U (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. *Plant Biotechnology Journal* 6:379-391

Gasser B, Maurer M, Gach J, Kunert R and Mattanovich D (2006) Engineering of *Pichia pastoris* for improved production of antibody fragments. *Journal of Biotechnology and Bioengineering* 4:353-361

Hellwig S, Drossard J, Twyman RM and Fischer R (2004) Plant cell cultures for the production of recombinant proteins. *Nature Biotechnology* 22:1415-1422

Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, Forthal DN, Koff WC, Poignard P, Watkins DI and Burton DR (2010) Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. *Journal of Virology* 84:1302-1313

Huang J, Dong X, Liu Z, Qin L and Chen YH (2002) A predefined epitope-specific monoclonal antibody recognizes ELDEWA-epitope just presenting on gp41 of HIV-1 O clade. *Immunology Letters* 84:205-209

Johansen LK and Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiology* 126:930-938

Joos B, Trkola A, Kuster H, Aceto L, Fischer M, Stiegler G, Armbruster C, Vcelar B, Katinger H and Günthard FH (2006) Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (mAbs) against human immunodeficiency virus type 1 envelope gp120 (mAb 2G12) and gp41 (mAbs 4E10 and 2F5). *Journal of Antimicrobial Chemotherapy* 50:1773-1779

Khan I, Twyman RM, Arcalis E and Stoger E (2012) Using storage organelles for the accumulation and encapsulation of recombinant proteins. *Biotechnology Journal* 7:1099-1108

Koff WC (2012) HIV vaccine development: Challenges and opportunities towards solving the HIV vaccine-neutralizing antibody problem. *Vaccine* 30:4310-4315

Kohli A, Gonzalez-Melendi P, Abranches R, Capell T, Stoger E and Christou P (2006) The quest to understand the basis and mechanisms that control expression of introduced transgenes in crop plants. *Plant Signal Behavior* 1:185-195

Kunert R, Rüker F and Katinger H (1998) Molecular characterization of five neutralizing anti-HIV type 1 antibodies: Identification of nonconventional D segments in the human monoclonal antibodies 2G12 and 2F5. *AIDS Research and Human Retroviruses* 14:1115-11128

Kunert R, Steinfellner W, Purtscher M, Assadian A and Katinger H (2000) Stable recombinant expression of the anti HIV-1 monoclonal antibody 2F5 after IgG3/IgG1 subclass switch in CHO cells. *Journal of Biotechnology and Bioengineering* 67:97-103

Lombardi R, Villani ME, Di Carli M, Brunetti P, Benvenuto E and Donini M (2010) Optimisation of the purification process of a tumourtargeting antibody produced in *N. benthamiana* using vacuum agroinfiltration. *Transgenic Research* 19:1083-1097

Ma JKC, Drake PMW and Christou P (2003) The production of recombinant pharmaceutical proteins in plants. *Nature Review Genetics* 4:794-805

Manrique A, Rusert P, Joos B, Fischer M, Kuster H, Leemann C, Niederöst B, Weber R, Stiegle G, Katinger H, Günthard HF and Trkola A (2007) *In vivo* and *In vitro* escape from neutralizing antibodies 2G12, 2F5 and 4E10. *Journal of Virology* 16:8793-8808

Mascola JR, Lewis MG, Stiegler G, Harris D, Van Cott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu YC, Robb ML, Katinger H and Birx DL (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *Journal of Virology* 73:4009-4018

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL and Lewis MG (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nature Medicine* 6:207-210

Mehandru S, Wrin T, Galovich J, Stiegler G, Vcelar B, Hurley A, Hogan C, Vasan S, Katinger H, Petropoulos CJ and Markowitz M (2004) Neutralization profiles of newy transmitted human immunodeficiency virus type 1 by monoclonal antibodies 2G12, 2F5 and 4E10. *Journal of Virology* 78:14039-14042

Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler H, Ruker F and Katinger H (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *Journal of Virology* 67:6642-6647

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T and Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proceedings of the National Academy of Sciences USA* 106:7762-7767

Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C and Christou P (2011) High-value products from transgenic maize. *Biotechnology Advances* 29:40-53

Nicholson L, Gonzalez-Melendi P, Van Dolleweerd C, Tuck H, Ma JKC, Fischer R, Christou P and Stoger E (2005) A recombinant multimeric immunoglobulin expressed in rice shows assembly-dependent subcellular localization in endosperm cells. *Plant Biotechnology Journal* 3:115-127

Parren PW and Burton DR (2001) The antiviral activity of antibodies *in vitro* and *in vivo*. A definitive review on how antibodies inhibit viral infection, HIV-1 infection in particular. *Advances in Immunology* 77:195-262

Peremarti A, Twyman RM, Gómez-Galera S, Naqvi S, Farré G, Sabalza M, Miralpeix B, Dashevskaya S, Yuan D, Ramessar K, Christou P, Zhu C, Bassie L and Capell T (2010) Promoter diversity in multigene transformation. *Plant Molecular Biology* 73:363-378

Platis D, Drossard J, Fischer R, Ma JKC and Labrou NE (2008) New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. *Journal of Chromatography A* 1211:80-89

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

Radomsky ML, Whaley KJ, Cone RA and Saltzman WM (1992) Controlled vaginal delivery of antibodies in the mouse. *Biology of Reproduction* 47:133-140

Ramessar K, Capell T and Christou P (2008a) Molecular pharming in cereal crops. *Phytochemistry Review* 7:579-592

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma JKC, Stoger E, Capell T and Christou P (2008b) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proceedings of the National Academy of Sciences USA* 105:3727-3732

Ramessar K, Sabalza M, Capell T and Christou P (2008c) Maize plants: An ideal production platform for effective and safe molecular pharming. *Plant Science* 174:409-419

Ramessar K, Sabalza M, Miralpeix B, Capell T and Christou P (2010) Can microbicides turn the tide against HIV? *Current Pharmaceutical Design* 16:468-485

Rogers SG, Klee HJ, Horsch RB and Fraley RT (1987) Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. *Methods in Enzymology* 153:253-277

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoger E and Rademacher T (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB Journal* 21:1655-1664

Sainsbury F and Lomonossoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiology* 148:1212-1218

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schillberg S, Zimmermann S, Voss A and Fischer R (1999) Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum*. *Transgenic Research* 8:255-263

Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock RJ and Ma JKC (2009) Transgenic plant production of cyanovirin-N, an HIV microbicide. *FASEB Journal* 20:356-368

Shattock RJ and Moore JP (2003) Inhibiting sexual transmission of HIV- 1 infection. *Nature Review Microbiology* 1:25-34

Shen R, Drelichman ER, Bimczok D, Ochsenbauer C, Kappes JC, Cannon JA, Tudor D, Bomsel M, Smythies LE and Smith PD (2010) gp41-specific antibody blocks cell-free HIV type 1 transcytosis through human rectal mucosa and model colonic epithelium. *Journal of Immunology* 184:3648-3655

Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, Lehrman JK, Boaz M, Tarragona-Fiol T, Miiro G, Birungi J, Pozniak A, McPhee DA, Manigart O, Karita E, Inwoley A, Jaoko W, Dehovitz J, Bekker LG, Pitisuttithum P, Paris R, Walker LM, Poignard P, Wrin T, Fast PE, Burton DR and Koff WC (2009) Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent

neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *Journal of Virology* 83:7337-7348

Stamatatos L, Morris L, Burton DR and Mascola JR (2009) Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nature Medicine* 15:866-870

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P and Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Molecular Biology* 42:583-590

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P and Fischer R (2002) Practical considerations for pharmaceutical antibody production in different crop systems. *Molecular Breeding* 9:149-152

Stoger E, Ma JKC, Fischer R and Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Current Opinion in Biotechnology* 16:167-173

Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, Mach L, Glossl J, Weterings K, Pabst M and Steinkellner H (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnology Journal* 6:392-402

Torres E, Vaquero C, Nicholson L, Sack M, Stoger E, Drossard J, Christou P, Fischer R and Perrin Y (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Research* 8:441-449

UNAIDS (2010) Fact Sheet: The Global AIDS Epidemic. UNAIDS, Geneva, Switzerland.

Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, Ketas T, Marx PA, Klasse PJ, Burton DR and Moore JP (2003) Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nature Medicine* 9:343-346

Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E and Donini M (2008) Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. *Plant Biotechnology Journal* 7:59-72

Voinnet O, Rivas S, Mestre P and Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* 33:949-956

Walker LM and Burton DR (2010) Rational antibody-based HIV-1 vaccine design: current approaches and future directions. *Current Opinion in Immunology* 22:358-366

Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Principal Investigators Protocol G., Koff WC, Wilson IA, Burton DR and Poignard P (2011) Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477:466-470

Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD and Shaw GM (2003) Antibody neutralization and escape by HIV-1. *Nature* 422:307-312

Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P and Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proceedings of the National Academy of Sciences USA* 105:18232-18237

Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR and Parre PW (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *Journal of Virology* 75:10892-10905

CHAPTER 4

NEXT GENERATION COMBINATION MICROBICIDES TO COMBAT HIV THROUGH MULTI-GENE ENGINEERING

CHAPTER 4: NEXT-GENERATION COMBINATION MICROBICIDES TO COMBAT HIV BY MULTI-GENE ENGINEERING

4.1 Abstract

Effective microbicides should comprise a cocktail of anti-HIV molecules to provide sufficient cross-clade protection and prevent the rapid evolution of resistance. It is unlikely that such drugs could be produced using conventional platforms because the production and distribution costs are too high, but plants provide a low-cost alternative for the manufacture of protein-based microbicide candidates. We explored the feasibility of producing three microbicide candidates (cyanovirin-N, griffithsin and the human HIV-neutralizing monoclonal antibody 4E10) in plants. In preliminary experiments, we transiently expressed cyanovirin-N and/or griffithsin with 4E10 and confirmed the presence and activity of these proteins by ELISA. We then carried out stable transformation using endosperm-specific maize expression vectors but we were unable to identify any plants containing the input transgenes.

4.2 Introduction

New HIV infections may already involve strains that are resistant to many drugs and microbicides. In order to reduce the emergence of resistant HIV-1 isolates, two or more compounds that target separate viral structures or functions are needed in microbicide formulations. Therefore, the development of effective microbicides will probably 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).

Combination microbicides are advantageous because they potentially have a wider antiviral spectrum, a higher genetic barrier to resistance, greater potency and broader cell/tissue coverage than individual microbicides. Combination microbicides maximize their activity through synergistic effects, potentially allowing lower effective concentrations to be used, thus reducing toxicity and other harmful side effects (De Clercq 2007, 2011; McMahon et al. 2009; Anderson et al. 2011; Herrera et al. 2011). Combinations are standard for highly active antiretroviral therapy (HAART), and the resulting suppression of HIV replication leads to low virus levels and delayed resistance. Typical HAART regimens use at least three antiretroviral drugs: two nucleoside reverse transcriptase inhibitors (NRTIs) and either a protease inhibitor (PI), non-NRTIs (NNRTIs) or an integrase inhibitor.

Microbicide combinations may also be used to target two diseases simultaneously, not only because some HIV microbicides are active against other viruses such as *Herpes simplex virus type 2* (HSV-2) 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-1 acquisition (Wald and Link 2002; Renzi et al. 2003; Reynolds et al. 2003; Freeman et al. 2006).

Truvada (Gilead Sciences, Inc., Foster City, CA) was approved in July 2012 for HIV pre-exposure prophylaxis. It is a combination of two antiretroviral drugs (the NRTI emtricitabine plus tenofovir disoproxil fumarate) and is taken daily to reduce the risk of infection (please see Chapter 1 for more details).

The synergistic inhibition of HIV-1 infection by combinations of potential microbicides 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 cyanovirin-N and showed synergistic activity, although the degree of synergy depends on the inhibitor concentration and combination (Ramessar et al. 2010; Pirroni et al. 2011).

For all these reasons, combination microbicides are a promising strategy to prevent HIV infection. Some combination microbicides have already been formulated and developed for phase I trials, e.g. MTN-013/IPM 026, the first clinical trial of a vaginal ring containing two antiretroviral (ARV) drugs: dapivirine and maraviroc (AVAC, 2012). Further preclinical studies of combination microbicide are discussed by Pirrone et al. (2011). The EU FP7 CHAARM project (Combined Highly Active Antiretroviral Microbicides) supports collaborative work among 31 institutions in 12 countries for the development of combinations of inhibitors of reverse transcriptase and/or integrase and/or fusions for vaginal and rectal applications (CHAARM, 2012).

4.2.1 Antiviral lectins

Lectins are proteins that bind carbohydrates without enzymatic chemical modification (Weis and Drickamer, 1996). Lectins are found in most organisms, including viruses and bacteria, sea corals, algae, fungi, plants and animals (Gabius, 1997; Lis and Sharon, 1998; De Mejia and Prisecaru, 2005). Proteins classified as 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-cell communication, induction of apoptosis, cancer metastasis and differentiation (Ziolkowska et al. 2006). However, their function in plants is unclear (Etzler, 1986; Goldstein and Poretz, 1986; Kijne et al. 1986; 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, 2010). Lectins that bind virus glycans can disrupt interactions between proteins on the viral envelope and their receptors (Sacchettini et al. 2001; Shenoy et al. 2002; Botos and Wlodawer, 2005; Balzarini, 2006a). Although many lectins have been characterized, their extraction from natural sources is challenging because large amounts of biomass are required, yields are low and purified lectins are often contaminated with other molecules (Oliveira et al. 2012). Therefore, molecular pharming can be used to produce specific anti-viral lectins because plants are cost-effective for large-scale production (see Chapter 1, section 1.4).

In order to understand how lectins bind to HIV, it is necessary to describe the virus envelope. HIV glycoproteins gp120 and gp41 are present on the envelope and are heavily glycosylated. After infection, the host cell synthesizes viral envelope glycoproteins encoded by the *env* gene, and the precursor is N-glycosylated to form the gp160 glycoprotein in the rough endoplasmic reticulum (ER). After oligomerization to form a trimer, gp160 is cleaved in the Golgi apparatus by a cellular protease (producing non-covalently linked gp120 and gp41) and the glycans are processed further (Helenius and Aebi, 2001).

The gp120 protein comprises five variable regions (V1-V5) interspersed by more conserved amino acid sequences (Leonard et al. 1990; Gallaher et al. 1995; Wyatt et al. 1998). The conserved gp120 regions form structures important for interaction with the gp41 ectodomain and cellular receptors/coreceptors such as CD4, CXCR4 and CCR5. Both the conserved and variable regions of gp120 are extensively glycosylated (Geyer et al. 1988; Mizuochi et al. 1988a,b; Ji et al. 2006) which helps to mask neutralization-sensitive epitopes and evade antibodies (Alexander and Elder, 1984; Oloffson and Hansen, 1989; Losman et al. 2001). This mechanism allows the virus to persist in the presence of an evolving antibody repertoire. The protein gp120 contains 18-33 predicted N-glycan acceptor sites (Leonard et al. 1990), 13 containing complex-type glycans and 11 containing hybrid and/or high-mannose glycans (Figure 4.1). The acceptor sites in the gp120 amino acid sequence are well conserved, despite significant variation in the

gp120 amino acid sequence from different viral isolates. Structural studies reveal gp120 has neutralizing, non-neutralizing and immunologically silent faces as shown in Figure 4.2 (Wyatt et al. 1998; Poignard et al. 2001). The large silent face has poor immunogenicity because it is protected against antibody responses by rapid mutations in the variable loops and the dense array of carbohydrates that block antibody access to conserved regions of the gp120 core (Wyatt et al. 1998). In contrast to gp120, gp41 is poorly glycosylated with 4-5 potential glycosylation sites (Chakrabarti et al. 1987; Dedera et al. 1992; Lee et al. 1992; Fenouillet et al. 1993).

Many lectins that bind high-mannose carbohydrates have antiviral activity, and they are classified according to source (plants, cyanobacteria, sea coral, actinomycetes, algae, invertebrates, vertebrates). Even though 2G12 is a human neutralizing monoclonal antibody, it is also considered a lectin of the innate immune system because it binds to high-mannose glycans (Balzarini, 2007a,b; François and Balzarini, 2010). The most widely studied prokaryotic antiviral lectins are cyanovirin-N (CV-N) and griffithsin (GRFT).

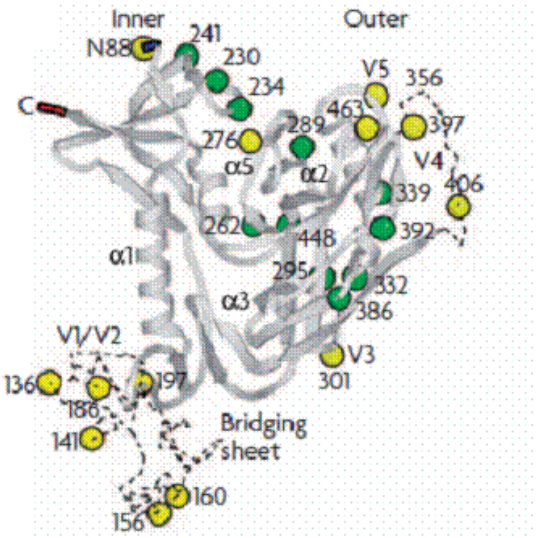

Figure 4.1: The HIV envelope glycoprotein gp120. Ribbon diagram shows 24 putative N-glycosylation sites (colored circles) in the HIV-1(IIIB) envelope glycoprotein gp120. These contain high-mannose-type (green) and complex or hybrid-type (yellow) glycans (adapted from Balzarini, 2007a).

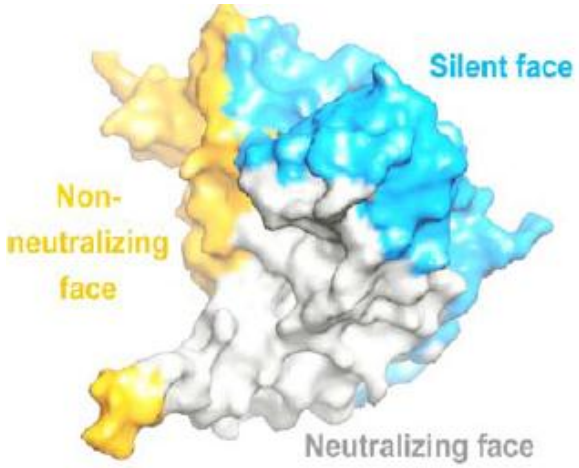

Figure 4.2: The approximate locations of the non-neutralizing (yellow), neutralizing (gray) and immunologically silent (light blue) faces of gp120. The silent face roughly corresponds to the highly glycosylated outer domain surface (adapted from Wyatt et al. 1998).

4.2.1.1 *Cyanovirin-N*

Cyanovirin-N (CV-N) was originally discovered and isolated from the aqueous extract of the cyanobacterium *Nostoc ellipsosporum* (Boyd et al. 1997). CV-N can permanently and efficiently inactivate an extensive range of HIV strains, preventing cell-to-cell fusion and transmission. It acts by binding with high affinity to N-linked high-mannose glycans on gp120 and gp41 (Dey et al. 2000; Shenoy et al. 2001) thus inhibiting attachment and fusion of the virus particle to the target cell. The predominant form of CV-N in solution is the monomeric form, but because it contains two carbohydrate recognition sites on symmetrically opposed domains of the protein, it can cross-link branched oligomannosides to form dimers or higher-order structures (Figure 4.3) (Bewley et al. 1998, Yang et al. 1999; Bewley, 2001). All reported crystal structures are domain-swapped dimers of the protein as shown in Figure 4.4 (Yang et al. 1999).

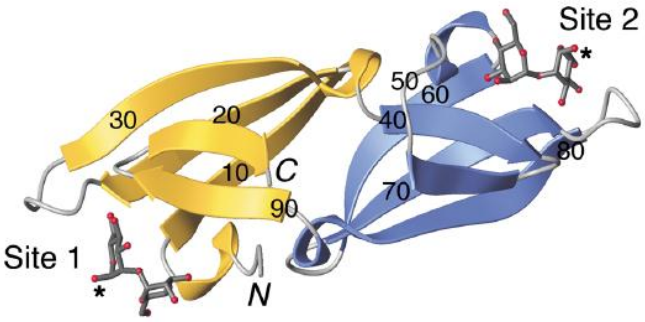

Figure 4.3: Structure of cyanovirin-N and carbohydrate ligands shown as a ribbon drawing. The opposing carbohydrate-binding sites are colored yellow and blue (Bewley, 2001).

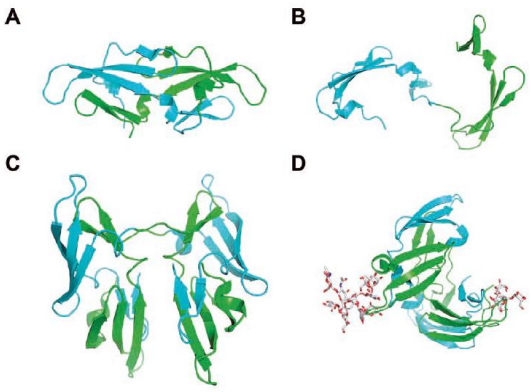

Figure 4.4: Different structural forms reported for CV-N. Domain A of each monomer is colored cyan and domain B green. A) Compact monomer observed so far only in (nuclear magnetic resonance) NMR structures; B) Extended monomer that cannot exist by itself, but was seen in all crystal structures and in some NMR structures as part of a dimer; C) Domain-swapped dimer comprising two monomers shown in panel B; D) A complex of CV-N with Man_9 (Ziółkowska et al. 2006).

Apart from HIV, CV-N is also active against rhinoviruses, herpes simplex virus (HSV-1), hepatitis C virus (HCV), Ebola virus, and influenza A and B viruses (Barrientos et al. 2003; O'Keefe et al. 2003; Helle et al. 2006; Smee et al. 2008). Furthermore, CV-N is insensitive to denaturants, detergents, organic solvents and extreme temperatures. Its potent anti-HIV activity and physicochemical properties make CV-N suitable for use as a topical microbicide. It has a molecular weight of 11 kDa and a sequence of 101 amino acid residues (Boyd et al. 1997). Although the two halves in the monomeric CV-N share a high degree of sequence homology, CV-N has no homology greater than eight

contiguous amino acids or 20% of the total sequence to any other known protein (Yang et al. 1999). In addition, it contains two intramolecular disulfide bonds (Bewley et al. 1998; Yang et al. 1999). These properties make the artificial production of CV-N challenging because of the complexity of the proteins, i.e. it is difficult to synthesize folded domains and then to stitch these domains together to build complex protein molecules (Dawson and Kent, 2000).

The expression of recombinant CV-N has been attempted in *Escherichia coli* (Boyd et al. 1997; Mori et al. 1997; Colleluori et al. 2005), *Pichia pastoris* (Mori et al. 2002) and transgenic tobacco (Sexton et al. 2006), but the efficient expression of biologically functional CV-N is still challenging due to the low yield, aggregation and specifically abnormal modifications when CV-N was produced in *E.coli* (Gao et al. 2010).

CV-N has shown promising *in vivo* efficacy in cellular and cervical explant models (Buffa et al. 2009). In addition, it can also inhibit viral infections in a vaginal and rectal transmission model, e.g. female and male *Macaca fascicularis* infected with a pathogenic recombinant chimeric SIV-HIV-1 (Tsai et al. 2003, 2004). Based on these data, it was suggested that CV-N could be evaluated as a candidate anti-HIV topical microbicide in humans. Liu et al. (2006) reported the development of a common vaginal strain of *Lactobacillus jensenii* to deliver CV-N. The modified bacteria were able to grow intravaginally in mice and to produce at least 10 ng/ml CV-N in vaginal washes (Liu et al. 2006). The cervicovaginal mucosa in humans is coated with a biofilm including *Lactobacillus* spp., which maintain a healthy mucosa and can be genetically engineered to produce antiviral peptides. Recently, the activity of *Lactobacillus jensenii* expressing CV-N was tested *in vivo* in macaques, reducing the transmission of a chimeric simian/HIV by 63% after repeated vaginal challenges (Lagenaur et al. 2011). In another preclinical test, CV-N was evaluated in a novel penile tissue explant model (Fischetti et al. 2009) where it conferred 95% protection against HIV-1 at 1 μM, which is similar to that seen in cervical explants (Fischetti et al. 2009).

Another characteristic of CV-N is that it inhibits HIV-1 binding to the DC-SIGN receptor and the DC-SIGN mediated transfer of the virus to target cells (Alexandre et al. 2010). 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).

These studies show that CV-N is a promising microbicide candidate, but *in vitro* toxicity studies have raised concerns about the safety of CV-N microbicides. CV-N can promote the secretion of pro-inflammatory cytokines and chemokines from human peripheral blood mononuclear cells (PBMCs), activate quiescent $CD4^+$ T lymphocytes and promote T-cell proliferation (Balzarini et al. 2006a; Huskens et al. 2007, 2010). CV-N toxicity was milder in treated cervical explants than PBMCs (Balzarini et al. 2006a; Huskens et al. 2007, 2010). Furthermore, other studies have suggested that the pro-inflammatory activity of CV-N is low and that the mitogenic activity seen in PBMC cultures is unlikely to be representative of the responses to topically applied CV-N *in vivo* (Buffa et al. 2009). No adverse effects were seen in macaques following vaginal or rectal application of 5 mg of CV-N formulated in HMC gel (Tsai et al. 2003). Nevertheless, repeat application studies are required to exclude its potential to induce chronic inflammation (Buffa et al. 2009).

4.2.1.2 *Griffithsin*

Griffithsin (GRFT) was isolated from the red alga *Griffithsia* spp. collected from the waters off New Zealand (Mori et al. 2005). GRFT has a molecular weight of 12.7 kDa and a sequence of 121 amino acids (Mori et al. 2005; Ziółkowska et al. 2006). Residue 31 does not appear to correspond to any standard amino acid and its identity is still unknown (Mori et al. 2005). The GRFT sequence shows less than 30% identity to other plant lectins (Aucouturier et al. 1987; Bourne et al. 1999; Jeyaprakash et al. 2004; Raval et al. 2004; Chandra, 2006). GRFT binds high-mannose oligosaccharides, targeting terminal mannose residues found on Man5-9-GlcNAc2 (Mori et al. 2005; Moulaei et al.

2010). In addition to inhibiting HIV-1, GRFT can also inhibit the replication and cytopathy of the coronavirus that causes SARS (Ziółkowska et al. 2006) and HCV (Meuleman et al. 2011).

GRFT exists exclusively as a 25 kDa dimer and has been crystallized as a domain-swapped dimer, with several high-resolution structures showing three putative carbohydrate-binding sites on each monomer as shown in Figure 4.5 (Moulaei et al. 2010; Ziolkowska et al. 2006, 2007a,b). Each site can bind only one glycan unit, but affinity studies with monosaccharides show only a micromolar binding potential. Therefore, some combination of glycan binding by the multiple sites on GRFT, rather than by individual sites, seems to be necessary to achieve high levels of virus inhibition (Xue et al. 2012). GRFT offers six binding sites for mannose per dimer, so there is significant binding potential for high-mannose glycans on gp120. This is why the reported biological activity of GRFT against HIV is much higher than the activities reported for several monosaccharide-specific lectins as listed in Table 4.1 (Charan et al. 2000; Ziółkowska et al. 2006).

GRFT has no mitogenic effect on PBMCs (O'Keefe et al. 2009). It is also fully active in the presence of macaque vaginal secretions (Emau et al. 2007) and was shown to have 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 HIV-1 infection of human cervical explants, and dissemination of HIV-1 infection from cells resident in the explants to donor T-cells (O'Keefe et al. 2009). Like CV-N, GRFT can also inhibit HIV-1 binding to the DC-SIGN receptor and DC-SIGN mediated transfer of the virus to target cells (Alexandre et al. 2010).

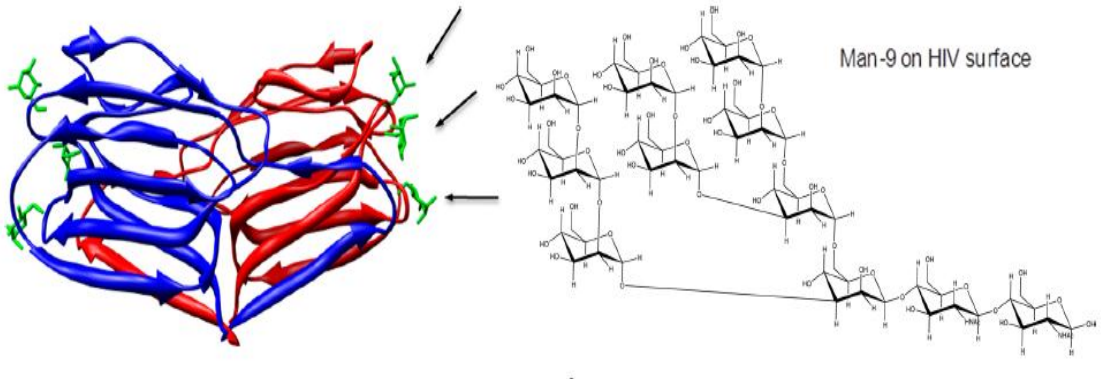

Figure 4.5: Left: The structure of the GRFT dimer in complex with mannose. The arrows denote the three mannose-binding sites of one monomer. Right: The structure of Man-9, an oligosaccharide on the surface of gp120 that is probably bound by GRFT (adapted from Xue et al. 2012).

Table 4.1: 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 halfway 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}) represents the effectiveness of a compound in inhibiting 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%.

Lectin	EC_{50} (nM)
Jacalin	>227
Myrianthus holstii lectin	150
Urtica diocia agglutinin	105
Concanavalin A	98
N. pseudonarcissus lectin	96
P. tetragonolobus lectin	52
MVL*	30
SVN	0.3
CV-N	0.1
GRFT	0.04

*IC_{50} rather than EC_{50} was reported.

4.2.2 Human monoclonal HIV-neutralizing antibody 4E10

Approximately 25 HIV-neutralizing monoclonal antibodies have been identified thus far (Walker et al. 2011) but only 2G12, 4E10 and 2F5 have undergone clinical evaluation (Armbruster et al. 2002, 2004; Mehandru, 2004; Joos, 2006). We discuss 2F5 and 2G12 in Chapters 3 and 5, respectively.

Antibody 4E10 targets a highly-conserved contiguous epitope at the C-terminal, membrane proximal region (MPER) of gp41, indicating that gp41 is not completely masked from antibody recognition by gp120 (Zwick et al. 2001, 2005; Kunert et al. 2004). Antibody 4E10 has the broadest HIV-1-neutralizing activity described to date, including clades A, B, C, D, E and G, albeit sometimes with less potency than 2G12 and 2F5 (Binley et al. 2004). The conserved C-terminal region of the gp41 extracellular domain including the 4E10 epitope is critical for Env-mediated membrane fusion and virus infectivity (Muñoz-Barroso et al. 1999; Salzwedel et al. 1999). Both 4E10 and 2F5 target a transient intermediate of gp41, which is difficult to reach.

HIV-1 infection is initiated when the virus fuses with the target-cell membrane, and during this process the envelope can exist in at least three distinct conformational states: a pre-fusion conformation; a pre-hairpin extended intermediate; and a post-fusion trimer-of-hairpins (Figure 4.6) (Weissenhorn et al. 1997; Chan et al. 1997; Chan and Kim, 1998; Harrison, 2008). The gp120 protein changes conformation when it engages with CD4 and co-receptor CCR5 or CXCR4, causing it to dissociate from gp41, which in turn undergoes a cascade of refolding events (Harrison et al. 2005). In the course of these rearrangements, the N-terminal fusion peptide of gp41 translocates and inserts into the target-cell membrane. A proposed extended conformation of the gp41 ectodomain, with its fusion peptide thus inserted and the transmembrane anchor still in the viral membrane, is known as the pre-hairpin intermediate (Chan and Kim, 1998). Subsequent gp41 rearrangements from the intermediate to the post-fusion state involve folding back each of the three chains into a hairpin-like conformation. This process brings the fusion peptide and transmembrane anchor, and hence the two membranes, close together at the same end of the refolded protein (Frey et al. 2008).

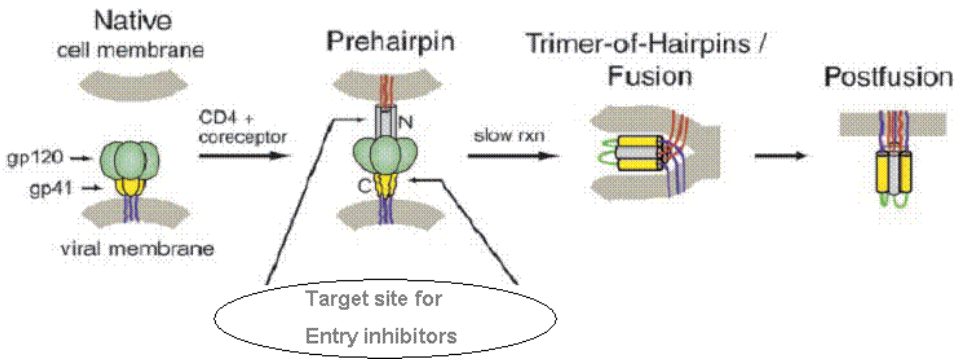

Figure 4.6: Working model of HIV-1 membrane fusion and its inhibition (adapted from Eckert and Kim, 2001). Before exposure to cellular receptors, Env exists in a native state (Native) on the surface of the virus (pre-fusion state). After interaction with CD4 and the coreceptor, a conformational change allows gp41 to insert its N-terminal fusion-peptide domain into the cell membrane, forming a transient pre-hairpin intermediate, in which the N-peptide region (gray) and possibly the C-peptide region (yellow) are exposed and vulnerable to entry inhibitors such as neutralizing antibodies. In the absence of inhibitors, the pre-hairpin intermediate slowly resolves to the trimer-of-hairpins structure that juxtaposes the virus and cell membranes and leads to fusion (post-fusion).

Both 4E10 and 2F5 inhibit HIV-1 infection by binding to epitopes on the pre-hairpin intermediate conformation of gp41, thereby blocking a crucial step in the conformational transition required for membrane fusion (Ofek et al. 2004; Cardoso et al. 2005). The antibodies have long, hydrophobic heavy-chain CDR3 loops that bind MPER peptides only at their base, and it has been proposed that they also interact with the viral envelope (Ofek et al. 2004; Cardoso et al. 2005). It appears that the viral lipid bilayer has a role in neutralization in addition to providing extra contacts for the antibody. One possibility is that 4E10 initially attaches to the viral membrane by lipid interaction, concentrating the antibody on the surface and providing a kinetic head start for capturing a transiently-exposed gp41 epitope (Figure 4.7) (Frey et al. 2008). Without first attaching to the surface, 4E10 might not be able to find gp41 targets during the short duration of the fusion-promoting structural transition (Alam et al. 2009).

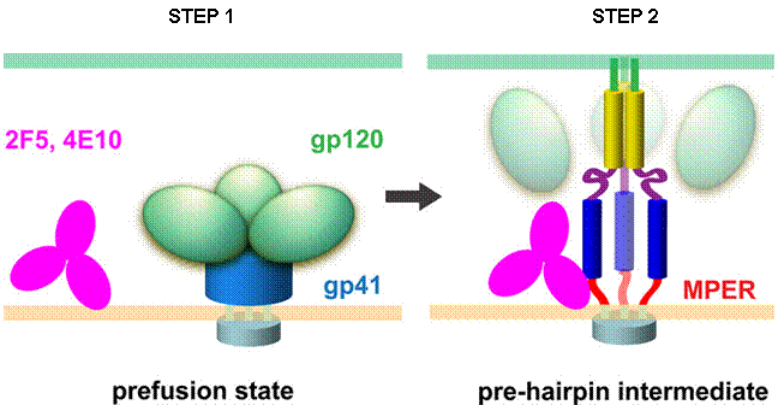

Figure 4.7: Two-step model for HIV-1 neutralization by MPER-directed antibodies such as 4E10 or 2F5 (Alam et al. 2009). Step 1: The antibody attaches to the viral membrane through its long, hydrophobic CDR H3 loop. The MPER is either concealed or configured inappropriately for antibody recognition until the virus encounters CD4 and co-receptor. Step 2: Once triggered by association of gp120 with CD4 and co-receptor, gp41 undergoes a cascade of conformational changes, leading to the pre-hairpin intermediate. The MPER target is exposed only briefly. Once the antibody has docked onto the gp41 epitope, it can prevent further gp41 structural rearrangements required for membrane fusion.

4.2.3 Combination microbicide candidate containing CV-N, GRFT and 4E10

Although current research and clinical development is focused on microbicides that contain antiretroviral drugs, protein microbicides such as human HIV-neutralizing monoclonal antibodies and anti-viral lectins (individually or in combination) are also promising candidates. As well as providing synergy, combination microbicides take advantage of the lectin pressure on viral evolution. To escape the lectins, HIV-1 must delete the N-linked glycans on the viral envelope under increasing lectin pressure (Figure 4.8). Several factors affect the dynamics and evolution of lectin resistance. More than one lectin can bind at several sites on one viral envelope, and because high-mannose type glycans are abundant on gp120, the lectins have several binding options. The deletion of a few N-glycans may create more space for other lectins to bind to the remaining glycans on the mutated gp120 glycoprotein (Huskens et al. 2007). This is why microbicides containing combinations of lectins are valuable. However deleting an individual N-linked glycan would not be sufficient for the virus to efficiently escape the antiviral action of lectins, indeed multiple oligosaccharide deletions are needed for the virus to evolve resistance (Balzarini et al. 2005, 2006b, 2007a,b; Witvrouw et al. 2005).

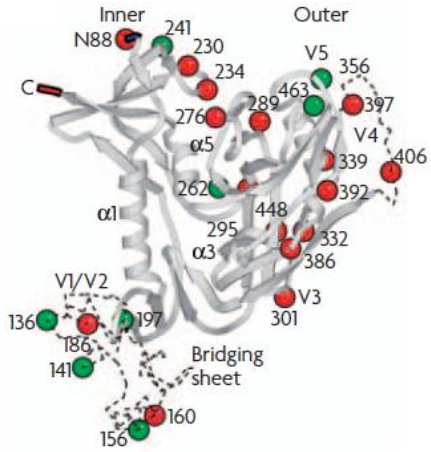

Figure 4.8: The HIV envelope glycoprotein gp120, shown as a ribbon diagram with the 24 putative N-glycosylation sites (colored circles). The red circles indicate the deleted N-glycosylation sites that appear under pressure from carbohydrate-binding agents such as *Galanthus nivalis* agglutinin (GNA), *Hyppeastrum* hybrid agglutinin (HHA), *Urtica dioica* agglutinin (UDA), cyanovirin-N (CV-N), pradimicin A (PRM-A) and the monoclonal antibody 2G12, in more than 30 different mutant virus isolates. The green circles represent glycosylation sites that have not yet been found to be deleted under pressure (adapted from François and Balzarini, 2010).

Reitter et al. (1998) showed that two glycan deletions in SIV gp120 can dramatically increase the neutralizing antibody response in mutant HIV-infected monkeys. Specific N-linked glycan modifications in the envelope V1 domain of SIV or in a SIV-HIV hybrid have also been shown to evolve in the host and alter recognition by neutralizing antibodies (Chackerian et al. 1997; Cheng-Mayer et al. 1999). In this sense, lectins may represent a new mode of antiviral therapy that actively involves the immune system to attack the virus and virus-infected cells. Therefore, it seems likely that lectin exposure to HIV strains forces the virus to progressively delete envelope glycans, allowing the immune system to become actively involved in inhibiting the infection. Microbicides containing a combination of lectins and human HIV-neutralizing monoclonal antibodies are promising because the lectins have a unique dual mechanism of antiviral activity, one involving direct inhibition of virus uptake and the other by indirectly triggering an immune response against previously hidden immunogenic epitopes by stripping the virus of its glycan shield (Figure 4.8) (Balzarini, 2007). Lectins bound to the viral envelope might also opsonize gp120, resulting in complement activation and a cellular

immune response (François and Balzarini, 2010). The number of N-linked glycans that can be deleted from gp120 is limited because they serve critical functions such as ensuring protein stability, providing resistance to proteolytic degradation, regulating enzyme activity and signaling, and ensuring correct folding in the ER, thus avoiding precipitation (Helenius and Aebi, 2001). The removal of too many glycans to escape lectin pressure would disrupt protein folding, rendering the virus unable to interact productively with its receptor and co-receptors (François and Balzarini, 2010).

4.3 Aim

The main aim of this investigation was to co-express two antiviral lectins (CV-N and GRFT) and the antibody 4E10 in maize endosperm tissue by multigene engineering and then carry out expression analysis to identify a lead transgenic event for further characterization and development.

4.4 Materials and methods

4.4.1 Transformation vectors

The CV-N gene (obtained from Prof. Julian Ma, St George's Hospital, London, UK, under an MTA with Dr. Barry R. O'Keefe, National Cancer Institute, Frederick, MD, USA), the GRFT gene (obtained from Dr. Barry R. O'Keefe) and the 4E10 heavy chain (HC) and light chain (LC) genes (obtained from Prof. Julian Ma) were amplified by PCR using primers containing restriction sites to facilitate cloning. Two groups of transformation vectors were used: test constructs for transient constitutive expression in *Nicotiana benthamiana* leaves, and constructs for stable endosperm-specific expression in transgenic maize plants.

The transient expression constructs were based on vector pL32 and contained the constitutive *Cauliflower mosaic virus* 35S promoter, the murine immunoglobulin signal peptide sequence, the CV-N or GRFT coding region, and the nopaline synthase (*nos*) terminator (Figures 4.9 and 4.10). A binary vector (pBI) containing the *Artichoke*

mottled crinkle virus (AMCV) p19 silencing suppressor gene (obtained from Prof. Julian Ma; Figure 4.11) was used to avoid any potential post-transcriptional gene silencing (PTGS) in the infiltrated plants (Johansen and Carrington, 2001). The stable transformation constructs pHor-4E10HC and pHor-4E10LC were generated by transferring the 4E10 HC and LC genes to the pHor vector, containing the endosperm-specific barley D-hordein promoter (Naqvi et al. 2009a) and a rice α-amylase 3A signal peptide sequence to target the secretory pathway (GenBank CAA39776; Figure 4.12). Different promoters were used for stable transformation with CV-N and GRFT: pRP5-CVN was generated by transferring the CV-N gene to the pRP5 vector, containing the endosperm-specific rice prolamin promoter, and pgZ63-GRFT was generated by transferring the GRFT gene to the pgZ63 vector, containing the endosperm-specific maize zein promoter (Naqvi et al. 2009a) and the rice α-amylase 3A signal peptide sequence described above (Figure 4.13). pTRAuxbar (obtained from Prof. Eva Stoger, BOKU, Vienna, Austria, and Dr Thomas Rademacher, Fraunhofer IME, Aachen, Germany) was used for the selection of plants on the basis of phosphinothricin resistance (Zhu et al. 2008). This comprised the constitutive maize ubiquitin-1 promoter, the maize ubiquitin-1 first intron, the *bar* selectable marker gene and the CaMV 35S terminator (Figure 4.14).

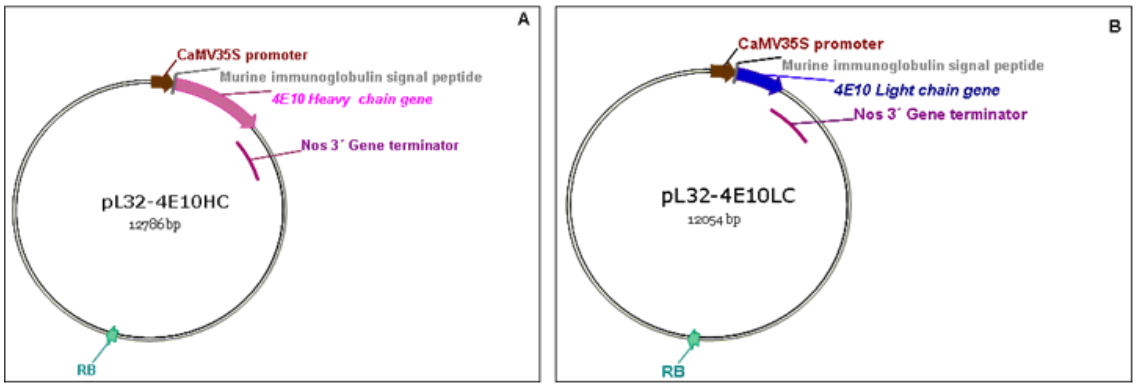

Figure 4.9: Transformation constructs for transient expression in *Nicotiana benthamiana*. A) pL32-4E10HC containing the 4E10 heavy chain (HC) gene; B) pL32-4E10LC containing the 4E10 light chain (LC) gene (adapted from Rogers et al. 1987).

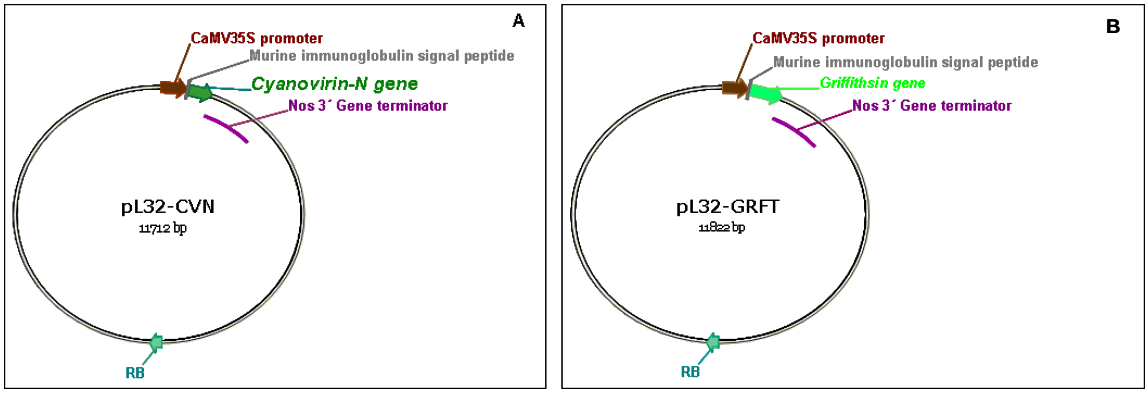

Figure 4.10: Transformation constructs for transient expression in *Nicotiana benthamiana*. A) pL32-CVN containing the CV-N gene; B) pL32-GRFT containing the GRFT gene (adapted from Rogers et al. 1987).

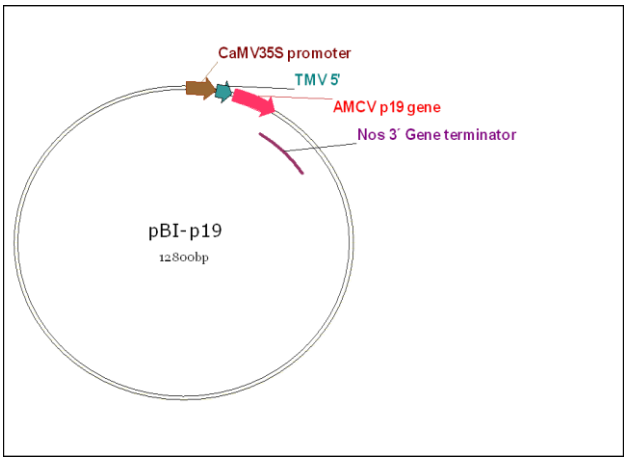

Figure 4.11: p35:AMCV-p19 construct for transient expression containing the AMCV-p19 gene silencing suppressor (Lombardi et al. 2010).

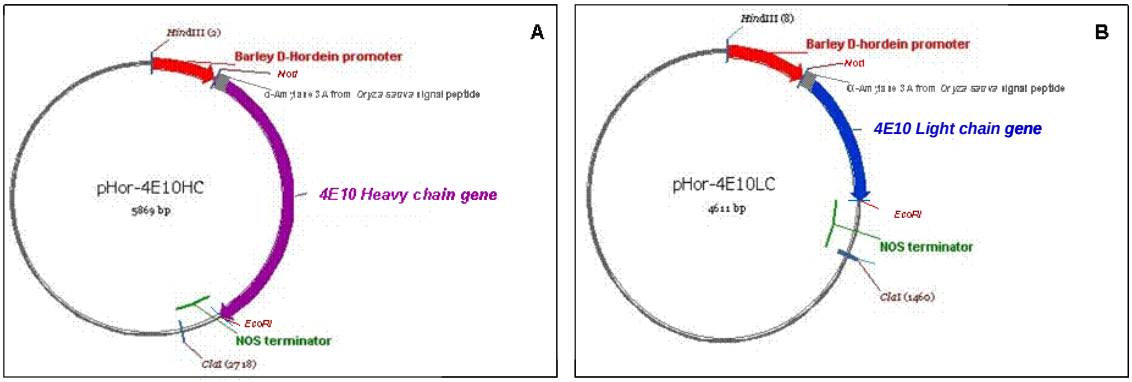

Figure 4.12: Transformation constructs for stable expression in maize seeds. A) pHor-4E10HC containing the 4E10 heavy chain (HC) gene; B) pHor-4E10LC containing the 4E10 light chain (LC) gene (Naqvi et al. 2009a).

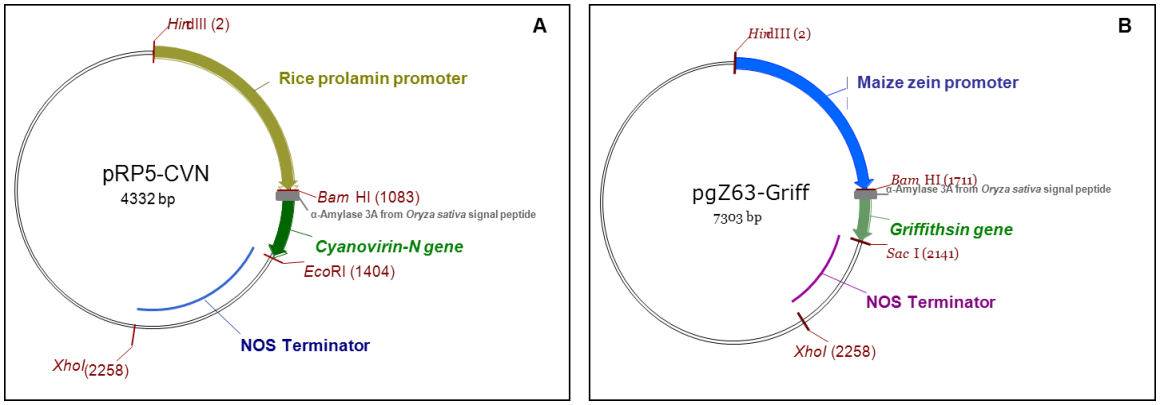

Figure 4.13: Transformation constructs for stable expression in maize seeds. A) pRP5-CVN containing the CV-N gene; B) pgZ63-GRFT containing the GRFT gene (Naqvi et al. 2009a).

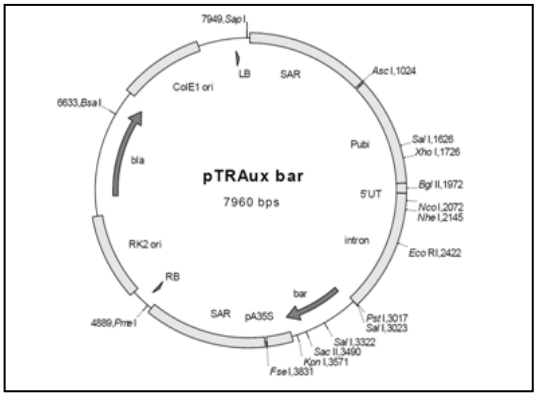

Figure 4.14: Transformation construct pTRAux-bar containing the *bar* selectable marker gene (Zhu et al. 2008).

4.4.2 Transient expression of CV-N and GRFT individually, together, and combined with 4E10, in *N. benthamiana* leaves

Individual bacterial clones containing the expression vectors were maintained in liquid culture until the OD_{600} reached 0.6, before mixing into five different combinations as follows: CV-N mix (CV-N and p19); GRFT mix (GRFT and p19); combination 1 mix (CV-N+GRFT+p19); combination 2 mix (CV-N+4E10HC+4E10LC+p19); and combination 3 mix (GRFT+4E10HC+4E10LC+p19). The five different mixtures at 0.6 OD_{600} were infiltrated in five different experiments into *N. benthamiana* leaves as described (Lombardi et al. 2010). Samples were taken for analysis 6 days post infiltration (dpi), when expression levels are typically highest (Villani et al. 2008).

4.4.3 Transformation, selection and regeneration of putative transgenic plants

This is described in detail in Chapter 3, section 3.4.3**.**

4.4.4 Enzyme-linked immunosorbent assay (ELISA)

Agroinfiltrated *N. benthamiana* leaves (harvested 6 dpi) were ground in two volumes of PBS and centrifuged twice at 20,000 x *g* for 10 min at 4°C to remove plant debris. The specific antigen binding activities of CV-N and GRFT alone or in combination were determined by coating ELISA wells with either 100 ng recombinant gp120 from HIV strain IIIB or 100 ng recombinant gp41 from HIV strain HXB2, both provided by the MRC Centralized Facility for AIDS Reagents, Potters Bar, UK. After washing and blocking with BSA, serial dilutions of each plant extract from the five transient expression experiments were added. The binding activity of each molecule and their combinations was detected using a primary rabbit anti-CVN or anti-GRFT polyclonal antiserum and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antiserum (The Binding Site, Birmingham, UK; 1:1000 dilution). After washing, HRP was detected using 3,3',5,5' tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

4.4.5 DNA blot analysis

This is described in detail in Chapter 3, section 3.4.4. In these DNA blot analyses specific probes for the CV-N, GRFT, 4E10HC, 4E10LC and *bar* genes were used.

4.5 Results

4.5.1 Construction of transformation vectors

The CV-N and GRFT sequences were individually cloned into vector pL32 for transient expression in *N. benthamiana*, and in vectors pRP5 and pgZ63 respectively for the

stable transformation of maize plants. The resulting plasmids were named pL32-CVN, pL32-GRFT, pRP5-CVN and pgZ63-GRFT.

The stable transformation constructs pHor-4E10HC and pHor-4E10L were generated by transferring the 4E10 HC and LC genes separately from the two pL32-based vectors in which they were housed, and inserting them into the pHor vector containing the endosperm-specific barley D-hordein promoter. The resulting plasmids were named pHor-4E10HC and pHor-4E10LC.

4.5.2 Transient expression of CV-N, GRFT and 4E10 in *N. benthamiana* leaves

Preliminary experiments in which *N. benthamiana* leaves were infiltrated with either CV-N or GRFT, or both lectins together, or each lectin with 4E10, demonstrated that the molecules were correctly expressed and functional individually or in combination. The leaf extracts were shown to bind soluble gp120 and gp41 to the same extent as purified lectin controls produced in *E.coli.* Plants agroinfiltrated with each protein individually were used as agroinfiltration positive controls. Extracts from wild-type plants did not bind to either gp120 or gp41, confirming that all activity in the plant extracts reflected the presence of CV-N and GRFT (Figure 4.15).

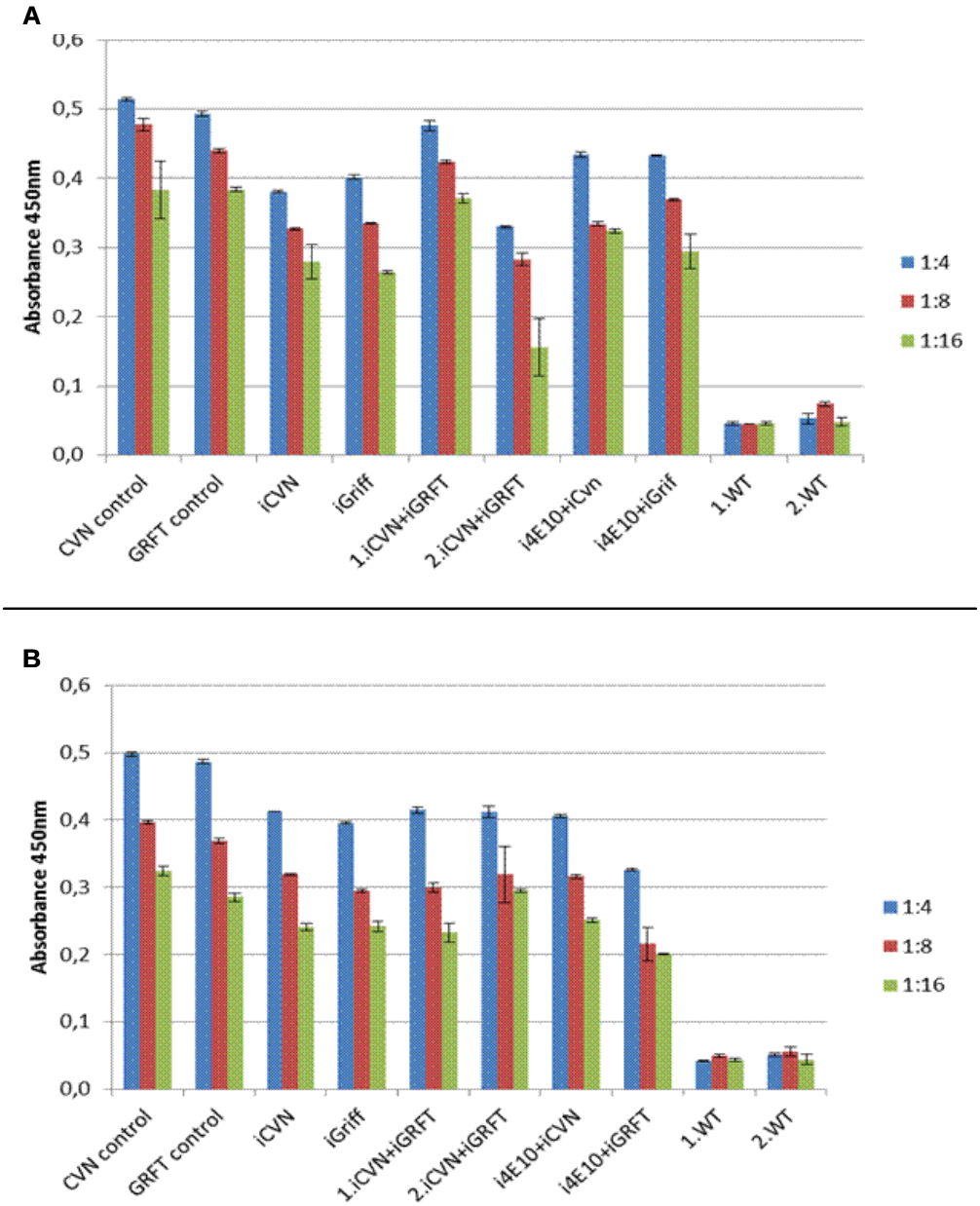

Figure 4.15: Structural and functional analysis of CV-N and GRFT produced individually or in combination by transient expression in *N. benthamiana* leaves. A) Leaf extract binding to gp120 measured by ELISA; B) Leaf extract binding to gp41 measured by ELISA. Purified recombinant CV-N (rCV-N) and GRFT (rGRFT) produced in *E. coli* were used as positive controls at a maximum concentration of 200 ng/ml. iCVN and iGriff: agroinfiltrated plants with each protein individually were used as agroinfiltration positive control. WT: *N. benthamiana* wild type; 1. iCVN+iGRFT: leaf extract detected with a rabbit anti-CVN polyclonal antiserum; 2. iCVN+iGRFT: leaf extract detected with a rabbit anti-GRFT polyclonal antiserum. iCVN+4E10: leaf extract detected with a rabbit anti-CVN polyclonal antiserum. iGRFT+4E10 leaf extract detected with a rabbit anti-GRFT polyclonal antiserum. Data represent neat extracts and dilutions, each a mean ± SD of triplicate readings.

4.5.3 Maize transformation

The selection and regeneration of putative transgenic plants was performed as described in section 4.4.3 (Figure 4.16). 48 putative transgenic events were generated in the elite maize inbred M37W by particle bombardment with pRP5-CVN, pgZ63-GRFT, pHor-4E10HC, pHor-4E10LC and pTRAux-Bar (with *bar* under constitutive expression to allow for phosphinothricin selection). All plants were either selfed or cross-pollinated with wild-type M37W depending on pollen availability.

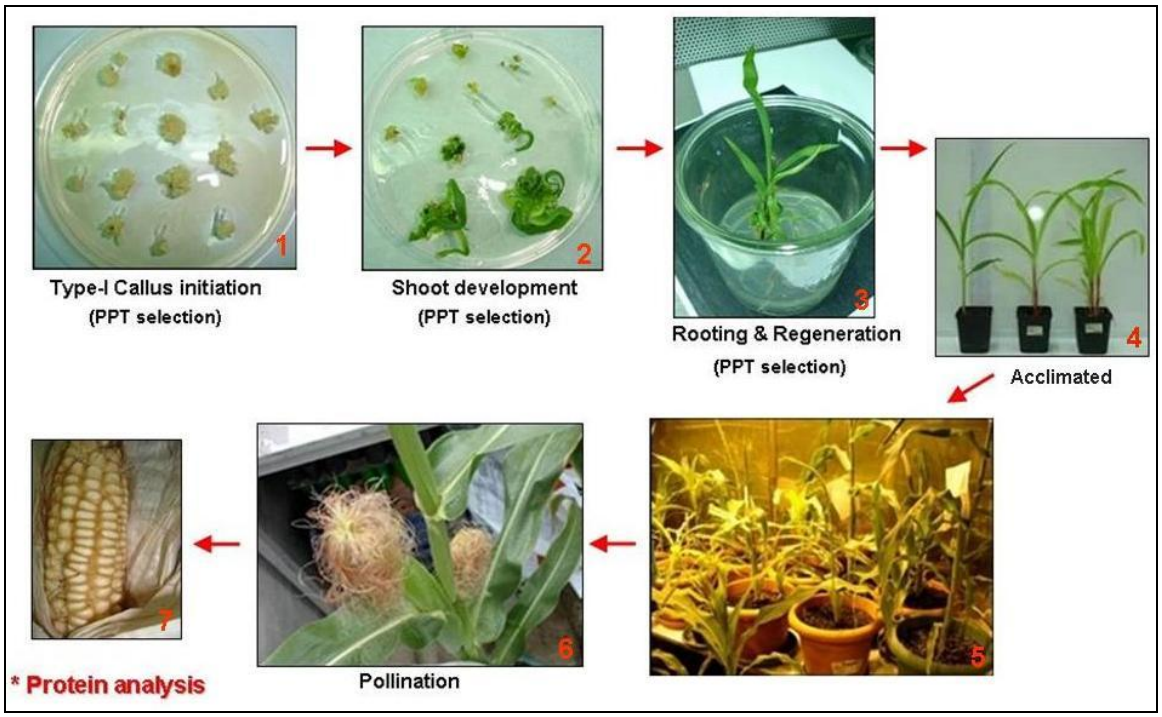

Figure 4.16: Selection and regeneration of putative transgenic plants: (1) type I callus on N6/PPT medium; (2) regenerating callus on MR1 medium containing PPT and cytokinin for shoot development; (3) plantlet regeneration on MR2 medium for rooting; (4) developing plantlets acclimated in soil; (5) putative transgenic plants in a growth chamber; (6) cob on transgenic plant; (7) dried cob after harvest.

4.5.4 DNA analysis of putative transgenic plants

DNA blot analysis indicated that none of the plants contained any of the input genes including the selectable marker *bar*. We thus concluded that none of the regenerated plants were transformed. Possible explanations for this result are discussed below.

4.6 Discussion

Protein microbicides against HIV are promising alternatives to the current generation of small molecule drugs. A number of such proteins have performed well, individually or in combination, in preclinical and clinical studies (De Clercq, 2007, Ramessar et al. 2010; Pirrone et al. 2011; AVAC, 2012). However, not all agents or drugs can be combined effectively, because whereas the hope is that combined microbicides would function additively or synergistically, they may also function antagonistically (Golan et al. 2008; Pirrone et al. 2011).

Synergy occurs when one drug potentiates another, resulting in a combined effect that could not be achieved by either drug acting alone even in the same formulation. In contrast, additive effects occur when two or more drugs used in combination produce a total effect the same as the sum of the individual effects. Antagonism occurs when there is a chemical or biological incompatibility between drugs, reducing their combined activity relative to the expected additive effects. Microbicide combinations must be analyzed on a case-by-case basis to determine the way in which the components interact (Mesquita et al. 2008; Lackman-Smith et al. 2008; Pirrone et al. 2011).

Although clinical research and development is currently focused on combination microbicides containing antiretroviral drugs, preclinical research is also considering potent combinations of entry inhibitors, such lectins and human HIV-neutralizing monoclonal antibodies. Studies with CV-N and GRFT have demonstrated that these lectins can act synergistically with other drugs and with neutralizing antibodies, e.g. GRFT combined with tenofovir, maraviroc or enfuvirtide increases the antiviral potency of the combination beyond the additive effects (Férir et al. 2011), and CV-N, GRFT or the neutralizing antibody 2G12 have all been shown to synergize with the peptide triazole HNG-156 (McFadden et al. 2012). It has also been shown that GRFT synergizes with the neutralizing antibodies b12 and b6, by increasing their affinity for the CD4-recognition site of gp120. This was not due to lectin cross-linking of viruses particles because HIV-1 binding to gp41- and V3-specific monoclonal antibodies was unaffected, so it probably reflects the ability of GRFT to expose the CD4 binding site

and facilitate access to it (Alexandre et al. 2011). Cocktails containing lectins and other entry inhibitors can also promote the emergence of lectin-resistant strains with glycans deleted from the HIV envelope, making space for other lectins in the cocktail that can bind gp120, and opening gaps in the protective glycan shield, thereby exposing hidden epitopes to the immune system and making the virus more vulnerable to a neutralizing antibody response. For example, a partially-deglycosylated gp120 resulting from exposure to CV-N became more sensitive to human HIV-neutralizing monoclonal antibodies and to sera from HIV patients (Hu et al. 2007).

Competition for binding sites on the HIV envelope is an important factor to take into account when combination microbicides are designed. CV-N and GRFT do not compete for binding sites but they do compete with 2G12 (Esser et al. 1999; Mori et al. 2005). In some cases, such competition may be advantageous. For example the ability of CV-N and GRFT to prevent HIV transmission can be overcome when resistant HIV strains emerge, but these strains are more susceptible to 2G12 which binds gp120 glycans or epitopes that were initially masked.

These data show that a combination of lectins and other entry inhibitors can increase antiviral potency suggesting that further clinical research on these combinations is needed. However, such microbicide compounds individually or in combination will not be broadly available because of their high production and distribution costs (Sexton et al. 2009). Molecular pharming is a cost-effective alternative production platform for such proteins because of the many advantages of plants in terms of cost, scalability and safety (Chapter 1, section 1.4). However, an additional advantage specifically in the context of topically applied combination microbicides is that different recombinant proteins can be produced simultaneously to produce combination microbicides directly rather than by mixing separate extracts. The cost of producing each component separately can be prohibitive, but by engineering plants to produce multiple proteins the costs would be reduced significantly by using seed extracts rather than purified separately seed-derived proteins. For such applications, the presence of additional plant proteins and metabolites would not present a significant risk because people are

routinely exposed to such compounds by contact and in their food (Twyman et al. 2003; Stoger et al. 2005). There are already many examples of transgenic plants co-expressing different enzymes in the context of metabolic engineering (Zhu et al. 2008; Naqvi et al. 2009a,b) so it only remains to ensure that the yields and stoichiometry of the microbicidal components can be regulated productively.

To date, no combination microbicides have been produced in plants, although a fusion protein has been expressed in tobacco containing the monoclonal HIV-neutralizing antibody b12 linked to CV-N, and this had greater potency against HIV than each component on its own (Sexton et al. 2009). In contrast, there are many examples of single microbicide compounds expressed successfully in plants, including 2G12 in maize seeds (Chapter 5; Ramessar et al. 2008; Rademacher et al. 2008); 2F5 in tobacco suspension cells (Sack et al. 2007), tobacco leaves (Floss et al. 2008) and maize seeds (Chapter 3; Sabalza et al. 2012); 4E10 secreted from the roots of hydroponic tobacco plants (Drake et al. 2009); CV-N in tobacco leaves (Sexton et al. 2006) and directed to tobacco chloroplasts (Elghabi et al. 2011); and GRFT transiently expressed in *N. benthamiana* (O'Keefe et al. 2009).

Based on the promising results when CV-N or GRFT were combined with other entry inhibitors, we carried out the first attempt to express CV-N, GRFT and 4E10 simultaneously in plants. First it was necessary to confirm the suitability of the CV-N and GRFT sequences for expression in plants, which we achieved by transient expression in *N. benthamiana* leaves followed functional analysis of the recombinant proteins. This was unnecessary for 4E10 because its binding activity was previously confirmed by Prof. Julian Ma. We co-infiltrated *N. benthamiana* leaves with three microbicide mixes, i.e. CV-N+GRFT, CV-N+4E10 and GRFT+4E10, in order to demonstrate that transgene expression and protein activity were unchanged by co-expression. These experiments showed conclusively that CV-N and GRFT were expressed and were able to bind their antigens (gp120 and gp41) individually or in combination, and there was no cross-reaction to endogenous plant proteins. It was difficult to speculate upon the potency of the mixtures because the efficiency of

infiltration cannot be controlled resulting in variable yields. Therefore, we used the transient expression experiments simply to confirm that the genes were expressed to yield functional products, with the intention of studying the potency of corresponding extracts from transgenic maize seeds.

The transient expression data suggested the constructs were suitable for transgenic maize plants and the transgenes were duly transferred to expression vectors promoting endosperm-specific expression in maize. We recovered 48 putative transgenic lines. DNA blot analysis were carried out to determine the transgene integration patterns but none of the plants contained any transgenes, not even the marker gene used for selection. We identified three factors that could have resulted in this large number of escapes, including the starting material (the immature maize embryos used as explants), the particle bombardment process and the tissue culture reagents and procedure.

Our established protocols for the transformation and regeneration of maize plants were adapted from the somatic embryogenesis procedure described by O'Kennedy et al. (2011) and the bombardment parameters developed by Christou et al. (1987, 1991). Immature embryos have been widely used for maize transformation and regeneration (Green and Phillips, 1975) because of their ability to produce embryogenic callus (Gordon-Kamm, 2002). Embryo size is a critical factor for maize transformation and must be 0.8-1.1 mm in length, corresponding to 10-14 days after pollination (dap). It is difficult to achieve the optimal embryo size in all experiments because the maturity of the embryo differs depending on its position on the cob and according to external factors such as the weather if the plants are not sourced from the greenhouse.

The physical parameters of the bombardment process also have a significant impact on the efficiency of transformation, including the helium pressure required to accelerate the metal particles through the plant cell wall and into the nucleus so that the DNA can integrate into the genome. The physical parameters that control this process, such as velocity, degree of vacuum and distance to the embryo, need to be optimized to achieve a high transformation frequency. However, these factors were unlikely to have caused

the high frequency of escapes because identical parameters were used successfully to generate the plants described in Chapters 3 and 5, where the transformation frequency was high.

Tissue culture and regeneration is another critical step in the transformation of maize plants, which involves the procedures described in section 4.4.3. The selectable marker is important for the isolation of transformed cells, and although antibiotics such as hygromycin are probably better selectable marker systems than herbicide tolerance genes, transformed maize cells tend to be difficult to select using antibiotics (Hoisington et al. 1996; Jones, 2009). We use the bialaphos resistance gene (*bar)* from *Streptomyces hygroscopicus* as a selectable marker, which encodes the enzyme phosphinothricin acetyltransferase (PAT) and confers resistance to BASTA (DL-phosphinothricin, PPT) by catalyzing the addition of an acetyl group to the free amino group (D'Halluin et al. 1992). The production of a large number of escapes when using *bar* has been described in wheat (Rasco-Gaunt et al. 2001), maize (Sawahel, 2002) and sorghum (Grootboom et al. 2010) and may reflect the multicellular structures present in organogenic callus and the nursing effect of transformants on untransformed cells. For example, the upper part of the callus may not be in direct contact with the medium and PPT and may therefore regenerate even though lacking the selectable marker. This is why the medium is replaced several times during PPT selection and dead cells are removed, ensuring that untransformed cells come into contact with the medium.

We consider it unlikely that the *bar* gene or the PPT selection strategy was the cause of the escapes in the experiments described above because the *bar* gene was not actually present in the recovered plants and the same selection procedure was used successfully to regenerate the transgenic plants described in Chapters 3 and 5. However, it is possible that the incorrect selection of callus tissue during the *in vitro* regeneration steps could have contributed to the high frequency of escapes. We therefore propose that the inability to recover transgenic plants expressing CV-N, GRFT and 4E10 is likely to reflect a combination of suboptimal embryo selection and *in vitro* regeneration. The immature zygotic embryos used in these experiments may not have been selected at

their optimal stage, reducing the efficiency of transformation, combined with the incorrect selection of untransformed callus during regeneration. The experiments will therefore be repeated, taking the above considerations into account.

When transgenic maize plants are available, we anticipate recovering multimeric forms of GRFT as reported in *N. benthamiana* (O'Keefe et al. 2009) and *E. coli* (Giomarelli et al. 2006) and multimeric forms of CV-N as reported in tobacco (Sexton et al. 2006; Elghabi et al. 2011) and *E. coli* (Boyd et al. 1997; Mori et al. 1998; Colleluori et al. 2005; Gao et al. 2010). The predominant form of CV-N in solution is the monomeric form, but all reported crystal structures are domain-swapped dimers of the protein (Yang et al. 1999; Barrientos et al. 2002) and these allow much more elaborate crosslinking with HIV, thus enhancing their antiviral potency (Moulaei et al. 2010; O'Keefe et al. 2009). Although it would therefore be beneficial to produce oligomers of CV-N and GRFT in plants, some aggregate forms are nonfunctional because disulfide bonds do not form correctly (Gao et al. 2010) therefore it is necessary to ensure that the recombinant protein is folded and assembled correctly and efficiently in plants.

4.7 Conclusions

We did not achieve our goals in these experiments because we could not regenerate transgenic plants co-expressing CV-N, GRFT and 4E10 in maize endosperm. This outcome is likely the consequence of technical difficulties in the selection of optimal immature zygotic embryos and the correct management of tissue culture procedures during regeneration. Transient expression in *N. benthamiana* leaves showed that the expression constructs were suitable for plants and that the recombinant proteins were functional when expressed alone and in combination. These preliminary results suggested that the expression of a combination of lectins, or one lectin and one antibody, should be achievable in plants. However the transient expression experiments cannot accurately predict what will happen when these components are stably co-expressed in maize seeds. We will therefore carry out a further round of transformation experiments, paying particular attention to the selection of optimal immature zygotic embryos and the tissue culture procedures during regeneration. These are the first

experiments addressing the production of combination microbicides in plants and it marks the beginning of further studies to develop a plant-based production system for combination microbicide candidates for the effective, affordable and durable treatment of HIV/AIDS in the developing world.

4.8 References

Alam SM, Morelli M, Dennison SM, Liao HX, Zhang R, Xia SM, Rits-Volloch S, Sun L, Harrison SC, Haynes BF and Chen B (2009) Role of HIV membrane in neutralization by two broadly neutralizing antibodies. *Proceedings of the National Academy of Science USA* 106:20234-20239

Alexander S and Elder JH (1984) Carbohydrate dramatically influences immune reactivity of antisera to viral glycoprotein antigens. *Science* 226:1328-1330

Alexandre KB, Gray ES, Mufhandu H, McMahon JB, Chakauya E, O'Keefe BR, Chikwamba R and Morris L (2010) The lectins griffithsin, cyanovirin-N and scytovirin inhibit HIV-1 binding to the DC-SIGN receptor and transfer to CD4(+) cells. *Virology* 423:175-186

Alexandre KB, Gray ES, Pantophlet R, Moore PL, McMahon JB, Chakauya E, O'Keefe BR, Chikwamba R and Morris L (2011) Binding of the mannose-specific lectin, griffithsin, to HIV-1 gp120 exposes the CD4-binding site. *Journal of Virology* 85:9039-9050

Anderson PL, Kiser JJ, Gardner EM, Rower JE, Meditz A and Grant RM (2011) Pharmacological considerations for tenofovir and emtricitabine to prevent HIV infection. *Journal of Antimicrobial Chemotherapy* 66:240-250

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Michael NL, Vetter N and Katinger HW (2002) A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. *AIDS* 16:227-233

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Köller U, Jilch R, Ammann CG, Pruenster M, Stoiber H and Katinger HWD (2004) Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. *Journal of Antimicrobial Chemotherapy* 54:915-920

Aucouturier P, Mihaesco E, Mihaesco C and Preud'Homme JL (1987) Characterization of jacalin, the human IgA and IgD binding lectin from jackfruit. *Molecular Immunology* 24:503-511

AVAC, Global advocacy for HIV prevention (2012) Ongoing and planned clinical trials of microbicide candidates (April 2012), accessed online 10 October 2012, http://www.avac.org/ht/a/GetDocumentAction/i/3109

Balzarini J, Van Laethem K, Hatse S, Froeyen M, Peumans W, Van Damme E and Schols D (2005) Carbohydrate-binding agents cause deletions of highly conserved glycosylation sites in HIV GP120: A new therapeutic concept to hit the achilles heel of HIV. *Journal of Biological Chemistry* 280:41005-41014

Balzarini J (2006a) Inhibition of HIV entry by carbohydratebinding proteins. *Antiviral Research* 71:237-247

Balzarini J, Van Laethem K, Peumans WJ, Van Damme EJ, Bolmstedt A, Gago F and Schols D (2006b) Mutational pathways, resistance profile, and side effects of cyanovirin relative to human immunodeficiency virus type 1 strains with N-glycan deletions in their gp120 envelopes. *Journal of Virology* 80:8411-8421

Balzarini J (2007a) Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. *Nature reviews microbiology* 5:583-597

Balzarini J, Van Laethem K, Daelemans D, Hatse S, Bugatti A, Rusnati M, Igarashi Y, Oki T, Schols D and Pradimicin A (2007b) A carbohydrate-binding nonpeptidic lead compound for treatment of infections with viruses with highly glycosylated envelopes, such as human immunodeficiency virus. *Journal of Virology* 81:362-373

Barrientos LG, Louis JM, Botos I, Mori T, Han ZZ, O'Keefe BR, Boyd MR, Wlodawer A and Gronenborn AM (2002) The domain-swapped dimer of cyanovirin-N is in a metastable folding intermediate. Reconciliation of X-ray and NMR structures. *Structure* 10:673-686

Bewley CA, Gustafson KR, Boyd MR, Covell DG, Bax A, Clore GM and Gronenborn AM (1998) Solution structure of cyanovirin-N, a potent HIV-inactivating protein. *Nature Structural Biology* 5:571-578

Bewley CA (2001) Solution structure of a cyanovirin-N: Man alpha 1-2Man alpha complex: structural basis for high-affinity carbohydrate-mediated binding to gp120. *Structure* 9:931-940

Binley JM, Wrin T, Korber B, Zwick MB, Wang M, Chappey C, Stiegler G, Kunert R, Zolla-Pazner S, Katinger H, Petropoulos CJ and Burton DR (2004) Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *Journal of Virology* 78:13232-13252

Botos I and Wlodawer A (2005) Proteins that bind high-mannose sugars of the HIV envelope. *Progress in Biophysics and Molecular Biology* 88:233-282

Bourne Y, Zamboni V, Barre A, Peumans WJ, Van Damme EJ and Rouge P (1999) *Helianthus tuberosus* lectin reveals a widespread scaffold for mannose-binding lectins. *Structure* 7:1473-1482

Boyd MR, Gustafson KR, McMahon JB, Shoemaker RH, O'Keefe BR, Mori T, Gulakowski RJ, Wu L, Rivera MI, Laurencot CM, Currens MJ, Cardellina JH 2nd, Buckheit RW Jr, Nara PL, Pannell LK, Sowder RC 2nd and Henderson LE (1997) Discovery of cyanovirin-N, a novel human immunodeficiency virusinactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. *Antimicrobial Agents and Chemotherapy* 41:1521-1530

Buffa V, Stieh D, Mamhood N, Hu Q, Fletcher P and Shattock RJ (2009) Cyanovirin-N potently inhibits human immunodeficiency virus type 1 infection in cellular and cervical explant models. *Journal of General Virology* 90:234-243

Cardoso RMF, Zwick MB, Stanfield RL, Kunert R, Binley JM, Katinger H, Burton DR and Wilson IA (2005) Broadly neutralizing anti-HIV antibody, 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* 22:163-173

CHAARM (2012) Combined Highly Active Anti-Retroviral Microbicides, accessed online 10 October 2012, http://chaarm.eu/

Chackerian B, Rundensey LM and Overbaugh J (1997) Specific N-linked and O-linked glycosylation modifications in the envelope VI domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. *Journal of Virology* 71:7719-7727

Chakrabarti L, Guyader M, Alizon M, Daniel MD, Desrosiers RC, Tiollais P and Sonigo P (1987) Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* 328:543-547

Chan DC, Fass D, Berger JM and Kim PS (1997) Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 89:263-273

Chan DC and Kim PS (1998) HIV entry and its inhibition. *Cell* 93:681-684

Chandra N (2006) Common scaffolds, diverse recognition profiles. *Structure* 14:1093-1094

Charan RD, Munro MH, O'Keefe BR, Sowder RCII, McKee TC, Currens MJ, Pannell LK and Boyd MR (2000) Isolation and characterization of *Myrianthus holstii* lectin, a potent HIV-1 inhibitory protein from the plant *Myrianthus holstii*. *Journal of Natural Products* 63:1170-1174

Cheng-Mayer C, Brown A, Harouse J, Luciw PA and Mayer AJ (1999) Selection for neutralization resistance of the simian human immunodeficiency virus sHIVSF33A variant *in vivo* by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. *Journal of Virology* 73:5294-5300

Christou P, McCabe DE and Swain WF (1987) Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology* 87:671-674

Christou P, Ford TL and Kofron M (1991) Production of transgenic rice plants from agronomically important indica and japonica via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Biotechnology* 9:957-962

Colleluori DM, Tien D, Kang F, Pagliei T, Kuss R, McCormick T, Watson K, McFadden K, Chaiken I, Buckheit RW Jr and Romano JW (2005) Expression, purification, and characterization of recombinant cyanovirin-N for vaginal anti-HIV microbicide development. *Protein Expression and Purification* 39:229-236

D'Halluin K, De Block M, Denecke J, Janssens J, Leemans J, Reynaerts A and Botterman J (1992) The bar gene as selectable and screenable marker in plant engineering. *Methods in Enzymology* 216:415-426

Dawson PE and Kent SB (2000) Synthesis of native proteins by chemical ligation. *Annual Review of Biochemistry* 69:923-960

De Clercq E (2007) AIDS in the Third World: How to stop the HIV infection? *Verhandelingen - Koninklijke Academie voor Geneeskunde van België* 69:65-80

De Clercq E (2011) A 40-year journey in search of selective antiviral chemotherapy. *Annual Review of Pharmacology and Toxicology* 51:1-24

De Hoff PL, Brill LM and Hirsch AM (2009) Plant lectins: the ties that bind in root symbiosis and plant defense. *Molecular Genetics and Genomics* 282:1-15

De Mejia EG and Prisecaru VI (2005) Lectins as bioactive plant proteins: a potential in cancer treatment. *Critical Reviews in Food Science and Nutrition* 45:425-445

Dedera DA, Gu R and Ratner L (1992) Role of asparagine linked glycosylation in human immunodeficiency virus type 1 transmembrane envelope function. *Virology* 187:377-382

Dey AK, David KB, Ray N, Ketas TJ, Klasse PJ, Doms RW and Moore JP (2008) N-terminal substitutions in HIV-1 gp41 reduce the expression of non-trimeric envelope glycoproteins on the virus. *Virology* 372:187-200

Drake PMW, Barbi T, Sexton A, McGowan E, Stadlmann J, Navarre C, Paul MJ and Ma JKC (2009) Development of rhizosecretion as a production system for recombinant proteins from hydroponic cultivated tobacco. *FASEB Journal* 23:3581-3589

Eckert DM and Kim PS (2001) Design of potent inhibitors of HIV-1 entry from the gp41 N-peptide region. *Proceedings of the Natural Academy of Science USA* 98:11187-11192

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

Emau P, Tian B, O'Keefe BR, Mori T and McMahon JB (2007) Griffithsin, a potent HIV entry inhibitor, is an excellent candidate for anti-HIV microbicide. *Journal of Medical Primatology* 36:244-253

Esser MT, Mori T, Mondor I, Sattentau QJ, Dey B, Berger EA, Boyd MR and Lifson JD (1999) Cyanovirin-N binds to gp120 to interfere with CD4-dependent human immunodeficiency virus type 1 virion binding, fusion, and infectivity but does not affect the CD4 binding site on gp120 or soluble CD4-induced conformational changes in gp120. *Journal of Virology* 73:4360-4371

Etzler ME (1986) Distribution and function of plant lectins. *In:* Liener IE, Sharon N, (eds) The lectins: properties, functions, and applications in biology and medicine. Academic Press, New York, pp:371-435

Fenouillet E, Jones IM, Powell B, Schmitt D, Kieny MP and Gluckman JC (1993) Functional role of the glycan cluster of the human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) ectodomain. *Journal of Virology* 67:150-160

Férir G, Palmer KE and Schols D (2011) Synergistic activity profile of griffithsin in combination with tenofovir, maraviroc and enfuvirtide against HIV-1 clade C. *Virology* 417:253-258

Fischetti L, Barry SM, Hope TJ and Shattock RJ (2009) HIV-1 infection of human penile explant tissue and protection by candidate microbicides. *AIDS* 23:319-328

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R and Conrad U (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. *Plant Biotechnology Journal* 6:379-391

François KO and Balzarini J (2010) Potential of carbohydrate-binding agents as therapeutics against enveloped viruses. *Medicinal Research Reviews* 32:349-387

Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA and Hayes RJ (2006) Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS* 20:73-83

Frey G, Peng H, Rits-Volloch S, Morelli M, Cheng Y and Chen B (2008) A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. *Proceedings of the Natural Academy of Science USA* 105:3739-3744

Gabius HJ (1997) Animal lectins. *European Journal of Biochemistry* 243:543-576

Gallaher WR, Ball JM, Garry RF, Martin-Amedee AM and Montelaro RC (1995) A general model for the surface glycoproteins of HIV and other retroviruses. *AIDS Research and Human Retroviruses* 11:191-202

Gantlett KE, Weber JN and Sattentau QJ (2007) Synergistic inhibition of HIV-1 infection by combinations of soluble polyanions with other potential microbicides. *Antiviral Research* 75:188-197

Gao X, Chen W, Guo C, Qian C, Liu G, Ge F, Huang Y, Kitazato K, Wang Y and Xiong S (2010) Soluble cytoplasmic expression, rapid purification, and characterization of cyanovirin-N as a His-SUMO fusion. *Applied Microbiology and Biotechnology* 85:1051-1060

Geyer H, Holschbach C, Hunsmann G and Schneider J (1988) Carbohydrates of human immunodeficiency virus. Structures of oligosaccharides linked to the envelope glycoprotein 120. *Journal of Biological Chemistry* 263:11760-11767

Giomarelli B, Schumacher KM, Taylor TE, Sowder RC 2nd, Hartley JL, McMahon JB and Mori T (2006) Recombinant production of anti-HIV protein, griffithsin, by auto-induction in a fermentor culture. *Protein Expression and Purification* 47:194-202

Golan DE, Tashjian AH Jr, Armstrong EJ and Armstrong AW (2008) Principles of pharmacology. *In*: The pathophysiologic basis of drug therapy, 2nd edn. Philadelphia, Pa.: Lippincott Williams & Wilkins, pp:25

Goldstein IJ and Poretz RD (1986) Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. *In:* Liener IE, Sharon N, Goldstein IJ (eds) The lectins: properties, functions, and applications in biology and medicine. Academic Press, New York, pp:33-245

Gordon-Kamm W, Dilkes BP, Lowe K, Hoerster G, Sun X, Ross M, Church L, Bunde C, Farrell J, Hill P, Maddock S, Snyder J, Sykes L, Li Z, Woo YM, Bidney D and Larkins BA (2002) Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. *Proceedings of the National Academy of Sciences USA* 99:11975-11980

Green CE and Phillips RL (1975) Plant regeneration from tissue cultures of maize. *Crop Science* 15:417-421

Grootboom AW, Mkhonza NL, O`Kennedy MM, Chakauya E, Kunert K and Chikwamba RK (2010) Biolistic Mediated Sorghum (Sorghum bicolor *L. Moench*) Transformation via Mannose and Bialaphos Based Selection Systems. *International Journal of Botany* 6:89-94

Hansen JE, Nielsen CM, Nielsen C, Heegaard P, Mathiesen LR and Nielsen JO (1989) Correlation between carbohydrate structures on the envelope glycoprotein gp120 of HIV-1 and HIV-2 and syncytium inhibition with lectins. *AIDS* 3:635-641

Harrison SC (2005) Mechanism of membrane fusion by viral envelope proteins. *Advances in Virus Research* 64:231-259

Harrison SC (2008) Viral membrane fusion. *Nature Structural and Molecular Biology* 15:690-698

Helenius A and Aebi M (2001) Intracellular functions of N-linked glycans. *Science* 291:2364-2369

Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C and Dubuisson J (2006) Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. *The Journal of Biological Chemistry* 281:25177-25183

Herrera C, Cranage M, McGowan I, Anton P and Shattock RJ (2011) Colorectal microbicide design: Triple combinations of reverse transcriptase inhibitors are optimal against HIV-1 in tissue explants. *AIDS* 25:1971-1979

Hladik F and Hope TJ (2009) HIV infection of the genital mucosa in women. *Current HIV/AIDS Report* 6:20-28

Hoisington DA (1996) The state of the art in maize transformation Memoria del Foro, El Batan, Tex. (Mexico), 21-25 Sep 1995. CIMMYT, Mexico, DF (Mexico)

Hu Q, Mahmood N and Shattock RJ (2007) High-mannose-specific deglycosylation of HIV-1 gp120 induced by resistance to cyanovirin-N and the impact on antibody neutralization. *Virology* 368:145-154

Huskens D, Van Laethem K, Vermeire K, Balzarini J and Schols D (2007) Resistance of HIV-1 to the broadly HIV-1-neutralizing, anti-carbohydrate antibody 2G12. *Virology* 360:294-304

Huskens D, Ferir G, Vermeire K, Kehr JC and Balzarini J (2010) Microvirin, a novel alpha(1,2)-mannose-specific lectin isolated from Microcystis aeruginosa, has anti-HIV-1 activity comparable with that of cyanovirin-N but a much higher safety profile. *Journal of Biological Chemistry* 285:24845-24854

Jeyaprakash AA, Srivastav A, Surolia A and Vijayan M (2004) Structural basis for the carbohydrate specificities of artocarpin: variation in the length of a loop as a strategy for generating ligand specificity. *Journal of Molecular Biology* 338:757-770

Ji X, Chen Y, Faro J, Gewurz H, Bremer J and Spear GT (2006) Interaction of human immunodeficiency virus (HIV) glycans with lectins of the human immune system. *Current Protein and Peptide Science* 7:317-334

Johansen LK and Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiology* 126:930-938

Jones TJ (2009) Maize tissue culture and transformation: the first 20 years. *In:* Kriz AL and Larkins BA (eds) Biotechnology in agriculture and forestry, vol 63 (genetic approaches to maize improvement). Springer-Verlag, Berlin, pp:7-23

Joos B, Trkola A, Kuster H, Aceto L, Fischer M, Stiegler G, Armbruster C, Vcelar B, Katinger H and Günthard FH (2006) Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (MAbs) against human immunodeficiency virus type 1 envelope gp120 (MAb 2G12) and gp41 (MAbs 4E10 and 2F5). *Journal of Antimicrobial Chemotherapy* 50:1773-1779

Kijne JW, Díaz CL and Bakhuizen R (1986) The physiological function of lectins. *In:* Bsg-Hanson TC, Van Driessche E (eds) Lectins: biology, biochemistry, clinical biochemistry, vol 5. Walter de Gruyter, Berlin, pp:45-52

Kouokam JC, Huskens D, Schols D, Johannemann A, Riedell SK, Walter W, Walker JM, Matoba N, O'Keefe BR and Palmer KE (2011) Investigation of griffithsin's interactions with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. *PLoS One* 6:*e*22635

Kunert R, Wolbank S, Stiegler G, Weik R and Katinger H (2004) Characterization of molecular features, antigen-binding, and in vitro properties of IgG and IgM variants of 4E10, an anti-HIV type 1 neutralizing monoclonal antibody. *AIDS Research and Human Retroviruses* 20:755-762

Lackman-Smith C, Osterling C, Luckenbaugh K, Mankowski M, Snyder B, Lewis G, Paull J, Profy A, Ptak RG, Buckheit RW Jr, Watson KM, Cummins JE Jr and Sanders-Beer BE (2008) Development of a comprehensive human immunodeficiency virus type 1 screening algorithm for discovery and preclinical testing of topical microbicides. *Antimicrobial Agents and Chemotherapy* 52:1768-1781

Lagenaur LA, Sanders-Beer BE, Brichacek B, Pal R, Liu X, Liu Y, Yu R, Venzon D, Lee PP and Hamer DH (2011) Prevention of vaginal SHIV transmission in macaques by a live recombinant Lactobacillus. *Mucosal Immunology* 4:648-657

Lee WR, Yu XF, Syu WJ, Essex M and Lee TH (1992) Mutational analysis of conserved N-linked glycosylation sites of human immunodeficiency virus type 1 gp41. *Journal of Virology* 66:1799-1803

Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN and Gregory TJ (1990) Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *Journal of Biological Chemistry* 265:10373-10382

Lis H and Sharon N (1998) Lectins: Carbohydrate-specific proteins that mediate cellular recognition. *Chemistry Review* 98:637-674

Liu X, Lagenaur LA, Simpson DA, Essenmacher KP, Frazier-Parker CL, Liu Y, Tsai D, Rao SS, Hamer DH, Parks TP, Lee PP and Xu Q (2006) Engineered vaginal Lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. *Antimicrobial Agents and Chemotherapy* 50:3250-3259

Lombardi R, Villani ME, Di Carli M, Brunetti P, Benvenuto E and Donini M (2010) Optimisation of the purification process of a tumourtargeting antibody produced in N. benthamiana using vacuum agroinfiltration. *Transgenic Research* 19:1083-1097

Losman B, Bolmstedt A, Schönning K, Björndal A, Westin C, Fenyö EM and Olofsson S (2001) Protection of neutralization epitopes in the V3 loop of oligomeric human immunodeficiency virus type 1 glycoprotein 120 by N-linked oligosaccharides in the V1 region. *AIDS Research and Human Retroviruses* 17:1067-1076

McFadden K, Fletcher P, Rossi F, Kantharaju, Umashankara M, Pirrone V, Rajagopal S, Gopi H, Krebs FC, Martin-Garcia J, Shattock RJ and Chaiken I (2012) Antiviral Breadth and Combination Potential of Peptide Triazole HIV-1 Entry Inhibitors. *Antimicrobial Agents and Chemotherapy* 56:1073-1080

McMahon MA, Shen L and Siliciano RF (2009) New approaches for quantitating the inhibition of HIV-1 replication by antiviral drugs in vitro and in vivo. *Current Opinion in Infectious Diseases* 22:574-582

Mehandru S, Wrin T, Galovich J, Stiegler G, Vcelar B, Hurley A, Hogan C, Vasan S, Katinger H, Petropoulos CJ and Markowitz M (2004) Neutralization profiles of newy transmitted human immunodeficiency virus type 1 by monoclonal antibodies 2G12, 2F5 and 4E10. *Journal of Virology* 78:14039-14042

Mesquita PMM, Wilson SS, Manlow P, Fischetti L, Keller MJ, Herold BC and Shattock RJ (2008) Candidate microbicide PPCM blocks human immunodeficiency virus type 1 infection in cell and tissue cultures and prevents genital herpes in a murine model. *Journal of Virology* 82:6576-6584

Meuleman P, Albecka A, Belouzard S, Vercauteren K, Verhoye L, Wychowski C, Leroux-Roels G, Palmer KE and Dubuisson J (2011) Griffithsin has antiviral activity against hepatitis C virus. *Antimicrobial Agents and Chemotherapy* 55:5159-5167

Mizuochi T, Spellman MW, Larkin M, Solomon J, Basa LJ and Feizi T (1988a) Carbohydrate structures of the human immunodeficiency virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese hamster ovary cells. *Biochemical Journal* 254:599-603

Mizuochi T, Spellman MW, Larkin M, Solomon J, Basa LJ, and Feizi T (1988b) Structural characterization by chromatographic profiling of the oligosaccharides of human immunodeficiency virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese hamster ovary cells. *Biomedical Chromatography* 2:260-270

Mori T, Shoemaker RH, Gulakowski RJ, Krepps BL, McMahon JB, Gustafson KR, Pannell LK and Boyd MR (1997) Analysis of sequence requirements for biological activity of cyanovirin-N, a potent HIV (human immunodeficiency virus)-inactivating protein. *Biochemical and Biophysical Research Communications* 238:218-222

Mori T, Barrientos LG, Han Z, Gronenborn AM, Turpin JA and Boyd MR (2002) Functional homologs of cyanovirin-N amenable to mass production in prokaryotic and eukaryotic hosts. *Protein Expression and Purification* 26:42-49

Mori T, O'Keefe BR, Sowder 2nd RC, Bringans S, Gardella R, Berg S, Cochran P, Turpin JA, Buckheit RW Jr, McMahon JB and Boyd MR (2005) Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp. *Journal of Biological Chemistry* 280:9345-9353

Moulaei T, Shenoy SR, Giomarelli B, Thomas C, McMahon JB, Dauter Z, O'Keefe B and Wlodawer A (2010) Monomerization of viral entry inhibitor griffithsin elucidates the relationship between multivalent binding to carbohydrates and anti- HIV activity. *Structure* 18:1104-1115

Muñoz-Barroso I, Salzwedel K, Hunter E and Blumenthal R (1999) Role of the membrane-proximal domain in the initial stages of human immunodeficiency virus type 1 envelope glycoprotein-mediated membrane fusion. *Journal of Virology* 73:6089-6092

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T and Christou P (2009a) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proceedings of the National Academy of Science USA* 108:1-6

Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C and Christou P (2009b) When more is better: multigene engineering in plants. *Trends in Plant Science* 15:48-56

O'Keefe BR, Smee DF, Turpin JA, Saucedo CJ, Gustafson KR, Mori T, Blakeslee D, Buckheit R and Boyd MR (2003) Potent anti-influenza activity of cyanovirin-N and interactions with viral hemagglutinin. *Antimicrobial Agents and Chemotherapy* 47:2518-2525

O'Keefe BR, Vojdani F, Buffa V, Shattock RJ, Montefiori DC, Bakke J, Mirsalis J, D'Andrea A, Hume SD, Bratcher B, Saucedo CJ, McMahon JB, Pogue GP and Palmer KE (2009) Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. *Proceedings of the National Academy of Sciences USA* 106:6099-6104

O'Kennedy MM, Stark HC and Dube N (2011) Biolistic-mediated transformation protocols for maize and pearl millet using pre-cultured immature zygotic embryos and embryogenic tissue. *Methods in Molecular Biology* 710:343-354

Ofek G, Tang M, Sambor A, Katinger H, Mascola JR, Wyatt R and Kwong PD (2004) Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with Its gp41 epitope. *Journal of Virology* 78:10724-10737

Oliveira C, Teixeira JA and Domingues L (2012) Recombinant lectins: an array of tailor-made glycan-interaction biosynthetic tools. *Critical Reviews in Biotechnology* DOI: 10.3109/07388551.2012.670614

Oloffson S and Hansen JE (1998) Host cell glycosylation of viral glycoproteins-a battefield for host defence and viral resistance. *Scandinavian Journal of Infectious Diseases* 30:435-440

Pirrone V, Thakkar N, Jacobson JM, Wigdahl B and Krebs FC (2011) Combinatorial approaches to the prevention and treatment of HIV-1 infection. *Antimicrobial Agents and Chemotherapy* 55:1831-1842

Poignard P, Saphire EO, Parren PW and Burton DR (2001) gp120: Biologic aspects of structural features. *Annual Reviews of Immunology* 19:253-274

Pusztai A (1991) Plant Lectins. Cambridge University Press, New York

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

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma JKC, Stoger E, Capell T and Christou P (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proceedings of the National Academy of Sciences USA* 105:3727-3732

Ramessar K, Sabalza M, Miralpeix B, Capell T and Christou P (2010) Can microbicides turn the tide against HIV? *Current Pharmatheutical Design* 16:468-485

Rasco-Gaunt S, Riley A, Cannell M, Barcelo P and Lazzeri PA (2001) Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum L.*) varieties via particle bombardment. *Journal of Experimental Botany* 52:865-874

Raval S, Gowda SB, Singh DD and Chandra NR (2004) A database analysis of jacalin-like lectins: sequence-structurefunction relationships. *Glycobiology* 14:1247-1263

Reitter JN, Means RE and Desrosiers RC (1998) A role for carbohydrates in immune evasion in AIDS. *Nature Medicine* 4:679-684

Renzi C, Douglas JM, Foster M, Critchlow CW, Ashley-Morrow R, Buchbinder SP, Koblin BA, McKirnan DJ, Mayer KH and Celum CL (2003) Herpes simplex virus type 2 infection as a risk factor for human immunodeficiency virus acquisition in men. *Journal of Infectious Diseases* 187:19-25

Reynolds SJ, Risbud AR, Shepherd ME, Zenilman JM, Brookmeyer RS, Paranjape RS, Divekar AD, Gangakhedkar RR, Ghate MV, Bollinger RC and Mehendale SM (2003) Recent herpes simplex virus type 2 infection and the risk of human immunodeficiency virus type 1 acquisition in India. *Journal of Infectious Diseases* 187:1513-1521

Rogers SG, Klee HJ, Horsch RB and Fraley RT (1987) Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. *Methods in Enzymology* 153:253-277

Sabalza M, Madeira L, Van Dolleweerd C, Ma JKC, Capell T and Christou P (2012) Functional characterization of the recombinant HIV-neutralizing monoclonal antibody 2F5 produced in maize seeds. *Plant Molecular Biology* 80:477-488

Sacchettini JC, Baum LG and Brewer CF (2001) Multivalent protein-carbohydrate interactions. A new paradigm for supramolecular assembly and signal transduction. *Biochemistry* 40:3009-3015

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoger E and Rademacher T (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB Journal* 21:1655-1664

Salzwedel K, West JT and Hunter E (1999) A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. *Journal of Virology* 73:2469-2480

Sawahel WA (2002) Production of herbicide-resistant transgenic maize plants using electroporation of seed-derived embryos. *Plant Molecular Biology Reporter* 20**:**303-304

Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock RJ and Ma JKC (2006) Transgenic plant production of cyanovirin-N, an HIV microbicide. *FASEB Journal* 20:356-358

Sexton A, Harman S, Shattock RJ and Ma JKC (2009) Design, expression, and characterization of a multivalent, combination HIV microbicide. *FASEB Journal* 23:3590-3600

Shenoy SR, Barrientos LG, Ratner DM, O'Keefe BR, Seeberger PH, Gronenborn AM and Boyd MR (2002) Multisite and multivalent binding between cyanovirin-N and branched oligomannosides: calorimetric and NMR characterization. *Chemistry and Biology* 9:1109-1118

Smee DF, Wandersee MK, Checketts MB, O'Keefe BR, Saucedo C, Boyd MR, Mishin VP and Gubareva LV (2007) Influenza A (H1N1) virus resistance to cyanovirin-N arises naturally during adaptation to mice and by passage in cell culture in the presence of the inhibitor. *Antimicrobial Agents and Chemotherapy* 18:317-327

Stoger E, Ma JKC, Fischer R and Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Current Opinion in Biotechnology* 16:167-173

Tsai CC, Emau P, Jiang Y, Tian B, Morton WR, Gustafson KR and Boyd MR (2003) Cyanovirin-N gel as a topical microbicide prevents rectal transmission of SHIV89.6P in macaques. *AIDS Research and Human Retroviruses* 19:535-541

Tsai CC, Emau P, Jiang Y, Agy MB, Shattock RJ, Schmidt A, Morton WR, Gustafson KR and Boyd MR (2004) Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models. *AIDS Research and Human Retroviruses* 20:11-18

Twyman RM, Stoger E, Schillberg S, Christou P and Fischer R (2003) Molecular farming in plants: host systems and expression technology. *Trends in Biotechnology* 21:570-578

Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E and Donini M (2008) Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. *Plant Biotechnology Journal* 7:59-72

Wald A and Link K (2002) Risk of human immunodeficiency virus infection in herpes simplex virus type 2 seropositive persons: A meta-analysis. *Journal of Infectious Diseases* 185:45-52

Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Principal Investigators Protocol G., Koff WC, Wilson IA, Burton DR and Poignard P (2011) Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477:466-470

Weis WI and Drickamer K (1996) Structural basis of lectin-carbohydrate recognition. *Annual Review of Biochemistry* 65:441-473

Weissenhorn W, Dessen A, Harrison SC, Skehel JJ and Wiley DC (1997) Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 387:426-430

Witvrouw M, Fikkert V, Hantson A, Pannecouque C, O'Keefe BR, McMahon J, Stamatatos L, De Clercq E and Bolmstedt A (2005) Resistance of human immunodeficiency virus type 1 to the high-mannose binding agents cyanovirin-N and concanavalin A. *Journal of Virology* 79:7777-7784

Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA and Sodroski JG (1998) The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* 393:705-711

Xue J, Gao Y, Hoorelbeke B, Kagiampakis I, Zhao B, Demeler B, Balzarini J and Liwang PJ (2012) The role of individual carbohydrate-binding sites in the function of the potent anti-HIV lectin griffithsin. *Molecular Pharmaceutics* 9:2613-2625

Yang F, Bewley CA, Louis JM, Gustafson KR, Gronenborn AM, Clore GM and Wlodower A (1999) Crystal structure of cyanovirin-N, a potent HIV-inactivating protein, shows unexpected domain swapping. *Journal of Molecular Biology* 288:403-412

Zhu C, Naqvi S, Breitenbach J, Sandman G, Christou P and Capell T (2008) Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. *Proceedings of the National Academy of Science USA* 105:18232-18237

Ziolkowska NE, O'Keefe BR, Mori T, Zhu C, Giomarelli B, Vojdani F, Palmer KE, McMahon JB and Wlodawer A (2006) Domain swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding. *Structure* 14:1127-1135

Ziolkowska NE, Shenoy SR, O'Keefe BR and Wlodawer A (2007a) Crystallographic studies of the complexes of antiviral protein griffithsin with glucose and N-acetylglucosamine. *Protein Science* 16:1485-1489

Ziolkowska NE, Shenoy SR, O'Keefe BR, McMahon JB, Palmer KE, Dwek RA, Wormald MR and Wlodawer A (2007b) Crystallographic, thermodynamic, and molecular modeling studies of the mode of binding of oligosaccharides to the potent antiviral protein griffithsin. *Proteins* 67:661-670

Zwick MB, Wang M, Poignard P, Stiegler G, Katinger H, Burton DR and Parren PW (2001) Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. *Journal of Virology* 75:12198-12208

Zwick MB, Jensen R, Church S, Wang M, Stiegler G, Katinger H and Burton DR (2005) Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *Journal of Virology* 79:1252-1261

CHAPTER 5

HUMAN NEUTRALIZING MONOCLONAL ANTIBODY 2G12 AS A MODEL TO STUDY INTERACTIONS WITH MAIZE SEED STORAGE PROTEINS

CHAPTER 5: HUMAN NEUTRALIZING MONOCLONAL ANTIBODY 2G12 AS A MODEL TO STUDY INTERACTIONS WITH MAIZE SEED STORAGE PROTEINS

5.1 Abstract

Cereal seed endosperm tissue provides an ideal biochemical environment for the stable accumulation of recombinant proteins, and this is generally achieved by the formation of specialized storage compartments such as protein bodies and protein storage vacuoles derived from the secretory pathway. We targeted a recombinant form of the HIV-neutralizing human monoclonal antibody 2G12 to the maize secretory pathway and analyzed its subcellular localization during seed development by immunoelectron microscopy. In agreement with previous investigations, we confirmed that 2G12 accumulated in protein bodies, mostly within PSVs. We also observed a chemical interaction between the antibody and maize zein protein bodies causing them to adopt an amorphous structure. We confirmed these results by analyzing a second event, in which 2G12 accumulated at a lower level and the number of amorphous zein bodies was much lower than in the first event. These results suggest that the amount of antibody influences the induction of morphological changes in the protein bodies. Therefore, according to the results obtained in both events, we propose a positive correlation between the accumulation of 2G12 in maize seeds and chemical interactions with the zein bodies. Our findings confirm that maize seeds are suitable for the production of recombinant antibodies, but further studies are needed to determine whether these interactions influence their structure and functionality.

5.2 Introduction

Seeds are valuable for the production of recombinant proteins because they are physiologically and morphologically adapted to store proteins (Stoger et al. 2005). Seed endosperm cells are unique in their natural ability to store large amounts of protein (Galili, 2004). Although most of the individual proteins present in mature seeds have either metabolic or structural roles, seeds also contain abundant storage proteins whose only function appears to be the provision of amino acids for use during germination and seedling growth (Shewry et al. 1995). Because these proteins appear to have no other biological function, the accumulation of recombinant proteins in protein storage organelles is desirable. The investigation of seed storage proteins and the cell compartments in which they accumulate is important when recombinant proteins are produced in seeds, because this allows the control of protein quality in terms of folding and posttranslational modifications such as N-glycosylation. There are only limited data on the trafficking of recombinant proteins in seed storage tissues, particularly proteins expressed in specialized tissues such as the endosperm (Hood, 2004). This chapter describes the expression of the HIV-neutralizing human monoclonal antibody 2G12 at high levels in maize seeds (Ramessar et al. 2008a) as a model to study the precise intracellular localization and accumulation of recombinant proteins.

Immunoelectron microscopy is a fundamental tool for the investigation of protein expression and deposition at the ultrastructural level. Furthermore, because 2G12 is a glycoprotein, the subcellular destination and the path taken by the protein through the endomembrane system will determine the final structure of the N-glycans (Arcalis et al. 2010). The analysis of 2G12 N-glycan profiles is therefore another useful approach to gain insight into the trafficking, processing and deposition of recombinant proteins.

5.2.1 Seed stored proteins: trafficking and deposition

Storage proteins travel along specific routes within the endomembrane system (Muntz, 1998) and are translocated cotranslationally into the lumen of the endoplasmic reticulum (ER). Some of these proteins continue to accumulate therein, and eventually

form discrete protein bodies that bud off from the ER and become independent compartments (Shewry and Halford, 2002). Others are transported to protein storage vacuoles (PSVs) after passing through the Golgi complex (Muntz, 1998) (Figure 5.1). Within the ER, the neutral pH, oxidizing environment and the presence of numerous chaperones optimize protein folding and assembly. Incorrectly folded proteins do not proceed along the secretory pathway, but are instead exported into the cytosol where they are degraded by the proteasome (Vitale and Ceriotti, 2004).

Secretory proteins possess an N-terminal signal peptide which ensures translocation into the ER, from where they pass through the Golgi complex and are either secreted or transported to hydrolytic compartments (vacuoles). Therefore, recombinant proteins such as antibodies can also be secreted (by default) from plant cells into the cell wall and the intercellular space (collectively termed the apoplast) where they may accumulate to high levels (Ma et al. 1995; Woodward et al. 2003).

The ER constitutively exchanges membrane and protein components with the Golgi complex, which is the key sorting compartment of the secretory pathway (Lee et al. 2004; Hanton et al. 2005). This allows newly synthesized proteins destined for the cell surface or different types of vacuoles to be trafficked correctly and also allows the retrieval of proteins that function within the ER itself. Although the ER is a transit compartment for most secretory proteins, those with signals that promote retrieval from the Golgi complex, such as the KDEL/HDEL tetrapeptide present in soluble proteins and the dilysine or diarginine motifs on the cytosolic tails of transmembrane proteins, are efficiently retained (Lee et al. 2004). Storage proteins accumulating in protein bodies in the ER may use controlled aggregation as a way to escape the final degradation phase of quality control and thus accumulate in the ER lumen without exposing this compartment to excessive stress (Vitali and Ceriotti, 2004). Furthermore, protein bodies are insoluble in low-salt aqueous buffers, so they can be purified by centrifugation (Mainieri et al. 2004). When KDEL/HDEL tetrapeptides are introduced by engineering into other proteins in addition to the N-terminal signal peptide, these recombinant proteins can also be retained in the ER. This often increases the

accumulation of recombinant proteins in transgenic plants by one or two orders of magnitude, a highly desirable achievement (Wandelt et al. 1992). This strategy has been used to boost the accumulation of immunoglobulins, vaccine antigens and other recombinant proteins in transgenic plants (Arakawa et al. 1998; Stoger et al. 2000; Vaquero et al. 2002; Ko et al. 2003).

Plants naturally use the ER to accumulate proteins in specific storage bodies, in the form of large aggregates or oligomers, as part of the developmental process of seed maturation (Herman and Larkins, 1999; Vitale and Ceriotti, 2004). Prolamins do not contain the KDEL or HDEL ER-localization signals and form protein bodies within the ER lumen. The molecular interactions that lead to the retention of prolamins and protein body formation are still not fully understood, but a number of prolamins can also form protein bodies when transiently synthesized in animal cells (Altschuler et al. 1994; Hurkman et al. 1981) or permanently expressed in vegetative (non-seed) tissues of transgenic plants (Geli et al. 1994; Bagga et al. 1995).

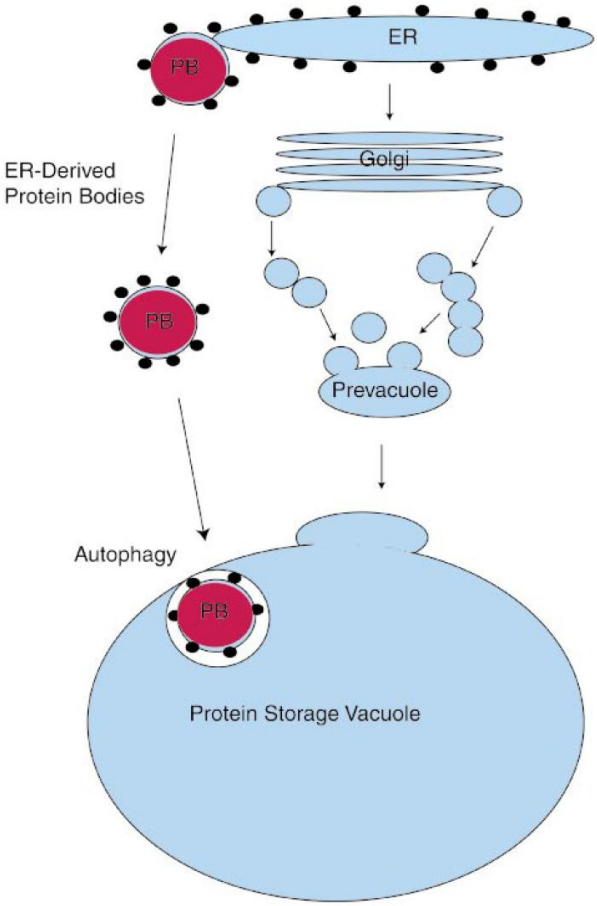

Figure 5.1: Ontogeny of plant protein bodies (PBs) and protein storage vacuoles (PSVs). PBs form through the aggregation of storage proteins within the ER. After formation, PBs can either remain attached to the ER or bud from the ER and form separate organelles known as PSVs. PBs can accumulate in the cytosol or become sequestered into PSVs by autophagy. PSVs are formed as the consequence of ER-synthesized storage proteins progressing through the endomembrane secretory system to specialized vacuoles (PSVs) for accumulation (Herman and Larkins, 1999).

The storage proteins that do not accumulate in the ER as protein bodies pass through the secretory pathway to the PSVs. Plants possess functionally distinct vacuolar compartments that can exist simultaneously in the same cell: lytic vacuoles and storage vacuoles (Paris et al. 1996). Whereas storage vacuoles are mainly found in storage tissue such as seeds and tubers (Hermana and Larkins, 1999), lytic vacuoles are normally present in all tissues and share some of their basic properties with the lysosomes of animal cells. The mechanisms which direct soluble proteins from the secretory pathway to the different vacuolar compartments in plant cells are not fully

understood. There are two routes from the ER to PSVs. One passes through the Golgi complex (Sanderfoot and Raikhel, 1999) and PSVs originate from post-Golgi central vacuoles that are devoid of significant protein accumulation in both embryo and vegetative cells. Storage proteins are added and gradually fill the vacuole. This contrasts with the formation of ER-derived protein bodies, which form as proteins accumulate and do not undergo further modification. Golgi-derived PSVs are structurally differentiated as transient subdivisions of the pre-existing vacuole, and this occurs co-ordinately with the onset of storage protein synthesis and accumulation.

The second route may be particular to storage proteins and occurs by autophagy, the route by which plant cells dispose of cytoplasmic constituents and materials internalized from the extracellular space by multivesicular endosomes (Herman, 1994; Robinson et al. 1998). In some cereals, autophagy is also used to accumulate storage proteins, bypassing the conserved mechanism of Golgi-mediated targeting and transport to the vacuole (Levanony et al. 1992). Storage proteins assembled in ER are polymerized into protein bodies (Shimoni and Galili, 1996) that are directly secreted from the ER. The protein bodies do not remain as separate cytosolic structures but are instead sequestered into provacuoles (Rubin et al. 1992). The provacuoles containing sequestered protein bodies fuse, forming one or more large central vacuoles that contain numerous storage protein aggregates. The limiting membrane of the sequestered protein bodies appears to be digested by vacuolar enzymes, releasing the naked protein bodies into the vacuolar sap.

5.2.2 Maize seed storage proteins

Seed storage proteins can be classified according to their solubility and extractability as described in the classical fractionation procedure of Osborne (1907), which distinguished proteins soluble in water (albumins), dilute saline (globulins), alcohol/water mixtures (prolamins) and dilute acid or alkali (glutens). Albumins and globulins are widely distributed in the seeds of dicotyledonous species, whereas prolamins are the major storage proteins in cereals (Boyer et al. 1992). Maize prolamins

are called zeins and they accumulate in protein bodies (Krishnan et al. 1986). Maize endosperm cells contain mainly ER-derived (pre-Golgi) prolamin bodies, and only a few globulin-like storage proteins that accumulate in post-Golgi PSVs (Woo et al. 2001).

Maize prolamins are synthesized 10–40 days after pollination (dap) and constitute approximately 50% of the total protein in the mature seed (Lee et al. 1976). There are three sub-families (α-, β- and γ-zeins) with the α-zeins classified as the major group (Wilson, 1991). Zeins are synthesized by membrane-bound polyribosomes and are transported into the ER lumen, where they assemble to form protein (or zein) bodies. The underlying mechanisms are poorly understood, as for other types of protein bodies. Zein protein bodies consist of a central core of α- and β-zeins surrounded by peripheral γ-zeins (Lending and Larkins, 1989) (Figure 5.2). Prolamins often undergo homophilic and heterophilic protein-protein interactions (Kim et al. 2002) and are often linked by intermolecular disulfide bridges. The γ-zeins are particularly rich in cysteine residues leading to the formation of covalently-linked multimeric aggregates, which initiate protein body formation in the ER (Conley et al. 2011).

Two domains within γ-zein confer its ability to be retained in the ER and to assemble into protein bodies. The repetitive proline-rich sequence adopts an amphipathic helical conformation, which can self-assemble and may be responsible for ER retention (Geli et al. 1994; Kogan et al. 2004). The γ-zein protein can induce the formation of ER-derived protein bodies in seeds and in vegetative tissues of transgenic dicot species in the absence of other zein subunits (Geli et al. 1994; Coleman et al. 1996). The γ-zein sequences involved can therefore be used as an alternative to the KDEL signal to accumulate recombinant proteins in the ER.

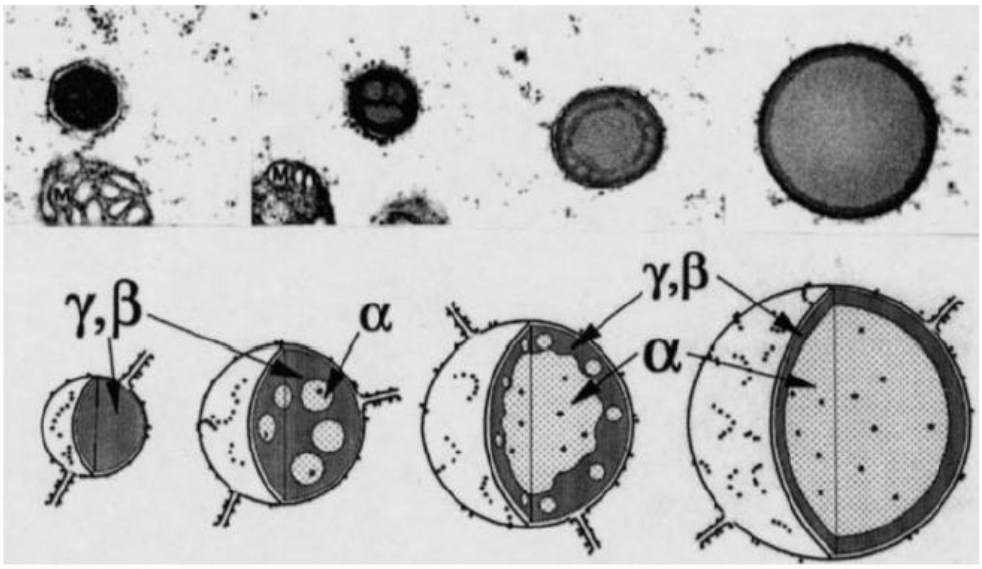

Figure 5.2: Developmental profile (left to right) of protein body formation in maize endosperm. Greek letters indicate the location of the corresponding zein classes as determined by immunolocalization (Coleman et al. 1999).

5.2.3 Plant glycosylation in the context of molecular pharming

Most proteins undergo co-translational and/or post-translational modifications. In the context of molecular pharming, glycosylation is the most important post-translational modification in the secretory pathway because it can affect the physical and chemical properties of a recombinant protein (charge and size), its structure, function and activity (by determining its capacity to fold and interact with substrates and ligands) and its stability.

Glycosylation is an enzyme-directed site-specific process that attaches glycans to proteins, lipids and other molecules. Protein glycosylation occurs only in the endomembrane system. Plant glycans can be either N-linked or O-linked. N-glycosylation begins in the ER but the primary oligosaccharide chain is processed further during its exit from the ER and passage through the Golgi complex which is also the site of protein O-glycosylation. Most N-linked oligosaccharides are classified as either high-mannose type (HMT) or complex type, although there are also hybrid and paucimannosidic type N-glycans (low-mannose and modified N-glycans carrying only

α(1,3)-fucose and/or β(1,2)-xylose residues linked to the core Man3–2GlcNAc) (Lerouge et al. 1998) (Figure 5.3). HMT N-glycans are a subset of the oligosaccharides attached to secreted proteins and membrane proteins. Complex type N-glycans are formed after the modification of HMT glycans in the Golgi complex and are characterized by the presence of α(1,3)fucose attached to the proximal GlcNac and/or β(1,2)xylose linked to the β-mannose residues of the core. These modifications are specific to plant glycoproteins because mammalian N-glycans have α(1,6)fucose attached to the proximal GlcNac and do not contain any xylose residues (Priem et al. 1993; Fitchette et al. 1994).

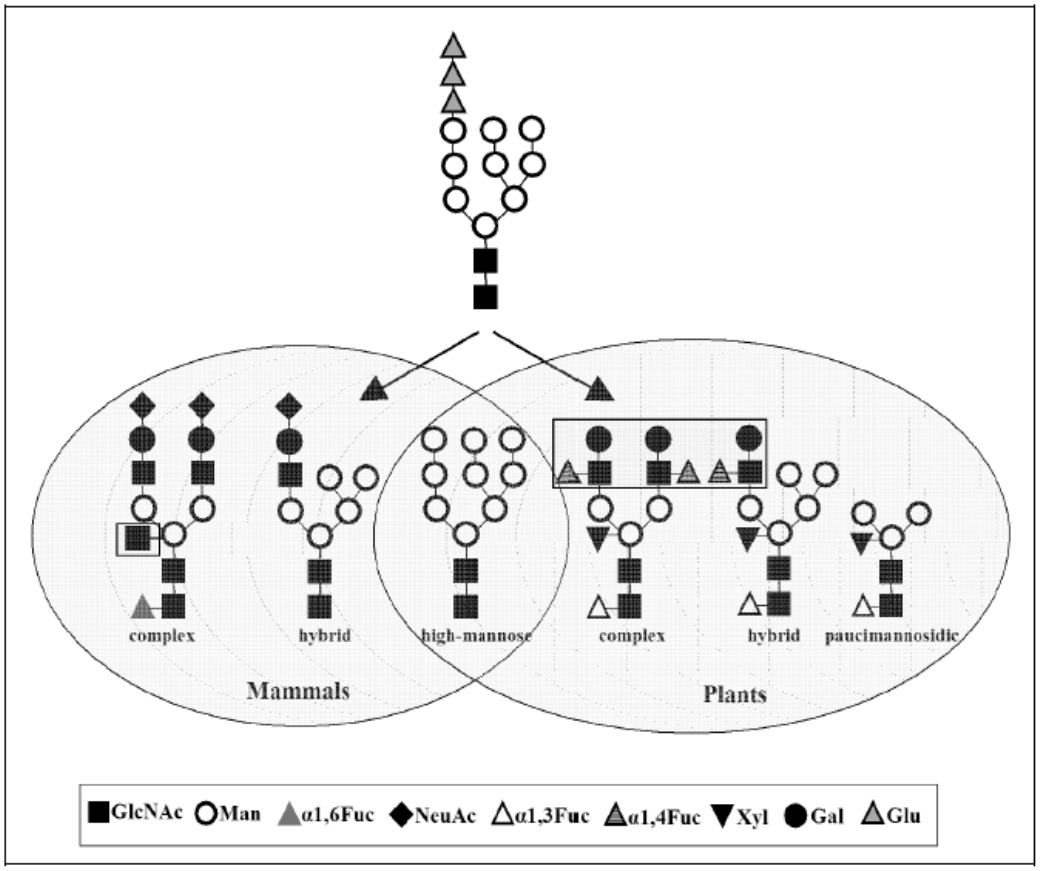

Figure 5.3: Differences in N-glycan structures between plants and mammals. The high-mannose type N-glycans have the same structure in plants and mammals, whereas complex, hybrid and paucimannosidic type N-glycans are structurally distinct. Complex and hybrid types contain α(1,3)-Fuc and β(1,2)-Xyl. Paucimannosidic type N-glycans, which contain only α(1,3)-Fuc and/or β(1,2)-Xyl linked to the core, are typical of the glycans found in plant vacuoles and they have not been detected in mammals (reproduced from Balen and Krsnik-Rasol, 2007).

Several studies have demonstrated that plants can produce complex mammalian glycoproteins efficiently, including monoclonal antibodies (Stoger et al. 2002; Paul and Ma, 2011). The secretory pathway is conserved between plants and mammals thus providing the post-translational modifications and correct folding needed to assemble functional antibodies in plants (Gomord et al. 2010). Although plants can synthesize N-glycan core structures identical to those of mammals (GnGn), the terminal residues differ, mainly because plant complex N-glycans lack β(1,4)galactose (and sialic acid) and core α(1,6)fucose. Instead they carry β(1,2)xylose and core α(1,3)fucose, which are not found in mammals (Gomord and Faye, 2004; Faye et al. 2005). Plant glycans are immunogenic in several mammals although their role in allergy has not yet been clarified (Garcia-Casado et al. 1996; Van Ree et al. 2000; Bardor et al. 2003) but curiously not in mice and only after multiple exposures in rats (Gomord et al 2005; Faye et al. 2005) (Figure 5.4). New strategies have therefore been developed to remove plant-specific glycans and "humanize" the glycan profiles of recombinant human glycoproteins (Yang et al. 2012). Thus far, such efforts have mainly focused on the targeted expression of therapeutic proteins, e.g. the use of ER-retention signals such as KDEL to prevent proteins being transported through the Golgi complex. Plants have also been engineered to abolish the genes encoding enzymes that carry out plant-specific modifications and to introduce the enzymatic machinery needed to humanize the glycan profiles of recombinant proteins (Gomord et al. 2010; Yang et al. 2012).

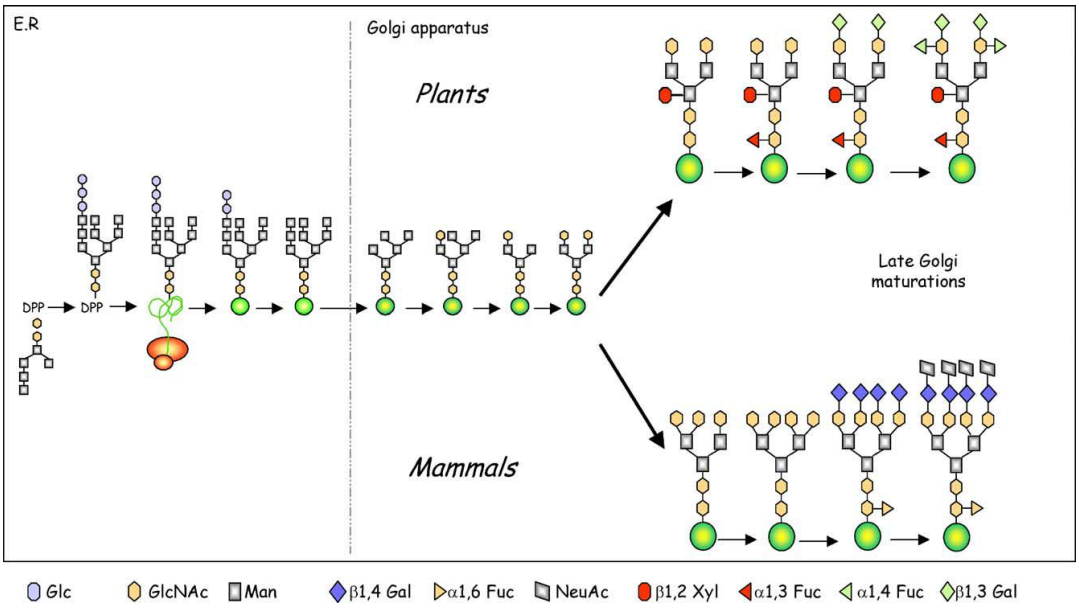

Figure 5.4: Addition and processing of N-linked glycans in the ER and Golgi apparatus of plants and mammals. A precursor oligosaccharide assembled onto a lipid carrier is transferred to specific Asn residues of the nascent polypeptide. The N-glycan is then trimmed to remove glucosyl and most mannosyl residues. Differences in the processing of plant and mammal N-glycans reflect late Golgi maturation events (Faye et al. 2005).

5.3 Aims

Lead event 3C, a transgenic maize line expressing 2G12 lacking an ER-retrieval signal (Ramessar et al. 2008a), was used as a model to investigate the precise subcellular localization of the antibody during maize seed development. We also tested the hypothesis that antibody localization or interactions with zein bodies might differ between lead event 3C, which accumulates high levels of 2G12 (Ramessar et al. 2008a), and another event (1F) which accumulates lower levels of the same molecule. Immunolocalization microscopy at different developmental stages was therefore used to compare lines 3C and 1F.

5.4 Materials and methods

5.4.1 Transformation vectors

All transformation constructs were based on the binary vector pTRA, a derivative of pPAM (GenBank accession no. AY027531) containing two tobacco RB7 scaffold attachment regions flanking the expression cassette (Sack et al. 2007). The coding regions of the 2G12 heavy and light chains (obtained from Polymun SA, Vienna, Austria) contained an N-terminal signal peptide targeting the secretory pathway. The expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the *Tobacco etch virus* 5' leader, the coding region, and the *Cauliflower mosaic virus* (CaMV) 35S terminator, resulting in final constructs pTRAgtiGH and pTRAgtiGL. The third construct (pTRAuxbar) contained the *bar* gene between the constitutive maize ubiquitin-1 promoter and the 35S terminator. All expression cassettes contained the maize ubiquitin-1 first intron (Figures 3.7 and 3.8 in Chapter 3, section 3.4.1).

5.4.2 Transformation, selection and regeneration of transgenic plants

The pTRAgti-GH and pTRAgti-GL vectors were introduced together with the pTRAux-bar vector into maize immature zygotic embryos. The DNA–gold mix was prepared as described by Drakakaki et al. (2005) with the pTRAgti-GH, pTRAgti-GL and pTRAux-bar plasmids present as a 3:3:1 molecular weight ratio (18.60:17.57:3.83 μg). Transformation, selection and regeneration were carried out as outlined in section 3.4.3. Putative transgenic plants were either selfed or cross pollinated depending on pollen availability.

5.4.3 Transgenic plants

Transgenic maize plants contained the pTRAgti-GH and pTRAgti-GL vectors for antibody expression and the pTRAux-bar vector for selection. Two different events were used for these experiments: the lead event 3C (described by Ramessar et al. 2008a) and event 1F which produces 2G12 at lower levels.

5.4.4 Electron microscopy

Immature maize seeds expressing 2G12 were collected at 14, 20 and 24 dap, fixed and processed for microscopy as described by Arcalis et al. (2004). The endosperm was sliced with a razor blade under phosphate buffer (0.1 M, pH 7.4). Tissue pieces were fixed in 4% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) overnight at 4°C. After several washing steps with phosphate buffer (0.1 M, pH 7.4), the samples were dehydrated through an ethanol series (50%, 70%, 90%, 100%) at 4°C and finally embedded progressively in LRWhite resin (25% in ethanol, 50%, 75% and pure resin for 3h 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 and were pre-incubated in 5% (w/v) bovine serum albumin (BSA) in phosphate buffer (0.1 M, pH 7.4) for 15 min at room temperature and then incubated with either polyclonal goat anti-HC or anti-LC (1:100 in phosphate buffer, 0.1 M, pH 7.4) for 2h 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 with phosphate buffer (0.1 M, pH 7.4) containing 0.5% Tween-20, sections were incubated with the secondary antibody (donkey anti-goat IgG coupled to 15-nm colloidal gold) diluted 1:30 in phosphate buffer (0.1 M, pH 7.4) and incubated for 1h at room temperature. After two washes for 10 min with phosphate buffer (0.1 M, pH 7.4) and two with distilled water, samples were air dried and the sections were observed using a Philips EM-400 transmission electron microscope.

5.5 Results

5.5.1 Subcellular localization of 2G12 during seed development

The subcellular localization of recombinant 2G12 in immature seed endosperm tissue was investigated by electron microscopy at three developmental stages. The secreted antibody chains were localized in the PSV where a strong signal was detected (red arrow, Figure 5.5A). In addition, both antibody chains were also detected within zein

bodies (black arrow, Figure 5.5D). The signal was not restricted to the peripheral γ-zein layer but was also present in the central core of the zein aggregates (black arrow). One significant observation was that the zein bodies had an abnormal structure. In stage 1, both antibody chains were detected in zein bodies that had lost their normal composition, with the normally uniform electron-dense γ-zein layer (dark grey) varying in thickness and interrupted (Figure 5.5A,D). This contrasts with the known structure of wild-type zein bodies (Figure 5.2) (Lending and Larkins, 1989). In stage 2, although the antibody chains were still found within zein bodies, the structure of the bodies was amorphous and disorganized, with no separation of the α-zein and γ-zein layers. The size of the bodies also varied considerably (Figure 5.5B,E). Greater numbers of amorphous bodies were observed in deeper layers of endosperm, where the cells are older. In stage 3, the number of amorphous zein bodies increased further. There was therefore a strong correlation between cell age and the loss of structural consistency among the zein bodies (Figure 5.5C,F).

5.5.2 Subcellular comparison of lead event 3C and the lower-yielding event 1F

The electron microscopy results for event 1F showed that the antibody HC and LC were localized in PSVs and also within zein bodies (Figure 5.5G), and as in the lead event (3C), the HC and LC signal was not restricted to the periphery of the zein bodies but was also present in the central core of the zein aggregates (Figure 5.5G). Fewer amorphous zein bodies were found in event 1F at 24 dap than event 3C (Figure 5.5D,F) suggesting that there is a negative correlation between the accumulation of 2G12 in maize seeds and the structural conservation of the zein body.

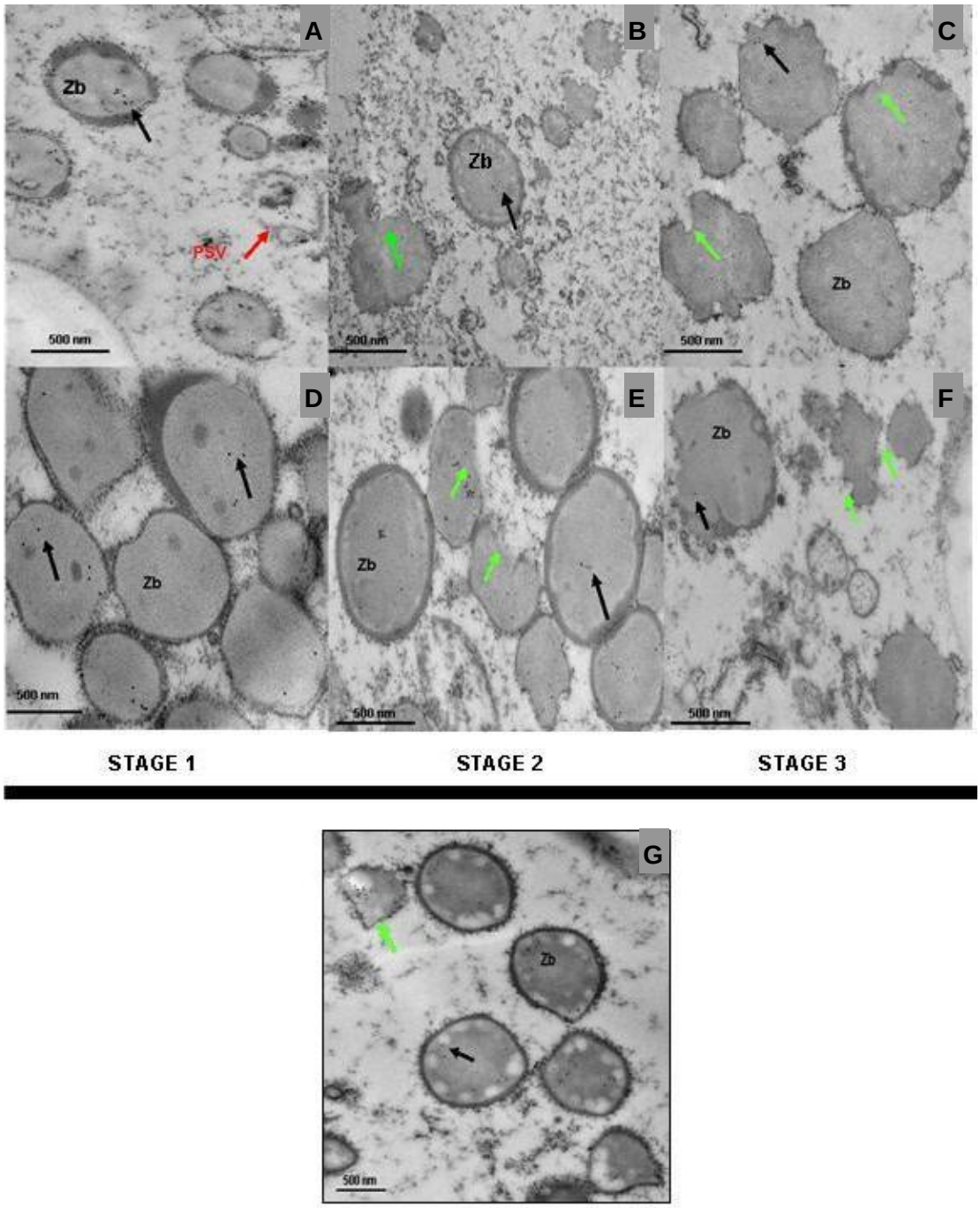

Figure 5.5: Localization of 2G12 during maize endosperm development by electron microscopy. A to C, detection of HC and LC in lead event 3C (primary antibody is not specific and binds both chains); D to F, detection of HC in lead event 3C. The antibody chains were localized in the PSVs where a strong signal was detected in all stages. Figure 5.5A shows PSVs containing the antibody (red arrow). In addition, both antibody chains (HC and LC) were also detected within zein bodies (black arrow) in all three stages. A and D (stage 1) HC and LC signal within the zein bodies, some of which have lost their normal structure, with an interrupted or non-uniform γ-zein electrondense layer (dark grey). B and E (stage 2) HC and LC signals are found within the zein bodies, but they now appear amorphous (green arrow). C and F (stage 3) More amorphous zein bodies containing HC and LC (green arrow) appear at the oldest stage. G) Event 1F (lower 2G12 accumulation), at 24 dap; HC and LC signal within the zein bodies (Zb) (black arrow) and less amorphous zein bodies (green arrow) than in event 3C at the same stage (D and F). PSV (protein storage vacuole); Zb (zein body); Bars = 500 nm.

5.6 Discussion

Subcellular localization has a significant impact on the structure, stability and yield of recombinant proteins expressed in plants. This is particularly true for multimeric proteins, which need to be folded and assembled before they can achieve their native state. Therefore, the yield of recombinant proteins in transgenic plants is strongly affected by the subcellular compartment in which they accumulate (Fischer et al. 2004; Doran, 2006; Streatfield, 2007). When the objective is to maximize recombinant protein yields, the native subcellular compartment of the protein is not always the best choice (De Virgilio et al. 2008). The best compartment is therefore a major strategic decision for the production of recombinant proteins in plants (Fischer et al. 2004; Doran, 2006; Streatfield, 2007).

A widely-used strategy in molecular pharming is to target recombinant proteins to the endomembrane system, which is a rich source of molecular chaperones. Signal peptides are used to target recombinant proteins into the ER, where they are folded, acquire N-linked glycans and assemble into multimers (Nicholson et al. 2005). The ER is linked to the Golgi complex, vacuoles, the plasma membrane and the extracellular environment. The principal routes for protein secretion are from the ER to the Golgi complex and from there to vacuoles (e.g. PSVs) or secretion to the apoplast (Vitale and Denecke, 1999; Jurgens, 2004).

One of the most compelling advantages of cereal seeds is that they naturally accumulate large amounts of protein in the endosperm and are thus an ideal vehicle for the stable accumulation of recombinant proteins. This stability is generally achieved by the formation of specialized storage compartments such as protein bodies and PSVs (Ramessar et al. 2008b). Various strategies have been developed to induce the formation of protein body-like structures by adding polypeptide sequence tags, such as the γ-zein coding region, elastin-like polypeptides (ELPs) and fungal hydrophobins (Conley et al. 2011). The inclusion of these sequences has a stabilizing effect on recombinant proteins by conferring protection from degradation, thus increasing yields. All the sequences

have similar properties, namely the ability to self-assemble via hydrophobic interactions (Conley et al. 2011).

The fusion of γ-zein to recombinant proteins has been tested in a wide range of expression platforms, including plants, insect cells, mammalian cells and filamentous fungi (Torrent et al. 2009; Conley et al. 2011). In plants, γ-zein has also been fused to the bean storage protein phaseolin to create a hybrid named zeolin (Mainieri et al. 2004). Hydrophobins are hydrophobic proteins produced by fungi (Linder et al. 2004). If they are fused to a KDEL-tagged recombinant protein in plants, they promote the formation of protein body-like aggregates that not only increase the recombinant protein yield but also provide a simplified purification strategy based on surfactant-based aqueous two-phase extraction, which can recover up to 90% of the protein in a single step (Joensuu et al. 2010). ELPs comprise numerous repeats of a short peptide such as VPGXG (where X is any amino acid except proline) which is reminiscent of the structure of the mammalian connective tissue protein elastin. The repeated domains allow self-assembly, conferring stability on fusion partners by packing them efficiently. ELPs also provide a simple purification method known as inverse transition cycling (ITC) which exploits their temperature-dependent solubility (Phan et al. 2011).

The subcellular localization of the recombinant proteins can also be investigated by glycan analysis, because particular glycans are specific to certain subcellular compartments. Plant glycans could increase the immunogenicity of injected biopharmaceuticals but for topical applications such as microbicides, the potential immunogenicity of plant glycans should not be an issue because humans are constantly exposed to plant glycoproteins in their diet and the environment (Twyman et al. 2005). Furthermore, plant glycans do not contain sialic acid residues, and monoclonal antibodies do not require these residues for successful topical passive immunization (Ma et al. 1998; Zeitlin et al. 1998). For topical applications is more important to ensure that the recombinant protein has correctly processed N-linked glycans because N-glycosylation is required for many proteins to function correctly, as it affects protein folding, quality control, sorting, degradation, secretion and modulates immune responses (Spiro, 2002; Helenius and Aebi, 2004).

Monoclonal antibody 2G12 has been produced in maize in two separate studies, in one case by secretion to the apoplast and in the other by retention in the ER (Ramessar et al. 2008a; Rademacher et al. 2008). In both cases, an N-terminal signal peptide was included in the transformation constructs for the heavy and light chains, facilitating cotranslational import into the ER and transport through the endomembrane system.

Rademacher et al. (2008) also added a C-terminal ER retrieval signal to both antibody chains in order to generate ER-derived protein bodies. Thus 2G12 lacking plant-specific complex-type N-glycans was produced in maize seeds and accumulated in the prolamin bodies, but not in the PSV-like structures. Immunofluorescence and electron microscopy confirmed the accumulation of the antibody in zein bodies that bud from the ER. N-glycans attached to the heavy chain were mostly devoid of Golgi-specific modifications, such as fucose and xylose. Small amounts of complex glycan structures typical of vacuolar proteins were detected, but their total amount rarely exceeded 10%. The accessibility of the KDEL sequence may explain differences in the retrieval efficiency for different KDEL-tagged antibodies (Sriraman et al. 2004). However, most of the glycans were trimmed extensively indicating that a significant endoglycanase activity was present in maize endosperm. The existence of single GlcNAc residues suggests that HMT glycans are processed by an endoglycanase (ENGase). The specific antigen-binding function of the purified antibody was verified by surface plasmon resonance spectroscopy, and *in vitro* assays demonstrated that its HIV-neutralizing properties were equivalent to or even better than those of its counterpart produced in Chinese hamster ovary (CHO) cells. The maximum yield was 40 µg/g dry seed weight (Rademacher et al. 2008).

Ramessar et al. (2008a) produced 2G12 without a retrieval signal, and one homozygous line (event 3C) was identified after screening over two generations that expressed 2G12 at levels exceeding 75 µg/g dry seed weight, which is considerably higher than the typical levels for recombinant proteins expressed from the plant nuclear genome. Event 3C was characterized as a lead event. The purified maize-derived 2G12 was physically identical to its CHO-derived counterpart with the exception of its glycan structure,

comprising a mixture of HMT and plant-specific complex-type glycans. Many antibodies contained single GlcNAc residues, as also noted by Rademacher et al. (2008). In these experiments, the subcellular localization of recombinant 2G12 in endosperm tissue was analyzed by fluorescence and electron microscopy at different developmental stages chosen according to the age of the cells rather than to the age of the seed (dap). The localization of the heavy chain (HC) was used as a benchmark for the assembled antibody, and was initially localized within the PSV although some zein bodies were also labeled. In older cells, the PSVs and zein bodies fused (the zein bodies were incorporated by autophagy) and the HC was found in deposits around the zein bodies, giving a peripheral ring-like structure. Later in development, the fused protein storage compartments tended to aggregate, leaving 2G12 at the periphery and producing masses with unclear signals. No significant changes were observed in the distribution of HC. Electron microscopy experiments revealed that secreted 2G12 accumulates in protein bodies, mostly within PSVs but also within the zein bodies. Glycan analysis for event 3C (Ramessar et al. 2008a) corroborated the immunolocalization data by showing that approximately 6% of the N-glycans were HMT (Man5-9) perhaps corresponding to the 2G12 detected in ER-derived protein bodies, whereas 28% contained typical vacuolar complex N-glycans (GnGnXF/MUXF) suggesting that 2G12 had traveled via the Golgi complex to the PSV. In functional terms, 2G12 showed identical antigen-binding activity to its CHO cell counterpart but nearly three times the efficacy in HIV-neutralization assays, probably because of the presence of aggregates.

We used lead event 3C expressing 2G12 without an ER retrieval signal as a model to investigate the precise subcellular localization of the antibody during maize seed development. Our results corroborate the earlier data and our analysis of a second event with low accumulation levels (event 1F) provides further confirmation. Proteins in the cereal endosperm endomembrane system proceed to the Golgi complex and eventually accumulate in the PSV if they carry signal peptide but lack additional targeting signals, and this is regarded as the default pathway in seeds (Drakakaki et al. 2006) rather than secretion to the apoplast which is the default pathway in other tissues (Denecke et al. 1990). We found that 2G12 was found predominantly in the PSV in both of the events

we analyzed, which was anticipated because the antibody chains do not carry targeting signals other than the N-terminal signal peptide. A recombinant version of fungal phytase expressed in maize endosperm without further targeting signals was also deposited in the PSV (Arcalis et al. 2010). However, we also found that 2G12 was localized within zein bodies, and was present in the central core as well as the peripheral layer. There are two possible explanations for this observation.

First, 2G12 might be trapped within the zein network by passive interactions as the bulk flow of zein proteins bypasses the Golgi complex. Zein bodies containing 2G12 may either accumulate in the cytosol or fuse directly with the PSV (Herman and Larkins, 1999). The expression of recombinant phytase in rice seeds provides an example of this mechanism (Takahashi et al. 2005). Rice endosperm PSVs consist mainly of glutelins that have passed through the Golgi complex (Okita and Rogers, 1996), although recent evidence suggests that recombinant phytase may have reached the PSVs directly from the ER without passing through the Golgi complex (Takahashi et al. 2005). This mechanism was observed in rice, but when recombinant phytase was produced in maize seeds the phytase was only found at the periphery of the zein bodies (Arcalis et al. 2010). Time-dependent protein trafficking in maize was also observed, resulting in two pools of phytase, an early pool that reflects trafficking through the ER and Golgi complex to the PSV, and a later pool accumulating in the ER and in ER-derived compartments. This was consistent with the changing N-glycan profiles of recombinant phytase (Arcalis et al. 2010). Therefore, the 2G12 we observed in maize zein bodies may not be passively trapped because its uniform distribution does not match the peripheral distribution of recombinant phytase.

The second possible explanation is that there is a specific interaction between zein proteins and the 2G12 HC, perhaps reflecting the ability of both proteins to form disulfide bonds. This is supported by the presence of 2G12 in the core zein clusters, where recombinant proteins do not usually accumulate. Lending and Larkins (1989) showed that young zein bodies consist only of γ- and β-zeins, whereas later in development α-zeins and β-zeins penetrate the network of γ-zeins and form isolated

inclusions that fuse in maturity so that mature zein bodies consist of a central core of α-zeins surrounded by γ-zeins. This is a conserved structure and, as demonstrated by Coleman et al. (1996, 2004), the presence of all zein subfamilies is necessary for its formation, with any disturbance in the zein balance leading to the formation of unstable and amorphous organelles (Lending and Larkins, 1989; Coleman et al. 1996, 2004).

In previous immunolocalization experiments, amorphous zein bodies were found in maize endosperm samples at 15 and 20 dap. We analyzed samples at 10, 20 and 24 dap in order to corroborate the previous observations and complement them, and to construct a developmental profile. In young endosperm cells, the normally uniform γ-zein electron-dense layer (Lending and Larkins, 1989) was non-uniform and interrupted whereas the core appeared normal. However, later in development, the entire structure became disorganized, with no clear zones, no defined shape and an unusually diverse size range. This disorganization becomes more common and more profound later in development.

Rademacher et al. (2008) observed that 2G12 was localized within zein bodies (as expected because of the presence of an ER-retrieval signal) but the zein bodies in this case remained normal even at 25 dap, corresponding to the oldest cells we analyzed. It is possible that the difference may be the timing of antibody assembly, i.e. if the HC and LC interact first, then the zein bodies retain their normal structure because there are fewer free cysteine residues available to form disulfide bonds with zeins (Rademacher et al. 2008). Immunolocalization experiments in event 1F at 24 dap revealed a much lower number of amorphous zein bodies compared to event 3C. The amount of available antibody therefore appears to influence the morphological changes in the protein bodies, which is consistent with the above hypothesis because this would also reduce the number of free cysteine residues available to form disulfide bonds. Similarly, the older the seed, the more antibodies are present, which could explain the worsening structural phenotype in older cells. This hypothesis could be addressed in more detail in future studies.

Coleman et al. (1996, 2004) showed by expressing one or more zeins in tobacco seeds that any kind of alteration in the zein ratio resulted in the formation of amorphous protein bodies accompanied by increase in binding proteins (BiP) levels (Zhang and Boston, 1992). The latter is usually associated with the accumulation of abnormal proteins in the ER, which would certainly be the case for HC-zein heterodimers, so it will be interesting in future studies to address the level of BiP in maize endosperm cells expressing 2G12.

Maize mutants with abnormal protein bodies (e.g. *floury-2*, *Mucronate*, *Defective endosperm B30*) often display the opaque phenotype accompanied by increases in BiP, so it would be interesting to investigate the phenotype of these transgenic events in detail to see if the opaque phenotype is also present (Kim et al. 2004).

From a molecular pharming perspective, the interaction between HC and zein is undesirable because some of the antibody produced in maize seeds would be non-functional. However, 2G12 accumulated to 75 mg/kg dry seed weight in the PSV, but to only 40 mg/kg dry seed weight when retained in the ER (Ramessar et al. 2008a; Rademacher et al. 2008). Therefore, the higher yields of the secreted antibody probably compensate for the loss of functional protein due to interactions with zeins. Even so, further studies are required to develop strategies to minimize or eliminate such interactions and produce even higher levels of functional recombinant antibodies in maize seeds.

5.7 Conclusions

Subcellular localization influences on the yield, structure and modification of recombinant proteins, and when these factors are combined with the cost of protein recovery during downstream processing, the ideal platform for recombinant protein expression can only be established on a case by case basis. Molecular pharming has many advantages for the production of recombinant proteins in terms of economy, scalability and safety, and seeds are particularly useful because they have evolved as

protein storage organs and have additional benefits such as their ability to accumulate proteins in a stable environment and maintain them in an inert state (thus making cold chain infrastructure unnecessary), and their suitability for minimal processing in relation to oral and topical application routes (thus making extensive downstream processing unnecessary). The accumulation of recombinant proteins in major seed protein storage organelles is therefore an attractive strategy. The storage proteins of cereals account for approximately 50% of the total protein in mature cereal grains, making cereal seeds ideal for recombinant protein expression. It is necessary to study recombinant protein trafficking, deposition and storage in cereal seeds to take full advantage of the unique morphological storage characteristics of cereal endosperm cells and to improve the yield and quality of recombinant proteins. Furthermore, this knowledge can be used to induce ectopic storage compartments in vegetative tissues, offering further opportunities for the large-scale and economic production of recombinant pharmaceutical proteins. The combination of microscopy and N-glycan analysis in this study provides evidence that the HIV-neutralizing human monoclonal antibody 2G12 is produced correctly in maize seeds with the exception of its glycan structure, but this does not affect its antigen-binding properties and it is up to three times more effective in HIV-neutralization assays than its CHO-derived counterpart. Immunolocalization experiments during maize endosperm development show that the recombinant protein is localized mainly in the PSVs in the endosperm, although there are also interactions between 2G12 and zein bodies. Even though 2G12 expression levels in maize endosperm were high, further improvements are required to minimize such interactions and achieve high yields, taking full advantage of maize seeds as a production platform for pharmaceutical proteins.

5.8 References

Altschuler Y and Galili G (1994) Role of conserved cysteines of a wheat gliadin in its transport and assembly into protein bodies in Xenopus oocytes. *Journal of Biology and Chemistry* 269:6677-6682

Arakawa T, Yu J, Chong DK, Hough J, Engen PC and Langridge WH (1998) A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nature Biotechnology* 16:934-938

Arcalis E, Marcel S, Altmann F, Kolarich D, Drakakaki G, Fischer R, Christou P and Stoger E (2004) Unexpected deposition patterns of recombinant proteins in post-endospermic reticulum compartments of wheat endosperm. *Plant Physiology* 136:3457-3466

Arcalis E, Stadlmann J, Marcel S, Drakakaki G, Winter V, Rodriguez J, Fischer R, Altmann F and Stoger E (2010) The changing fate of a secretory glycoprotein in developing maize endosperm. *Plant Physiology* 153:693-702

Bagga S, Adams H, Kemp JD and Sengupta-Gopalan C (1995) Accumulation of 15-kilodalton zein in novel protein bodies in transgenic tobacco. *Plant Physiology* 107:13-23

Balen B and Krsnik-Rasol M (2007) Plant specific N-glycosylation. *Food Technology and Biotechnology* 45:1-10

Bardor M, Faveeuw C, Fitchette AC, Gilbert D, Galas L, Trottein F, Faye L and Lerouge P (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)- fucose and core xylose. *Glycobiology* 13:427-434

Boyer SK, Shotwell MA and Larkins BA (1992) Evidence for the translational control of storage protein gene expression in oat seeds. *Journal of Biological Chemistry* 267:17449-17457

Coleman CE, Herman EM, Takasaki K and Larkins BA (1996) The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies in transgenic tobacco endosperm. *Plant Cell* 8:2335-2345

Coleman CE and Larkins BA (1999) The prolamins from maize. *In*: Shewry PR, Casey R, (eds) Seed Proteins. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp:109-139

Coleman CE, Yoho PR, Escobar S and Ogawa M (2004) The accumulation of alpha-zein in transgenic tobacco endosperm is stabilized by co-expression of beta-zein. *Plant Cell Physiology* 45:864-871

Conley AJ, Joensuu JJ, Richman A and Menassa R (2011) Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. *Plant Biotechnology Journal* 9:419-433

De Virgilio M, Weninger H and Ivessa NE (1998) Ubiquitination is required for the retrotranslocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *Journal of Biological Chemistry* 273:9734-9743

Denecke J, Botterman J and Deblaere R (1990) Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2:51-59

Doran PM (2006) Foreign protein degradation and instability in plants and plant tissue cultures. *Trends in Biotechnology* 24:426-432

Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P and Stoger E (2005) Endosperm specific co-expression of recombinant soybean ferritin and *Aspergillus phytase* in maize results in significant increases in the levels of bioavailable iron. *Plant Molecular Biology* 59:869-880

Drakakaki G, Marcel S, Arcalis E, Altmann F, Gonzalez-Melendi P, Fischer R, Christou P and Stoger E (2006) The intracellular fate of a recombinant protein is tissue dependent. *Plant Physiology* 141:578-586

Faye L, Boulaflous A, Benchabane M, Gomord V and Michaud D (2005) Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. *Vaccine* 23:1770-1778

Fischer R, Stoger E, Schillberg S, Christou P and Twyman R (2004) Plant-based production of biopharmaceuticals. *Current Opinion in Plant Biology* 7:152-158

Fitchette AC, Gomord V, Chekkafi A and Faye L (1994) Distribution of xylosylation and fucosylation in the plant Golgi apparatus. *The Plant Journal* 5:673-682

Galili G (2004) ER-derived compartments are formed by highly regulated processes and have special functions in plants. *Plant Physiology* 136:3411-3413

Garcia-Casado G, Sanchez-Monge R, Chrispeels MJ, Armentia A, Salcedo G and Gomez L (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* 6:471-477

Geli MI, Torrent M and Ludevid D (1994) Two structural domains mediate two sequential events in γ-zein targeting: protein endoplasmic reticulum retention and protein body formation. *Plant Cell* 6:1911-1922

Gomord V and Faye L (2004) Post-translational modification of therapeutic proteins in plants. *Current Opinion in Biotechnology* 7:171-181

Gomord V, Chamberlain P, Jefferis R and Faye L (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. *Trends in Biotechnology* 23:559-565

Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D and Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnology Journal* 8:564-587

Hanton SL, Bortolotti LE, Renna L, Stefano G and Brandizzi F (2005) Crossing the divide transport between the endoplasmic reticulum and Golgi apparatus in plants. *Traffic* 6:267-277

Helenius A and Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annual Review Biochemistry* 73:1019-1049

Herman EM and Larkins BA (1999) Protein storage bodies and vacuoles. *Plant Cell* 11:601-603

Herman EM (1994) Multiple origins of intravacuolar protein accumulation of plant cells. *Advances in Structural Biology* 3:243-283

Hood EE (2004) Where, oh where has my protein gone? *Trends in Biotechnology* 22:53-55

Hurkman WJ, Smith LD, Richter J and Larkins BA (1981) Subcellular compartmentalization of maize storage proteins in Xenopus oocytes injected with zein messenger RNAs. *Journal of Cell Biology* 89:292-299

Joensuu JJ, Conley AJ, Lienemann M, Brandle JE, Linder MB and Menassa R (2010) Hydrophobin fusions for high-level transient protein expression and purification in *Nicotiana benthamiana*. *Plant Physiology* 152:622-633

Jurgens G (2004) Membrane trafficking in plants. *Annual Review of Cell Development Biology* 20:481-504

Kim CS, Woo YM, Clore AM, Burnett RJ, Carneiro NP and Larkins BA (2002) Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. *Plant Cell* 14:655-672

Kim CS, Hunter BG, Kraft J, Boston RS, Yans S, Jung R and Larkins BA (2004) A defective signal peptide in a 19-kD alpha-zein protein causes the unfolded protein response and an opaque endosperm phenotype in the maize De*-B30 mutant. *Plant Physiology* 134:380-387

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M and Koprowski H (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proceedings of the National Academy of Sciences USA* 100:8013-8018

Kogan MJ, López O, Cocera M, López-Iglesias C, De La Maza A and Giralt E (2004) Exploring the interaction of the surfactant N-terminal domain of gamma-zein with soybean phosphatidylcholine liposomes. *Biopolymers* 73:258-268

Krishnan HB, Franceschi VR and Okita TW (1986) Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. *Planta* 169:471-480

Lee CW, Erickson HT and Janick J (1976) Inheritance of cleistogamy in *Salpiglossis sinuata*. *Journal of Heredity* 67:267-270

Lee MCS, Miller EA, Goldberg J, Orci L and Schekman R (2004) Bi-directional protein transport between the ER and the Golgi. *Annual Review of Cell and Developmental Biology* 20:87-123

Lending CR and Larkins BA (1989) Changes in the zein composition of protein bodies during maize endosperm development. *Plant Cell* 1:1011-1023

Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Laine AC, Gomord V and Faye L (1998) N-Glycoprotein biosynthesis in plants: Recent developments and future trends. *Plant Molecular Biology* 38:31-48

Levanony H, Rubin R, Altschuler Y and Galili G (1992) Evidence for a novel route of wheat storage proteins to vacuoles. *Journal of Cell Biology* 119:1117-1128

Linder MB, Qiao M, Laumen F, Selber K, Hyytiä T, Nakari-Setälä T and Penttilä ME (2004) Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. *Biochemistry* 43:11873-11882

Ma JKC, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, Van Dolleweerd C, Mostov K and Lehner T (1995) Generation and assembly of secretory antibodies in plants. *Science* 268:716-719

Ma JKC, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB and Lehner T (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nature Medicine* 4:601-606

Mainieri D, Rossi M, Archinti M, Bellucci M, De Marchis F, Vavassori S, Pompa A, Arcioni S and Vitale A (2004) Zeolin. A new recombinant storage protein constructed using maize γ-zein and bean phaseolin. *Plant Physiology* 136:3447-3456

Muntz K (1998) Deposition of storage proteins. *Plant Molecular Biology* 38:77-99

Nicholson L, Gonzalez-Melendi P, Van Dolleweerd C, Tuck H, Perrin Y, Ma JKC, Fischer R, Christou P and Stoger E (2005) A recombinant multimeric immunoglobulin expressed in rice shows assembly dependent subcellular localization in endosperm cells. *Plant Biotechnology Journal* 3:115-127

Okita TW and Rogers JC (1996) Compartmentation of proteins in the endomembrane system of plant cells. *Annual Review of Plant Physiology Plant Molecular Biology* 47:327-350

Osborne TB (1907) The proteins of the wheat kernel. Carnegie Institute, Washington Publ. 84:1-119

Paris N, Stanley CM, Jones RL and Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. *Cell* 85:563-572

Paul M and Ma JKC (2011) Plant-made pharmaceuticals: Leading products and production platforms. *Biotechnology and Applied Biochemistry* 58:58-67

Phan HT and Conrad U (2011) Membrane-based inverse transition cycling: an improved means for purifying plant-derived recombinant protein-elastin-like polypeptide fusions. *International Journal of Molecular Science* 12:2808-2821

Priem B, Gitti R, Bush CA and Gross KC (1993) Structure of ten free N-Glycans in ripening tomato fruit. *Plant Physiology* 102:445-458

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

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma JKC, Stoger E, Capell T and Christou P (2008a) Cost-effective production of a

vaginal protein microbicide to prevent HIV transmission. *Proceedings of the National Academy of Sciences USA* 105:3727-3732

Ramessar K, Capell T and Christou P (2008b) Molecular pharming in cereal crops. *Phytochemistry Review* 7:579- 592

Robinson DG, Galili G, Herman E and Hillmer S (1998) Topical aspects of vacuolar protein transport: autophagy and prevacuolar compartments. *Journal of Experimental Botany* 49:1263-1270

Rubin R, Levanony H and Galili G (1992) Characterization of two types of protein bodies in developing wheat endosperm. *Plant Physiology* 99:718-724

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoger E and Rademacher T (2007) Functiona analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic tobacco BY2 suspension cells. *FASEB Journal* 21:1655-1664

Sanderfoot AA and Raikhel NV (1999) The specificity of vesicle trafficking: coat proteins and SNAREs. *Plant Cell* 11:629-642

Shewry PR, Napier JA and Tatham AS (1995) Seed storage proteins: structures and biosynthesis. *Plant Cell* 7:945-956

Shewry PR and Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany* 53:947-958

Shimoni Y and Galili G (1996) Intramolecular disulfide bonds between conserved cysteines in wheat gliadins control their deposition into protein bodies. *Journal of Biological Chemistry* 271:18869-18874

Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12:43-56

Sriraman R, Bardor M, Sack M, Vaquero C, Faye L, Fischer R, Finnern R and Lerouge P (2004) Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-α(1,3)-fucose residues. *Plant Biotechnology Journal* 2:279-287

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P and Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Molecular Biology* 42:583-590

Stoger E, Sack M, Fischer R and Christou P (2002) Plantibodies: applications, advantages and bottlenecks. *Current Opinion in Biotechnology* 13:161-166

Stoger E, Ma JKC, Fischer R and Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Current Opinion in Biotechnology* 16:167–173

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnology Journal* 5:2-15

Takahashi H, Saito Y, Kitagawa T, Morita S, Masumura T and Tanaka K (2005) A novel vesicle derived directly from endoplasmic reticulum is involved in the transport of vacuolar storage proteins in rice endosperm. *Plant Cell Physiology* 46:245-249

Torrent M, Llompart B, Lasserre-Ramassamy S, Llop-Tous I, Bastida M, Marzabal P, Westerholm-Parvinen A, Saloheimo M, Heifetz BP and Ludevi MD (2009) Eukaryotic protein production in designed storage organelles. *BMC Biology* 7:5

Twyman RM, Schillberg S and Fischer R (2005) Transgenic plants in the biopharmaceutical market. *Expert Opinion on Emergening Drugs* 10:185-218

Van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L and Lerouge P (2000) Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. Journal of Biological Chemistry 275:11451–11458

Vaquero C, Sack M, Schuster F, Finnern R, Drossard J, Schumann D, Reimann A and Fischer R (2002) A carcinoembryonic antigen specific diabody produced in tobacco. *FASEB Journal* 16:408-410

Vitale A and Denecke J (1999) The endoplasmic reticulum-gateway of the secretory pathway. *Plant Cell* 11:615-628

Vitale A and Ceriotti A (2004) Protein quality control mechanisms and protein storage in the endoplasmic reticulum. A conflict of interests? *Plant Physiology* 136:3420–3426

Wandelt CI, Khan MR, Craig S, Schroeder HE, Spencer D and Higgins TJ (1992) Vicilin with carboxyterminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant Journal* 2:181-192

Wilson CM (1991) Multiple zeins from maize endosperms characterized by reversed-phase high performance liquid chromatography. *Plant Physiology* 95:777

Woo YM, Hu DW, Larkins BA and Jung R (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. *Plant Cell* 13:2297–2317

Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, Lane JR, Delaney DE, McComas-Wagner JM, Mallubhotla HD, Hood EE, Dangott LJ, Tichy SE and Howard JA (2003) Maize (Zea mays)-derived bovine trypsin: Characterization of the first large-scale, commercial protein product from transgenic plants. *Biotechnology and Applied Biochemistry* 38:123-130

Yang Z, Bennett EP, Jorgensen B, Drew DP, Arigi E, Mandel U, Ulvskov P, Levery SB, Clausen H and Petersen BL (2012) Toward stable genetic engineering of human O-glycosylation in plants. Plant Physiology 160:450–463

Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA and Whaley KJ (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nature Biotechnology* 16:1361-1364

Zhang F and Boston RS (1992) Increases in binding protein (BiP) accompany changes in protein body morphology in three high-lysine mutants in maize. *Protoplasma* 171:142-152

CHAPTER 6

NON-TECHNICAL BARRIERS FOR THE ADOPTION OF GE CROPS IN EUROPE

CHAPTER 6: NON-TECHNICAL BARRIERS FOR THE ADOPTION OF GE CROPS IN EUROPE

6.1 Abstract

The commercial cultivation of genetically engineered (GE) crops began in 1996 and has been expanding continuously ever since, both in industrialized and developing countries. In contrast, the European Union (EU) has limited the cultivation of GE crops through the establishment of complex, inconsistent and non-science/evidence-based regulatory frameworks that actively slow down socioeconomic development and strongly discourage farmers from adopting the technology. The EU recognizes the potential of GE crops and has developed a series of research and development funding policies based on the Lisbon Strategy to establish the most competitive knowledge-based bioeconomy (KBBE) in the world. However, these are actively opposed by EU agricultural policies, which have created an environment in which the aims of the Lisbon Strategy can never be achieved by favoring the economic privileges of organic farmers and supporting the Common Agricultural Policy (CAP). There is also a long and complex road to gain authorization for cultivation and import of GE crops and their products, and the EU has recently approved a plan to allow member states to opt-out of the cultivation of approved GE crops with no scientific justification. As a consequence, these non-technical barriers to the adoption of GE crops are damaging the EU's global scientific standing and making the economy increasingly uncompetitive and isolated in the international markets, which thrive on innovation and technological development in agriculture.

6.2 Global adoption of GE crops

GE crops continued to flourish globally in 2011 and the upward trend in the number of countries adopting the technology, the amount of land set aside for GE crops and the number and diversity of products, shows no sign of abating (James, 2012). In contrast, GE agriculture in the EU is moribund and declining rapidly. The public in Europe have adopted a predominantly anti-GE stance, which is fuelled by politicians eager to pander to a cause with obvious popular support and a media eager to exploit public outrage. This vicious cycle seems set to continue for the foreseeable future (Farre et al. 2011). The rules governing the commercial cultivation of GE crops in Europe are obstructive and arbitrary, making it virtually impossible for a farmer to make an independent decision to adopt the technology on his or her land even if the crop in question has been approved for cultivation (Ramessar et al. 2010). In a misguided attempt to break out of this deadlock, the European Commission (EC) has proposed that member states should be given an unconditional opt-out, i.e. the opportunity to ban the planting of approved GE crops on a case-by-case basis, without having to provide any rational scientific evidence of risk (European Commission, 2010a).

The EC claims that the intention of the opt-out is benign, i.e. it aims to remove the current opportunity for member states to vote against the adoption of individual GE crops and prevent their cultivation throughout the EU. However, we argue that the opt-out will have an unintentional negative impact, by further increasing public confusion surrounding GE crops and allowing countries to become GE exclusion zones for reasons of political expediency rather than genuine concerns about public safety (Morris and Spillane, 2010; Sabalza et al. 2011). The suppression of GE agriculture in Europe is widely known to be purely an economic phenomenon, since GE agriculture is regarded as a threat to organic farming which attracts premium prices (Ramessar et al. 2010). However, allowing a globally successful and beneficial technology to be outlawed for artificial reasons and short-term economic gains shows the EU to be extremely short sighted, and actively working against its own goals as set out in the Lisbon Agenda. The potential damage to EU agriculture, the economy and to the future of industrial

investment in scientific research and development is the elephant in the room that no-one in Europe wants to see.

6.2.1 Global area of GE crops

In 1996 the first GE crops were commercialized in the USA and five other countries. Since then, millions of farmers in 29 countries worldwide have chosen to grow GE crops over a cumulative area of more than 1.25 billion hectares (25% larger than the total land mass of the United States) (Figure 6.1). The most widely grown GE crops were herbicide-tolerant varieties (Figure 6.2) (James, 2012).

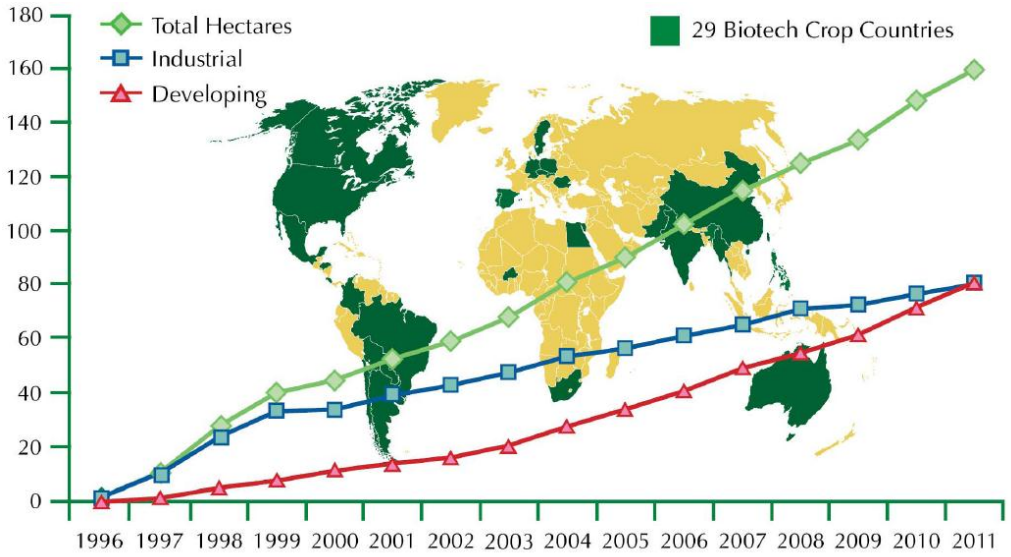

Figure 6.1: Global area of GE crops (millions of hectares 1996-2011) (James, 2011).

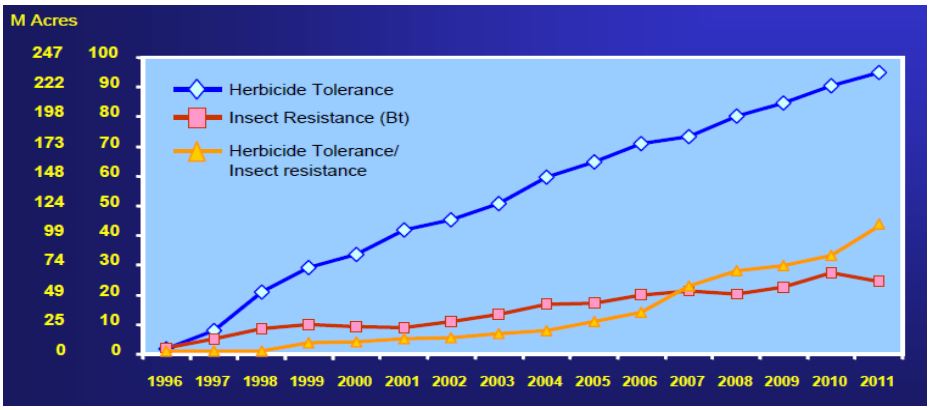

Figure 6.2: Global area of GE crops, 1996 to 2011, by trait (millions of hectares/acres) (James, 2011).

In 2011, more GE crops were grown in the USA than any other country (70 million hectares). Maize, soybean and cotton were the main GE crops grown in the USA, and more than half of the maize and cotton contained stacked traits (principally herbicide tolerance and pest resistance). There were also half a million hectares of sugar beet, and more limited areas of canola, alfalfa, squash and papaya (Figure 6.3) (James, 2012).

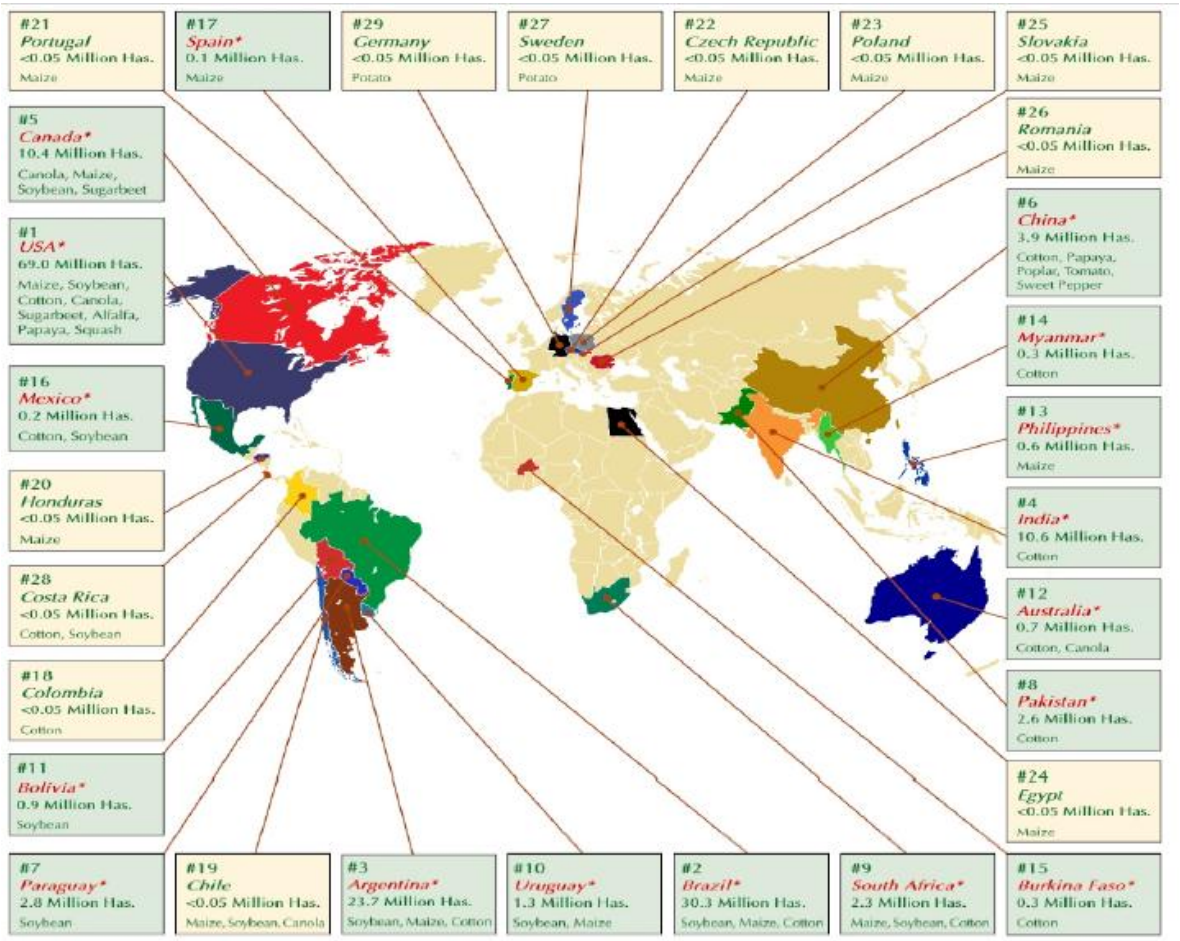

Figure 6.3: Global map of GE crop countries and mega-countries in 2011 (James, 2011).
Cultivation of the Amflora potato was prohibited in Germany in September 2010 (GMO Compass, 2010).

More developing countries than industrialized nations adopted GE crops in 2011, with China and India leading the way in Asia, Brazil and Argentina leading the way in South America, and South Africa leading the way in Africa (Figure 6.3). Approximately 50% of GE crops in 2011 were cultivated in developing countries and their GE land area is expected to exceed that of industrial countries in 2012 (James, 2012).

6.2.2 A brief history of GE crops in Europe

The first commercial GE crop to be cultivated in the EU was the MON810 maize variety, incorporating an insecticidal transgene from the bacterium *Bacillus thuringiensis* (Bt). This was planted for the first time in 1998, two years after the same variety was adopted in the USA, with approximately 20,000 hectares planted in Spain. By 2006, this had expanded to ~54,000 hectares in Spain (14% of the available land for maize cultivation), with 5000 hectares also cultivated in France and smaller areas (less than 1000 hectares) also planted in Germany, Portugal, the Czech Republic, Romania, Poland and Slovakia (James, 2011). Since then, the prevalence of GE agriculture has fallen in the EU, a phenomenon that is unique in the world. Only six EU countries (Spain, Portugal, the Czech Republic, Poland, Slovakia and Romania) planted Bt maize in 2011, falling from seven since the decision by Germany to discontinue cultivation in 2008. These six EU countries grew 114,490 hectares of Bt maize, a substantial 26% hectares more than in 2010 (James, 2011). The most enthusiastic adopter of Bt maize in Europe is Spain, which in 2011 had an adoption rate of 28% and accounted for 85% of the GE maize grown in Europe (James, 2011). As well as MON810, there is a second GE crop approved for commercial release in the EU, i.e. Amflora potato with modified starch, which is approved for non-food/feed use (GMO Compass, 2012). Cultivation of the Amflora potato was prohibited in Germany in September 2010 because intermixture with another GE potato variety occurred during its cultivation in Sweden (GMO Compass, 2010). These barriers eventually persuaded the developer BASF to move production abroad because "there is still a lack of acceptance for this technology in many parts of Europe" (BASF, 2012).

The authorization for a further two crops (one food crop and one ornamental flower crop) has expired although renewals have been submitted, which means the products can still be cultivated in lieu of a new license, and a further 22 crops have new applications pending (Tables 6.1-6.4) (GMO Compass, 2012).

TABLE 6.1: Crops authorized for cultivation (current and expired)

Trait	Genes	Event	Current status	Regional veto
CARNATION				
Modified flower color	*surB,bp40, dfr, acc*	959A, 988A, 1226A, 1351A, 1363A, 1400A (*surB, bp40, dfr*)	Approval expired in October 2008. Application for renewal filed.	
Modified flower color and Increased shelf-life		66 (*acc, surB*)	Authorization no longer valid in October 2008	
MAIZE				
Insect resistance	*cry1Ab*	MON810 (*cry1Ab*)	Valid authorization	Austria, Hungary, France, Greece, Germany, Luxembourg
		Bt176 (*cry1Ab*)	Authorization no longer valid, April 2007	
Herbicide tolerance	*pat*	T25 (*pat*)	Notified as an existing product on 1 October 2004. Approval expired on 18 April 2007. Application for renewal filed.	
POTATO				
Altered composition	*antisense GBSS*	EH92-527-1 (*antisense GBSS*)	Valid authorization	Austria, Hungary, Luxembourg
OILSEED RAPE				
Male sterility, herbicide tolerance	*barnase, barstar*	MS1 x RF2 (*barnase, barstar*), MS1 x RF1 (*barnase, barstar*)	Authorization no longer valid, April 2007	
CHICORY				
Male sterility, herbicide tolerance	*barnase, bar*	RM3-3, RM3-4, RM3-6 (*barnase, bar*)	Authorization no longer valid, 27 May 2003	

Table 6.2: Crops with authorization pending for cultivation.

Trait	Genes	Event
COTTON		
Insect resistance	*cry1Ac*	MON 531 (*cry1Ac)*
Herbicide tolerance	*cp4 epsps*	MON 1445 (*cp4 epsps*)
OILSEED RAPE		
Herbicide tolerance	*pat*	Liberator pHoe6/Ac (*pat)*, GS40/90pHoe6 / Ac (*pat)*
SOYBEAN		
Herbicide tolerance	*cp4 epsps*	MON40-3-2 (*cp4 epsps*)
SUGAR BEET		
Herbicide tolerance	*cp4 epsps*	H7-1 (*cp4 epsps*), A5-15 (*cp4 epsps)*
MAIZE		
Insect resistance and herbicide tolerance	*cry1A.105, cry2Ab2, cry3Bb1, cp4epsps, cryIF, cry34Ab1, cry35Ab1, pat, cry1A2, mcry3A, , m epsps*	MON89034 x NK603 (cry1A.105, cry2Ab2), MON89034 x MON88017 (cry1A.105,cry2Ab2, cry3Bb1, cp4 epsps), MON88017 (cry3Bb1, cp4 epsps), 1507 x 59122 (cryIF, cry34Ab1, cry35Ab1, pat), 59122 x 1507 x NK603 (cryIF, cry34Ab1, cry35Ab1, cp4 epsps), 59122 (Cry34Ab1, Cry35Ab1, pat), NK603 x MON810 (cry1A2, cp4 epsps), 1507 x NK603 (cp4 epsps, cryIF, pat), Bt11 x MIR604 x GA21 (pat, m epsps, cry 1AB, mcry3A)
Insect resistance	*cry1Ab, pat, cryIF, mcry3A*	Bt11 (*cry1Ab, pat*), 1507 (*cryIF, pat*), MIR604 *(mcry3A*)
Herbicide tolerance	*cp4 epsps, m epsps, pat*	NK603 (*cp4 epsps)*, GA21 (*m epsps*), T25 (*pat*)

Table 6.3: Crops authorized for import (current and expired).

Trait	Genes	Event	Current status	Cultivated where?
COTTON				
Insect resistance and herbicide tolerance	*cp4 epsps, cry1Ac, cry2Ab2*	MON531 x MON1445 (*cp4 epsps, cry1Ac*)	Notified as an existing product on 18 April 2005. Approval expired on 18 April 2007. An application for renewal has been filed. These products remain marketable thereby.	Argentina, Australia, Brazil, South Africa
		MON 15985 x MON 1445 (*cry1Ac, cry2Ab2 and cp4 epsps*)		Australia
Insect resistance	*cry2ab, cry1Ac*	MON15985 (*cry2ab, cry1Ac,*)		Australia (New South Wales and southern Queensland only), Brazil, Burkina Faso, India, South Africa, United States
		MON531 (*cry1Ac*)		Argentina, Australia, Brazil, Colombia, India , Japan, Mexico, South Africa, United States
Herbicide tolerance	*cp4 epsps, pat*	MON 1445 (*cp4 epsps*)		Argentina, Australia, Brazil, Colombia, Japan, South Africa, United States
		LL Cotton 25 (*pat*)	Valid authorization	Australia, Brazil, United States
OILSEED RAPE				
Fertility restored and herbicide tolerance	*bar, barnase, barstar*	MS8 x RF3 (*bar, barnase, barstar*)	Valid authorization	Australia, Canada, Japan, United States
Herbicide tolerance	*cp4 epsps, gox, pat*	Gt 73 (CP4 *epsps, gox*)	Valid authorization	Australia, Canada, Japan, United States
		T45 (*pat*)		Australia, Canada, Japan, United States
POTATO				
Increased amylopectin content	Inhibited *GBSS*	EH92-527-1 (inhibited *GBSS*)	Valid authorization	
SOYBEAN				
Herbicide tolerance	*cp4 epsps, pat*	MON40-3-2 (*cp4 epsps*)	Notified as an existing product on 13 July 2004. Approval expired on 18 April 2007. An application for renewal has been filed. These products remain marketable thereby	Argentina, Brazil, Canada, Japan, Mexico, Paraguay, South Africa, United States and Uruguay
		A2704-12 (*pat)*	Valid authorization	Brazil, Canada, Japan, United States
		MON89788 (*cp4 epsps*)		Canada, Japan, United States
SUGAR BEET				
Herbicide tolerance	*cp4 epsps*	H7-1 (*cp4 epsps)*	Valid authorization	Canada, Japan, United States

Table 6.3 (continued)

MAIZE				
Insect resistance and herbicide tolerance	*cry35Ab1, cry34Ab1, cp4 epsps, cry3Bb1 , pat, cry1Fa2, cry1Ab, cry1A.105, cry2Ab, nptII*	59122 (*cry35Ab1, cry34Ab1, pat*)	Valid authorization	Argentina, Brazil, Canada, Japan, Philippines and United States
		MON88017 (*cp4 epsps, cry3Bb1*)		Canada, Japan and United States
		59122 x NK603 (*cp4 epsps, cry35Ab1 , cry34Ab, pat*)		Canada and Japan
		1507 x 59122 (*pat, cry1Fa2, cry34Ab1, cry35Ab1)*		Canada and Japan
		MON88017 x MON810 (*cp4 epsps, cry1Ab, cry3Bb1)*		Canada
		MON89034 x NK603 (*cp4 epsps, cry1A.105, cry2Ab)*		
		Bt11 x GA21 (*pat, cp4 epsps)*		Brazil, Canada and Japan
		Bt11 (*pat, cry1Ab*)		Argentina, Brazil, Canada, Colombia, Japan, Philippines, South, Africa, United States, Uruguay
		MON863 x MON810 x NK603 (*cp4 epsps, cry3Bb1, cry1Ab, nptII)*		Canada and Japan
		MON863 x NK603 (*cp4 epsps, cry3Bb1, nptII)*	Notified as an existing product on 13 October 2004. An application for renewal has been filed. These products remain marketable thereby.	Japan
		NK603 x MON810 (*cp4 epsps, cry1Ab)*	Valid authorization	Argentina, Brazil, Canada, Japan Philippines, South Africa
		1507 (*pat, cry1Fa2)*	Valid authorization	Argentina, Brazil, Canada, Japan, United States
		1507 X NK603 (*cry1Fa2, cp4 epsps , pat*)		Argentina, Brazil, Canada, Japan
		59122 x 1507 x NK603 (*cp4 epsps, cry35Ab1, cry1Fa2, cry34Ab1, pat)*		Canada and Japan
Herbicide tolerance	*Pat, cp4 epsps*	GA21 (*cp4 epsps*)	Valid authorization	Argentina, Brazil, Canada, Japan, Philippines, United States
		T25 (*pat*)	Notified as an existing product on 1 October 2004. Approval expired on 18 April 2007. Application for renewal has been filed. These products are marketable.	Philippines, Taiwan, China, South Africa and Korea
		NK603 (*cp4 epsps*)	Valid authorization	Argentina, Brazil, Canada, Japan, Philippines, South Africa and United States
Insect resistance	*cry3Bb1, crya1A. 105, cry2AB, cry3A, cry1Ab*	*MON863 (cry3Bb1)*	Valid authorization	Canada and United States
		MON89034 (*crya1A. 105, cry2AB)*		Brazil, Canada, Japan and United States
		MIR 604 (*cry3A*)		Canada, Japan and United States
		MON863 x MON810 (*cry3Bb1, cry1Ab)*		Japan
		MON810 (*cry1Ab*)	Notified as an existing product on 18 April 2004. Approval expired on 18 April	Argentina, Brazil, Canada, Japan, Philippines, South Africa, United States
CARNATION				
Flower color and Herbicide tolerance	surB, dfr, hfl	4, 11, 15, 16 (*surB, dfr, hfl*)	Valid authorization	Australia
Flower color	*surB,bp40, dfr, acc*	Carnation Moondust (FLO-07442-4) (*surB, bp40, dfr)* 959A, 988A, 1226A, 1351A, 1363A, 1400A	Notified as "existing product". Approval expired in October 2007. Application for renewal filed, and products remain marketable.	Colombia
		Carnation Moonlite (FLO-40644-4)(*surB, bp40, dfr),* 959A, 988A, 1226A, 1351A, 1363A, 1400A	Valid authorization	

Table 6.4: Crops with authorizations pending for import.

Trait	Genes	Event
COTTON		
Insect resistance and herbicide tolerance	*cry1Ac,cry1F, pat, cry2Ab2, cp4 epsps*	281-24-236 x 3006-210-23 x MON88913 (*cry1Ac, cry1F, pat*), MON 15985 x MON 1445 *(cryIAc, cry2Ab2, cp4 epsps)*, MON88913x MON15985 *(cryIAc, cry2Ab2, cp4 epsps)*, MON531 x MON1445 (*cry1Ac, cp4 epsps*)
Insect resistance	*cry1Ac, cry1F, cry2ab*	281-24-236 x 3006-210-23 (*cry1Ac, cry1F*), MON15985 (*cryIAc, cry2ab*), MON 531 (*cry1Ac*),
Herbicide tolerance	*2m epsps, pat, cp4 epsps*	GHB614 x LLCotton25 (*2mepsps,pat*), GHB614 (*2mepsps*), MON88913 (*cp4 epsps*), MON 1445 (*cp4 epsps*)
CARNATION		
Modified flower color	*dfr, bp40*	Carnation Moonaqua (*dfr, bp40*)
OILSEED RAPE		
Herbicide tolerance	*pat, bar, barnase, barstar, cp4 epsps, gox*	Liberator pHoe6/Ac (*pat),* GS40 / 90pHoe6 / Ac (*pat),* MS8 x RF3 x GT73 (*pat, bar, barnase, barstar, cp4 epsps, gox)*
RICE		
Herbicide tolerance	*pat*	LL Rice 62 (*pat*)
SOYBEAN		
Herbicide tolerance	*csr1-2, pat, gat, cp4 epsps*	BPS-CV127-9 (*csr1-2*), A5547-127 (*pat*), 356043 (*gat*), A2704-12 (*pat*), MON89788 (*cp4 epsps*)
Insect resistance	*cry1Ac*	MON87701 (*cry1Ac*)
Altered composition	*Pj.D6D, Nc.Fad3, gm-fad2-1*	MON87769 (*Pj.D6D, Nc.Fad3*), 305423 (*gm-fad2-1*)
Altered composition and herbicide tolerance	*cp4 epsps, FAD2-1A/ FATB1-A, gm-fad2-1*	MON87705 (*FAD2-1A/ FATB1-A, cp4 epsps*), 305423 x 40-3-2 (*gm-fad2-1, cp4 epsps*)
Insect resistance and herbicide tolerance	*cry1Ac, cp4 epsps*	MON87701 x MON89788 (*cry1Ac, cp4 epsps)*
SUGAR BEET		
Herbicide tolerance	*cp4 epsps*	H7-1 (*cp4 epsps*), A5-15 (*cp4 epsps*)
MAIZE		
Altered composition	*amy797E*	3272 (*amy797E*)
Drought tolerance	*cspB*	MON87460 (*cspB*)
Insect resistance	*cry3Bb1, cry1Ab, vip3Aa20, cryIF*	MON863 x MON810 (*cry3Bb1, cry1Ab*), MIR162 (*vip3Aa20*), Bt11 (*cry1Ab, pat*) 1507 (*cryIF, pat*)
Herbicide tolerance	*pat, cp4 epsps, als, gat*	NK604 x T25 (*pat, cp4 epsps*), 98140 (*als, gat*), NK603 (*cp4 epsps*), NK603 (*cp4 epsps*), GA21 (*m epsps*), T25 (*pat*)
Insect resistance and herbicide tolerance	*cp4 epsps, cryIF, cry34Ab1, cry35Ab1, pat, cry1Ab, mcry3A, cry3Bb1, cry2Ab, cry1A.105, vip3Aa20*	1507 x 59122 (*cryIF, cry34Ab1, cry35Ab1, pat*), 59122 x 1507 x NK603 (*cryIF, cry34Ab1, cry35Ab1, cp4 epsps*) 59122 (*cry34Ab1, cry35Ab1, pat*), 1507 x NK603 (*cp4 epsps, cryIF, pat*), Bt11 x MIR604 (*cry1Ab, mcry3A, pat*), MIR604 x GA21 (*mcry3A, cp4epsps*), Bt11 x MIR604 x GA21 (*cry1Ab, mcry3A, Ccp4epsps*), MON89034 x MON88017 (*cry3Bb1, cry2Ab, cry1A.105, cp4epsps*), Bt11 x MIR162 x GA21 (*cry1Ab, vip3Aa20, pat*), MON89034 x 1507 x MON88017 x 59122 (*cry1A.105, cry2Ab2, cryIF-gene., pat, cry3Bb1, cp4epsps, cry34Ab1, cry35Ab1*), Bt11 x MIR162 x MIR604 x GA21 (*cry1Ab, pat, vip3Aa20, cry3A, cp4epsps*) NK603 x MON810 (*cry1Ab, cp4epsps*)

Source GMO compass website (www.gmo-compass.org); AGBIOS website (www.cera-gmo.org)

6.3 EU policy on GE crops

6.3.1 The knowledge-based bioeconomy (KBBE) in Europe

The Lisbon Strategy was launched in 2000 by the European Council to increase the productivity and competitiveness of the EU and aspiring to turn the EU into "the most dynamic and competitive knowledge-based bioeconomy in the world" (European Council, 2000). The Lisbon Strategy explicitly considered knowledge as a valuable resource for economic growth and social welfare, highlighting the importance of investment into research and development (Webb, 2009). Among the wide range of knowledge areas, biotechnology and the life sciences were singled out as essential components in the development of the Lisbon Strategy through the establishment of a dynamic and cutting-edge KBBE, a sector that accounts for €1.5-2 trillion of the EU gross domestic product (European Commission, 2005).

A bioeconomy comprises all the industries that produce, manage or exploit biological resources. Because crops are a major source of biomass, the European Commission has recognized the potential of agricultural biotechnology as a means to increase the yield and quality of economically-relevant crops (European Commission, 2010b). Since 1982, the EU has invested more than €300 million into more than 130 biosafety-focused research projects concentrating on GE agriculture and its related issues, eventually concluding that "...genetically modified organisms are not *per se* more risky than e.g. conventional plant breeding technologies..." (Kessler and Economidis, 2001; Economidis et al. 2010).

Despite official discourse acknowledging the potential benefits of agricultural biotechnology and the generous funding of research in this area, little has been done to promote the commercialization of this technology to recoup its achievements at the farm and consumer levels. Only the two GE crops described above (MON810 maize and the Amflora potato) have been authorized in the past 13 years (GMO Compass, 2012) and a number of additional crops are still mired in the system. Farmers outside Europe have upwards of 20 GE varieties to choose from (Ramessar et al. 2010).

6.3.2 Common Agricultural Policy (CAP)

The CAP was created in 1957 under the Treaty of Rome and was implemented in 1962. Initially it sought to increase agricultural productivity in Europe and secure the availability of food supplies during the Cold War. The CAP was successful in moving the European Economic Community (EEC) toward self-sufficiency from the 1980s onwards, to the extent that almost permanent surpluses of the major farm commodities became a problem. Some of the surplus was exported, with the help of subsidies (European Commission, 2010c). The EEC created a controversy by adopting policy measures to limit the production of surplus products, but gradually these policies succeeded and surpluses were reduced (European Commission, 2010c).

The CAP is currently one of the most controversial EU policies not only because of its huge cost as a proportion of the EU budget, but also because it is an unfair way of protecting European agriculture. The stated objectives of the CAP are to ensure a stable supply of high-quality food for the EU population at fair prices while providing farmers with a reasonable standard of living, and to preserve rural heritage (EEC, 1957). This is achieved by subsiding agricultural production. However, most of the subsidies available under the CAP are used to benefit large producers rather than family-based agriculture (Wiggerthale, 2005). This appears to contradict one of the explicit CAP objectives, i.e. to ensure a fair standard of living to all EU farmers.

Another major criticism is that direct payments and export refunds promote the practice of dumping CAP-subsidized EU products (exporting at prices below the cost of production), which is holding back the development of agriculture in developing countries (Goodison, 2011). It is also clear that the CAP helps to undermine GE agriculture and prevent its adoption in the EU, since the cost savings brought about by GE crops in other markets are hidden by the CAP subsidies (Van Mejil and Van Tongeren, 2003; European Commission, 2010d; Sanders et al. 2011). Organic farms receive on average higher subsidies in absolute terms and per hectare than conventional farms (European Commission, 2010d).

Maintaining the CAP in its current state means the EU will continue to waste a large proportion of its budget on the artificial support of uncompetitive producers and processers, while leaving poorer farmers in poverty and depressing the development of agriculture in developing countries. GE agriculture provides a potential solution to this dilemma by offering a competitive advantage to food producers and processers based on the adoption of new technology rather than artificial subsidies. However, this solution goes against the CAP objectives.

6.3.3 GE crop authorization process and the route to commercial cultivation in EU

The authorization process for GE crops in Europe is straightforward in principle, but authorization is only the first of several hurdles that must be overcome before farmers can cultivate GE crops (Figure 6.4). Once authorization has been received, farmers must ensure they comply with the conditions laid down by the authorities in their member state and/or local region, often finding that arbitrary (and arguably illegal) national or regional bans on GE agriculture have been imposed (Table 6.5). Even if no such impediments exist, farmers must abide by the coexistence measures that have been implemented in each member state or region, and the complexity of these regulations and their strict implementation often means that it is impossible to comply. The four major post-authorization obstacles to GE agriculture in the EU are:

- Public field registers showing the location of commercially grown GE crops are compulsory in almost all member states, and tend to discourage farmers from adopting GE agriculture.
- Six member states use a ‘safeguard clause’ nominally based on environmental or health concerns, to implement national cultivation bans for GE crops (Austria, France, Germany, Greece, Luxemburg and Hungary).

- Stringent coexistence measures have been implemented in Belgium, the Czech Republic, Germany, Hungary, Portugal, Romania and Slovakia, which make it almost impossible to grow GE crops without risking litigation from surrounding farms.

- The negative publicity surrounding GE agriculture in Europe, which means farmers are ostracized, intimidated and have GE crops destroyed by activists.

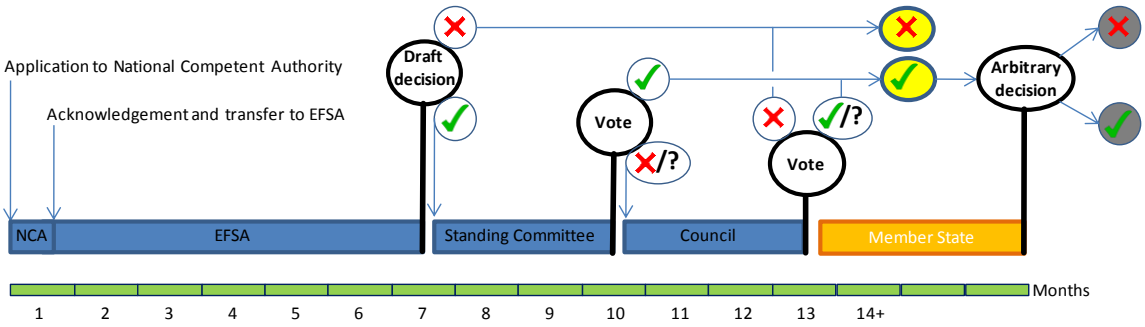

Figure 6.4: The authorization process for GE crops in the EU before and that one proposed after July 2010. Before (blue sections) the pathway to authorization involved national competent authorities (NCA), the European Food Safety Authority (EFSA) and the EU Standing Committee on the Food Chain and Animal Health. A draft decision from EFSA to refuse authorization on safety grounds would be effective as a final rejection, because the application would have to be adjusted and resubmitted. Alternatively, EFSA could request further documentation, which would delay but not kill the application. Approval by EFSA followed by approval by the Standing Committee would result in authorization throughout the EU (yellow circle). Approval by EFSA followed by either rejection by the Standing Committee or no qualified majority (indicated by a question mark) would result in the application being reviewed by the EC Council of Ministers. Here a rejection would effectively kill the application because it would be returned to the Standing Committee for revision, which would likely involve further involvement from EFSA and a return to the early part of the application pathway. Either approval or no qualified majority would in this case result in the application being approved throughout the EU. The 2010 Recommendation subverts this process by proposing that member states should be allowed to use arbitrary reasons to overrule EFSA, the Standing Committee and the Council of Ministers for applications related to their territory. Therefore, even if a GE crop has been authorized by EFSA, it can be banned on a national basis (gray circles). The timeline shown is the maximum permitted time taken by each agency or committee, although EFSA consultations may be extended if further documentation is required. There is no time limit for member states to implement national provisions, which means crops that have been approved for years can still be banned on a national basis under the proposed rule changes.

Table 6.5: National bans currently implemented under the 'safeguard clause' (Sabalza et al. 2011).

Country	Event	Date	Coverage
Austria	Bt176 maize	1997*	Cultivation
	MON810 maize	1999*	Cultivation
	T25 maize	2000*	Cultivation
	GT73 rapeseed	2007*	Import
	MON863 maize	2008	Import
	Ms8 rapeseed	2008	Import
	Rf3 rapeseed	2008	Import
	Ms8/Rf3 rapeseed hybrid	2008	Import
	EH92-527-1 potato	2010	Import
France	Topas 19/2 rapeseed	1998	Import
	MS1/Rf1 rapeseed hybrid	1998	Import
	MON810 maize	2008	Cultivation
Germany	Bt176 maize	2000	Cultivation
	MON810 maize	2009	Cultivation
Greece	Bt176 maize	1997	Cultivation
	T25 maize	1997	Import
	Topas 19/2 rapeseed	1998	Import
	MS1/Rf1 rapeseed hybrid	1998	Import
	MON810 maize	2001	Cultivation
Luxemburg	Bt176 maize	1997	Cultivation
	MON810 maize	2009	Cultivation
Hungary	MON810 maize	205	Cultivation

* These bans were amended in 2008

6.3.3.1 *Risk assessment*

The first stage in the approval process for GE crops is risk assessment, which is initially carried out by the competent authorities in each member state in collaboration with the developers of each GE product. When an application is received, the competent authority has 14 days to inform European Food Safety Authority (EFSA), which must then inform the other member states and make the application public. EFSA then carries out its own evaluation, a process that may take up to six months, and even longer if further documentation is requested. If the product is to be used as seeds, EFSA collaborates with the national competent authorities to carry out the appropriate environmental assessment.

EFSA is an independent organization that evaluates the risks of GE crops based on scientific evidence using a panel of experts, and testing is carried out at an EU reference laboratory. As such, EFSA is best placed to advise individual member states and the EC as a whole on safety issues, and their recommendations may encompass post-market

environmental monitoring and labeling suggestions. Applications for food and feed crops are submitted under Regulation EC1829/2003 if the plants are to be used as source material in food and feed production. Non-food/feed crops can also be authorized under Directive 2001/18/EC (deliberate release of GMOs into the environment). The existing regulations ensure that any crops destined for human consumption must undergo extensive safety testing, even if they contain the same genes as crops that are already approved. This remains the case even though every national agency tasked with determining the safety of GE food has yet to find any quantifiable hazard, and after more than 15 years of GE agriculture in North America there has been not one substantiated case of either health or environmental damage caused by GE crops or their products. Notably, new crops generated by non-GE means (including other biotechnology approaches such as mutagenesis) are not subject to any regulatory oversight and can be cultivated without regulatory involvement.

The new authorization process proposed by the EC does not affect the risk assessment process directly, although it does impact on the authorization itself. However, in 2010 EFSA developed new guidance on the environmental risk assessment of genetically modified plants by expanding and completing most sections of the previous GMO Panel Guidance Document (EFSA, 2006a,b) in accordance with current legislation and in response to a mandate from the European Commission (EFSA, 2010). The new EFSA guidance includes substantial modifications, such as the addition of long-term effects on non-target organisms, field trials and additional guidance on stacked events.

6.3.3.2 *The role of member states*

Once a GE crop receives a favorable assessment from EFSA, authorization is considered on a case-by-case basis. EFSA submits its opinion to the European Commission, which then has three months to come to a draft decision. This decision is submitted to the Standing Committee on the Food Chain and Animal Health (SCoFCAH) comprising representatives from all member states. This committee may approve or reject the draft decision with a qualified majority, which is defined in the Treaty of Nice. Each member state is allotted a certain number of votes according to its

population, e.g. Germany 29, France 29, Czech Republic 12, Malta 3 (GMO compass, 2006). In order to reach a qualified majority, 232 out of 321 votes are needed. Additionally, a qualified majority must represent at least 62 percent of the EU population. If the decision is approved, the GE crop is duly authorized and remains so for 10 years. However, if the decision is rejected, or if there is no qualified majority, then the European Parliament must be informed and the decision goes to the European Council of Ministers, which has 90 days to approve or reject the draft decision, also on the basis of a qualified majority. If the Council rejects the Commission's draft, the Commission must revise the document and re-present it. If the Council approves the draft, or if the Council cannot reach a qualified majority, the draft decision comes into effect. This process of moving through the committee stages is formally known as commitology.

On 13th July 2010, the European Commission officially proposed to give member states the freedom to veto the cultivation of GE crops on their own territory without having to provide any scientific evidence of new risks. The first reading of the proposal was on 5th July 2011 and now the European Parliament, the European Commission and the European Council of Ministers need to build a consensus before it becomes law or returns to the European Parliament for a second reading (Gómez-Galera et al. 2012). As stated above, the objective of this proposal is to make individual member states responsible for their own policy on GE crops, and therefore speed up pending authorizations by removing the ability of member states to block approval by avoiding a qualified majority. Despite its stated intent, this amendment effectively serves to legalize the (currently illegal) practice of individual member states arbitrarily declaring GE-free zones within their borders, or banning GE crops all together. Six member states (Austria, Hungary, France, Greece, Germany and Luxembourg) have already prohibited the cultivation of the MON810 maize variety on dubious safety grounds, and three (Austria, Luxembourg and Hungary) have similarly banned the cultivation of the Amflora potato. Poland is currently drawing up legislation to ban all GE seeds. The amendment means that such exclusion policies can be implemented without scientific justification. If the legal amendment enters into force, member states will be free to

restrict or prohibit the cultivation of all or particular GE crops within their territory, including crops that have already been authorized for cultivation under Directive 2001/18/EC and Regulation EC 1829/2003. According to this proposal member states are only allowed to adopt measures against the cultivation of GE crops, but not the import and/or the marketing in the EU of authorized GE seeds, which therefore allows GE products from abroad to be imported, sold and consumed in the EU even though the same products cannot be cultivated there.

6.3.3.3 *Coexistence measures*

EU policy officially supports the coexistence of GE agriculture and conventional agriculture, and lays down regulations to govern this coexistence by allowing member states to establish minimum distances between fields of GE and conventional crops in order to prevent adulteration. However, there is no scientific basis for these coexistence thresholds, which means that member states can and do implement such absurd minimum distances and such draconian penalties for the adulteration of conventional crops that farmers dare not risk adopting GE agriculture and their choice is effectively removed (Ramessar et al. 2010).

Coexistence refers to the ability of farmers to make a practical choice between conventional, organic and GE crops, in compliance with legal obligations for labeling and/or purity standards defined in European Commission legislation (European Commission, 2003a). The possibility of adventitious presence (the unintended presence of low levels of GE material in conventional crops) cannot be entirely excluded (Directive 2001/29/EC). The European Commission has published detailed and pragmatic recommendations for the development of coexistence regulations to be implemented at national or regional levels, based on a tolerance threshold for adventitious presence above which a conventional crop must be labeled as containing GE material. The regulations were developed because adventitious presence could reduce the value of a conventional or organic crop, particularly if sold at a premium, thus acknowledging that coexistence measures are concerned with the economic impact of adulteration and not the health or environmental safety of the product, given that no

GE crops can be grown without a positive safety evaluation from EFSA (European Commission, 2003a; Brookes, 2004; Kalaitzandonakes, 2005).

Adventitious presence thresholds in the EU are the strictest in the world (Ramessar et al. 2010). In the United States, Canada and Japan, non-GE products may contain up to 5% GE material before they must be labeled as GE. Other countries have lower tolerance thresholds (e.g., 1% in Australia, New Zealand, South Africa, Brazil and China). The EU has a two-tier tolerance policy (EC 1830/2003), with a 0.9% limit applied to approved products and a zero tolerance threshold applied to unapproved products, replacing the temporary 0.5% second-tier limit previously approved by EFSA. Additionally, Recommendation 2003/556/EC and the new Recommendations 2010/C 200/01 provide guidelines for the development of national coexistence strategies and best practices that, where necessary, can be applied to prevent non-GE products exceeding the labeling threshold. This means coexistence is also reduced to a matter of national competence, where each member state is responsible for the establishment of a legislative framework on a crop-by-crop basis. The practical effect of these recommendations is that member states can impose excessive and arbitrary coexistence rules that effectively suppress GE agriculture over large areas of Europe (Dobbs, 2011; Sabalza et al. 2011) (Table 6.2). Austria, Belgium, Bulgaria, Czech Republic, Denmark, Germany, Hungary, Latvia, Luxemburg, Portugal, Romania and the Slovak Republic have already started adopting regulations governing the planting and handling of GE crops, whereas others are still in the process of developing their regulations. The lack of EU-wide regulations means that member states can impose minimum isolation distances that are completely disconnected from the needs indicated by scientific evidence, and serve only as an artificial strategy to suppress GE agriculture (Sabalza et al. 2011). This contrasts starkly with the EU's stated policy to develop national coexistence measures to avoid the unintended presence of GE material in conventional and organic crops through transparency (the rules are unfathomable and those implementing them are unaccountable), cross-border co-operation (member states act independently), stakeholder involvement (GE farmers are sidelined), proportionality (current coexistence rules are far from proportional), and the strict liability rules mean that GE

farmers are always responsible for any admixture and will be fined accordingly (Table 6.6).

Table 6.6: Paradoxes among the EU's coexistence measures.

Principle	Intention	Consequence
Transparency	National strategies and best practices for coexistence should be developed in a transparent manner.	Coexistence requirements are confusing and unfathomable, and those implementing them are unaccountable (Ramessar et al. 2009, 2010).
Cross-border cooperation	Member states should ensure cross-border cooperation with neighboring countries to guarantee the effective functioning of coexistence measures in border areas.	There is no cooperation because member states act independently and national governments are responsible for coexistence policies. Each member state establishes a legislative framework on a crop-by-crop basis (Ramessar et al. 2009, 2010).
Stakeholder involvement	National strategies and best practices for coexistence should be developed in cooperation with all relevant stakeholders.	GE farmers are sidelined. Farmers who choose to grow GE crops have to invest extra money to comply with the excessive coexistence measures (Messean et al. 2006; Demont and Devos, 2008).
Based on scientific evidence	Management measures for coexistence should reflect the best available scientific evidence on the probability and sources of admixture between GE and non-GE crops.	The thresholds for adventitious presence are far stricter than for conventional crops and the isolation distances enforced to achieve such thresholds are arbitrary, excessive and are politically motivated rather than reflecting scientific reality (Ramessar et al. 2010).
Proportionality	Measures to avoid the unintended presence of GE material in other crops and vice versa should be proportionate to the intended objective (protection of the particular needs of conventional, organic and GE farmers).	Measures are neither regionally nor economically proportionate. Proportionality is still linked to economic loss even if not necessarily to the labeling thresholds (Ramessar et al. 2010).
Liability	The instruments adopted may have an impact on national liability rules in the event of economic damage resulting from admixture.	Strict liability regulations mean that GE farmers are always responsible for any admixture and risk fines or litigation from surrounding farms (Ramessar et al. 2009, 2010; Messean et al. 2006; Demont and Devos, 2008)

The exaggerated coexistence measures implemented by member states are often justified in the name of safety despite the stated economic purpose of coexistence regulations and the fact that only GE crops with a positive safety evaluation from EFSA are approved for commercial release, but this nevertheless damages the perception of safety because most consumers only note the nature of the regulations, not the underlying justification. Therefore it is clear that coexistence measures are being used

as a convenient and politically expedient proxy for the unilateral prohibition of GE agriculture (Ramessar et al. 2010; Sabalza et al. 2011).

6.3.3.4 *GE crop cultivation vs imports*

EU policy on GE food imports is less restrictive than the regulations covering GE agriculture at home because the EU is dependent on imported GE feed to sustain the livestock industry. This explains the vast difference between the numbers of crops approved for import and cultivation. As of August 2012, 39 different GE crops were approved for import (22 varieties of maize, 6 cotton, 3 rapeseed, 3 soybean, 1 sugar beet, 1 potato and 3 carnation) whereas only two products are approved for cultivation, the pest-resistant maize variety MON810 and the Amflora potato variety, which as stated above is used for industrial starch production rather than food (GMO Compass, 2012) (Table 6.1 and 6.3).

A similar contrast between the regulations on import and cultivation is shown by the member state opt-out discussed above, which applies to cultivated GE crops only. Member states are not legally permitted to block the marketing of approved imported GE products. Although less stringently regulated than cultivation, the import of GE products is nevertheless still heavily regulated, causing logistical and economic problems for foreign exporters and EU importers alike, reducing the flow of commodities and placing the EU at a significant risk of crisis in the livestock industry. The complex import approval process summarized in Figure 6.4 (Davison, 2010; Wager and McHughen, 2010; USDA GAIN Report, 2011) causes delayed and asynchronous authorizations in which products are authorized in the supplier's country but not in the EU (Figure 6.5). The zero tolerance for adventitious presence results in shipments containing traces of GE material, which are approved under the more relaxed thresholds in the supplier's country, being rejected at the point of import, which can have significant economic consequences for the EU and the supplier, especially if the supplier is a developing country (Stein and Rodríguez-Cerezo, 2009; Davison, 2010; Wager and McHughen, 2010).

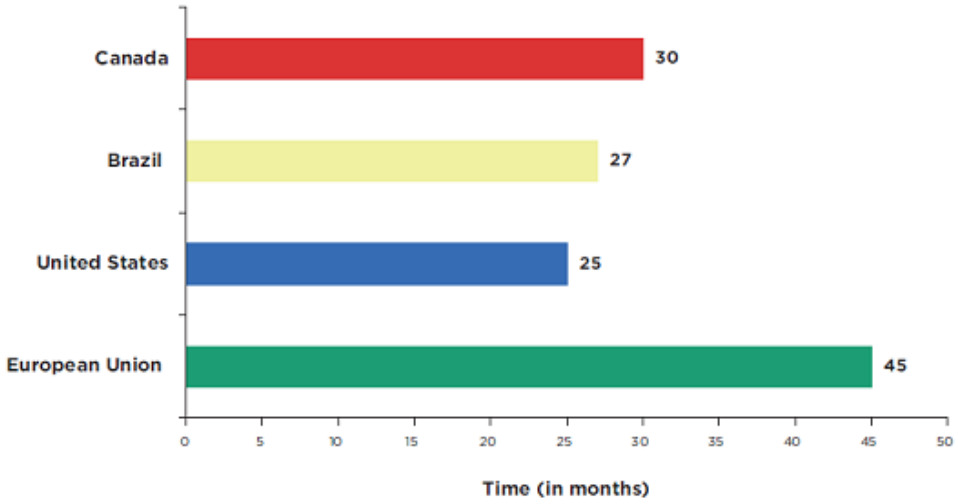

Figure 6.5: Average time required for a GE product approval (EuropaBio, 2011).

6.4 Consequences of EU policy on GE crops

6.4.1 Consequences for the future of European agriculture

EU policy on agriculture has evolved over the last 50 years as the continent has developed from a net importer struggling to feed its population at the end of a devastating war, to the current situation of near trade parity with the rest of the world. However, the EU is still a net importer of agricultural raw materials and 55% of imports come from 10 countries, with Brazil, the USA and Argentina ranking in the top three positions (European Commission, 2009). The same three countries also happen to be the world's largest adopters of GE technology, with the US planting 70 million hectares of transgenic crops in 2011 and both Brazil and Argentina planting just over 50 million hectares (James, 2012). The new measures now being considered by the EU will allow member states to restrict or prohibit the cultivation of all or particular GE crops, in parts of or in their entire territory, but they will not be allowed to adopt measures prohibiting the import and/or the marketing in the EU of authorized GE seeds (European Commission, 2010a) which means that EU markets could be flooded with imported GE produce that cannot be grown domestically. Therefore, member states can forbid their

own farmers from growing GE crops but need GE crops to sustain their livestock industries and could not legally prevent the import of such GE crops even if they wanted to on principle, even if those crops are identical to the ones that cannot be cultivated. The absurdity of this position also debunks any concocted safety basis for the moratorium on GE agriculture in Europe since any safety concerns should apply to a product regardless of its origin, and the artificial distinction only serves to highlight the double standard being employed in this context (Sabalza et al. 2011). Furthermore EU farmers are not only denied the right to choose their own crops, but are forced to adopt unsustainable and uncompetitive farming practices, making them unwitting coconspirators to maintain the CAP. Any farmers that go ahead with GE agriculture lose any economic advantages the technology might bring through the costs of compliance, negotiations with surrounding farms and insurance to cover litigation costs if neighboring crops are found to be adulterated; there are no obligations placed on farmers growing conventional or organic crops (Messean et al. 2006; Demont and Devos, 2008).

This same schizophrenic approach is apparent in the EU's treatment of imported maize and soybean from the US and elsewhere, and has a significant knock-on effect on animal agriculture. In this context, the EU is deficient for feed protein and is ultimately dependent on soybean meal imports. However, EU imports have declined considerably (e.g. from $2.8 billion in 1997 to $1.9 billion in 2008 (USDA GAIN Report, 2009)), predominantly because of the complex and onerous process for approving imported GE products, causing asynchronous authorizations. This situation may worsen with the increasing adoption of GE agriculture as a mainstream technology outside Europe, leading to deficits in the soybean meal demand and supply chain and then in the feed industry more generally, with a knock-on effect on the livestock, poultry and dairy industries and economic decline throughout the EU (Wager and McHughen, 2010). As a consequence, Europe is increasingly being perceived as a risky export market, resulting in preferential trading between other countries and EU importers bearing high prices

and insurance premiums to offset risks undertaken by the supplier (Brookes, 2008; Krueger and Le Buanec, 2008; UK DEFRA and FSA, 2009; Stein and Rodríguez-Cerezo, 2009).

The USA, Brazil, Argentina and other countries with significant GE adoption rates have also recognized that biotechnology can be used to limit the environmental footprint of conventional agriculture, i.e. reducing pesticide use and also fuel consumption by reducing the need for spraying, and eliminating tillage (Brookes and Barfoot, 2010). If the EU continues obstructing the deployment of GE crops in Europe, it will force farmers to continue to use environmentally hazardous, expensive and unsustainable agricultural practices, spend unnecessary resources on fossil fuels and agrochemicals, while at the same time letting the same products be imported from the USA and South America further tipping the balance of trade in the wrong direction.

6.4.2 Consequences for the future European economy

The reasons for the low take up of GE crops in Europe include the onerous compliance regulations, the threat of litigation from neighboring farmers reflecting the irrational coexistence policies currently in place (Ramessar et al. 2010), and the absence of a market due to the public distrust of GE technology. The last point is especially hard to understand. Despite claims that GE crops are not used as food, it is actually the case that more than 70% of processed foods consumed by humans in the USA and Canada contain GE ingredients, a similar proportion of white maize in Africa for human consumption is transgenic and several GE products are consumed by humans in China, all with no reported ill effects after 10 years. These countries also export GE seeds to other markets (including the EU) with no reported incidents.

The failure of the EU to support the adoption of GE crops is symptomatic of the largely unsuccessful Lisbon Agenda, which set out in 2000 to change the EU into a highly competitive knowledge-based economy with innovation, economic, social, and environmental renewal and sustainability as its core values. These are laudable goals, but GE crops are an innovation supported by many EU research organizations that have

already proven successful in other countries in terms of economic growth, environmental sustainability and competition. Yet every possible obstacle has been erected in the EU to prevent this beneficial form of agriculture from being adopted, leading to economic stagnation, trade disputes, and the continued destruction of the environment through chemical use and intensive tillage (Sabalza et al. 2011).

6.4.3 Consequences for European R&D, biotechnology SMEs and larger corporations

One additional impact of EU policy is to discourage research into agricultural biotechnology and drive the researchers overseas where the value chain can be realized in terms of commercial GE crops. Within the EU, researchers working on transgenic plants know that the best they can expect is for their products is cultivation in containment greenhouses and that, despite their benefits, the plants they produce are unlikely to be deployed in any setting where they could perform a useful function. The EU policy on GE crops is therefore attacking its own foundations as a competitive bioeconomy, because on one hand the EC offers funding for innovative biotechnology research and values (or even requires) the participation of SMEs and large industry partners, but with the other hand they are slamming the door in the face of the same companies by offering them little hope of commercial realization. Many large corporations with ambitious GE research projects have decamped to the USA or elsewhere to continue their work, and promising European SMEs are stifled because they are unable to find investment partners (BASF is an excellent example, as already discussed; BASF 2012). No agrobiotechnology company is likely to invest significantly in Europe unless there is an opportunity to recoup their R&D costs by offering their products to farmers. This reveals the immense divide between the rational evaluation of science and business, and the panicky, expedient politics pandering to a populist media and activists.

In addition, the onerous GE crop regulation process blocks the approval pathway to all but the most committed and well-funded agro-industry companies, preventing the realization of innovation generated by public sector institutions and SMEs unless they

agree to collaborate with major industry players (Kalaitzandonakes et al. 2007; Lemaux, 2009; Gómez-Galera et al. 2012). If this situation should continue, it will damage the EU economy far beyond the agricultural sector, reducing the scientific standing of EU research and leaving the continent heavily reliant on the import of products that could easily be grown within its own borders (Sabalza et al. 2011).

6.4.4 Global consequences for developing countries and emerging economies

Currently, private companies in the USA or Europe develop most GE crops, which are generally first authorized and cultivated in the USA (Stein and Rodríguez-Cerezo, 2010). However, more GE crops will be supplied by private and public entities from Asia in the near future, particularly China and India, and in the longer-term, even more developing countries may commercialize GE crops (FAO, 2009). This changing pattern will influence the zero tolerance towards the adventitious presence of traces of GE material in Europe. In contrast to the Americans, GE crops developed in Asia are usually intended for domestic consumption rather than export, and therefore the events are less likely to be submitted for approval in the EU (Stein and Rodríguez-Cerezo, 2010). As a consequence, asynchronous approvals could become more common. The strict regulations governing the import of GE products in EU may have a detrimental impact on the development of potentially welfare-enhancing crops in developing countries. If these countries fear the loss of markets for economically important export crops because of possible but unavoidable traces of unrelated GE crops, developing countries may reject GE crops for domestic use that could potentially enhance productivity, increase socioeconomic welfare and even save lives (Graff et al. 2009).

The EU is undermining its own competitiveness in the agricultural sector, not only threatening the global economy but also humanitarian activities in the developing world. Approximately half the world's population lacks access to food with sufficient levels of micronutrients, which means that malnutrition is one of the major global health challenges (Stein, 2010; Gómez-Galera et al. 2010). Even in Europe, where most people have access to a diverse diet, up 5% of the population may be malnourished, particularly the elderly (Ljungqvist, 2009). The biofortification of crops with essential minerals and

vitamins using GE technology can yield nutritious foods more rapidly and more sustainably by equipping plants with the means to synthesize, absorb and accumulate nutrients at source. For example, the development of Golden Rice enriched with β-carotene (Paine et al. 2005), multivitamin corn enriched with ascorbate, β-carotene and folate (Naqvi et al. 2009), and folate-biofortified rice (Storozhenko et al. 2007) are key examples of successful biofortification achieved using EU public sector research (Khush et al. 2012).

GE crops also provide an alternative for pharmaceutical production because they produce large amounts of biomass that can be scaled up and down as required to meet demand (Hood et al. 2002; Ramessar et al. 2008a,b; Ma et al. 2003; Twyman et al. 2005). Diverse pharmaceutical products have been produced in plants including vaccines, antibodies and enzymes (Ma et al. 2003; Twyman et al. 2005). However, EU agricultural policy hampers the adoption of GE crops and prevents the deployment of cost-effective technologies that could provide more food, higher-quality food and more accessible medicines, not only for EU citizens but also for the developing world.

The CAP has also a serious impact on developing and emerging countries. Maintaining the CAP in its current state undermines global food security and the fight against poverty. European tariffs and export subsidies, in particular, lower world food prices. This damages farmers and depresses wages for less-skilled labor in developing countries. EU funding could instead be spent on agricultural research and development adapted to developing country needs (European Commission, 2010e).

6.5 Conclusions

The EU has enacted a series of policies whose stated aim is to develop the most competitive knowledge-based bioeconomy in the world. So far this has failed. One reason for this disappointing performance is the paradoxical nature of the agricultural policies for example GE crop policy. This has led to the bizarre situation in which these policies actually promote the very practices they are aiming to avoid. The new EC

Recommendations relating to the approval of GE crops in Europe aim to combine the EU science-based authorization system with freedom for member states to decide on their own cultivation policies. These concepts are irreconcilable because the freedom of member states to ban GE crops is granted with no conditions, allowing decisions to be taken based on irrational criteria ("any grounds") and essentially rendering the whole process of risk assessment obsolete. The new recommendation on coexistence recognizes that member states may adopt measures to avoid the unintended presence of GE material in other products below the labeling threshold of 0.9%, and such measures may include the restriction or outright banning of GE agriculture within their borders. Such restriction measures should be proportionate to the objective, but obviously are not given the arbitrary and excessive isolation distances that are enforced without any scientific data to show that such distances are necessary. The recommendations apply to cultivated GE crops but not to imported GE products even if the two are identical. This is clearly an illogical position to adopt. Any safety concerns about GE products should apply equally to cultivated and imported material, and the artificial distinction only serves to highlight the double standard. The EC states that "…ensuring a safety assessment following the highest scientific standards and a reinforcement of the monitoring function were and remain priorities for the Commission as concerns GMO cultivation…" while at the same time stating that no grounds are required at all for member states to limit or ban GE agriculture, which is clearly not a high scientific standard by any stretch of the imagination. This self-contradictory proposal does nothing to smooth the authorization process for GE crops in Europe, but does provide activists and the media with fuel for their anti-GE propaganda and will no doubt cause confusion among the public. This EC proposal contrasts with one of the EU's key goals, i.e. the creation of a free market economy without border controls, since it imposes arbitrary segregation with respect to GE and non-GE agriculture. Although the authorization system is still science-based, the EC has rendered this system meaningless by giving member states the power to ban GE crops without any scientific justification. As a consequence of this illogical and contradictory policy on GE crops, EU is falling behind technologically, economically and in terms of its humanitarian policies, to the detriment of the EU population and the rest of the world.

6.6 References

BASF (2012) BASF to concentrate plant biotechnology activities in main markets in North and South America. News Release, accessed online 27 August 2012,

http://www.basf.com/group/pressrelease/P-12-109

Brookes G (2004) Coexistence of GM and Non-GM crops. Current experience and key principles. PG Economics Ltd, Dorchester, UK

Brookes G (2008) Economic impacts of low level presence of not yet approved GMOs on the EU food sector, accessed online 29 August 2012,

http://www.agrodigital.com/images/estudio.pdf

Brookes G and Barfoot P (2010) GM crops: global socio-economic and environmental impacts 1996- 2008. PG Economics Ltd, Dorchester, UK

Davison J (2010) GM plants: Science, politics and EC regulations. *Plant Science* 178:94-98

Demont M and Devos Y (2008) Regulating coexistence of GM and non-GM crops without jeopardizing economic incentives. *Trends in Biotechnology* 26:353-358

Dobbs M (2011) Excluding Coexistence of GMOs? The Impact of the EU commission's 2010 recommendation on coexistence. *Review of European Community and International Environmental Law* 20:180-193

Economidis I, Cichocka D and Högel J (2010) A decade of EU-funded GMO research (2001-2010) European Commission (EUR 24473) Luxembourg: publications office of the European Union

EFSA (2006a) Guidance document for the risk assessment of genetically modified microorganisms and their derived products intended for food and feed use by the scientific panel on genetically modified organisms (GMO). *The EFSA journal* 374:1-115

EFSA (2006b) Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. *The EFSA Journal* 99:1-100

EFSA (2010) Guidance on the environmental risk assessment of genetically modified plants. EFSA panel on genetically modified organisms (GMO) *The EFSA Journal* 8:1879

EuropaBio (2011) Aprovals of GMOs in the European Union. EuropaBio, Brussels, accessed online 29 August 2012,

http://www.europabio.org/sites/default/files/position/approval_of_gmos_in_the_european_union.pdf

European Commission (2003a) Official Journal European Community. Guidelines for the development of national strategies and best practices to ensure the coexistence of genetically modified crops with conventional and organic farming, 2003/557/EC. *Official Journal European Community* L189:36-47

European Commission (2005) Conference report: New perspectives on the knowledge-based bio-economy. Brussels, Belgium, on 15-16 September 2005, accessed online 29 August 2012, http://ec.europa.eu/research/conferences/2005/kbb/pdf/kbbe_conferencereport.pdf

European Commission (2009) EU trade in agricultural products 2009, accessed online 29 August 2012, http://ec.europa.eu/agriculture/statistics/agricultural/2011/pdf/full-report_en.pdf

European Commission (2010a) Memo 10/325: Questions and answers on the EU's new approach to the cultivation of GMOs, accessed online 29 August 2012,

http://europa.eu/rapid/pressReleasesAction.do?reference=MEMO/10/325

European Commission (2010b) The knowledge-based bio-economy (KBBE) in Europe: achievements and challenges, accessed online 29 August 2012, http://www.bio-economy.net/reports/files/KBBE_2020_BE_presidency.pdf

European commission (2010c) The Common Agricultural Policy explained, accessed online 29 August 2012, http://ec.europa.eu/agriculture/publi/capexplained/cap_en.pdf

European Commission (2010d) An analysis of the EU organic sector, accessed online 29 August 2012, http://ec.europa.eu/agriculture/analysis/markets/organic_2010_en.pdf

European Commission (2010e) The Common Agricultural Policy after 2013 Public debate, accessed online 29 August 2012, http://ec.europa.eu/agriculture/cap-post-2013/debate/report/executive-summary_en.pdf

European Council (2000) Lisbon European Council 23 and 24 March 2000. Presidency Conclusions, accessed online 27 August 2012,

http://www.europarl.europa.eu/summits/lis1_en.htm

EEC (European Economic Community) (1957) The Treaty of Rome. 25 March 1957, Rome

Farre G, Twyman RM, Zhu C, Capell T and Christou P (2011) Nutritionally enhanced crops and food security: scientific achievements versus political expediency.

Current Opinion in Biotechnology 22:245-251

Food and Agriculture Organization of the United Nations (FAO) (2009) FAO-BioDeC: Biotechnologies in developing countries, accessed online 29 August 2012, http://www.fao.org/biotech/en/

GMO compass (2006) Food and Feed from GMOs: The long road to authorisation, accessed online 29 August 2012,

http://www.gmocompass.org/eng/regulation/regulatory_process/157.eu_gmo_authorisation_procedures.html

GMO compass (2010), accessed online 27 August 2012,

http://www.gmocompass.org/eng/news/536.iamflorai_potato_intermixing_sweden_ban_germany.html

GMO compass (2012), accessed online 27 August 2012,

http://www.gmo-compass.org/eng/gmo/db/

Gómez-Galera S, Rojas E, Sudhakar D, Zhu C, Pelacho AM, Capell T and Christou P (2010) Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Research* 19:165-180

Gómez-Galera S, Twyman RM, Sparrow PAC, Van Droogenbroeck B, Custers R, Capell T and Christou P (2012) Field trials and tribulations-making sense of the regulations for experimental field trials of transgenic crops in Europe. *Plant Biotechnology Journal* 10:511-523

Goodison P (2011) EU's Common Agricultural Policy (CAP): Tools protecting European farmers. South Centre, Geneva, Switzerland. Analytical note: SC/TDP/AN/AG/13 March 2011

Graff GD, Zilberman D and Bennett AB (2009) The contraction of agbiotech product quality innovation. *Nature Biotechnology* 27:702-704

Hood EE, Woodard SL and Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants - Myths and realities. *Current Opinion in Biotechnology* 13:630-635

James C (2011) Global status of commercialized biotech/GM crops: 2011. ISAAA Brief No. 43. ISAAA: Ithaca, NY

James C (2012) USDA crop acreage report. ISAAA press release, accessed online 20 August 2012, http://www.isaaa.org/kc/cropbiotechupdate/pressrelease/2012/default.asp

Kalaitzandonakes N (2005) *In:* Proceedings to the second international conference on co-existence between GM and non-GM-based agricultural supply chains, Montpellier, France, November 14-15 (ed. Messéan, A.); Agropolis Productions, pp:29-30

Kalaitzandonakes N, Alston JM and Bradford KJ (2007) Compliance costs for regulatory approval of new biotech crops. *Nature Biotechnology* 25:509-511

Kessler C and Economidis I (2001) EC-sponsored research on safety of genetically modified organisms. European Communities. EUR 19884

Khush GS, Lee S, Cho JI and Jeon JS (2012) Biofortification of crops for reducing malnutrition. *Plant Biotechnology Report* 3:195-202

Krueger R and Buanec BL (2008) Action needed to harmonize regulation of low-level presence of biotech traits. *Nature Biotechnology* 26:161-162

Lemaux PG (2009) Genetically engineered plants and foods: a scientist's analysis of the issues (part II). *Annual Review Plant Biology* 60:511-559

Ljungqvist O, Van Gossum A, Sanz ML and De Man F (2010) The European fights against malnutrition. *Clinical Nutrition* 29:149-150

Ma JKC, Drake PMW and Christou P (2003) The production of recombinant pharmaceutical proteins in plants. *Nature Review Genetics* 4:794-805

Messean A, Angevin F, Gomez-Barbero M, Menard K and Rodríguez-Cerezo E (2006) in technical report series of the Joint Research Center of the European Commission. Institute for Prospective Technological Studies, Sevilla, Spain, accessed online 29 August 2012, http://ftp.jrc.es/EURdoc/eur22102en.pdf

Morris HS and Spillane C (2010) EU GM crop regulation: a road to resolution or a regulatory roundabout? Symposium on the EU's GMO Reform. *The European Journal of Risk Regulation* 4:359-369

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T and Christou P (2009) Transgenic multivitamin corn through

biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proceedings of the National Academy of Science USA* 106:7762-7767

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL and Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnology* 23:482-487

Ramessar K, Sabalza M, Capell T and Christou P (2008a) Maize plants: An ideal production platform for effective and safe molecular pharming. *Plant Science* 174:409-419

Ramessar K, Capell T and Christou P (2008b) Molecular pharming in cereal crops. *Phytochemistry Review* 7:579-592

Ramessar K, Capell T, Twyman RM, Quemada H and Christou P (2009) Calling the tunes on transgenic crops: The case for regulatory harmony. *Molecular Breeding* 23: 99-112

Ramessar K, Capell T, Twyman RM and Christou P (2010) Going to ridiculous lengths-European coexistence regulations for GM crops. *Nature Biotechnology* 28:133-136

Sabalza M, Miralpeix B, Twyman RM, Capell T and Christou P (2011) EU legitimizes GM crop exclusion zones. *Nature Biotechnology* 29:315-317

Sanders J, Stolze M and Padel S (2011) Final report: External study: Use and efficiency of public support measures addressing organic farming. Johann Heinrich von Thünen Institut (vTI) - Institute of Farm Economics

Stein AJ and Rodríguez-Cerezo E (2009) The Global Pipeline of New GM Crops. Implications of asynchronous approval for international trade. European Commission, Joint Research Centre. 23846 EN

Stein AJ (2010) Global impacts of human mineral malnutrition. *Plant Soil* 335:133-154

Stein AJ and Rodríguez-Cerezo E (2010) Low-Level Presence of new GM crops: an issue on the rise for countries where they lack approval. *AgBioForum* 13:173-182

Storozhenko S, De Brouwer V, Volckaert M, Navarrete O, Blancquaert D, Zhang GF, Lambert W and Van Der Straeten D (2007) Folate fortification of rice by metabolic engineering. *Nature Biotechnology* 25:1277-1279

Twyman RM, Schillberg S and Fischer R (2005) Transgenic plants in the biopharmaceutical market. *Expert Opinion on Emerging Drugs* 10:185-218

UK DEFRA and FSA (2009) GM crops and foods: follow-up to the food matters, accessed online 29 August 2012,

http://www.food.gov.uk/multimedia/pdfs/foodmattergmreport.pdf

US Department of Agriculture (USDA) (2009) GAIN Report: EU-27 Biotechnology. GM Plants and Animals (USDA, Washington, DC)

Van Mejil H and Van Tongeren F (2003) International diffusion of gains from biotechnology and the European Union's Common Agricultural Policy. International Association of Agricultural Economists, 2003 Annual Meeting, August 16-22 2003, Durban, South Africa

Wager R and McHughen A (2010) Zero sense in European approach to GM. *EMBO Reports* 11:258-262

Webb D (2009) The Lisbon Strategy (No.SN/EP/3404) Retrieved 15th Septemer 2010, from www.parliament.uk/briefing-papers/SN03404.pdf

Wiggerthale M (2005) What's wrong with EU agricultural subsidies? Attac-netzwerk, accessed online 29 August 2012,

http://www.attacnetzwerk.de/fileadmin/user_upload/AGs/Agrarnetz/EUAgrarpolitik/marita_eusubsidies.pdf

CHAPTER 7

GENERAL CONCLUSIONS

CHAPTER 7: GENERAL CONCLUSIONS

HIV-neutralizing human monoclonal antibodies (such as 2G12, 4E10 and 2F5) and antiviral lectins such as cyanovirin-N and griffithsin are among the most promising microbicide candidates currently undergoing evaluation. Combinations of these compounds can be used as microbicide ingredients along with other molecules that protect against secondary infections and other sexually transmitted diseases (STDs) and this would result in a complete and effective microbicide that would inhibit all HIV clades. However, because of the high production and distribution costs for each protein, they would not be available in developing countries, where HIV infection rates are the highest in the world. Furthermore, the cost of manufacturing more than one product is prohibitive. Plants could provide a reliable, low-cost platform for the manufacture of protein microbicide candidates. Maize in particular has a number of important advantages for molecular pharming applications, including its unique physiology, its efficient biomass production based on C4 photosynthesis, its large seed size compared to other cereals, and the presence of an outer layer (husk) protecting kernels from microbial infections and preventing seed loss during harvesting.

In this thesis, I demonstrated the potential of maize as a platform for the large-scale production of recombinant pharmaceutical proteins such as HIV-neutralizing human monoclonal antibodies (2F5 and 2G12). The 2F5 antibody was assembled efficiently in maize seeds and its antigen-binding (gp41) activity was similar to that of its CHO counterpart, but the yields were much lower than previously achieved with 2G12 in the same genetic background. The low yields of 2F5 in maize seeds are consistent with those obtained in tobacco suspension cells. Therefore, I concluded that such yield constraints reflect the intrinsic properties of the antibody and not the limitations of the production platform.

I also carried out the first experiments to demonstrate the production of a combination of microbicide components in plants, i.e. the simultaneous production of two anti-viral lectins (cyanovirin-N and griffithsin) and the human HIV-neutralizing monoclonal antibody 4E10. Preliminary results from agroinfiltration experiments in *Nicotiana benthamiana* showed that these combinations are functional when produced *in planta*.

However, transient expression experiments cannot accurately predict what will happen when these components are stably co-expressed in maize seeds. I will therefore carry out a further round of transformation experiments to determine the potential of combinatorial expression compared to the expression of a single compound.

Maize event 3C producing the HIV-neutralizing human monoclonal antibody 2G12 at high levels was used as a model to study the interactions of a recombinant antibody with maize seed storage proteins during seed development. Immunolocalization studies demonstrated that 2G12 was localized mainly in protein storage vacuoles (PSVs) in the endosperm, and also interacted within zein bodies causing them to adopt an amorphous structure. I concluded that cell age and 2G12 yields in maize correlate positively with the loss of structural consistency among the zein bodies. Even though 2G12 expression levels in maize endosperm were high, further strategies are required to minimize such interactions and increase yields by taking full advantage of maize seeds as a production platform for recombinant proteins. These results can be applied not only to 2G12 but also to other recombinant proteins expressed in maize seeds.

Despite the advantages of GE crops there remain hurdles to overcome before they are broadly adopted for the commercial or humanitarian production of biopharmaceuticals on a large scale. One of the most important hurdles reflects the onerous regulations that differ widely from country to country, some of which are stringent but fair, whereas others are irrational and unnecessarily restrictive. In particular, not only does the EU have the most restrictive regulatory policies for GE crops in the world, the regulations are contradictory and based on political expediency rather than rational scientific grounds. This has led to a bizarre situation in which the policies actually promote the very practices they are aiming to avoid, e.g. the new EC recommendations relating to the approval of GE crops in Europe which if implemented will give member states the authority to ban GE crops without any scientific justification.

Concluding Statements

1. The HIV-neutralizing human monoclonal antibody 2F5 is assembled efficiently in maize seeds and is functional, with an antigen-binding activity similar to that of its CHO counterpart.

2. The HIV-neutralizing human monoclonal antibody 2F5 accumulated to lower levels than previously achieved with 2G12 in the same genetic background, consistent with the results obtained in tobacco suspension cells. This suggests that 2F5 yield constraints reflect the intrinsic properties of the antibody and not the limitations of the production platform.

3. 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. The experiments described in this thesis are a first attempt to create such combinations at source.

4. Preliminary results from agroinfiltration experiments in *Nicotiana benthamiana*, combining cyanovirin-N, griffithsin and the HIV-neutralizing human monoclonal antibody 4E10, demonstrate that these combinations are functional when produced *in planta*. However transient expression experiments cannot accurately predict what will happen when these components are stably co-expressed in maize seeds.

5. Stable co-expression of cyanovirin-N, griffithsin and 4E10 in maize seeds is necessary to assess the potential microbicidal activity of such combinations.

6. Immunoelectron microscopy experiments showed that 2G12 is localized mainly in the protein storage vacuoles during maize endosperm development, although there are also interactions between 2G12 and zein bodies causing them to adopt amorphous structures.

7. During seed development there is a strong correlation between cell age and the loss of structural consistency among the zein bodies. In addition, there is a negative correlation between the accumulation of 2G12 in maize seeds and the structural conservation of the zein body. Therefore, I conclude berries a positive

correlation between the accumulation of 2G12 in maize seeds and interactions with the zein bodies.

8. Immunolocalization studies confirmed that maize seeds are a suitable system for the production of recombinant antibodies, but further studies are needed to determine whether interactions within the zein bodies influence the expression levels and functionality of the antibody.

9. The EU has the most restrictive regulatory policies regarding GE crops in the world. These policies are contradictory and are based on political expediency rather than rational scientific grounds. The policies promote the very practices they are aiming to avoid and therefore represent a hurdle in the commercialization of GE crop applications such as molecular pharming.

10. New European Commission recommendations relating to the approval of GE crops in Europe, if approved and implemented, will allow member states to ban GE crops without any scientific justification.

ANNEX 1

Sabalza M, Miralpeix B, Twyman RM, Capell T, Christou P (2011) EU legitimizes GM crop exclusion zones. Nature Biotechnology 29: 315-317

EU legitimizes GM crop exclusion zones

To the Editor:

On July 13, 2010, the European Commission (EC) officially proposed to give member states the freedom to veto the cultivation of genetically modified (GM) crops on their own territory without having to provide any scientific evidence relating to new risks[1]. The objective of the legislation is ostensibly to make individual member states responsible for their own policy on GM crops, and therefore to speed up pending authorizations by removing the ability of those member states to veto approval throughout the European Union by avoiding a qualified majority (**Fig. 1**). However, we argue that the opt-out will have exactly the opposite effect to that intended, allowing the creation of arbitrary GM-free zones in Europe that will cause untold damage to the EU economy and its global scientific standing.

The removal of any need for scientific justification in decisions concerning GM crops effectively serves to legalize the currently illegal practice in which individual member states arbitrarily declare GM-free zones within their borders, or ban GM crops altogether[2]. The only GM crops currently grown in Europe are the pest-resistant maize variety MON810 and the Amflora potato variety engineered to produce modified starch. Both are banned in Austria, Hungary and Luxembourg, and the MON810 event is also banned in France, Greece and Germany (**Table 1**). Poland is currently drawing up legislation to ban all GM seeds, and other member states are considering similar proposals. Although these existing and proposed bans are technically in breach of EU regulations, at least those member states implementing a ban have to make some sort of effort to justify their decision on scientific grounds, even if the evidence used in such cases is dubious (the 'safeguard clause'). When the legal amendment enters into force, member states will be free to restrict or prohibit the cultivation of all or particular GM crops within their territory, including crops that have already been authorized for cultivation under Directive 2001/18/EC and Regulation EC 1829/2003, and will be able to do so without explanation. This places the future of GM agriculture in Europe at the whim of politicians who may feel compelled to act in response to the media or activist propaganda.

EU policy on agriculture has evolved over the past 50 years as the continent has moved from the position of a net importer struggling to feed its population at the end of a devastating war, to today's near trade parity with the rest of the world. Even so, the EU is still a net importer of agricultural raw materials and 55% of imports come from ten countries, with Brazil, the United States and Argentina ranking in the top three positions[3]. The same three countries also happen to be the world's largest adopters of GM technology, with the United States planting 64 million hectares of transgenic crops in 2009 and both Brazil and Argentina planting just over 21 million hectares[4]. Paradoxically, although the proposed amendment will allow member states to adopt measures against the cultivation of GM crops, they will not be allowed to adopt measures prohibiting the import or marketing in the European Union of authorized GM products from elsewhere, which means that EU markets are likely to be flooded with imported GM products that could just as easily be homegrown. However, the import of GM products is also heavily regulated, as is particularly apparent in the EU's treatment of imported maize and soybean from the United States and elsewhere, which has a substantial knock-on effect on animal agriculture. In this context, the European Union is deficient in feed protein and is ultimately dependent on soybean meal imports. However, imports have declined considerably (from $2.8 billion in 1997 to $1.9 billion in 2008)[5], predominantly because of the complex and onerous process for approving imported GM products, which is administrated at the member state level after the European Food Safety Authority (Parma, Italy) has issued opinions declaring that products are safe. The United States Department of Agriculture states that GM events take on average 15 months to approve in the

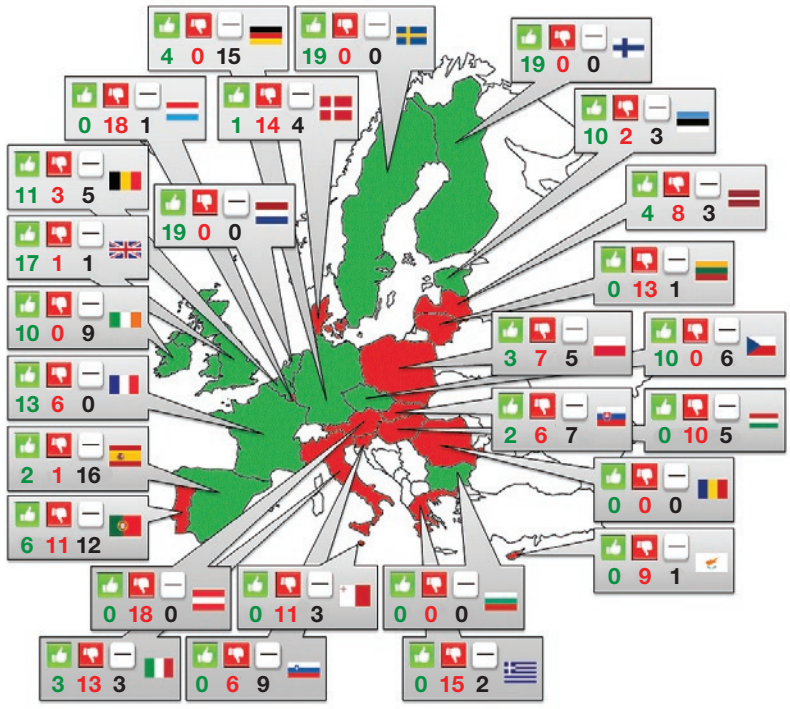

Figure 1 The previous voting habits of the EU ministers on matters concerning GM crops. Europe divides into two roughly equal camps (pro-GM in green and anti-GM in red) based on votes cast on 19 GM proposals between 2003 and 2005. In 2005, the pro-GM camp had a slight advantage because several of the most populous member states were pro-GM. More recently, there has been a slip towards the anti-GM stance with Germany and France now adopting an anti-GM position, although they are more likely to abstain on proposals rather than actively oppose. This is reflected in the voting for the approval of the Amflora GM potato on February 18, 2008. (Data from Friends of the Earth; symbols represent votes for, against and abstentions; not all countries voted on every proposal; the authors thank Michael Green for assistance in the preparation of this figure.)

United States but 40 months in the European Union[5].

Implementation of the July 13, 2010, recommendations also means that co-existence measures for registered GM products will be deferred to national competent authorities, making each member state responsible for the establishment of a legislative framework on a crop-by-crop basis. The practical effect of this recommendation is that member states can impose excessive and arbitrary co-existence rules that effectively suppress GM agriculture over large areas of Europe. Austria, Belgium, Bulgaria, the Czech Republic, Denmark, Germany, Hungary, Latvia, Luxembourg, Portugal, Romania and the Slovak Republic have already started adopting regulations governing the planting and handling of GM crops, whereas others are still in the process of developing their regulations. The lack of EU-wide regulations means that member states can impose minimum isolation distances that are completely removed from the requirements necessary to meet Europe's strict adventitious-presence thresholds and serve only as an artificial strategy to suppress GM agriculture. The EU's stated policy on co-existence is to develop measures that avoid the unintended presence of GM material in conventional and organic crops through transparency, cross-border cooperation, stakeholder involvement and proportionality. Instead, co-existence requirements are confusing and unfathomable, those implementing them are unaccountable, there is no cooperation because member states act independently, those farmers who choose to plant GM crops are sidelined and the rules are as far from proportional as it is possible to be. Furthermore, the strict liability regulations mean that GM farmers are always responsible for any admixture and will be fined accordingly, and risk litigation from surrounding farms.

The United States, Brazil, Argentina and other countries with substantial GM adoption rates have also recognized that biotech can be used to limit the environmental footprint of conventional agriculture (that is, by reducing pesticide use and also fuel consumption by reducing the need for spraying, and by eliminating tillage). In 2008, these savings amounted to 35 million metric tons of pesticides, over 1.2 million metric tons of CO_2 saved from the use of fossil fuels and an additional 13.2 million metric tons of CO_2 sequestered into the soil through the implementation of no-tillage policies[6]. If the EU continues obstructing the deployment of GM crops in Europe, it will force farmers to use environmentally hazardous, expensive and unsustainable agricultural practices, spend unnecessary resources on fossil fuels and agrochemicals, while at the same time letting the same products be imported from the United States and South America, further tipping the balance of trade in the wrong direction.

One of the reasons for the low take-up of GM crops in Europe is low consumer demand and public trust in the technology compared with conventional or organic crops. Mistrust of GM crops by the European public is hard to rationalize, given that >70% of processed foods consumed by humans in the United States and Canada contain GM ingredients, a similar proportion of white maize in Africa is transgenic and several GM products are consumed by humans in China, all with no reported ill effects after 10 years. These countries also export GM seeds to other markets (including the European Union) with no reported incidents.

The failure of the EU to support the adoption of GM crops is symptomatic of the largely unsuccessful Lisbon Agenda, which set out in 2000 to change the EU into a highly competitive knowledge-based economy with innovation, economic vitality, social and environmental renewal and sustainability as its core values. GM crops are an innovation supported by many EU research organizations and they have already proven successful in other countries in terms of economic growth, environmental sustainability and competition. Yet every possible obstacle has been erected in the European Union to prevent this beneficial form of agriculture from being adopted, leading to economic stagnation, trade disputes and the continued destruction of the environment through chemical use and intensive tillage.

One additional impact of the EU's policy on GM agriculture is to discourage homegrown research in agbiotech and drive researchers overseas where the value chain can be realized in terms of released GM crops. Within the European Union, researchers working on transgenic plants know that the best they can expect for their products is greenhouse cultivation, and that despite their benefits, transgenic plants are unlikely to be deployed in any setting where they could perform a useful function. Here the EU policy on GM crops is attacking its own foundations as a competitive bioeconomy because with one hand the EC offers funding for innovative biotech research and values (or even requires) the

Table 1 National bans currently implemented under the 'safeguard clause'

Country	Event	Date	Coverage
Austria	Bt176 maize	1997[a]	Cultivation
	MON810 maize	1999[a]	Cultivation
	T25 maize	2000[a]	Cultivation
	GT73 rapeseed	2007[a]	Import
	MON863 maize	2008	Import
	Ms8 rapeseed	2008	Import
	Rf3 rapeseed	2008	Import
	Ms8/Rf3 rapeseed hybrid	2008	Import
	EH92-527-1 potato	2010	Import
France	Topas 19/2 rapeseed	1998	Import
	MS1/Rf1 rapeseed hybrid	1998	Import
	MON810 maize	2008	Cultivation
Germany	Bt176 maize	2000	Cultivation
	MON810 maize	2009	Cultivation
Greece	Bt176 maize	1997	Cultivation
	T25 maize	1997	Import
	Topas 19/2 rapeseed	1998	Import
	MS1/Rf1 rapeseed hybrid	1998	Import
	MON810 maize	2001	Cultivation
Luxembourg	Bt176 maize	1997	Cultivation
	MON810 maize	2009	Cultivation
Hungary	MON810 maize	2005	Cultivation

[a]These bans were amended in 2008.

participation of small- to medium-sized enterprises (SMEs) and large industry partners, but with the other hand they slam the door in the face of the same companies by offering them little hope of commercial realization. Many large corporations with ambitious GM research projects have already decamped to the United States or elsewhere to continue their work, and promising European SMEs have been stifled because they are unable to find investment partners. No agbiotech company is likely to invest significantly in Europe unless there is an opportunity to recoup their R&D costs by offering their products to farmers. The attitude of European policymakers reveals the immense divide between the rational evaluation of science and business, and the panicky, expedient politics pandering to a populist media and activists.

The new EC recommendations relating to the approval of GM crops in Europe aim to combine the EU science-based authorization system with freedom for member states to decide on their own cultivation policies. We firmly believe that these concepts are irreconcilable because the freedom of member states to ban GM crops is granted with no conditions, allowing decisions to be made based on irrational criteria ('any grounds') and essentially rendering the whole process of risk assessment obsolete. The new recommendation on co-existence recognizes that member states may adopt measures to avoid the unintended presence of GM material in other products below the labeling threshold of 0.9%, and such measures may include the restriction or outright banning of GM agriculture within their borders. Such measures should be proportionate to the objective, but obviously they are not, given the arbitrary and excessive isolation distances that are enforced without any scientific data to show that such distances are necessary[2]. The recommendations apply to cultivated GM crops but not to imported GM products, even if the two represent identical events. This is clearly an illogical position to adopt. Any safety concerns about commercialized GM products should apply equally to cultivated and imported material, and the artificial distinction only serves to highlight the double standard that is being employed in this context. The EC states that "…ensuring a safety assessment following the highest scientific standards and a reinforcement of the monitoring function were and remain priorities for the Commission as concerns GMO cultivation…" while at the same time stating that no grounds are required at all for member states to limit or ban GM agriculture[1]. This clearly is not a high scientific standard by any stretch of the imagination. The proposed legislation does nothing to smooth the authorization process for GM crops in Europe but does provide activists and the media with fuel for their anti-GM propaganda and will no doubt cause massive confusion among the public. The EC proposal also contrasts with one of the EU's key goals (that is, the creation of a free market economy without border controls) because it imposes arbitrary segregation with respect to GM and non-GM agriculture. Although the authorization system is still science-based, the EC has rendered this system toothless by giving member states the means to ignore or overturn regulatory guidance and allow the implementation of policies that have no rational basis by people who have no accountability.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. European Commission. Memo 10/325: *Questions and Answers on the EU's New Approach to the Cultivation of GMOs* (European Commission, Brussels, 2010).
2. Ramessar, K. *et al. Nat. Biotechnol.* **28**, 133–136 (2010).
3. European Commission. *EU Trade in Agricultural Products 2009* (European Commission, Brussels, 2009).
4. Peng, W. *Nat. Biotechnol.* **29**, 302 (2011).
5. US Department of Agriculture. *GAIN Report: EU-27 Biotechnology. GE Plants and Animals* (USDA, Washington, DC, 2009).
6. Brookes, G. & Barfoot, P. *GM Crops: Global Socio-economic and Environmental Impacts 1996–2008.* (PG Economics Ltd., 2010).

ANNEX 2

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Functional characterization of the recombinant HIV-neutralizing monoclonal antibody 2F5 produced in maize seeds

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Abstract Monoclonal antibodies (mAbs) that neutralize human immunodeficiency virus (HIV) can be used as microbicides to help prevent the spread of HIV in human populations. As an industry standard, HIV-neutralizing mAbs are produced as recombinant proteins in mammalian cells, but the high manufacturing costs and limited capacity reduce the ability of target populations in developing countries to gain access to these potentially life-saving medicines. Plants offer a more cost-effective and deployable production platform because they can be grown inexpensively and on a large scale in the region where the products are required. Here we show that the maize-derived HIV-neutralizing mAb 2F5 is assembled correctly in planta and binds to its antigen with the same affinity as 2F5 produced in mammalian cells. Although 2F5 has been produced at high levels in non-plant platforms, the yield in maize seeds is lower than previously achieved with another HIV-neutralizing mAb, 2G12. This suggests that the intrinsic properties of the antibody (e.g. sensitivity to specific proteases) and the environment provided by the production host (e.g. the relative abundance of different proteases, potential transgene silencing) may combine to limit the accumulation of some antibodies on a case-by-case basis.

Keywords Anti-HIV monoclonal antibodies · Microbicides · Molecular pharming · Maize seeds

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Introduction

At least 34 million people are currently infected with human immunodeficiency virus (HIV) and are therefore at risk of acquired immunodeficiency syndrome (AIDS) (UNAIDS 2010). More than 65 % of these individuals live in sub-Saharan Africa (UNAIDS 2011). There is currently no effective vaccine against HIV despite promising initial results (Fauci et al. 2008; Dolgin 2010; Walker and Burton 2010). There is also no cure, although the latest generation of antiretroviral drugs can slow the disease and improve quality of life. Low-cost microbicides that prevent the spread of HIV are therefore considered the best alternative strategy to fight the current pandemic (Ramessar et al. 2010; Shattock and Rosenberg 2012).

Many potential microbicide candidates are undergoing tests but those containing HIV-neutralizing monoclonal antibodies (mAbs) are particularly advantageous because they target conserved but distinct epitopes on the HIV-1 envelope glycoproteins gp120 and gp41, thus inhibiting the uptake of HIV into human cells (Shattock and Moore 2003; Cardoso et al. 2005; reviewed by Ramessar et al. 2010). Such mAbs as passive immune therapy may inhibit virus replication not only by preventing uptake but also by inducing the clearance of virus particles and infected cells by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Armbruster et al. 2004). A number of broadly HIV-neutralizing human

mAbs have been described (Cardoso et al. 2005; Burton and Weiss 2010; Walker et al. 2011) but thus far only 2G12, 4E10 and 2F5 have undergone clinical evaluation (Armbruster et al. 2002, 2004, Mehandru et al. 2004; Joos et al. 2006), including a triple microbicide formulation developed as part of the European Microbicides Project [EMPRO; Dr. Charles Kelly-Hull York Medical School (HYMS) York, United Kingdom; and Dr. Charles Lacey-King's College London (London, United Kingdom), personal communication].

Monoclonal antibody 2F5 demonstrates broad neutralizing activity against different HIV-1 strains in vitro and in animal models (Huang et al. 2002; Cardoso et al. 2005) because it binds to the highly-conserved linear epitope ELDKWA found on the C-terminus of the gp41 heptad (Muster et al. 1993; Binley et al. 2008). Binding inhibits the gp41 conformational changes required for HIV to interact with human cells (Parren and Burton 2001; Zwick et al. 2001). Furthermore, in combination with 2G12 and/or 4E10, 2F5 has been shown to confer protection against intravenous, intrarectal, intravaginal and oral challenge with simian-human immunodeficiency virus (SHIV) in Rhesus macaques (Mascola et al. 1999, 2000; Baba et al. 2000; Hessell et al. 2010; Shen et al. 2010). Phase I clinical studies using 2F5 produced in Chinese hamster ovary (CHO) cells showed no evidence of toxicity or immunogenicity in human subjects alone or in combination with other antibodies (Armbruster et al. 2002, 2004; Mehandru et al. 2004). Phase II clinical studies with the three antibodies indicate that 2F5 is effective as part of a monoclonal antibody cocktail to prevent the evolution of virus escapes (Joos et al. 2006; Manrique et al. 2007).

The inclusion of 2F5 in a broadly-accessible microbicide entails the use of a large-scale production platform because effective prevention is likely to require the administration of up to 5 mg of the active pharmaceutical ingredient twice weekly per individual, which equates to 5,000 kg of antibody per year per 10 million women (Shattock and Moore 2003). This far exceeds the capacity of current CHO-based infrastructure, and even if the capacity were available the costs would be prohibitive (Hofmann-Lehmann et al. 2001; Stiegler et al. 2002). Plants offer a more cost-effective and scalable manufacturing platform that could be used to produce mAbs locally (Ma et al. 2003; Ramessar et al. 2008a). Maize is particularly suitable in developing country settings that lack a cold chain and distribution network because recombinant proteins remain stable for months or years at ambient temperatures when produced and stored in seeds (Stoger et al. 2005; Ramessar et al. 2008a,b,c).

Several HIV-neutralizing mAbs have already been produced in plants. The first was b12, which was expressed in tobacco as an IgG and also as a fusion (Sexton et al. 2009) with the anti-HIV lectin cyanovirin-N (Boyd et al. 1997; Dey et al. 2000). 4E10 mAb was produced by secretion from hydroponic tobacco cultures and was shown to assemble and function correctly although the overall yields were low (Drake et al. 2009). 2G12 mAb has been expressed successfully in tobacco and was the first plant-derived mAb to complete a phase I clinical study in Europe (Fischer et al. 2012). This mAb 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 (Ramessar et al. 2008b; Rademacher et al. 2008). In both cases the yields were high (40–100 mg/kg dry seed weight) and the purified antibody demonstrated HIV-1 neutralization activity that was similar to or greater than the same antibody produced in CHO cells. The 2G12 mAb was also expressed transiently in *Nicotiana benthamiana* with yields of up to 50 mg/kg fresh biomass (Sainsbury and Lomonossoff 2008; Strasser et al. 2008).

2F5 mAb has been produced in tobacco plants as a fusion protein with an elastin like peptide (Floss et al. 2008). Free 2F5 has also been expressed in tobacco suspension cells, where it was reported to have similar antigen-binding affinity to its CHO counterpart but less efficient HIV-1 neutralization (Sack et al. 2007).

We wished to test the hypothesis that the intrinsic properties of a given macromolecule determine the level of its accumulation in a given production platform, rather the production platform itself. To that effect we expressed mAb 2F5 in maize seeds to determine whether it was possible to match the high yields and neutralization activity previously achieved with 2G12. We found that 2F5 in maize seeds was assembled efficiently and could bind gp41, but that the yields were much lower than previously achieved with 2G12 in the same genetic background. The low 2F5 accumulation levels obtained in maize seeds are consistent with those obtained in tobacco suspension cells (Sack et al. 2007). We therefore concluded that such yield constraints reflect the intrinsic properties of the mAb and not limitations of the production platform.

Materials and methods

Transformation constructs

The 2F5 heavy chain (HC) and light chain (LC) genes (obtained from Polymun Scientific Immunobiologische Forschung, Vienna, Austria) were amplified by PCR using primers containing (1) restriction sites to facilitate cloning and (2) a murine immunoglobulin chain signal peptide sequence to target the secretory pathway (Schillberg et al. 1999) and achieve appropriate post-translational modification. Two groups of transformation vectors were used,

i.e. test constructs for transient constitutive expression in *N. benthamiana* leaves, and constructs for stable endosperm-specific expression in transgenic maize plants.

The transient expression constructs were based on vector pL32 and contained the constitutive *Cauliflower mosaic virus* 35S promoter, the murine immunoglobulin leader peptide sequence, the coding region of the HC/LC genes and the nopaline synthase (*nos*) gene terminator. Matching constructs were generated with the 2G12 HC and LC genes for comparison. A binary vector (pBI) containing the *Artichoke mottled crinkle virus* (AMCV) p19 silencing suppressor gene was used to avoid any potential post-transcriptional gene silencing in the infiltrated plants (Johansen and Carrington 2001).

The stable transformation constructs pHor-2F5H and pHor-2F5L were generated by transferring the 2F5 HC and LC genes to the pHor vector, containing the endosperm-specific barley D-hordein promoter (Naqvi et al. 2009), the mouse immunoglobulin signal peptide and the rice *ADPGPP* gene terminator. A third construct (pTRAuxbar; obtained from Drs E. Stoger-BOKU, Vienna, Austria and T. Rademacher-Fraunhofer IME, Aachen, Germany) comprising the constitutive maize ubiquitin-1 promoter, the maize ubiquitin-1 first intron, the *bar* selectable marker gene and the CaMV 35S terminator was used to select transgenic plants on the basis of phosphinothricin resistance (Zhu et al. 2008). Maize seeds expressing 2G12 antibody were generated previously and are described in (Ramessar et al. 2008b).

Transient expression assays

Individual bacterial clones were maintained in liquid culture until the OD_{600} reached 0.6, before mixing into one of two bacterial mixtures: the 2F5 mix (2F5HC, 2F5LC and p19) and the 2G12 mix (2G12HC, 2G12LC and p19). *N. benthamiana* leaves were infiltrated as previously described (Lombardi et al. 2010) with one of two bacterial mixtures described above. Samples were taken for analysis 6 days post infiltration (dpi), when transient accumulation levels are typically highest (Villani et al. 2008).

Stable expression of mAb 2F5 in transgenic maize plants

Immature zygotic embryos of the South African elite white maize (*Zea mays*) inbred M37W were transformed by particle bombardment 10–14 days after pollination as described by Drakakaki et al. (2005) and modified by Naqvi et al. (2009) using 3:1 (by molecular weight) DNA ratio for the vector containing the gene of interest: *bar* selectable marker. Bombarded embryos were incubated in the dark and transferred every 2 weeks to fresh N6-based medium containing 3 mg/L phosphinothricin (PPT). After 4–6 weeks, pieces of PPT-resistant embryogenic type I callus were transferred to regeneration medium containing 3 mg/L PPT for 2–4 weeks with a 16h photoperiod. All experiments were performed at 25 °C. Developing plantlets with well-formed shoots and roots were hardened off and transferred to soil.

Southern blot analysis

Genomic DNA was isolated from maize leaves by phenol extraction (Edwards et al. 1991) and 20 µg aliquots were digested overnight with restriction enzymes recognizing either a single site in the transformation vector (HindIII) or two sites flanking the 2F5 HC and LC transgenes (NotI/EcoRI). The DNA was fractionated by 0.8 % agarose gel electrophoresis (Sambrook et al. 1989), transferred to a positively-charged nylon membrane (Roche, UK) and fixed by UV cross-linking. DIG-labeled probes were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and denatured at 90 °C for 10 min prior to hybridization overnight at 42 °C. Membranes were washed at high stringency (twice for 5 min in 2 × SSC, 0.1 % SDS at room temperature, twice for 25 min in 0.5 × SSC, 0.1 % SDS at 68 °C, once for 15 min in 0.2 × SSC, 0.1 % SDS at 68°C, and once for 10 min in 0.1 × SSC, 0.1 % SDS at 68 °C) prior to chemiluminescent detection using the DIG Luminescent Detection Kit (Roche, UK) according to the manufacturer's instructions. After washing, the membranes were incubated with CSPD chemiluminescent substrate (Roche, UK) and exposed on BioMax light film (Kodak, Rochester, NY, USA) at 37 °C.

Enzyme-linked immunosorbent sandwich assay (ELISA)

Agroinfiltrated *N. benthamiana* leaves (harvested 6 dpi) and transgenic maize endosperm tissue (embryo removed) were ground in two volumes of PBS and centrifuged twice at 20,000×*g* for 10 min at 4 °C to remove plant debris.

The presence of assembled mAb was determined by coating Nunc Maxisorp 96-well Immunoplates (Nunc, Roskilde, Denmark) with anti-human γ-chain antiserum (The Binding Site, Birmingham, UK) and blocking with 2.5 % (w/v) bovine serum albumin (BSA). Serial dilutions of the supernatant from plant tissue samples were then added, and captured 2F5 mAb was detected after a brief wash step by adding horseradish peroxidase (HRP)-conjugated sheep anti-human K-chain antiserum (The Binding Site, Birmingham, UK; 1:1000 dilution), washing, developing the signal with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO, USA) and reading the absorbance at 450 nm.

The specific antigen binding activities of 2F5 and 2G12 were detected by coating ELISA wells with either 100 ng recombinant gp41 from HIV strain HXB2 (2F5) or 100 ng recombinant gp120 from HIV strain IIIB (2G12), both provided by the MRC Centralized Facility for AIDS reagents, Potters Bar, UK. After washing and blocking with BSA as above, we added serial dilutions of the 2F5 and 2G12 mAbs and determined the amount of bound antibody using HRP-conjugated anti γ-chain and HRP-conjugated anti κ-chain antiserum as appropriate (The Binding Site, Birmingham, UK; 1:1000 dilution) with signal detection as above.

Western blot analysis

Proteins were separated under non-reducing conditions on 4–12 % pre-cast Bis-Tris Nu- PAGE gels (Invitrogen, Carlsbad, CA, USA) using Precision Plus protein standards (BioRad, Hercules, USA). Each sample comprised 15 µl of maize 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). Separated proteins were blotted onto nitrocellulose membranes using the Hoefer TE70 semidry transfer system (Amersham Biosciences, Piscataway, NJ, USA) 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), mAbs were detected with HRP-conjugated IgG γ-chain or HRP-conjugated IgG κ-chain antiserum (The Binding Site, Birmingham, UK; 1:1,000 dilution). After further washes, signals were detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, Little Chalfont, UK).

Results

Transient expression of 2F5 and 2G12 in *N. benthamiana* leaves produces correctly-assembled and functional antibodies

We produced 2F5 and 2G12 in *N. benthamiana* leaves by transient expression to test the expression constructs and to confirm the assembly of functional antibodies *in planta*. In separate experiments, leaves were infiltrated with two bacterial preparations, namely the 2F5 mix (2F5HC, 2F5LC and p19) and the 2G12 mix (2G12HC, 2G12LC and p19). In both cases, the presence of correctly assembled antibody molecules was confirmed using an immunoglobulin-specific sandwich ELISA procedure in which the plates were coated with a HC-specific capture antibody, and bound 2F5 and 2G12 was detected with a LC-specific antibody conjugated to HRP. This ensured that only correctly assembled antibodies were detected and free HC and LC molecules were excluded. The concentrations of antibodies were calculated using titration curves compared with the positive controls at known concentrations and no significant difference was observed (Fig. 1a). We used corresponding mAbs produced in CHO cells as positive controls and non-infiltrated tobacco leaves as a negative control to rule out cross-reaction with endogenous plant proteins.

Having confirmed mAb expression and assembly, we used an antigen-specific ELISA procedure to investigate antigen binding. In this assay, the capture reagent was gp41HXB2 for 2F5 and gp120IIIB for 2G12, and a mixture of anti-human gamma and anti-human kappa antibodies (which bind HC and LC respectively) was used for detection (Fig. 1b, c). This experiment showed that the plant-derived antibodies bound to their specific antigens as expected.

Particle bombardment generates independent transgenic lines containing the 2F5 HC and LC genes at the same locus

Having established that the HC and LC constructs were suitable for expression in plants, we bombarded maize embryos with the vectors pHor-2F5HC, pHor-2F5LC and pTRAuxbar, and regenerated transgenic plants from the resulting callus material. These plants were either selfed or crossed with wild-type M37W plants depending on pollen availability to generate 20 putative independent transgenic lines.

Southern blot analysis confirmed the presence of the antibody transgenes in most of the lines, and the unique integration patterns revealed by cleaving genomic DNA with HindIII (which has a single restriction site in each transgene) confirmed that each line represented an independent transformation event (representative results for the 2F5 HC are shown in Fig. 2a, results for the 2F5 LC are not shown but are similar to the results for the HC). We anticipated linkage of the three input transgenes based on the tendency of separate transformation constructs to integrate at the same locus (Nicholson et al. 2005; Drakakaki et al. 2005; Altpeter et al. 2005; Kohli et al. 2006) and this was confirmed in the T1 generation by the Mendelian segregation of the transgenes. Finally, digestion with NotI and EcoRI released the 1,453-bp the 2F5HC cassette in the same 5 maize lines (Fig. 2b) and the 713-bp 2F5LC cassette confirming their integrity (data not shown).

Immunoglobulin-specific ELISA and native western blot demonstrate that 2F5 is correctly assembled in maize endosperm but is expressed at lower levels than 2G12

Six T1 generation seeds each from five representative 2F5 transgenic lines (as described above) and five T3

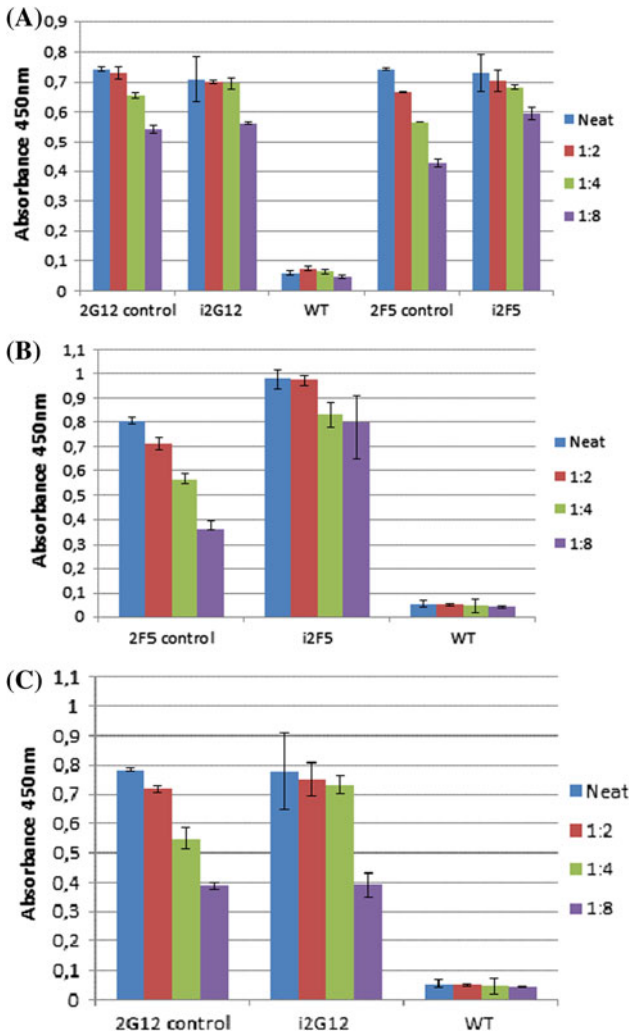

Fig. 1 Structural and functional analysis of mAbs 2F5 and 2G12 produced by transient expression in *N. benthamiana* leaves (i2F5 and i2G12). **a** Assembled 2F5 and 2G12 in leaf extracts were detected using a sandwich ELISA, comprising a heavy chain-specific anti-human IgG γ-chain capture antibody and a light chain-specific HRP-conjugated anti-human IgG κ-chain detection antibody (absorbance measured at 450 nm). Data represent neat extracts and dilutions as well as 1 μg/ml CHO-derived reference antibodies and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings. **b** Antigen-specific ELISA using gp41 as capture reagent for 2F5 and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies. Data represent neat extracts and dilutions as well as 1 μg/ml CHO-derived reference antibody and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings. **c** Antigen-specific ELISA using gp120 as capture reagent for 2G12 and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies. Data represent neat extracts and dilutions as well as 1 μg/ml CHO-derived reference antibody and wild-type leaf extract as a negative control, each a mean ± SD of triplicate readings

generation seeds from a previously-described transgenic line expressing 2G12 (Ramessar et al. 2008b) were separated into embryo and endosperm. We tested endosperm extracts by ELISA and native western blot to investigate antibody expression, folding and assembly. Embryos from seeds with the highest 2F5 yields in the endosperm were germinated, and the best-performing T1 lines were selected and grown to maturity. The resulting plants were either selfed or crossed with wild-type M37W depending on pollen availability and we identified the T2 homozygous lines.

The endosperm extracts were tested using the immunoglobulin-specific ELISA procedure discussed above to confirm the presence of assembled antibody. Representative results from 8 plants from 3 different lines are shown in Fig. 3a, with corresponding antibodies produced in CHO cells as positive controls and non-transgenic endosperm extract as a negative control to rule out cross-reactivity. These experiments confirmed the presence of assembled 2F5 (Fig. 3a) and 2G12 (Fig. 3b). Overall we found that 2F5 accumulated to a consistently lower level (0.80 ± 0.33 μg/ml) than 2G12 and the best 2F5 seeds produced three-fold lower amounts of antibody compared to the best 2G12 seeds (3.98 ± 0.83 μg/ml).

Non-reducing western blots of seed extracts from the three best-performing 2F5 transgenic lines revealed the presence of a 150-kDa product in all lines, which is the expected size of a tetrameric mAb comprising two 50-kDa HCs and two 25-kDa LCs and is identical to the size of the assembled CHO-derived 2F5 antibody which was used as a control (Fig. 4). The 150-kDa assembled antibody is also the dominant product in the 2G12 seeds (Fig. 5). In both cases, there is also a ~25-kDa product, which corresponds to excess LC, and additional bands (indicated by red arrows in Fig. 4) that are likely to be degradation products. Excess HC molecules are present in the CHO 2F5 control sample whereas the LC product is absent (Fig. 4).

Antigen-specific ELISA demonstrates that 2F5 from maize endosperm is functional but is expressed at lower levels than 2G12

Having established that 2F5 is correctly assembled in maize endosperm, we used the antigen-specific ELISA procedure described above with crude extracts to estimate the binding activity of plant-derived mAb against gp41 (Fig. 6). All extracts from transgenic seeds generated a signal greater than the wild type extract background, indicating that all the seeds produced a functional 2F5 antibody. There was no cross-reactivity between 2F5 and endogenous plant proteins showing that the plant-derived antibody was specific. The binding activity between plant-derived 2F5 and gp41 was similar to that between the control 2F5 antibody produced in CHO cells and gp41 and to that between plant-derived 2G12 and gp120 (Fig. 7). However, these experiments confirmed that the abundance of functional antibody was lower than previously achieved

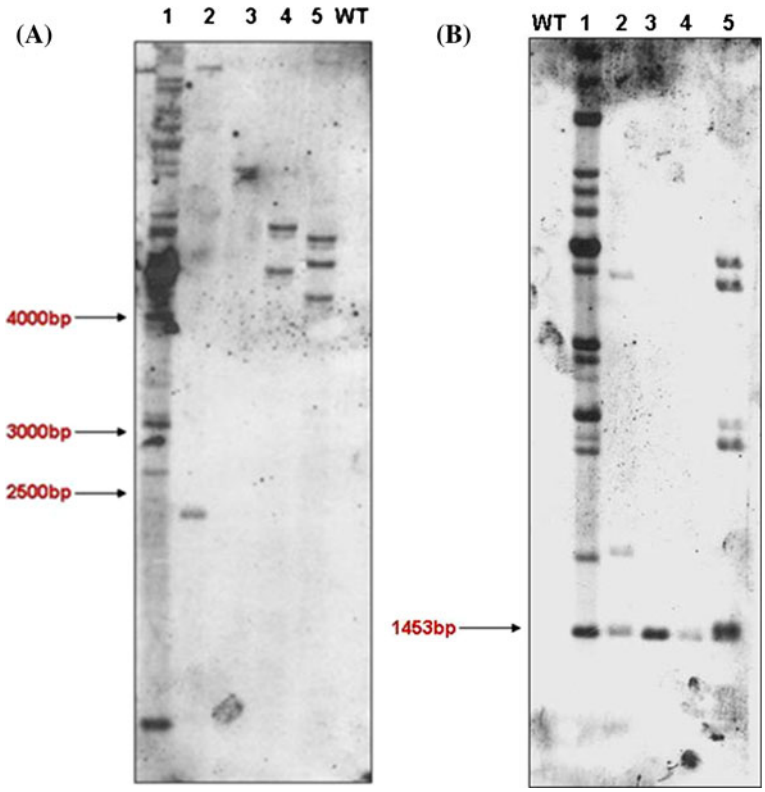

Fig. 2 Southern blot analysis to verify the independent origin of five transgenic maize lines (*lanes 1–5*) and the integrity of the integrated transgene cassette. **a** Digestion of genomic DNA with HindIII, which cuts once within the transgene cassette, and detection with a heavy chain-specific probe. The different sized fragments in each transgenic line confirm their independent origin. **b** Digestion of genomic DNA with NotI and EcoRI, which flank the heavy chain cassette, and detection with a heavy chain-specific probe. The released fragment is consistent with the expected size of the transgene (1453 bp). Wild type maize genomic DNA was used as a control. Similar results were obtained with a light chain-specific probe (not shown)

by expressing 2G12 using the same platform. The highest concentration of functional 2F5 antibody in the seed extract was 0.61 ± 0.28 μg/ml, whereas for 2G12 the equivalent value was 3.68 ± 0.56 μg/ml.

Discussion

HIV/AIDS is an ongoing pandemic that has caused more than 30 million deaths since the virus was first recognized in 1981 (UNAIDS 2010). There is no cure for HIV/AIDS and efficacious vaccines against HIV are currently unavailable (Walker and Burton 2010). Microbicides that block the transmission of HIV are considered one of the most promising strategies to slow the HIV/AIDS pandemic in developing countries (where 20–30 % of the population is infected) because it allows women to play a proactive role in sexual health rather than relying on men to cooperate in the use of condoms.

Human antibodies are preferred over humanized or murine antibodies because they tend not to induce an immune response against the microbicide itself even after repeated applications (Radomsky et al. 1992). HIV-neutralizing mAbs are also persistent, and therefore not only reduce male-to-female HIV-1 transmission at the time of intercourse, but also neutralize virus particles in vaginal secretions and thus also reduce HIV-1 transmission from women to men (Veazey et al. 2003).

The advantageous properties of HIV-neutralizing mAbs are offset by the costs of manufacture. The current industry standard is to produce antibodies by fermentation in mammalian cells, which is suitable for products required in relatively small amounts (e.g. for the treatment of cancer), but far too expensive for the manufacture of 'commodity' antibodies that are required on a much larger scale. Plants have emerged as a suitable alternative to mammalian cells where cost-effective large-scale production is necessary (Ma et al. 2003; Fischer et al. 2004; Stoger et al. 2002). 2G12 was the first HIV-neutralizing mAb to be manufactured in plants under good manufacturing practice (GMP) guidelines (Fischer et al. 2012) and recently completed a phase I clinical trial.

The clinical-grade 2G12 was produced in transgenic tobacco plants, which grow rapidly and produce large amounts of biomass but require a conventional processing infrastructure including a cold chain. In developing country settings where such facilities are generally not available, seed-based production systems maintain the economy of scale enjoyed by tobacco but also allow crude bulk drug substance to be stockpiled at ambient temperatures without product degradation (Stoger et al. 2005). Maize is advantageous because it is widely cultivated in developing countries and has already been developed as a commercial platform for recombinant protein production (Naqvi et al. 2011).

We have previously expressed 2G12 in maize seeds and found that the antibody was expressed at high levels, was

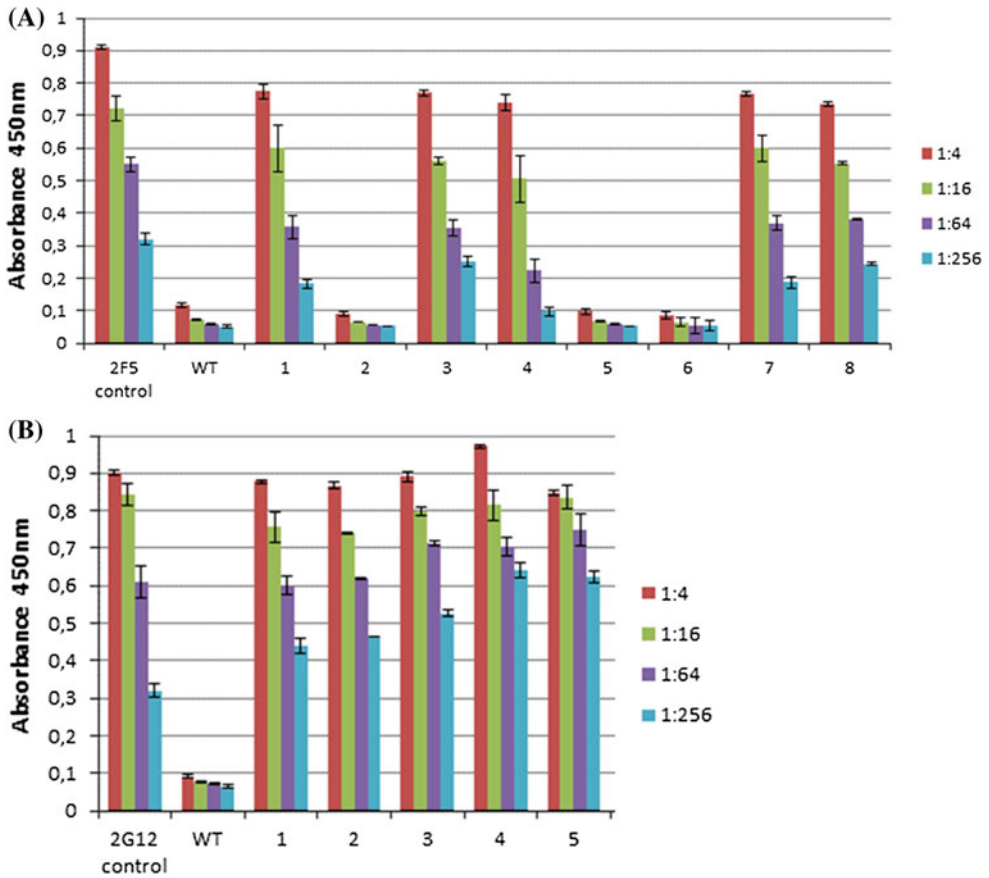

Fig. 3 Structural analysis of mAbs 2F5 and 2G12 produced by stable expression in maize. Assembled **a** 2F5 and **b** 2G12 in endosperm extracts were detected using a sandwich ELISA, comprising a heavy chain-specific anti-human IgG γ-chain capture antibody and a light chain-specific HRP-conjugated anti-human IgG κ-chain detection antibody (absorbance measured at 450 nm). In **a** *lanes 1–8* represent three independent transgenic lines expressing 2F5, three seeds from one event in *lanes 1–3*, three seeds from a second event in *lanes 4–6* and two seeds from a third event in *lanes 7–8*. In **b** *lanes 1–5* represent five seeds from a single previously-described transgenic maize line expressing 2G12. Data represent extract dilutions as well as 1 μg/ml CHO-derived reference antibodies and wild-type endosperm extract as a negative control, each a mean ± SD of triplicate readings

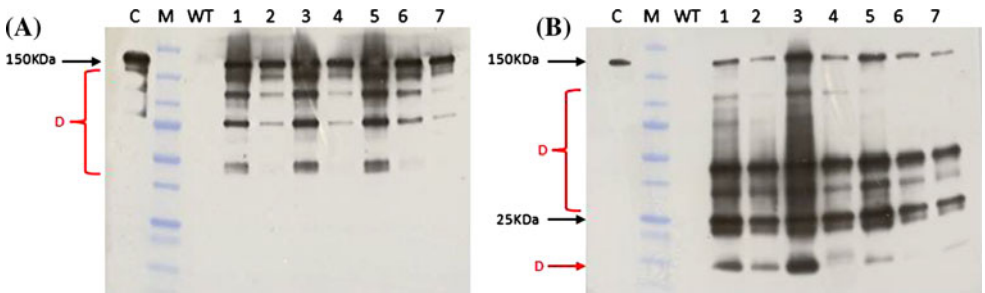

Fig. 4 Western blot analysis of maize-derived 2F5 under non-reducing conditions using **a** heavy chain-specific anti-human IgG γ-chain and **b** light chain-specific anti-human IgG κ-chain antibodies. C = positive control (50 ng 2F5 produced in CHO cells), M = protein size markers, WT = wild type seed extract as a negative control. *Lanes 1–7* represent three independent transgenic lines expressing 2F5, three seeds from one event in *lanes 1–3*, three seeds from a second event in *lanes 4–6* and two seed a from a third event in lane 7

easy to purify from ground endosperm tissue and demonstrated similar or even better HIV-neutralization activity than 2G12 derived from CHO cells (Ramessar et al. 2008b). This established the principle of efficient functional antibody expression in maize, but to reduce the likelihood of viral escape, microbicides must contain a cocktail of mAbs

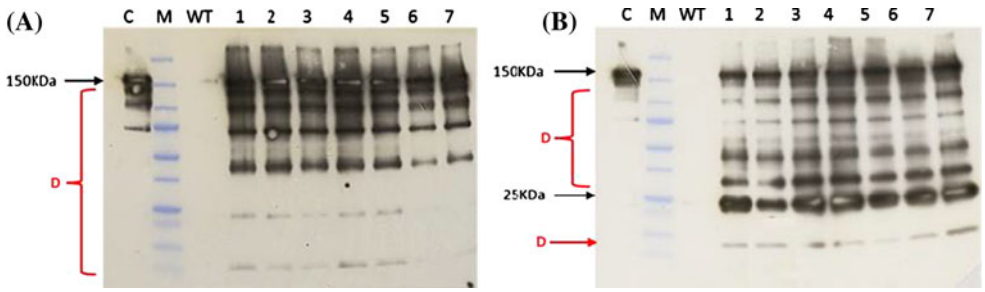

Fig. 5 Western blot analysis of maize-derived 2G12 under non-reducing conditions using **a** heavy chain-specific anti-human IgG γ-chain and **b** light chain-specific anti-human IgG κ-chain antibodies. C = positive control (50 ng 2G12 produced in CHO cells), M = protein size markers, WT = wild type seed extract as a negative control. *Lanes 1–7* represent one independent transgenic line expressing high levels of 2G12. *Lanes 1–7* represent 7 seeds from a single transgenic line maize seed

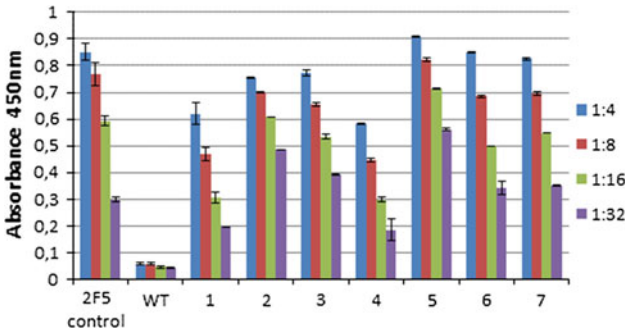

Fig. 6 Functional analysis of 2F5 produced in transgenic maize seeds using gp41 as the capture reagent and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies (absorbance measured at 450 nm). Data represent crude extract dilutions as well as 1 µg/ml CHO-derived reference 2F5 and wild-type seed extract as a negative control, each a mean ± SD of triplicate readings. We show three independent events; *1–3* represent three seeds from one event; *4–5* represent two seeds from a second event; and *6–7* represent two seeds from a third event

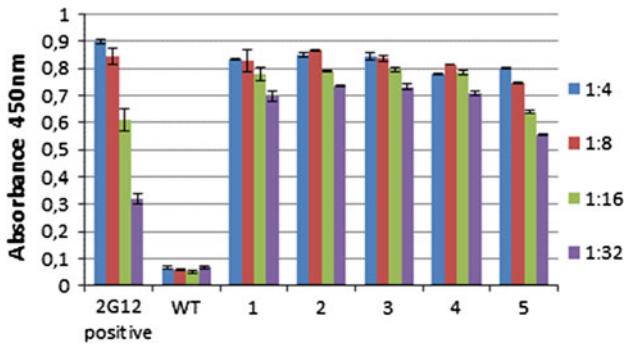

Fig. 7 Functional analysis of 2G12 produced in transgenic maize seeds using gp120 as the capture reagent and detection with a mixture of HRP-conjugated anti-human IgG γ-chain and κ-chain antibodies (absorbance measured at 450 nm); *1–5* represent five seeds from a single previously-described transgenic maize line expressing 2G12. Data represent crude extract dilutions as well as 1 µg/ml CHO-derived reference 2G12 and wild-type seed extract as a negative control, each a mean ± SD of triplicate readings

recognizing different epitopes. Therefore it is necessary to establish whether the same platform can be used to produce additional HIV-neutralizing mAbs.

The production of 2F5 in plants has previously been achieved using two different tobacco-based systems, in one case suspension cells expressing the free antibody (Sack et al. 2007) and in the other case transgenic plants expressing a fusion protein comprising the antibody joined to an elastin like peptide to increase its stability and facilitate extraction (Floss et al. 2008). The suspension cells produced low yields of 2F5 and although the antibody demonstrated a similar affinity to its antigen as a CHO-derived counterpart, its HIV-neutralization activity was reduced (Sack et al. 2007). Two fusion constructs were produced in transgenic plants, one with the fusion tag on the HC and one with the tag on the LC, both strategies achieving higher yields than the untagged protein, which accumulated to only low levels (Floss et al. 2008). A comparison between the untagged 2F5 expressed in transgenic tobacco and the similarly untagged 2G12 expressed in the same platform (Fischer et al. 2012) revealed significant differences in yield which could reflect differences in the host plants, the strategy for product development (i.e. the breeding program to create the production line) or intrinsic properties of the antibody.

As a first step towards developing a generic maize-based platform for the production of different HIV-neutralizing antibodies for a microbicidal cocktail, we therefore attempted to express 2F5 in maize using precisely the same strategy as previously adopted for 2G12. First it was necessary to confirm that the cDNA constructs containing the antibody HC and LC sequences were suitable for expression in plants, and this was achieved by transient expression in *N. benthamiana* leaves to demonstrate transgene expression and functional antibody assembly. All steps were carried out in parallel for both 2F5 and 2G12, the latter as a control. These experiments showed conclusively that all transgenes were expressed, the antibodies assembled correctly and were able to bind their antigens and there was no cross-reaction to endogenous plant proteins.

The transient expression data suggested that the constructs were suitable for the production of transgenic maize plants and the transgenes were duly transferred to expression vectors promoting endosperm-specific expression in maize. We recovered 20 independent transgenic lines as verified by unique transgene integration patterns and selected a representative number for expression analysis. The 2F5 transgenic maize lines were tested in parallel with our previously-reported transgenic line expressing 2G12 to compare their performance. We repeated the experiments to confirm antibody expression, assembly and functionality, but found that whereas the transient expression data reliably predicted the structural and functional integrity of the 2F5 antibody, there was no relationship between the yields achieved in the two sets of experiments. Indeed, we found that the yield of 2F5 in the best-performing plants was consistently lower than the best 2G12 lines.

The differences between 2F5 and 2G12 levels achieved in transgenic tobacco plants could reflect differences in experimental conditions, breeding and/or the intrinsic properties of the antibody. Yields of the same recombinant antibody can vary substantially in different plant systems, particularly when comparing transient and stable expression, whole plants and suspension cultures, or transgenic and transplastomic plants (Daniell et al. 2001; Hellwig et al. 2004; Strasser et al. 2008). For example, 2G12 accumulates to different levels when transiently expressed in *N. benthamiana* and stably expressed in tobacco plants and suspension cells (Platis et al. 2008; Dr. Stefan Schillberg (Fraunhofer IME, Aachen, Germany, personal communication).

The plant production species may also affect yields, partly because common regulatory elements may differ in efficiency in different genetic backgrounds, partly because the efficiency of protein synthesis may reflect species-dependent codon preferences, and partly because the intracellular environment may affect protein stability in a species-dependent context (Torres et al 1999; Stoger et al. 2000; Desai et al. 2010; Khan et al. 2012). Yields in the same production platform can vary when different promoters are used to drive transgene expression (Peremarti et al. 2010) or when different targeting strategies are employed (Khan et al. 2012). For example, 2G12 accumulated to 75 mg/kg dry seed weight in maize when secreted to the apoplast, but to only 40 mg/kg dry seed weight when retained in the endoplasmic reticulum (Ramessar et al. 2008b; Rademacher et al. 2008).

The track record for 2F5 shows that, in three different transgenic plant systems, the yields are lower than 2G12. Our results in transgenic maize are consistent with transgenic tobacco (Floss et al. 2008; Fischer et al. 2012) and tobacco suspension cells (Sack et al. 2007). Our transgenic maize lines expressing 2F5 and 2G12 were prepared using the same techniques and selection procedures, allowing head-to-head comparison between the antibodies with other factors taken into account. The discordant transient expression and stable expression data in tobacco suggest that the 2F5 and 2G12 molecules probably do not differ significantly in terms of intrinsic stability and that the difference in yields in transgenic systems may reflect a combination of host-dependent interactions, such as the abundance of different proteases and the potential for unique sequences within the transgene to induce silencing. The intrinsic stability of 2F5 is also supported by the high yields achieved in other platforms such as *Pichia pastoris* (Gasser et al. 2006), hybridoma lines (Kunert et al. 1998) and CHO cells (Kunert et al. 2000) and also the sequence homology between 2F5 and 2G12 in the constant region domains of the antibodies. It therefore seems likely that the variable region of the 2F5 antibody may render it more susceptible than 2G12 to the particular spectrum of proteases found in maize seeds (comparing Figs. 4b with 5b shows more degradation in the 2F5 lane, supporting this hypothesis) and/or that certain unique aspects of the 2F5 cDNA sequence render it more susceptible to silencing in maize. These hypotheses are not mutually exclusive—indeed the accumulation of any recombinant protein is always dependent on the combined influences affecting transgene expression (product synthesis) and protein stability (product degradation).

In conclusion, we have achieved the production of the HIV-neutralizing mAb 2F5 in transgenic maize seeds. Thus maize could be used to produce multiple antibodies as components of a microbicidal cocktail to prevent the spread of HIV. The maize-derived antibody was assembled efficiently *in planta* and bound its corresponding antigen in the same way as a control 2F5 preparation manufactured in CHO cells. Although the antibody appeared stable and the degradation was not significantly greater than 2G12 produced using the same platform, the overall yield of the antibody was consistently more than threefold lower than 2G12 produced in an identical background, suggesting 2F5 is more sensitive than 2G12 to platform-specific factors such as maize proteases or transgene silencing. We found that 2F5 accumulated to similar levels as 2G12 by transient expression in *N. benthamiana* leaves suggesting that 2F5 is not intrinsically less stable than 2G12 and that the cDNA does not contain codons that reduce the efficiency of protein synthesis. The low levels of 2F5 expression achieved in other transgenic plant systems including tobacco suggest that the bottleneck may involve a combination of factors including sensitivity to specific proteases, the influence of transgene silencing or some form of incompatibility between the 2F5 transgene and the adjacent regulatory elements in the construct.

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References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Michael NL, Vetter N, Katinger HW (2002) A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. AIDS 16:227–233

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Köller U, Jilch R, Ammann CG, Pruenster M, Stoiber H, Katinger HWD (2004) Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. J Antimicrob Chemother 54:915–920

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu WD, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu YC, Wright JE, Chou TC, Ruprecht RM (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 6:200–206

Binley JM, Lybarger EA, Crooks ET, Seaman MS, Gray E, Davis KL, Decker JM, Wycuff D, Harris L, Hawkins N, Wood B, Nathe C, Richman D, Tomaras GD, Bibollet-Ruche F, Robinson JE, Morris L, Shaw GM, Montefiori DC, Mascola JR (2008) Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. J Virol 82:11651–11668

Boyd M, Gustafson K, McMahon J, Shoemaker R, O'Keefe B, Mori T, Gulakowski R, Wu L, Rivera M, Laurencot C, Currens M, Cardellina J, Buckheit R Jr, Nara P Jr, Pannell L, Sowder R Jr, Henderson L (1997) Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. Antimicrob Agents Chemother 41:1521–1530

Burton DR, Weiss RA (2010) A boost for HIV vaccine design. Science 329:770–773

Cardoso RMF, Zwick MB, Stanfield RL, Kunert R, Binley JM, Katinger H, Burton DR, Wilson IA (2005) Broadly neutralizing anti-HIV antibody, 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. Immunity 22:163–173

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219–226

Desai P, Shrivastava N, Padh H (2010) Production of heterologous protein in plants: strategies for optimal expression. Biotechnol Adv 28:427–435

Dey B, Lerner DL, Lusso P, Boyd MR, Elder J, Berger EA (2000) Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. J Virol 74:4562–4569

Dolgin E (2010) Lack of uptake threatens promise of HIV vaccines. Nat Med 16:937

Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P, Stoger E (2005) Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59:869–880

Drake PMW, Barbi T, Sexton A, McGowan E, Stadlmann J, Navarre C, Paul MJ, Ma JKC (2009) Development of rhizosecretion as a production system for recombinant proteins from hydroponic cultivated tobacco. FASEB J 23:3581–3589

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19:1349

Fauci AS, Johnston MI, Dieffenbach CW, Burton DR, Hammer SM, Hoxie JA, Martin M, Overbaugh J, Watkins DI, Mahmoud A, Greene WC (2008) HIV vaccine research: the way forward. Science 321:530–532

Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM (2004) Plant-based production of biophramaceuticals. Curr Opin Plant Biotechnol 7:152–158

Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv 30:434–439

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R, Conrad U (2008) Biochemical and functional characterization of anti-HIV antibody–ELP fusion proteins from transgenic plants. Plant Biotechnol J 6:379–391

Gasser B, Maurer M, Gach J, Kunert R, Mattanovich D (2006) Engineering of *Pichia pastoris* for improved production of antibody fragments. Biotechnol Bioeng 94:353–361

Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422

Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, Forthal DN, Koff WC, Poignard P, Watkins DI, Burton DR (2010) Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. J Virol 84:1302–1313

Hofmann-Lehmann R, Vlasak RJ, Rasmussen BA, Smith TA, Baba VW, Liska F, Ferrantelli D, Montefiori C, McClure HM, Anderson DC, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Katinger H, Stiegler G, Cavacini LA, Posner MR, Chou TC, Andersen J, Ruprecht RM (2001) Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. J Virol 75:7470–7480

Huang J, Dong X, Liu Z, Qin L, Chen YH (2002) A predefined epitope-specific monoclonal antibody recognizes ELDEWA-epitope just presenting on gp41 of HIV-1 O clade. Immunol Lett 84:205–209

Johansen LK, Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. Plant Physiol 126:930–938

Joos B, Trkola A, Kuster H, Aceto L, Fischer M, Stiegler G, Armbruster C, Vcelar B, Katinger H, Günthard FH (2006) Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (MAbs) against human immunodeficiency virus type 1 envelope gp120 (MAb 2G12) and gp41 (MAbs 4E10 and 2F5). J Antimicrob Chemother 50:1773–1779

Khan I, Twyman RM, Arcalis E, Stoger E (2012) Using storage organelles for the accumulation and encapsulation of recombinant proteins. Biotechnol J (online)

Kohli A, Gonzalez-Melendi P, Abranches R, Capell T, Stoger E, Christou P (2006) The quest to understand the basis and mechanisms that control expression of introduced transgenes in crop plants. Plant Signal Behav 1:185–195

Kunert R, Rüker F, Katinger H (1998) Molecular characterization of five neutralizing anti-HIV type 1 antibodies: identification of nonconventional D segments in the human monoclonal antibodies 2G12 and 2F5. AIDS Res Hum Retroviruses 14:1115–1128

Kunert R, Steinfellner W, Purtscher M, Assadian A, Katinger H (2000) Stable recombinant expression of the anti HIV-1 monoclonal antibody 2F5 after IgG3/IgG1 subclass switch in CHO cells. Biotechnol Bioeng 67:97–103

Lombardi R, Villani ME, Di Carli M, Brunetti P, Benvenuto E, Donini M (2010) Optimisation of the purification process of a tumourtargeting antibody produced in N. benthamiana using vacuum agroinfiltration. Transgeni Res 19:1083–1097

Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Manrique A, Rusert P, Joos B, Fischer M, Kuster H, Leemann C, Niederöst B, Weber R, Stiegle G, Katinger H, Günthard HF, Trkola A (2007) In vivo and In vitro escape from neutralizing antibodies 2G12, 2F5 and 4E10. J Virol 16:8793–8808

Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu YC, Robb ML, Katinger H, Birx DL (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol 73:4009–4018

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 6:207–210

Mehandru S, Wrin T, Galovich J, Stiegler G, Vcelar B, Hurley A, Hogan C, Vasan S, Katinger H, Petropoulos CJ, Markowitz M (2004) Neutralization profiles of newy transmitted human immunodeficiency virus type 1 by monoclonal antibodies 2G12, 2F5 and 4E10. J Virol 78:14039–14042

Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler H, Ruker F, Katinger H (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 67:6642–6647

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. PNAS USA 106:7762–7767

Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C, Christou P (2011) High-value products from transgenic maize. Biotechnol Adv 29:40–53

Nicholson L, Gonzalez-Melendi P, van Dolleweerd C, Tuck H, Ma JKC, Fischer R, Christou P, Stoger E (2005) A recombinant multimeric immunoglobulin expressed in rice shows assembly-dependent subcellular localization in endosperm cells. Plant Biotechnol J 3:115–127

Parren PW, Burton DR (2001) The antiviral activity of antibodies in vitro and in vivo. A definitive review on how antibodies inhibit viral infection, HIV-1 infection in particular. Adv Immunol 77:195–262

Peremarti A, Twyman RM, Gómez-Galera S, Naqvi S, Farré G, Sabalza M, Miralpeix B, Dashevskaya S, Yuan D, Ramessar K, Christou P, Zhu C, Bassie L, Capell T (2010) Promoter diversity in multigene transformation. Plant Mol Biol 73:363–378

Platis D, Drossard J, Fischer R, Ma JKC, Labrou NE (2008) New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. J Chromatogr A 1211:80–89

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

Radomsky ML, Whaley KJ, Cone RA, Saltzman WM (1992) Controlled vaginal delivery of antibodies in the mouse. Biol Reprod 47:133–140

Ramessar K, Capell T, Christou P (2008a) Molecular pharming in cereal crops. Phytochem Rev 7:579–592

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma JKC, Stoger E, Capell T, Christou P (2008b) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. PNAS USA 105:3727–3732

Ramessar K, Sabalza M, Capell T, Christou P (2008c) Maize plants: an ideal production platform for effective and safe molecular pharming. Plant Sci 174:409–419

Ramessar K, Sabalza M, Miralpeix B, Capell T, Christou P (2010) Can microbicides turn the tide against HIV? Curr Pharm Des 16:468–485

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoger E, Rademacher T (2007) Functional analysis of the broadly neutralizing human anti- HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J 21:1655–1664

Sainsbury F, Lomonossoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 148:1212–1218

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schillberg S, Zimmermann S, Voss A, Fischer R (1999) Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum*. Transgenic Res 8:255–263

Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock RJ, Ma JKC (2009) Transgenic plant production of Cyanovirin-N, an HIV microbicide. FASEB J 20:356–368

Shattock RJ, Moore JP (2003) Inhibiting sexual transmission of HIV-1 infection. Nat Rev Microbiol 1:a007385

Shattock RJ, Rosenberg Z (2012) Microbicides: topical prevention against HIV. Cold Spring Harb Perspect Med 2:1–17

Shen R, Drelichman ER, Bimczok D, Ochsenbauer C, Kappes JC, Cannon JA, Tudor D, Bomsel M, Smythies LE, Smith PD (2010) GP41-specific antibody blocks cell-free HIV type 1 transcytosis through human rectal mucosa and model colonic epithelium. J Immunol 184:3648–3655

Stiegler G, Armbruster C, Vcelar B, Stoiber H, Kunert R, Michael NL, Jagodzinski LL, Ammann C, Jager W, Jacobson J, Vetter N, Katinger H (2002) Antiviral activity of the neutralizing antibodies 2F5 and 2G12 in asymptomatic HIV-1-infected humans: a phase I evaluation. AIDS 16:2019–2025

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 42:583–590

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R (2002) Practical considerations for pharmaceutical antibody production in different crop systems. Mol Breed 9:149–152

Stoger E, Ma JKC, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, Mach L, Glossl J, Weterings K, Pabst M, Steinkellner H (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J 6:392–402

Torres E, Vaquero C, Nicholson L, Sack M, Stoger E, Drossard J, Christou P, Fischer R, Perrin Y (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. Transgenic Res 8:441–449

UNAIDS (2010) Fact sheet: the global AIDS epidemic. UNAIDS, Geneva

UNAIDS (2011) Report on the global AIDS epidemic, 2011. http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf

Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, Ketas T, Marx PA, Klasse PJ, Burton DR, Moore JP (2003) Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 9:343–346

Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E, Donini M (2008) Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. Plant Biotechnol J 7:59–72

Walker LM, Burton DR (2010) Rational antibody-based HIV-1 vaccine design: current approaches and future directions. Curr Opin Immunol 22:358–366

Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P (2011) Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 477:466–470

Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. PNAS USA 105:18232–18237

Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR, Parre PW (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol 75:10892–10905

ANNEX 3

Sabalza M, Vamvaka E, Christou P and Capell T (2012) Seeds as a production system for molecular pharming applications: status and prospects. Current Pharmaceutical Design. *In press*

SEEDS AS A PRODUCTION SYSTEM FOR MOLECULAR PHARMING APPLICATIONS: STATUS AND PROSPECTS

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Abstract:

The production of recombinant proteins in seeds is achieved by driving transgene expression using promoters and protein targeting sequences derived from genes encoding abundant seed storage proteins. This approach is advantageous because high yields, stability and containment are conferred by the accumulation of recombinant proteins in specialized storage compartments such as protein bodies and protein storage vacuoles. Seeds are particularly suitable for the production of pharmaceutical proteins in developing country settings because they reduce the costs of production and distribution by avoiding the need for fermenter-based production capacity and a cold chain for storage and distribution, thus increasing access to critical medicines for the poor in rural areas. Seeds are also ideal for the production of oral vaccine antigens, because encapsulation within the seed provides protection that prolongs exposure to the gastric immune system and thus increases the potency of the immune response. In this review we discuss the current state of the art in seed-based molecular pharming and the future potential of production platforms based on seeds.

1. Seed-based production platforms:

1.1 The advantages of seeds for the production of recombinant proteins

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 [1], [2], [3]. 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 [1], [4]. 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 [5]. 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 compounds such as phenolics and alkaloids, which are often present in leaves and which interfere with downstream processing by fouling membranes and chromatography media [6]. 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 use of the oil bodies found specifically in oilseeds (e.g. safflower and rapeseed) for economic purification [6].

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 [7] and plant lines can be stored indefinitely and in an easily accessible form simply by storing seeds [2].

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 [8].

1.2 The challenges of seed based systems:

1.2.1 Product quantity and quality

In order to determine whether any production system is economically viable there are two major critical factors: the amount of product that accumulates per unit biomass (productivity, specific yield) and the quality of the final product in terms of homogeneity and functionality. The accumulation of pharmaceutical proteins in seeds

can be enhanced in several ways, e.g. by using promoters derived from the genes encoding abundant seed storage proteins [9], the optimization of codon usage, the use of a KDEL tag to retain proteins in storage compartments derived from the secretory pathway, and the use of other targeting signals derived from storage proteins [10]. The restriction of recombinant proteins to seeds and the control of protein targeting within seeds increases product homogeneity and therefore the overall quality of protein batches produced in seeds.

1.2.2 Purification

More than 80% of the total production costs of a recombinant protein are associated with downstream processing, which tend to be similar across platforms thus reducing the comparative economic advantages of upstream production using plants [11]. Strategies to reduce the downstream production costs of plant-based systems have therefore received considerable attention, and the limited number of competing molecules (proteins and small-molecule metabolites) in seeds means that separation and extraction strategies that purify the recombinant protein are simpler to implement [12]. The development of purification strategies based on oleosin fusions in oil bodies or polymer fusions in other compartments provides a convenient method for the isolation of even recalcitrant proteins [13].

1.3 Production of protein-based medicine in seeds

Many different recombinant proteins have been successfully expressed in seeds, including **therapeutic antibodies** [5], **antimicrobial proteins** [14], **cytokines** [15], **growth factors** [16], **blood products** [17], **dietary factors** [18], **vaccine antigens** [19], [20], [21], structural components such as **collagen** [22], and **industrial enzymes** [23]. Typical seed crops used for molecular pharming include the cereals **rice** [5], **wheat** [5], **barley** [24] and **maize** [25], the legumes **pea** [26], **cowpea** and **soybean** [27], **tobacco** [28], [29], **Arabidopsis** [30] and the oil crops **canola** and **safflower** [31].

M**aize** seeds are the most widely used platform and have been developed commercially in several independent ventures both for pharmaceutical products and industrial proteins [32-53]. Highlights include the production of the heat-labile enterotoxin B subunit

(LTB) as an early example of an oral vaccine candidate [32], the spike protein N-terminal domain from *Transmissible gastroenteritis coronavirus* (TGEV), which was one of the first plant-derived oral vaccines to be tested in animals [35], HIV-neutralizing antibody 2G12 with homogeneous glycans, providing an inexpensive source of a microbicide to prevent the spread of HIV/AIDS [43], [44], gastric lipase, one of the first plant-derived pharmaceuticals in clinical development [47], and β-glucuronidase, which was commercially released as a laboratory reagent [49]. **Rice** seeds have also been widely used [54-71], particularly for the production of dietary proteins such as lactoferrin [56], [57] and lysozyme [57], [58], and pollen antigens such as Japanese cedar pollen Cryj1 and Cryj2 [60] and a mite allergen [70].

Whereas **tobacco** seeds have been used for a small number of experimental products usually on the laboratory scale [72-79], **cowpea** seeds have been extensively used for the production of multivalent vaccine antigen candidates often in combination with Cowpea mosaic virus structural proteins subunits [80-82]. **Soybean** seeds [83-86] have also been used to produce primarily vaccine candidates, including the heat-labile enterotoxin B subunit (LTB) vaccine [83], a nucleocapsid protein from *Porcine reproductive and respiratory syndrome virus* (PRRSV) [85], and full length humanized IgG recognizing herpes simplex virus 2 (HSV-2) [27]. Like tobacco, **Arabidopsis** seeds have been used for mainly experimental products [87-94], whereas **wheat** has been pioneered not only for the production of antibodies, but also heterologous seed storage proteins and the nutrient enhancer phytase [54, 95, 96]. **Safflower** seeds have been used to produce apolipoprotein [97], hirudin [98] and human insulin [99] and have been developed as an alternative to **canola/rapeseed** [87], [99]. **Pea** seeds have been used for the experimental production of bean alpha-amylase inhibitor [100] and carcinoembryonic antigen scFv (CEA) [54]. B**arley** seeds have been used to produce the human growth factor Flt3 ligand [101], collagen [22], HIV-neutralizing antibodies [24] and human lactoferrin [102].

An exhaustive list of molecules produced in seeds for molecular pharming applications is given in Table 1.

2. Plant species that provide seed-based production platforms for molecular pharming applications

2.1 *Arabidopsis thaliana*

Arabidopsis seeds have been used as a production system for recombinant proteins but for experimental purposes rather than commercial exploitation because Arabidopsis seeds are tiny and the biomass is low [103-106]. The advantage of Arabidopsis is its amenability to transformation and regeneration, the short generation cycle and the production of thousands of seeds, which means it can be used to test the efficiency of molecular pharming strategies ahead of deployment in more expensive species [103].

2.2 Tobacco

Tobacco has a long history as both a model system and as a commercial platform for molecular pharming, which makes it the most widely used species for the production of recombinant pharmaceutical proteins in the research laboratory and commercially, but platforms based on tobacco are typically based on leaves or cultured cells [3-5]. Although some recombinant proteins have been produced in tobacco seeds for experimental purposes (e.g. to investigate differences in protein targeting between leafy plants and cereals) the morphology of tobacco seeds is somewhat similar to those of Arabidopsis plants, with the same disadvantages in terms of size and biomass.

2.3 Cereals

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 [107]. Commercial development has focused on three staple cereal crops, i.e. maize, rice and barley, with maize as a clear market leader [1], [4], [108]. Wheat is potentially advantageous as a pharmaceutical production crop because it has a low producer price, but commercial development is hampered by its

low transformation efficiency and the low yields of recombinant proteins that have been achieved [5].

2.3.1 Maize

Maize has many advantages over other plant seeds which make it ideal for the production of recombinant pharmaceutical proteins [108]. Compared to other cereals, maize has a larger grain size and a higher proportion of endosperm, which can occupy up to 82% of the seed [109]. Maize plants have undergone selective breeding for both food and feed production, and have been optimized for increased seed yield. Therefore maize has a high biomass yield per hectare and lower production costs than other major cereals [110]. Unlike other cereals, the use of the C4 photosynthetic pathway in maize increases the efficiency of biomass production. On a technological level, maize benefits from relatively straightforward and efficient in vitro culture, transformation and regeneration protocols as well as a suite of seed-specific promoter systems that can be used to drive transgene expression alone or in combination. Maize was therefore used to produce the first commercially successful plant-derived recombinant proteins, namely avidin and β-glucuronidase marketed by ProdiGene Inc. [39], [111].

One potential drawback of maize is that, unlike the other cereals discussed below, it is a cross-pollinating species, which means the cultivation of pharmaceutical crops without containment can lead to gene flow into non-pharmaceutical crops [112]. Even so, this can be addressed by appropriate cultivation management practices that mitigate gene flow, and which are already widely established to prevent gene flow between GM and conventional food crops. None of the other cereal crops discussed below can presently match the yields of recombinant protein achieved in maize [4].

2.3.2 Rice

Rice shares many of the advantages discussed above for maize, including the high grain yield (albeit lower than maize), 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 crops to non-pharmaceutical 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 [4].

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 bud from the endoplasmic reticulum when sufficient protein has accumulated [69]. 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 [113], [114]. 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 [69]. 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.

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 [14], [58], [107], [115-117].

2.3.3 Barley

Barley is advantageous because it is self-pollinating, the producer price is low, and the seed protein content is high. It is not as widely grown as maize, rice and wheat which means that genetic isolation can be achieved simply by growing the crop in an area where it is not traditionally cultivated. However, techniques for in vitro culture and transformation are not as widely established as in other cereals [4, 117].

The Icelandic company ORF Genetics has adopted barley as their production platform because barley is not grown for food use on Iceland and therefore there is no risk of outcrossing [107]. The company has produced several pharmaceutical proteins in barley seeds, including the marketed products ISOkine™ and DERMOkine™ (human-like growth factors) which are approved for laboratory use and as cosmetic ingredients, but

nevertheless demonstrate that seed-based systems can achieve GMP-like batch-to-batch reproducibility and quality [118].

2.4 Legumes

Leguminous plants are potentially attractive production systems because their seeds have a protein content of 20–40% (compared to 10–12% in cereals) which could favor high yields of recombinant protein if the protein biosynthesis machinery is harnessed efficiently [4]. Only soybean and pea have been explored for their potential in molecular pharming applications, but the lack of straightforward in vitro culture and transformation methods for these species has hampered progress [26, 27]. Apart from a recent study where high yields of a human growth hormone (hGH) were obtained in soybean [84], to date yields of recombinant proteins in legume seeds have been low[119-122]. The higher protein content of legume seeds compared to cereals is also balanced by the lower biomass yields per hectare [4].

2.5 Oilseed crops

Oilseeds are so named because they contain a large amount of stored oils, which accumulate in compartments known as oil bodies that can be exploited to simplify the purification of recombinant proteins. The primary example is the oleosin fusion platform, which has been developed commercially in safflower and rapeseed by the Canadian company SemBioSys Genetics [123], [124]. The recombinant protein is expressed as a fusion with oleosin, an endogenous protein that normally localizes within oil bodies. Therefore, the fusion protein becomes concentrated in the oil bodies, which can be separated from the bulk seed homogenate in the lipid fraction. Treatment of the lipid fraction with an endoprotease then separates the recombinant protein from its fusion partner, allowing recovery in the aqueous phase [123], [124].

As stated above, the olesion fusion platform has been developed primarily in rapeseed and safflower, the latter chosen for commercial development because it is self-pollinating and has a higher protein yield than rapeseed, which is an outcrossing plant. However, both oilseed crops have a lower overall biomass yield compared to cereals [4]. The anti-coagulant hirudin was produced in rapeseed by SemBioSys to demonstrate the principle of the oleosin fusion platform [97], but commercial development has

focused on human insulin produced in safflower (this completed phase I and II clinical trials in 2009) with several other products in the pipeline, although the company is currently struggling to secure further financing [125-127].

3. Future prospects

Seeds provide a number of clear advantages for molecular pharming, particularly the enhanced stability achieved by exploiting a natural storage organ, the large biomass yields that can be achieved using cereals seeds (particularly maize), the ease of purification and the potential to use seeds for oral administration of vaccines, but reducing the cost of downstream processing remains a significant challenge [128]. Downstream purification costs are negligible when seeds are used for oral administration because processing is reduced to simple procedures such as crushing or grinding [78] [117], [129]. Seed-based expression platforms will therefore play a prominent role in the development of future oral vaccine candidates as demonstrated most recently with Japanese cedar pollinosis vaccines [130]. However dosage will need to be monitored carefully because differences in expression levels between batches would affect the amount of ingested recombinant protein. Some form of quality control involving the testing of homogenized seed samples would be required to ensure that doses remain within a certain tolerance.

Downstream purification costs can also be reduced by using seed extracts rather than purified seed-derived proteins. This would be suitable for topical products, such as griffithsin, cyanovirin-N and HIV-neutralizing antibodies, which are envisaged for passive immunization against the spread of HIV. For such applications, the presence of additional plant proteins and metabolites would not present a significant risk because people are routinely exposed to such compounds by contact and in their food [2], [44], [131], [132]. For proteins intended for injection, conventional production including extraction and purification according to GMP would still be required. As well as the economic benefits of minimal processing, the ability to use powdered seeds or seed extracts directly would solve a major current challenge in the distribution and storage of vaccines and other medicines in developing countries, namely the reliance on a cold chain [133].

Another potential benefit of seeds is their ability to produce different recombinant proteins simultaneously, particularly by exploiting different targeting strategies and

storage compartments [see Stoger et al., this issue]. One potential application of this approach is the simultaneous production of different microbicide components in a single formulation, since combination microbicides are more effective than single molecules and help to prevent the evolution of resistant pathogens [134], [135]. The cost of producing each component separately can be prohibitive, but by engineering seeds to produce multiple proteins then the costs of single and multiple formulations would be the same. There are already many examples of transgenic seeds co-expressing different enzymes in the context of metabolic engineering [136], [137], [138], so it only remains to ensure that the yields and stoichiometry of the components can be regulated productively.

Proteins that need to be purified from seeds are often expressed with affinity tags that allow them to be isolated by affinity chromatography, e.g. polyhisitidine tags that can be captured by metal ions and specific epitopes such as FLAG that can be captured with their corresponding antibodies, so that generic purification processes can be used with diverse proteins [1], [139]. However, these affinity tags must be removed before a plant-derived pharmaceutical product can be approved because they may alter its properties compared to the native protein, and this in vitro enzymatic or chemical process reduces the final product yield and introduces additional excipients that must be removed in subsequent purification steps [128]. The oleosin fusion method discussed above avoids some of these challenges by providing a facile way to isolate the (hydrophilic) native protein from the lipid fraction of the seeds following endoprotease cleavage [124]. A similar concept is exploited when proteins are expressed as fusions with an integral membrane-spanning domain derived from the human T-cell receptor, allowing protein extraction in a small volume using appropriate buffers and detergents [140]. Other separation methods have been devised that rely neither on fusions nor on chromatographic separations, e.g. the purification of recombinant collaged from low-pH maize seed extracts by cross-flow filtration, which is possible because of the distinct molecular properties of collagen compared to endogenous endosperm proteins [141].

The many advantages provided by seed-based molecular pharming platforms will not be realized unless the general barriers to the adoption of genetically engineered crops are overcome, and these are political rather than technical [142]. Genetically engineered crops are burdened with onerous regulations that differ widely from country to country,

some of which are stringent but fair, whereas others (particularly within the European Union) are irrational and unnecessarily restrictive [143-145]. The benefits of molecular pharming using seeds would be promoted by the adoption of a rational, science-based and globally harmonious regulatory framework that removes trade barriers and embraces risk/benefit awareness rather than the current precautionary approach focusing on the elimination of all risks [146], [147].

4. Conclusions

The advantages of seeds (and particularly cereal seeds) as a production platform for molecular pharming reflect two key properties, namely their ability to accumulate proteins in a stable environment and maintain them in an inert state (thus making cold chain infrastructure unnecessary), and their suitability for minimal processing in relation to oral and topical application routes (thus making extensive downstream processing unnecessary). Downstream processing accounts for much of the cost of drug manufacturing, whereas the need for a cold chain accounts for much of the cost of drug distribution and makes distribution next to impossible in countries where a cold chain infrastructure is unavailable. By eliminating these requirements, seeds can reduce the costs of drug manufacture and supply by over 90% and enable the distribution of drugs to remote areas without refrigeration, making them ideal for deployment in developing-country settings. Because many rural communities in developing countries rely on subsistence agriculture and have limited access to medicines, the use of staple cereals to provide such medicines could have a powerful impact on the health and wellbeing of the world's poorest people.

In the developed world, seed-based production systems still provide an economical alternative to conventional manufacturing processes, because they are much more scalable than fermenter-based platforms and the seed proteome is simple, which means that innovative purification schemes can be developed to reduce the number of chromatography steps required for purification and therefore reduce the overall costs of production. The production of recombinant pharmaceutical proteins in leafy crops has been achieved under GMP conditions, but processing requires a complex series of filtration and chromatography steps to remove the large number of endogenous plant proteins and metabolites found in leaves. Seeds offer several opportunities to remove

such requirements by simplifying the processing steps, including the isolation of protein bodies and the expression of fusion proteins targeted to oil bodies, essentially performing a biological prefractionation before processing. This indicates that pharmaceutical proteins could be extracted from seeds using streamlined processes with simple steps, suggesting that even proteins intended for injection could eventually be produced inexpensively in developing country settings perhaps using portable and self-contained processing suites.

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References:

[1] Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. Nature Rev Genet 2003;4:794-805.

[2] Twyman RM, Stöger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. Trends Biotechnol 2003;21:570-8.

[3] Fischer R, Stöger E, Schillberg S, Christou P, Twyman RM. Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 2004;7:152-8.

[4] Stöger E, Ma JK, Fischer R, Christou P. Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotech 2005;16:167-73.

[5] Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, *et al*. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 2000;42:583-90.

[6] Moloney M, Boothe J, van Rooijen G. Oil bodies and associated proteins as affinity matrices Canada: SemBioSys Genetics Inc, Calgary 1999: Patent: 5856452:1-17.

[7] Schillberg S, Emans N, Fischer R. Antibody molecular farming in plants and plant cells. Phytochem Rev 2002;1:45-54.

[8] Peters J, Stöger E. Transgenic crops for the production of recombinant vaccines and anti-microbial antibodies. Hum Vaccines 2011;7:367-74.

[9] Potenza C, Aleman L, Sengupta-Gopalan C. Targeting transgene expression in research, agricultural, and environmental applications: Promoters used in plant transformation. In Vitro Cell Dev-Pl 2004;40:1-22.
[10] Petruccelli S, Otegui MS, Lareu F, Tran Dinh O, Fitchette A-C, Circosta A, *et al*. A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnol J 2006;4:511-27.
[11] Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE. Considerations for the recovery of recombinant proteins from plants. Biotechnol Progr 2004;20:1001-14.
[12] Baez J, Russell D, Craig J, Cass PL. Corn seed production of therapeutic proteins moves forward. Biopharm Int 2000;13:50-4.
[13] Daniell H, Streatfield SJ, Wycoff K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 2001;6:219-26.
[14] Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, Bartley G, Huang N, Lonnerdal B. Expression of human lactoferrin in transgenic rice grains for the application in infant formula. Plant Sci 2002;163:713-22.
[15] Zhu Z, Hughes KW, Huang L, Sun B, Liu C, Li Y. Expression of human α-interferon cDNA in transgenic rice plants. Plant Cell Tiss Org 1994;36:197-204.
[16] Huang N, Yang D. Production of human growth factors in monocot seeds. USA, 2004: Patent: 20040078851.
[17] Dieryck W, Pagnier J, Poyart C, Marden MC, Gruber V, Bournat P, *et al*. Human haemoglobin from transgenic tobacco. Nature 1997;386:29-30.
[18] Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, *et al*. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 2000;287:303-5.
[19] Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, *et al*. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. P Natl Acad Sci USA 2007;104:10986-91.
[20] Streatfield SJ, Howard JA. Plant-based vaccines. Int J Parasitol 2003;33:479-93.
[21] Wu J, Yu L, Li L, Hu J, Zhou J, Zhou X. Oral immunization with transgenic rice seeds expressing VP2 protein of infectious bursal disease virus induces protective immune responses in chickens. Plant Biotechnol J 2007;5:570-8.
[22] Eskelin K, Ritala A, Suntio T, Blumer S, Holkeri H, Wahlström EH, *et al*. Production of a recombinant full-length collagen type I α-1 and of a 45-kDa collagen type I α-1 fragment in barley seeds. Plant Biotechnol J 2009;7:657-72.
[23] Hood EE, Love R, Lane J, Bray J, Clough R, Pappu K, *et al*. Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. Plant Biotechnol J 2007;5:709-19.
[24] Schünmann PHD, Coia G, Waterhouse PM. Biopharming the SimpliRED™ HIV diagnostic reagent in barley, potato and tobacco. Mol Breeding 2002;9:113-21.
[25] Hood EE, Woodard SL, Horn ME. Monoclonal antibody manufacturing in transgenic plants--myths and realities. Curr Opin Biotech 2002;13:630-5.
[26] Perrin Y, Vaquero C, Gerrard I, Sack M, Drossard J, Stöger E, *et al*. Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. Mol Breeding 2000;6:345-52.
[27] Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, *et al*. A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nat Biotechnol 1998;16:1361-4.
[28] Tackaberry ES, Dudani AK, Prior F, Tocchi M, Sardana R, Altosaar I, *et al*. Development of biopharmaceuticals in plant expression systems: cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. Vaccine 1999;17:3020-9.

[29] Scheller J, Leps M, Conrad U. Forcing single-chain variable fragment production in tobacco seeds by fusion to elastin-like polypeptides. Plant Biotechnol J 2006;4:243-9.
[30] Nykiforuk CL, Boothe JG, Murray EW, Keon RG, Goren HJ, Markley NA, *et al*. Transgenic expression and recovery of biologically active recombinant human insulin from Arabidopsis thaliana seeds. Plant Biotechnol J 2006;4:77-85.
[31] Szarka S, van Rooijen G, Moloney M. Methods for the production of multimeric immunoglobulins, and related compositions. SemBioSys Genetics Inc 2006: Patent: 7098383.
[32] Tacket CO, Pasetti MF, Edelman R, Howard JA, Streatfield S. Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 2004;22:4385-9.
[33] Monsanto Protein Technology and NeoRx Corporation. [Accessed online 27 August 2012]; Available at: http://www.mpt.monsanto.com/ and http://www.neorx.com/.
[34] Samyn-Petit B, Gruber V, Flahaut C, Wajda-Dubos JP, Farrer S, Pons A, *et al*. N-glycosylation potential of maize: The human lactoferrin used as a model. Glycoconjugate J 2001;18:519-27.
[35] Gomez N, Wigdorovitz A, Castanon S, Gil F, Ordas R, Borca MV, *et al*. Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. Arch Virol 2000;145:1725-32.
[36] Petolino JF, Young S, Hopkins N, Sukhapinda K, Woosley A, Hayes C, *et al*. Expression of murine adenosine deaminase (ADA) in transgenic maize. Transgenic Res 2000;9:1-9.
[37] Zhong G-Y, Peterson D, Delaney DE, Bailey M, Witcher DR, James C Register III, *et al*. Commercial production of aprotinin in transgenic maize seeds. Mol Breeding 1999;5:345-56.
[38] Gruber V, Berna PP, Arnaud T, Bournat P, Clément C, Mison D, *et al*. Large-scale production of a therapeutic protein in transgenic tobacco plants: effect of subcellular targeting on quality of a recombinant dog gastric lipase. Mol Breeding 2001;7:329-40.
[39] Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, *et al*. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breeding 1997;3 (4):291-306.
[40] Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, Lane JR, *et al*. Maize (Zea mays)-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. Biotechnol Appl Bioc 2003;38:123-30.
[41] Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, *et al*. Plant-based vaccines: unique advantages. Vaccine 2001;19:2742-8.
[42] Lamphear BJ, Barker DK, Brooks CA, Delaney DE, Lane JR, Beifuss K, *et al*. Expression of the sweet protein brazzein in maize for production of a new commercial sweetener. Plant Biotechnol J 2005;3:103-14.
[43] Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, *et al*. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 2008;6:189-201.
[44] Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, *et al*. Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. P Natl Acad Sci USA 2008;105:3727-32.
[45] Hayden CA, Streatfield SJ, Lamphear BJ, Fake GM, Keener TK, Walker JH, *et al*. Bioencapsulation of the hepatitis B surface antigen and its use as an effective oral immunogen. Vaccine 2012;30:2937-42.
[46] Srivastava HS, Asthana JS, Jain A. Induction of Nitrate Reductase and Peroxidase Activity in the Seedlings of Normal and High Lysine Maize. Physiol Plantarum 1979;47:119-23.
[47] Zhong Q, Gu Z, Glatz CE. Extraction of Recombinant Dog Gastric Lipase from Transgenic Corn Seed. J Agr Food Chem 2006;54:8086-92.

[48] EPlcyte [Accessed online 27 August 2012]; Available at: http://www.epicyte.com.
[49] Kusnadi AR, Evangelista RL, Hood EE, Howard JA, Nikolov ZL. Processing of transgenic corn seed and its effect on the recovery of recombinant beta-glucuronidase. Biotechnol Bioeng 1998;60:44-52.
[50] Guerrero-Andrade O, Loza-Rubio E, Olivera-Flores T, Fehérvári-Bone T, Gómez-Lim MA. Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. Transgenic Res 2006;15:455-63.
[51] Ludwig DL, Witte L, Hicklin DJ, Prewett M, Bassi R, Burtrum D, *et al*. Conservation of receptor antagonist anti-tumor activity by epidermal growth factor receptor antibody expressed in transgenic corn seed. Hum Antibodies 2004;13:81-90.
[52] Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, *et al*. Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 2005;59:869-80.
[53] Chen R, Xue G, Chen P, Yao B, Yang W, Ma Q, *et al*. Transgenic maize plants expressing a fungal phytase gene. Transgenic Res 2008;17:633-43.
[54] Stöger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, *et al*. Practical considerations for pharmaceutical antibody production in different crop systems. Mol Breeding 2002;9:149-58.
[55] Xie T, Qiu Q, Zhang W, Ning T, Yang W, Zheng C, *et al*. A biologically active rhIGF-1 fusion accumulated in transgenic rice seeds can reduce blood glucose in diabetic mice via oral delivery. Peptides 2008;29:1862-70.
[56] Rachmawati D, Mori T, Hosaka T, Takaiwa F, Inoue E, Anzai H. Production and characterization of recombinant human lactoferrin in transgenic Javanica rice. Breeding Sci 2005;55:213-22.
[57] Huang N. High-level protein expression system uses self-pollinating crops as hosts. Bioprocess Int 2004;2:54-9.
[58] Yang D, Guo F, Liu B, Huang N, Watkins S. Expression and localization of human lysozyme in the endosperm of transgenic rice. Planta 2003;216:597-603.
[59] Oszvald M, Kang TJ, Tomoskozi S, Tamas C, Tamas L, Kim TG, *et al*. Expression of a synthetic neutralizing epitope of porcine epidemic diarrhea virus fused with synthetic B subunit of Escherichia coli heat labile enterotoxin in rice endosperm. Mol Biotechnol 2007;35:215-23.
[60] Takaiwa F, Takagi H, Hirose S, Wakasa Y. Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice. Plant Biotechnol J 2007;5:84-92.
[61] Alli Z, Sardana RK, Pierre B, Andonov A, Robert LS, Schernthaner JP, *et al*. Pharming vaccines for hepatitis and cytomegalovirus: Towards the development of multivalent and subunit vaccines for oral delivery of antigens. Phytochemistry Rev 2002;1:55-66.
[62] Wakasa Y, Zhao H, Hirose S, Yamauchi D, Yamada Y, Yang L, *et al*. Antihypertensive activity of transgenic rice seed containing an 18-repeat novokinin peptide localized in the nucleolus of endosperm cells. Plant Biotechnol J 2011;9:729-35.
[63] Qian-feng L, Qiao-quan L, Da-jiang Z, Hong-mei W, Heng-xiu Y, Ming-hong G, *et al*. Expression of Recombinant Phytase in Transgenic Rice. Chinese J Rice Sci 2006;20:243-7.
[64] Capell T, Claparols I, Del Duca S, Bassie L, Miro B, Rodriguez-Montesinos J, *et al*. Producing transglutaminases by molecular farming in plants: Minireview article. Amino Acids 2004;26:419-23.
[65] Zhang D, Nandi S, Bryan P, Pettit S, Nguyen D, Santos MA, *et al*. Expression, purification, and characterization of recombinant human transferrin from rice (Oryza sativa L.). Protein Expres Purif 2010;74:69-79.
[66] Sardana R, Dudani AK, Tackaberry E, Alli Z, Porter S, Rowlandson K, *et al*. Biologically active human GM-CSF produced in the seeds of transgenic rice plants. Transgenic Res 2007;16:713-21.

[67] Nicholson L, Gonzalez-Melendi P, van Dolleweerd C, Tuck H, Perrin Y, Ma JK, *et al*. A recombinant multimeric immunoglobulin expressed in rice shows assembly-dependent subcellular localization in endosperm cells. Plant Biotechnol J 2005;3:115-27.

[68] Qiao-quan L, Qian-feng L, Li J, Da-jiang Z, Hong-mei W, Ming-hong G, *et al*. Transgenic expression of the recombinant phytase in rice (Oryza sativa). IRRN 2007;32:44.

[69] Takaiwa F, Hirose S, Takagi H, Yang L, Wakasa Y. Deposition of a recombinant peptide in ER-derived protein bodies by retention with cysteine-rich prolamins in transgenic rice seed. Planta 2009;229:1147-58.

[70] Suzuki K, Kaminuma O, Yang L, Takai T, Mori A, Umezu-Goto M, *et al*. Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression. Plant Biotechnol J 2011;9:982-90.

[71] Shigemitsu T, Ozaki S, Saito Y, Kuroda M, Morita S, Satoh S, *et al*. Production of human growth hormone in transgenic rice seeds: co-introduction of RNA interference cassette for suppressing the gene expression of endogenous storage proteins. Plant Cell Rep 2012;31:539-49.

[72] Wright KE, Prior F, Sardana R, Altosaar I, Dudani AK, Ganz PR, *et al*. Sorting of glycoprotein B from human cytomegalovirus to protein storage vesicles in seeds of transgenic tobacco. Transgenic Res 2001;10:177-81.

[73] Leite A, Kemper EL, da Silva MJ, Luchessi AD, Siloto RMP, Bonaccorsi ED, *et al*. Expression of correctly processed human growth hormone in seeds of transgenic tobacco plants. Mol Breeding 2000;6:47-53.

[74] Altenbach SB, Pearson KW, Meeker G, Staraci LC, Sun SM. Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. Plant Mol Biol 1989;13:513-22.

[75] Petruccelli S, Otegui MS, Lareu F, Tran Dinh O, Fitchette AC, Circosta A, *et al*. A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnol J 2006;4:511-27.

[76] Sardana R, Ganz PR, Dudani A,Tackaberry ES, Cheng X, Altosaar I. Synthesis of Recombinant Human Cytokine GM-CSF in the Seeds of Transgenic Tobacco Plants. In: Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds 1997;77-87.

[77] Fiedler U, Conrad U. High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. Biotechnology (NY) 1995;13:1090-3.

[78] Floss DM, Conrad U. Expression of Complete Antibodies in Transgenic Plants. In: Antibody Engineering 2010;489-502.

[79] Lombardi R, Donini M, Villani ME, Brunetti P, Fujiyama K, Kajiura H, *et al*. Production of different glycosylation variants of the tumour-targeting mAb H10 in Nicotiana benthamiana: influence on expression yield and antibody degradation. Transgenic Res 2012:1-17.

[80] Yasawardene SG, Lomonossoff GP, Ramasamy R. Expression & immunogenicity of malaria merozoite peptides displayed on the small coat protein of chimaeric cowpea mosaic virus. Indian J Med Res 2003;118:115-24.

[81] Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gómez N, *et al*. Protective Immune Response to Foot-and-Mouth Disease Virus with VP1 Expressed in Transgenic Plants. J Virol 1998;72:1688-90.

[82] Canizares MC, Lomonossoff GP, Nicholson L. Development of cowpea mosaic virus-based vectors for the production of vaccines in plants. Expert Rev Vaccines 2005;4:687-97.

[83] Moravec T, Schmidt MA, Herman EM, Woodford-Thomas T. Production of Escherichia coli heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine 2007;25:1647-57.
[84] Cunha N, Murad A, Cipriano T, Araújo A, Aragão F, Leite A, *et al*. Expression of functional recombinant human growth hormone in transgenic soybean seeds. Transgenic Res 2011;20:811-26.
[85] Vimolmangkang S, Gasic K, Soria-Guerra R, Rosales-Mendoza S, Moreno-Fierros L, Korban SS. Expression of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus in soybean seed yields an immunogenic antigenic protein. Planta 2012;235:513-22.
[86] Yamada Y, Nishizawa K, Yokoo M, Zhao H, Onishi K, Teraishi M, *et al*. Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin. Peptides 2008;29:331-7.
[87] Vandekerckhove J, van Damme J, van Lijsebettens M, Botterman J, De Block M, Vandewiele M, *et al*. Enkephalins Prouduced in Transgenic Plants Using Modified 2S Seed Storage Proteins. Nat Biotechnol 1989;7:929-32.
[88] Lau OS, Ng DW, Chan WW, Chang SP, Sun SS. Production of the 42-kDa fragment of Plasmodium falciparum merozoite surface protein 1, a leading malaria vaccine antigen, in Arabidopsis thaliana seeds. Plant Biotechnol J 2010;8:994-1004.
[89] Loos A, van Droogenbroeck B, Hillmer S, Grass J, Kunert R, Cao J, *et al*. Production of monoclonal antibodies with a controlled N-glycosylation pattern in seeds of Arabidopsis thaliana. Plant Biotechnol J 2011;9:179-92.
[90] Markley N, Nykiforuk C, Boothe J, Moloney M. Producing Proteins Using Transgenic Oilbody-Oleosin Technology. BioPharm Int 2006;19:34.
[91] van Droogenbroeck B, Cao J, Stadlmann J, Altmann F, Colanesi S, Hillmer S, *et al*. Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. P Natl Acad Sci 2007;104:1430-5.
[92] Wang B, Ma M, Wu T. Aptamers improve the expression of a human granulocyte-macrophage colony stimulating factor in transgenic Arabidopsis thaliana seeds. J Plant Biol 2007;50:29-37.
[93] Sullivan S, Ralet MC, Berger A, Diatloff E, Bischoff V, Gonneau M, *et al*. CESA5 is required for the synthesis of cellulose with a role in structuring the adherent mucilage of Arabidopsis seeds. Plant Physiol 2011;156:1725-39.
[94] Downing WL, Galpin JD, Clemens S, Lauzon SM, Samuels AL, Pidkowich MS, *et al*. Synthesis of enzymatically active human α-l-iduronidase in Arabidopsis cgl (complex glycan-deficient) seeds. Plant Biotechnol J 2006;4:169-81.
[95] Stöger E, Parker M, Christou P, Casey R. Pea legumin overexpressed in wheat endosperm assembles into an ordered paracrystalline matrix. Plant Physiol 2001;125:1732-42.
[96] Brinch-Pedersen H, Olesen A, Rasmussen SK, Holm PB. Generation of transgenic wheat (Triticum aestivum L.) for constitutive accumulation of an Aspergillus phytase. Mol Breeding 2000;6:195-206.
[97] Nykiforuk CL, Shen Y, Murray EW, Boothe JG, Busseuil D, Rheaume E, *et al*. Expression and recovery of biologically active recombinant Apolipoprotein AI(Milano) from transgenic safflower (Carthamus tinctorius) seeds. Plant Biotechnol J 2011;9:250-63.
[98] Boothe JG, Saponja JA, Parmenter DL. Molecular farming in plants: Oilseeds as vehicles for the production of pharmaceutical proteins. Drug Develop Res 1997;42:172-81.
[99] Boothe JG, Nykiforuk CL, Kuhlman PA, Whelan H, Pollock WBR, Clark S, *et al*. Analytical characterization, safety and clinical bioequivalence of recombinant human insulin from transgenic plants. American Diabetes Association, 69th Scientific Sessions 2009: Abstract 5-LB.

[100] Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, *et al*. Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. J Agr Food Chem 2005;53:9023-30.
[101] Erlendsson LS, Muench MO, Hellman U, Hrafnkelsdottir SM, Jonsson A, Balmer Y, *et al*. Barley as a green factory for the production of functional Flt3 ligand. Biotechnology J 2010;5:163-71.
[102] Stefanova G, Vlahova M, Atanassov A. Production of recombinant human lactoferrin from transgenic plants. Biol Plantarum 2008;52:423-8.
[103] TAIR (The Arabidopsis Information Resource) 2012; available at http://www.arabidopsis.org/ (accessed online 1 September 2012)
[104] Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Gorlach J. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell 2001; 13: 1499–1510.
[105] Ruebelt MC, Lipp M, Reynolds TL, Astwood JD, Engel KH, Jany KD. Application of two dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 2. Assessing natural variability. J Agric Food Chem 2006; 54: 2162–68.
[106] Demeyer R, De Loose M, Van Bockstaele E, Van Droogenbroeck B. Exploiting the natural variation of Arabidopsis thaliana for the seed-specific production of proteins. Euphytica 2012; 183: 83-93.
[107] Ramessar K, Capell T, Christou P. Molecular pharming in cereal crops. Phytochem Rev 2008; 7:579-92.
[108] Ramessar K, Sabalza M, Capell T, Christou P. An ideal production platform for effective and safe molecular pharming. Plant Science 2008; 174: 409-19.
[109] Watson SA. in: S.A. Watson, P.T. Ramstad (Eds.) Corn: Chemistry and Technology. Am. Assoc. Cereal Chemists, St. Paul, MN; 1987: 53–82.
[110] Giddings G, Allison G, Brooks D, Carter A. Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 2000; 18: 1151–55.
[111] Witcher DR, Hood EE, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh R. Commercial production of b-glucuronidase (GUS): a model system for the production of proteins in plants. Mol Breed 1998; 4: 301-412.
[112] Commandeur U, Twyman RM, Fischer R. The biosafety of molecular farming in plants. AgBiotechNet 2003; 5: ABN110.
[113] Yamagata H, Tanaka K. The site of synthesis and accumulation of rice storage proteins. Plant Cell Physiol 1986; 27: 135–45.
[114] Müntz K. Deposition of storage proteins. Plant Mol Biol 1998; 38: 77-99.
[115] Yang D, Wu L, Hwang YS, Chen L, Huang N. Expression of the REB transcriptional activator in rice grains improves the yield of recombinant proteins whose genes are controlled by a Reb-responsive promoter. Proc Natl Acad Sci USA (PNAS) 2001; 98: 11438-43.
[116] Hennegan K, Yang D, Nguyen D, Wu L, Goding J, Huang J, Guo F, Huang N, Watkins SC. Improvement of human lysozyme expression in transgenic rice grain by combining wheat (Triticum aestivum) puroindoline b and rice (Oryza sativa) Gt1 promoters and signal peptides. Transgenic Res 2005; 14: 583-92.
[117] Lau OS, Sun SSM. Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 2009; 27: 015–22.
[118] Faye L, Gomord V. Success stories in molecular farming—a brief overview. Plant Biotechnol J 2010; 8: 525–28.
[119] Philip R, Darnowski DW, Maughan PJ, Vodkin LO. Processing and localization of bovine b-casein expressed in transgenic soybean seeds under control of a soybean lectin expression cassette. Plant Science 2001; 161: 323–35.
[120] Moravec T, Schmidt MA, Herman EM, Woodford-Thomas T. Production of Escherichia coli heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine 2007; 25: 1647–57.

[121] Cunha NB, Murad A, Ramos GL, Maranhão AQ, Brígido MM, Cláudia A, Araújo G, Lacorte C, Aragão FJL, Covas DT, Fontes AM, Souza GHMF, Vianna GR, Rech EL. Accumulation of functional recombinant human coagulation factor IX in transgenic soybean seeds. Transgenic Res 2011; 20: 841–55.
[121] Saalbach I, Giersberg M, Conrad U. High-level expression of a single-chain Fv fragment (scFv) antibody in transgenic pea seeds. J Plant Physiol 2001; 158: 529–33.
[122] Mikschofsky H, Broer I. Feasibility of Pisum sativum as an expression system for pharmaceuticals. Transgenic Res 2011; DOI 10.1007/s11248-011-9573-z.
[123] Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM. Seed-based expression systems for plant molecular farming. Plant Biotechnol J 2010; 8: 588–606.
[124] Van Rooijen GJH, Moloney MM. Plant seed oil-bodies as carriers for foreign proteins. Nat Biotechnol 1995; 13: 72–77.
[125] Paul M, Ma JKC. Plant-made pharmaceuticals: Leading products and production platforms. Biotechnol Appl Biochem 2011; 58: 58–67.
[126] Spök A, Twyman RM, Fischer R, Ma JKC, Sparrow PAC. Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. Trends Biotechnol 2008; 26: 506–17.
[127] SemBioSys Genetics Inc. 2010; available at http://micro.newswire.ca/36078-0.html?Start=15 (Accessed online 1 September 2012)
[128] Wilken LR, Nikolov ZL. Recovery and purification of plant-made recombinant proteins. Biotechnol Adv 2012; 30: 419–33.
[139] Mason HS, Warzecha H, Mor T, Arntzen CJ. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol Med 2002; 8: 324–29.
[130] Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H, Takaiwa F. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. Proc Natl Acad Sci USA (PNAS) 2005; 102: 17525–30.
[131] Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock, RJ, Ma JK. Transgenic plant production of Cyanovirin-N, an HIV microbicide. FASEB J 2009; 20: 356-58.
[132] O'Keefe BR, Vojdani F, Buffa V, Shattock RJ, Montefiori DC, Bakke J, Mirsalis J, d'Andrea A, Hume SD, Bratcher B, Saucedo CJ, McMahon JB, Pogue GP, Palmer KE. Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. Proc Natl Acad Sci USA (PNAS) 2009; 106: 6099-104.
[133] Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C, Christou P. High-value products from transgenic maize. Biotechnol Adv 2011; 29: 40–53.
[134] Ramessar K, Sabalza M, Miralpeix B, Capell T, Christou P. Can microbicides turn the tide against HIV? Curr Pharm Design 2010; 4: 468-85.
[135] Shattock RJ, Rosenberg Z. Microbicides: Topical Prevention against HIV. Cold Spring Harbor Perspectives Medicine 2012; doi: 10.1101/cshperspect.a007385.
[136] Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C, Christou P. When more is better: multigene engineering in plants. Trends Plant Sci 2009; 15: 48-56.
[137] Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P. Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA (PNAS) 2009; 108: 1-6.
[138] Zhu C, Naqvi S, Breitenbach J, Sandman G, Christou P, Capell T. Combinatorial Genetic Transformation of Cereals and the Creation of Metabolic Libraries for the Carotenoid Pathway. Proc Natl Acad Sci USA (PNAS) 2008; 105: 18232-37.
[139] Chen Q. Expression and purification of pharmaceutical proteins in plants. Biol Engineering 2008; 2: 291–321.

[140] Schillberg S, Zimmermann S, Findlay K, Fischer R. Plasma membrane display of anti-viral single chain Fv fragments confers resistance to tobacco mosaic virus. Mol Breed 2000; 6: 317–26.
[141] Aspelund MT, Glatz CE. Purification of recombinant plant-made proteins from corn extracts by ultrafiltration. J Membr Sci 2010; 353: 103–10.
[142] Farre G, Twyman RM, Zhu C, Capell T, Christou P. Nutritionally enhanced crops and food security: scientific achievements versus political expediency. Curr Opi Biotechnol 2011; 22: 245–51.
[143] Ramessar K, Capell T, Twyman RM, Quemada H, Christou P. Trace and traceability - a call for regulatory harmony. Nat Biotehnol 2008; 26: 975-78.
[144] Ramessar K, Capell T, Twyman RM, Christou P. Going to ridiculous lengths - European coexistence regulations for GM crops. Nat Biotechnol 2010; 28: 133-36.
[145] Sabalza M, Miralpeix B, Twyman RM, Capell T, Christou P. EU legitimizes GM crop exclusion zones. Nat Biotechnol 2011; 29: 315-17.
[146] Twyman RM, Ramessar K, Quemada H, Capell T, Christou P. Plant biotechnology: the importance of being accurate. Trends Biotechnol 2009; 27: 609-12.
[147] Ramessar K, Capell T, Twyman RM, Quemada H, Christou P. Calling the tunes on transgenic crops: the case for regulatory harmony. Mol Breed 2009; 23: 99-112.

TABLES

Table 1: Molecules produced in seed-based platforms for molecular pharming applications.

Species (seeds)	***Molecules***	***Category***	***References***
Maize	Heat-labile enterotoxin B subunit (LTB)	Subunit vaccine	[32]
	Avicidin	Antibody	[33]
	Human lactoferrin	Glycoprotein	[34]
	Transmissible gastroenteritis coronavirus (TGEV) N-terminal domain of the spike glycoprotein	Vaccine antigen	[35]
	Adenosine deaminase	Enzyme	[36]
	Aprotinin	Proteinase inhibitor	[37]
	Pancreatic lipase	Pancreatic enzyme	[38]
	Avidin	Glycoprotein	[39]
	Trypsinogen (Trypsin)	Pancreatic enzyme	[40]
	Transmissible gastroenteritis virus/pigs (TGEV)- glycoprotein S	Vaccine	[41]
	Sweet brazzein	Sweet-tasting protein	[42]
	Recombinant antibody 2G12 contains predominantly single-GlcNAc N-glycans.	Antibody	[43], [44]

	Hepatitis B surface antigen	Detectable viral antigen	[45]
	Nitrate reductase and peroxidase	Industrial enzyme	[46]
	Laccase	Industrial enzyme	[53]
	Cellulase	Industrial enzyme	[23]
	Gastric lipase	Industrial enzymes	[47]
	Respiratory syncytial virus (RSV), IgG(Ab form)	Antibody	[48]
	Sperm (antigen) IgG(Ab form)	Antigen	[48]
	β-glucuronidase	Enzyme	[49]
	Newcastle Disease virus (NDV)-poultry, wild birds	Antigen	[50]
	Anti/EGFR antibody C225	Antibody	[51]
	Aspergillus phytase + ferritin	Enzyme + Protein	[52]
	Aspergillus phytase	Enzyme	[53]
Rice	Carcinoembryonic antigen scFv (CEA)	Antigen	[54]
	Insuline-like growth factor-1	Hormone	[55]
	Human lactoferrin	Glycoprotein	[56], [57]
	Lysozyme	Enzyme	[57], [58]
	Human a-interferon	Protein	[15]
	Porcine epidemic diarrhea virus (PEDV)	Vaccine	[59]
	Polypeptide consisting of 7 dominant human T-cell epitopes derived from the Japanese cedar pollen allergens, Cry j 1 and Cry j 2	Peptide for immunotherapy	[60]
	VP2 protein of infectious bursal disease virus (IBDV)/chicken	Antigen	[21]
	Hepatitis B virus (HBV) core particles	Antigen	[61]
	Novokinin (Arg-Pro-Leu-Lys-Pro-Trp, RPLKPW) plus KDEL	Hypotensive peptide	[62]
	Phytase	Enzyme	[63]
	Transglutaminases	Enzymes	[64]
	Cholera toxin B subunit	Mucosal vaccine	[19]

	Transferrin	Glycoprotein	[65]
	Herpes simplex virus	Antibody	[27]
	Provitamin A	Vitamin	[18]
	Recombinant human cytokine GM-CSF	Cytokine	[66]
	Antibody for Streptococcus mutans surface antigen I/II	Antibody	[67]
	Aspergillus niger phytase	Enzyme	[68]
	7Crp peptide	Peptide	[69]
	Mite allergene	Allergene	[70]
	Human growth hormone	Hormone	[71]
Tobacco	Glycoprotein B of human cytomegalovirus	Glycoprotein	[72]
	Human growth hormone	Hormone	[73]
	Human haemoglobin (a- and β-globin)	Blood substitute	[17]
	Methionine	Protein-based amino acid	[74]
	14D9 antibody	Antibody	[75]
	Recombinant human cytokine GM-CSF	Cytokine	[76]
	Carcinoembryonic antigen scFv (CEA)	Antigen	[77]
	Human monoclonal antibody 2G12	Antibody	[78]
	Tumor-targeting mAb H10	Antibody	[79]
Cowpea	Canine parvovirus VP2 epitope	Vaccine antigen	[80]
	Foot and mouth disease virus VP1 epitope	Vaccine antigen	[81]
	Human rhinovirus (type 14) VP1 epitope	Vaccine antigen	[82]
	Pseudomonas aeruginosa membrane protein F epitope	Vaccine antigen	[82]
	Mink enteritis virus VP2 epitope	Vaccine antigen	[82]
	Staphylococcus aureus D2 epitope of fibronectin-binding protein	Vaccine antigen	[82]

Soybean	Heat-labile enterotoxin B subunit (LTB)	Subunit vaccine	[83]
	Herpes simplex virus	Antibody	[27]
	Human growth hormone	Hormone	[84]
	Nucleocapsid protein of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)	Immunogenic antigenic protein	[85]
	Full length humanized IgG recognizing herpes simplex virus 2 (HSV-2)	Antibody	[27]
	Novokinin (Arg-Pro-Leu-Lys-Pro-Trp)	Hypotensive peptide	[86]
Arabidopsis	Human enkephalins	Antihyperanalgesic peptide	[87]
	42 k-Da fragment of P.falciparum merozoite surface protein 1	Vaccine antigen	[88]
	Recombinant antibody 2G12 (anti-HIV monoclonal antibody)	Antibody	[89]
	HA78 against Hepatitis A virus	Antibody	[89]
	Insulin	Hormone	[90], [30]
	Carcinoembryonic antigen scFv (CEA)	Antigen	[91]
	Human granulocyte-macrophage colony stimulating factor	Hormone	[92]
	Cellulose	Industrial enzyme	[93]
	Human α-L-iduronidase	Enzyme	[94]
Wheat	Carcinoembryonic antigen scFv (CEA)	Antigen	[54]
	Pea legumin	Storage protein	[95]
	Aspergillus phytase	Enzyme	[96]
Safflower	Apolipoprotein AI Milano (ApoAIMilano)	Protein	[97]
	Hirudin	Peptide-Anticlotting agent	[98]
	Chymosin	Enzyme	unpublished
	Human insulin	Hormone	[99]

Canola	Human enkephalins	Antihyperanalgesic peptide	[87]
	Hirudin	Peptide-Anticlotting agent	[99]
Pea	Bean alpha-amylase inhibitor	Enzyme inhibitor	[100]
	Carcinoembryonic antigen scFv (CEA)	Antigen	[54]
Barley	Human growth factor Flt3 ligand	Growth factor	[101]
	Collagen	Protein	[22]
	HIV antibodies	Antibodies	[24]
	Human lactoferrin	Glycoprotein	[102]

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