An Investigation into Improved HIV-1 Subtype C Envelope Based Vaccine Design.

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ABBREVIATIONS

Ad5	**Adenovirus serotype 5**
AIDS	**Acquired Immunodeficiency Syndrome**
ART	**Antiretroviral therapy**
CD4bs	**CD4 binding site**
CTL	**Cytotoxic T lymphocyte**
DMEM	**Dulbecco's Modified Eagle Medium**
DNA	**Deoxyribonucleic acid**
ER	**Endoplasmic reticulum**
gp	**Glycoprotein**
HIV	**Human Immunodeficiency Virus**
L	**Litre**
M	**Molar**
Mg	**Milligram**
ml	**Millilitre**
MPER	**Membrane-proximal external region**
Ng	**Nanogram**
Nm	**Nanometer**
Ori	**Origin of replication**

PCR	**Polymerase chain reaction**
PTGS	**Post transcriptional gene silencing**
SIV	**Simin Immunodeficiency Virus**
TSP	**Total soluble protein**
VLP	**Virus-like particle**
^{o}C	**Degrees celcius**

ABSTRACT

Sub-Saharan Africa is disproportionately affected by the HIV-1 pandemic and whilst antiretroviral therapy and social interventions are having a positive impact, a successful vaccine remains humanity's best hope to combat the virus. This study served to develop a heterologous DNA prime-protein boost vaccine combination by exploiting existing genetic immunization and plant expression platforms developed by our research group. An antigenically promising HIV-1 envelope, with known sensitivity to prototype broadly neutralizing monoclonal antibodies, was selected for development into 3 vaccine immunogens. The first antigen, gp150, comprised of a truncated derivative of the full length envelope protein designed to enhance expression and the exposure of antibody sensitive epitopes. The remaining 2 antigens, gp120-HA_2 and gp140-HA_2tr, comprised of chimeric HIV envelope antigens fused to portions of the influenza haemagglutinin (HA) transmembrane subunit.

DNA vaccines encoding each of the immunogens were constructed using a plasmid which exploits a porcine circovirus type 1 enhancer element to drive high levels of antigen expression. The expression and localization of each of the antigens was verified following transfection of HEK 293 cells. High levels of expression of all three immunogens were observed by both western blotting and immunostaining of HEK 293 cells transfected with the DNA vaccines. The parental envelope from which the vaccines were designed exhibited poor expression, possibly due to either the expression vector or intrinsic properties of the envelope gene itself. The gp150 and gp140-HA_2tr vaccine antigens displayed a diffuse subcellular localization pattern and the proteins were evident both intracellularly and at the cell periphery, similar to the HIV-1 Du151 envelope antigen encoded by the SAAVI-C2 DNA vaccine. Interestingly, the chimeric immunogen containing the influenza haemagglutinin HA_2 subunit translationally fused to HIV-1 gp120 (gp120-HA_2) appeared to be localized to the cell periphery, suggesting that this approach may enhance surface density of the glycoprotein.

Recombinant strains of *Agrobacterium tumefaciens* were generated for each immunogen and antigen expression optimized in plants, using a systematic approach to evaluate multiple variables known to influence expression and recovery of heterologous proteins. The most successful approach involved infiltrating high densities of the bacterial culture into plant leaves and extracting protein directly from the apoplast. The co-expression of a post-transcriptional gene silencing suppressor also had a profound influence on expression, enabling the duration and magnitude of expression to be improved. The use of denaturing conditions to extract protein proved successful for gp140-HA_2tr antigen only but this approach was deemed impractical as it would preclude the recovery of intact protein. Variation in antigen expression was seen when experiments were repeated highlighting the influence of plant development on recombinant protein expression. At best low level expression of the recombinant antigens were achieved in plants that was insufficient for further characterization or immunization studies.

Future studies of antigen expression in plants will likely benefit from the optimization of the gene coding sequences of the immunogens for both codon usage and GC content, as well as exploring different subcellular localizations for the accumulation of the proteins. The promising *in vivo* data generated for the DNA vaccines warrants further evaluation and immunogenicity studies are being planned in rabbits to evaluate the capacity of the immunogens to induce neutralizing antibodies.

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CONTENTS

CHAPTER 1: INTRODUCTION

1.1. Origin and diversity of the Human Immunodeficiency Virus (HIV)

HIV, a retrovirus of simian origin, is the etiological agent responsible for the global Acquired Immunodeficiency Syndrome (AIDS) pandemic (13, 99). The origin of the pandemic has been traced to multiple zoonotic transmissions of Simian Immunodeficiency Virus (SIV), from African primates to humans, in west and central Africa (371). Although the earliest documented sample of HIV dates back to 1959, it is suspected that the virus first entered humans in the early 20th century, most likely from the butchering of nonhuman primates during hunting (402, 420).

The virus is broadly classified into 2 lineages (HIV-1 and HIV-2) which are distinct in terms of both pathogenicity and geographical distribution (217). HIV-1, the more pathogenic of the 2 types, is globally distributed, whereas HIV-2 is largely confined to west Africa (213, 217, 271). The HIV-1 lineage comprises of 4 sub-lineages denoted as groups M, N, O and P, each of which arose through independent zoonotic events (**Figure 1.1**) (119, 162, 269, 329, 366, 367). Groups M and N are believed to have been derived from SIV_{CPZ}, a simian virus endemic to chimpanzees (*Pan troglodytes troglodytes*) in Southern Cameroon (100, 162). In contrast, groups O and P appear more closely related to an SIV strain occurring in gorillas (*Gorilla gorilla gorilla*) that inhabit western Cameroon (119, 269).

Whilst group M accounts for the majority of HIV-1 infections worldwide, the other HIV-1 lineages only make a minor contribution to the global pandemic. Group M is estimated to be responsible for approximately 33 million infections globally (135). In contrast group O infections are largely confined to Cameroon and account for less than 1 percent of the global pandemic (119, 424). Reports of group N and P infections have been rare and thus far only 13 group N and 2 group P infections have been

confirmed (269, 366, 367). Group M, the pandemic group, is further divided into 9 subtypes (A-D, F-H, J and K) and a myriad of circulating recombinant forms arising from genetic recombination in co-infected individuals (314). The pandemic is highly dynamic and each of these subtypes and recombinants differs in terms of both geographical distribution and prevalence. Notably subtype C is the predominating strain in Sub-Saharan Africa, including South Africa, and India and accounts for almost half of the worldwide HIV-1 infections (128, 137).

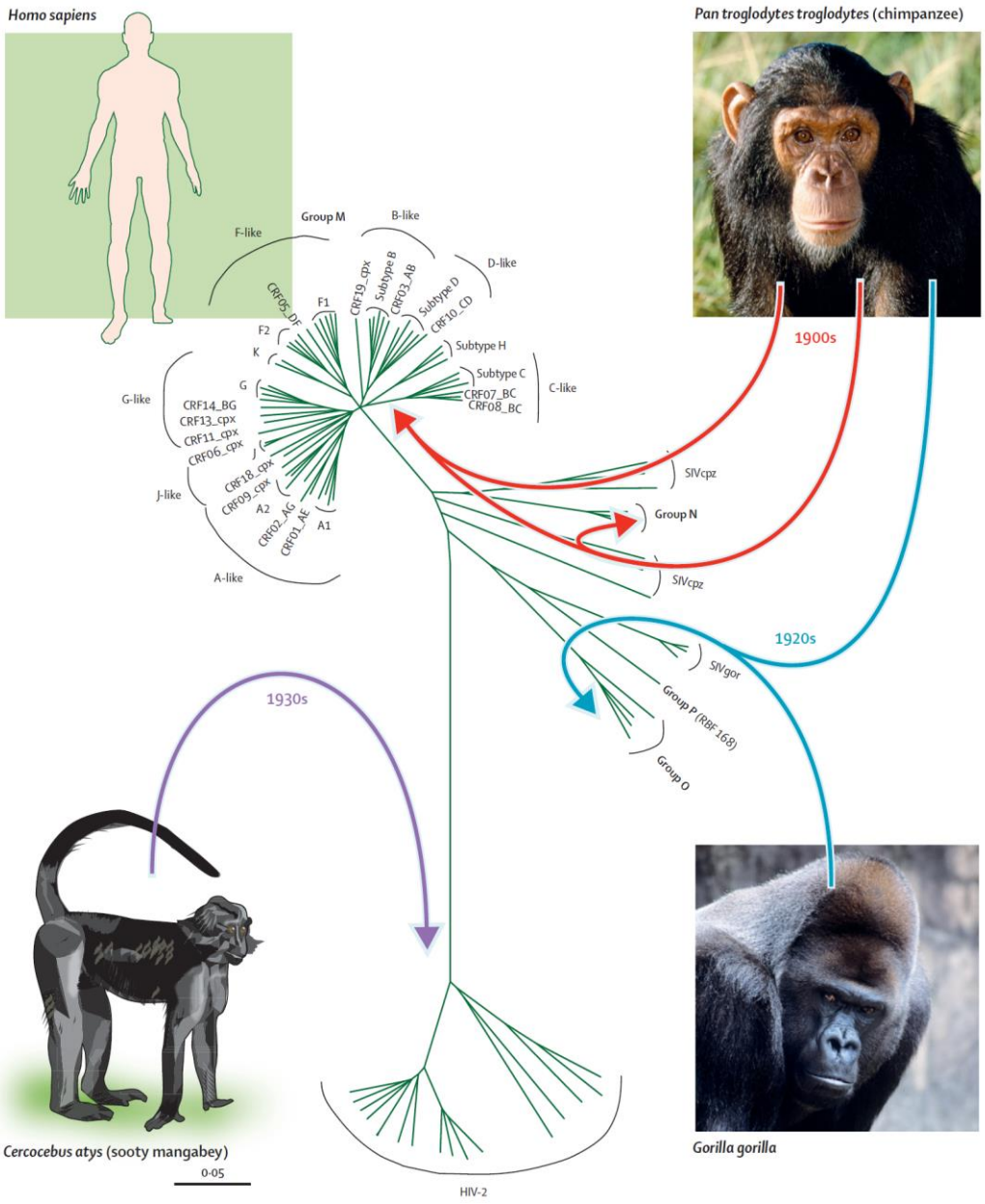

Figure 1.1: Phylogenetic relationships between circulating HIV-1 and HIV-2 isolates and the progenitor viruses from which they evolved (355).

The HIV-2 lineage is believed to have originated from SIV_{SM}, with the Sooty Mangabey implicated as the reservoir host (101, 141). HIV-2 is defined by 8 groups (A-H) and is estimated to cause less than 1 million infections, globally (355). Only groups A and B appear to have spread appreciably, with the other groups confined to single individuals (327). It is noteworthy that in recent years HIV-2 has exhibited a noticeable trend towards decreasing prevalence (185, 273, 328).

1.2. Gravity of the HIV pandemic in the context of Sub-Saharan Africa

The global dissemination of HIV-1 has culminated in an epidemic of unparalleled proportions. The virus has been implicated in over 60 million worldwide infections, of which 25 million have proven fatal. It is estimated that in the year 2012 alone, HIV-1 was responsible for 1.9 million new infections in middle and low income countries alone, with 1.6 million AIDS related deaths (423). The epidemic also has far reaching social and economic implications with low to middle income countries being disproportionately affected in the global context (314).

The severity of the epidemic and the failures of current intervention strategies to effectively combat the virus are particularly evident in Sub-Saharan Africa (**Figure 1.2**). The region remains the most heavily effected in the context of the global pandemic and accounted for 68% of all infected people in 2010, despite representing only 12% of the global population. Sub-Saharan Africa was responsible for an alarming 70% of new infections in 2010 and 22.9 million of the estimated 34 million people living with the virus resided in this region. The same year bore witness to 1.2 million AIDS related deaths in sub-Saharan Africa alone (363). Unsurprisingly, Swaziland, the country with the highest adult HIV prevalence (25.9%) and South Africa, the country with the highest number of people living with HIV-1 (5.6 million), are both located in Sub-Saharan Africa. Globally, the virus has orphaned 16.6 million children, almost 90% of whom live in sub-Saharan Africa (364).

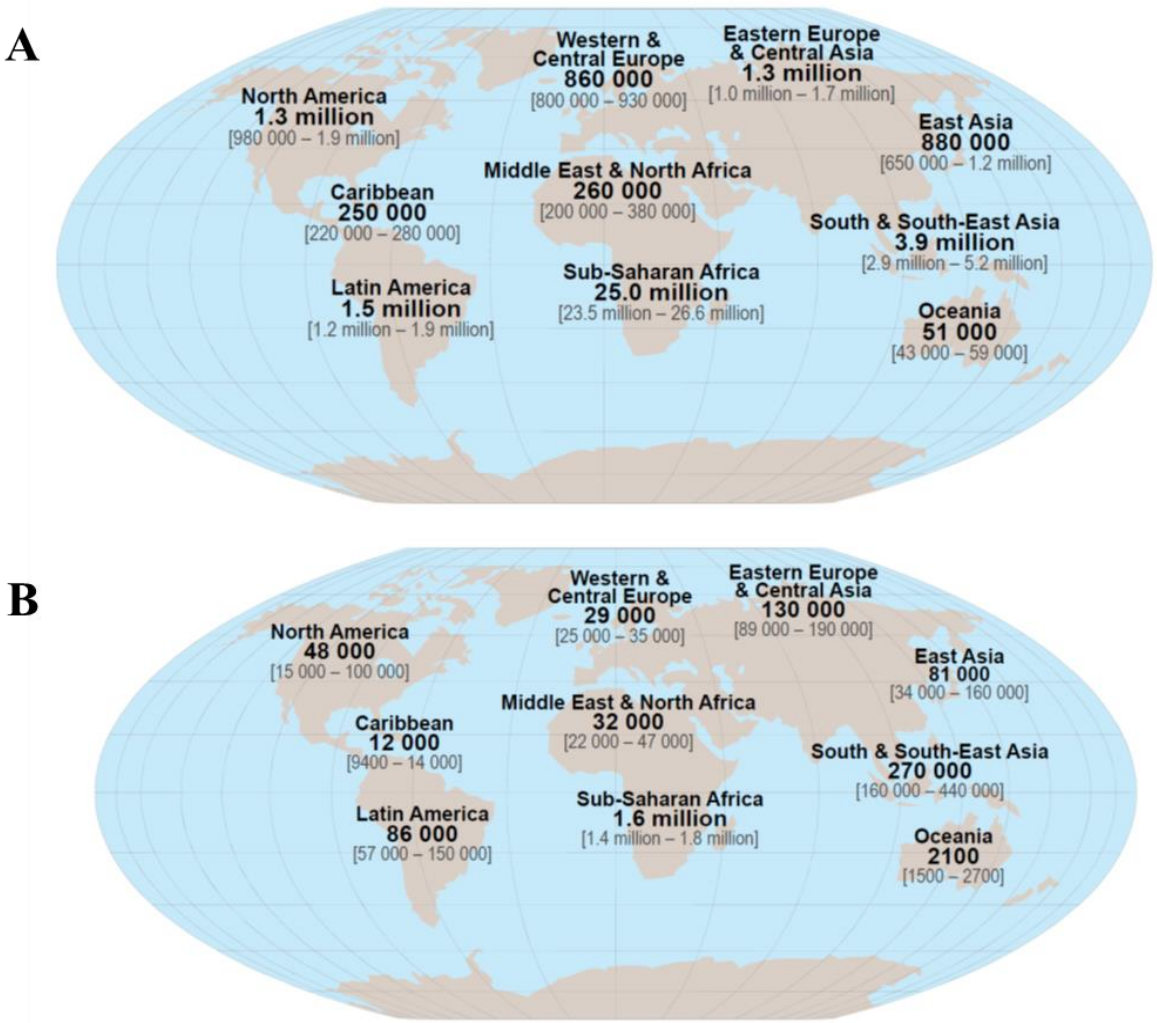

Figure 1.2: Estimates of the global HIV-1 prevalence (A) and incidence (B) in 2012 highlighting the disproportionate role of Sub-Saharan Africa in the global HIV-1 epidemic (423).

The HIV epidemic also has far reaching economic ramifications and estimates suggest that the annual HIV related expenditure in low to middle income countries exceeded US$ 4.1 billion in 2008 (364). It is estimated that the annual cost of achieving universal access to antiretroviral therapy (ART) and care will fall within the region of US$22 billion (309). However, the economic impact of HIV is not merely confined to HIV-associated costs but also extends to the concomitant loss of labour supply and reduction in productivity (77, 330).

Although a marked 30% decrease in the global incidence of HIV-1 has been witnessed since 2001, these figures underestimate the gravity of the epidemic (**Figure 1.3**) (423). In spite of the promising impact of antiretroviral therapy and the implementation of wide scale behavioural changes, current social interventions alone

are unlikely to eradicate the virus. In the light of the gravity of the global epidemic and the far reaching social, economic and medical ramifications it imposes, a successful vaccine is urgently needed to curb the relentless spread of the virus.

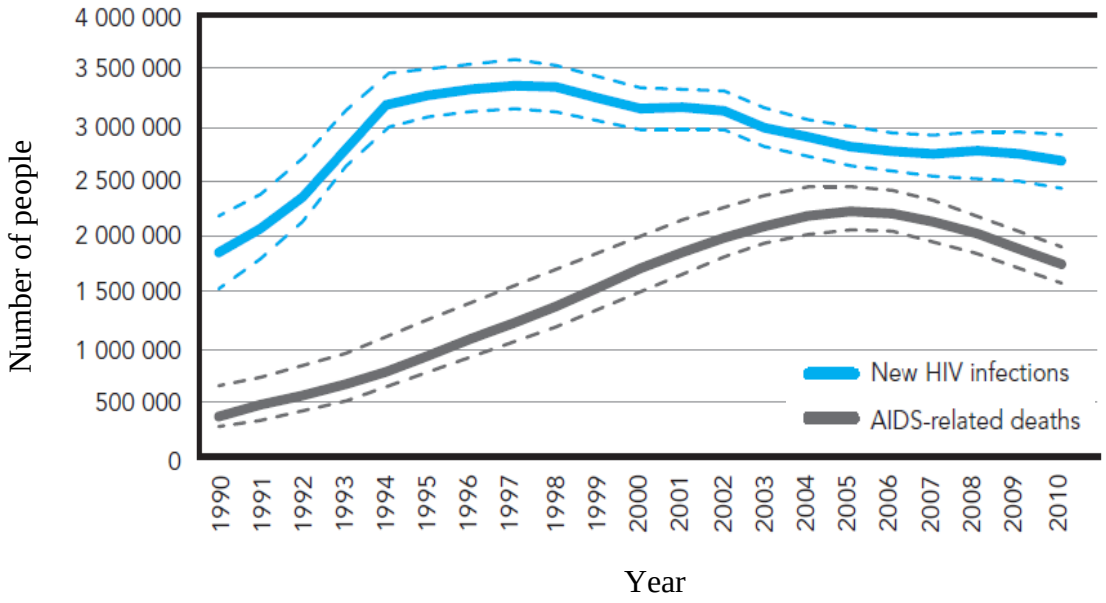

Figure 1.3: Global estimates of new HIV-1 infections and AIDS related deaths from 1990-2010 (363).

1.3. Synthesis and Structure of the HIV envelope Glycoprotein Spike

The surface-bound envelope glycoprotein of HIV-1 is the target of all known antibodies capable of mediating cross-clade neutralization of the virus and is therefore of considerable interest to immunogen design (178). The structure of the glycoprotein will be briefly reviewed in the text to follow in order to provide background to the vaccine antigens designed in this study. The HIV-1 envelope glycoprotein encodes an approximately 845-870 amino acid glycoprotein product that mediates entry into target cells (65, 408). Following translation, the gp160 precursor is subjected to N-linked glycosylation as it passes through the endoplasmic reticulum (ER) and proteolytic cleavage by furin proteases in the Golgi apparatus (76, 124, 340, 391). The envelope protein oligomerizes as it translocates through the ER to form trimeric complexes, although other oligomers have also been reported (81, 391). The transmembrane (gp41) and surface (gp120) envelope subunits remain non-covalently

attached to form heterodimers, which remain closely associated in membrane-bound glycoprotein complexes that populate the surface of the virion (82, 168, 391, 419).

The gp41 subunit protrudes through the virion membrane to form a compact mushroom shaped base of the glycoprotein trimer from which the surface unit, gp120, extends (**Figure 1.4**) (393). The transmembrane subunit is divided into 3 major structural regions; a long cytoplasmic tail extending into the virion core, a transmembrane domain anchoring the transmembrane glycoprotein in the virion lipid bilayer and an extracellular domain which protrudes to the exterior of the virion (168, 392). The extracellulular domain contains crucial functional elements involved in entry and fusion of the virus with target cells. These include the hydrophobic N-terminal fusion peptide, two disulphide-linked alpha helical coiled coils (Heptad Repeats 1 and 2) and a tryptophan-rich region referred to as the membrane-proximal external region (MPER) (29, 48, 94, 296, 352).

The surface envelope subunit is composed of 5 hypervariable regions, four of which form disulphide bonded loops, interspersed with 5 conserved regions (187, 338, 396). The core of gp120 comprises of an inner domain, an outer domain and an intermediate bridging sheet (146, 175). The inner domain is comparatively more conserved and devoid of glycans, unlike the outer domain which exhibits extensive variability and glycosylation (407). The first and second variable loops (V1/V2) are positioned at the apex of the glycoprotein trimer, in proximity to the point of contact between the virus and host cell (393). The third variable loop (V3) is in close proximity to the V1 and V2 regions with its tip pointing towards the trimer axis (156, 206).

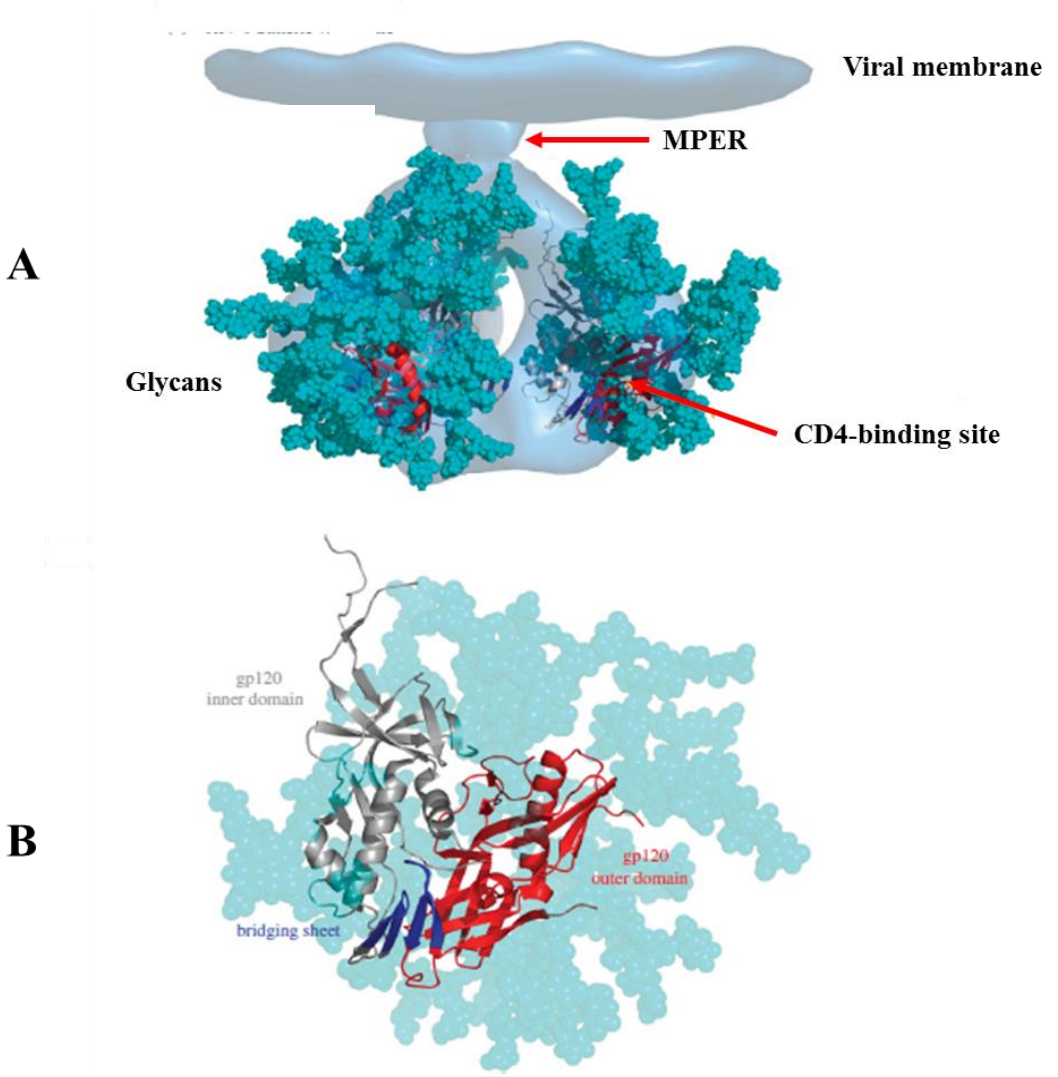

Figure 1.4: Structure of the HIV-1 envelope glycoprotein in its trimeric (A) and monomeric (B) forms (adapted from Nabel *et al*. (241)). The crystal structure of the envelope glycoprotein has been overlayed with the density plot of the glycoprotein spike as predicted by cryoelectron tomography. N-linked glycosylation is represented in cyan and the peptide backbone depicted using ribbon diagrams. The relative positions of the inner domain, outer domain and bridging sheet are indicated in gray, blue and red respectively.

1.4. The Neutralizing antibody response during natural HIV-1 infection

HIV-1 infection is associated with a robust humoral response which fails to prevent the inevitable onset of AIDS defining illness (283). The earliest detectable humoral response following infection takes the form of antigen-antibody complexes which are present approximately 8 days following the detection of plasma viremia (**Figure 1.5**). After a further delay; free serum binding antibodies targeting gp41 appear, followed

not long after by gp120 binding antibodies (358). Most individuals develop neutralizing antibodies to the autologous virus within several months of infection (1, 113, 186, 230, 280, 390). However, these antibodies are type specific and fail to neutralize heterologous primary viral isolates (113, 228, 230, 280). Antibodies targeting the V3 loop comprise a significant proportion of the early antibody response to gp120 and can be detected as early as 3 weeks, following the transmission event (69, 70, 231, 358). Yet despite developing high titers and demonstrating considerable cross reactivity, these antibodies do not contribute to autologous neutralization (69, 70, 231, 358). This is presumably due to the occlusion of neutralization sensitive epitopes in the context of the functional glycoprotein trimer (69, 70, 169, 170). The autologous neutralizing antibody response largely targets the hypervariable loops of the virus, particularly the V1/V2 regions, with V4 and V5 appearing to play a less prominent role (231, 234, 288). Consistent with these findings, experimental SHIV infection of rhesus macaques has been shown to be associated with autologous antibodies targeting the V1/V2 region of the envelope protein (179). In subtype C infection an epitope encompassing the C3-V4 region is commonly targeted by autologous neutralizing antibodies, but this appears to be subtype specific and has not been observed in infection with other clades (231). The detection of frequent escape mutations in the alpha-2 helix of C3 is further evidence of the selective pressure imposed on this region by the antibody response of the infected host (234, 287). Antibodies directed at epitopes induced by CD4 binding are often elicited prior to autologous neutralizing antibodies, but are of negligible functional significance (113). MPER-specific binding antibodies are less common in natural infection during the first year but when elicited also make no discernible contribution to autologous neutralization (113). Antibodies targeting the CD4-binding site usually develop within the first 16 weeks of infection but are usually of weak affinity and low potency (205).

Although the autologous neutralizing antibody response is usually of a high titer, it fails to successfully mediate clearance of the virus. Instead the antibody response imposes strong selective pressure on the virus and drives the evolution of the viral envelope (32, 234, 280). Interestingly, it has been demonstrated that the antibody response to HIV-1 infection, during the first year, is usually only directed at 1 or 2 epitopes of the envelope glycoprotein (234). The virus easily escapes antibody mediated immune pressure as highlighted by the inability of plasma to successfully

neutralize contemporaneous virus, despite neutralizing virus from earlier time points (32, 71, 230, 234, 288, 390). Escape from neutralizing antibodies is achieved through multiple mechanisms including single amino acid substitutions, insertions, deletions, expansion of variable loops, changes in N-linked glycosylation and conformational masking of vulnerable epitopes (32, 96, 176, 234, 288, 390). The development of antibodies with cross-neutralizing activity against heterologous primary isolates usually only develops later in infection (71, 228, 230). Such antibodies are typically evident after about 2.5 years of infection, but may be detected as early as 1 year in rare instances (228).

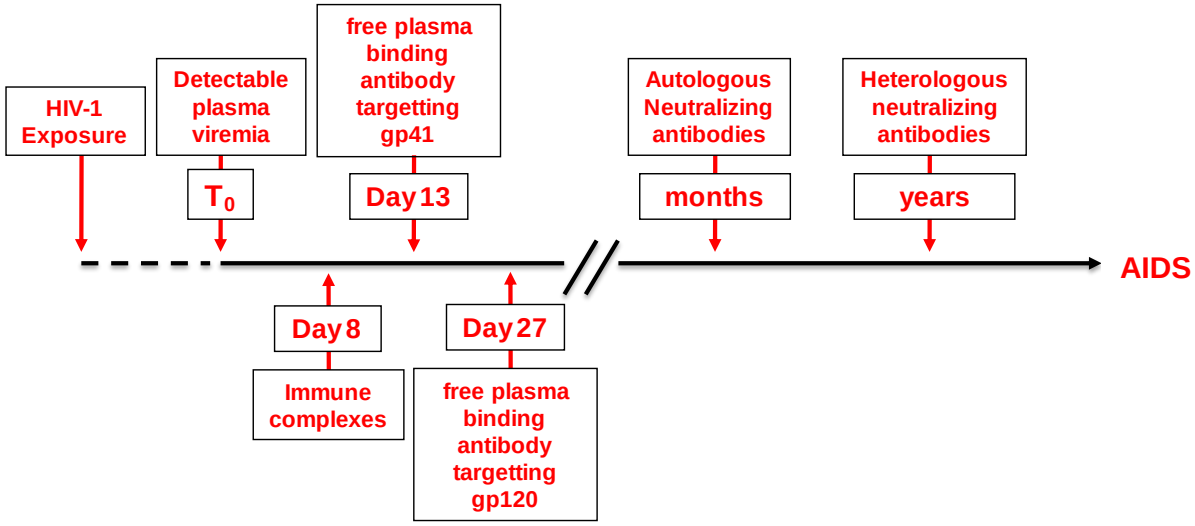

Figure 1.5: Development of the antibody response to HIV-1 after infection (358)**.**

1.5. Broadly neutralizing HIV-1 antibodies and sites of vulnerability on the HIV-1 envelope glycoprotein

Despite the perceived paucity of antibodies capable of mediating broad neutralization of heterologous primary isolates, it has become apparent that a subset of HIV-1 infected individuals develop antibodies with considerable cross-neutralizing activity (177). A series of studies have demonstrated that these responses occur in about 20% of infected individuals, but are only detectable late in infection and are of little clinical significance (25, 80, 193, 302, 337, 380). In the last several years a milieu of broadly neutralizing monoclonal antibodies have been isolated from HIV-1 infected individuals delineating 4 distinct sites of vulnerability present on the HIV-1 envelope

glycoprotein (**Figure 1.6**) (177). These include the CD4 binding site, the MPER of gp41 and glycan dependent epitopes in the V1/V2 and V3 regions of the HIV-1 envelope (193, 194, 379, 380, 422). Passive transfer experiments with these antibodies have demonstrated their protective efficacy against SHIV challenge, supporting the notion that if similar responses could be elicited by vaccination they would be protective (138, 139, 318).

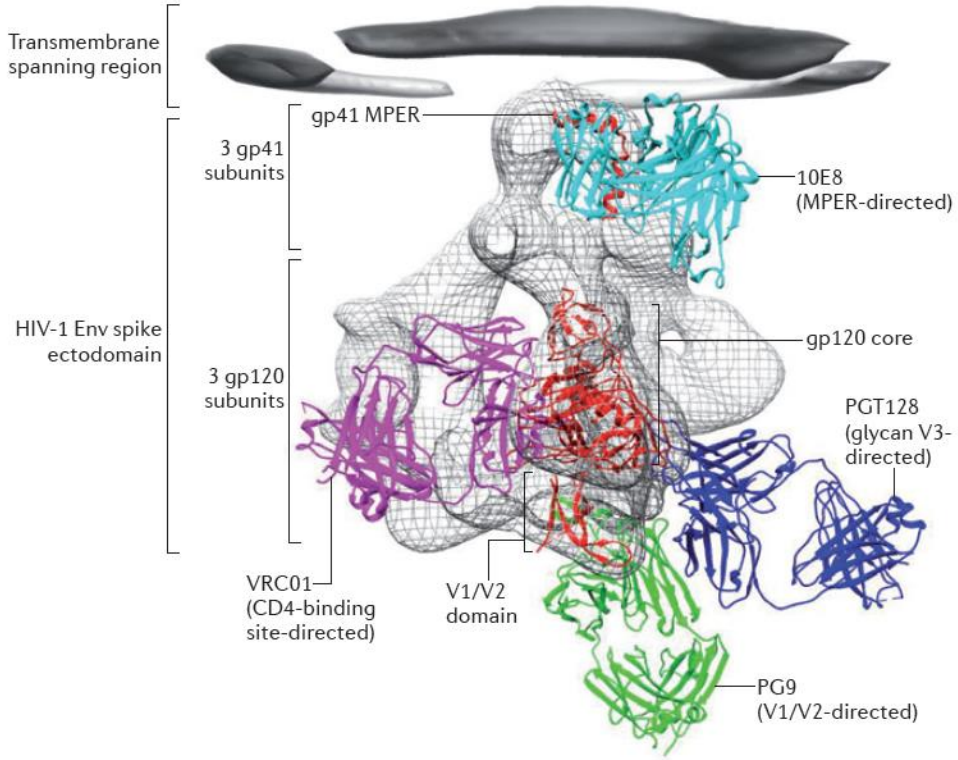

Figure 1.6: Regions of vulnerability of the HIV-1 envelope targeted by broadly neutralizing antibodies (adapted from Kwong *et al*., 2013) (178)**.** A model of the envelope glycoprotein, rendered by cryo-electron microscopy, is depicted with prototype broadly neutralizing antibodies that target each of the main regions of vulnerability.

1.5.1. The CD4 binding site (CD4bs)

The CD4-binding site is responsible for initiating entry into permissive target cells and therefore antibodies with the potential to block this interaction are likely to be protective (175). Several highly potent broadly neutralizing antibodies have been isolated that recognize the functionally conserved CD4 binding site including the likes of; VRC01, VRC-CH31, VRC-PG04, 3BNC117, 12A12 and PGV04 (84, 303, 404, 405). Several other antibodies that target the CD4 binding site with considerably less

breadth and potency have also been identified (37, 58). It has become apparent that the differences in neutralization breadth and potency between these groups of antibodies appears to be related to their modes of recognition (51). Antibodies, such as VRC01, achieve neutralization by partial mimicry of the CD4 receptor interaction whereby they target the initial site of CD4 attachment (404, 418). Deep sequence analysis has revealed that this is in fact a common mode of recognition for the development of broadly neutralizing CD4-binding site antibodies, achieved by convergent evolution in unrelated donors (303, 405).

Lynch and colleagues have reported that the CD4-binding site is immunogenic in natural infection and that 88% of seroconverters from a longitudinal study developed antibodies recognizing this target (205). However, broadly cross-neutralizing antibodies targeting the CD4-binding site are associated with unusually high levels of affinity maturation which poses a formidable challenge to their induction by vaccination (405, 418). Although at this venture it is unclear how these antibodies can be elicited by vaccination, it may be necessary to guide the evolution of the antibody response by mimicking the complex interplay between the virus and the immune system (197).

1.5.2 The Membrane-Proximal External region of gp41 (MPER)

Amongst the earliest broadly neutralizing antibodies identified, the gp41-specific 2F5 and 4E10 monoclonal antibodies defined a distinct region of vulnerability in the MPER region of the envelope glycoprotein (41, 240, 249). Other gp41-specific neutralizing antibodies have also been isolated but with considerably less potency and breadth, such as the CAP206-CH12, Z13 and m66 monoclonal antibodies (236, 421, 422). These MPER-specific broadly neutralizing antibodies recognize a linear epitope in gp41 which is considered an attractive vaccine target (41, 229, 240, 249). However, the MPER is poorly immunogenic and MPER-specific antibodies are often polyreactive preventing their induction via vaccination due to immunological tolerance mechanisms (131, 229). More recently a novel antibody, 10E8, was isolated and demonstrated to have a greater neutralization breadth than other previously

isolated MPER-specific antibodies. Additionally, 10E8 was not found to be autoreactive and antibodies of a similar specificity were reported in 8% of a 72 person cohort of HIV-1 infected donors (147).

1.5.3. V1/V2 glycans

The identification of antibodies PG9 and PG16, from the activated memory B cells derived from a clade A donor in 2009, defined a new site of envelope vulnerability in the V1/V2 region (379). Subsequent studies have resulted in the isolation of similar antibodies, such as PGT141-145 and CH01-4 (26, 377). This class of antibodies all recognizes a quaternary epitope that is preferentially expressed on trimeric envelope and is dependant on the glycan attached to the asparagine at amino acid 160 (N160) (26, 220, 377). Interestingly, a similar antibody (2909) has been identified that recognizes an epitope involving lysine at residue 160, instead of a glycan (403). This antibody, although similar to PG9 and PG16, is strain specific due to the conservation of asparagine at residue 160 (49, 403). Similar antibody specificities have also been reported in rhesus macaques after experimental SHIV infection (284). Broadly neutralizing antibodies targeting epitopes involving the glycan at N160 are commonly responsible for neutralization breadth in infected individuals who display cross neutralizing activity (380).

1.5.4. V3 glycans

A second class of glycan dependent antibodies was identified by the monoclonal antibody 2G12 which recognizes a cluster of glycans proximal to the V3 loop (299, 359). Although 2G12 was initially believed to represent a unique competition group, a number of broadly neutralizing antibodies of overlapping specificities were recently identified from Protocol G donors including; PGT121-123, PGT125-128, PGT130, PGT131 and PGT135-138 (299, 377). The majority of these antibodies all recognized epitopes involving glycans at N301 or N332 (377). Delineation of the crystal structures of the antigen binding fragments of PGT127 and PGT128 with synthetic glycan ligands have revealed that their epitopes encompass 2 glycans and a β-strand region of the V3 loop (259). It has been demonstrated that this unique class of

antibodies often displays diverse modes of interaction with their epitopes, although the key N301 and N332 glycans remain central for recognition (253). A recent study by Walker and colleagues revealed that broadly neutralizing antibodies targeting the N332 epitope was the most common specificity in a cohort of 19 donors selected for their potency of cross-clade serum neutralizing activity (380). Experimental SHIV infection in Rhesus monkeys has also been associated with the development of cross-clade neutralizing antibodies dependant on the N332 glycan (381). A recent study has suggested that these antibody responses target escape variants and that the N332 glycan is often not present on the transmitted founder virus, undermining the efficacy of these responses should they be elicited by vaccination (233).

1.6. Vaccine concepts evaluated in efficacy trials

Despite over three decades of scientific research since the discovery of HIV, only 4 vaccine concepts have completed evaluation in efficacy trials (30, 93, 115, 125, 267, 279). Whilst these trials have only demonstrated modest efficacy at best, they have played an important role in guiding future vaccine development and have highlighted the nature of immunological responses that may confer protection (132, 164, 279). Even efficacy trials that have failed to demonstrate any overt vaccine efficacy have proved enlightening in retrospect and can be viewed as a dynamic part of the vaccine development process (30, 93, 115, 267). Notably, these studies have highlighted shortcomings of nonhuman primate models and the limitations of *in vitro* assays to faithfully predict *in vivo* vaccine efficacy (30, 115, 388). The outcome of these studies has drawn attention to the potential impact of pre-existing immunity to viral vectors and the possibility of interference between mucosal immunity and antibody-mediated effector functions (30, 115, 132). Lastly, one of the largest caveats to these trials is the biased representation of immunogens derived from subtype B and CRF01_AE which collectively only comprise 16% of global infections (137).

1.6.1. VaxGen's AIDSVAX Recombinant gp120 envelope Vaccines

The majority of clinically licensed vaccines mediate their protective efficacy via the induction of serum or mucosal neutralizing antibodies (270). The first HIV-1 vaccine efficacy trials were therefore based on the premise that the induction of neutralizing antibodies directed at the envelope glycoprotein gp120 could confer sterilizing immunity (112). Preliminary studies in nonhuman primates demonstrated that envelope subunit vaccines could confer protection against both homologous and heterologous viral challenges, giving credence to the logic of this approach (21, 22, 97, 105). These studies were followed by promising phase 1 and 2 clinical trials suggesting that monomeric gp120 envelope-based vaccines, derived from subtype B and E strains, were safe and immunogenic (17, 266, 268) .

Based on these studies, two bivalent recombinant envelope vaccine regimens were conceived for efficacy testing. The first of these vaccines (AIDSVAX B/B) was evaluated in men who have sex with men (MSM) and women at high risk for heterosexual HIV-1 acquisition in North America and the Netherlands. The vaccine comprising of a bivalent formulation of the gp120 envelope subunits of the HIV-1 subtype B MN and GNE strains, achieved a mere 6% vaccine efficacy (93). The second efficacy trial evaluated a similar vaccine modality in injection drug users in Bangkok, Thailand. This time however, the vaccine (AIDSVAX B/E) was formulated from the envelope subunits derived from HIV-1 MN and CRF_01AE. This trial demonstrated a negligible 0.1% efficacy (267). A subsequent post-hoc analysis has suggested that despite the lack of apparent efficacy of either vaccine; the AIDSVAX B/B regimen elicited a greater magnitude of antibody responses in non-white and female vaccinees. Furthermore peak antibody titers appear to be inversely correlated with the incidence of HIV-1 infection (103). Notably, both vaccines failed to impact plasma viral load, maintance of peripheral CD4+ T cell count or other clinical outcomes following HIV-1 infection (93, 267). These failures have been attributed to the inability of either vaccine to generate antibodies capable of neutralizing diverse primary viral isolates (103). This may be partly due to the envelope immunogen not

accurately replicating the native structure of the envelope glycoprotein spike, which occurs as a trimer in the context of the HIV-1 virion (46).

1.6.2. Merck Replication-Defective Adenovirus Serotype 5 (Ad5) Vector Based Vaccine

In the aftermath of the failed AIDSVAX clinical trials, HIV-1 vaccine development gravitated towards vector based vaccines for the induction of cellular immunity (376). Although sterilizing immunity remains the holy grail of HIV vaccinology, this idealistic notion was largely perceived as unrealistic in the light of the outcome of the AIDSVAX clinical trials (190). Encouraged by the importance of cellular responses in temporal control of acute phase viremia in both humans and nonhuman primates and the presence of strong cellular responses in long term non-progressors, vaccines were developed in the hope of ameliorating the course of infection (27, 39, 95, 167, 254, 306). Although such a vaccine would be unable to prevent infection, a robust cellular response would be expected to limit acute phase viremia and lower the concomitant viral set point (154). Additionally, cell-mediated immune control would reduce the risk of HIV transmission as the viral load is directly related to the risk of transmission (116, 276). Therefore based on promising nonhuman primate vaccine studies demonstrating viremic control of chimeric SHIV 89.6P and partial control of SIVmac239, a prototype Ad5 vector based vaccine was developed for clinical trials (43, 195, 322, 398).

The vaccine was developed by Merck and comprised of a trivalent, replication-defective adenovirus serotype 5 vector expressing the *gag*, *polymerase* and *nef* genes derived from subtype B isolates (30, 115, 274). Based on promising immunogenicity results in early clinical trials, the vaccine was evaluated in two phase 2b proof-of-concept trials despite the high prevalence of pre-existing Ad5 vector immunity (30, 115, 216, 246, 274). The first, termed the STEP trial, tested the efficacy of the vaccine in the face of high risk heterosexual and homosexual behaviour in a predominantly subtype B epidemic (30). The second trial took place in South Africa (Phambili), to evaluate the cross reactivity of the vaccine to subtype C, the predominant viral subtype in the region (115).

However, despite the favourable immunogenicity of the vaccine in the trial participants, both trials were prematurely terminated after interim analysis showed that the STEP trial failed to meet predetermined efficacy criteria (30, 115, 218, 274). Not only did the MRK Ad5 vaccine fail to impact HIV-1 acquisition or viral load, but the vaccine appeared to enhance HIV infection in a subset of uncircumcised men and participants with pre-existing immunity to the Ad5 vector (30, 115). Subsequent attempts to identify the underlying mechanism for this phenomenon have raised a number of plausible hypotheses (20, 149, 247, 262). It is noteworthy that despite the lack of efficacy of the vaccine, an analysis of breakthrough infections has suggested the presence of cell-mediated selective pressure on the virus (286). In retrospect, the failures of the STEP and Phambili trials have highlighted the need for more fundamental scientific research, particularly with regard to the stringency of nonhuman primate challenge models and the need for more qualitative *in vitro* assessments of vaccine induced cellular responses (12, 312, 388).

1.6.3. RV144 Heterologous Poxvirus Prime – Recombinant Glycoprotein Boost Vaccination Regimen

The failure of the STEP and Phambili trials lead to the development of the heterologous prime-boost concept aimed at inducing both the humoral and cellular arms of immunity (279). A series of phase 1/2 clinical trials demonstrated the utility of combination vaccines, comprising of priming with poxvirus vectors followed by boosting with recombinant envelope proteins, at inducing both antibodies and cytotoxic T lymphocyte (CTL) responses (18, 55, 244, 291, 370). A candidate prime boost vaccine combination was advanced to phase 3 in Thailand in spite of cynicism and the cancellation of a similar trial in America due to a perceived lack of compelling evidence to justify such a study (16, 36, 221, 291). The priming vaccine comprised of a recombinant canarypox vector expressing HIV-1 CRF01_AE gp120 fused to the transmembrane region of subtype B gp41 (LAI strain) containing a deletion in the immunodominant region. The poxvirus vector also expressed the *gag* and *protease* genes from the subtype B LAI strain (244). This was later followed by a

boost with the bivalent AIDSVAX B/E vaccine that had completed efficacy testing in Thailand (267).

The RV144 trial was conducted in Thailand in a community based population at heterosexual risk of HIV-1 acquisition. The vaccine demonstrated a modest 31.2% efficacy at preventing HIV acquisition but had no discernable impact on either viral load or preservation of peripheral CD4+ T cell count in subsequently infected vaccinees. Despite the declining vaccine efficacy (from 59.9% after 12 months) and evidence suggesting that the vaccine efficacy was greater in people at low risk of infection, the RV144 trial was the first to demonstrate any meaningful protection from HIV-1 infection (279). A subsequent immune correlates analysis has revealed that plasma IgG binding antibodies to scaffolded variable regions 1 and 2 (V1/V2) of the HIV envelope protein was inversely correlated with risk of infection. In contrast, binding of plasma IgA to the envelope glycoprotein was correlated with infection risk. Secondary analysis suggested that envelope specific IgA may have negated the beneficial effect of other protective antibodies. Although the latter appears to undermine the rationale of vaccine-elicited mucosal antibodies, no mucosal samples were available from the study for analysis. Secondary analysis of plasma samples also suggested that in the presence of low levels of envelope-specific IgA, IgG avidity, antibody dependent cellular cytotoxicity (ADCC), neutralizing antibodies, and CD4+ T cells all correlated with reduced infection risk, albeit of borderline significance (132). Consistant with these observations the vaccination combination was shown to elicit strong ADCC activity in phase 1/2 trials (160). A sieve analysis of breakthrough infections has revealed the presence of selective pressure on residues 169 and 181 in the V1/V2 region of breakthrough viruses, suggesting that vaccine induced immunity to this region was responsible for the protective effect reported (285). Liao and colleagues went on to isolate 4 monoclonal antibodies from participants in the RV144 trial, targetting epitopes involving the K169 residue, and demonstrated their ability to mediate antibody-dependant cellular cytotoxicity (196). An analogous study to the RV144 vaccine regimen has partly recapitulated these findings by demonstrating a correlation between V2 antibodies and protection from SIV_{MAC251} challenge (257). Additional studies have also suggested that the vaccine regimen may have lowered the seminal viral load post infection and that vaccine-induced T cells preferentially targeted the V2 region of the envelope glycoprotein (333, 334). In the light of these

promising findings, a series of follow up studies have been conceived to evaluate the impact of RV144-like trials in South Africa (11).

1.6.4. Heterologous DNA prime-recombinant Adenovirus serotype 5 boost immunization regimen (HVTN 505)

The most recent vaccine concept to be evaluated for efficacy is the heterologous DNA prime-recombinant adenovirus boost vaccine, designed by the Betty Bumpers Vaccine Research Centre, with the aim of inducing polyfunctional T cell and cross-reactive antibody responses (125). The priming immunization comprised of an equimolar mixture of 6 plasmids encoding Subtype B Gag, Pol and Nef Proteins and truncated envelope antigens from Clade A, B and C viruses (45, 111). This was followed by boosting immunizations with replication-defective adenovirus serotype 5 vectors encoding a Subtype B Gag-Pol fusion protein and matched envelope antigens from clades A, B and C (44). Participants received 3 genetic immunizations at weeks 0, 4 and 8 followed by a single boosting vaccine at week 24 (125). Both vaccines were proven to be safe and immunogenic in early phase clinical assessments when administered alone or in combination (44, 54, 111, 152, 163, 258). Furthermore, the immunogenicity of the adenovirus vector did not appear to be negatively impacted by the presence of pre-existing vector immunity when this phenomenon was evaluated in an early phase 1 trial (152). However, the clinical trial was prematurely terminated due to a lack of efficacy. Additionally concerns were raised that the vaccination regimen may have enhanced HIV infection, although this aspersion was not statistically significant and was later disproved (125). Despite the lack of statistical evidence to confirm these concerns, this is the second adenovirus serotype 5-vectored vaccine which has raised concerns of increased infection risk (30, 115, 218). However, if the vaccination regimen did in fact enhance the risk of HIV-1 infection, the factors responsible were likely to be different as all participants in the HVTN 505 trial were all circumcised and did not have pre-existing vector immunity (125).

1.7. Genetic immunization as an *in vivo* expression platform

The potential to use naked DNA as a vaccine vector was first demonstrated by Tang and Johnston, in 1992, when they exploited a plasmid to induce immunity against human growth hormone in mice (353). This was followed shortly by several studies that reported similar results for viral antigens, giving further credence to the logic of genetic immunization (98, 362, 382). DNA vaccines boast the promise of an improved safety profile compared to conventional live and whole-killed vaccines, along with ease of manufacture for large scale production and the potential to induce both humoral and cellular immunity (173, 201). Additionally, plasmid DNA has intrinsic adjuvant properties due to the presence of unmethylated CpG DNA motifs which are recognized by toll-like receptor 9 (171).

DNA vaccines are minimalistic vaccines that comprise of a bacterially-derived plasmid that has been engineered to express a heterologous antigen in mammalian cells (79, 120, 201). The basic elements that constitute a DNA vaccine include: promoter and polyadenylation (Poly (A)) signals to regulate expression of the heterologous gene, a multiple cloning site to facilitate genetic manipulation of the plasmid, a bacterial origin of replication to enable large scale production of the plasmid in bacterial cells and a selectable marker to ensure the maintance of the plasmid during culturing (**Figure 1.7**) (120, 173).

The use of various transactivator and enhancer elements may also be included in the plasmid vector to increase the levels of antigen expression *in vivo* (10, 102, 151, 174). Our lab has developed an improved DNA vaccine vector, pTHPcapR, which employs a porcine circovirus enhancer element to improve the expression of heterologous genes. The use of this enhancer element has been demonstrated to improve the expression levels of an HIV-1 polyprotein antigen up to 2-fold, in transfected HEK 293 cells, relative to the parental vector which lacks the enhancer element. Consistent with these findings, immunization of mice with the improved vector achieved a 3-fold increase in cytotoxic T cell activity (354).

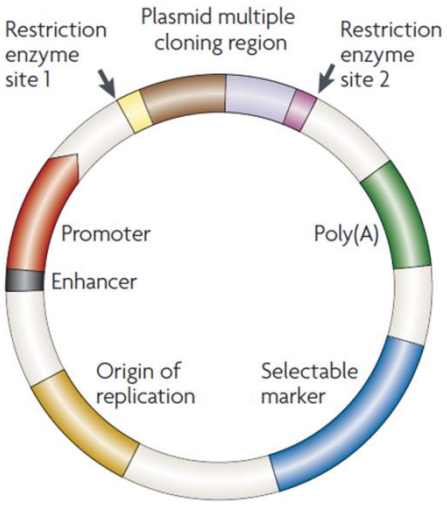

Figure 1.7: Schematic indicating the important functional features of a typical DNA vaccine (modified from Kutzler and Weiner, 2008) (173)**.**

However, despite the initial promise in small animal models, candidate DNA vaccines were found to be poorly immunogenic in clinical trials and high plasmid dosages were required for sub-optimal responses (79, 150, 201, 384). Several different delivery systems have been explored to improve immunogenicity including various needle free devices such as gene gun systems, patch systems, jet injections, electroporation (150). Modification of the antigen coding sequence by codon optimization and the removal of inhibitory sequences has been reported to improve immunogenicity (223). Lastly, the co-administration of cytokines along with the DNA vaccine has also shown promise (173).

To date however, the most promising approach has been the use of DNA vaccines in combination with either matched recombinant proteins or a viral vector encoding the same antigen (204, 304). This strategy, termed heterologous prime-boost, results in high levels of T cell memory and is often associated with a synergistic improvement in immune responses (401). Prime-boost vaccination strategies have been employed extensively against HIV as well as other pathogens including; *Mycobacterium tuberculosis*, Herpes Simplex virus-2, *Plasmodium falciparum*, *Listeria monocytogenes*, *Leishmania infantum* and Ebola virus, amongst others (87, 88, 110, 150, 222, 225, 307, 348, 349).

The DNA prime-protein boost combination is a particularly attractive approach to induce cross-neutralizing antibodies against the HIV-1 envelope glycoprotein (150).

Wang and colleagues reported the induction of antibodies capable of neutralizing JR-FL, a hard to neutralize HIV primary isolate, by immunizing rabbits with a DNA vaccine encoding envelope followed by a recombinant envelope protein boost. Neither approach on its own induced comparable antibody responses, even after multiple immunizations (385). A similar study by Vain and colleagues demonstrated that the DNA prime-protein boost approach could induce antibodies targeting a conformational epitope in the HIV envelope CD4 binding site, whereas immunization with the individual components could not (365). There is also evidence to suggest that DNA priming, followed by a protein boost may enhance antibody avidity (385). These studies strongly support the development of prime-boost vaccination regimens against HIV-1, particularly for the induction of neutralizing antibodies.

1.8. Plants as an expression system for heterologous proteins

The concept of using plants as an expression platform for heterologous protein production, although over 2 decades old, is only now receiving widespread acceptance as a commercially viable expression system (207). The idealistic ambition to exploit plants to produce edible antigens has remained elusive, but instead has been replaced by attempts to create formulatable vaccines that are amenable to standard delivery methods (292). The earliest documented example of a pharmaceutically relevant protein, produced in plants, was reported by Barta and Colleagues in 1986 who expressed a chimeric human growth hormone protein in transgenic tobacco (14). This was followed soon after by the successful expression of plant-derived mouse monoclonal antibodies and then the hepatitis B surface antigen which proved to be immunogenic in mice (140, 214, 356). Yet, in spite of the promise of this early work, only 3 plant produced products have achieved licensure to date (251, 275, 293). Although partly attributable to lack of big pharma buy-in and general public resistance, a major barrier is the low yields associated with many proteins expressed in plants which in most cases fails to reach the 1% total souble protein threshold considered to constitute a feasible product (207, 293). In the light of this, extensive effort has been devoted to understanding the variables that influence protein

expression *in planta*. These variables are discussed in the following sections with emphasis on how they may impact this study.

1.9. Advantages of plants as a heterologous expression system for vaccine antigens

Plants offer a number of distinct advantages over conventional expression platforms that make them a potentially promising source of recombinant proteins. The production of recombinant pharmaceuticals in plants is significantly cheaper than conventional cell culture and bacterial fermentation technology (66, 91, 256, 346). It has been estimated that the production of recombinant proteins in plants could potentially circumvent both batch and site production expenses, representing approximately 31% of the overall cost. Additionally, the raw material expenses are approximated to be 1000-fold less than traditional cell culture based protein production and 100-fold less than proteins produced by bacterial fermentation (293).

Another advantage of plant-based proteins is the potential to produce edible vaccines enabling oral administration, without the extensive purification associated with conventional vaccines (66, 67, 295, 346). Important proofs of concept include the use of transgenic tobacco containing a measles haemagglutinin antigen to boost antibodies induced by a matched DNA vaccine (389), the induction of mucosal neutralizing antibodies in mice against rotavirus VP7 by oral vaccination with transgenic potatoes (406) and the use of transgenic corn seed to boost lactogenic immunity in swine against a porcine transmissible gastroenteritis virus (180). Similar promise has been demonstrated in a clinical context where transgenic potato tubers, containing hepatitis B surface antigen, were reported to boost blood serum antibodies (357).

Additionally plant-based expression platforms are potentially infinitely scalable and uniquely suited to the rapid response required for pandemic disease outbreaks (292). This has been demonstrated best by Medicago who reported the production of a candidate virus-like particle (VLP) vaccine against influenza H5N1 within 3 weeks from the release of the viral sequence. Additionally, researchers at Medicago have

also illustrated the scalability of their expression platform by successfully producing 10 million doses of an H1N1 VLP vaccine in under a month (62).

Plants also have the capacity to mediate proper eukaryotic post-translational modifications and the assembly of VLPs has been observed for several antigens, as described for other expression systems (207, 311). Lastly, plant-produced pharmaceuticals offer an improved safety profile, relative to conventional pharmaceutical production platforms, as they do not support the growth of human pathogens or endotoxins (66, 207, 346).

1.10. The impact of plant-specific N-linked glycosylation

Although plants are capable of reproducing many of the post translation modifications of mammalian expression systems, they differ with respect to the glycosylation they impart onto recombinant proteins (109). Whilst the first phase of glycosylation occurring in the Endoplamic Reticulum (ER), is conserved amongst both plants and animals, the subsequent modifications that occur in the Golgi apparatus differ (256). Plant-derived N-glycan moieties are devoid of terminal galacatose and sialic acid residues and contain non-mammalian β (1,2)-xylose and α (1,3)-fucose carbohydrate groups resulting in a glycan profile that is distinct from conventional mammalian expression platforms (86, 109, 207, 415). An additional complication is the variability of glycosylation imparted by a given host plant depending on the subcellular localization of a recombinant protein. Heterologous proteins expressed in the plant chloroplast are not glycosylated (40, 415). Conversely heterologous proteins retained in the ER typically exhibit only high mannose glycans whereas proteins passing through the Golgi into the apoplast will undergo further modification resulting in plant-specific glycan structures (28, 92).

Concerns have been raised regarding the potential implications of the structural differences of plant glycans in a pharmaceutical context, particularly with regard to their potential immunogenicity (28). The detection of antibodies in human serum that recognize core xylose and α (1,3)-fucose moieties of plant glycans has raised concerns

that plant antigens may induce adverse reactions (9). Furthermore, a series of small animal studies have suggested that plant glycans may be immunogenic, although reports have been inconsistent between different models (9, 50, 153). However, as Ma and colleagues have succinctly reflected, the mere presence of antibodies recognizing plant carbohydrate moieties is not a sufficient indication that they are harmful, especially as these plant glycans are commonly encountered in our diets (207). A recent clinical trial of a candidate influenza haemagglutinin vaccine, produced in *Nicotiana benthamiana* plants, did not detect any increase in plant carbohydrate-specific antibodies following vaccination suggesting that concern is unwarranted (183). Furthermore, the inherent differences in plant glycosylation may even be beneficial in certain settings. Recombinant antibodies expressed in plants may exhibit enhanced effector functions due to the lack of fucose residues in the mammalian α (1,6)-linkage which is believed to limit these non-neutralizing activities (319). Additionally, the lack of terminal sialic acid residues in plant-produced antigens may promote increased binding to cellular receptors enhancing the immunogenicity of vaccines (243).

Despite the potential benefits of plant derived glycans, considerable effort has been devoted to generate plant produced proteins with a humanized glycosylation profile, that is more closely comparable to conventional expression platforms. Promising approaches have included using genetically engineered plants expressing β 1,4-galactosyltransferase to produce glycans with terminal β 1,4-galactose moieties (8, 344) and the generation of knockout mutants lacking the glycosyltransferases responsible for the addition of plant specific carbohydrate groups (343, 345).

1.11. Approaches to the expression of heterologous proteins in plants

A number of different approaches have been developed to express heterologous proteins in plants. These approaches can be broadly divided into either stable or transient expression (**Figure 1.8**) (75). Although much of the early research into using plants as an expression platform relied on stable expression, this approach is limited by the long time scale required to generate transgenic plants (415). Despite initially

being used merely as a vehicle for the validation of expression constructs, the speed and versatility of transient expression systems has seen their widespread acceptance as the method of choice for heterologous protein production in plants (75, 292).

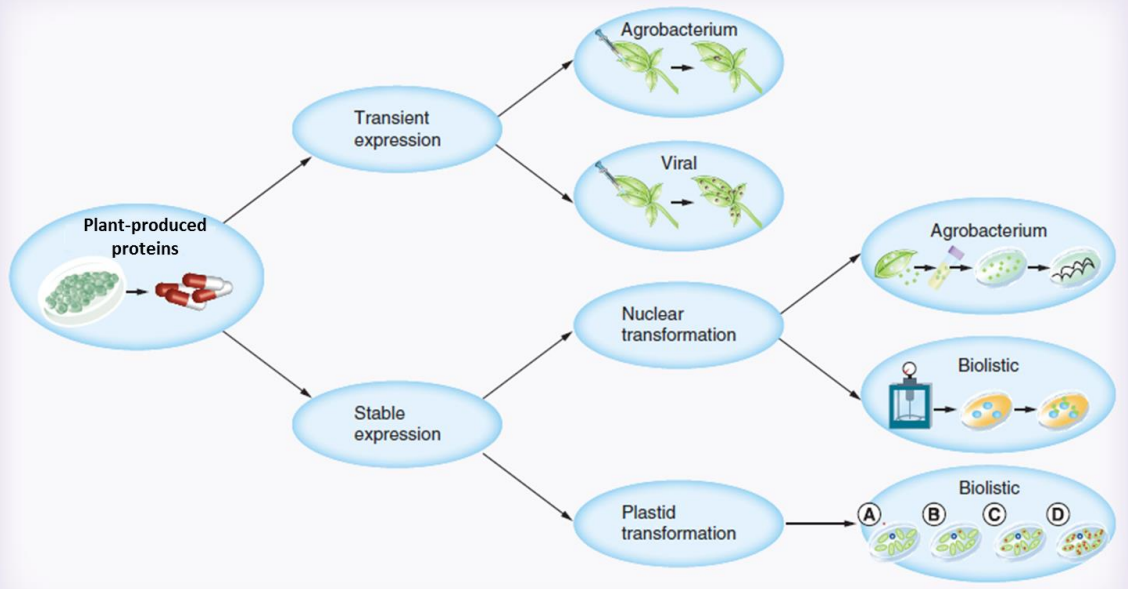

Figure 1.8: Summary of possible approaches to the expression of heterologous proteins in plants (adapted from Scotti and Rybicki, 2013) (311)**.**

Stable expression involves the generation of nuclear transgenic or transplastomic plants by the stable integration of a transgene into the nuclear or plastid genome respectively (248). This can be achieved by transformation with either *Agrobacterium tumefaciens* or by microparticle bombardment (188, 415). Conversely, the generation of transplastomic plants can only be achieved by biolistics or using polyethylene glycol, as *A. tumefaciens* can only deliver DNA to the plant nucleus (40).

Transient expression in plants is typically achieved using either *A. tumefaciens*, plant viral vectors or the *A. tumefaciens*-mediated delivery of viral replicons (166). *A. tumefaciens*-mediated transient expression is achieved by infiltrating a bacterial suspension into plant leaves to enable the transfer of the T-DNA region, encoding the gene of interest, to the plant nucleus (159, 292). This approach has been used routinely by our research group to express antigens from influenza, HPV and HIV amongst others (209, 227, 238, 264). Medicago have championed the use of a similar transient *A. tumefaciens*-mediated expression system for the production of influenza VLPs (63, 183).

The use of plant viral vectors is another promising tool for transient protein expression in plants, enabling rapid onset of transgene expression and systemic spread of the virus (75). This approach exploits a modified plant virus to deliver a transgene to the host plant nucleus for expression, without stable integration (272). Alternately, plant viruses can be used for the display of heterologous epitopes on the surface of chimeric particles (416). A range of plant viruses have been exploited as vectors including *Tobomaviruses*, *Potexviruses*, *Potyviruses*, *Bromoviruses*, *Comoviruses* and *Geminiviruses* (166).

A third approach to transient expression in plants employs *A. tumefaciens* as a vector to deliver viral replicons. The feasibility of this approach was first demonstrated by Icon Genetics (http://icongenetics.com/) who exploited agroinfiltration for the delivery of a "deconstructed" tobacco mosaic viral vector (TMV) (211, 212). This approach enables amplification of the viral replicons leading to expression leves of up to 80% of the total soluble protein (TSP) (106). The technology has subsequently been exploited for the expression of antigens from hepatitis B and *Yersinia pestis* (148, 301). A similar TMV replicon system delivered by agroinfiltration, termed the "launch vector", has also shown promise for the expression of antigens from *Bacillus anthracis*, HPV, *Y. pestis* and influenza (53, 215, 226, 239, 324). Our research group has pioneered the development of a novel bean yellow dwarf virus replicon system, pRIC, delivered by *A. tumefaciens* (278).

1.12. Factors influencing the expression of heterologous antigens *in planta*

The expression of heterologous proteins in plants is a complex process which is influenced by many factors. Although the optimization of expression for any given protein can only be determined impirically, several variables have been identified which can profoundly influence heterologous protein expression *in planta* (292). These variables are reviewed in the text to follow.

1.12.1. Host species

The choice of expression host is an important consideration that influences the yield of the final recombinant protein product. A variety of different plant species have been explored as potential hosts for the expression of pharmaceutical proteins including leafy crops, cereals and legumes, fruit and vegetables; as well as plant cell culture systems (91, 207). However, in spite of the extensive range of plant species that have been exploited for the experimental production of recombinant proteins, it is acknowledged that a limited number of proven systems exist that are capable of industrial scale protein production (292).

Tobacco species remain the workhorse of recombinant plant-derived pharmaceuticals and have been adapted as the expression host of choice by several established biotechnology companies including the likes of Medicago (http://www.medicago.com/), Planet Biotechnology (http://planetbiotechnology.com/) and Meristem Therapeutics (http://www.meristem-therapeutics.com/). Tobacco-based expression systems offer the advantages of high biomass yields, rapid scalability and existing infrastructure for large scale processing, with minimal concerns for contamination of food supplies (342). However, tobacco plants also contain high levels of nicotine and other undesirable alkaloids which complicate down-stream purification (91, 248). Menassa and colleagues have managed to partly circumvent this limitation of tobacco as a production platform by developing a hybrid tobacco platform with lower levels of toxic alkaloids (224). Other promising leafy crops include lettuce, alfalfa and clover although they all share the limitation of intrinsic protein instability (91, 315). Medicago's early stage research has demonstrated the feasibility of alfalfa as an expression host by exploiting novel promoters to achieve high level protein expression in leaf tissue (89). Alfalfa is a particularly attractive expression system for glycoproteins as recombinant proteins have homogenous glycan structures, ensuring batch-to-batch consistency (91).

The use of cereals crops as expression hosts offers the advantage of improved protein stability compared to leafy crops, which require freezing or immediate processing

after harvesting (207). The endosperm tissue of seeds enables high levels of protein accumulation in specialized protein bodies and storage organelles. Additionally, the dessicated environment of seeds ensures that recombinant proteins are protected from proteolytic degradation and enzymatic hydrolysis enabling prolonged storage (277). Recombinant proteins accumulating in seeds are associated with unusual stability and both enzymes and antibodies have been reported to retain full biological activity after refrigeration for more than 3 years (184). Maize appears to have been accepted as the commercial crop of choice for farming; most likely a reflection of its scalability, ease of genetic manipulation and high biomass yields (207). The now defunct, ProdiGene Corp championed maize as an expression host successfully producing proteins, such as avidin and β-glucuronidase, as well as exploring the feasibility of the system for antibodies, enzymes and vaccine antigens (6, 143, 144, 181, 400). Ventria biosciences (http://www.ventria.com/) have also adopted cereal crops as their platform of choice with promising rice and barley-based expression platforms (315). However, the inherent caveat of using food crops as expression hosts is the public concern of inadvertent contamination of the food supply chain (207). The highly publicized 2002 incident, whereby Prodigene Corp was fined for contaminating soybean and maize harvests in 2 states in America, did nothing to appease these concerns (293).

Fruit and vegetables are another popular expression host and boast the appeal of potentially serving as edible vaccines, with minimal processing (207). Potatoes have received a considerable amount of attention and have been evaluated for the expression of vaccine antigens such as the rotavirus VP6, Norwalk virus capsid, *Escherichia coli* heat labile enterotoxin (LT-B), human papillomavirus (HPV) L1 and hepatitis B surface antigen (24, 127, 357, 414, 417). Potatoes have also been exploited for the production of recombinant antibodies and proteins of potential therapeutic value including tumour necrosis factor-α and human serum albumin, amongst others (85, 250, 308, 395). Prototype potato-derived vaccine antigens for hepatitis B and *E. coli* LT-B have even been evaluated in early stage clinical studies, although licensure has not been acheived (350, 357). Tomatoes are also a promising host for edible antigens as they are more palatable than raw potatoes and offer a higher biomass yield (207). Numerous vaccine relevant antigens have been expressed in tomatoes including bacterial antigens from the likes of *Yersinia pestis, Corynebacterium diphteriae, Bordetella pertussis* and *Clostridium tetani* (4, 331) as well as viral antigens from

hepatits B, hepatitis E, Norwalk virus, respitatory syncytial virus and rabies (202, 208, 219, 300, 417). Other production hosts have also been investigated including bananas, carrots, and lettuce with varying degrees of success (91). Obstacles hindering plant-derived oral vaccines include the inability to ensure dose consistency without processing, concerns of antigenic tolerance and poor immunogenicity (293). This unfortunate limitation is best demonstrated by the clinical evaluation of transgenic potatoes containing hepatitis B surface antigen which demonstrated that 25 times the routinely administered parental dose was required to achieve similar immune responses by oral immunization (357).

Plant cell cultures have also been used to produce recombinant proteins, but have comprised only a small part of the overall biopharming effort (292). Although plant cell cultured-based expression offers the potential for ease of biological containment and production under Good Manufacturing Practise conditions, there is little advantage over conventional cell culture platforms (293). In spite of this, the most recently licenced plant-derived product is a recombinant glucocerebrosidase protein produced in plant cells for the treatment of Gauchers disease (313).

Clearly choosing the optimal plant host must take into account a number of variables and the best approach is often determined empirically. Studies conducted in our lab have reinforced the necessity for an empirical approach by demonstrating the differences in recombinant HPV 11 L1 protein yields between different plant host species. It has been the experience of our research group that *Arabidopsis thaliana* yields greater amounts of recombinant L1 than tobacco plants. Furthermore, we have also found that significant variations exist in the expression levels of closely related *Nicotiana tabacum* varieities (294).

1.12.2. Optimization of the gene coding sequence

Optimization of the gene coding sequence is another crucial consideration for the expression of foreign proteins in plants. The observation that tRNA abundance correlates with preferred codon usage and the belief that rare codons may be a rate limiting step in translation has lead researchers to explore different codon biases for

the expression of proteins in heterologous systems (31, 121). Perlak and colleagues demonstrated that optimizing the *Bacillus thuringiensis* insect control *cryIA (b)* coding sequence, for preferred plant codon usage, culminated in 100-fold increase in expression in transgenic tomato and tobacco plants (261). Similar findings were also reported for the expression of a plant codon optimized variant of the gene encoding the Green Fluorescent Protein in *Nicotiana tabacum* cv. SR1 (290).

However, the ideal codon usage for a given gene in plants is unpredictable and is best determined empirically (292, 294). This point is best illustrated by Biemelt and colleagues who described the successful expression of a humanized variant of the HPV-16 L1 protein in tobacco plants, but could not detect expression of either the wildtype or plantized variants (24). Our group has reiterated these findings by demonstrating higher levels of expression of the HPV-16 L1 protein optimized for the preferred human codon usage versus both native and plant codon usage. Interestingly, in this study the native gene sequence was observed to express at higher levels than the corresponding plant codon optimized version (209). Conversely, attempts to express variants of HIV *gag* demonstrated higher levels of expression of the plantized gene than the wildtype sequence (294).

These findings clearly highlight the profound, yet unpredictable, influence that codon usage can have on the expression of heterologous proteins in plants. Interestingly codon optimization of the terminal 40 amino acids of a gene alone, without alteration of the remainder of the sequence, may be sufficient to significantly increase gene expression (15). A further complication in optimizing the codon usage of a given gene is the observation that the preferred codon usage differs between monocotyledonous and dicotyledonous plants (15). In addition to the codon usage, other elements in the coding sequence also require optimization. These include the removal of message destabilizing sequences from the construct as well as modification of the region containing the translational start site to suit the expression host (346). Other important considerations during optimization of the gene coding sequence include avoiding extensive nucleotide runs or repeating codons to avoid the cognate tRNA becoming a rate limiting variable in protein expression. Lastly, the impact of codon optimization on secondary RNA structure should also be considered to avoid the formation of undesirable secondary structures that impede translation (75, 346).

1.12.3. Regulatory elements

The expression of recombinant proteins in a heterologous expression platform is largely governed by *cis* acting elements in the gene sequence. Therefore significant effort has been invested in improving the transcription and stability of foreign genes *in planta*. Heterologous promoters are often exploited *in planta* to increase transcription and manipulate the timing or site of recombinant protein expression. These promoters are typically divided into 3 main categories, namely: constitutive, inducible and tissue specific (75). Strong, constitutive promoters are most commonly used to drive transcription of a desired gene, with the cauliflower mosaic virus 35S RNA promoter (CaMV 35S) and the maize ubiquitin-1 promoter often being the preferred choice for dicotyledonous and monocotyledonous plant hosts respectively (248). However, whilst constitutive promoters are generally associated with the highest levels of protein expression, accumulation of excessive levels of the recombinant protein often has a deleterious affect on host metabolism. Alternatively, organ and tissue specific promoters, as well as inducible promoters may be employed to circumvent these negative effects on plant growth and development (75, 315).

In addition to the CaMV 35S promoter, other plant viral promoters have also shown promise including; the promotor of milk vetch dwarf virus component 8 (321), a cassava vein mosaic virus promoter (369) and the *C1* gene promoter of cotton leaf curl multan virus (409) amongst others. Promoters derived from *A. tumefaciens*, such as the nopaline synthetase and mannopine synthetase promoters, as well as various plant promoters have also been investigated (5, 75, 129). In some cases, researchers have even engineered hybrid promoters comprising of chimeric elements derived from different regulator sequences such as in the case of the synthetic Mac promoter (56, 242). Inducible promoters have also been used to regulate the expression of foreign genes *in planta* and offer the potential to minimize the toxicity associated with overexpression of heterologous proteins (315). The sucrose starvation inducible promoter of the rice *alpha amylase* gene has been exploited to produce a number of recombinant proteins with human therapeutic potential including; human growth hormone, human granulocyte macrophage-colony stimulating factor and human

interferon gamma (52, 165, 320). Another promising inducible promoter system is the stress responsive hydroxyl-3-methylglutaryl CoA reductase 2 promoter, used by CropTech Corp, to rapidly induce recombinant protein expression during the harvesting of plant leaves (207). Other inducible promoter systems have also been described that are regulated by chemicals such as ethanol, copper, ecdysone, oestrogen, tetracycline and glucocorticoids (252). It has also been reported that promoter activity can be further improved by stacking transcriptional activators in the promoter region (118).

In addition to the promoter, a number of other *cis* elements also influence recombinant gene expression in plants. Studies have shown that flanking the gene of interest with heterologous scaffold or matrix attachment regions can significantly improve protein expression in plants (2, 3, 411). Although their exact mechanism of action is undefined they are believed to function by interacting with plant nuclear scaffolds to promote the recruitment of transcription factors to drive gene expression (335). The use of heterologous polyadenylation sites has also been exploited to improve mRNA stability and ultimately levels of the recombinant protein (281). Similarly, the inclusion of 5' untranslated regions in the coding sequence, such as those from alfalfa mosaic virus mRNA 4 or the rice polyubiquitin gene, have also been shown to improve the translational efficiency of heterologous genes (68, 203).

1.12.4. Subcellular localization

The subcellular localization of a heterologous protein impacts both the stability and yield (90). Therefore the choice of localization is an important variable, especially considering that stability is cited as the single greatest factor limiting the yield of plant-produced recombinant proteins (305). The use of heterologous signal peptides can be exploited to target a recombinant protein to the specific subcellular localization that is best suited to its accumulation (346). Conversely, in the absence of any heterologous signal peptide mediating translocation through the cell secretory system or retention in a particular organelle, recombinant proteins will accumulate in the cytosol (207, 346). A number of different localization strategies have been explored involving targeting of recombinant proteins to the cytosol, ER, chloroplast, storage

vacuoles and oil bodies (315). The ER offers a protease free environment containing molecular chaperones to assist with protein folding and enzymes that mediate glycosylation (207, 245). Additionally the ER can tolerate high levels of heterologous protein accumulation without any adverse effect on the development of the plant host (394). Retention of recombinant proteins in the ER is typically achieved by fusing the carboxy terminal HDEL or KDEL sequence to the protein of interest (108, 260). Another ER retention signal derived from γ-zein, a maize prolamin, has also shown promise to increase recombinant protein yields. This particular signal also promotes the formation of ER-associated protein bodies, aiding downstream processing and recovery of the protein (210). A comparative study comparing the protein yields of recombinant Phaseolin, when using the KDEL and γ-zein ER retention signals, demonstrated the latter to be superior. The γ-zein tagged protein accumulated at approximately 3.5% of the TSP compared to the 0.5% achieved by the KDEL tagged protein (372).

Recombinant proteins can also be targeted to the apoplast by the use of a signal peptide that directs the recombinant proteins through the secretory pathway into the extracellular spaces between leaf cells (90). Antibodies generally exhibit lower stability in the apoplastic spaces than when retained in the ER, presumably a reflection of the higher levels of protease activity in the plant apoplast (57, 75). In spite of this, apoplastic localization appears to be amenable to high levels of influenza haemagglutinin production as demonstrated by our group and Medicago, who have advanced their candidate vaccine into clinical trials (63, 183, 238).

Unsurprisingly, considering their ancient prokaryotic origin, the chloroplast is well suited to the expression of bacterial antigens (292, 415). The expression of genes in the plant chloroplast can be achieved by biolistic transformation or by treatment with polyethylene glycol (40). This approach boasts the advantage of high transgene copy number, resulting from homologous recombination between the gene of interest and the plastid genome, and has been reported to result in high recombinant protein yields in excess of 45% of the TSP in the case of the *Bacillus thuringiensis* CRY2Aa2 protein (59). Chloroplasts have also been exploited to express heterologous proteins of viral, protozoan and human origin (40). Staub and colleagues reported the expression of human somatotropin in tobacco chloroplasts with the recombinant

protein accumulating to levels in excess of 7% of the TSP (339). Notably, expression of HIV-1 $pr55^{gag}$ VLPs have been reported in transformed tobacco chloroplasts at levels of between 7-8% TSP (310). Recombinant products expressed in the cytoplasm can be targeted to accumulate in the chloroplast by exploiting a chloroplast-transit sequence, such as the transit peptide derived from the potato *rbcs*1 gene (209). Our research group has also reported high transient expression levels of both HPV-16 L1 and truncated HIV Gag proteins in tobacco chloroplasts using this approach (209, 294). Although transformed chloroplasts also enable biological containment of transgenes in the absence of post transcriptional gene silencing (PTGS), their inability to mediate N-glycosylation is a major limitation (67, 415). In spite of this, a chloroplast-based expression platform has been adopted by Chlorogen Inc. for the development of therapeutic proteins and vaccines (315).

Recombinant proteins can also be targeted to oil bodies in seeds by constructing translational fusions with oleosin enabling ease of downstream centrifugation by floatation centrifugation (315). This approach has been exploited for the production of the anticoagulant, hirudin (255). SemBioSys Genetics Inc. (http://www.sembiosis.com/) has developed an oleosin fusion protein for the expression of heterologous proteins in safflower or oilseed rape (91).

The optimal subcellular localization for any given protein is governed by a number of variables including the origin of the protein, its inherent stability and the complexity of post translational modifications and glycosylation required. It is our experience that the ideal subcellular compartment is best determined empirically for each protein, due to the huge discrepancies in expression levels that exist between different compartments (292, 294). This phenomenon is best illustrated by a comparative expression study of the *E. coli* heat labile toxin which differed in expression of up to 4 orders of magnitude across 6 cellular compartments (347). It has been suggested that these differences are largely a reflection of differences in biochemical environment and space constraints inherent in different plant cellular compartments (346). Consistant with these findings, Wirth and colleagues expressed Human epidermal growth factor in the cytoplasm and apoplast by both nuclear transformation and a viral vector. In both cases, the recombinant protein was barely discernable in the plant cytoplasm but detectable levels were observed in the plant apoplast (399). Our group

has reported similar findings for a number of different antigens. A comparative expression study examining the influence of subcellular localization on the expression of the HPV-16 L1 capsid protein revealed higher levels of protein accumulation in the chloroplast of *N. benthamiana* plants, than in the ER or cytoplasm. Curiously, the poorest expression levels of the antigens were observed in the ER (209). A similar study revealed higher expression levels of the HIV Gag antigen in the chloroplast than in either the cytoplasm or ER (292). Similar variations were observed by our research group for influenza haemagglutinin antigens, this time however the apoplast was found to be optimal (238).

1.12.5. Post Transcriptional Gene Silencing (PTGS)

Transgene silencing is a major obstacle for the economic exploitation of plants as an expression platform for recombinant proteins (394). PTGS, the phenomenon whereby homologous double stranded RNA molecules mediate the destruction of specific mRNA transcripts, has been demonstrated to be responsible for the cessation of recombinant protein expression *in planta* after several days (42, 373, 387). The effects of PTGS appears most pronounced for genes present in multiple copies or under the influence of strong constitutive promoters suggesting that this silencing mechanism is directly correlated with gene expression levels (75).

Several strategies have been implemented to mitigate the effects of PTGS and increase the yield of recombinant proteins produced in plants. The most commonly used method involves the co-expression of a viral PTGS suppressor protein such as the HC-Pro suppressor from Tobacco potyvirus, the p19 supressor from Tomato bushy stunt virus and the NSs suppressor from tomato spotted wilt virus (155, 351, 373). Voinnet and colleagues have reported an excess of 50-fold increase in expression of a range of proteins in *N. benthamiana* plants when co-expressing the p19 silencing suppressor (373). This suppressor has also been adopted by Medicago as part of their expression platform and has enabled the production of a number of chimeric VLPs for Rabies, HIV and varicella zoster amongst others (61). Similarly, Rosenberg and colleagues exploited the p19 supressor to express high levels of broadly neutralizing anti-HIV monoclonal antibodies in plants (289). Our research

group routinely uses the NSs silencing suppressor protein to optimize transgene expression *in planta* and has successfully reported the expression of antigens from the human papillomavirus, influenza virus and HIV using this approach (209, 227, 238, 264). More recently, the 126K protein from Pepper mild mottle virus was demonstrated to be a superior suppressor of PTGS when compared to P19, HC-Pro and Begomovirus AC2 supressor proteins using a green fluorescent protein reporter gene in *N. benthamiana* (332).

A number of other strategies have also been explored to circumvent the influence of PTGS. It has been suggested that *Agrobacterium*–mediated transformation is likely to lead to lower levels of PTGS than biolistic transformation due to the lower transgene copy number (64). Lastly, the use of matrix attachment regions flanking the coding sequence of the gene of interest has also been reported to lower the levels of PTGS induced upon expression of the protein, presumably by interfering with the formation of antisense RNA (361).

1.13. Project rationale

Despite the promise of strategic intervention strategies and biomedical advances in HIV prevention, a prophylactic vaccine remains humanity's best hope to curb the relentless spread of the HIV-1 pandemic (265). The collective body of research suggests that a successful prophylactic vaccine will likely comprise of multiple components to induce the breadth of immunity required to prevent HIV-1 infection (178, 341, 378). The incumbent costs of such an immunization regimen may prove to be prohibitive; particularly in developing countries where the pandemic is disproportionately severe (314). This is particularly evident in the case of HPV vaccines which, despite irrefutable efficacy, are too expensive to be implemented in the extended immunization schemes of developing countries (104, 311). Similarly, in the case of the Hepatitis B Virus vaccine, 15 years elapsed after the development of a successful vaccine before the vaccine cost became affordable for its implementation into the health schemes of developing countries (311).

In this study the feasibility of developing a DNA prime-recombinant plant-derived protein vaccine combination will be evaluated. The priming DNA vaccines will be constructed from the pTHPcapR DNA vaccine vector which enables dose sparing by exploiting a porcine circovirus type 1 enhancer element, to drive high levels of antigen expression (354). The recombinant protein boost will be produced using a transient *A. tumefaciens* mediated plant-based expression platform to reduce production costs associated with heterologous expression systems (293). Additionally, this approach may result in an improved immunogen with the capacity to induce neutralizing antibodies with a greater breadth than associated with currently used vaccine antigens. The expression of these immunogens in plants may result in VLPs with an improved glycoprotein density or improve the accessibility of important antibody epitopes resulting from plant-specific glycosylation patterns. The DNA prime-recombinant protein boost vaccination is well established for the induction of neutralizing antibodies against the HIV-1 envelope glycoprotein. Several studies have demonstrated that the DNA prime-recombinant envelope protein boost combination is capable of inducing neutralizing antibodies of improved titer and longevity than either approach alone (378).

Plants are uniquely suited to the production of cheap recombinant proteins as plant expression platforms are estimated to reduce generic production costs by at least 31% (293). Additionally, plant-based expression platforms are infinitely scalable and would be able to accommodate production levels suitable to the implementation of a vaccine on a global scale, such as is necessitated by the HIV-1 pandemic (293, 410). Recent advances in plant biotechnology have demonstrated the viability of plant-based expression platforms for influenza haemagglutinin-based subunit vaccines, with high yields of recombinant proteins that are immunogenic in both pre-clinical and clinical contexts (157, 158, 183, 226, 323–326, 336). These studies, stemming largely from the inability of conventional influenza vaccine production platforms to respond to pandemic outbreaks, have demonstrated that plant-derived antigens are capable of competing with existing production platforms. Notably, Medicago, a clinical-stage biopharmaceutical company, reported the successful production of fully formulated haemagglutinin VLP vaccines within as little as 3 weeks (62). The research team responsible for these findings reported that, when targeted to the apoplast of plant leaves, the full length haemagglutinin protein had the capacity to form highly

immunogenic particulate structures. Although the mechanism of this assembly remains uncertain, it has been suggested that these proteins bud from the plasma membrane into the apoplastic spaces of the plant leaf, along with host derived lipids (63) (**Figure 1.9**). Further research by Medicago has demonstrated that this approach can be exploited to generate chimeric particles for the HIV-1 envelope glycoprotein, Rabies glycoprotein G and Varicella Zoster Virus glycoprotein, when the antigen of interest is fused to the transmembrane and cytoplasmic domains of the influenza H5 glycoprotein (61).

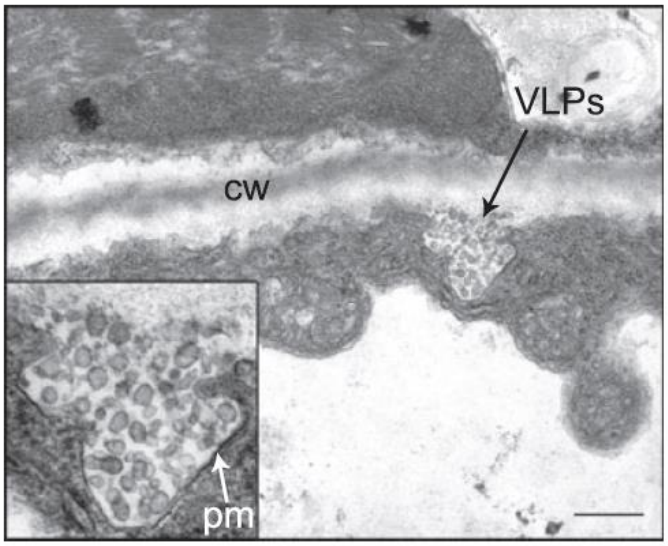

Figure 1.9: Positive staining transmission electron microscopy demonstrating H5 VLP accumulation in the apoplast of agroinfiltrated plant leaves (62)**.** The apoplastic space containing the VLPs is enlarged (bottom left) and position of the cell wall (CW), plasma membrane (pm) and VLPs indicated.

Our lab has developed a similar *A. tumefaciens*-mediated transient expression platform for recombinant haemagglutinin proteins which could potentially be adapted to the production of HIV-1 envelope subunit vaccines (238). Similar to Medicago's reports, we have also achieved the formation of haemagglutinin VLPs in the absence of other structural proteins, when the recombinant proteins are targeted to the apoplastic spaces of plant leaves (Ed Rybicki; personal correspondence) (63). To date this phenomenon has only been demonstrated for the full length haemagglutinin protein of influenza, or for antigens fused to the influenza H5 or H3 transmembrane and cytoplasmic domains (61, 63). However it is plausible that other structurally homologous glycoproteins, such as the HIV-1 envelope glycoprotein, may also

possess the ability to form similar enveloped particles. The HIV-1 envelope glycoprotein is structurally similar to influenza haemagglutinin, both of which form trimeric glycoprotein complexes, in the context of the native virion (134) (**Figure 1.10**). Therefor the construction of chimeric immunogens that retain the important structural features of these type 1 transmembrane glycoproteins may enable the formation of higher order oligomers, possibly even trimers. Ye *at al.* have reported the formation of trimeric oligomers when expressing a fusion protein comprising of gp41 and the HA_1 portion of the influenza HA protein (412). Similarly, the fusion of the transmembrane region from the Epstein-Barr Virus major envelope glycoprotein gp220/350 to the gp120 portion of HIV-1 envelope has been associated with the formation of oligomeric species, although the study in question did not determine whether these were in fact trimeric (72).

VLPs are a particularly promising approach for the induction of robust immune responses by vaccination. They are highly immunogenic due to their large size and dense repetitive arrays of protein which promote cross-linking of B cells (7, 47, 117). They also activate both MHC class I and MHC class II immune pathways culminating in the induction of both humoral and cellular immunity (47, 117, 172, 199). VLPs are efficiently taken up by professional antigen presenting cells, particularly dendritic cells (33, 47, 117, 172). The use of VLP-based vaccines is also considerably safer than other vaccine approaches, yet considerably more immunogenic than conventional subunit vaccines (33, 78, 199). Additionally, the formation of high density envelope particles would be a promising step towards developing a prophylactic HIV-1 VLP vaccine by circumventing the low envelope densities associated with conventional approaches (263).

The construction of chimeric HIV-1 envelope particles with heterologous domains from other viral proteins is not unprecedented. Deml and colleagues managed to successfully increase the surface density and stability of HIV-1 envelope glycoproteins on $Pr55^{gag}$ particles by fusing the exterior glycoprotein (gp120) to the Epstein-Barr virus major envelope glycoprotein gp220/350 transmembrane domain. The authors also confirmed the formation of higher order oligomeric species for the chimeric envelope proteins, consistent with the oligomeric structure of the native HIV-1 envelope glycoprotein (72). Preclinical evaluation of these vaccines confirmed

their immunogenicity in both murine and nonhuman primate models, with the induction of both humoral and cellular immunity (73, 375). Ye *et al.*, constructed a chimeric HIV-1 envelope antigen by fusing the gp41 subunit of the HIV-1 to the HA_1 subunit of influenza haemagglutinin. The resulting protein retained its ability to form trimers, demonstrated increased reactivity with selected monoclonal antibodies and induced neutralizing antibodies in immunized mice (412). The increased epitope exposure, arising from the altered conformation of the chimeric antigens, also culminated in the induction of MPER-specific neutralizing activity in guinea pigs (413). Lastly, Wang *et al.*, demonstrated that by substituting the native HIV-1 envelope transmembrane and cytoplasmic domains, with the analogous regions from influenza haemagglutinin, the envelope glycoprotein density on the surface of $Pr55^{gag}$ particles could be increased (383).

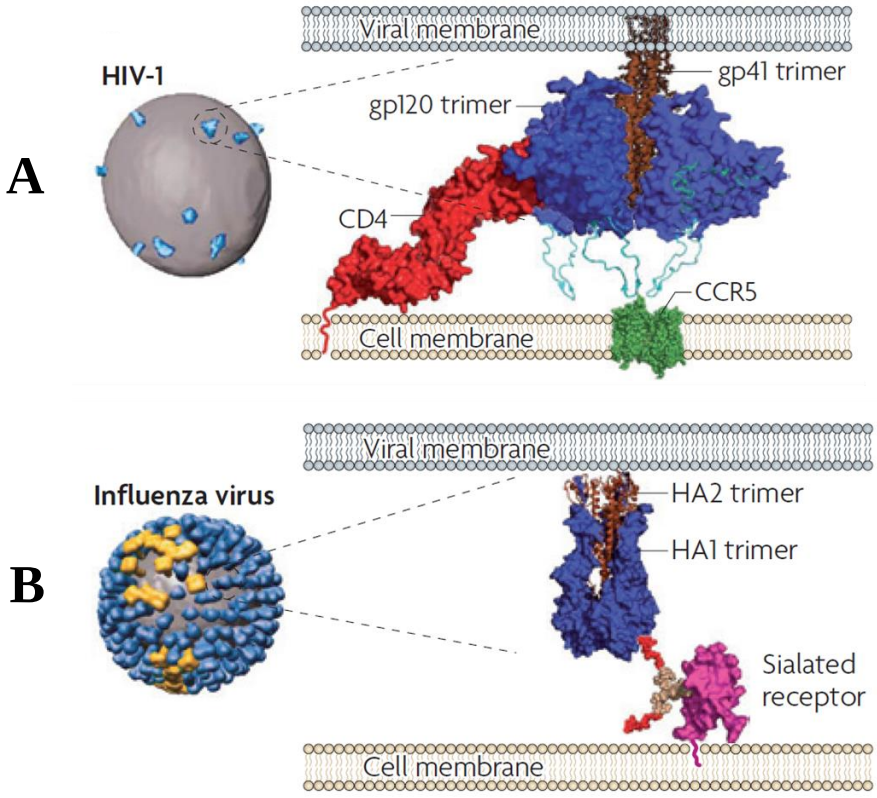

Figure 1.10: Schematic diagram of the virion bound glycoproteins of A) HIV-1 and B) influenza infecting their respective cells (modified from Hedestam *et al.*(134))**.** In both figure A and B surface rendered cryo-electron tomographic images are provided on the left. To the right of each figure the trimeric glycoprotein complex is depicted, as a structure based model, interacting with a host receptor to mediate cellular entry. The virion surface is depicted in grey and the glycoprotein spikes in blue.

Despite the extensive research into HIV-1 vaccine development, only 2 studies have reported the successful expression of an envelope candidate in plants which retains the neutralizing epitopes of the functional glycoprotein (61, 289). Despite successfully generating chimeric HIV-1 envelope-based particles Medicago are not pursuing these candidate vaccines further (61). More recently, Rosenberg and colleagues have reported the production of different glycoforms of an extensively engineered gp140 protein via both transient and transgenic expression. The recombinant antigen was antigenically representative of the native glycoprotein and demonstrated reactivity with selected prototype monoclonal antibodies (289). Immunogenicity studies for these vaccine candidates are ongoing and the scientific community eagerly awaits their outcome. Lastly, the analogous SIV protein, gp130, has been expressed in transgenic corn, although the immunogenicity of the vaccine has not been reported (145).

This project will explore the viability of 3 novel HIV-1 subtype C immunogens using genetic immunization and plant expression platforms. The DNA vaccines constructed in this study will be evaluated *in vivo* to evaluate expression levels and subcellular localization of the immunogen. We will also explore the feasibility of adapting our *A. tumefaciens*-transient expression platform, used for influenza haemagglutinin subunit vaccines, for the production of recombinant HIV-1 envelope proteins (238). In this study we will apply a systematic approach to investigate various parameters known to influence the expression of recombinant antigens in plants. We will also explore various purification strategies to recover our protein from contaminating plant proteins.We will specifically focus on the production of variants of an HIV-1 subtype C envelope protein, with the hope of generating HIV-1 envelope particles. To account for the possibility that the formation of glycoprotein particles *in planta* is an intrinsic property of the influenza transmembrane and cytoplasmic regions, we will also construct chimeric proteins containing these elements. It is noteworthy that Medicago were unable to detect expression of their HIV-1 envelope protein when expressed without being fused to the cytoplasmic and transmembrane regions of the influenza glycoprotein, suggesting that these elements may somehow aid with protein expression or stability (61).

1.14. Aims and Objectives

The ultimate aim of this project is to develop a heterologous DNA prime-recombinant protein boost vaccination scheme, that is both affordable and relevant to the HIV strains predominating in Sub-Saharan Africa. The secondary aim of this study will be to explore the feasibility of a plant-based expression platform for the production of recombinant HIV-1 envelope subunit vaccines, with the capacity to induce neutralizing antibodies. The study comprises a pilot project to investigate whether plants have the capacity to express the HIV-1 envelope glycoprotein and to investigate the various parameters that influence the expression of these immunogens *in planta*. Matched DNA vaccines will be constructed and characterized for each recombinant protein, enabling their potential use in a prime-boost immunization regimen at a later stage. The vaccines will be constructed from a subtype C HIV-1 isolate that conforms to a stringent series of criteria that are likely to favour the induction of cross-neutralizing antibodies. The study will also address the influence of substituting analogous regions of the influenza H5 transmembrane subunit with gp41 on recombinant protein expression. This approach will also be investigated in order to potentially improve the immunogenicity of the antigens by altering the exposure of antibody sensitive epitopes. Additionally, these influenza H5 elements may faciliate the formation of particulate structures *in planta*, containing high densities of the surface-bound antigens, which are expected to be highly immunogenic.

The recombinant proteins will be transiently expressed in the leaves of tobacco plants and targeted to the apoplastic spaces between leaf cells. Recombinant *A. tumefaciens* strains will be infiltrated into *N. benthamiana* plants and the influence of various parameters, on the expression of the recombinant antigens, will be systematically investigated:

- The timing and duration of protein expression in leaf tissue following infiltration of tobacco plants with recombinant *A. tumefaciens* strains.
- The influence of a PTGS suppressor on the yield and duration of recombinant protein expression.

- The use of a detergent to extract crude leaf protein under stringent denaturing conditions
- The influence of the density of the bacterial suspension infiltrated into tobacco leaves on recombinant antigen expression
- The feasibility of recovering recombinant protein directly from the apoplastic spaces of agroinfiltrated plant leaves.

DNA vaccines will be constructed for each immunogen using the pTHPcapR DNA vaccine vector and evaluated *in vivo* (354). As a preliminary indication of their feasibility, expression of the antigens from the pTHPcapR DNA vaccines will be verified by immunostaining and western blotting of transfected cells.

CHAPTER 2:
MATERIALS AND METHODS

2.1. Bacterial strains and culturing conditions

2.1.1. *Escherichia coli* DH5α

E. coli DH5α cells were used as a surrogate cloning host for the construction of the recombinant pTRA-A plant expression vectors and pTHPcapR vectored DNA vaccines used in this study. Liquid cultures were cultivated in Luria Bertani (LB) broth and incubated overnight at 37°C, with gentle agitation. *E. coli* DH5α cells grown on solid media were inoculated onto Luria Agar (LA) and incubated overnight at 37°C. Both liquid and solid media were supplemented with antibiotics, when appropriate, to impose selective pressure on the bacteria to ensure the maintenance of episomal plasmid vectors.

2.1.2. *Agrobacterium tumefaciens* GV3101::pMP90RK

A. tumefaciens GV3101::pMP90RK cells were obtained from Dr. Rainer Fischer (Fraunhofer Institute for Molecular Biology and Applied Ecology, IME, Germany). The *Agrobacterium* strain contains the pMP90RK helper plasmid which encodes the *vir* genes, enabling the transfer of foreign DNA from a disarmed Ti plasmid to the nucleus of the plant host. Collectively *A. tumefaciens* GV3101::pMP90RK and a disarmed Ti plasmid form a functional binary vector system capable of mediating expression of a foreign gene *in planta*.

Liquid cultures were propagated in LB media, supplemented with 50 µg/ml rifampicin (Sigma-Aldrich, South Africa) and 30 µg/ml Kanamycin (Sigma-Aldrich, South Africa), and shaken overnight at 27°C. In contrast, bacteria grown on solid

media were inoculated onto LA plates and incubated at 27°C for 2-3 days. Both solid and liquid media were further supplemented with antibiotics, when appropriate, to impose selective pressure on the recombinant bacteria.

2.1.3. *A. tumefaciens* LBA4404 (pBIN-NSs)

The *A. tumefaciens* LBA4404 (pBIN-NSs) strain was supplied by Dr Marcel Prins (Laboratory of virology, Wageningen University, Binnenhaven, Netherlands). This strain of *A. tumefaciens* has been transformed with an episomal plasmid vector that expresses NSs, an RNA silencing suppressor derived from Tomato spotted wilt virus (351). *A. tumefaciens* LBA4404 (pBIN-NSs) has been used extensively within our group to prolong the duration of protein expression and to improve the yield of recombinant proteins in plants, by mitigating the effect of PTGS (209, 238, 264).

Liquid cultures of *A. tumefaciens* LBA4404 (pBIN-NSs) were propagated in LB broth supplemented with 2mM $MgSO_4$, 50 µg/ml rifampicin (Sigma-Aldrich, South Africa) and 30 µg/ml kanamycin (Sigma-Aldrich, South Africa). The growing cultures were incubated overnight at 27°C, with agitation at 200 rpm. The propagation of *A. tumefaciens* LBA4404 (pBIN-NSs) on solid media was achieved by the transfer of a bacterial inoculum onto Luria agar plates, supplemented as for broth cultures, and their subsequent incubation at 27°C for 2-3 days.

Table 2.1: Summary table of selectable markers for *A. tumefaciens* and *E. coli* host strains used in this study.

Bacterial Strain	Resistance gene	Concentration (µg/ml)
E. coli DH5α	None	NA
A. tumefaciens GV3101::pMP90RK	Rifampicin	30
	Kanamycin	50
A. tumefaciens LBA4404 (pBIN-NSs)	Rifampicin	30
	Kanamycin	50

2.2. Preparation of competent bacterial cells for genetic manipulation

2.2.1. Preparation of competent *Escherichia coli* DH5α cells

Competent DH5α cells were prepared using an amended version of the DMSO method described by Chung and Miller (38). A single colony of DH5α cells was propagated overnight in 5 ml of LB media as described in **Section 2.1.1**. The following morning a 250 µl inoculum of the growing culture was transferred to 50 ml of fresh LB broth and the cells cultivated, as before, until early logarithmic phase growth (OD_{600} 0.2-0.4). The entire culture volume was transferred to a pre-chilled 50 ml falcon tube and the cells harvested by centrifugation at 4000 rpm, for 5 minutes. The supernatant was discarded and the bacterial pellet was gently resuspended in 5 ml of ice cold transfer and storage buffer (TSB) (**Appendix B**) by aspiration with a Gilson pipette. The bacterial suspension was incubated on ice for 10 minutes and then stored at -70°C in 100 µl aliquots. The competency of the cells was assessed by transformation with 0.1 ng and 1 ng aliquots of pSK plasmid DNA and determined as the number of colony forming units yielded per microgram of DNA transformed.

2.2.2. Preparation of electrocompetent *Agrobacterium tumefaciens* GV3101::pMP90RK cells

Electrocompetent *Agrobacterium tumefaciens* GV3101::pMP90RK were prepared as described by Shen and Forde, 1989 (316). A glycerol stock of *Agrobacterium tumefaciens* GV3101::pMP90RK cells was propagated in 100 ml LB media and the culture cultivated until logarithmic phase growth (OD_{600} 0.5-1.0), as outlined in **Section 2.1.2**. The following morning, the entire culture volume was equally distributed between 2 sterile falcon tubes and the bacterial cells harvested by centrifugation at 4000 rpm, for 10 minutes. The supernatant was discarded and the cells resuspended in 50 ml sterile water. The cells were pelleted by centrifugation, as before, and the rinse step repeated. The bacterial suspension was then subjected to centrifugation at 4000 rpm for 10 minutes, the supernatant discarded and the cell pellet resuspended in 10 ml 10% glycerol. The glycerol wash was repeated and the

pellet resuspended in a final volume of 5 ml sterile 10% glycerol. The bacterial suspension was then divided into 500 µl aliquots and frozen at -70 ^{0}C until required.

2.3. Transformation of bacterial cells

2.3.1. Transformation of competent *E. coli* DH5α cells by heat shock

The transformation of competent *E. coli* DH5α cells was achieved using the protocol outlined by Chung and Miller (38). Aliquots of 100 µl of competent *E. coli* cells were thawed on ice prior to the transformation procedure. An aliquot of 0.1-10 ng of plasmid DNA was added to the competent cells and the contents gently mixed by aspiration with a Gilson pipette. The bacterial suspension was incubated on ice for 20-30 minutes and then heat shocked at 42°C for 30 seconds. The samples were then incubated on ice for a further 2 minutes prior to the addition of 900 µl of TSGB media (**Appendix B**). The cells were incubated at 37°C, with shaking, for 1 hour to enable induction of the antibiotic resistance gene encoded by the plasmid. Aliquots of 100 µl and 200 µl of each transformation sample were plated on LA media, supplemented with the appropriate antibiotics. The remaining sample volume was subjected to centrifugation at 5000 rpm, for 5 minutes, the cell pellets resuspended in 200 µl of fresh LB broth and plated as before. All experimental transformations were performed in parallel with a positive control transformation comprising of 0.1 ng of pSK plasmid DNA and a negative control transformation whereby plasmid DNA was omitted.

2.3.2. Transformation of *A. tumefaciens* GV3101::pMP90RK by electroporation

Recombinant *A. tumefaciens* GV3101::pMP90RK strains were generated by electroporation using the method described by Shen and Forde, 1989 (316). Electrocompetent *A. tumefaciens* GV3101::pMP90RK cells, prepared as indicated in **Section 2.2.2**, were thawed on ice prior to the transformation procedure. An aliquot of

100 µl of competent cells was transfered to a sterile eppendorf tube containing 400 ng of plasmid DNA. The contents of the eppendorf were gently mixed and the sample incubated on ice for 5 minutes. The sample was then transferred to a chilled 1 mm gap electroporation curvette and electroporated at 1.8 kV, 25 µF and 200 Ω using the GenePulser (BioRad), before being returned to ice. The samples were then transferred to sterile eppendorf tubes and supplemented with 900 µl of LB media. The cells were incubated at 27^0 C, for 2 hours with gentle agitation prior to plating. Aliquots of 100 µl and 200 µl of the transformation reaction were plated on LA media, containing the appropriate antibiotics, as indicated in **Section 2.1.2**. The remaining cells were harvested by centrifugation at 5000 rpm, for 5 minutes, resuspended in 200 µl of fresh LB media and plated as before. All transformations were performed in parallel with a positive control of pTRA-A: H5 plasmid DNA and a negative control whereby plasmid DNA was omitted.

2.4. Recovery of HEK 293 cells from liquid nitrogen

A cryovial of HEK 293 cells, stored in liquid nitrogen, was obtained from the ATCC® (CRL-1573™). The cells were rapidly thawed by gentle agitation in a 37^0C water bath. The outside of the cryovial was decontaminated by swabbing with 70% ethanol and the contents transferred to a sterile tube containing 9 ml complete Growth Media (GM) (**Appendix B**). The cells were harvested by centrifugation at 125 ×g for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 5 ml complete GM and the cell suspension dispensed into a 25 cm^2 culture flask.

2.5. Growth and maintenance of HEK 293 cells

HEK 293 cells were cultivated at 37^0C, in an incubator with 5% atmospheric CO_2 and 95% humidity. The growth medium was replaced every 2-3 days, with fresh GM, until the cell monolayers were confluent. Confluent monolayers were passaged approximately every 3 days with a subculture ratio of 1:6. The culture medium was discarded and residual serum removed by rinsing the monolayers with 5 ml sterile Gibco® Dulbecco's Phosphate Buffered Saline (dPBS) (without magnesium or

calcium) (Gibco®, South Africa). A 2 ml aliquot of 1× Trypsin-EDTA solution (Gibco®, South Africa) was gently dispensed over the cell monolayer and the flask incubated at 37^{0}C for 2 minutes to facilitate detachment of the cells. The reaction was terminated by the addition of 8 ml of complete GM and the cells gently resuspended in the medium. An aliquot of 20 µl of the cell suspension was diluted 2-fold in 0.4% Trypan Blue (Gibco®, South Africa) and 10 µl transferred to a haemocytometer. The number of viable cells in the 4 major quadrants were determined and multiplied by 5000 to obtain an estimate of the number of cells per ml and an appropriate volume used for sub-culturing.

2.6. Growth of *N. benthamiana* biomass

N. benthamiana seeds were propagated in flat trays filled with soil. The plants were incubated at 22°C, under a 16 h light/8 h dark photocycle. Individual seedlings were transplanted to pots after 3 weeks, containing a 2:1 ratio of peat to vermiculite, and left to grow under the same environmental conditions. Plants were infiltrated with recombinant *A. tumefaciens* 6-8 weeks after germination and returned to the greenhouse until the optimal time for protein extraction.

2.7. Isolation of recombinant plasmid DNA

2.7.1. Small scale isolation of plasmid DNA from recombinant *E. coli* cells

Small scale plasmid DNA isolations were performed using the method described by Sambrook and Russel (297). A single recombinant *E. coli* colony was inoculated into 800 µl of LB media and cultured overnight as outlined in **Section 2.1.1**. The following morning the cells were harvested by centrifugation at 5000 rpm for 3 minutes, the supernatant discarded and the bacterial pellet resuspended in 200 µl of solution I (**Appendix B**). A 400 µl aliquot of freshly prepared solution II (**Appendix B**) was added to the bacterial suspension and the samples incubated on ice for 5

minutes to lyse the cells. The lysis reaction was terminated by the addition of 300 µl of solution III (**Appendix B**). The samples was inverted several times to mix the contents and the contaminating protein and residual bacterial contaminants pelleted by centrifugation at 14000 rpm, for 5 minutes. The supernatant was retained and transferred to a sterile eppindorf tube containing 600 µl of isopropanol. The tube was inverted several times and the DNA was precipitated by subjecting the sample to 10 minutes of centrifugation at 14000 rpm. The supernatant was discarded and 200 µl of 70% ethanol was added to the DNA pellet. The pellet was rinsed to remove any residual salts by centrifugation at 14000 rpm for 5 minutes. The ethanol was carefully removed with a Gilson pipette and the pellet resuspended in 50 µl sterile water. The DNA was stored at -20° C until needed.

2.7.2. Medium scale isolation of plasmid DNA from recombinant *E. coli* cells

Medium scale plasmid DNA isolations were performed using the Genopure Plasmid Midi Kit (Roche, South Africa), in accordance with the protocol described for low copy number plasmid DNA. A single recombinant *E. coli* colony was inoculated into 50 ml of LB media and cultured as described in **Section 2.1.1**. The bacterial cells were harvested by centrifugation at 4000 rpm, for 10 minutes, and the spent media discarded. The resulting cell pellet was gently resuspended in 8 ml of chilled Resuspension buffer, supplemented with RNAse A. An aliquot of 8 ml of Lysis buffer was added to the bacterial suspension and the tubes inverted gently. The sample was incubated at room temperature for 2 minutes, prior to termination of the lysis reaction by the addition of 8 ml chilled Neutralization buffer. The cell lysate was inverted gently several times and incubated on ice for 5 minutes. The sample was then applied to an equilibrated piece of folded filter paper, placed in the mouth of a sterile 50 ml falcon tube, and allowed to drain through under the influence of gravity. A NucleoBond AX 100 Column was mounted in the mouth of a sterile 50 ml falcon tube and equilibrated with 2.5 ml of Equilibration Buffer. The flow through was discarded and the bacterial lysate applied to the column. The flow through was retained and reapplied to the column for a second time followed by 3 sequential wash steps of the membrane, to which the DNA had bound, with 4 ml Wash Buffer. The column was

transferred to another sterile 50 ml conical tube and the DNA eluted in 5 ml of prewarmed Elution buffer. Aliquots of 1 ml of the eluate were transferred to sterile eppendorfs containing 700 µl isopropanol. The DNA was precipitated by centrifugation for 10 minutes and the pellet rinsed by the addition of 200 µl 70% ethanol. The sample was centrifuged at 14000 rpm for 5 minutes and the residual ethanol drawn off with a Gilson pipette. Lastly, a 20 µl aliquot of sterile water was added to each sample and the samples left at 4°C for an hour to resuspend. The samples were pooled together and then stored at -20°C until needed.

2.7.3. Small scale isolation of crude plasmid DNA from recombinant *A. tumefaciens*

Crude plasmid DNA isolations from recombinant *A. tumefaciens* were achieved using the Biospin Plasmid DNA Extraction Kit (Bioflux, China). A single recombinant colony or glycerol stock was propagated in 8-10 ml of liquid both, in accordance with **Section 2.1.2**. The bacterial cells were harvested by centrifugation, for 30 seconds at 10 000 rpm, the supernatant discarded and the cells gently resuspended in 250 µl chilled Resuspension Buffer. A 250 µl aliquot of Lysis Buffer was added to the sample and the cells incubated for 5 minutes, before the reaction was terminated by the addition of 350 µl of Neutralization Buffer. The sample was gently inverted several times and the contaminating cell debris pelleted by centrifugation at 13 000 rpm for 10 minutes. The supernatant was retained and applied to a spin column. The sample was subjected to 1 minute of centrifugation at 5 000 rpm and the flow through discarded. An aliquot of 650 µl Wash Buffer was applied to the column and the membrane bound DNA washed by centrifugation at 12 000 rpm, for 1 minute. The flow through was discarded and the wash step repeated again. The spin column was centrifuged for an additional minute, as before, to remove any residual reagents trapped in the column. The column was transferred to a sterile 1.5 ml eppindorf tube and 50 µl of Elution Buffer added directly to the membrane of the spin column. The sample was incubated for 1 minute at room temperature prior to the elution of the DNA from the membrane; by subjecting the column to 1 minute of centrifugation at 13 000 rpm. The plasmid DNA was either used immediately or stored at -20°C until required.

2.8. Enzymatic manipulation of DNA

2.8.1. Enzymatic cleavage of plasmid DNA with restriction endonucleases

Plasmid DNA was enzymatically cleaved for the purposes of cloning and screening of recombinant DNA using Fermentas Fastdigest® enzymes (Fermentas, South Africa), in accordance with the manufacturer's instructions. Briefly, all reactions were conducted in a volume of 20 µl comprising of 250 ng - 1 µg of plasmid DNA, 2 µl of 10× FastDigest® Green Buffer, 1 µl FastDigest® enzyme and an appropriate volume of sterile water to make up the reaction volume. The samples were incubated in a 37°C water bath for 30 minutes to allow the reaction to occur to completion. The reaction was either terminated by heat inactivation, as indicated by the manufacturer, or the resulting DNA fragments purified from contaminating restriction enzymes by means of the Wizard® SV Gel and PCR Clean-up System (Promega, South Africa); as per the protocol outlined for PCR products (to follow in **Section 2.11**)

2.8.2. Covalent linkage of DNA fragments by ligation

DNA fragments were ligated into plasmid vector backbones using either the Clonejet PCR Cloning Kit (Thermo Scientific, South Africa) for PCR products or Fermentas T4 DNA ligase (Thermo Scientific, South Africa) for DNA fragments derived from other sources. DNA fragments amplified by PCR were subjected to a blunt end ligation into the pJET1.2/blunt plasmid provided with the CloneJET™ PCR Cloning Kit. The enzymatic reactions were carried out in a 20 µl reaction volume containing 2 µl purified PCR product, 10 µl 2× Reaction buffer, 50 ng pJET1.2/blunt plasmid DNA, 1 µl T4 DNA ligase and an aliquot of sterile water to make up the reaction volume. The samples were incubated at room temperature for 30 minutes prior to heat inactivation of the T4 DNA ligase at 70°C for 5 minutes.

Sticky end cloning of DNA was mediated by T4 DNA ligase in a 20 µl volume; using a 3:1 molar ratio of insert DNA to plasmid backbone. In addition to 1 µl of T4 DNA ligase and appropriate volumes of the 2 DNA species, the reaction also comprised of 2 µl 10× DNA ligase reaction buffer and an aliquot of sterile water to make up the reaction volume. The reaction was allowed to proceed overnight, at room temperature, prior to heat inactivation of the enzyme at 70°C for 5 minutes. In the case of both blunt and sticky end ligations; the reactions were conducted in parallel with controls whereby a) insert DNA was omitted or b) both insert DNA and DNA ligase were omitted.

2.9. Separation of DNA fragments by agarose gel electrophoresis

DNA samples were resolved on the basis of their molecular weight by agarose gel electrophoresis in 1× TBE running buffer. Aliquots of 20-50 µl of DNA were supplemented with 1×Fast Digest Green Buffer and loaded into each well of an agarose gel. Unless otherwise indicated; The samples were electrophoresed at 100V, for 2 and half hours alongside 10 µl Fermentas O'GeneRuler™ 1kb DNA ladder (Thermo Scientific, South Africa). The TBE running buffer was further supplemented with 6 µl of Ethidium Bromide (Sigma-Aldrich, South Africa) to enhance visualization of the DNA. Agarose gels were visualized using the UviPro Silver Gel Documentation System and the resulting images captured with UviPro software.

2.10. Excision and purification of DNA fragments from agarose gels

The desired DNA fragment was excised from the agarose gel using a scalpel, over a UV light box, and purified from contaminating agarose using the Wizard® SV Gel and PCR Clean-up System (Promega, South Africa). The agarose slice was weighed and a volume of Membrane Binding Solution added to the agarose at a ratio of 10 µl Membrane Binding Solution to 10 mg agarose. The sample was vortexed and incubated at 55°C for 10 minutes to allow the agarose to dissolve completely. The sample was then applied to a SV Minicolumn assembly and incubated at room

temperature for 1 minute enabling the DNA to bind to the column. The SV Minicolumn assembly was centrifuged at 14 000 rpm for 1 minute and the flow through discarded from the collection tube. An aliquot of 700 µl of Membrane Wash Solution was applied to the membrane and the column centrifuged at 14 000 rpm, for 1 minute. The supernatant was discarded and the wash step repeated, but for 5 minutes this time, with 500 µl of Membrane Wash Solution. The supernatant was discarded and the column centrifuged as before, for an addition minute, to ensure the removal of any residual ethanol. Lastly, the column was transferred to a sterile 1.5 ml eppendorf tube and a 20-30 µl aliquot of nuclease-free water added directly to the membrane to which the DNA was adsorbed. The sample was incubated at room temperature for 5 minutes and the DNA eluted by centrifuging the sample for 1 minute at 14 000 rpm. The plasmid DNA was either used directly or stored at -20°C for further use.

2.11. Purification of PCR products after amplification

Following the amplification of DNA by PCR, the products of the reaction were purified from contaminating reagents using the Wizard® SV Gel and PCR Clean-up System (Promega, South Africa). An equal volume of Membrane Binding Solution was added to the PCR reaction and the PCR products purified as outlined for DNA fragments excised from agarose gels (**Section 2.10**).

2.12. Plasmid Vectors used for the construction of recombinant plant expression vectors and DNA vaccines

2.12.1. pJET1.2/blunt cloning vector

The commercially available cloning vector pJET1.2/blunt (Thermo Scientific, South Africa) was used in this study, as a cloning intermediate, for the construction of both the recombinant pTRA-A expression vectors and pTHPcapR DNA vaccines. The

plasmid is amenable to highly efficient cloning of PCR fragments by exploiting a lethal selection system to select for recombinant clones.

2.12.2. pBluescript SK (pSK) cloning vector

The commercially available cloning vector pSK (Agilent Technologies, South Africa) was used in this study as a positive control during transformations and to assess the competency of *E. coli* DH5α cells.

2.12.3. pTRA-A plant expression vector

The pTRA-A plasmid comprises of a disarmed Ti plasmid that targets the expression of heterologous proteins to the apoplastic spaces of plant leaves (238). The Ti plasmid backbone has been augmented with a number of additional features to enhance the expression of heterologous genes (**Figure 2.1**) (209). Collectively *A. tumefaciens* GV3101::pMP90RK and the disarmed Ti plasmid form a functional binary vector system enabling the expression of heterologous genes *in planta*.

The expression of the foreign gene is regulated by the Cauliflower mosaic virus (CaMV) 35S promoter (p35SS), with a duplicated transcriptional enhancer and the CaMV 35S polyadenylation signal (pA35S). Upstream of the gene of interest; the vector encodes a plant codon optimized signal peptide sequence (LPH), derived from murine mAb24 heavy chain, to ensure the translocation of the recombinant protein through the ER of the plant cell. Additionally, the insert is flanked by tobacco *rb7* derived scaffold attachment regions (SAR) to enhance transgene expression and stability. The left border (LB) and right border (RB) elements are located on either end of the scaffold attachment regions; defining the termini of the DNA region that will be transferred from *A. tumefaciens* to the plant nucleus. The plasmid contains origins of replication for both *E. coli* (ColE1 ori) and *A. tumefaciens* (Rk2 ori) as well as ampicillin/carbenicillin (*bla*) and kanamycin (*npt*II) resistance genes which serve as selectable markers. The expression of the *npt*II gene is regulated by the *nopaline synthase* promoter (Pnos) and polyadenylation signal (pAnos). Lastly the plasmid also

contains the Chalcone synthase 5' untranslated region (CHS) to enhance mRNA stability and translation efficiency (209).

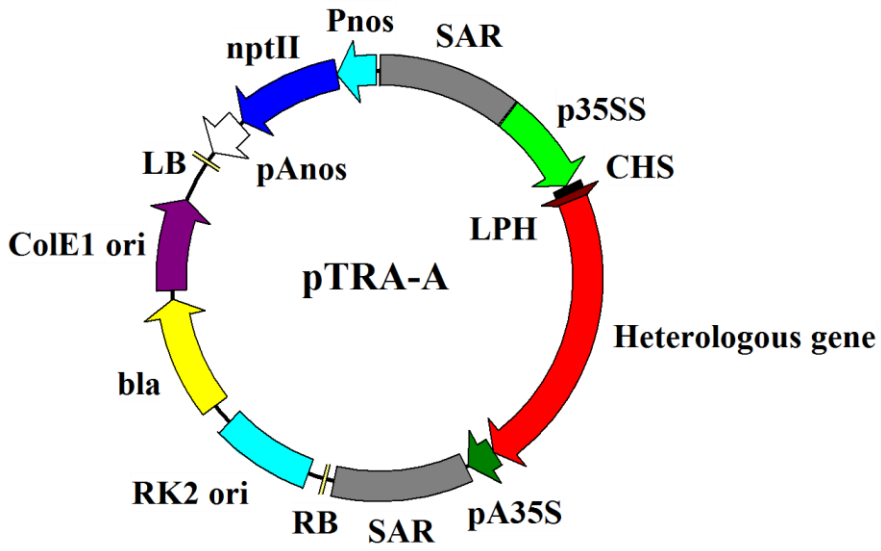

Figure 2.1: Annotated plasmid map of the pTRA-A binary plant expression vector. The disarmed Ti plasmid backbone has been augmented with numerous additional features to enhance the expression and stability of a foreign gene and to direct its translocation to the apoplast of plant leaves (209, 238). (RK2 ori = *A. tumefaciens* origin of replication, ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, SAR = Scaffold attachment region, LB = left border, RB = Right border, p35SS = CaMV duplicated transcriptional enhancer, pA35S = CaMV polyadenylation signal, LPH = signal peptide, CHS = chalcone synthase 5' untranslated region, Pnos = *nopaline synthase* promoter, pAnos = *nopaline synthase* polyadenylation signal, *npt*II = kanamycin resistance gene).

2.12.4. pTHPcapR DNA vaccine vector

The pTHPcapR DNA vaccine vector was obtained from Tsungai Jongwe (PhD candidate, Department of Medical Virology, University of Cape Town) as pTJDNA4, a recombinant construct used in an independent study. The recombinant plasmid encodes a full length HIV-1 subtype C *gag* mosaic immunogen flanked by *Hind*III and *Xba*I restriction enzyme recognition sequences. The pTHPcapR DNA vaccine has ultimately been derived from the pRc/CMV commercial expression vector (Invitrogen, South Africa). In an unrelated study Thomas Hanke and colleagues developed a minimalistic pTH DNA vaccine vector from the pRc/CMV expression vector. The resulting plasmid retained the ColE1 origin of replication and the β-lactamase genes from pRc/CMV facilitating cloning in *E. coli* cells. The expression of the foreign gene is regulated by the Human CMV enhancer/promoter/intron region

(pCMV) and bovine growth hormone polyadenylation signal (BGH PolyA) (126). Fiona Tanzer *et al*., reported further modifying the pTH vector backbone by incorporating a porcine circovirus type 1 enhancer element (PcapR) upstream of the multiple cloning site culminating in the formation of the pTHPcapR vaccine vector used in this study (354) (**Figure 2.2**).

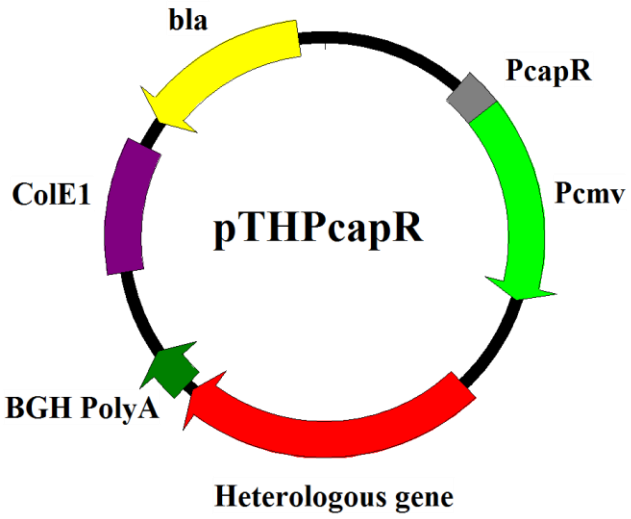

Figure 2.2: Annotated plasmid map of the pTHPcapR DNA vaccine vector. The DNA vaccine vector has been designed to maximize antigen expression by employing the Human CMV enhancer/promoter/intron region (Pcmv) and a porcine circovirus type 1 enhancer element (PcapR) to drive expression of a heterologous gene (126, 354). Additional elements include an *E. coli* origin of replication (ColE1), the polyadenylation signal from bovine growth hormone (BGH PolyA) and a β-lactamase gene for use as a selectable marker (bla).

Table 2.2: Summary table of selectable markers on the plasmid vectors used in this study.

Plasmid	Resistance gene	Concentration (µg/ml)
pSK	Ampicillin	100 µg/ml
pJET1.2/blunt	Ampicillin	100 µg/ml
pTHPcapR	Ampicillin	100 µg/ml
pTRA-A	Ampicillin/carbenicillin	50 µg/ml

2.13. Amplification of HIV-1 envelope antigen coding sequences

The gene coding sequences designed in this study were constructed by PCR using plasmid DNA templates encoding the HIV-1 envelope from the CAP256 superinfecting virus (pLM1) and the full length influenza H5 haemagglutinin gene of

H5N1 (pTRA-A:H5). The pLM1 plasmid comprised of the pcDNA3.1D/V5-His-Topo® plasmid vector (Invitrogen, South Africa) encoding the CAP256 superinfecting virus envelope (clone 256.3mo.9C) as part of an HIV-1 expression cassette. In addition to the envelope gene sequence, the plasmid also encoded the 3' end of *rev* exon 1, the *vpu* gene and the 5' end of the *nef* coding sequence. All PCR reactions were conducted in a 50 µl reaction volume using Fermentas *Pfu* DNA Polymerase (Thermo Scientific, South Africa) in accordance with the manufacturer's instructions. Unless indicated otherwise; 0.5 µM of both the forward and reverse primer were used to initiate amplification from a 10 ng template of plasmid DNA in the presence of 0.2 mM dNTPs. The PCR reactions were buffered by 1 × *Pfu* Buffer and the reaction volume adjusted to 50 µl with sterile dH_2O. The Mg^{2+} ion concentration was titrated and the annealing temperatures optimized for each reaction; prior to the amplification of the products used for cloning. *Pfu* DNA Polymerase was only added once the sample temperature had reached 95°C. The reaction was then allowed to proceed for 25 cycles. Control samples lacking the forward primer, reverse primer or template DNA were subjected to the same experimental conditions alongside the experimental samples. The primers used for the amplification of the envelope antigens, used in the construction of the recombinant pTRA-A plasmid vectors, are indicated below in **Table 2.3**.

Table 2.3: Summary table of the primers used for the amplification and assembly of the plant antigen coding sequences designed in this study. The *Nco*I (red) and *Spe*I (blue) restriction enzyme sites that were engineered into the primers are underlined.

Primer	Orientation	Sequence (5'-3')	Use
$FWD_{CAP256}1$	Forward	CGCG**CCATGG**TCAAT GGCTTGTGGGTTACA	PCR amplification of gp150, gp140 and gp120 coding sequences as well as the assembly of the chimeric gp120-HA_2 and gp140-HA_2tr genes.
$RVS_{CAP256}1$	Reverse	ATGGCGCCGAACAGGCCT CTTTTCTCTTTCTGCAC	PCR amplification of the gp120 coding sequence.
$RVS_{CAP256}2$	Reverse	CGC**ACTAGT**AGTCTCTGTC TTGCTCTCCACCTTCTTC	PCR amplification of the gp150 coding sequence.
$RVS_{CAP256}3$	Reverse	GGCCACGGTGCTGTAGATG CTCAGTTTTATATACCACA GCCACTTTGA	PCR amplification of the gp140 coding sequence.
$FWD_{H5}1$	Forward	GTGCAGAAAGAGAAAAGA GGCCTGTTCGGCGCCATC	PCR amplification of the HA_2 coding sequence.

$FWD_{H5}2$	Forward	TCAAAGTGGCTGTGGTATA TAAAACTGAGCATCTACAG CACCGTGGCC	PCR amplification of the HA_{2tr} coding sequence.
$RVS_{H5}1$	Reverse	CCGCACTAGTTTATGCGGC CGCTCTACACTGC	PCR amplification of the HA_2 and HA_{2tr} coding sequences as well as the assembly of the chimeric gp120-HA_2 and gp140-HA_2tr genes.

2.13.1. PCR amplification of the gp150 coding sequence

The gp150 coding sequence was amplified directly from 1 ng of pLM1 template DNA using the $FWD_{CAP256}1$ and $RVS_{CAP256}2$ primers (**Table 2.3**). The reaction was conducted at 3.0 mM $MgSO_4$ using the cycling parameters outlined in **Table 2.4** below. A 20 µl aliquot of the resulting PCR products were resolved on a 0.8% agarose gel to verify the successful amplification of the desired DNA fragment.

Table 2.4: PCR cycling parameters used for the amplification of the HIV-1 gp150 coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	65.5	30 seconds	25
Extension	72	4.5 minutes	25
Final Extension	72	7 minutes	1

2.13.2. Assembly of the chimeric gp120-HA_2 coding sequence by overlap extension PCR

The gp120 DNA fragment was amplified from 10 ng of pLM1 template DNA using the $FWD_{CAP256}1$ and $RVS_{CAP256}1$ primers (**Table 2.3**). The reaction was conducted at 2.5 mM $MgSO_4$ in accordance with the cycling conditions outlined in **Table 2.5**. The successful amplification of the desired DNA fragment was verified by resolving a 20µl aliquot of the PCR products on a 0.8% agarose gel (**Section 2.9**) alongside a 10 µl aliquot of Fermentas O'GeneRuler™ 100 bp Plus DNA ladder.

Table 2.5: PCR cycling parameters used for the amplification of the HIV-1 gp120 coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	70	30 seconds	25
Extension	72	3 minutes, 5 seconds	25
Final Extension	72	7 minutes	1

The full length HA_2 coding sequence was amplified from 10 ng of pTRA-A: H5 template DNA using the $FWD_{H5}1$ and $RVS_{H5}1$ primers (**Table 2.3**). The DNA fragment was amplified at 2.5 Mm $MgSO_4$, using the PCR parameters described in **Table 2.6**, and the amplification of the desired product confirmed by 1.5% agarose gel electrophoresis (**Section 2.9**).

Table 2.6: PCR cycling parameters used for the amplification of the full length HA_2 coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	63	30 seconds	25
Extension	72	1 minute, 30 seconds	25
Final Extension	72	7 minutes	1

The HIV-1 gp120 and influenza HA_2 DNA fragments were excised from their respective agarose gels and recovered using the Wizard® SV Gel and PCR Clean-up System in accordance with **Section 2.10**. The chimeric gp120-HA_2 gene was assembled from an equimolar ratio of the purified gp120 and HA_2 PCR products using the $FWD_{CAP256}1$ and $RVS_{H5}1$ primers (**Table 2.3**). The reaction was conducted at 1.75 mM $MgSO_4$ using the cycling parameters outlined in **Table 2.7**. The PCR products were subjected to 0.8% agarose gel electrophoresis to verify the successful amplification of the chimeric gene (**Section 2.9**).

Table 2.7: PCR cycling parameters used for the amplification of the chimeric gp120-HA_2 coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	63	30 seconds	25
Extension	72	1 minute, 30 seconds	25
Final Extension	72	7 minutes	1

2.13.3. Assembly of the chimeric gp140-HA_2tr coding sequence by overlap extension PCR

The gp140 coding sequence was amplified from 2 ng pLM1 using the $FWD_{CAP256}1$ and $RVS_{CAP256}3$ primers (**Table 2.3**). The reaction was conducted at 3.0 mM $MgSO_4$ using the cycling parameters delineated in **Table 2.8**. The resulting PCR products were resolved on a 0.8% agarose gel as outlined in **Section 2.9**.

Table 2.8: PCR cycling parameters used for the amplification of the gp140 coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	62.4	30 seconds	25
Extension	72	4 minute, 35 seconds	25
Final Extension	72	7 minutes	1

The HA_2tr coding sequence was amplified from 1 ng pTRA-A: H5 plasmid DNA template using the $FWD_{H5}2$ and $RVS_{H5}1$ primers (**Table 2.3**). The PCR reaction was performed at 3.0 mM $MgSO_4$ in accordance with the cycling parameters described in **Table 2.9**. A 10 µl aliquot of the PCR product was resolved on a 1.5% agarose gel (**Section 2.9**) alongside 10 µl of Fermentas O'GeneRuler™ 100 bp Plus DNA Ladder.

Table 2.9: PCR cycling parameters used for the amplification of the HA_2tr coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	62.6	30 seconds	25
Extension	72	22 seconds	25
Final Extension	72	7 minutes	1

The HIV-1 gp140 coding sequence and the influenza HA_2tr DNA fragments were separately amplified by PCR and resolved by agarose gel electrophoresis, as described in **Section 2.9**. The desired fragments were recovered from the agarose gels using the Wizard® SV Gel and PCR Clean-up System (**Section 2.10**) and an equimolar amount of the purified PCR products used as template to assemble the gp140-HA_2tr chimera; as indicated in **Table 2.10**. The reaction was conducted at 3.0 mM $MgSO_4$ and allowed to proceed for 5 cycles prior to the addition of the $FWD_{CAP256}1$ and $RVS_{H5}1$ primers (**Table 2.3**).

Table 2.10: PCR cycling parameters used for the assembly of the gp140-HA_2tr chimera coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	72	30 seconds	25
Extension	72	4 minutes (+5 seconds)*	25
Final Extension	72	7 minutes	1

*An additional 5 seconds were added to the extension time after each successive cycle

2.14. Construction of recombinant pTRA-A:gp150, pTRA-A:gp120-HA_2 and pTRA-A:gp140-HA_2tr plant expression vectors.

The gp150 coding sequence was amplified as described in **Section 2.13.1** and the 2 chimeric genes assembled as delineated in **Section 2.13.2** and **Section 2.13.3**. The resulting PCR products were purified using the Wizard® SV Gel and PCR Clean-up System in accordance with **Section 2.11**. The purified PCR products were then ligated into the pJET1.2/blunt cloning vector as outlined in **Section 2.8.2**. Following heat

inactivation of the residual DNA Ligase; half the reaction volume was transformed into competent *E. coli* DH5α cells (**Section 2.3.1**) that were prepared using the DMSO method reported by Chung and Miller (38) (**Section 2.2.1**). Ten recombinant colonies for each construct were propagated in 800 µl of LB media (**Section 2.1.1**) and the bacterial cultures subjected to a small scale DNA extraction using the alkaline-lysis method described by Sambrook and Russel (297) (**Section 2.7.1**). An aliquot of 10 µl of each DNA sample was screened by *Xho*I- *Xba*I restriction analysis, and the resulting restriction fragments resolved on a 0.8% agarose gel (**Section 2.8.1** and **Section 2.9** respectively). A single recombinant colony, containing the desired recombinant pTRA-A plasmid, was cultivated in 100 ml of liquid broth (**Section 2.1.1**). A medium scale DNA isolation was performed on the bacterial culture (**Section 2.7.2**) and the antigen coding sequence excised from a 1 µg aliquot of the pJET1.2/blunt vector backbone by dual *Nco*I- *Spe*I enzymatic digestion (**Section 2.8.1**). The products of the restriction digest reaction were separated on a 0.8% agarose gel and the band corresponding to the antigen coding sequence excised and purified (**Section 2.9** and **Section 2.10** respectively).

The pTRA-A plasmid vector backbone was recovered from the recombinant pTRA-A:H5 plasmid construct by digesting 1 µg of plasmid DNA with *Nco*I and *Xba*I (**Section 2.8.1**). The products of the reaction were separated on a 0.8% agarose gel in accordance with **Section 2.9**. In order to ensure optimal separation of the pTRA-A backbone and H5 insert the samples were electrophoresed at 80 V for 20 minutes, followed by 15 V overnight. The DNA fragments corresponding to the pTRA-A backbone were excised from the agarose slab and purified as outlined in **Section 2.10**. The purified PCR product was then ligated into the pTRA-A plasmid backbone (**Section 2.8.2**) and half the ligation reaction transformed into *E. coli* DH5α cells (**Section 2.3.1**). Ten putative recombinant colonies were cultivated in 800 µl of LB media (**Section 2.1.1**) and DNA isolated using the small scale extraction protocol described in **Section 2.7.1**. A 10 µl aliquot of each sample was subjected to restriction analysis to identify samples containing the final recombinant plasmid (**Section 2.8.1**) and the resulting DNA fragments resolved by 0.8% agarose gel electrophoresis (**Section 2.9**). Putative pTRA-A: gp150 samples were screened by separate *Nco*I and *Pst*I restriction enzyme digests. In contrast putative pTRA-A: gp120-HA_2 and pTRA-A:gp140-HA_2tr samples were screened by dual *Nco*I- *Not*I restriction analysis.

A single recombinant colony, containing the recombinant plasmid, was cultivated in 100 ml of LB (**Section 2.1.1**) and subjected to a medium scale DNA isolation procedure using the Genopure Plasmid Midi Kit (**Section 2.7.2**). Aliquots of 250 ng of the plasmid DNA were analysed by restriction analysis (**Section 2.8.1**) and the resulting DNA fragments resolved on a 0.8% agarose gel (**Section 2.9**) to verify that the reaction yielded fragments of the desired size. The genetic integrity of the final constructs was independently verified by sequencing using the complete gp160 primer set along with the pTRA-kc F and pTRA-kc R primers (indicated below in **Table 2.11**). The sequence data was generated by Stellenbosch sequencing unit and aligned to a hypothetical reference sequence, generated *in silico*, using CLC Main Workbench (Version 6).

Table 2.11: Summary table of the primers used for sequencing of the recombinant plasmid vectors constructed in this study.

Primer	Orientation	Sequence (5'-3')	Use
pTRA-kc F	Forward	CATTTCATTTGGAGAG GACACG	Sequencing of recombinant pTRA-A plasmid DNA.
pTRA-kc R	Reverse	GAACTACTCACACATT ATTCTGG	Sequencing of recombinant pTRA-A plasmid DNA.
FOR14	Forward	TATGGGACCAAAGCCT AAAGCCATGTG	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
FOR16	Forward	TTTAATTGTGGAGGAG AATTTTTCTA	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF00	Forward	GGGAAAGAGCAGAAG ACAGTGGCAATGA	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF15	Reverse	TGCTCTCCACCTTCTTC TTC	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF55	Reverse	GCCCCAGACCGTGAGT TGCAACATATG	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF115	Reverse	AGAAAAATTCTCCTCT ACAATTAA	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF175	Reverse	TTTAGCATCTGATGCA CAGAATAG	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.

EF200	Forward	GGGATAACATGACCTG GATGCAGTGGG	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
EF260	Forward	TTCAGCTACCACCGAT TGAGAGACT	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
REV15	Reverse	CTGCCATTTAACAGCA GTTGAGTTGA	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.
REV19	Reverse	ACTTTTTGACCACTTG CCACCCAT	Sequencing of recombinant pTRA-A and pTHPcapR plasmid DNA.

2.15. Construction of matched pTHPcapR-vectored DNA vaccines

The coding sequences of the 3 antigens were amplified, from the recombinant pTRA-A expression vectors constructed in **Section 2.14**. Unless indicated otherwise; all PCR reactions were conducted using *Pfu* DNA Polymerase; in accordance with **Section 2.13**. The gp150 coding sequence was amplified from 1ng of pTRA-A: gp150 plasmid DNA template using the $FWD_{DNA}1$ and $RVS_{DNA}1$ primers (**Table 2.12**). The reaction was conducted at 1.75 mM $MgSO_4$ as outlined in **Table 2.13**. The amplification of the desired DNA fragment was confirmed by subjecting a 25 µl aliquot of the PCR product to agarose gel electrophoresis on a 0.8% agarose gel (**Section 2.9**).

Table 2.12: Summary table of the primers used for the construction of the pTHPcapR DNA vaccines designed in this study. The *Hind*III (red) and *Xba*I (blue) restriction enzyme sites that were engineered into the primers are underlined.

Primer	Orientation	Sequence (5'-3')	Use
$FWD_{DNA}1$	Forward	GCA**AAGCTT**ATGGAG TGGAGCTGGATC	PCR amplification of gp150, gp120-HA_2 and gp140-HA_2tr coding sequences.
$RVS_{DNA}1$	Reverse	GCG**TCTAGA**TTAGTCT CTGTCTTGCTCTCC	PCR amplification of the gp150 coding sequence
$RVS_{DNA}2$	Reverse	AAC**TCTAGA**TTATGC GGCCGCTCTACACTG	PCR amplification of the gp120-HA_2 and gp140-HA_2tr coding sequences.

Table 2.13: PCR cycling parameters used for the amplification of the gp150 DNA vaccine antigen coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	65	30 seconds	25
Extension	72	4 minutes, 12 seconds	25
Final Extension	72	7 minutes	1

Similarly, the gp120-HA_2 coding sequence was amplified from 1ng pTRA-A: gp120-HA_2 plasmid DNA template using the $FWD_{DNA}1$ and $RVS_{DNA}2$ primers (**Table 2.12**). The reaction was carried out at 1.75 mM using the cycling parameters elucidated in **Table 2.14**. The entire reaction volume was resolved on a 0.8% agarose gel to verify that the desired DNA fragment had been successfully amplified (**Section 2.9**).

Table 2.14: PCR cycling parameters used for the amplification of the gp120-HA_2 DNA vaccine coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	71	30 seconds	25
Extension	72	4 minutes, 20 seconds	25
Final Extension	72	7 minutes	1

Lastly, the gp140-HA_2tr PCR product was amplified from 1 ng of pTRA-A: gp140 plasmid DNA template using the $FWD_{DNA}1$ and $RVS_{DNA}2$ primers (**Table 2.12**). The reaction was conducted at 3.0 mM $MgSO_4$ in accordance with **Table 2.15**. The entire reaction volume was subjected to 0.8% agarose gel electrophoresis to confirm that the desired DNA fragment had been amplified.

Table 2.15: PCR cycling parameters used for the amplification of the gp140-HA_2tr DNA vaccine coding sequence.

Reaction stage	Temperature (°C)	Duration	Number of cycles
Initial Denaturing	95	3 minutes	1
Denaturing	95	30 seconds	25
Annealing	66	30 seconds	25
Extension	72	4 minutes, 16 seconds	25
Final Extension	72	7 minutes	1

The gp150, gp120-HA_2 and gp140-HA_2tr antigen coding sequences were amplified by PCR and the gene products purified using the Wizard® SV Gel and PCR Clean-up System (Promega, South Africa) (**Section 2.11**). Each PCR product was ligated into the pJET1.2/blunt cloning vector and the reaction allowed to occur overnight, at room temperature (**Section 2.8.2**). A 10 µl aliquot from each ligation reaction was then transformed into competent *E. coli* DH5α cells (**Section 2.3.1**); prepared in accordance with **Section 2.2.1**. Five to ten putative recombinant colonies were cultivated overnight in 800 µl of LB broth (**Section 2.1.1**) and subjected to a small scale DNA isolation (**Section 2.7.1**). A 10 µl aliquot of plasmid DNA from each sample was screened by *Hind*III-*Xba*I dual restriction enzyme digestion (**Section 2.8.1**) to identify recombinant pJET1.2/blunt samples containing the desired insert. The resulting restriction fragments were resolved on a 0.8% agrose gel (**Section 2.9**) alongside a 10 µl aliquot of undigested plasmid DNA, prepared in accordance with **Section 2.7.1**. A single colony containing the desired insert for each plasmid construct was propagated overnight in 50 ml LB broth (**Section 2.1.1**) and subjected to a medium scale DNA isolation using the Genopure Plasmid Midi Kit (Roche, South Africa) (**Section 2.7.2**). A 1 µg aliquot of each recombinant pJET1.2/blunt plasmid construct was subjected to a *Hind*III-*Xba*I dual restriction enzyme digest to liberate the PCR products from the cloning vector backbone (**Section 2.8.1**). The resulting restriction fragments were resolved on a 0.8% agarose gel (**Section 2.9**) and the desired DNA fragments were excised and purified from contaminating agarose (**Section 2.10**).

A 1 µg aliquot of pTJDNA4 was simultaneously digested with *Hind*III-*Xba*I (**Section 2.8.1**) and the resulting DNA fragments resolved on a 0.8% agarose gel (**Section 2.9**). The DNA fragments corresponding to the pTHPcapR vaccine vector backbone were

recovered from the agarose gel and purified (**Section 2.10**). The purified DNA fragments corresponding to the antigen coding sequences were then ligated into the pTHPcapR backbone (**Section 2.8.2**) and half the ligation reaction transformed into competent *E. coli* DH5α cells (**Section 2.3.1**). Ten putative recombinant colonies for each construct were propagated overnight in 800 µl of LB broth (**Section 2.1.1**), and subjected to a small scale DNA isolation procedure as described in **Section 2.7.1**. A 10 µl aliquot of the resulting plasmid DNA was screened by *Hind*III-*Xba*I restriction digestion (**Section 2.8.1**) and the restriction fragments separated on a 0.8% agarose gel to identify samples containing the desired ligation products (**Section 2.9**). A single recombinant colony for each construct was cultivated overnight in 100 ml of liquid broth (**Section 2.1.1**) and the culture subjected to a medium scale DNA isolation procedure (**Section 2.7.2**). The structural integrity of the 3 recombinant DNA vaccines was then verified by restriction analysis and sequencing as described for the recombinant pTRA-A plasmid vectors in **Section 2.15**.

2.16. Medium scale transfection of HEK 293 cells with recombinant pTHPcapR DNA vaccines

Medium scale transfections of HEK 293 cells were performed, in triplicate, in 6 well culture plates (Nest Biotech Co., Ltd). Each well was seeded with 0.4×10^6 cells in a volume of 2 ml complete GM (**Appendix B**). The cells were incubated for 24 hours at 37°C, prior to the transfection, to allow the cells to reach 70-90% confluence. Aliquots of 2 µg of recombinant plasmid DNA were gently mixed with 50 ng pGL4.13 (luc2/SV40) reporter plasmid in a sterile 1.5 ml eppendorf tube (Sigma-Aldrich, South Africa) containing 100 µl unsupplemented Gibco ®DMEM (Life Technologies, South Africa).

A 3 µl aliquot of X-tremeGENE HP DNA Transfection Reagent (Roche, South Africa) was added to the DNA suspension and the sample incubated at room temperature for 30 minutes to facilitate the formation of complexes between the transfection reagent and the recombinant plasmid DNA. The transfection complex was then added directly to the cells in a drop wise manner and the 6 well plates briefly agitated to evenly distribute the complexes across the surface of the wells. The cells

were incubated at 37°C for 48 hours to enable protein expression. All transfections were performed in parallel with controls comprising cells alone, cells with transfection reagent and cells transfected with the SAAVI C2 DNA vaccine (positive control).

2.17. Harvesting of transfected HEK293 cell lysate for Western blotting

The spent media was discarded and the transfected cells gently washed with 1 ml sterile Gibco® dPBS (without magnesium or calcium) (Life Technologies, South Africa). An aliquot of 200 µl 1× Glo Lysis Buffer (Promega, South Africa), supplemented with 1× Complete EDTA-free Protease Inhibitor (Roche, South Africa), was dispensed over the cell monolayer and the cells incubated at room temperature for 5 minutes. The cell lysate, from triplicate samples, was gently mixed by pipette aspiration and pooled in a single 1.5 ml eppendorf tube. The cell debris was collected by centrifugation for 10 minutes at 13000 rpm and the supernatant retained at -20°C, as 50 µl aliquots. The cell lysate was quantified (**Appendix A.1**) and subjected to a luciferase assay (**Appendix A.2**) to measure the transfection efficiency of the pGL4.13 (luc2/SV40) reporter plasmid.

2.18. Western blotting to detect recombinant HIV-1 envelope in transfected cell lysate

The transfected cell lysate samples were analysed for the presence of the recombinant antigens by Western blotting; under denaturing conditions. Aliquots of 10 µl of XT Sample Buffer (Bio-Rad, South Africa) and 2 µl of XT Reducing Agent (Bio-Rad, South Africa) were added to 28 µl of unquantified cell lysate; in a sterile 1.5 ml eppendorf tube. A positive control sample, comprising of 100 ng of recombinant CN54 gp120 was also prepared in this way. The CN54 envelope was obtained from the AIDS reagent programme as a recombinant HIV-1 Subtype C protein expressed in insect cells via recombinant baculovirus (#7749). The protein was produced as a

translational fusion with maltose binding protein and a C terminal histidine tag culminating in an approximate molecular weight of 130 kDa (386). The samples were boiled at 95 °C for 5 minutes and the entire volume loaded onto a precast 7% Criterion Tris-Acetate Gel, suspended in 1 × XT Tricine Running buffer (Bio-Rad, South AfricaO. The samples were electrophoresed at 150V for 1 hour, alongside a 10 µl aliquot of either Thermo Scientific PageRuler Prestained Protein Ladder (Fermentas, South Africa) or Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad, South Africa).

A pre-cut sheet of Immun-Blot™ PVDF membrane (Bio-Rad, South Africa) was soaked in methanol for 2 minutes and then equilibrated in 20 ml Transfer buffer for 20 minutes. Similarly, following electrophoresis the Criterion Tris-Acetate Gel and 2 pre-cut pieces of Extra Thick Blot Paper (Bio-Rad, South Africa) were also equilibrated in 20-30 ml Transfer buffer for 20 minutes. A piece of the saturated Extra Thick Blot Paper (Bio-Rad) was placed on top of a Semi-dry Blot apparatus (Bio-Rad, South Africa), followed by the equilibrated PVDF membrane and then the Criterion Tris-Acetate Gel. A second piece of saturated Extra Thick Blot Paper was placed on top of the gel essentially sandwiching the gel between the membrane and 2 pieces of saturated blotting paper. The proteins were electrophoretically transferred from the gel to the membrane at 15V for 1 hour. The successful electrophoretic transfer of protein, from the gel to the PVDF membrane, was verified by staining the membrane with Ponceau S solution for 2 minutes.

Following Ponceau S staining, the membrane was rinsed in RO water and incubated with 30 ml Block/Wash buffer for 4 hours, with gentle shaking. The Block/Wash buffer was discarded and the membrane incubated overnight at 4° C with a 1: 1000 dilution of either the Serotech Polyclonal anti-gp120 primary antibody or the MRC ADP 408/5104 polyclonal anti-gp160 primary antibody. The antibody suspension was then discarded and the membrane rinsed for 4×15 minute intervals with Block/Wash buffer (**Appendix B**). The membrane was then incubated with a 1:10 000 dilution of GT34 anti-Sheep/Goat secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich). The wash was repeated 4 times, as before, and the HIV-1 envelope

detected by incubating the membrane with 5 ml of NBT/BCIP (Roche) solution for 5 minutes.

2.19. Small-scale transfection of HEK293 cells

Small scale transfections of HEK293 cells were performed, in duplicate, in 8 well Permanox® Chamber slides (Lab-tek®). Each well was seeded with 80 000 cells, in a volume of 200 µl complete GM, and incubated at 37°C overnight. An aliquot of 400 ng of plasmid DNA was suspended in 100 µl of DMEM and X-tremeGENE HP DNA Transfection Reagent added at a 1:1 ratio to plasmid DNA. The DNA suspension was gently agitated and incubated at room temperature for 20-30 minutes. The transfection complex was then added, dropwise, to each well and the microchamber plate gently agitated to distribute the transfection reaction in the wells. A further 200 µl of complete GM was added to each well and the microchamber plate incubated at 37°C for 48 hours before detection of recombinant protein. All transfections were performed in duplicate alongside controls comprising cells alone, cells with transfection reagent and cells transfected with a positive control plasmid; the SAAVI C2 DNA vaccine. The SAAVI DNA-C2 vaccine comprises of a bivalent mixture of 2 plasmids. The first plasmid encodes a truncated HIV-1 envelope protein (gp150), whereas the second encodes a heavily engineered fusion protein (Grttn) comprising of Gag, Reverse Transcriptase, Tat and Nef (397).

2.20. Immunodetection of recombinant HIV-1 envelope protein in transfected HEK293 cells

The excess medium was removed from each well and the cells fixed in 500 µl acetone; for 10 minutes at 4^0 C. The acetone was removed and the cells left to air dry at room temperature. Each well was briefly rinsed with PBS and then blocked with 500 µl 2% Bovine Serum Albumin (BSA); for 20 minutes at room temperature. The residual blocking solution was removed and the cells subjected to 2 ten minute washes with PBS. This was followed by the addition of 500 µl of MRC ADP

408/5104 polyclonal anti-gp160 primary antibody (1:200), suspended in 1.5% BSA solution. The cells were incubated with the primary antibody for 1 hour at 37^0C. The antibody dilution was then removed and the cells washed twice with PBS, as before, to remove any unbound antibody. An aliquot of 500 µl of Donkey anti-goat CY3 secondary antibody (1:500), suspended in 1.5% BSA solution, was added to each well. The cells were incubated at 37^0 C for 1 hour. The residual antibody was removed and the cells washed twice with PBS, as before. An aliquot of 500 µl of Hoechst nuclear stain (diluted 1:10 000 in PBS) was dispensed into each well and the microchamber plate incubated for 10 minutes, at room temperature. The Hoechst stain was removed and the cells washed twice with PBS. The wells were then removed from the microchamber slide and the cells gently rinsed with sterile dH_2O. The cells were allowed to air dry and then mounted with a coverslip using Mobial. The slides were viewed for fluorescence, at the blue and red light emission spectra, using the Zeiss Axiovert 200M LSM 510 Meta Confocal microscope.

2.21. Generation of recombinant *A. tumefaciens* GV3101::pMP90RK strains

Electrocompetent *A. tumefaciens* GV3101::pMP90RK cells were transformed with each of the recombinant pTRA-A plasmid vectors (constructed in **Section 2.14**), in accordance with **Section 2.3.2**. Putative recombinants were screened by back-transformation of crude DNA extract into *E. coli* cells to assess the integrity of the recombinant plasmid DNA. A single colony of each putative *A. tumefaciens* recombinant was inoculated into 10 ml of LB media and cultured as outlined in **Section 2.1.2**. A 1 ml aliquot of the bacterial culture was retained and stored as a 25% glycerol stock, and the remaining culture volume was subjected to a small scale DNA isolation procedure using the Biospin Plasmid DNA Extraction kit (Bioflux) (**Section 2.7.3**). An aliquot of 5 µl of the crude plasmid DNA sample was transformed into competent *E. coli* DH5α cells and plated onto LA media supplemented with 50 µg/ml carbenicillin; as outlined in **Section 2.3.1**. Five putative recombinant colonies, derived from each *A. tumefaciens* clone, were cultured overnight in 800 µl of LB media and subjected to a small scale DNA isolation procedure as outlined in **Section 2.1.1** and

Section 2.7.1 respectively. Aliquots of 10 µl of each sample were screened by restriction enzyme digestion to verify the genetic integrity of the plasmid samples after growth *in vitro*. Recombinant pTRA-A: gp150 plasmid DNA was screened by *Nco*I-*Sph*I dual enzyme digestion. The pTRA-A:gp120-HA_2 and pTRA-A:gp140-HA_2tr plasmid samples were screened by *Nco*I- *Not*I enzyme digestion.

The resulting restriction fragments were resolved by electrophoresis on a 0.8% agarose gel (**Section 2.9**). Once the success of the transformation had been verified and the genetic integrity of the plasmid DNA confirmed; the original *A. tumefaciens* glycerol stocks were cultivated in 10 ml of selective media, in accordance with **Section 2.1.2**, and stored as 25% glycerol stocks.

2.22. Syringe infiltration of *N. benthamiana* leaves with recombinant *A. tumefaciens* strains

Transient protein expression time trials were conducted by syringe infiltration of *N. benthamiana* plant leaves using the method described by Maclean *et al*., 2007 (209).
All recombinant strains were infiltrated alone or co-infiltrated with the LBA4404 (pBIN-NSs) *Agrobacterium* strain. A 1 ml glycerol stock of recombinant *A. tumefaciens* GV3101::pMP90RK or LBA4404 (pBIN-NSs) was propagated in 10 ml LB media; as described in **Section 2.1.2** or **Section 2.1.3 respectively**. After overnight incubation a 1 ml inoculum of the growing culture was transferred to a 10 ml aliquot of Induction medium (**Appendix B**), augmented with antibiotics as before. The growing LBA4404 (pBIN-NSs) culture was further supplemented with 2mM $MgSO_4$ to prevent the bacterial cells from aggregating together. The cells were cultivated overnight, harvested by centrifugation at 4000 rpm, for 10 minutes and resuspended in 500 µl freshly prepared Infiltration medium (**Appendix B**). The bacterial suspension was adjusted to an OD_{600} of 0.5-1.5 with infiltration medium and incubated at room temperature for 2-3 hours. In the case of recombinant strains that were co-infiltrated with LBA4404 (pBIN-NSs); the OD_{600} was individually adjusted for each culture and the 2 strains mixed to give the same final OD_{600}.

Aliquots of 1-2 ml of the bacterial suspension were infiltrated into the abaxial airspaces on the ventral side of the leaves of 6-8 week old tobacco plants. The plants were returned to the greenhouse and incubated under the same environmental conditions, as delineated in **Section 2.6**, for the duration of the experiment. Five leaves were infiltrated for each construct and separate plants were used for different recombinant strains of *A. tumefaciens*. Additionally, a negative control infiltration was performed alongside the experimental infiltrations whereby *N. benthamiana* leaves were infiltrated with infiltration media only.

2.23. Harvesting of crude protein extract from agroinfiltrated leaves

Six leaf discs were harvested from agroinfiltrated plant leaves, using the cap of an eppendorf tube, and finely ground in liquid nitrogen. The leaf matter was resuspended in 300 µl PBS containing Complete EDTA-free Protease Inhibitor (Roche). The residual plant matter was collected by centrifugation at 13000 rpm, for 5 minutes and the supernatant stored at -20°C. The pellet comprising of residual plant matter was also retained and stored at -20°C for later use.

2.24. Protein extraction from agroinfiltrated plant tissue under denaturing conditions

The agroinfiltrated leaf matter, retained from **Section 2.23**, was resuspended in 300 µl PBS containing 8M Urea and Complete EDTA-free Protease Inhibitor (Roche, South Africa). The plant matter was vigorously vortexed to ensure the complete resuspension of agroinfiltrated leaf tissue and the samples incubated at 4ºC for 1 hour; with gentle shaking. The samples were clarified by 10 minutes of centrifugation, at 14000 rpm, and the supernatant stored at -20 °C.

2.25. Direct protein extraction from the apoplastic spaces of agroinfiltrated plant leaves

Agroinfiltrated leaves were syringe infiltrated with 1 ml of sterile PBS supplemented with 1× Complete EDTA-free Protease Inhibitor (Roche, South Africa); as indicated in **Section 2.22**. The leaves were sliced vertically, with a scalpel, on either side of the leaf midrib. The leaf slices were carefully rolled and placed inside the mouth of a spin column from which the silica membrane had been removed (QIAprep Spin Miniprep Kit) (QIAGEN, South Africa). The spin column, containing the agroinfiltrated leaf tissue was transferred to a sterile 2 ml eppindorf tube. The samples were centrifuged at 5000 rpm for 5 minutes; to draw protein from the apoplastic spaces of plant leaves. Residual plant debris remaining in the flow through was collected by centrifugation, at 14 000 rpm for 15 minutes and the supernatant retained at -20°C.

2.26. Western blotting to detect recombinant HIV-1 envelope in extract from agroinfiltrated plant leaves

The crude leaf extract samples were analysed for the presence of the recombinant antigens by western blotting as indicated in **Section 2.18** or using the Mini-PROTEAN® Tetra SDS-PAGE System (Bio-Rad, South Africa). In the case of the latter approach, an 8 µl aliquot of 5× Loading dye (**Appendix B**) was added to 32 µl of leaf extract and the samples resolved on a 7% Acrylamide suspended in 10× Electrophoresis running buffer (**Appendix B**). The protein samples were electrophoresed for 2 hours, at 130 V. The remainder of the western blotting protocol was conducted in accordance with **Section 2.18**. Aliquots of 8 µl of crude *E. coli* inclusion body-derived H5, was used as a positive control during western blotting of the recombinant H5 and H5-ELP antigens (infiltration controls).

Recombinant envelope antigens were detected using a 1:1000 dilution of either Polyclonal anti-gp120 primary antibody (Serotec) or MRC ADP 408/5104 polyclonal anti-gp160 primary antibody. In turn the primary antibody was detected with 1:10 000 dilution of GT34 anti-Sheep/Goat antibody conjugated to alkaline phosphatase

(Sigma-Aldrich). The protocol was later refined by increasing the primary antibody concentration to 1:500, decreasing the secondary antibody concentration to 1:20 000 and increasing the incubation time of the PVDF membrane with the substrate to 1 hour. Additionally, the amount of the recombinant CN54 envelope positive control was decreased to 20 ng.

In contrast recombinant H5-ELP and full length H5 proteins were detected with 1:2000 dilution of anti-his mouse monoclonal primary antibody (AD1.1.10) (Serotec) and 1:5000 dilution of mouse monoclonal H5N1 primary antibody [8D2] (Abcam) respectively. Both primary antibodies were detected with a 1:10 000 dilution of poly-clonal anti-mouse IgG conjugated to alkaline phosphatase (Sigma).

CHAPTER 3: RESULTS

3.1. Selection of an antigenically promising HIV-1 envelope protein for vaccine development

The first stage of this study comprised a highly rational selection process to identify a suitable HIV-1 envelope protein for the development of candidate HIV-1 subunit vaccines. Several candidate viruses were identified from the CAPRISA Acute Infection cohort from participants who developed broadly neutralizing antibodies. All of these viruses were of subtype C origin and had been isolated within the first year post infection, ensuring that the vaccine immunogen was representative of transmitted viruses that are likely to be encountered by sexual exposure (114). An HIV-1 Subtype C viral isolate was specifically chosen for the design of the vaccine immunogens as this clade represents the predominant strain in South Africa and accounts for the majority of infections worldwide (128, 136, 137).

A viral isolate from participant CAP256 was selected for the development of the candidate vaccine immunogens described in this study. The virus of interest was responsible for a secondary HIV-1 infection, approximately 13-15 weeks after primary infection, ultimately leading to the development of broadly cross-neutralizing antibodies that overlapped the PG9/16 antibody epitope (232, 235). It was further demonstrated, by Penny Moore and colleagues, that the viral envelope was highly sensitive to prototype broadly cross-neutralizing monoclonal antibodies comprising all known regions of vulnerability of the envelope glycoprotein (summarized below in **Table 3.1**) (235). The use of this envelope therefore ensured the structural integrity and exposure of important antibody epitopes and allowed us to partly account for any virological variables that may have been responsible for the induction of such a broadly cross-neutralizing antibody response.

Table 3.1: Neutralization sensitivity of the CAP256 superinfecting envelope glycoprotein to prototype broadly neutralizing monoclonal antibodies (modified from (235)**.**

Specificity	CD4bs	MPER	N332	Anti-V2, N160		
Prototype mAb	VRC01	4E10	PGT128	PG9	PG16	PGT145
Sensitivity (µg/ml)	0.6811	3.59	0.0447	0.0453	0.0055	1.25

3.2. Overview of immunogen design

Three novel HIV-1 envelope antigens were designed for use in a heterologous DNA prime-protein boost immunization regimen. The coding sequences of all 3 recombinant proteins were derived from the native viral sequence of the superinfecting CAP256 envelope glycoprotein (*gp160*) (114, 232, 235). The envelope clone (clone 256.3mo.9C) was provided by Dr Penny Moore (Senior Medical Scientist, Centre for HIV and STIs, National Institute for Communicable Diseases, Johannesburg) in the form of an expression cassette cloned into the pcDNA3.1D/V5-His-TOPO® plasmid vector (pLM1). In addition to the CAP256 envelope coding sequence, the plasmid also encoded the 3' end of *rev* exon 1, the *vpu* coding sequence in its entirety and the 5' end of the *nef* gene.

In the case of all 3 antigens, the native HIV-1 envelope signal peptide was substituted with a heterologous signal peptide (LPH) derived from the heavy chain of murine mAb24. The rationale underlying this decision was largely due to the retarded maturation of the HIV-1 envelope glycoprotein during its biosynthesis which has been attributed to the inefficient cleavage of the signal peptide (60, 82, 192). This ultimately results in prolonged retention of the glycoprotein in the ER and hinders its efficient production (182, 191). Additionally, several studies have shown that the expression of the glycoprotein could be dramatically improved in heterologous systems by the use of foreign signal peptides in place of the native HIV-1 envelope signal sequence (23, 107, 189). Furthermore the LPH signal peptide ensures the translocation of the recombinant protein through the ER and into the apoplastic spaces of plant leaves via the natural cell secretory pathway.

The first antigen comprised of a truncated derivative of the full length HIV-1 envelope glycoprotein, gp160. The coding sequence was prematurely terminated by means of a truncation in the C terminal cytoplasmic tail (Cyt) to generate gp150, as described for the SAAVI DNA and MVA vaccines currently being evaluated in clinical trials (34, 35, 317). The rationale underlying this approach was based on reports that truncation of the HIV-1 envelope cytoplasmic tail results in a partially triggered conformation that favours the exposure of conserved neutralization sensitive epitopes (83). Additionally, Vzorov *et al.* have reported that truncation of the SIV envelope cytoplasmic tail culminated in enhanced expression by DNA vaccines *in vitro* which correlated with improved immunogenicity *in vivo* (374). The gp150 immunogen retained the exterior subunit region of the HIV envelope glycoprotein (gp120) along with the native ectodomain (Ecto), transmembrane domain (TM) and a portion of the cytoplasmic tail of the gp41 transmembrane subunit. The putative structure of the monomeric form of the gp150 glycoprotein is shown in the schematic in **Figure 3.1**. It should be noted, however that the gp150 glycoprotein normally exists as a trimer.

The other 2 immunogens comprised of chimeric fusion proteins encompassing elements of the HIV-1 envelope glycoprotein (gp160) and the transmembrane subunit (HA_2) of H5N1 influenza haemagglutinin glycoprotein. The first chimera, gp120-HA_2, retained the full length exterior subunit of the HIV-1 envelope glycoprotein (gp120) translationally fused to the full length transmembrane subunit of influenza H5 (HA_2). The transmembrane subunit, HA_2, comprised the cytoplasmic tail, transmembrane and ectodomain portions of the influenza haemagglutinin; essentially replacing the analogous regions of the HIV-1 gp41 subunit (**Figure 3.2**).

The second chimera, gp140-HA_2tr, comprised of the exterior subunit (gp120) and ectodomain of HIV-1 gp160 translationally fused to a truncated derivative of the HA_2 subunit of haemagglutinin. The HA_2 subunit was truncated at the N terminus to remove the ectodomain of the protein which protrudes beyond the exterior of the virion. The chimeric glycoprotein retained both the transmembrane region and cytoplasmic tail of haemagglutinin with the ectodomain of HIV-1 envelope replacing the truncated portion of HA_2 (**Figure 3.2**).

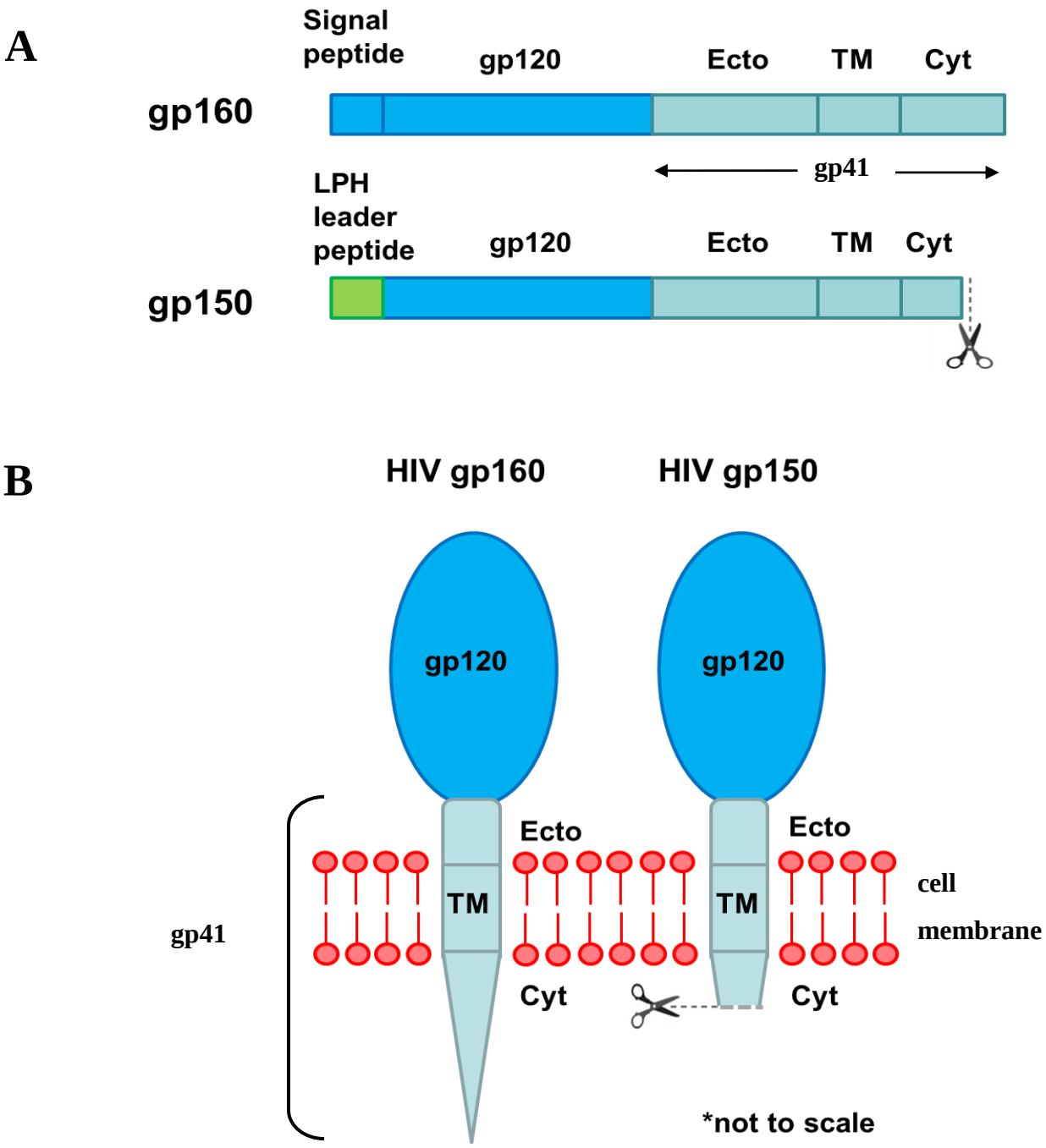

Figure 3.1: Annotated schematic of A) the coding sequence and B) the putative structure of the recombinant gp150 glycoprotein in its monomeric form. The gp150 immunogen was derived from the native coding sequence of the CAP256 superinfecting virus which has been truncated in the cytoplasmic tail region and the native signal sequence substituted for the LPH signal peptide. The ectodomain (Ecto), transmembrane domain (TM) and cytoplasmic tail (Cyt) of the antigens are indicated in the figure above.

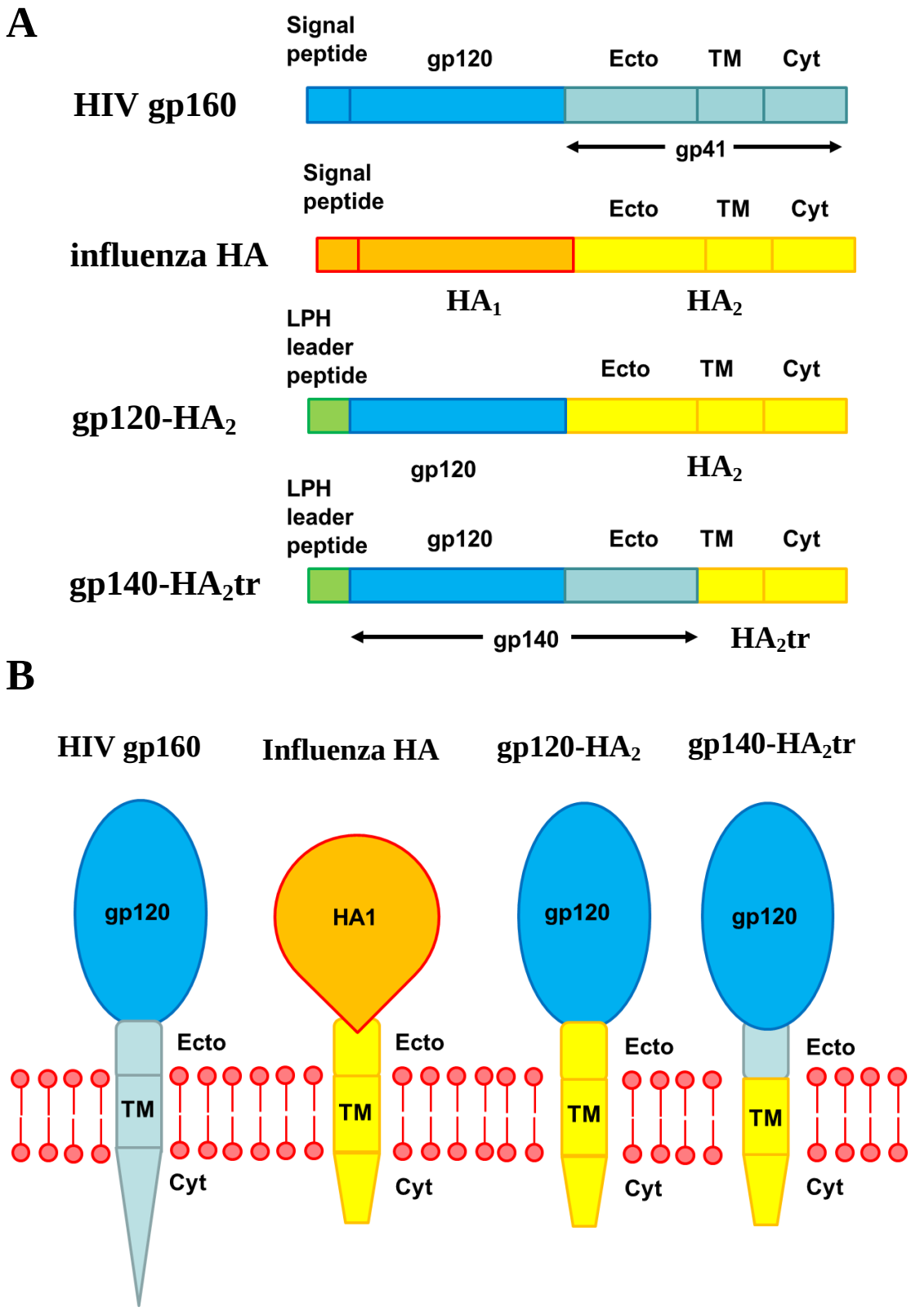

Figure 3.2: Annotated schematic of A) the coding sequence and B) the putative structure of the chimeric gp120-HA_2 and gp140-HA_2tr antigens in their monomeric form. The chimeric antigens comprised of translation fusions between elements of the HIV-1 CAP256 superinfecting virus envelope glycoprotein and the influenza transmembrane subunit (HA_2) of influenza H5 glycoprotein. The ectodomain (Ecto), transmembrane domain (TM) and cytoplasmic tail (Cyt) of the antigens are indicated in the figure above.

3.3. Cloning strategy used for the construction of recombinant pTRA-A expression vectors encoding novel HIV-1 envelope antigens

The coding sequences of the antigens were constructed by PCR using plasmid DNA templates encoding the HIV-1 CAP256 superinfecting virus envelope and the full length influenza H5 haemagglutinin gene of H5N1. As alluded to earlier, the superinfecting envelope clone was received as a *rev-vpu-env-nef* expression cassette encoded by the pcDNA3.1D/V5-His-TOPO® plasmid vector (pLM1). In the case of the influenza H5 haemagglutinin template; the *A. tumefaciens* GV3101::pMP90RK (pTRA-H5) clone, generated in an independent study, was obtained from Sandiswa Mbewana (PhD candidate, Department of Molecular and Cell Biology, University of Cape Town) (238). This recombinant strain of *A. tumefaciens* had been transformed with recombinant pTRA-A encoding a humananized version of the influenza H5 haemagglutinin gene (A/Viet Nam/1194/2004).

The gp150 PCR product was amplified directly from the CAP256 envelope coding sequence in a single PCR reaction. In contrast, the 2 chimeric glycoproteins were constructed by overlap-extension PCR (**Figure 3.3**). This technique allowed the in frame fusion of 2 DNA fragments from unrelated sources, without the introduction of synthetic restriction enzyme recognition sequences which would alter the amino acid sequence of the protein (133). The overlap-extension PCR involved 3 sequential PCR reactions to generate a translational fusion between 2 DNA fragments. The 2 fragments were first amplified in separate reactions using primers that generated a complementary overlap at the 3' end of the HIV-1 envelope PCR product and the 5' end of the influenza HA_2 PCR product. The 2 DNA fragments were then used as templates in a third reaction where the templates were denatured and annealed to each other by their complementary overlap to prime the final reaction (360). The coding sequences of each of the antigens were then cloned into the pJET1.2/blunt vector. The PCR fragment was excised from the plasmid backbone by means of a *Nco*I-*Spe*I dual restriction enzyme digest and cloned into the pTRA-A plasmid vector, in place of the H5 insert which had been liberated from the plasmid using *Nco*I and *Xba*I restriction enzymes (**Figure 3.4-3.6**).

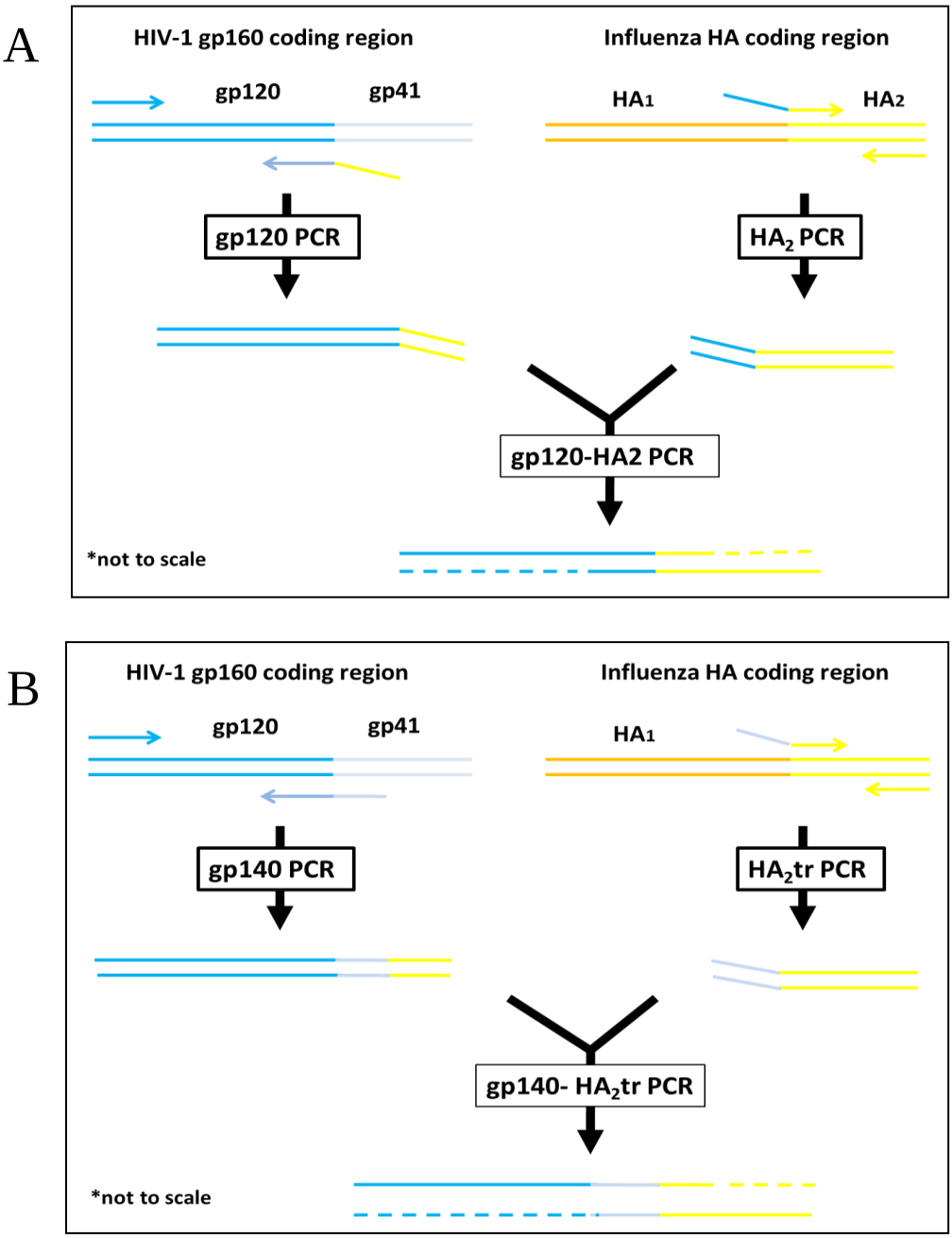

Figure 3.3: Construction of; A) the gp120-HA_2 and B) gp140-HA_2tr chimeric glycoprotein gene sequences by overlap extension PCR.

In order to maintain the reading frame a single nucleotide was incorporated into the coding sequence of each of the recombinant proteins resulting in the addition of a valine residue at the N terminus of the protein, immediately after the LPH signal peptide. Synthetic *Nco*I and *Spe*I restriction enzyme recognition sites were incorporated into the primers that annealed to the terminal ends of the coding sequences in order to facilitate the cloning of all 3 PCR products into the pTRA-A expression vector.

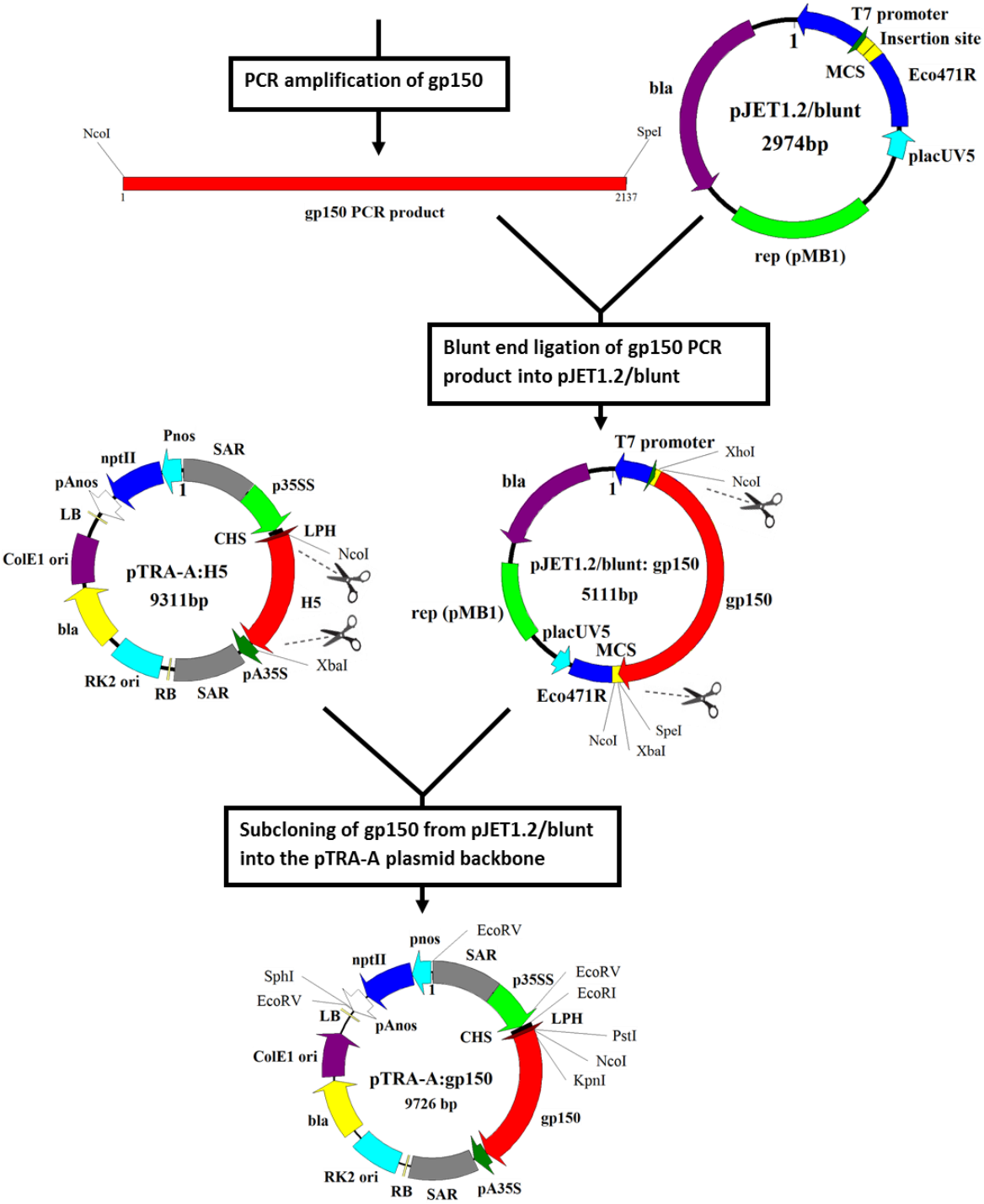

Figure 3.4: Overview of the cloning strategy used for the construction of the recombinant pTRA-A: gp150 plant expression vector. (RK2 ori = *A. tumefaciens* origin of replication, ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, SAR = Scaffold attachment region, LB = left border, RB = Right border, p35SS = CaMV duplicated transcriptional enhancer, pA35S = CaMV polyadenylation signal, LPH = signal peptide, CHS = chalcone synthase 5' untranslated region, Pnos = *nopaline synthase* promoter, pAnos = *nopaline synthase* polyadenylation signal, *npt*II = kanamycin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E.coli* origin of replication, MCS = multiple cloning site).

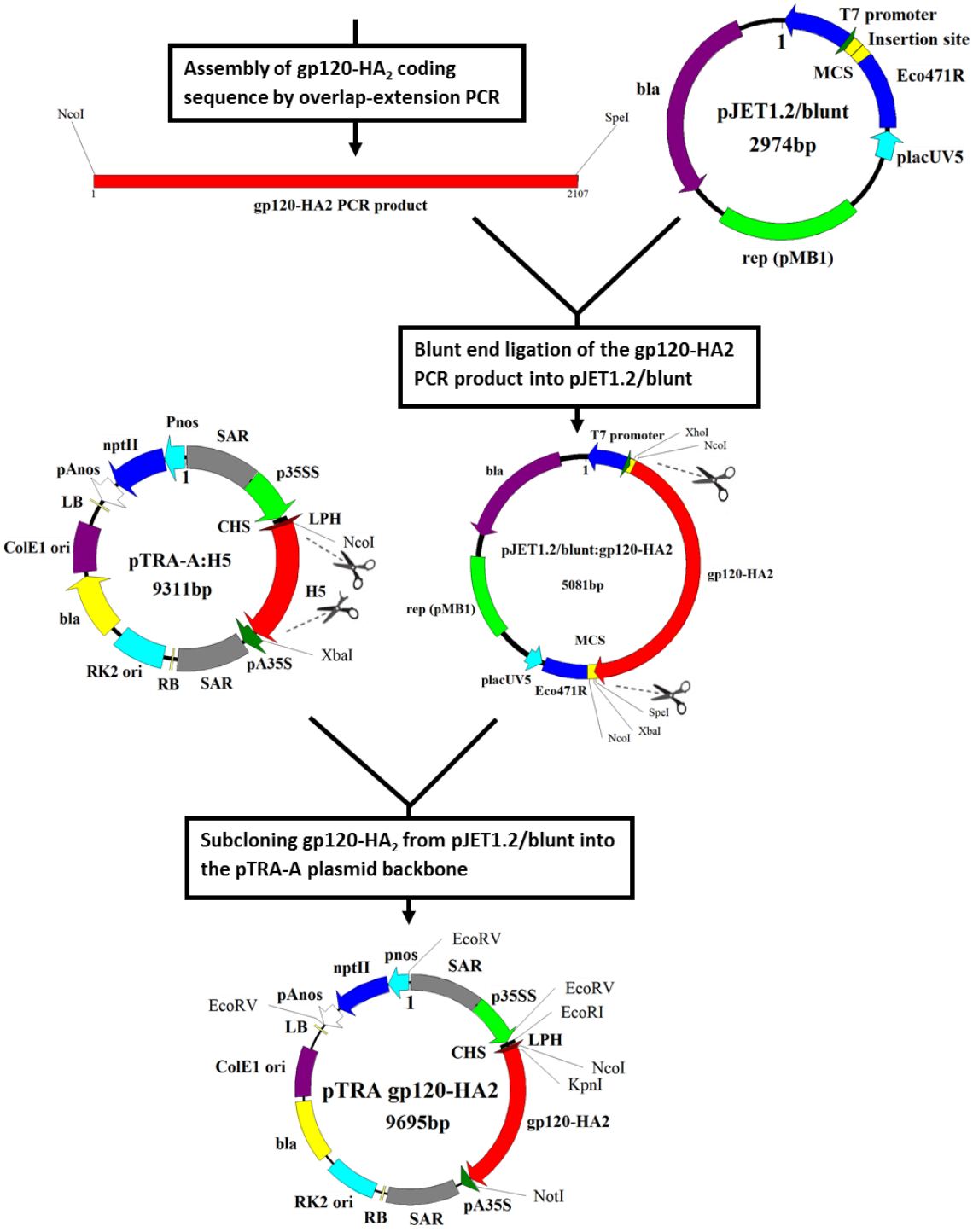

Figure 3.5: Overview of the cloning strategy used for the construction of the recombinant pTRA-A: gp120-HA$_2$ plant expression vector. (RK2 ori = *A. tumefaciens* origin of replication, ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, SAR = Scaffold attachment region, LB = left border, RB = Right border, p35SS = CaMV duplicated transcriptional enhancer, pA35S = CaMV polyadenylation signal, LPH = signal peptide, CHS = chalcone synthase 5' untranslated region, Pnos = *nopaline synthase* promoter, pAnos = *nopaline synthase* polyadenylation signal, *npt*II = kanamycin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E.coli* origin of replication, MCS = multiple cloning site).

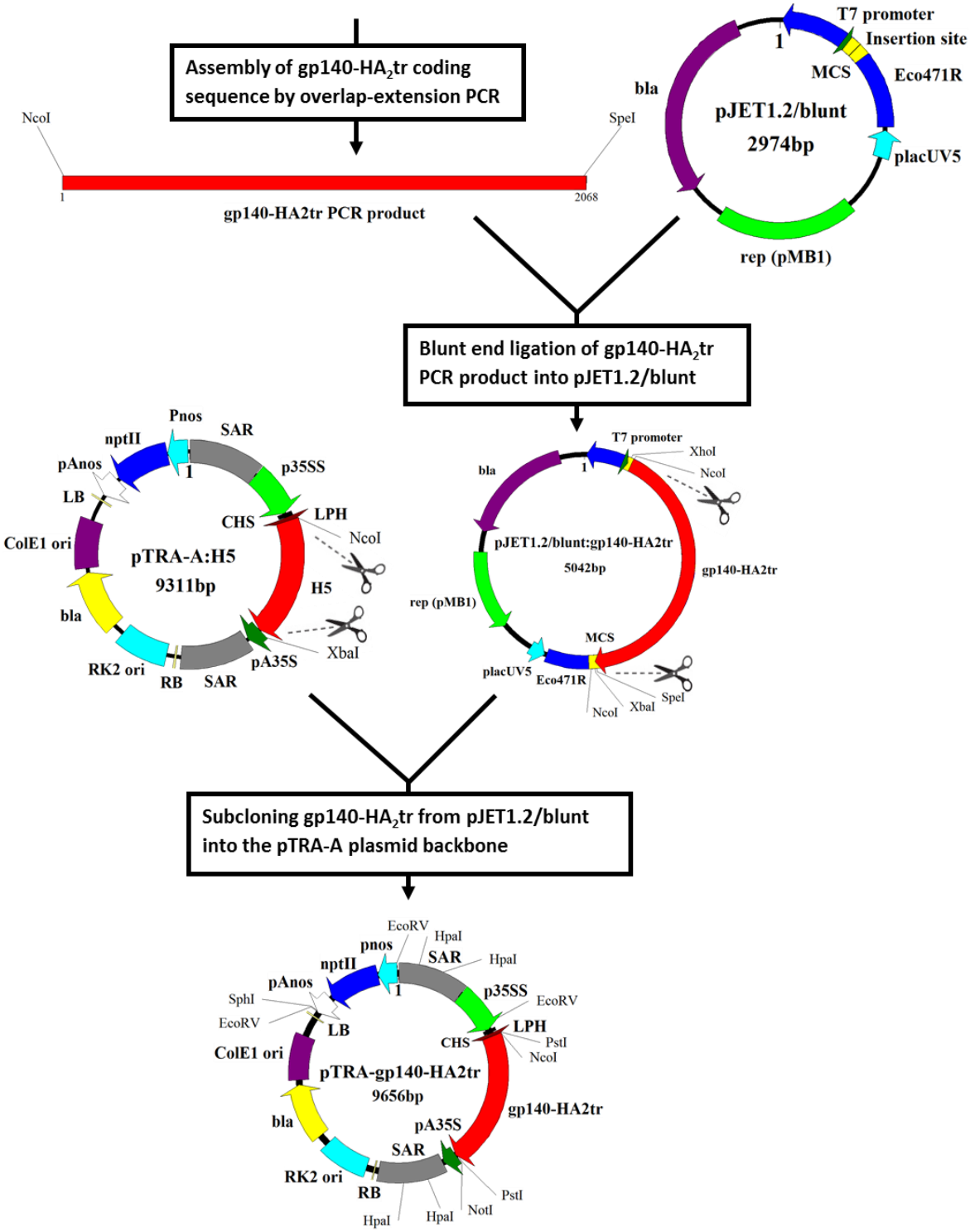

Figure 3.6: Overview of the cloning strategy used for the construction of the recombinant pTRA-A: gp140-HA$_2$tr plant expression vector. (RK2 ori = *A. tumefaciens* origin of replication, ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, SAR = Scaffold attachment region, LB = left border, RB = Right border, p35SS = CaMV duplicated transcriptional enhancer, pA35S = CaMV polyadenylation signal, LPH = signal peptide, CHS = chalcone synthase 5' untranslated region, Pnos = *nopaline synthase* promoter, pAnos = *nopaline synthase* polyadenylation signal, *npt*II = kanamycin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E.coli* origin of replication, MCS = multiple cloning site).

3.4. PCR amplification of the gp150 coding sequence and assembly of the chimeric gp120-HA_2 and gp140-HA_2tr genes

A 2137 bp DNA fragment, comprising of the gp150 coding sequence, was amplified directly from the pLM1 plasmid DNA template (**Figure 3.7)**.

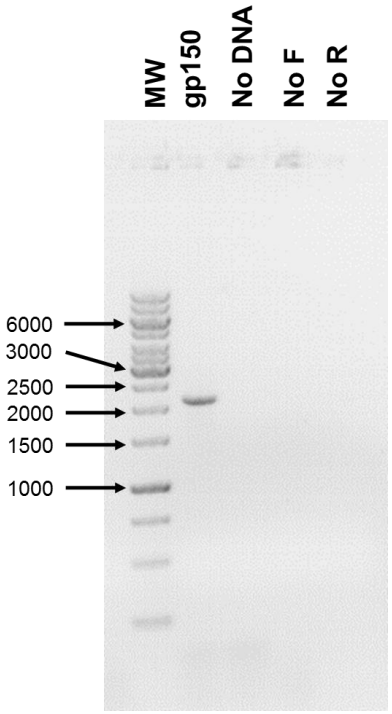

Figure 3.7: Agarose gel electrophoresis of the gp150 PCR product along with controls whereby template DNA (No DNA), Forward primer (No F) and reverse primer (No R) were omitted. The PCR products were resolved on a 0.8% agarose gel alongside a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA ladder.

The gp120-HA_2 and gp140-HA_2tr coding sequences were both assembled by overlap extension PCR using sequential PCR reactions. In the case of the chimeric gp120-HA_2 antigen; the gp120 and HA_2 fragments were first amplified in separate PCR reactions giving rise to DNA fragments of 1445 bp and 697 bp respectively (**Figure 3.8 A** and **Figure 3.8 B**). The 2 PCR products were then denatured and annealed to each other in a third reaction to prime the amplification of the chimeric gp120-HA_2 gene. The final reaction yielded a 2107 bp DNA fragment corresponding to the chimeric gene (**Figure 3.8 C**).

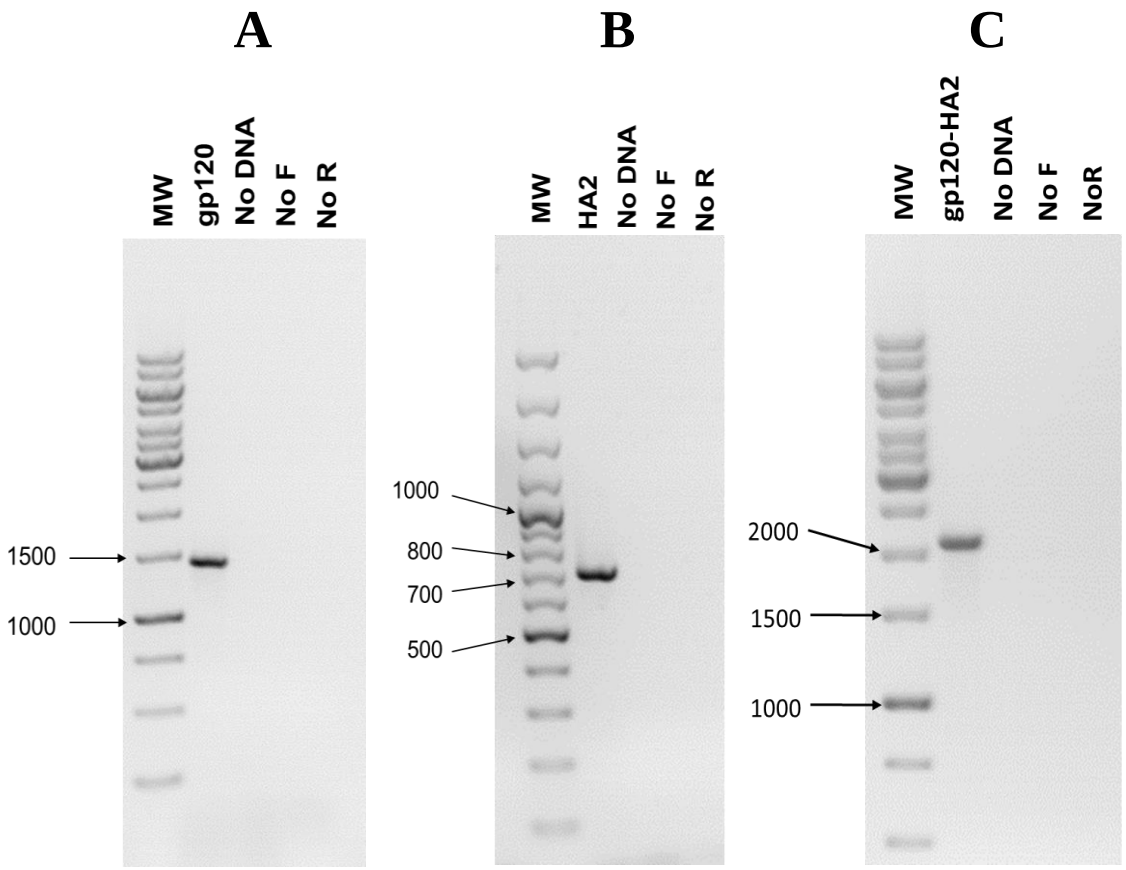

Figure 3.8: PCR amplification of A) the gp120 coding sequence, B) the HA_2 coding sequence and C) the chimeric gp120-HA_2 antigen. Each PCR reaction was performed in parallel with controls whereby template DNA (No DNA), forward primer (No F) and reverse primer (No R) were omitted. The PCR products were resolved on a 0.8% agarose gel alongside either 10 µl of Fermentas O'GeneRuler™ 1kb DNA ladder for the gp120 and gp120-HA_2 PCR products or Fermentas O'GeneRuler™ 100 bp Plus DNA ladder for the HA_2 PCR products.

Similarly, the gp140-HA_2tr gene sequence was also assembled by overlap extension PCR. The gp140 and HA_2tr coding sequences were first individually amplified giving rise to PCR products of 1971 bp and 145 bp respectively (**Figure 3.9 A** and **Figure 3.9 B**). The 2 products were then used as templates to assemble the 2168 bp chimeric gp140-HA_2tr gene sequence (**Figure 3.9 C**).

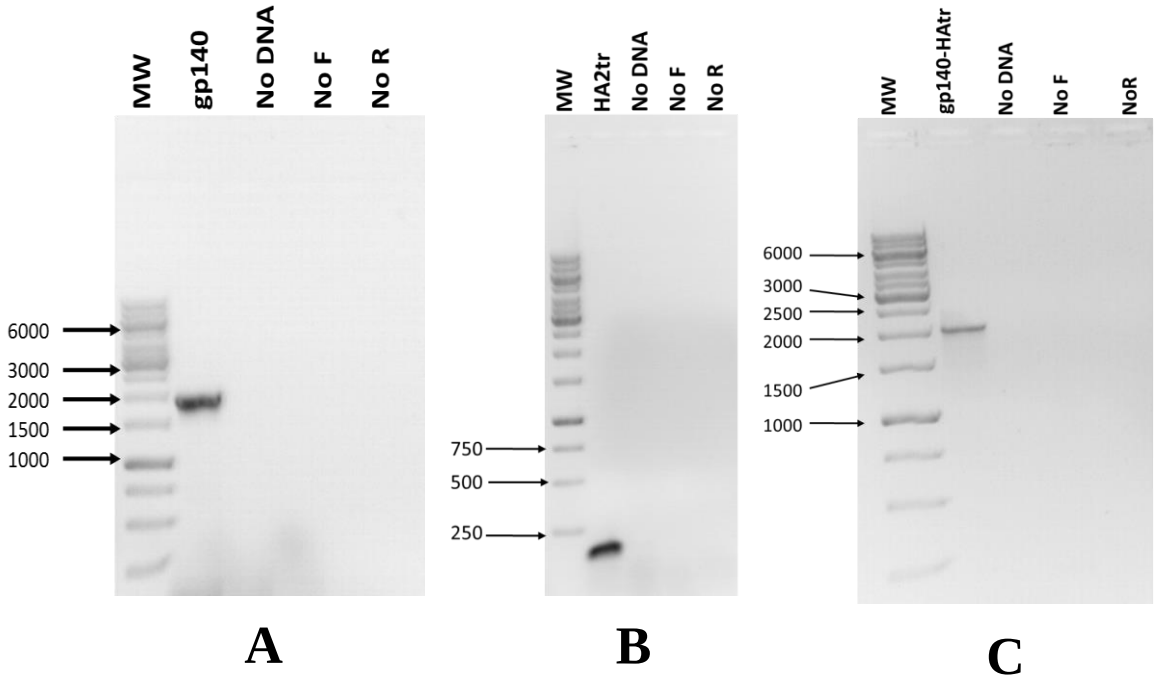

Figure 3.9: PCR amplification of A) the gp140 coding sequence, B) the HA_2tr coding sequence and C) the coding sequence of gp140-HA_2tr chimeric antigen. Each PCR reaction was performed in parallel with controls whereby template DNA (No DNA), Forward primer (No f) and reverse primer (No R) were omitted. The PCR products were resolved on a 0.8% agarose gel alongside a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA.

3.5. Construction of recombinant pTRA-A vectors encoding the gp150, gp120-HA2 and gp140-HA_2tr antigens

The antigen coding sequence for each immunogen was amplified by PCR and the resulting PCR products purified and ligated into the pJET1.2/blunt cloning vector. The ligation products were transformed into *E. coli* DH5α cells and putative recombinants screened by restriction analysis. The PCR product was then excised from the pJET1.2/blunt vector backbone and ligated into the pTRA-A: H5 plasmid vector, in place of the H5 insert. Products of the ligation reaction were transformed into *E. coli* DH5α cells and putative recombinants screened by restriction enzyme digestion. A single recombinant colony was propagated and subjected to a medium scale DNA isolation to obtain sufficient DNA for expression studies *in planta*.

The genetic integrity of each of the recombinant pTRA-A expression vectors was verified by restriction analysis using the restriction enzymes outlined in **Table 3.2**

(**Figure 3.10 A-C**) and independently confirmed by sequencing using the primers outlined in **Section 2.14; Table 2.11**.

Table 3.2: Summary of the enzymes used to verify the genetic integrity of the recombinant pTRA-A expression vectors designed in this study.

Plasmid	Enzyme (s)	Expected products (bp)
pTRA-A:gp150	*Eco*RV	1405, 1652, 6669
	*Kpn*I	9726
	*Eco*RI- *Sph*I	3142, 6584
	*Nco*I	9726
	*Pst*I	9726
pTRA-A:gp120-HA$_2$	*Eco*RI	9695
	*Eco*RV	1405, 1652, 6638
	*Kpn*I- *Not*I	2041, 7654
	*Nco*I	9695
pTRA-A:gp140-HA$_2$tr	*Eco*RV	1405, 1652, 6599
	*Pst*I	2045, 7611
	*Nco*I- *Not*I	2043, 7613
	*Sph*I- *Kpn*I	3292, 6364
	*Eco*RI	9656
	*Hpa*I	348, 348, 3769, 5191

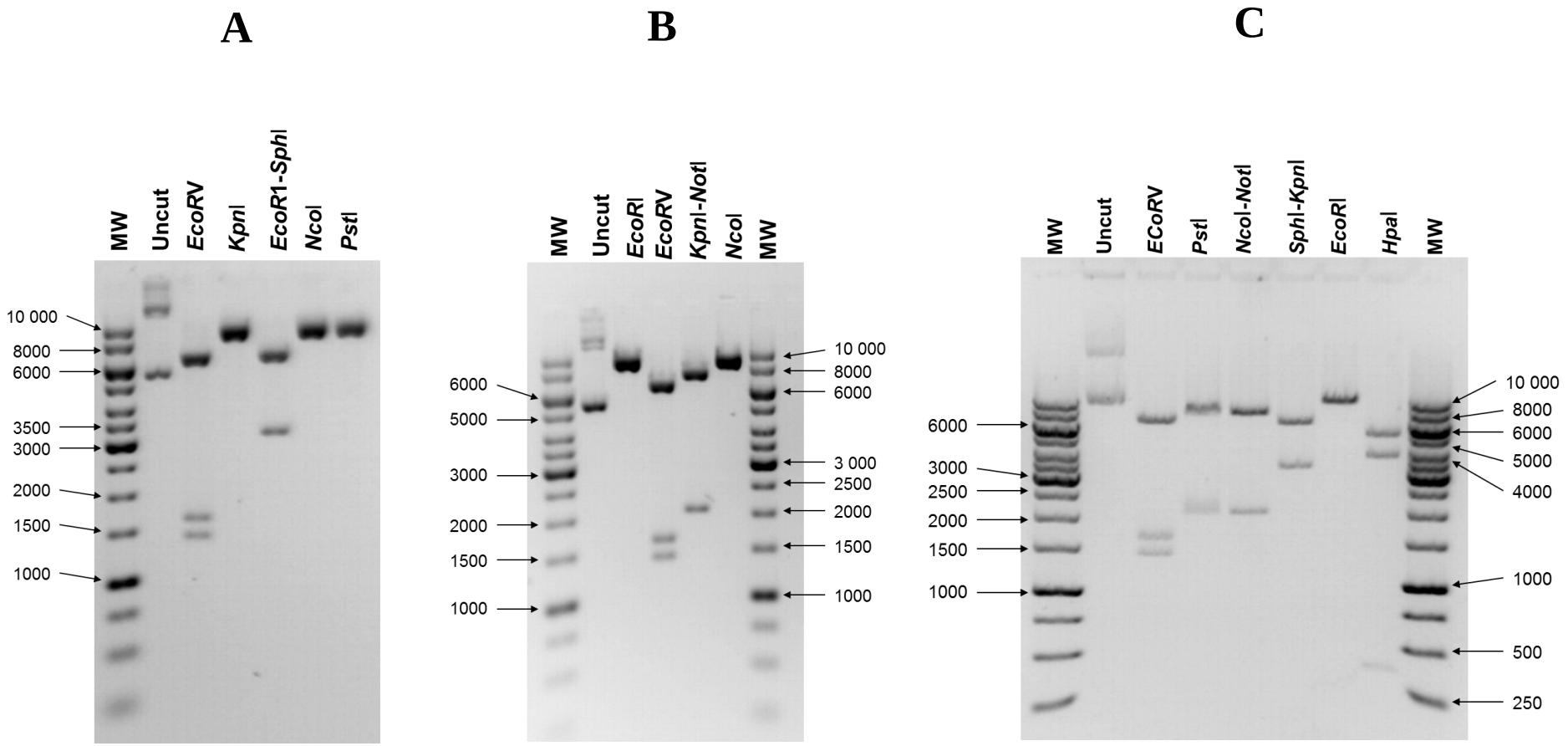

Figure 3.10: Restriction analysis to verify the genetic integrity of A) pTRA-A: gp150, B) pTRA-A: gp120-HA$_2$ and C) pTRA-A: gp140-HA$_2$tr. The samples were electrophoresed on a 0.8% agarose gel alongside a 250 ng aliquot of undigested plasmid DNA and a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA (MW). The restriction enzymes used are indicated above each lane on the agarose gel.

3.6. Cloning strategy used for the construction of matched pTHPcapR-vectored DNA vaccines

Three matched DNA vaccines were constructed, using the pTHPcapR DNA vaccine vector system, enabling their potential use in a heterologous prime-boost vaccination regimen with the recombinant plant-produced antigens. The antigen coding sequence of each of the 3 vaccines was amplified directly by PCR, in a single reaction, from the recombinant pTRA-A vectors assembled in **Section 3.5**. Each of the immunogens retained the LPH signal peptide, which was substituted for the native HIV-1 envelope signal sequence for expression *in planta*, as well as the same transcriptional initiation and termination signals. The immunogens were amplified with primers containing synthetic 5' *Hind*III and 3' *Xba*I restriction enzyme recognition sequences enabling the antigens to be cloned into the pTHPcapR DNA vaccine backbone.

The pTJDNA4 plasmid, developed in an independent study, was obtained from Tsungai Jongwe (PhD candidate, Department of Medical Virology, University of Cape Town). The plasmid vector, comprising of the pTHcapR DNA vaccine vector with porcine circovirus-derived enhancer element, encodes a full length HIV-1 subtype C *gag* mosaic immunogen flanked by *Hind*III and *Xba*I restriction enzyme recognition sequences. The coding sequences of each of the three antigens were amplified by PCR and cloned into the pJET1.2/blunt cloning vector. The gene sequences were then excised from the pJET1.2/blunt plasmid backbone by means of a *Hind*III-*Xba*I dual restriction enzyme digest and cloned into the pTHPcapR plasmid vector, in place of the HIV-1 mosaic insert which had been liberated from the plasmid using *Hind*III and *Xba*I restriction enzymes (depicted in **Figure 3.11**-**3.13**).

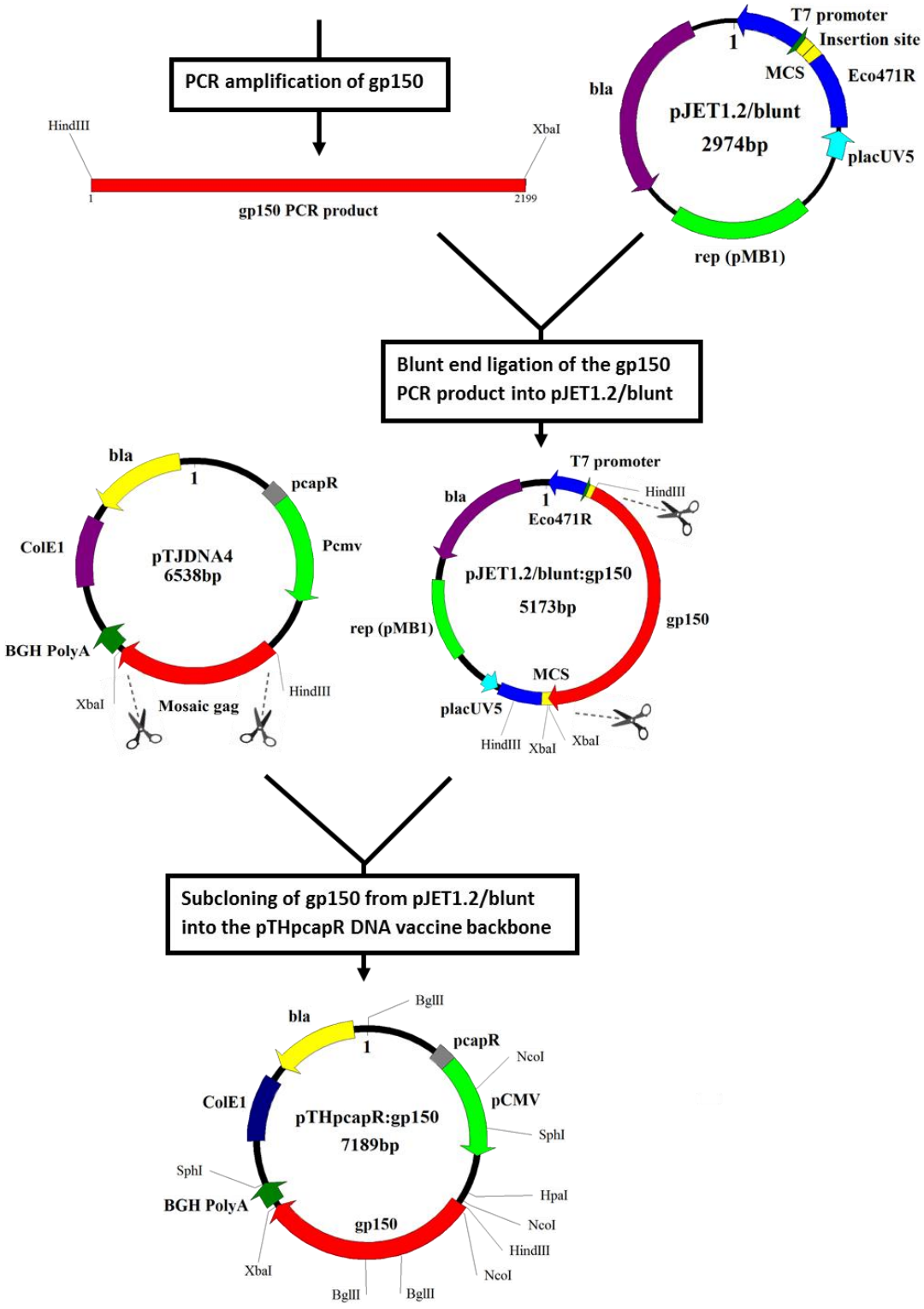

Figure 3.11: Overview of the cloning strategy used for the construction of the pTHPcapR: gp150 DNA vaccine. (ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E. coli* origin of replication, MCS = multiple cloning site, PcapR = porcine circovirus type 1 enhancer element, Pcmv = human CMV enhancer/promoter/intron region, BGH PolyA = bovine growth hormone polyadenylation signal).

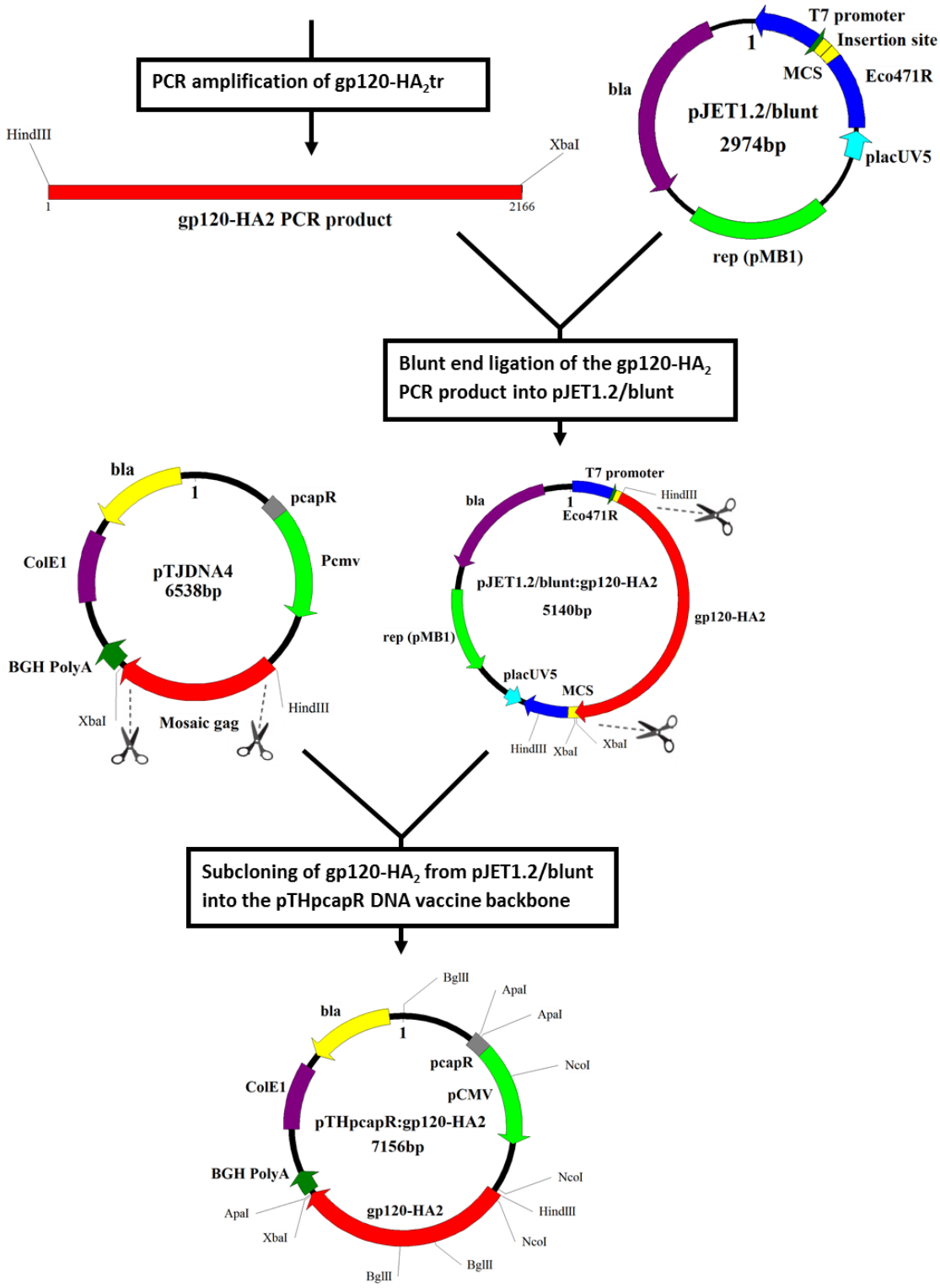

Figure 3.12: Overview of the cloning strategy used for the construction of the pTHPcapR: gp120-HA$_2$ DNA vaccine. (ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E. coli* origin of replication, MCS = multiple cloning site, PcapR = porcine circovirus type 1 enhancer element, Pcmv = human CMV enhancer/promoter/intron region, BGH PolyA = bovine growth hormone polyadenylation signal).

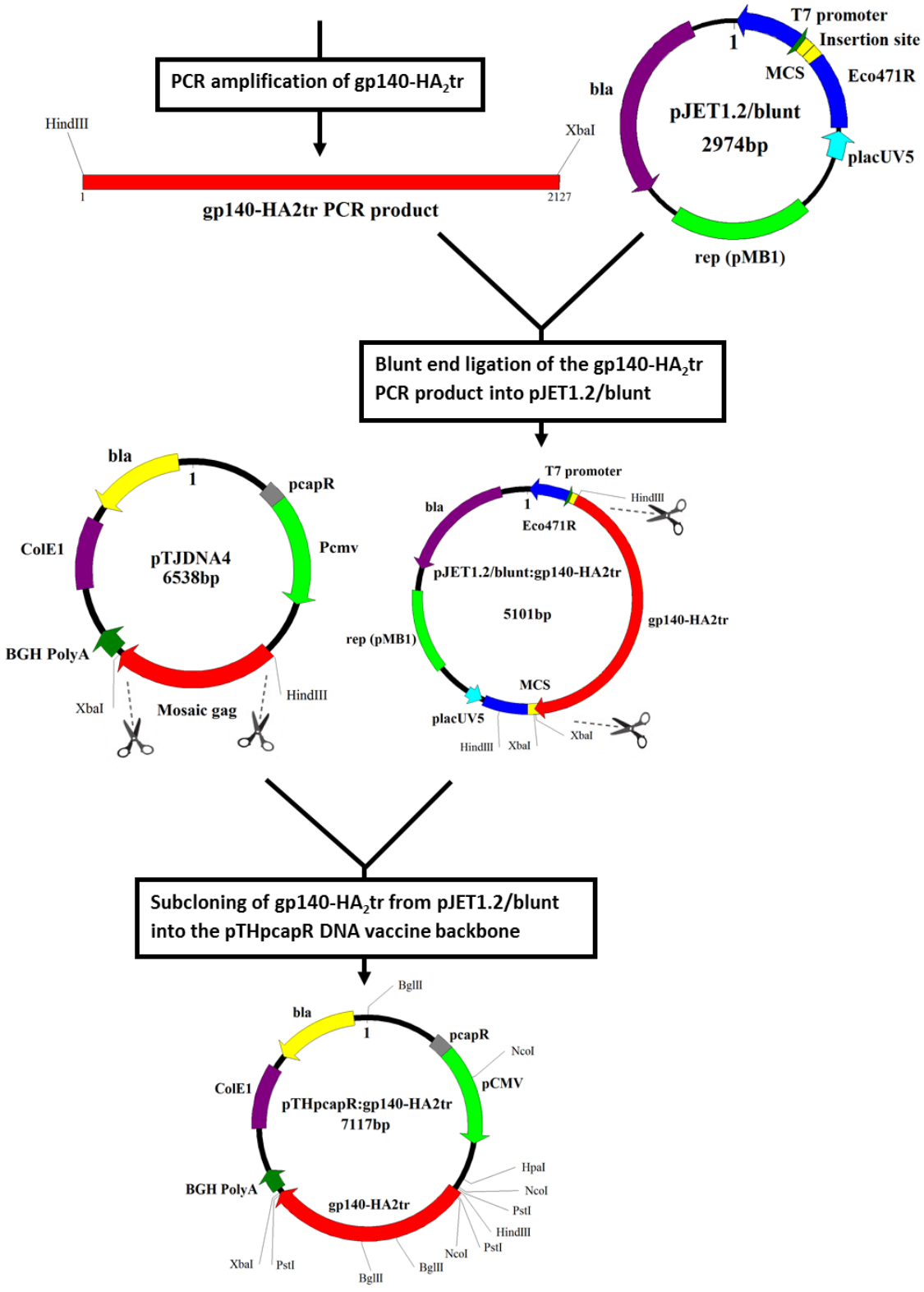

Figure 3.13: Overview of the cloning strategy used for the construction of the pTHPcapR: gp140-HA$_2$tr DNA vaccine. (ColE1 = *E. coli* origin of replication, bla = ampicillin/carbenicillin resistance gene, Eco471R = lethal selection gene, PlacUV5 = modified Plac promoter, rep(pMB1) = *E. coli* origin of replication, MCS = multiple cloning site, PcapR = porcine circovirus type 1 enhancer, Pcmv = human CMV enhancer/promoter/intron region, BGH PolyA = bovine growth hormone polyadenylation signal).

3.7. PCR amplification of the gp150, gp120-HA_2 and gp140-HA_2tr antigen coding sequences for the construction of pTHPcapR-vectored DNA vaccines

The coding sequence of each of the vaccine antigens was amplified by PCR from the recombinant pTRA-A vectors in order to add synthetic restriction sites to the terminal ends of the genes for cloning. A 2199 bp fragment, comprising the gp150 coding sequence, was amplified directly from the pTRA-A: gp150 plasmid DNA template (**Figure 3.14 A**). Similarly the 2 chimeric antigens were amplified directly from their corresponding pTRA-A expression vectors in a single step PCR reaction giving rise to PRC products of 2166 bp and 2127 bp for the gp120-HA_2 and gp140-HA_2tr products respectively (**Figure 3.14 B** and **Figure 3.14 C**).

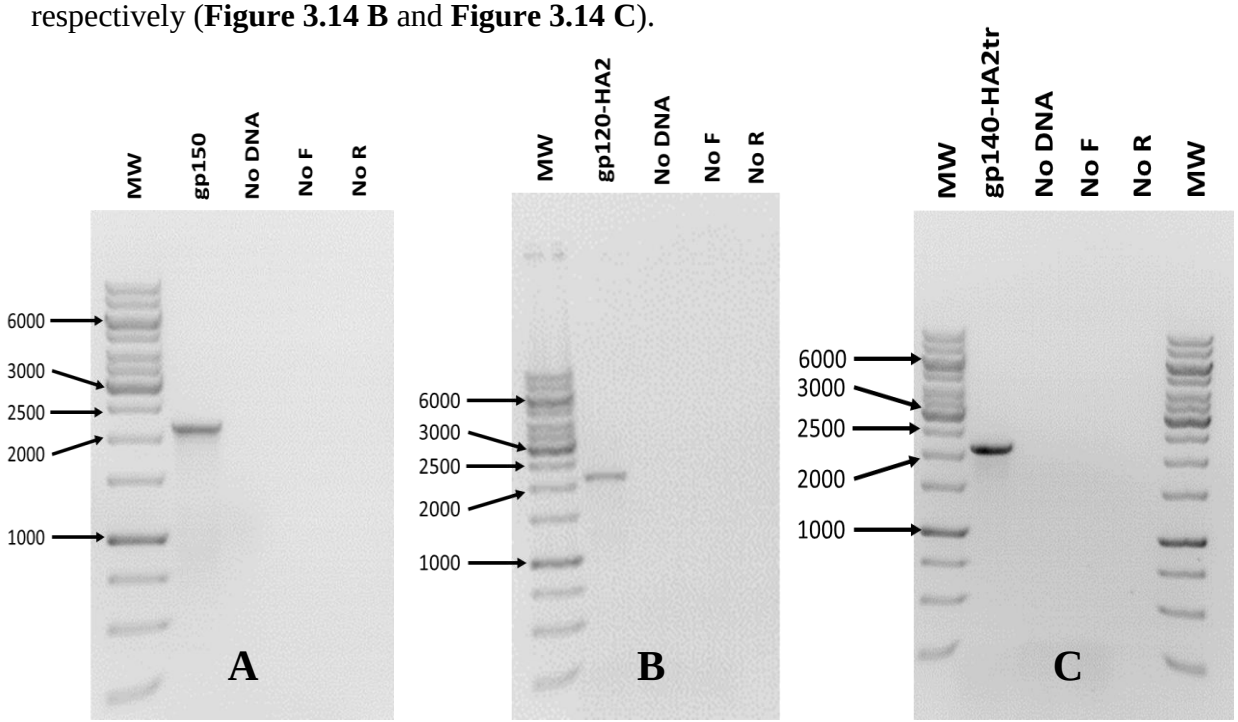

Figure 3.14: PCR amplification of A) the gp150 coding sequence, B) the gp120-HA_2 coding sequence and C) the gp140-HA_2tr coding sequence. Each PCR reaction was performed in parallel with controls whereby template DNA (No DNA), Forward primer (No F) and reverse primer (No R) were omitted. The PCR products were resolved on a 0.8% agarose gel alongside a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA.

3.8. Construction of recombinant pTHPcapR DNA vaccines encoding the gp150, gp120-HA_2 and gp140-HA_2tr antigens

The pTHPcapR-vectored DNA vaccines were constructed using the same approach described for the construction of the recombinant pTRA-A expression vectors (**Section 3.3**). Briefly, the antigen coding sequences were PCR amplified and cloned into the pJET1.2/blunt plasmid backbone. The PCR product was then excised from the plasmid backbone and cloned into the pTHPcapR backbone in place of the HIV subtype C mosaic *gag* gene. The genetic integrity of the DNA vaccines were verified by restriction analysis using the enzymes outlined in **Table 3.3** (**Figure 3.15**).

Table 3.3: Restriction enzymes used to verify the genetic integrity of the recombinant pTHPcapR DNA vaccines.

Plasmid	Enzyme (s)	Expected products (bp)
pTHPcapR:gp150	*Hind*III-*Xba*I	97, 1152, 2126, 3814
	*Bgl*II	338, 3258, 3593
	*Nco*I	97, 1152, 5940
	*Sph*I	3238, 3951
	*Hpa*I	7189
pTHPcapR:gp120-HA_2	*Hind*III-*Xba*I	5002, 2154
	*Bgl*II	338, 3258, 3260
	*Nco*I	97, 1152, 5907
	*Apa*I	29, 3296, 3831
pTHPcapR:gp140-HA_2tr	*Hind*III-*Xba*I	5002, 2115
	*Bgl*II	338, 3521, 3258
	*Nco*I	97, 1152, 5868
	*Hpa*I	7117
	*Pst*I	69, 2045, 5003

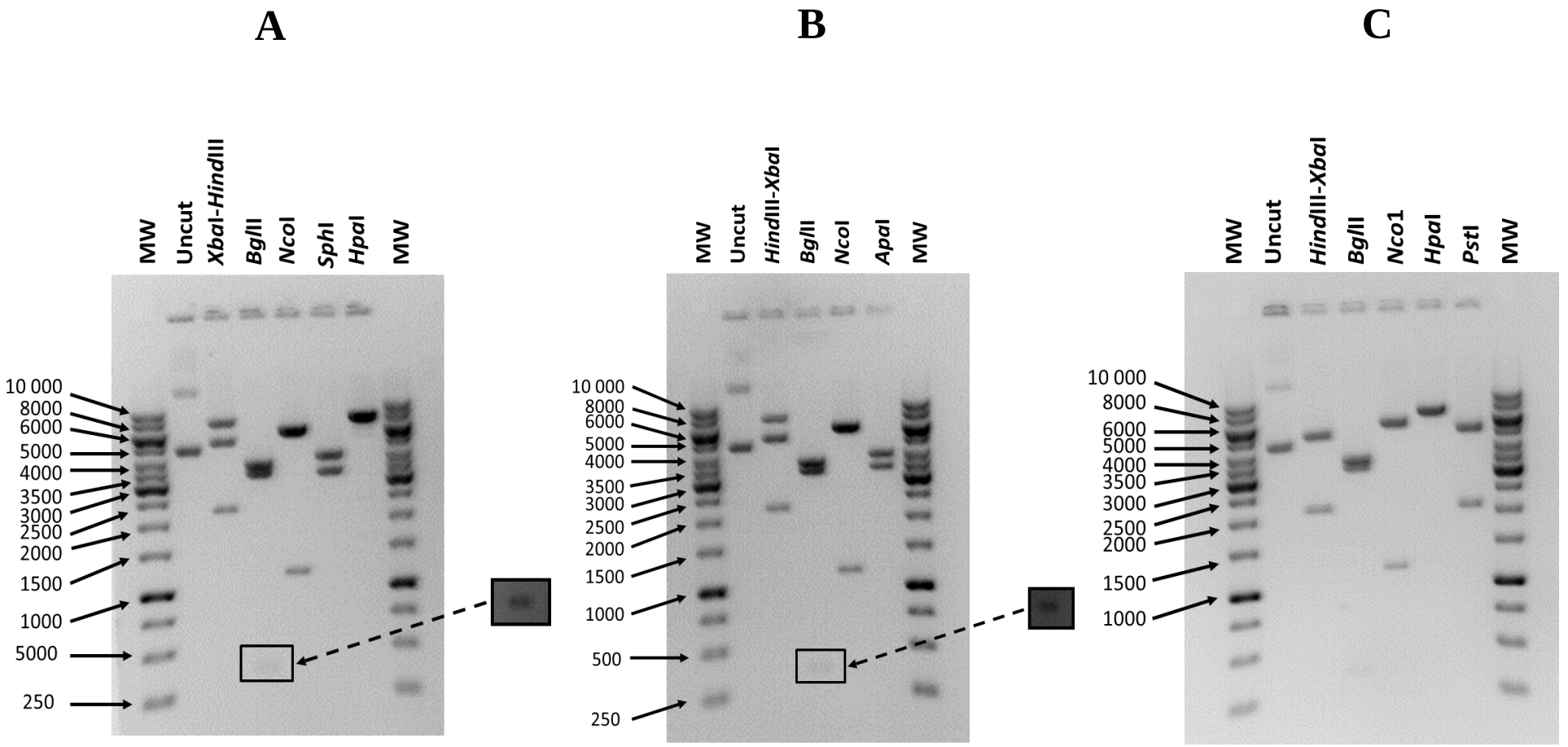

Figure 3.15: Restriction analysis to verify the structural integrity of; A) pTHPcapR: gp150, B) pTHPcapR: gp120-HA_2 and C) pTHPcapR: gp140-HA_2tr plasmid DNA. The samples were electrophoresed on a 0.8% agarose gel alongside a 250 ng aliquot of undigested plasmid DNA (uncut) and a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA (MW). The restriction enzymes used are indicated above each lane on the agarose gel.

In the case of all 3 plasmids, the products yielded by the enzymatic reactions were of the expected sizes (**Figure 3.15 A-C**). The integrity of the 3 DNA vaccines was independently verified by sequencing using the primers outlined in **Section 2.15; Table 2.11.**

3.9. Characterization of pTHPcapR DNA vaccines *in vitro*

3.9.1. Western blotting of transfected HEK293 cell lysate

Western blotting was used to confirm expression of the immunogens in transfected HEK 293 cells. Both the the MRC ADP 408/5104 polyclonal anti-gp160 primary antibody (**Figure 3.16 A**) and the Serotec polyclonal anti-gp120 primary antibody (**Figure 3.16 B**) reacted with the CAP256 derived antigens present in transfected HEK 293 cell lysate samples. In both western blots high levels of all 3 immunogens were detected with negligible non-specific reactivity. Both antibodies also yielded distinct bands for the recombinant CN54 positive control and the SAAVI-C2 DNA vaccine at the expected sizes of 130 kDa and 150 kDa respectively. However, neither antibody detected any discernible expression of the parental CAP256 envelope glycoprotein in HEK 293 cells transfected with pLM1, from which the vaccine antigens were derived.

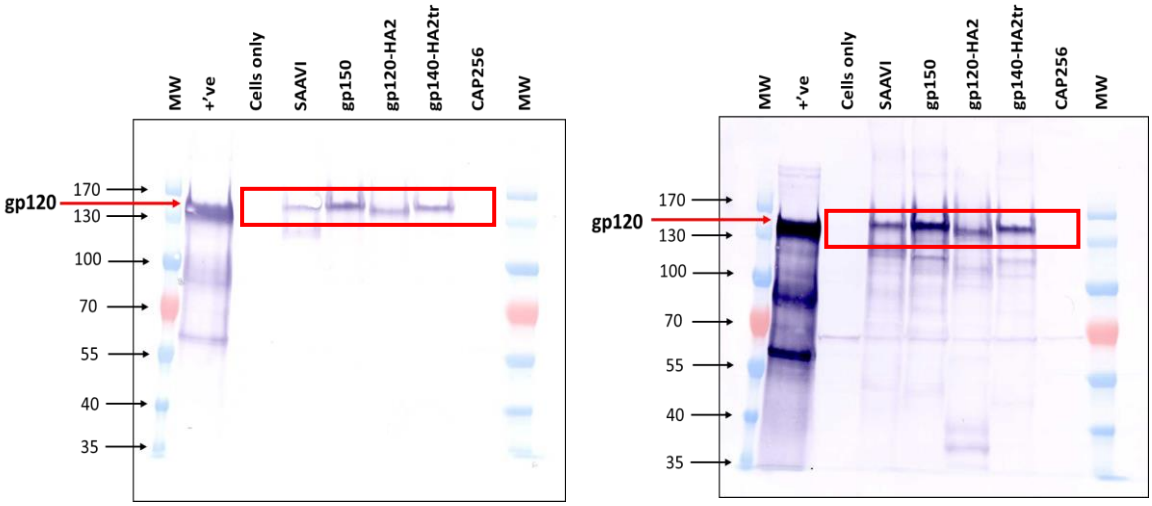

Figure 3.16: Western blotting of transfected HEK293 cell lysate using; A) MRC ADP 408/5104 polyclonal anti-gp160 primary antibody and B) Serotec polyclonal anti-gp120 primary antibody. The cell lysate was harvested 48 hours after transfection and loaded onto a 7% Criterion Tris-Acetate Gel. The samples were electrophoresed in parallel with 100 ng of recombinant CN54 envelope (+'ve) and a 10 µl aliquot of Thermo Scientific PageRuler Prestained Protein Ladder (MW).

The experiment was repeated to account for any biological variation but the CAP256 envelope could still not be detected by western blotting, despite verifying that the transfection was successful. A standard curve was generated for luciferase standards and the transfection efficiency of the pLM1 (encoding the CAP256 superinfecting envelope) and SAAVI DNA C2 plasmids extrapolated based on the level of luciferase expression of the co-transfected pGL4.13 (luc2/SV40) reporter plasmid. The lysate was determined to contain 258.8 pg/ml and 39.0 pg/ml of luciferase for the pLM1 and SAAVI DNA C2 samples respectively, confirming that the transfection was successful (**Figure 3.17**).

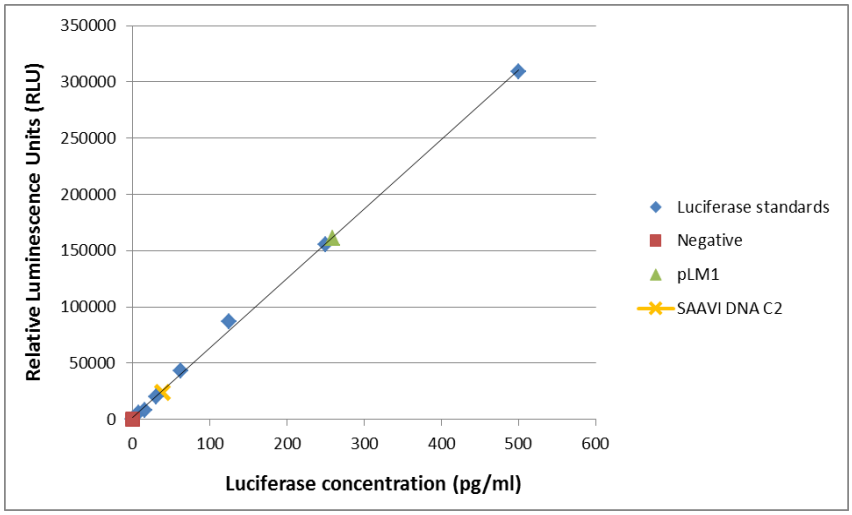

Figure 3.17: Standard curve of relative luminescence units generated by luciferase enzyme standards. The transfection efficiency was extrapolated from the standard curve and determined to fall within the linear range of the standard curve.

3.9.2. Immunostaining of transfected HEK293 cells

After successfully demonstrating expression of each of the recombinant antigens by western blotting, immunofluorescence assays were conducted on fixed HEK293 cells following transfection with the DNA vaccines. These experiments were conducted not only to confirm expression but also to determine the subcellular localization of antigens and to determine if expression of the full length CAP256 envelope glycoprotein could be demonstrated. The 3 DNA vaccines were transfected into HEK293 cells alongside the SAAVI DNA-C2 vaccine which was used as a positive control (SAAVI), the pLM1 expression plasmid encoding the parental envelope from which the vaccines were designed (CAP256) and a negative control whereby plasmid DNA was omitted from the transfection reaction (Negative). The cells were fixed 48

hours after transfection and the cell nuclei were stained blue using Hoechst nuclear stain. The recombinant HIV-1 antigens were stained red using MRC ADP 408/5104 polyclonal anti-gp160 primary antibody and Donkey anti-Goat CY3 secondary antibody.

The SAAVI DNA-C2 vaccine yielded high levels of HIV-1 envelope expression demonstrated by the strong red fluorescence signal of transfected cells (**Figure 3.18 B**). The "cells only" control and the experimental controls whereby primary antibody was omitted didn't yield any fluorescence, confirming the specificity of the antibodies (**Figure 3.18 A**, **Figure 3.18 D-F** and **J-L**). Faint fluorescence was determined from cells transfected with the pLM1 plasmid suggesting very poor expression of the full length CAP 256 envelope glycoprotein (**Figure 3.18 C**). This is consistent with the results from the western blotting experiment, described in **Section 3.9.1** (**Figure 3.16**), and implies that the level of CAP256 envelope expression is below the threshold detectable by western blotting. High levels of expression were witnessed for each of the recombinant HIV-1 envelope antigens (**Figure 3.18 G-I**).

Although the fluorescent signals were not quantified, the gp120-HA_2 protein appeared to yield the highest apparent levels of protein expression. However it is noteworthy that this could be a reflection on variation in the transfection efficiency between experimental samples or the localization of the signal. The transfection efficiency could not be determined for the immunofluorescence assay as the cells were fixed and the assay requires lysis of transfected cells. The fluorescent signal for the gp120-HA_2 antigen appeared to be localized to the cell periphery suggesting that the protein may be membrane-associated (**Figure 3.18 H**). In contrast the other 2 antigens (gp150 and gp140-HA_2tr) (**Figure 3.18 G and Figure 3.18 H**) appeared more diffuse in subcellular localization; reminiscent of the expression witnessed for the SAAVI DNA-C2 vaccine (**Figure 3.18 B**). The recombinant gp150 and gp140-HA_2tr proteins appeared to be distributed within the cell, as well as at the cell periphery (**Figure 3.18 G and Figure 3.18 I**). These observations suggest that the presence of the influenza transmembrane glycoprotein subunit (HA_2) may have improved the surface density of the recombinant antigen. The lack of a similar observation for the gp140-HA_2tr antigen, whereby the influenza HA_2 subunit was truncated, suggests that the influenza ectodomain may promote this membrane association.

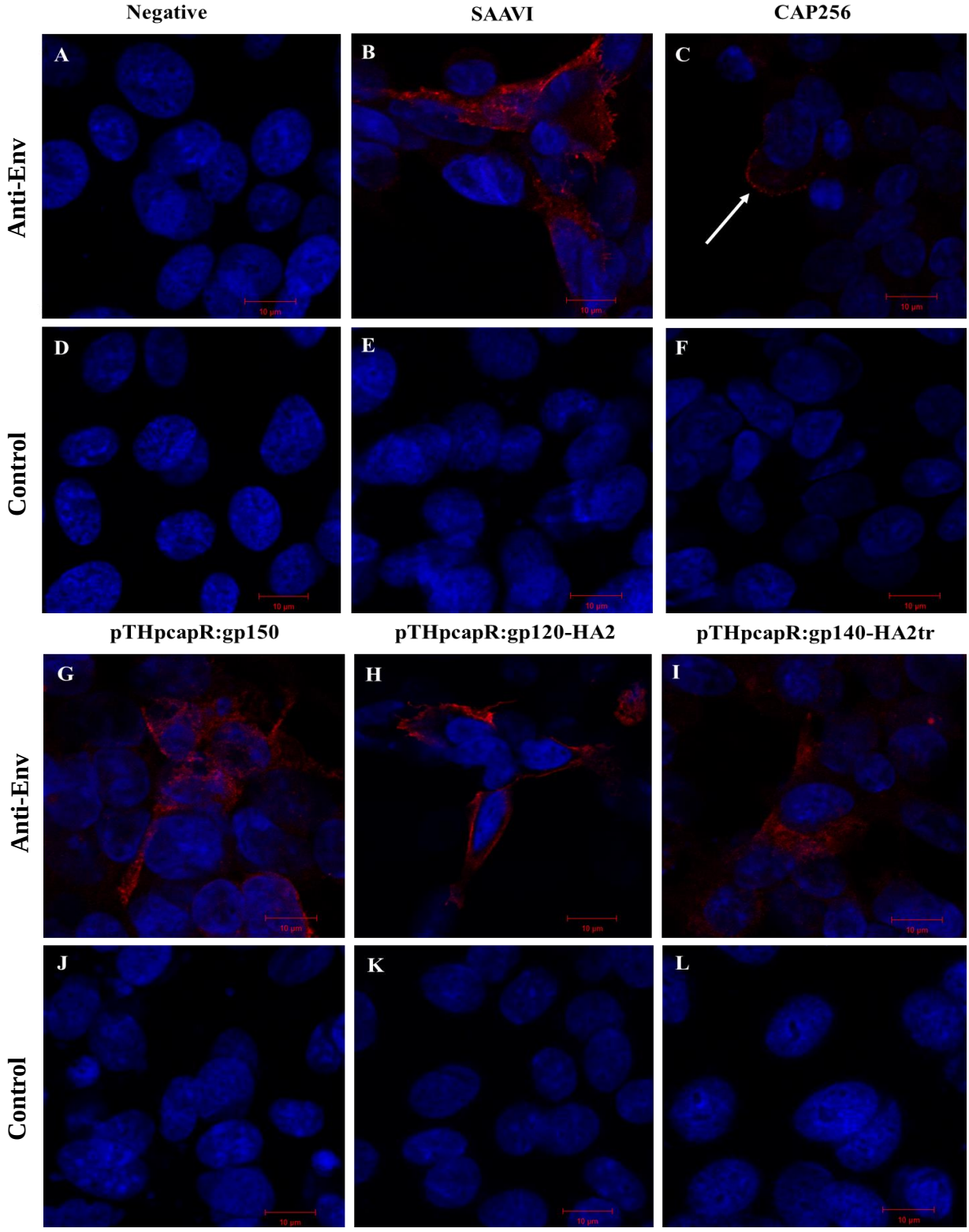

Figure 3.18: Immunostaining to detect recombinant antigen expression following transfection of HEK 293 cells with recombinant pTHPcapR-vectored DNA vaccines. In the figure above, for each experimental sample (A-C & G-I), the corresponding control whereby the primary antibody was omitted is included below (D-F & J-L). Control transfections were performed alongside the experimental samples comprising of the SAAVI DNA-C2 vaccine (SAAVI) (B & E), a negative transfection control (cells only) (A & D) and transfection with a plasmid encoding the CAP256 envelope expression cassette (pLM1) from which the vaccines were derived (CAP 256) (C & F). The white arrow indicates faint fluorescence which may be difficult to distinguish.

3.10. Generation of stable recombinant *A. tumefaciens* GV3101::pMP90RK stocks for agroinfiltration of tobacco plants

Recombinant *A. tumefaciens* strains were generated by electroporating competent GV3101::pMP90RK cells with the pTRA-A plant expression vectors designed in this study. The genetic integrity of the recombinant plasmids were verified following electroporation, by back-transformation of plasmid DNA isolated from recombinant *A. tumefaciens*, into *E. coli* DH5α cells. The DNA derived from the *E. coli* colonies was then transformed into *E. coli* DH5α cells and the DNA derived from the recombinant colonies screened by restriction analysis in accordance with **Table 3.4**.

Table 3.4: Summary of the enzymes used for screening the stability of *A. tumefaciens*-derived plasmid DNA after *in vitro* culturing.

Plasmid	Restriction enzyme (s)	Expected products (bp)
pTRA-A:gp150	*Nco*I- *Sph*I	3251, 6475
pTRA-A:gp120-HA$_2$	*Nco*I- *Not*I	2082, 7613
pTRA-A:gp140-HA$_2$tr	*Nco*I- *Not*I	7613, 2043

Restriction analysis revealed bands of the expected sizes for all of the putative recombinant clones that were screened verifying that the transformation had been successful and that the plasmid DNA was stable after culturing *in vitro* (**Figure 3.19 A-C**).

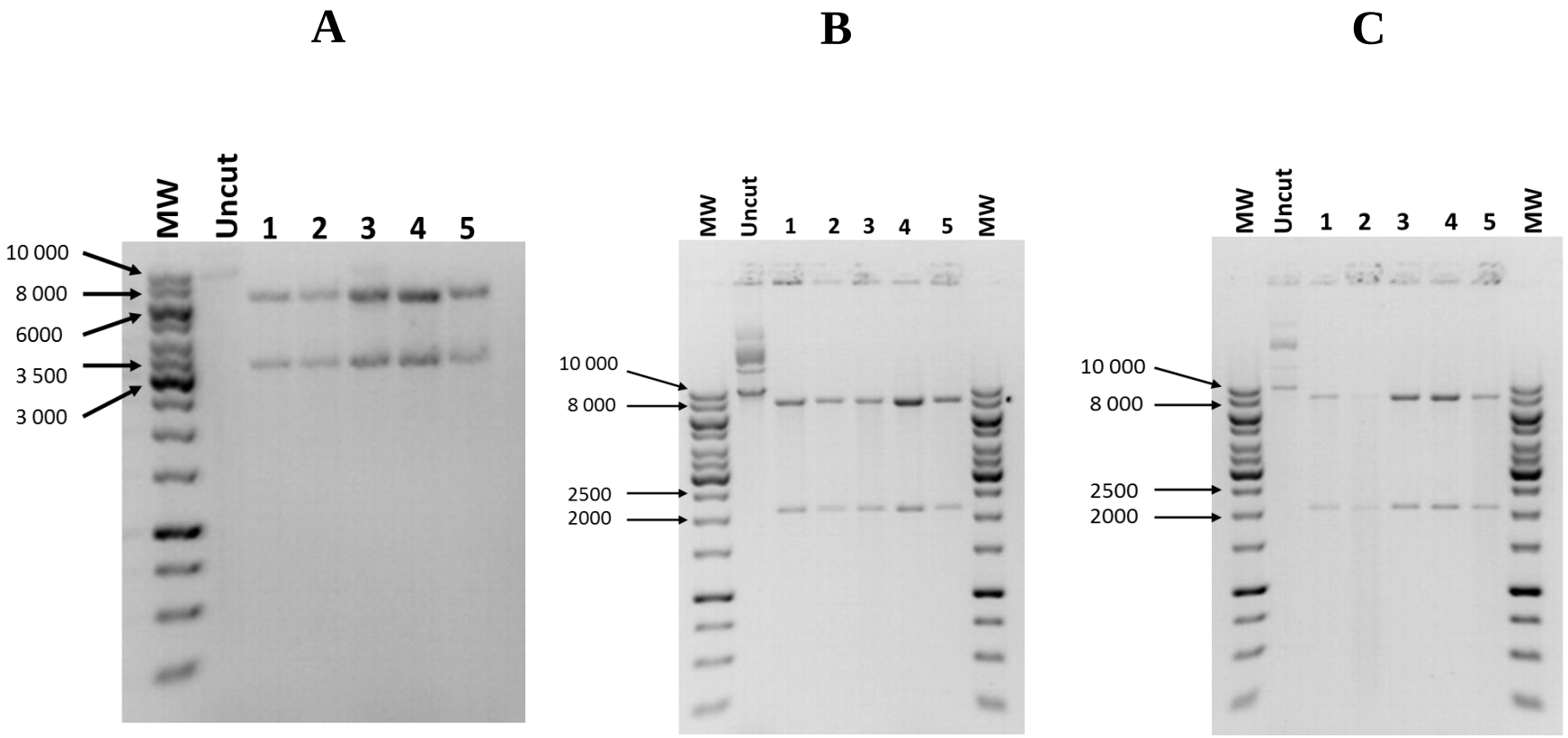

Figure 3.19: Screening of recombinant; A) pTRA-A: gp150, B) pTRA-A: gp120-HA$_2$ and C) pTRA-A: gp140-HA$_2$tr plasmid DNA derived from putative recombinant *A. tumefaciens* clones. DNA derived from 5 *E. coli* colonies was screened by restriction analysis and the resulting restriction fragments resolved on a 0.8% agarose gel (1-5). The samples were electrophoresed alongside 10 µl of undigested pTRA-A: gp150 plasmid DNA (uncut) and a 10 µl aliquot of Fermentas O'GeneRuler™ 1kb DNA ladder (MW). pTRA: gp150 plasmid DNA was screened with *Nco*I and *Sph*I restriction enzymes, whereas pTRA-A: gp120-HA$_2$ and pTRA-A: gp140-HA$_2$tr samples were screened with *Nco*I- *Not*I restriction enzymes.

3.11. Pilot experiment to assess the transient expression of recombinant HIV-1 envelope antigens *in planta*

As a proof-of-concept experiment to verify that plants had the capacity to express the newly designed HIV-1 envelope proteins, 6-8 week old tobacco plants were subjected to a 9 day protein expression time trial. Tobacco plants were infiltrated with recombinant *A. tumefaciens* strains expressing the desired HIV-1 antigen and the dynamics of protein expression followed over a 9 day period. A crude extract of leaf protein was harvested on days 3, 5, 7 and 9 post agroinfiltration and subjected to western blotting, to establish both the duration of protein expression and to determine when protein expression peaked.

The western blots failed to reveal any discernible expression of any of the 3 antigens, at any of the time points sampled, over the 9 day period following agroinfiltration (**Figure 3.20 A-C**). In each case the positive control (recombinant CN54 envelope protein) was detected, verifying the reactivity of the antibodies used and that the western blotting experimental procedure had been performed succesfully.

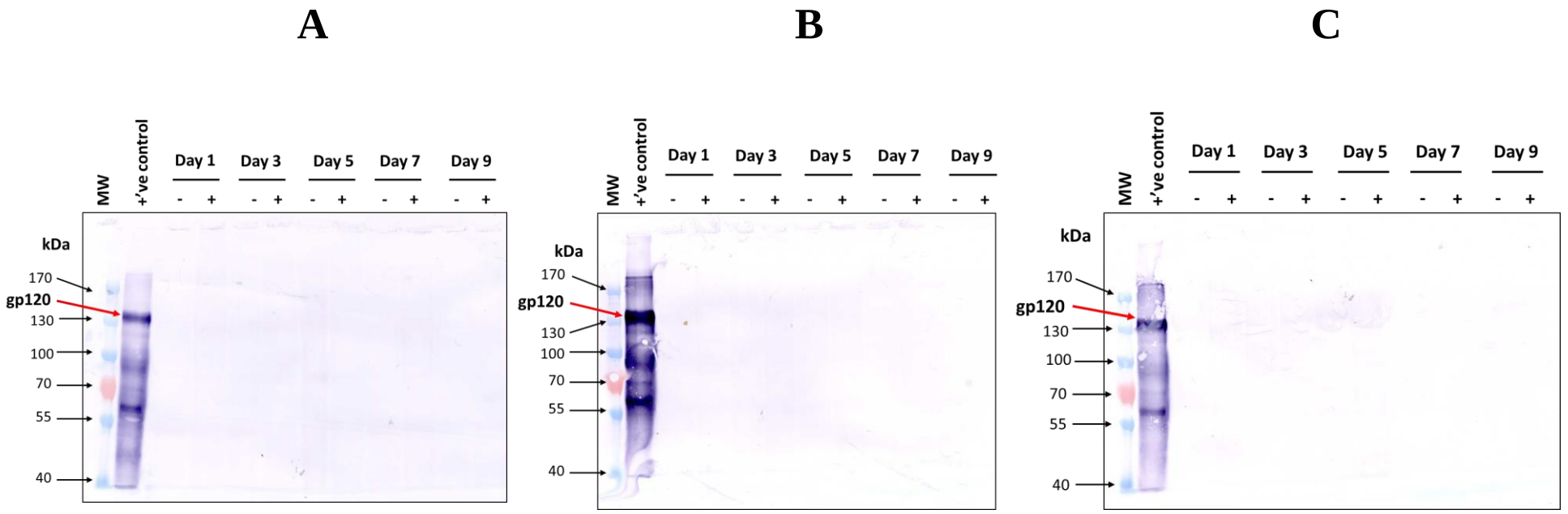

Figure 3.20: Western blotting to detect recombinant A) gp150, B) gp120-HA_2 and C) gp140-HA_2tr protein expression in crude leaf extract for a 9 day period following agroinfiltration of tobacco plants. At each time point; a negative control sample comprising of leaf tissue infiltrated with infiltration media (-) was loaded onto a 7% Criterion Tris Acetate Gel alongside the experimental sample (+). The samples were resolved in parallel with 100 ng of recombinant CN54 envelope (+'ve control) and a 10 µl aliquot of Thermo Scientific PageRuler Prestained Protein Ladder.

3.12. Influence of a PTGS suppressor on the yield and duration of antigen expression

The lack of discernible protein expression following western blotting of the leaf lysate from agroinfiltrated tobacco plants could potentially be attributed to a number of variables that are known to influence the expression of heterologous antigens *in planta*. However, one of the factors most likely to be responsible for the lack of expression is post transcriptional gene silencing (PTGS), a phenomenon that is associated with the antiviral plant immune response (368). In order to determine the influence of PTGS on the expression of the recombinant proteins, the transient expression time trial was repeated with the co-infiltration of the LBA4404 (PBIN-NSs), which contains the p19 supressor from Tomato spotted wilt virus, along with each recombinant *A. tumefaciens* strain generated in this study. Additionally, a positive control comprising of *A. tumefaciens* (pTRA-A: H5-ELP) was infiltrated alongside the experimental expression, in both the presence and absence of the LBA4404 (pBINSs) strain. The recombinant strain encodes a 66 kDa protein product comprising of the full length H5 protein fused in frame to a histidine tag and a 51 bp ELP fragment. This was included in the experimental procedure to corroborate that the infiltration was successful. This particular construct has been extensively characterized and has been demonstrated to achieve high levels of H5-ELP antigen expression.

The co-infiltration of LBA4404 (pBIN-NSs) along with the recombinant *A. tumefaciens* strains generated in this study failed to have any impact on the expression of the recombinant antigens. No expression of the antigen was detected at any of the time points sampled, in either the presence or absence of the NSs silencing suppressor (**Figure 3.21 B, Figure 3.21 C** and **Figure 3.21 D**). In contrast, expression of the recombinant H5-ELP protein was discernible at 3 days post infiltration in both the presence and absence of the NSs suppressor. On day 5 the magnitude of expression in the presence of the NSs suppressor was noticeably greater than when the *A. tumefaciens* (H5-ELP) construct was infiltrated alone. Additionally, on days 7 and 9 expression of the recombinant antigen was only detectable when the LBA4404

(pBINSs) strain had been co-infiltrated (**Figure 3.21 A**). These results highlight the profound influence of mitigating PTGS in order to enhance the duration and magnitude of foreign antigens *in planta*. Furthermore, the successful expression of the H5-ELP protein confirmed the validity of the transient expression system used in this study.

The lack of detectable antigen expression prompted rescreening recombinant *A. tumefaciens* stocks that were used to inoculate cultures to infiltrate plants. The plasmid DNA was screened by back-transformation, as outlined in **Section 3.10**,
and demonstrated to be stable (data not shown). This confirmed that the lack of envelope expression was not merely a reflection of PTGS and was subject to other variables warranting further investigation.

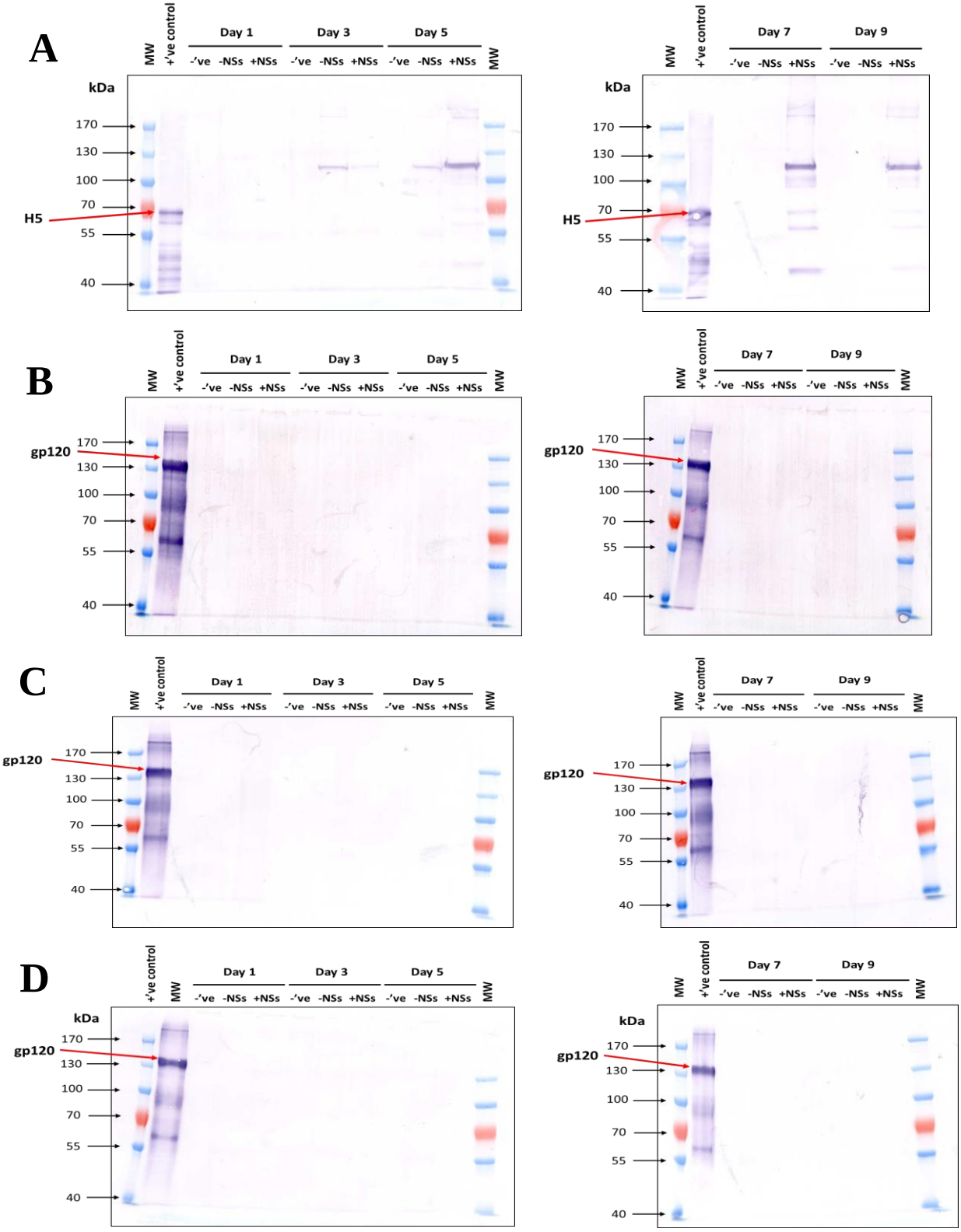

Figure 3.21: Western blotting to detect; A) H5-ELP, B) gp150, C) gp120-HA$_2$ and D) gp140-HA$_2$tr protein expression in crude leaf extract for a 9 day period following agroinfiltration of tobacco plants. At each time point; In addition to the experimental sample (+NSs) negative control samples were included whereby leaves were infiltrated with infiltration media only (-'ve) or the LBA4404 (pBINSs) strain was not coinfiltratrated with the recombinant *A.tumefaciens* strain encoding the antigen (-NSs).

3.13. Impact of solubilizing plant cell membranes during protein extraction on the yield of recombinant antigens

Another possible explanation for the lack of detectable expression by western blotting is that the antigens may have been sequestered in the plant cell membranes due to the presence of a transmembrane domain in the protein structure. This may have prevented antigens from being liberated from leaf tissue during the crude purification procedure resulting in the immunogens being retained in the insoluble protein fraction. The gp150 protein retained the native HIV-1 envelope transmembrane region whereas the gp120-HA_2 and gp140-HA_2tr antigens retained the analogous transmembrane domain from the H5 glycoprotein (**Section 3.2; Figure 3.1** and **Figure 3.2**). The leaf pellet retained from the protein extraction time trials (**Section 3.12**) was solubilized in extraction buffer containing 8M urea, to disrupt the cell membranes of the plant leaf cells. The resulting supernatant was probed for expression of the HIV-1 envelope antigens by western blotting.

The altered extraction protocol failed to make any apparent difference for the gp150 and gp120-HA_2 antigens with neither antigen detected by western blotting at any of the time points sampled (**Figure 3.22 A** and **Figure 3.22 B**). Expression of the gp140-HA_2tr antigen could be detected in urea-solubilized leaf extract 24 hours post infiltration, with optimal expression occurring after 3 days. Faint expression of the antigen was also evident on days 5-9. Notably, expression of the antigen was only evident when LBA4404 (pBIN-NSs) was co-infiltrated with *A. tumefaciens* (pTRA-A: gp140-HA_2tr). The band corresponding to the recombinant antigen appeared significantly larger than expected and was estimated to be in excess of 170 kDa.

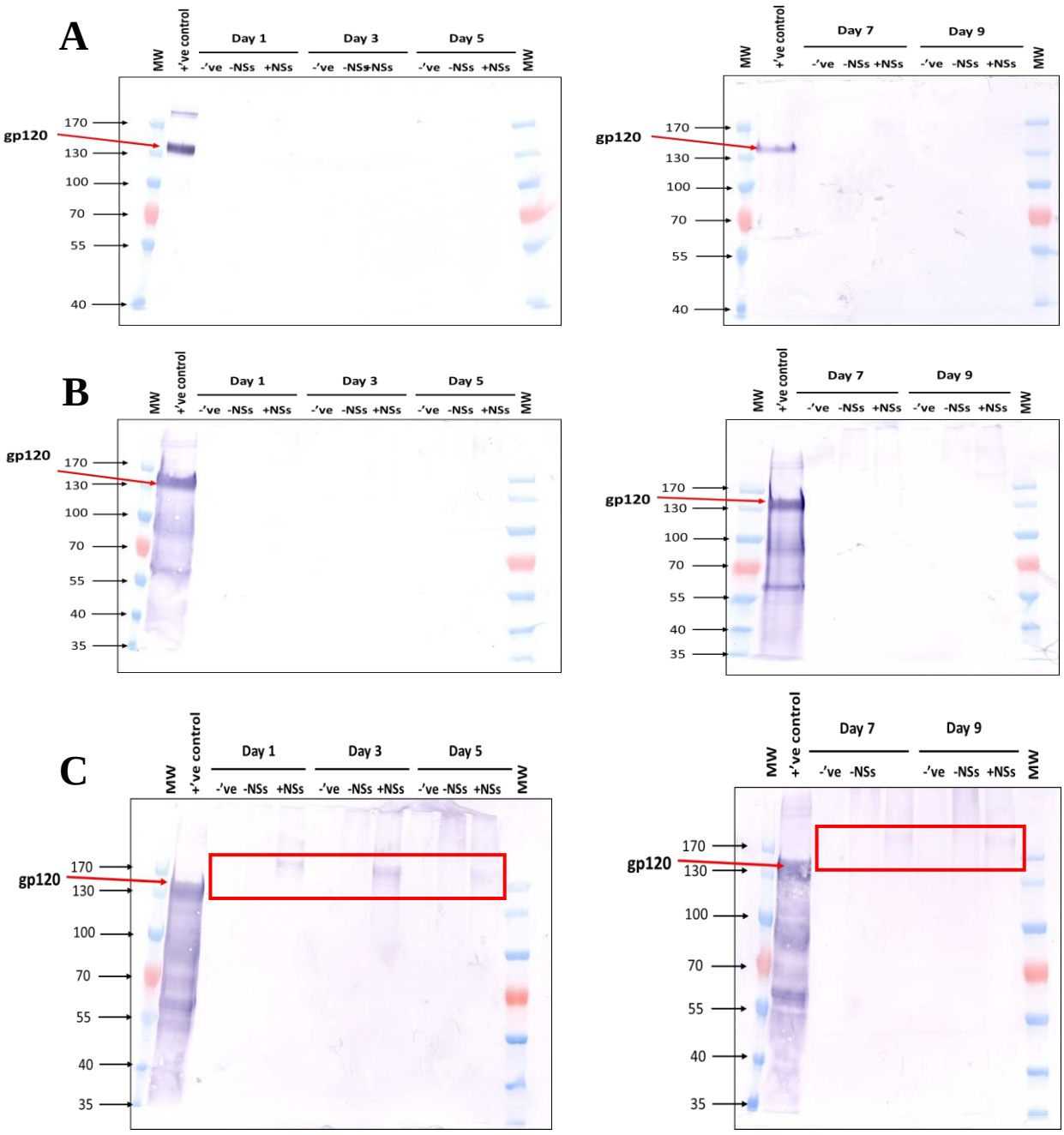

Figure 3.22: Western blotting to detect; A) gp150 and B) gp120-HA_2 and C) gp140-HA_2tr protein expression in urea-solubilized crude leaf extract for a 9 day period following agroinfiltration of tobacco plants. At each time point, in addition to the experimental sample (+NSs); negative control samples were included whereby leaves were infiltrated with infiltration media only (-'ve) or the LBA4404 (pBINSs) strain was not coinfiltrated with the recombinant *A. tumefaciens* strain encoding the antigen (-NSs). The samples were resolved on a 7% Criterion Tris Acetate Gel alongside 100 ng of recombinant CN54 envelope (+'ve control) and a 10 µl aliquot of Thermo Scientific PageRuler Prestained Protein Ladder.

3.14. Impact of varying culture densities used for infiltration on recombinant protein expression levels

Despite the promising results yielded by the inclusion of urea in the extraction buffer, this approach is not suitable for the extraction of proteins where the protein conformation is important for biological activity. The inherent caveat with using urea, or other detergents, during the protein extraction process is their propensity to denature higher order protein conformations. This is particularly unhelpful in the case of the HIV-1 envelope glycoprotein which is functional only as a trimeric oligomer (200). Additionally, certain neutralization sensitive antibody epitopes, such as the PG9/16 epitopes, are preferentially recognized in the context of the trimeric envelope (379). We therefore continued to explore other methods to optimize expression of the recombinant plant-produced HIV-1 subunit vaccines.

Another variable that has been shown to influence the levels of expression of heterologous antigens, using the transient expression system exploited in this study, is the density of the bacterial culture at the time of infiltration. The expression time trials were repeated for each recombinant strain of *A. tumefaciens* designed in this study, at 3 different culture densities (OD_{600} of 0.5, 1 and 1.5). In each case, LBA4404 (pBIN-NSs) was co-infiltrated with the recombinant *A. tumefaciens* strain of interest based on the profound improvement in heterologous antigen expression levels demonstrated for the recombinant H5-ELP antigen demonstrated in **Section 3.12**, **Figure 3.21 A**. Additionally, *A. tumefaciens* (pTRA: H5-ELP) and LBA4404 (pBIN-NSs) were co-infiltrated into tobacco plants, at an OD_{600} of 0.5, to verify that the infiltration was successful.

In the case of all 3 antigens, faint expression was evident 1 day post infiltration at all 3 culture densities, even at OD_{600} of 0.5 which had not yielded any apparent protein expression previously (**Figure 3.23** and **Figure 3.24**). It is noteworthy that for the gp150 and gp120-HA_2 antigens, the levels of expression at OD_{600} 0.5 were barely discernible after detection of the western blot and may not be apparent in this

manuscript due to the loss of detail incumbent with capturing the image (**Figure 3.23**).

Expression of all 3 antigens was also detected on day 3 and the apparent band intensity appeared greater at higher culture densities. Expression of recombinant gp150 in the crude plant extract was only evident at OD_{600} of 1 and 1.5 on day 3. In the case of gp120-HA_2 expression was only detectable at the highest culture density (OD_{600} 1.5). In constrast gp140-HA_2tr expression occured at all 3 culture densities on day 3. Furthermore, expression of the gp140-HA_2tr antigen was also detected 5 days postinfiltration at the lowest culture density (OD_{600} 0.5) but not at any of the other time points sampled (**Figure 3.24**). Lastly high levels of recombinant H5-ELP expression was detected at both time points sampled indicating that the infiltration procedure was successful (**Figure 3.25**).

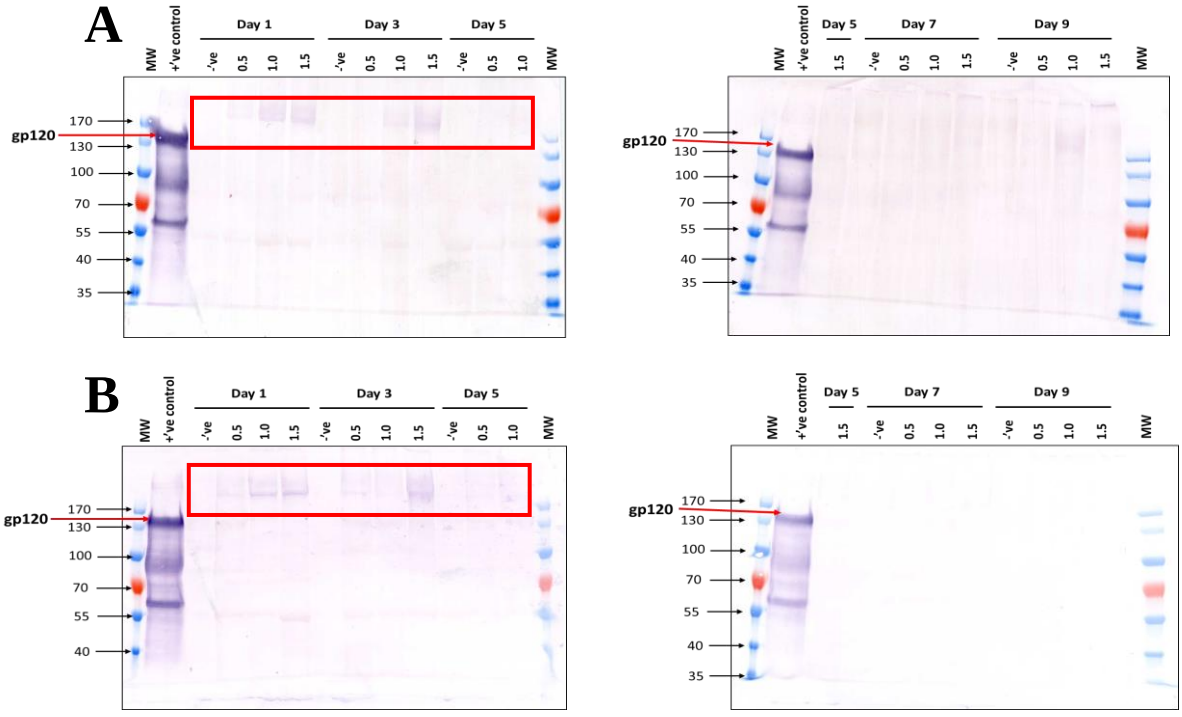

Figure 3.23: Western blotting to detect; A) gp150 and B) gp120-HA_2 protein expression in crude leaf extract following agroinfiltration of tobacco plants at different culture densities. At each time point, recombinant *A. tumefaciens* was co-infiltrated into tobacco leaves along with LBA4404 (pBINSs) at culture densities (OD_{600}) of 0.5, 1 and 1.5. The experiment was performed in parallel with a negative control comprising of leaf tissue infiltrated with infiltration media.

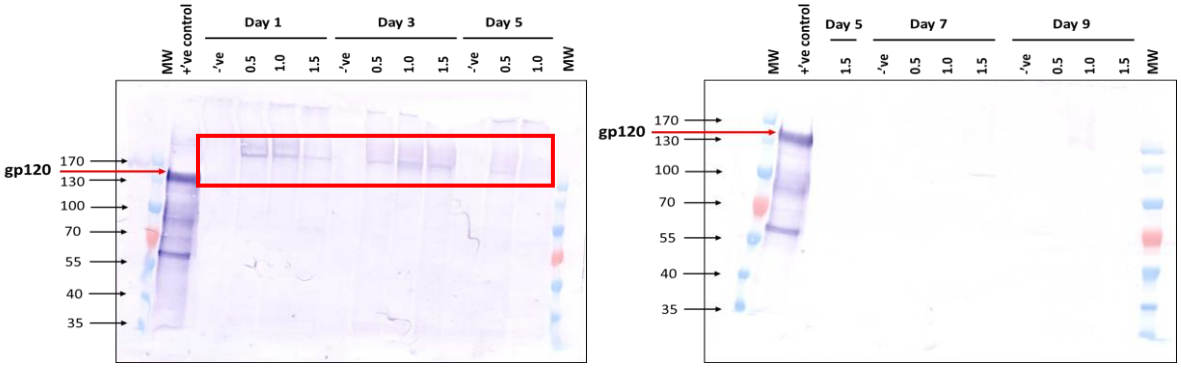

Figure 3.24: Western blotting to detect recombinant gp140-HA$_2$tr protein expression in crude leaf extract following agroinfiltration of tobacco plants at different culture densities. At each time point, recombinant *A. tumefaciens* was co-infiltrated into tobacco leaves along with LBA4404 (pBINSs) at culture densities (OD_{600}) of 0.5, 1 and 1.5. The experiment was performed in parallel with a negative control comprising of leaf tissue infiltrated with infiltration media (-'ve).

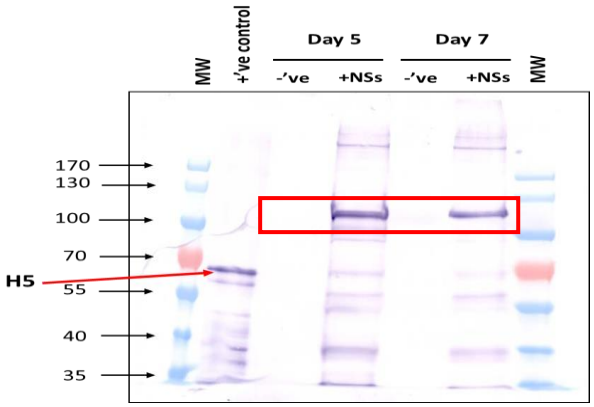

Figure 3.25: Western blotting to detect recombinant H5-ELP protein expression in crude leaf extract following agroinfiltration of tobacco plants. *A. tumefaciens* (H5-ELP) was infiltrated into tobacco plants at an OD_{600} of 0.5 as a positive infiltration control. At each time point, in addition to the experimental sample (+NSs) a negative control sample was included whereby leaves were infiltrated with infiltration media only (-'ve).

In order to account for the possibility that the bands observed in **Figure 3.23** and **Figure 3.24** were due to non-specific reactivity of the antibody, the western blot was repeated using a different negative control. The negative control used in **Figure 3.23** and **Figure 3.24** comprised of leaf tissue infiltrated with infiltration media and therefore may not reflect whether the antibody was reacting non-specifically with some *A. tumefaciens* protein produced early after infiltration. The western blot was repeated using a negative control comprising of leaf lysate derived from tobacco plants agroinfiltrated with *A. tumefaciens* GV3101::pMP90RK (pRIC: N) at an OD_{600} of 1. This recombinant strain of *A. tumefaciens* encodes a full length, human codon optimized, Crimean-Congo Hemorrhagic Fever N protein from the pRIC plasmid vector. The agroinfiltrated leaf lysate was obtained from Richard Atkinson (M.Sc.

student, Biopharming Research Unit, Department of Molecular and Cell Biology, University of Cape Town). It is noted that the use of material from a separate experiment is not an ideal control due to the potential influence of other variables that may not have been accounted for.

The day 3 lysate samples for each antigen at OD_{600} of 1.5 (+NSs) were subjected to western blotting alongside the negative lysate sample. The western blot procedure was also refined to allow the incubation time between the membrane bound proteins and the substrate to be prolonged to 1 hour without non-specific reactivity of the antibodies. The primary antibody concentration was increased to 1:500, the secondary antibody concentration decreased to 1:20 000 and the concentration of recombinant CN54 positive control was decreased to 20 ng. Consistent with **Figure 3.23** and **Figure 3.24** all 3 lysate samples yielded a band slightly larger than 170 kDa that was not present in the negative control (**Figure 3.26**). This confirmed low expression levels of the 3 recombinant envelope antigens *in planta*.

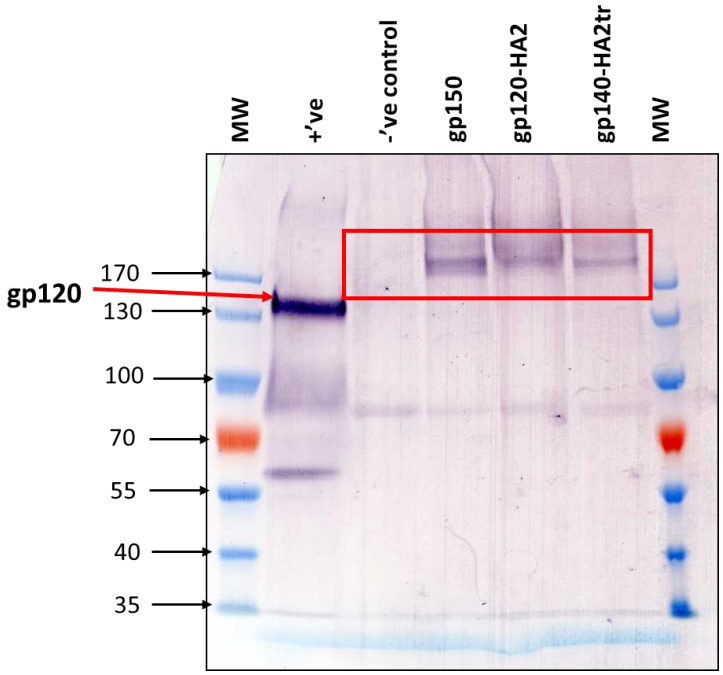

Figure 3.26: Western blotting to detect recombinant HIV-1 antigen expression in day 3 crude leaf extract following agroinfiltration of tobacco plants at an OD_{600} of 1.5.
Agroinfiltrated leaf lysate samples from Day 3 (OD_{600} of 1.5) were resolved on a 7% Criterion Tris Acetate Gel alongside leaf lysate derived from tobacco plants infiltrated with *A. tumefaciens* (pRIC: N) (-'ve). The samples were elecrophoresed in parallel with a 20 ng µl aliquot of recombinant CN54 envelope glycoprotein and a 10 µl aliquot of Thermo Scientific PageRuler Prestained Protein Ladder (MW).

3.15. Direct extraction of recombinant antigens from the apoplastic spaces of plant leaves

The successful expression of the recombinant envelope subunit vaccines, demonstrated in **Section 3.14**, confirmed that plants have the capacity to express the novel HIV-1 proteins designed in this study. However, the yields observed by western blotting were not suitable for further characterization or immunization studies. Having evaluated several different strategies to maximize expression of the antigens, our focus shifted to improving the recovery of the proteins from the plant leaves. Based on promising reports by Medicago and our lab at purifying recombinant proteins directly for the apoplastic spaces in plant leaves, we sought to apply this approach to our HIV-1 subunit vaccines (63, 238). The experimental infiltrations described in **Section 3.14** were repeated at OD_{600} of 1.5 for the experimental strains and 0.25 for the *A. tumefaciens* (pTRA-A: H5) strain. A negative control infiltration of LBA4404 (pBIN-NSs) was conducted in parallel with the experimental samples.

Once again protein samples were harvested at day 3 for the HIV-1 antigens and day 7 for the H5 protein. PBS was infiltrated into the abaxial airspaces on the ventral side of agroinfiltrated leaves and protein from the apoplastic spaces purified by low speed centrifugation. Protein was also purified for each of the recombinant strains using the crude homogenization method employed in **Section 3.14**. The purified apoplastic protein samples were subjected to western blotting alongside their corresponding samples purified by homogenization, to determine whether the apoplastic extraction method improved protein recovery.

In the case of all 3 antigens, purification of protein directly from the apoplast yielded a band of greater intensity than the corresponding sample purified by homogenization (**Figure 3.27**). The gp150 and gp120-HA_2 proteins were barely detectable when purified by homogenizing leaf tissue and may not be apparent in this manuscript due to the loss of detail associated with capturing the image (**Figure 3.27**). Interestingly the bands corresponding to the antigens of interest are located higher up on the gel than expected. The apparent size anomaly of the recombinant antigens may be due to their aggregation with inclusion bodies during their egress through the ER into the

apoplastic space (**Figure 3.27**). It is unlikely that these bands are artifacts of non-specific antibody binding as they are not present in the NSs control and are darker when the samples were enriched for apoplastic proteins. These findings suggest that extracting protein directly from the leaf apoplast significantly improves the apparent yield of the recombinant antigens.

Consistent with these findings, purification of recombinant H5 in this manner yielded a sharply defined band of the expected size (**Figure 3.28 A**). The corresponding western blot for H5 purified by homogenizing agroinfiltrated leaves is included alongside in **Figure 3.28 B** for comparison. Although the lysate samples were unquantified the images strongly suggest that the apoplastic method employed here culminated in a significant improvement in recovery of the desired protein.

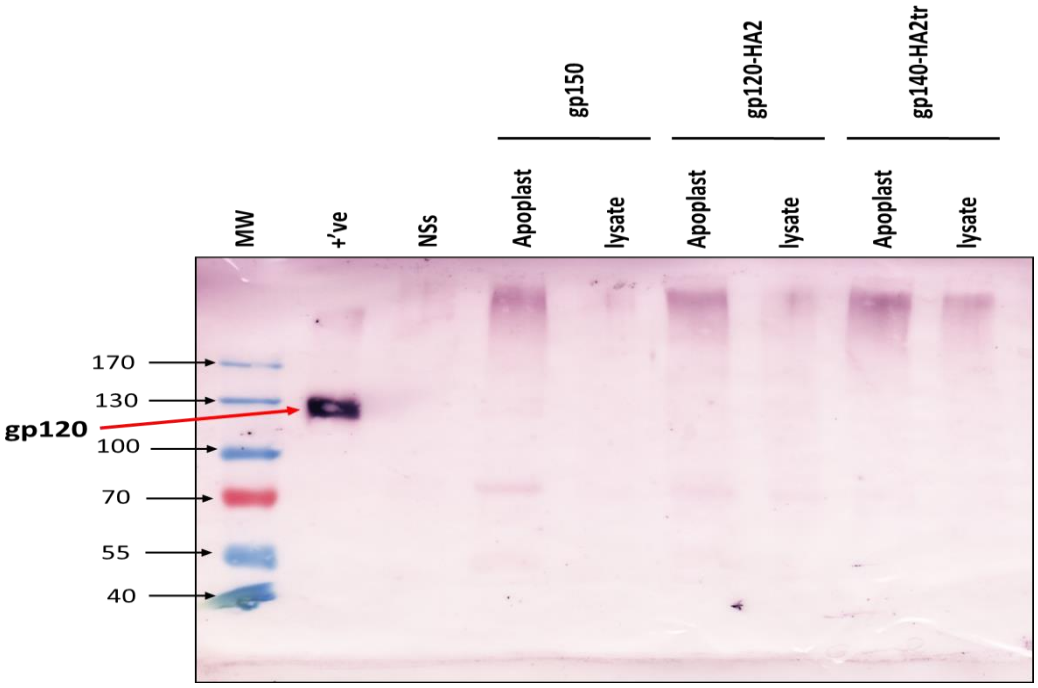

Figure 3.27 Western blotting to detect recombinant HIV-1 antigen expression in apoplastic protein lysate. Apoplastic protein was harvested 3 days post agroinfiltration of leaf tissue and subjected to western blotting alongside protein samples harvested by homogenization (lysate). In addition to the experimental sample, a negative control sample (NSs) was included whereby leaves were infiltrated with LBA4404 (pBIN-NSs). The samples were elecrophoresed in parallel with a 20 ng µl aliquot of recombinant CN54 envelope glycoprotein and 10 µl Thermo Scientific PageRuler Prestained Protein Ladder (MW).

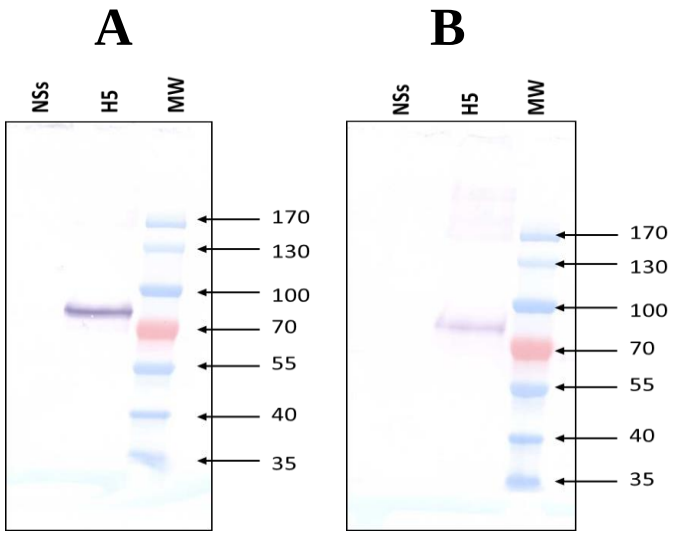

Figure 3.28: Western blotting to detect recombinant H5 antigen expression in A) apoplastic protein lysate and B) crude protein lysate. Protein was harvested 7 days post agroinfiltration of leaf tissue, by either direct apoplastic proteinextraction or leaf homogenization, and subjected to western blotting alongside crude LBA4404 (pBIN-NSs) agroinfiltrated leaf lysate.

3.16. Summary of plant expression parameters

In summation, preliminary attempts to express the newly designed antigens were unsuccessful even when a PTGS was co-expressed along with the proteins. The extraction of the antigens under denaturing conditions was partially successful, although impractical for the recovery of intact proteins. Increasing the culture density of *A. tumefaciens* infiltrated into plant leaves and direct extraction of the antigens from the apoplast allowed the recovery of low levels of the recombinant proteins, although insufficient for further characterization **(Table 3.5)**.

Table 3.5: Summary of the expression levels achieved by varying protein expression and extraction conditions.

Reaction stage	Expression
Preliminary expression studies	-
Influence of PTGS suppressor	-
Protein extraction under denaturing conditions	+
Impact of infiltration culture density	++
Direct apoplastic protein extraction	++

(- = no detectable expression, + = very low expression, ++ = low expression)

CHAPTER 4:
DISCUSSION & CONCLUSION

The development of a heterologous DNA prime-protein boost immunization regimen is a promising approach to induce neutralizing antibodies against the HIV-1 envelope glycoprotein. However, conventional vaccine production platforms are expensive and therefore represent an additional complication to developing a globally implemented HIV-1 vaccine. In this study two vaccine platforms, namely a novel DNA vaccine and a transient plant expression system, were investigated to produce 3 HIV-1 envelope immunogens.

We have developed DNA vaccines based on the pTHPcapR vector system which enables dose sparing by employing a porcine circovirus type 1 enhancer element, to drive high levels of antigen expression (354). We have also exploited a transient *A. tumefaciens* mediated plant-based expression platform for the production of recombinant HIV-1 envelope proteins in order to reduce production costs associated with heterologous expression systems and to potentially improve the immunogenicity of the antigens (293). Plant-derived pharmaceutical proteins are cheaper than both conventional cell culture and bacterial fermentation production platforms, largely due to the reduction in generic production costs associated with this system (66, 91, 256, 293, 346). Additionally, plant-based expression platforms are potentially infinitely scalable and could be exploited for the large scale production levels required for the implementation of a vaccine on a global scale (293, 410). The feasibility of this expression system is best demonstrated for influenza haemagglutinin subunit vaccines, with commercially viable yields of subunit vaccines having been reported by a number of different research groups (157, 158, 183, 226, 323–326, 336). Lastly, there is evidence to suggest that glycoproteins passing through the plant cell secretory pathway may bud into the apoplastic spaces, along with plant-derived lipids, to form highly immunogenic particulate structures (61, 63).

4.1. Characterization of pTHPcapR DNA vaccines encoding novel HIV-1 envelope antigens

A HIV-1 subtype C envelope gene, derived from a secondary HIV-1 infection, was obtained from a CAPRISA Acute Infection Cohort donor who later developed cross neutralizing PG9/16-like antibodies (114, 232). The envelope glycoprotein in question was selected for vaccine development on the basis of its documented sensitivity to prototype monoclonal antibodies comprising the main regions of vulnerability of the HIV-1 glycoprotein, ensuring both the integrity and exposure of the cognate epitopes on the parental virus (235). These included antibodies PG9, PG16 and PGT145 which target an epitope involving residue N160, PGT128 which recognizes the glycan at N332, 4E10 which interacts with the MPER of gp41 and VRC01 which binds to the CD4bs (41, 235, 377, 379, 418).

This is in stark contrast to the approach employed to select vaccine immunogens for efficacy trials which have been chosen to represent the predominating subtypes in the given geograpical region where the trial was taking place (30, 93, 115, 125, 279). Recently, a more rationale approach has been applied to developing an immunogen capable of inducing cross-neutralizing antibodies. Hoffenberg and colleagues have reported the identification of an HIV-1 subtype A envelope protein (BG505.W6M.ENV.C2) that is recognized by most known HIV-1 envelope broadly cross-neutralizing monoclonal antibodies. Furthermore immunogenicity studies in rabbits with an adjuvanted recombinant gp120 antigen, derived from this envelope, resulted in the induction of antibodies recognizing the CD4-binding site, the N160 epitope defined by PG9 and a glycan dependent epitope defined by PG126 (142). Further development of this envelope variant as an antigen has resulted in the production of stable, soluble, cleaved trimers that retain the epitopes recognized by broadly neutralizing antibodies without exposing non-neutralizing epitopes (298).

Three novel antigens were designed in this study, based on the CAP256 envelope coding sequence, for use in genetic immunizations and as recombinant protein immunogens. The first antigen, gp150, comprised of a truncated form of the envelope protein whereby the cytoplasmic tail region had been shortened to improve the

exposure of antibody-sensitive epitopes and to enhance expression of the protein (83, 374). This approach has also been employed by the South African AIDS Vaccine Initiative for the expression of an HIV-1 envelope antigen from both DNA and MVA vaccine modalities that are currently being evaluated in clinical trials (34, 35, 317).

The other 2 antigens, gp120-HA_2 and gp140-HA_2tr, comprised of chimeric proteins that contained elements of the HIV-1 envelope glycoprotein (gp160) and the transmembrane subunit (HA_2) of H5N1 influenza haemagglutinin glycoprotein. The main rationale underlying this approach is the possibility that chimeric glycoproteins containing the influenza transmembrane subunit may form particulate structures, along with plasma membrane-derived lipids, when passing through the plant cell secretory pathway (61). This approach has been successfully demonstrated by Medicago who have reported the formation of chimeric VLPs for HIV-1, Varicella Zoster and Rabies virus antigens when translationally fused to the transmembrane and cytoplasmic domains of influenza H5 haemagglutinin (61). The formation of such VLPs could potentially circumvent the low envelope densities associated with conventional vaccine approaches (263). Lastly, these chimeras may also promote increased surface density following genetic immunization which could be expected to improve antibody responses to the vaccine (383).

The DNA vaccines were characterized *in vivo* to verify expression of the antigens and to obtain an indication of their subcellular localization. Western blotting experiments of transfected HEK293 cells verified promising expression of each of the antigens in the DNA vaccines constructed for this project. However expression of the full length CAP256 envelope from which the vaccines were designed could not be detected. This plasmid differed from the vaccines in that it had coding sequence for additional elements of the HIV-1 genome which will have altered the proximity of the envelope gene to the plasmid's promoter. These elements included the 3' region of *rev* exon 1, the 5' end of the *nef* gene and the full length *vpu* gene. Although the pcDNA3.1 plasmid, encoding the parental CAP256 envelope glycoprotein, had the same Cytomegalovirus-promoter/enhancer and bovine polyadenylation signal as the pTHPcapR DNA vaccine, it lacks the porcine enhancer element. A luciferase assay, performed to determine the transfection efficiency of the co-transfected pGL4.13

(luc2/SV40) reporter plasmid, verified that the transfection was successful. These findings preclude the possibility that the lack of detectable CAP256 envelope protein is a reflection on either poor transfection efficiency or a lack of antibody reactivity. Additionally, the genetic integrity of the plasmid had previously been confirmed by sequencing and expression of the protein and indirectly confirmed by the use of CAP256 envelope-derived pseudovirus in TZM-bl luciferase assays (114, 232, 235). Collectively this suggests that the CAP256 envelope glycoprotein is poorly expressed from plasmid pLM1 and that the levels of protein in the lysate may be below the threshold of detection by western blotting.

The DNA vaccines were also characterized by immunostaining of transfected HEK293 cells, followed by confocal microscopy. Consistant with western blotting of transfected HEK293 cell lysate, high levels of antigen expression were observed for the gp150, gp120-HA_2 and gp140-HA_2tr antigens. In contrast, poor expression of the full length CAP256 envelope was observed for cells transfected with the pLM1 expression construct. Interestingly, the expression of the gp120-HA_2 antigen appeared to be predominantly localized to the periphery of transfected cells. In contrast, the fluorescent signal for the gp150 and gp140-HA_2tr proteins appeared to be more diffuse in subcellular localization, as witnessed for the SAAVI DNA-C2 envelope protein. These observations suggest that the presence of the full length influenza HA_2 glycoprotein subunit may promote increased surface density of the glycoprotein antigen.

Further work will be required to validate these assertions by demonstrating co-localization of the fluorescent signal for the antigen with a plasma membrane-specific stain, such as Nile Red. If this hypothesis can be confirmed it would suggest that the ectodomain of the HA_2 subunit may somehow promote the association of the glycoprotein with the plasma membrane as the gp140-HA_2tr immunogen (which is identical except for its ectodomain) did not give rise to a similar localization pattern. In contradiction to this theory, Wang *et al.*, have proposed that the discrepancy in glycoprotein densities between HIV-1 and influenza virions may be a reflection on the increased length of the HIV-1 envelope cytoplasmic tail compared to influenza haemagglutinin (383). However, even when the cytoplasmic tail of the HIV-1

antigens was truncated, in the case of the gp150 antigen and the SAAVI DNA-C envelope, there was no evidence to suggest comparable levels of membrane association. The detection of the gp150 antigen but not the full length CAP256 envelope may be a reflection of inhibitory signals in the cytoplasmic region that preclude high levels of expression. Alternatively, the higher levels of gp150 expression may be due to the porcine enhancer element in the pTHPcapR DNA vaccine which is not present in pLM1 (354). In contrast to the CAP256 envelope, the high expression levels observed for the gp150 antigen, encoded by the SAAVI-C2 DNA vaccine, suggest that this envelope is efficiently produced.

Consistent with the observations from the immunostaining experiment, influenza virions are known to have a greater glycoprotein density than HIV-1 viral particles (134). A recent cryoelectron tomography study of influenza virions suggested that each virion contained approximately 375 glycoprotein spikes compared to the estimated 14 glycoprotein trimers per HIV-1 virion (130, 419). Furthermore, it has been reported that substitution of the HIV-1 envelope transmembrane and cytoplasmic domains with analogous regions from influenza virus culminates in increased glycoprotein density on the surface of $pr55^{gag}$ particles, in insect cell culture (383).

In essence, the *in vivo* characterization of the 3 DNA vaccines constructed for this project demonstrated high levels of expression for each antigen and immunofluorescence assays suggested that the presence of the full length influenza HA_2 subunit may promote increased surface density. The low level expression of the parental CAP256 envelope is possibly a reflection on the selective pressures acting on the transmission bottleneck prior to the establishment of infection (74, 122, 161, 282). It is plausible that low glycoprotein density on the CAP256 virion may have enabled the virus to evade antibody responses, in the donor, prior to the transmission event to CAPRISA participant 256. Unfortunately no information regarding the donor's immune response to infection is available and this hypothesis is purely speculative. The CAP256 envelope was derived from the superinfecting virus which is believed to have established infection 13-15 weeks after the primary infection, prior to the development of neutralizing antibodies (114, 232, 235). Therefor the low

envelope density on the transmitted virus is unlikely to have conferred a selective advantage to the virus during the transmission event.

4.2. Expression of recombinant HIV-1 envelope antigens *in planta*

The expression of a viable plant-produced HIV-1 envelope vaccine candidate will constitute a significant development in biopharming and HIV-1 vaccine development alike. To date a limited number of studies have reported the expression of vaccine relevant HIV-1 envelope proteins in plants and therefore a major aim of this study was also to investigate the capacity of plants to express HIV-1 envelope immunogens. In this study we have systematically investigated various parameters that have been shown to influence the expression of recombinant proteins *in planta*, with the aim of optimizing recombinant protein expression and downstream purification (**Figure 4.1**).

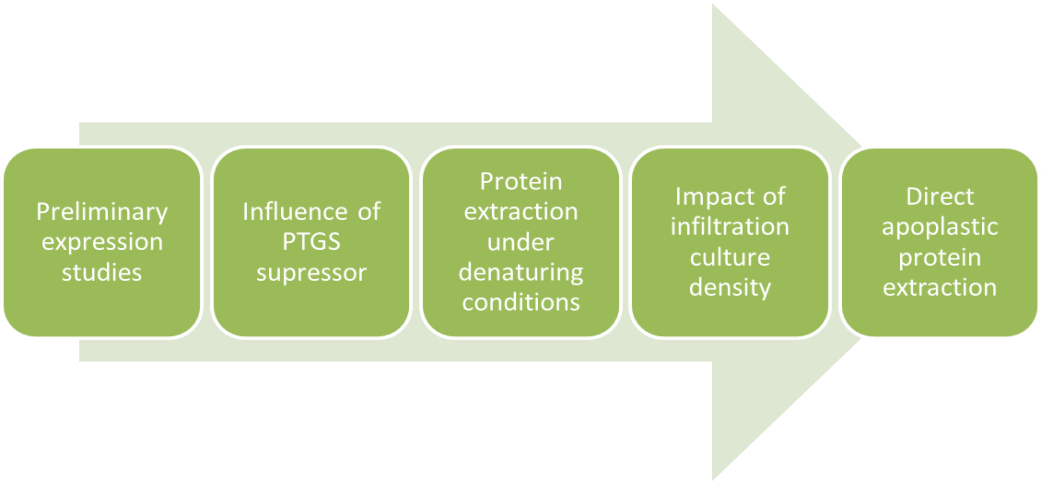

Figure 4.1: Schematic diagram outlining the systematic approach used to optimize recombinant HIV-1 envelope antigen expression *in planta*.

Recombinant *A. tumefaciens* strains were generated, with specialized pTRA-A expression vectors, encoding matched antigens to the priming DNA vaccines. Initial attempts to transiently express the 3 recombinant immunogens by agroinfiltation of *N. benthamiana* leaves failed to yield any discernible protein expression, even when a PTGS suppressor was co-expressed with the antigens. However based on the expression time trial of the *A. tumefaciens* (pTRA-A: H5-ELP) strain, used as an infiltration control, it was apparent that supressing PTGS could profoundly improve the expression of heterologous proteins in plants. Western blotting of crude

agroinfiltrated leaf lysate suggested that the NSs suppressor enhanced both the magnitude and duration of H5-ELP antigen expression suggesting that circumventing PTGS was a promising approach to enhance the yield of recombinant antigens.

In order to account for the possibility that the antigens were sequestered in the cell membranes and not liberated into the soluble protein fraction during protein extraction, the experiments were repeated and leaf protein extracted under denaturing conditions. This approach enabled the detection of faint expression of gp140-HA_2tr, but not the other antigens, suggesting that the presence of the influenza haemagglutinin transmembrane domain on the gp140-HA_2tr antigen precluded efficient recovery of the antigen by homogenization of leaf tissue. Despite the promising results yielded by including urea in the extraction buffer, the use of detergents precludes the recovery of proteins in their native conformations rendering it unsuitable for the purification of our antigens. We therefore compared the influence of different culture densities of the recombinant *A. tumefaciens* strains, on heterologous protein expression, when infiltrated into *N. benthamiana* plants.

Variation of the culture density at infiltration appeared to have a positive impact on the expression of each of the recombinant antigens, with detectable levels of antigen expression witnessed within 3 days post infiltration. Interestingly, a faint band was detected for all 3 antigens on days 1 and 3 at an OD_{600} of 0.5 despite being unable to detect any protein expression at this culture density under identical experimental conditions previously. This observation raises the possibility that considerable variation may exist between experimental infiltration procedures. In this vein, it is well documented that senescing leaves are associated with higher levels of protease activity and agroinfiltration of older leaves may result in higher levels of protein degradation (19).

In order to verify that the putative bands yielded by western blotting were not an artifact of non-specific antibody reactivity, the western blotting procedure was repeated with a different negative control and the experimental procedure refined. Each of the experimental lysate samples yielded a single defined band above the 170 kDa molecular weight standard which was absent from the negative control. These

findings support the assertion that the bands of interest do in fact correspond to the antigens of interest, although the expression levels were poor.

Lastly, in an attempt to improve recovery of the recombinant proteins from agroinfiltrated leaf tissue a direct apoplastic extraction procedure was conducted on agroinfiltrated leaves. Western blotting of the samples revealed a blurry, yet promising, band for each of the antigens that was absent from the negative control. The increased band intensity when the antigens were enriched for by the apoplastic protein extraction method supports the assertion that the band of interest corresponds to the desired antigens. Additionally, the band of interest was also absent from the negative control. Collectively these observations suggest that the 3 recombinant antigens were successfully expressed in plants, albeit at very low levels.

The plant-derived envelope antigens migrated at a noticeably higher molecular weight, following western blotting, than when the identical antigens had been expressed in transfected HEK 293 cells. The detection of the gp140-$HA2_{tr}$ product in the presence of urea, under denaturing western blotting conditions, precludes the possibility that the size discrepancy could be attributed to the formation of oligomers. It is also unlikely that the size anomaly is a reflection of the inherent differences in the glycosylation patterns of plants as neither Medicago nor Rosenberg and colleagues reported similar observations (61, 289). The coding sequence of the antigens had also been verified, after cloning and *in vitro* culturing of *A. tumefaciens,* confirming that the unexpected size difference was not due to an error in the DNA sequence of the genes. The unexpected size difference of the antigens could possibly be attributed to modification of the proteins as they passed through the cell secretory pathway. It is possible that the gene coding sequence may have contained processing signals that were recognized by plants but not mammalian cells. However, further work will be required to determine the exact cause of this size anomaly.

Medicago, a Quebec-based plant biotechnology company, have reported promising expression levels of similar HIV-1 envelope antigens comprising of translational fusions between the envelope coding sequence and the transmembrane and cytosolic domains of influenza H5 or H3 (61). Several disparities could account for the

conflicting results arising from our study when compared to the data generated by Medicago; pertaining both to the choice of envelope and the expression systems used (**Table 4.1**). Firstly, it is noteworthy that the HIV-1 envelope coding sequence used by Medicago was based on a group M consensus sequence as opposed to a naturally derived sequence. Furthermore the envelope protein was extensively engineered by shortening the variable regions and deleting the cleavage site, fusion peptide and immunodominant region of gp41. In stark contrast, the envelope protein chosen for this study was derived from a naturally occurring viral isolate whereby the structure was preserved as far as possible. Additionally, the coding sequences of the immunogens used by Medicago were optimized to represent preferred human codon usage whereas the gene sequences used in this study were based on the native viral sequence (198). The expression system used by Medicago is also considerably different to the system used in this study. Notably; the Medicago expression system employs the Alfalfa Protein disulphide isomerase signal sequence to direct the recombinant antigens into the leaf apoplast. Lastly, the Medicago expression cassette exploits the P19 silencing suppressor as opposed to the NSs suppressor that was used in this study.

An independent study by Rosenberg *et al*., also reported promising expression of recombinant HIV-1 envelope in plants using a truncated protein whereby the cleavage site, fusion peptide and immunodominant region of gp41 had been removed. The study reported the successful expression of the gp140 ΔCFI protein in both the cytoplasm and ER, using a similar pTRA vector system to that described in this study, along with the P19 suppressor. The codon usage of the gene sequence in the study was not disclosed. Interestingly, in addition to the expected band for the antigen, western blotting of agroinfiltrated plant extracted yielded an additional higher order band which the authors attributed to the formation of oligomers (289).

Table 4.1: Comparative summary of the differences in antigens and expression systems used in this study and the Medicago study (61)

	pTRA-A: gp150	pTRA-A: gp120-HA2	pTRA-A: gp140-HA_2tr	995	997	999
Insert	gp150: CAP 256 HIV-1 envelope glycoprotein truncated in C terminal cytoplasmic tail.	gp120-HA_2: Full length exterior subunit (gp120) of CAP 256 HIV-1 envelope glycoprotein fused in frame to full length HA_2 subunit of influenzaH5.	gp140-HA_2tr: Full length exterior subunit and ectodomain of CAP 256 HIV-1 envelope glycoprotein fused in frame to truncated HA_2 subunit of influenza H5	ConS-ΔCFI : Group M consensus sequence containing shortened variable loops, deletions in the cleavage site, fusion peptide and immunodominant region of gp41	ConS-ΔCFI-H3: ConS-ΔCFI lacking the native TM and cytosolic domains fused to the transmembrane and cytosolic domains of H3/A/Brisbane/10/2007	ConS-ΔCFI-H5: ConS-ΔCFI lacking the native TM and cytosolic domains fused to the transmembrane and cytosolic domains of H5 A/Indonesia/5/2005
Codon usage	Native	Native for HIV Humanized for HA	Native for HIV Humanized for HA	Humanized	Humanized	Humanized
Signal peptide	LPH	LPH	LPH	Alfalfa protein disulphide isomerase	Alfalfa protein disulphide isomerase	Alfalfa protein disulphide isomerase
Expression vector	pTRA-A	pTRA-A	pTRA-A	2X35S-CPMV-HT	2X35S-CPMV-HT	2X35S-CPMV-HT
localization	Apoplast	Apoplast	Apoplast	Apoplast	Apoplast	Apoplast
Bacterial strain	GV3101::pMP90RK	GV3101::pMP90RK	GV3101::pMP90RK	AGL1	AGL1	AGL1
Silencing suppressor	NSs	NSs	NSs	P19	P19	P19

In the light of these studies, it is likely that the poor expression of the antigens observed in this project may be a reflection on the codon usage of the gene sequences and that codon optimization of the DNA may be beneficial. The coding sequences of our 3 antigens were based on the CAP256 envelope coding sequences and were therefore representative of the wild type codon usage. It has been suggested that the native HIV-1 codon usage is not amenable to high levels of expression and has been reported to hinder the expression of a model protein, Thy-1, when expressed using the preferred HIV-1 codon bias (123).

Codon optimization is often exploited to achieve optimal levels of expression of heterologous proteins in plants, although the ideal codon usage is largely empirically determined rather than being governed by any pre-existing rationale (292, 293). To illustrate this point, early work by Biemelt and colleagues demonstrated that human codon optimization of the HPV L1 protein achieved superior expression to plantized and native sequences in transgenic plants (24). A similar study conducted by our research group corroborated these findings using a transient *A. tumefaciens* mediated expression system (209). Initial attempts by our group to express both plantized and native HIV-1 Gag particles in transgenic plants were unsuccessful and no discernable Gag expression could be discerned using standardized diagnostic assays (294). A subsequent study managed to achieve detectable levels of Gag particles derived from plantized, but not native, gene sequences (227). In some cases, it has been our experience that mycobacterial codon optimized genes are amenable to higher levels of expression in plants than their native counterparts, presumably attributable to the improved mRNA stability conferred by their high GC content (Personal communication; Ann Meyers). Post hoc analysis of the gene sequences used in this study revealed that they had surprisingly low GC contents, especially when compared to the full H5 protein that was used as an expression control (**Table 4.2**).

Another strategy that could be exploited to improve the levels of heterologous protein expression is to alter the subcellular localization of the antigens. Once again the outcome of this approach is unpredictable and whether this may confer any benefit can only be established emperically (292, 293). Lastly, it cannot be discounted that

the poor levels of protein expression may be an intrinsic property of the CAP256 envelope glycoprotein itself, rather than a reflection on the expression system.

Table 4.2: Summary of the GC content of the antigen coding sequences used in this study.

Antigen	GC content (%)
gp150	39.1
gp120-HA$_2$	44.2
gp140-HA$_2$tr	40
H5	60.6

4.3. Future prospects

The promising expression of each of the HIV-1 envelope antigens from the pTHPcapR DNA vaccine vector strongly supports their further development. A recent study reported the induction of robust antibody responses against influenza H5N1 Haemagglutinin using the pTH DNA vaccine vector system from which the DNA vaccine vector in this study was derived (237). Immunization studies are currently being planned in rabbits to determine whether these candidate vaccines have the capacity to induce neutralizing antibodies against the HIV-1 envelope glycoprotein. Concurrent with these studies, co-localization experiments are planned to determine whether the influenza HA_2 transmembrane subunit promotes increased association with the plasma membrane.

The low expression levels of the plant-derived antigens are not sufficient for further characterization or immunogenicity studies. Unfortunately due to the time constraints inherent in this study the expression of these proteins could not be further optimized. The low expression levels of the recombinant protein expression will be addressed by optimizing both the GC content and codon usage of the gene sequences. Additionally, the influence of targeting the antigens to different subcellular locations may be explored using a panel of pTRA plant expression vectors available in our lab (209). The antigenicity of each of the immunogens will also need to be evaluated in order to determine whether the broadly neutralizing epitopes, that are present on the parental virus, are reproduced in the context of the synthetic proteins that were designed in this study. This will be particularly important in the case of the plant-derived antigens due

to the inherent differences in plant glycosylation which may influence the conformation of the protein (109). It is possible that different conformations of the antigens may alter the exposure of neutralizing antibody epitopes, influencing both the antigenicity and immunogenicity of the recombinant subunit vaccines. In conclusion 3 promising DNA vaccines have been developed in this study warranting further characterization and immunogenicity studies. A highly systematic process was employed to optimize the expression of matching recombinant proteins in plants. The levels of the recombinant proteins, expressed in plants were low at best and further optimization will be required to utilize these antigens as subunit vaccines.

REFERENCES

1. **Aasa-Chapman M. M., A. Hayman, P. Newton, D. Cornforth, I. Williams, P. Borrow, P. Balfe, and A. McKnight**. 2004. Development of the antibody response in acute HIV-1 infection. Aids **18**:371–381.

2. **Allen G. C., G. E. Hall, L. C. Childs, A. K. Weissinger, S. Spiker, and W. F. Thompson**. 1993. Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. Plant Cell **5**:603–13.

3. **Allen G. C., G. Hall, S. Michalowski, W. Newman, S. Spiker, A. K. Weissinger, and W. F. Thompson**. 1996. High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell **8**:899–913.

4. **Alvarez M., H. Pinyerd, J. Crisantes, R. MM, P. J, W. AM, M. HS, and C. GA**. 2006. Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice. Vaccine **24**:2477–2490.

5. **Arakawa T., J. Yu, and W. Langridge**. 2001. Synthesis of a cholera toxin B subunit-rotavirus NSP4 fusion protein in potato. Plant Cell Rep. **20**:343–348.

6. **Azzoni A. R., A. R. Kusnadi, E. A. Miranda, and Z. L. Nikolov**. 2002. Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. Biotechnol. Bioeng. **80**:268–76.

7. **Bachmann M. F., U. H. Rohrer, T. M. Kündig, K. Bürki, H. Hengartner, and R. M. Zinkernagel**. 1993. The influence of antigen organization on B cell responsiveness. Science **262**:1448–51.

8. **Bakker H., M. Bardor, J. Molthoff, V. Gomord, I. Elbers, L. Stevens, W. Jordi, A. Lommen, L. Faye, P. Lerouge, and D. Bosch**. 2001. Galactose-extended glycans of antibodies produced by transgenic plants. Proc. Natl. Acad. Sci. **98**:2899–904.

9. **Bardor M., C. Faveeuw, A.-C. Fitchette, D. Gilbert, L. Galas, F. Trottein, L. Faye, and P. Lerouge**. 2003. Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose. Glycobiology **13**:427–34.

10. **Barouch D. H., Z. Yang, W. Kong, B. Korioth-Schmitz, S. M. Sumida, D. M. Truitt, M. G. Kishko, J. C. Arthur, A. Miura, J. R. Mascola, N. L. Letvin, and G. J. Nabel**. 2005. A Human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. J. Virol. **79**:8828–8834.

11. **Barouch D.** 2013. The quest for an HIV vaccine - moving forward. N. Engl. J. Med. **369**:2073–2076.

12. **Barouch D., and B. Korber**. 2010. HIV-1 vaccine development after STEP. Annu. Rev. Med. **61**:153–167.

13. **Barré-Sinoussi F., J. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier**. 1983. Isolation of a T-Lymphotrophic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). Science (80-.). Wiley Online Library **220**:868–871.

14. **Barta A., K. Sommergruber, D. Thompson, K. Hartmuth, M. A. Matzke, and A. J. M. Matzke**. 1986. The expression of a nopaline synthase - human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol. Biol. **6**:347–357.

15. **Batard Y., a Hehn, S. Nedelkina, M. Schalk, K. Pallett, H. Schaller, and D. Werck-Reichhart**. 2000. Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. Arch. Biochem. Biophys. **379**:161–9.

16. **Belshe R., G. Franchini, M. Girard, G. F, P. Kaleebu, M. Marthas, M. MB, R. McCullough, F. Mhalu, D. Salmon-Ceron, R.-P. Sekaly, K. van Rompay, B. Verrier, B. Wahren, and M. Weissenbacher**. 2004. Support for the RV144 HIV vaccine trial. Science (80-.). **305**:177–180.

17. **Belshe R. B., B. S. Graham, M. C. Keefer, M. Gorse, P. Wright, R. Dolin, T. Matthews, K. Weinhold, D. P. Bolognesi, R. Sposto, D. M. Stablein, T. Twaddell, P. W. Berman, T. Gregory, A. E. Izu, M. C. Walker, and P. Fast**. 1994. Neutralizing Antibodies to HIV-1 in Seronegative Volunteers Immunized With Recombinant gp 120 From the MN Strain of HIV-1. J. Am. Med. Assoc. **272**:475–480.

18. **Belshe R., G. Gorse, M. Mulligan, T. Evans, M. Keefer, J.-L. Excler, A.-M. Duliege, J. Tartaglia, W. Cox, J. McNamara, K.-L. Hwang, A. Bradney, D. Montefiori, and K. Weinhold**. 1998. Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. Aids **12**:2407–2415.

19. **Benchabane M., C. Goulet, D. Rivard, L. Faye, V. Gomord, and D. Michaud**. 2008. Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol. J. **6**:633–48.

20. **Benlahrech A., J. Harris, A. Meiser, T. Papagatsias, J. Hornig, P. Hayes, A. Lieber, T. Athanasopoulos, V. Bachy, E. Csomor, R. Daniels, K. Fisher, F. Gotch, L. Seymour, K. Logan, R. Barbagallo, L. Klavinskis, G. Dickson, and S. Patterson**. 2009. Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. Proc. Natl. Acad. Sci. U. S. A. **106**:19940–19945.

21. **Berman P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg**. 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature **345**:622–625.

22. **Berman P. W., K. K. Murthy, T. Wrin, J. C. Vennari, E. K. Cobb, D. J. Eastman, M. Champe, G. R. Nakamura, D. Davison, M. F. Powell, J. Bussiere, D. P. Francis, T. Matthews, T. J. Gregory, and J. F. Obijeski**. 1996. Protection of MN-rgp120-immunized chimpanzees from heterologous infection with a primary isolate of human immunodeficiency virus type 1. J. Infect. Dis. **173**:52–59.

23. **Berman P. W., W. M. Nunes, and O. K. Haffar**. 1988. Expression of membrane-associated and secreted variants of gp160 of human immunodeficiency virus type 1 in vitro and in continuous cell lines. J. Virol. **62**:3135–42.

24. **Biemelt S., U. Sonnewald, P. Galmbacher, L. Willmitzer, and M. Müller**. 2003. Production of Human Papillomavirus Type 16 Virus-Like Particles in Transgenic Plants. J. Virol. **77**:9211–9220.

25. **Binley J. M., T. Wrin, B. Korber, M. B. Zwick, M. Wang, C. Chappey, G. Stiegler, R. Kunert, S. Zolla-pazner, H. Katinger, C. J. Petropoulous, and D. R. Burton**. 2004. Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies. J. Virol. **78**:13232–13252.

26. **Bonsignori M., K.-K. Hwang, X. Chen, C.-Y. Tsao, L. Morris, E. Gray, D. J. Marshall, J. A. Crump, S. H. Kapiga, N. E. Sam, F. Sinangil, M. Pancera, Y. Yongping, B. Zhang, J. Zhu, P. D. Kwong, S. O'Dell, J. R. Mascola, L. Wu, G. J. Nabel, S. Phogat, M. S. Seaman, J. F. Whitesides, M. A. Moody, G. Kelsoe, X. Yang, J. Sodroski, G. M. Shaw, D. C. Montefiori, T. B. Kepler, G. D. Tomaras, S. M. Alam, H.-X. Liao, and B. F. Haynes**. 2011. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. J. Virol. **85**:9998–10009.

27. **Borrow P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone**. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68**:6103–6110.

28. **Bosch D., and A. Schots**. 2010. Plant glycans: friend or foe in vaccine development? Expert Rev. Vaccines **9**:835–842.

29. **Bosch M. L., P. L. Earl, K. Fargnoli, S. Picciafuoco, F. Giombini, F. Wong-staal, and G. Franchini**. 1989. Identification of the Fusion Peptide of Primate Immunodeficiency Viruses. Science (80-.). **244**:694–697.

30. **Buchbinder S. P., D. V Mehrotra, A. Duerr, D. W. Fitzgerald, R. Mogg, D. Li, P. B. Gilbert, J. R. Lama, M. Marmor, C. Del Rio, M. J. McElrath, D. R. Casimiro, K. M. Gottesdiener, J. A. Chodakewitz, L. Corey, and M. N. Robertson**. 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet **372**:1881–1893.

31. **Bulmer M.** 1987. Coevolution of codon usage and transfer RNA abundance.pdf. Nat. Lett. **325**:328–330.

32. **Bunnik E., L. Pisas, A. Van Nuenen, and H. Schuitmaker**. 2008. Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. J. Virol. **82**:7932–7941.

33. **Buonaguro L., M. Tagliamonte, M. L. Tornesello, and F. M. Buonaguro**. 2011. Developments in virus-like particle-based vaccines for infectious diseases and cancer. Expert Rev. Vaccines **10**:1569–1583.

34. **Burgers W. A., J. H. van Harmelen, E. Shephard, C. Adams, T. Mgwebi, W. Bourn, T. Hanke, A.-L. Williamson, and C. Williamson**. 2006. Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. J. Gen. Virol. **87**:399–410.

35. **Burgers W. a, E. Shephard, J. E. Monroe, T. Greenhalgh, A. Binder, E. Hurter, J. H. Van Harmelen, C. Williamson, and A.-L. Williamson**. 2008. Construction, characterization, and immunogenicity of a multigene modified vaccinia Ankara (MVA) vaccine based on HIV type 1 subtype C. AIDS Res. Hum. Retroviruses **24**:195–206.

36. **Burton D. R., R. C. Desrosiers, R. W. Woms, M. B. Feinberg, R. C. Gallo, B. Hahn, J. A. Hoxie, E. Hunter, B. Korber, A. Landay, M. M. Lederman, J. Lieberman, J. M. McCune, J. P. Moore, N. Nathanson, L. Picker, L. Richman, D. Richman, C. Rinaldo, M. Stevenson, D. I. Watkins, S. M. Wolinsky, and J. A. Zack**. 2004. A sound rationale needed for phase III HIV-1 vaccine trials. Science (80-.). **303**:120.

37. **Burton D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, and P. L. Nara**. 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science (80-.). **266**:1024–1027.

38. **C.T Chung and R.H. Miller**. 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16**:20892–20892.

39. **Cao Yy., L. Qin, L. Zhang, J. Safrit, and D. D. Ho**. 1995. Virological and Immunological Characterization of long-term survivors of Human Immunod eficiency Virus Type 1 Infection. N. Engl. J. Med. **332**:201–208.

40. **Cardi T., P. Lenzi, and P. Maliga**. 2010. Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev. Vaccines **9**:893–911.

41. **Cardoso R. M. F., M. B. Zwick, R. L. Stanfield, R. Kunert, J. M. Binley, H. Katinger, D. R. Burton, and I. A. Wilson**. 2005. Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. Immunity **22**:163–173.

42. **Carthew R. W.** 2001. Gene silencing by double-stranded RNA. Curr. Opin. Cell Biol. **13**:244–248.

43. **Casimiro D. R., F. Wang, W. A. Schleif, X. Liang, Z.-Q. Zhang, T. W. Tobery, M.-E. Davies, A. B. McDermott, D. H. O'Connor, A. Fridman, A. Bagchi, L. G. Tussey, A. J. Bett, A. C. Finnefrock, T. Fu, A. Tang, K. A. Wilson, M. Chen, H. C. Perry, G. J. Heidecker, D. C. Freed, A. Carella, K. S. Punt, K. J. Sykes, L. Huang, V. I. Ausensi, M. Bachinsky, U. Sadasivan-Nair, D. I. Watkins, E. A. Emini, and J. W. Shiver**. 2005. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. J. Virol. **79**:15547–15555.

44. **Catanzaro A. T., R. A. Koup, M. Roederer, R. T. Bailer, M. E. Enama, Z. Moodie, L. Gu, J. E. Martin, L. Novik, B. K. Chakrabarti, B. T. Butman, J. G. D. Gall, C. R. King, C. A. Andrews, R. Sheets, P. L. Gomez, J. R. Mascola, G. J.**

Nabel, and B. S. Graham. 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. J. Infect. Dis. **194**:1638–1649.

45. **Catanzaro A. T., M. Roederer, R. A. Koup, R. T. Bailer, M. E. Enama, M. C. Nason, J. E. Martin, S. Rucker, C. A. Andrews, P. L. Gomez, J. R. Mascola, G. J. Nabel, and B. S. Graham**. 2007. Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. Vaccine **25**:4085–4092.

46. **Center R., R. Leapman, J. Lebowitz, L. Arthur, P. Earl, and M. B**. 2002. Oligomeric structure of the human immunodeficiency virus type 1 envelope protein on the virion surface. J. Virol. **76**:7863–7867.

47. **Chackerian B.** 2007. Virus-like particles: flexible platforms for vaccine development. Expert Rev. Vaccines **6**:381–390.

48. **Chan D. C., D. Fass, J. M. Berger, and P. S. Kim**. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89**:263–273.

49. **Changela A., X. Wu, Y. Yang, B. Zhang, J. Zhu, G. A. Nardone, S. O'Dell, M. Pancera, M. K. Gorny, S. Phogat, J. E. Robinson, L. Stamatatos, S. Zolla-Pazner, J. R. Mascola, and P. D. Kwong**. 2011. Crystal structure of human antibody 2909 reveals conserved features of quaternary structure-specific antibodies that potently neutralize HIV-1. J. Virol. **85**:2524–2535.

50. **Chargelegue D., N. D. Vine, C. J. van Dolleweerd, P. M. Drake, and J. K.-C. Ma**. 2000. A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. Transgenic Res. **9**:187–194.

51. **Chen L., Y. Do Kwon, T. Zhou, X. Wu, S. O'Dell, L. Cavacini, A. J. Hessell, M. Pancera, M. Tang, L. Xu, Z.-Y. Yang, M.-Y. Zhang, J. Arthos, D. R. Burton, D. S. Dimitrov, G. J. Nabel, M. R. Posner, J. Sodroski, R. Wyatt, J. R. Mascola, and P. D. Kwong**. 2009. Structural basis of immune evasion at the site of CD4 attachment on HIV-1 gp120. Science (80-.). **326**:1123–1127.

52. **Chen T.-L., Y.-L. Lin, Y.-L. Lee, N.-S. Yang, and M.-T. Chan**. 2004. Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures. Transgenic Res. **13**:499–510.

53. **Chichester J. A., K. Musiychuk, P. de la Rosa, A. Horsey, N. Stevenson, N. Ugulava, S. Rabindran, G. A. Palmer, V. Mett, and V. Yusibov**. 2007. Immunogenicity of a subunit vaccine against Bacillus anthracis. Vaccine **25**:3111–3114.

54. **Churchyard G. J., C. Morgan, E. Adams, J. Hural, B. S. Graham, Z. Moodie, D. Grove, G. Gray, L.-G. Bekker, M. J. McElrath, G. D. Tomaras, P. Goepfert, S. Kalams, L. R. Baden, M. Lally, R. Dolin, W. Blattner, A. Kalichman, J. P. Figueroa, J. Pape, M. Schechter, O. Defawe, S. C. De Rosa, D. C. Montefiori, G. J. Nabel, L. Corey, and M. C. Keefer**. 2011. A phase IIA randomized clinical trial of a multiclade HIV-1 DNA prime followed by a multiclade rAd5 HIV-1 vaccine boost in healthy adults (HVTN204). PLoS One **6**:e21225.

55. **Clements-Mann M., K. Weinhold, T. J. Matthews, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, R.-H. Hsieh, J. Mestecky, S. Zolla-Pazner, J. Mascola, D. Schwartz, R. Siliciana, L. Corey, P. F. Wright, R. Belshe, R. Dolin, S. Jackson, S. Xu, P. Fast, M. C. Walker, D. Stablein, J.-L. Excler, J. Tartagia, A.-M. Duliege, F. Sinangil, and E. Paoletti**. 1998. Immune responses to human immunodeficiency virus (HIV) type 1 induced by canarypox expressing HIV-1MN gp120, HIV-1SF2 recombinant gp120, or both vaccines in seronegative adults. J. Infect. **177**:1230–1246.

56. **Comai L., P. Moran, and D. Maslyar**. 1990. Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol. Biol. **15**:373–81.

57. **Conrad U., and U. Fiedler**. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol. Biol. **38**:101–109.

58. **Corti D., J. P. M. Langedijk, A. Hinz, M. S. Seaman, F. Vanzetta, B. M. Fernandez-Rodriguez, C. Silacci, D. Pinna, D. Jarrossay, S. Balla-Jhagjhoorsingh, B. Willems, M. J. Zekveld, H. Dreja, E. O'Sullivan, C. Pade, C. Orkin, S. A. Jeffs, D. C. Montefiori, D. Davis, W. Weissenhorn, A. McKnight, J. L. Heeney, F. Sallusto, Q. J. Sattentau, R. A. Weiss, and A. Lanzavecchia**. 2010. Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. PLoS One **5**:e8805.

59. **Cosa B. De, W. Moar, S. B. Lee, M. Miller, and H. Daniell**. 2001. Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat. Biotechnol. **19**:71–74.

60. **Crooks E. T., T. Tong, K. Osawa, and J. M. Binley**. 2011. Enzyme digests eliminate nonfunctional Env from HIV-1 particle surfaces, leaving native Env trimers intact and viral infectivity unaffected. J. Virol. **85**:5825–5839.

61. **D'Aoust M.-A., M. Couture, and P.-O. Lavoie**. 2012. Virus like particle production in plants. U.S. Patent PCT/ CA2011/ 001427.

62. **D'Aoust M.-A., M. M.-J. Couture, N. Charland, S. Trépanier, N. Landry, F. Ors, and L.-P. Vézina**. 2010. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol. J. **8**:607–619.

63. **D'Aoust M., P. Lavoie, M. M. Couture, S. Trépanier, J.-M. Guay, M. Dargis, S. Mongrand, N. Landry, B. J. Ward, and L.-P. Vézina**. 2008. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol. J. **6**:930–940.

64. **Dai S., P. Zheng, P. Marmey, S. Zhang, W. Tian, R. N. Beachy, and C. Fauquet**. 2001. Comparative analysis of transgenic rice plants obtained by Agrobacterium -mediated transformation and particle bombardment. Mol. Breed. **7**:25–33.

65. **Dalgleish A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss**. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312**:763–767.

66. **Daniell H., S. J. Streatfield, and K. Wycoff**. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. **6**:219–226.

67. **Daniell H., N. D. Singh, H. Mason, and S. J. Streatfield**. 2009. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci. **14**:669–679.

68. **Datla R. S. S., F. Bekkaoui, J. K. Hammerlindl, G. Pilate, D. I. Dunstan, and W. L. Crosby**. 1993. Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. **94**:139–149.

69. **Davis K. L., F. Bibollet-Ruche, H. Li, J. M. Decker, O. Kutsch, L. Morris, A. Salomon, A. Pinter, J. A. Hoxie, B. H. Hahn, P. D. Kwong, and G. M. Shaw**. 2009. Human immunodeficiency virus type 2 (HIV-2)/HIV-1 envelope chimeras detect high titers of broadly reactive HIV-1 V3-specific antibodies in human plasma. J. Virol. **83**:1240–1259.

70. **Davis K. L., E. S. Gray, P. L. Moore, J. M. Decker, A. Salomon, D. C. Montefiori, B. S. Graham, M. C. Keefer, A. Pinter, L. Morris, B. H. Hahn, and G. M. Shaw**. 2009. High titer HIV-1 V3-specific antibodies with broad reactivity but low neutralizing potency in acute infection and following vaccination. Virology. Elsevier Inc. **387**:414–26.

71. **Deeks S. G., B. Schweighardt, T. Wrin, J. Galovich, R. Hoh, E. Sinclair, P. Hunt, J. M. McCune, J. N. Martin, C. J. Petropoulos, and F. M. Hecht**. 2006. Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. J. Virol. **80**:6155–6164.

72. **Deml L., G. Kratochwil, N. Osterrieder, R. Knüchel, H. Wolf, and R. Wagner**. 1997. Increased incorporation of chimeric human immunodeficiency virus

type 1 gp120 proteins into Pr55gag virus-like particles by an Epstein-Barr virus gp220/350-derived transmembrane domain. Virology **235**:10–25.

73. **Deml L., R. Schirmbeck, J. Reimann, H. Wolf, and R. Wagner**. 1997. Recombinant human immunodeficiency Pr55gag virus-like particles presenting chimeric envelope glycoproteins induce cytotoxic T-cells and neutralizing antibodies. Virology **235**:26–39.

74. **Derdeyn C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen, and E. Hunter**. 2004. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science (80-.). **303**:2019–2022.

75. **Desai P. N., N. Shrivastava, and H. Padh**. 2010. Production of heterologous proteins in plants: strategies for optimal expression. Biotechnol. Adv. Elsevier Inc. **28**:427–435.

76. **Dimarzo F., A. L. Devico, T. D. Copeland, S. Oroszlan, R. C. Gallo, M. G. Sarngadharan, and F. D. Veronse**. 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV Envelope gene. Science (80-.). **229**:1402–1405.

77. **Dixon S., S. Mcdonald, and J. Roberts**. 2001. The impact of HIV and AIDS on Africa's economic development. Br. Med. J. **234**:232–234.

78. **Doan L. X., M. Li, C. Chen, and Q. Yao**. 2005. Virus-like particles as HIV-1 vaccines. Rev. Med. Virol. **15**:75–88.

79. **Donnelly J. J., B. Wahren, and M. A. Liu**. 2005. DNA vaccines: progress and challenges. J. Immunol. **175**:633–639.

80. **Doria-Rose N. a, R. M. Klein, M. G. Daniels, S. O'Dell, M. Nason, A. Lapedes, T. Bhattacharya, S. a Migueles, R. T. Wyatt, B. T. Korber, J. R. Mascola, and M. Connors**. 2010. Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. J. Virol. **84**:1631–6.

81. **Earl P. L., R. W. Doms, and B. Moss**. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. **87**:648–52.

82. **Earl P. L., B. Moss, and R. W. Doms**. 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. **65**:2047–2055.

83. **Edwards T. G., S. Wyss, J. D. Reeves, S. Zolla-pazner, J. A. Hoxie, R. W. Doms, and F. Baribaud**. 2002. Truncation of the Cytoplasmic Domain Induces Exposure of Conserved Regions in the Ectodomain of Human Immunodeficiency Virus Type 1 Envelope Protein. J. Virol. **76**:2683–2691.

84. **Falkowska E., A. Ramos, Y. Feng, T. Zhou, S. Moquin, L. M. Walker, X. Wu, M. S. Seaman, T. Wrin, P. D. Kwong, R. T. Wyatt, J. R. Mascola, P. Poignard, and D. R. Burton**. 2012. PG V04, an HIV-1 gp120 CD4 binding site antibody, is broad and potent in neutralization but does not induce conformational changes characteristic of CD4. J. Virol. **86**:4394–4403.

85. **Farran I., J. J. Sánchez-Serrano, J. F. Medina, J. Prieto, and A. M. Mingo-Castel**. 2002. Targeted expression of human serum albumin to potato tubers. Transgenic Res. **11**:337–346.

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

87. **Feng C. G., U. Palendira, C. Demangel, J. M. Spratt, A. S. Malin, and W. J. Britton**. 2001. Priming by DNA Immunization Augments Protective Efficacy of Mycobacterium bovis Bacille Calmette-Guerin against Tuberculosis. Infect. Immun. **69**:4174–4176.

88. **Fensterle J., L. Grode, J. Hess, and S. H. Kaufmann**. 1999. Effective DNA vaccination against listeriosis by prime/boost inoculation with the gene gun. J. Immunol. **163**:4510–4518.

89. **Fischer R., S. Schillberg, P. Christou, and H. Klee**. 2004. Molecular farming: Plant-made pharmaceuticals and technical proteins., 1st ed. Wiley-CVH Verlag GmbH & Co., Weiheim.

90. **Fischer R., S. Schillberg, S. Hellwig, R. M. Twyman, and J. Drossard**. 2012. GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnology. Elsevier Inc. **30**:434–439.

91. **Fischer R., E. Stoger, S. Schillberg, P. Christou, and R. M. Twyman**. 2004. Plant-based production of biopharmaceuticals. Curr. Opin. Plant Biol. **7**:152–8.

92. **Floss D. M., M. Sack, E. Arcalis, J. Stadlmann, H. Quendler, T. Rademacher, E. Stoger, J. Scheller, R. Fischer, and U. Conrad**. 2009. Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus-neutralizing antibody. Plant Biotechnol. J. **7**:899–913.

93. **Flynn N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, and M. F. Para**. 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J. Infect. Dis. **191**:654–665.

94. **Freed E. O., D. J. Myers, and R. Risser**. 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc. Natl. Acad. Sci. U. S. A. **87**:4650–4654.

95. **Friedrich T. C., L. E. Valentine, L. J. Yant, E. G. Rakasz, S. M. Piaskowski, J. R. Furlott, K. L. Weisgrau, B. Burwitz, G. E. May, E. J. León, T. Soma, G. Napoe, S. V Capuano, N. A. Wilson, and D. I. Watkins**. 2007. Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication. J. Virol. **81**:3465–3476.

96. **Frost S. D. W., T. Wrin, D. M. Smith, K. S. L. Pond, Y. Liu, E. Paxinos, C. Chappey, J. Galovich, J. Beauchaine, C. J. Petropoulos, S. J. Little, and D. D. Richman**. 2005. Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. Proc. Natl. Acad. Sci. U. S. A. **102**:18514–18519.

97. **Fultz P. N., P. Nara, F. Barre-Sinoussi, a Chaput, M. L. Greenberg, E. Muchmore, M. P. Kieny, and M. Girard**. 1992. Vaccine protection of chimpanzees against challenge with HIV-1-infected peripheral blood mononuclear cells. Science **256**:1687–90.

98. **Fynan E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson**. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. U. S. A. **90**:11478–11482.

99. **Gallo R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham**. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science (80-.). **224**:500–503.

100. **Gao F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn**. 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature **397**:436–441.

101. **Gao F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw, and B. H. Hahn**. 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature **358**:495–499.

102. **Garg S., A. E. Oran, H. Hon, and J. Jacob**. 2004. The hybrid cytomegalovirus enhancer/ chicken Beta-actin promoter along with woodchuck hepatitis virus posttranscriptional regulatory element enhances the protective efficacy of DNA vaccines. J. Immunol. **173**:550–558.

103. **Gilbert P. B., M. L. Peterson, D. Follmann, M. G. Hudgens, D. P. Francis, M. Gurwith, W. L. Heyward, D. V Jobes, V. Popovic, S. G. Self, F. Sinangil, D. Burke, and P. W. Berman**. 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J. Infect. Dis. **191**:666–677.

104. **Giorgi C., R. Franconi, and E. P. Rybicki**. 2010. Human papillomavirus vaccines in plants. Expert Rev. Vaccines **9**:913–924.

105. **Girard M., M. P. Kieny, A. Pinter, F. Barre-Sinoussi, P. Nara, H. Kolbe, K. Kusumi, A. Chaput, T. Reinhart, E. Muchmore, J. Ronco, M. Kaczorek, E. Gomard, J.-C. Cluckman, and P. N. Fultz**. 1991. Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. Proc. Natl. Acad. Sci. U. S. A. **88**:542–546.

106. **Gleba Y., V. Klimyuk, and S. Marillonnet**. 2005. Magnifection--a new platform for expressing recombinant vaccines in plants. Vaccine **23**:2042–2048.

107. **Golden A., D. A. Austen, M. R. van Schravendijk, B. J. Sullivan, E. S. Kawasaki, and M. S. Osburne**. 1998. Effect of promoters and signal sequences on the production of secreted HIV-1 gp120 protein in the baculovirus system. Protein Expr. Purif. **14**:8–12.

108. **Gomord V., A.-C. Fitchette-Laine, B. Satiat-Jeunemaitre, C. Hawes, and L. Faye**. 1997. The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targetting of proteins that escape the ER. plant J. **11**:313–325.

109. **Gomord V., A.-C. Fitchette, L. Menu-Bouaouiche, C. Saint-Jore-Dupas, C. Plasson, D. Michaud, and L. Faye**. 2010. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol. J. **8**:564–587.

110. **Gonzalo R. M., G. del Real, J. R. Rodriguez, D. Rodriguez, R. Heljasvaara, P. Lucas, V. Larraga, and M. Esteban**. 2002. A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the Leishmania infantum P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. Vaccine **20**:1226–1231.

111. **Graham B. S., R. A. Koup, M. Roederer, R. T. Bailer, M. E. Enama, Z. Moodie, J. E. Martin, M. M. McCluskey, B. K. Chakrabarti, L. Lamoreaux, C. a Andrews, P. L. Gomez, J. R. Mascola, and G. J. Nabel**. 2006. Phase 1 safety and

immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. J. Infect. Dis. **194**:1650–1660.

112. **Graham B. S., and J. R. Mascola**. 2005. Lessons from failure - preparing for future HIV-1 vaccine efficacy trials. J. Infect. Dis. **191**:647–649.

113. **Gray E. S., P. L. Moore, I. A. Choge, J. M. Decker, F. Bibollet-Ruche, H. Li, N. Leseka, F. Treurnicht, K. Mlisana, G. M. Shaw, S. S. A. Karim, C. Williamson, and L. Morris**. 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. J. Virol. **81**:6187–6196.

114. **Gray E. S., M. C. Madiga, T. Hermanus, P. L. Moore, C. K. Wibmer, N. L. Tumba, L. Werner, K. Mlisana, S. Sibeko, C. Williamson, S. A. Karim, and L. Morris**. 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. J. Virol. **85**:4828–4840.

115. **Gray G. E., M. Allen, Z. Moodie, G. Churchyard, L.-G. Bekker, M. Nchabeleng, K. Mlisana, B. Metch, G. de Bruyn, M. H. Latka, S. Roux, M. Mathebula, N. Naicker, C. Ducar, D. K. Carter, A. Puren, N. Eaton, M. J. McElrath, M. Robertson, L. Corey, and J. G. Kublin**. 2011. Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. Lancet Infect. Dis. Elsevier Ltd **11**:507–515.

116. **Gray R. H., M. J. Wawer, R. Brookmeyer, N. K. Sewankambo, D. Serwadda, F. Wabwire-Mangen, T. Lutalo, X. Li, T. VanCott, and T. C. Quinn**. 2001. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. Lancet **357**:1149–1153.

117. **Grgacic E. V. L., and D. A. Anderson**. 2006. Virus-like particles: passport to immune recognition. Methods **40**:60–65.

118. **Guerineau F., A. Lucy, and P. Mullineaux**. 1992. Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. Plant Mol. Biol. **18**:815–818.

119. **Gurtler L. G., P. H. Hauser, J. Eberle, A. von Brunn, S. Knapp, L. Zekeng, J. M. Tsague, and L. Kaptue**. 1994. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. J. Virol. **68**:1581–1585.

120. **Gurunathan S., D. M. Klinman, and R. A. Seder**. 2000. DNA vaccines: immunology, application, and optimization*. Annu. Rev. Immunol. **18**:927–974.

121. **Gustafsson C., S. Govindarajan, and J. Minshull**. 2004. Codon bias and heterologous protein expression. Trends Biotechnol. **22**:346–353.

122. **Haaland R. E., P. A. Hawkins, J. Salazar-Gonzalez, A. Johnson, A. Tichacek, E. Karita, O. Manigart, J. Mulenga, B. F. Keele, G. M. Shaw, B. H. Hahn, S. A. Allen, C. A. Derdeyn, and E. Hunter**. 2009. Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. PLoS Pathog. **5**:e1000274.

123. **Haas J., E.-C. Park, and B. Seed**. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr. Biol. **6**:315–324.

124. **Hallenberger S., V. Bosch, H. Angliker, E. Shaw, H. D. Klenk, and W. Garten**. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature **360**:358–361.

125. **Hammer S. M., M. E. Sobieszczyk, H. Janes, S. T. Karuna, M. J. Mulligan, D. Grove, B. A. Koblin, S. P. Buchbinder, M. C. Keefer, G. D. Tomaras, N. Frahm, J. Hural, C. Anude, B. S. Graham, M. E. Enama, E. Adams, E. DeJesus, R. M. Novak, I. Frank, C. Bentley, S. Ramirez, R. Fu, R. A. Koup, J. R. Mascola, G. J. Nabel, D. C. Montefiori, J. Kublin, M. J. McElrath, L. Corey, and P. B. Gilbert**. 2013. Efficacy Trial of a DNA/rAd5 HIV-1 Preventive Vaccine. N. Engl. J. Med. **309**:2083–2092.

126. **Hanke T., J. Schneider, S. C. Gilbert, a V Hill, and A. McMichael**. 1998. DNA multi-CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice. Vaccine **16**:426–435.

127. **Haq T., H. Mason, J. Clements, and C. Arntzen**. 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science (80-.). **268**:714–716.

128. **Harmelen J. H. van, E. van der Ryst, A. S. Loubser, D. York, S. Madurai, S. Lyons, R. Wood, and C. Williamson**. 1999. A predominantly HIV type 1 subtype C-restricted epidemic in South African urban populations. AIDS Res. Hum. Retroviruses **15**:395–398.

129. **Harpster M. H., J. A. Townsend, J. D. G. Jones, J. Bedbrook, and P. Dunsmuir**. 1988. Relative strengths of the 35S cauliflower mosaic virus, 1', 2', and nopaline synthase promoters in transformed tobacco sugarbeet and oilseed rape callus tissue. Mol. Gen. Genet. **212**:182–190.

130. **Harris A., G. Cardone, D. C. Winkler, J. B. Heymann, M. Brecher, J. M. White, and A. C. Steven**. 2006. Influenza virus pleiomorphy characterized by cryoelectron tomography. Proc. Natl. Acad. Sci. U. S. A. **103**:19123–19127.

131. **Haynes B. F., J. Fleming, E. W. St Clair, H. Katinger, G. Stiegler, R. Kunert, J. Robinson, R. M. Scearce, K. Plonk, H. F. Staats, T. L. Ortel, H.-X. Liao, and S. M. Alam**. 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science **308**:1906–1908.

132. **Haynes B., P. Gilbert, M. McElrath, S. Zolla-Pazner, G. Tomaras, S. Alam, D. Evans, D. Montefiori, C. Karnasuta, R. Sutthent, H.-X. Liao, A. DeVico, G. Lewis, C. Williams, A. Pinter, Y. Foung, H. Janes, A. DeCamp, Y. Huang, M. Rao, E. Billings, N. Karasavvas, M. Robb, V. Ngauy, M. de Souza, R. Paris, G. Ferrarri, R. Bailer, K. Soderberg, C. Andrews, P. Berman, N. Frahm, S. De Rosa, M. Alpert, N. Yates, X. Shen, R. Koup, P. Pitisuttithum, J. Kaewkungwal, S. Nitayaphan, S. Rerks-Ngarm, N. Michael, and J. Kin**. 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N. Engl. J. Med. **366**:1275–1286.

133. **Heckman K. L., and L. R. Pease**. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat. Protoc. **2**:924–932.

134. **Hedestam G. B. K., R. A. M. Fouchier, S. Phogat, D. R. Burton, J. Sodroski, and R. T. Wyatt**. 2008. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat. Rev. Microbiol. **6**:143–155.

135. **Hemelaar J.** 2012. The origin and diversity of the HIV-1 pandemic. Trends Mol. Med. Elsevier Ltd **18**:182–192.

136. **Hemelaar J., E. Gouws, P. D. Ghys, and S. Osmanov**. 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS **20**:13–23.

137. **Hemelaar J., E. Gouws, P. D. Ghys, and S. Osmanov**. 2011. Global trends in molecular epidemiology of HIV-1 during 2000–2007. Aids **25**:679–689.

138. **Hessell A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. H. I. Parren, P. A. Marx, and D. R. Burton**. 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat. Med. Nature Publishing Group **15**:951–954.

139. **Hessell A. J., E. G. Rakasz, P. Poignard, L. Hangartner, G. Landucci, D. N. Forthal, W. C. Koff, D. I. Watkins, and D. R. Burton**. 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog. **5**:e1000433.

140. **Hiatt A., R. Cafferkey, and K. Bowdish**. 1989. Production of antibodies in transgenic plants. Nature **342**:76–78.

141. **Hirsch V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson**. 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature **339**:389–392.

142. **Hoffenberg S., R. Powell, A. Carpov, D. Wagner, A. Wilson, S. Kosakovsky Pond, R. Lindsay, H. Arendt, J. Destefano, S. Phogat, P. Poignard, S. P. Fling, M. Simek, C. Labranche, D. Montefiori, T. Wrin, P. Phung, D. Burton, W. Koff, C. R. King, C. L. Parks, and M. J. Caulfield**. 2013. Identification of an HIV-1 clade

A envelope that exhibits broad antigenicity and neutralization sensitivity and elicits antibodies targeting three distinct epitopes. J. Virol. **87**:5372–5383.

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

144. **Hood E. E., S. L. Woodard, and M. E. Horn**. 2002. Monoclonal antibody manufacturing in transgenic plants - myths and realities. Curr. Opin. Biotechnol. **13**:630–635.

145. **Horn M. E., K. M. Pappu, M. R. Bailey, R. C. Clough, M. Barker, J. M. Jilka, J. A. Howard, and S. J. Streatfield**. 2003. Advantageous features of plant-based systems for the development of HIV vaccines. J. Drug Target. **11**:539–545.

146. **Huang C.-C., M. Tanng, S. Majeed, E. Montabana, R. L. STanfield, D. S. Dimitrov, B. Korber, J. Sodroski, I. A. Wilson, R. Wyatt, and P. D. Kwong**. 2005. Structure of a V3-Containing HIV-1 gp120 Core. Science (80-.). **310**:1025–1028.

147. **Huang J., G. Ofek, L. Laub, M. K. Louder, N. A. Doria-Rose, N. S. Longo, H. Imamichi, R. T. Bailer, B. Chakrabarti, S. K. Sharma, S. M. Alam, T. Wang, Y. Yang, B. Zhang, S. A. Migueles, R. Wyatt, B. F. Haynes, P. D. Kwong, J. R. Mascola, and M. Connors**. 2012. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature. Nature Publishing Group **491**:406–412.

148. **Huang Z., K. LePore, G. Elkin, Y. Thanavala, and H. S. Mason**. 2008. High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression system. Plant Biotechnol. J. **6**:202–209.

149. **Hutnick N. A., D. G. Carnathan, S. A. Dubey, G. Makedonas, K. S. Cox, L. Kierstead, S. J. Ratcliffe, M. N. Robertson, D. R. Casimiro, H. C. J. Ertl, and M. R. Betts**. 2009. Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific CD4+ T cells. Nat. Med. **15**:876–878.

150. **Hutnick N. A., D. J. F. Myles, C. B. Bian, K. Muthumani, and D. B. Weiner**. 2011. Selected approaches for increasing HIV DNA vaccine immunogenicity in vivo. Curr. Opin. Virol. Elsevier B.V. **1**:233–240.

151. **Ito H., M. H. Sommer, L. Zerboni, H. He, D. Boucaud, J. Hay, W. Ruyechan, and A. M. Arvin**. 2003. Promoter Sequences of Varicella-Zoster Virus Glycoprotein I Targeted by Cellular Transactivating Factors Sp1 and USF Determine Virulence in Skin and T Cells in SCIDhu Mice In Vivo. J. vir **77**:489–498.

152. **Jaoko W., E. Karita, K. Kayitenkore, G. Omosa-Manyonyi, S. Allen, S. Than, E. M. Adams, B. S. Graham, R. A. Koup, R. T. Bailer, C. Smith, L. Dally, B. Farah, O. Anzala, C. M. Muvunyi, J. Bizimana, T. Tarragona-Fiol, P. J. Bergin, P. Hayes, M. Ho, K. Loughran, W. Komaroff, G. Stevens, H. Thomson, M. J. Boaz, J. H. Cox, C. Schmidt, J. Gilmour, G. J. Nabel, P. Fast, and J. Bwayo**. 2010. Safety and immunogenicity study of Multiclade HIV-1 adenoviral vector vaccine alone or as boost following a multiclade HIV-1 DNA vaccine in Africa. PLoS One **5**:e12873.

153. **Jin C., F. Altmann, R. Strasser, L. Mach, M. Schähs, R. Kunert, T. Rademacher, J. Glössl, and H. Steinkellner**. 2008. A plant-derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. Glycobiology **18**:235–241.

154. **Johnston M. I., and A. S. Fauci**. 2007. An HIV vaccine-evolving concepts. N. Engl. J. Med. **356**:2073–2081.

155. **Jolla L.** 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. **95**:13079–13084.

156. **Julien J.-P., A. Cupo, D. Sok, R. L. Stanfield, D. Lyumkis, M. C. Deller, P.-J. Klasse, D. R. Burton, R. W. Sanders, J. P. Moore, A. B. Ward, and I. A. Wilson**. 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science **342**:1477–1483.

157. **Jul-Larsen A., A. S. Madhun, K. A. Brokstad, E. Montomoli, V. Yusibov, and R. J. Cox**. 2012. The human potential of a recombinant pandemic influenza vaccine produced in tobacco plants. Hum. Vaccin. Immunother. **8**:653–661.

158. **Kalthoff D., A. Giritch, K. Geisler, U. Bettmann, V. Klimyuk, H.-R. Hehnen, Y. Gleba, and M. Beer**. 2010. Immunization with plant-expressed hemagglutinin protects chickens from lethal highly pathogenic avian influenza virus H5N1 challenge infection. J. Virol. **84**:12002–12010.

159. **Kapila J., R. De Rycke, M. Van Montagu, and G. Angenon**. 1997. An Agrobacterium-mediated transient gene expression system for intact leaves. Plant Sci. **122**:101–108.

160. **Karnasuta C., R. M. Paris, J. H. Cox, S. Nitayaphan, P. Pitisuttithum, P. Thongcharoen, A. E. Brown, S. Gurunathan, J. Tartaglia, W. L. Heyward, J. G. McNeil, D. L. Birx, and M. S. de Souza**. 2005. Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. Vaccine **23**:2522–9.

161. **Keele B. F., E. E. Giorgi, J. F. Salazar-Gonzalez, J. M. Decker, K. T. Pham, M. G. Salazar, C. Sun, T. Grayson, S. Wang, H. Li, X. Wei, C. Jiang, J. L. Kirchherr, F. Gao, J. A. Anderson, L.-H. Ping, R. Swanstrom, G. D. Tomaras, W. A. Blattner, P. A. Goepfert, J. M. Kilby, M. S. Saag, E. L. Delwart, M. P. Busch, M. S. Cohen, D. C. Montefiori, B. F. Haynes, B. Gaschen, G. S. Athreya, H. Y. Lee, N. Wood, C. Seoighe, A. S. Perelson, T. Bhattacharya, B. T. Korber, B. H. Hahn, and G. M. Shaw**. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc. Natl. Acad. Sci. U. S. A. **105**:7552–7557.

162. **Keele B. F., F. Van Heuverswyn, Y. Li, E. Bailes, J. Takehisa, M. L. Santiago, F. Bibollet-Ruche, Y. Chen, L. V Wain, F. Liegeois, S. Loul, E. M. Ngole, Y. Bienvenue, E. Delaporte, J. F. Y. Brookfield, P. M. Sharp, G. M. Shaw, M. Peeters, and B. H. Hahn**. 2006. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science (80-.). **313**:523–526.

163. **Kibuuka H., R. Kimutai, L. Maboko, F. Sawe, M. S. Schunk, A. Kroidl, D. Shaffer, L. A. Eller, R. Kibaya, M. A. Eller, K. B. Schindler, A. Schuetz, M. Millard, J. Kroll, L. Dally, M. Hoelscher, R. Bailer, J. H. Cox, M. Marovich, D. L. Birx, B. S. Graham, N. L. Michael, M. S. de Souza, and M. L. Robb**. 2010. A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172). J. Infect. Dis. **201**:600–607.

164. **Kim J. H., S. Rerks-ngarm, J. Excler, and N. L. Michael**. 2011. HIV Vaccines-lessons learnt and the way forward. Curr. Opin. HIV AIDS **5**:428–434.

165. **Kim T.-G., M.-Y. Baek, E.-K. Lee, T.-H. Kwon, and M.-S. Yang**. 2008. Expression of human growth hormone in transgenic rice cell suspension culture. Genet. Transform. Hybrid. **27**:885–891.

166. **Komarova T. V, S. Baschieri, M. Donini, C. Marusic, E. Benvenuto, and Y. L. Dorokhov**. 2010. Transient expression systems for plant-derived biopharmaceuticals. Expert Rev. Vaccines **9**:859–876.

167. **Koup R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho**. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68**:4650–4655.

168. **Kowalski M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski**. 1987. Functional regions of the envelope glycoprotein of Human Immunodeficiency Virus type 1. Science (80-.). **237**:1351–1355.

169. **Krachmarov C. P., W. J. Honnen, S. C. Kayman, M. K. Gorny, S. Zolla-Pazner, and A. Pinter**. 2006. Factors Determining the Breadth and Potency of Neutralization by V3-Specific Human Monoclonal Antibodies Derived from Subjects Infected with Clade A or Clade B Strains of Human Immunodeficiency Virus Type 1. J. Virol. **80**:7127–7135.

170. **Krachmarov C., A. Pinter, W. Honnen, M. K. Gorny, P. N. Nyambi, S. Zolla-Pazner, and S. C. Kayman**. 2005. Antibodies that are cross-reactive for human immunodeficiency virus type 1 clade A and clade B V3 domains are common in patient sera from Cameroon, but their neutralization activity is usually restricted by epitope masking. J. Virol. **79**:780–790.

171. **Krieg A. M.** 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. **20**:709–760.

172. **Kushnir N., S. J. Streatfield, and V. Yusibov**. 2012. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. Vaccine. Elsevier Ltd **31**:58–83.

173. **Kutzler M. A., and D. B. Weiner**. 2008. DNA vaccines: ready for prime time? Nat. Rev. Genet. **9**:776–788.

174. **Kwissa M., V. K. von Kampen, R. Zurbriggen, R. Glück, J. Reimann, and R. Schirmbeck**. 2000. Efficient vaccination by intradermal or intramuscular inoculation of plasmid DNA expressing hepatitis B surface antigen under desmin promoter/enhancer control. Vaccine **18**:2337–2344.

175. **Kwong P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson**. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393**:648–659.

176. **Kwong P. D., M. L. Doyle, D. J. Casper, C. Cicalak, S. A. Leavitt, S. Majeed, T. D. Steenbekek, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P. W. I. H. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt, J. Sodroski, W. A. Hendrickson, and J. Arthosk**. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. **420**:678–682.

177. **Kwong P. D., and J. R. Mascola**. 2012. Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. Immunity. Elsevier Inc. **37**:412–25.

178. **Kwong P. D., J. R. Mascola, and G. J. Nabel**. 2013. Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. Nat. Rev. Immunol. Nature Publishing Group **13**:693–701.

179. **Laird M. E., T. Igarashi, M. A. Martin, and R. C. Desrosiers**. 2008. Importance of the V1/V2 loop region of simian-human immunodeficiency virus envelope glycoprotein gp120 in determining the strain specificity of the neutralizing antibody response. J. Virol. **82**:11054–11065.

180. **Lamphear B. J., J. M. Jilka, L. Kesl, M. Welter, J. A. Howard, and S. J. Streatfield**. 2004. A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine **22**:2420–2424.

181. **Lamphear B. J., S. J. Streatfield, J. M. Jilka, C. A. Brooks, D. K. Barker, D. D. Turner, D. E. Delaney, M. Garcia, B. Wiggins, S. L. Woodard, E. E. Hood, I. R. Tizard, B. Lawhorn, and J. A. Howard**. 2002. Delivery of subunit vaccines in maize seed. J. Control. release **85**:169–80.

182. **Land A., D. Zonneveld, and I. Braakman**. 2003. Folding of HIV-1 envelope glycoprotein involves extensive isomerization of disulfide bonds and conformation-dependent leader peptide cleavage. FASEB J. **17**:1058–67.

183. **Landry N., B. J. Ward, S. Trépanier, E. Montomoli, M. Dargis, G. Lapini, and L.-P. Vézina**. 2010. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PLoS One **5**:e15559.

184. **Larrick J. W., and D. W. Thomas**. 2001. Producing proteins in transgenic plants and animals. Curr. Opin. Biotechnol. **12**:411–418.

185. **Larsen O., Z. da Silva, A. Sandström, P. K. Andersen, S. Andersson, a G. Poulsen, M. Melbye, F. Dias, A. Nauclér, and P. Aaby**. 1998. Declining HIV-2 prevalence and incidence among men in a community study from Guinea-Bissau. AIDS **12**:1707–1714.

186. **Legrand E., I. Pellegrin, D. Neau, J. L. Pellegrin, J. M. Ragnaud, M. Dupon, B. Guillemain, and H. J. A. Fleury**. 1997. Course of specific T lymphocyte cytotoxicity, plasma and cellular viral loads, and neutralizing antibody titers in 17 recently seroconverted HIV type 1-infected patients. AIDS Res. Hum. Retroviruses **13**:1383–1394.

187. **Leonard C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory**. 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. J. Biol. Chem. **265**:10373–10382.

188. **Lessard P. A., H. Kulaveerasingam, G. M. York, A. Strong, and A. J. Sinskey**. 2002. Manipulating gene expression for the metabolic engineering of plants. Metab. Eng. **4**:67–79.

189. **Letvin N., D. Montefiori, Y. Yasumtoni, H. Perry, M.-E. Davies, C. Lekutis, A. Marianne, D. Freed, C. Lord, L. Handt, M. Lius, and J. Shiver**. 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proc. Natl. Acad. Sci. **94**:9378–9383.

190. **Letvin N. L.** 2007. Correlates of immune protection and the development of a human immunodeficiency virus vaccine. Immunity **27**:366–369.

191. **Li Y., J. J. M. Bergeron, L. Luo, W. J. Ou, D. Y. Thomas, and C. Y. Kang**. 1996. Effects of inefficient cleavage of the signal sequence of HIV-1 gp120 on its association with calnexin, folding, and intracellular transport. Proc. Natl. Acad. Sci. U. S. A. **93**:9606–9611.

192. **Li Y., L. Luo, D. Y. Thomas, and C. Y. Kang**. 2000. The HIV-1 Env protein signal sequence retards its cleavage and down-regulates the glycoprotein folding. Virology **272**:417–428.

193. **Li Y., S. A. Migueles, B. Welcher, K. Svehla, A. Phogat, M. K. Louder, X. Wu, G. M. Shaw, M. Connors, R. T. Wyatt, and J. R. Mascola**. 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat. Med. **13**:1032–1034.

194. **Li Y., K. Svehla, M. K. Louder, D. Wycuff, S. Phogat, M. Tang, S. A. Migueles, X. Wu, A. Phogat, G. M. Shaw, M. Connors, J. Hoxie, J. R. Mascola, and R. Wyatt**. 2009. Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals. J. Virol. **83**:1045–1059.

195. **Liang X., D. R. Casimiro, W. A. Schleif, F. Wang, M.-E. Davies, Z. Zhang, T. Fu, A. C. Finnefrock, L. Handt, P. Michael, G. Heidecker, A. Tang, M. Chen, K. A. Wilson, L. Gabryelski, M. Mcelhaugh, A. Carella, C. Moyer, L. Huang, D. Patel, J. Lin, E. A. Emini, W. John, M. P. Citron, S. Vitelli, and J. W. Shiver**. 2005. Vectored Gag and Env but Not Tat Show Efficacy against Simian-Human Immunodeficiency Virus 89.6P Challenge in Mamu-A * 01-Negative Rhesus Monkeys. J. Virol. **79**:12321–12331.

196. **Liao H.-X., M. Bonsignori, S. M. Alam, J. S. McLellan, G. D. Tomaras, M. A. Moody, D. M. Kozink, K.-K. Hwang, X. Chen, C.-Y. Tsao, P. Liu, X. Lu, R. J. Parks, D. C. Montefiori, G. Ferrari, J. Pollara, M. Rao, K. K. Peachman, S. Santra, N. L. Letvin, N. Karasavvas, Z.-Y. Yang, K. Dai, M. Pancera, J. Gorman, K. Wiehe, N. I. Nicely, S. Rerks-Ngarm, S. Nitayaphan, J. Kaewkungwal, P. Pitisuttithum, J. Tartaglia, F. Sinangil, J. H. Kim, N. L. Michael, T. B. Kepler, P. D. Kwong, J. R. Mascola, G. J. Nabel, A. Pinter, S. Zolla-Pazner, and B. F. Haynes**. 2013. Vaccine induction of antibodies against a structurally heterogeneous site of immune pressure within HIV-1 envelope protein variable regions 1 and 2. Immunity. Elsevier Inc. **38**:176–186.

197. **Liao H.-X., R. Lynch, T. Zhou, F. Gao, S. M. Alam, S. D. Boyd, A. Z. Fire, K. M. Roskin, C. A. Schramm, Z. Zhang, J. Zhu, L. Shapiro, J. C. Mullikin, S. Gnanakaran, P. Hraber, K. Wiehe, G. Kelsoe, G. Yang, S.-M. Xia, D. C. Montefiori, R. Parks, K. E. Lloyd, R. M. Scearce, K. A. Soderberg, M. Cohen, G. Kamanga, M. K. Louder, L. M. Tran, Y. Chen, F. Cai, S. Chen, S. Moquin, X. Du, M. G. Joyce, S. Srivatsan, B. Zhang, A. Zheng, G. M. Shaw, B. H. Hahn, T. B. Kepler, B. T. M. Korber, P. D. Kwong, J. R. Mascola, and B. F. Haynes**. 2013. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. Nature **496**:469–476.

198. **Liao H., L. L. Sutherland, S. Xia, M. E. Brock, R. M. Scearce, S. Vanleeuwen, S. M. Alam, M. Mcadams, E. A. Weaver, Z. T. Camacho, B. Ma, Y. Li, J. M. Decker, G. J. Nabel, D. C. Montefiori, B. H. Hahn, B. T. Korber, F. Gao, and B. F. Haynes**. 2006. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. Virology **353**:268–282.

199. **Liu F., S. Ge, L. Li, X. Wu, Z. Liu, and Z. Wang**. 2012. Virus-like particles: potential veterinary vaccine immunogens. Res. Vet. Sci. Elsevier Ltd **93**:553–559.

200. **Liu J., A. Bartesaghi, M. Borgnia, G. Sapiro, and S. Subramaniam**. 2008. Molecular architecture of native HIV-1 gp120 trimers. Nature **455**:109–113.

201. **Liu M.** 2003. DNA vaccines: a review. J. Intern. Med. **253**:402–410.

202. **Lou X., Q. Yao, Z. Zhang, R. Peng, A. Xiong, and H. Wang**. 2007. Expression of the Human Hepatitis B Virus Large Surface Antigen Gene in Transgenic Tomato Plants. Clin. vaccine Immunol. **14**:464–469.

203. **Lu J., E. Sivamani, K. Azhakanandam, P. Samadder, X. Li, and R. Qu**. 2008. Gene expression enhancement mediated by the 5' UTR intron of the rice rubi3 gene varied remarkably among tissues in transgenic rice plants. Mol. Genet. genomics **279**:563–572.

204. **Lu S.** 2009. Heterologous prime-boost vaccination. Curr. Opin. Immunol. **21**:346–351.

205. **Lynch R., L. Tran, M. Louder, S. Schmidt, R. Dersimonian, Z. Euler, E. Gray, S. Karim, J. Kirchher, D. Montefiori, S. Sibeko, K. Soderberg, G. Tomaras, Z.-Y. Yang, G. Nabel, H. Schuitemaker, L. Morris, H. BF, and M. JR**. 2012. The Development of CD4 Binding Site Antibodies during HIV-1 Infection. J. **86**:7588–7595.

206. **Lyumkis D., J.-P. Julien, N. de Val, A. Cupo, C. S. Potter, P.-J. Klasse, D. R. Burton, R. W. Sanders, J. P. Moore, B. Carragher, I. A. Wilson, and A. B. Ward**.

2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science **342**:1484–1490.

207. **Ma J. K.-C., P. M. W. Drake, and P. Christou**. 2003. The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. **4**:794–805.

208. **Ma Y., S. Lin, Y. Gao, M. Li, W. Luo, J. Zhang, and N. Xia**. 2003. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. Viral Hepat. **9**:2211–2215.

209. **Maclean J., M. Koekemoer, A. J. Olivier, D. Stewart, I. I. Hitzeroth, T. Rademacher, R. Fischer, A.-L. Williamson, and E. P. Rybicki**. 2007. Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. J. Gen. Virol. **88**:1460–1469.

210. **Mainieri D., M. Rossi, M. Archinti, M. Bellucci, F. De Marchis, S. Vavassori, A. Pompa, S. Arcioni, and A. Vitale**. 2004. Zeolin: A New Recombinant Storage Protein Constructed Using Maize gamma -Zein and Bean Phaseolin. Plant Physiol. **136**:3447–3456.

211. **Marillonnet S., A. Giritch, M. GIls, R. Kandzia, V. Klimyuk, and Y. Gleba**. 2004. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proc. Natl. Acad. Sci. **101**:6852–6857.

212. **Marillonnet S., C. Thoeringer, R. Kandzia, V. Klimyuk, and Y. Gleba**. 2005. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat. Biotechnol. **23**:718–723.

213. **Marlink R., P. Kanki, I. Thior, K. Travers, G. Eisen, T. Siby, I. Traore, C. C. Hsieh, M. C. Dia, and E. H. Gueye**. 1994. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science (80-.). **265**:1587–1590.

214. **Mason H. S., Do. M.-K. Lam, and C. J. Arntzen**. 1992. Expression of hepatitis B surface antigen in transgenic plants. Proc. Natl. Acad. Sci. **89**:11745–11749.

215. **Massa S., R. Franconi, R. Brandi, A. Muller, V. Mett, V. Yusibov, and A. Venuti**. 2007. Anti-cancer activity of plant-produced HPV16 E7 vaccine. **25**:3018–3021.

216. **Mast T. C., L. Kierstead, S. B. Gupta, A. A. Nikas, E. G. Kallas, V. Novitsky, B. Mbewe, P. Pitisuttithum, M. Schechter, E. Vardas, N. D. Wolfe, M. Aste-Amezaga, D. R. Casimiro, P. Coplan, W. L. Straus, and J. W. Shiver**. 2010. International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. Vaccine **28**:950–957.

217. **McCutchan F.** 2006. Global epidemiology of HIV. J. Med. Virol. **12**:S7–S12.

218. **McElrath M. J., S. C. De Rosa, Z. Moodie, S. Dubey, L. Kierstead, H. Janes, O. D. Defawe, D. K. Carter, J. Hural, R. Akondy, S. P. Buchbinder, M. N. Robertson, D. V Mehrotra, S. G. Self, L. Corey, J. W. Shiver, and D. R. Casimiro**. 2008. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet. Elsevier Ltd **372**:1894–1905.

219. **Mcgarvey P. B., J. Hammond, M. M. Dienelt, D. C. Hooper, Z. F. Fu, B. Dietzschold, H. Koprowskim, and F. H. Michaels**. 1995. Expression of the Rabies Virus Glycoprotein in Transgenic Tomatoes. Biotechnology **13**:1484–1487.

220. **McLellan J. S., M. Pancera, C. Carrico, J. Gorman, J.-P. Julien, R. Khayat, R. Louder, R. Pejchal, M. Sastry, K. Dai, S. O'Dell, N. Patel, S. Shahzad-ul-Hussan, Y. Yang, B. Zhang, T. Zhou, J. Zhu, J. C. Boyington, G.-Y. Chuang, D. Diwanji, I. Georgiev, Y. Do Kwon, D. Lee, M. K. Louder, S. Moquin, S. D. Schmidt, Z.-Y. Yang, M. Bonsignori, J. A. Crump, S. H. Kapiga, N. E. Sam, B. F. Haynes, D. R. Burton, W. C. Koff, L. M. Walker, S. Phogat, R. Wyatt, J. Orwenyo, L.-X. Wang, J. Arthos, C. A. Bewley, J. R. Mascola, G. J. Nabel, W. R. Schief, A. B. Ward, I. A. Wilson, and P. D. Kwong**. 2011. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature. Nature Publishing Group **480**:336–343.

221. **McNeil J., M. Johnston, D. Birx, and E. Tramont**. 2004. HIV vaccine trial justified. Science (80-.). **303**:961.

222. **Mcshane H., R. Brookes, S. C. Gilbert, and A. V. S. Hill**. 2001. Enhanced Immunogenicity of CD4 + T-Cell Responses and Protective Efficacy of a DNA-Modified Vaccinia Virus Ankara Prime-Boost Vaccination Regimen for Murine Tuberculosis. Infect. Immun. **69**:681–686.

223. **Megede J. zur, M. Chen, B. Doe, M. Schaefer, C. E. Greer, M. Selby, G. R. Otten, and S. W. Barnett**. 2000. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. J. Virol. **74**:2628–2635.

224. **Menassa R., V. Nguyen, A. Jevnikar, and J. Brandle**. 2001. A self-contained system for the field production of plant recombinant interleukin-10. Mol. Breed. **8**:177–185.

225. **Meseda C. A., K. L. Elkins, M. J. Merchlinsky, and J. P. Weir**. 2002. Prime-boost immunization with DNA and modified vaccinia virus ankara vectors expressing herpes simplex virus-2 glycoprotein D elicits greater specific antibody and cytokine responses than DNA vaccine alone. J. Infect. Dis. **186**:1065–1073.

226. **Mett V., K. Musiychuk, H. Bi, C. E. Farrance, A. Horsey, N. Ugulava, Y. Shoji, P. de la Rosa, G. A. Palmer, S. Rabindran, S. J. Streatfield, A. Boyers, M. Russell, A. Mann, R. Lambkin, J. S. Oxford, G. C. Schild, and V. Yusibov**. 2008. A plant-produced influenza subunit vaccine protects ferrets against virus challenge. Influenza Other Respi. Viruses **2**:33–40.

227. **Meyers A., E. Chakauya, E. Shephard, F. L. Tanzer, J. Maclean, A. Lynch, A.-L. Williamson, and E. P. Rybicki**. 2008. Expression of HIV-1 antigens in plants as potential subunit vaccines. BMC Biotechnol. **8**:53–68.

228. **Mikell I., D. N. Sather, S. A. Kalams, M. Altfeld, G. Alter, and L. Stamatatos**. 2011. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathog. **7**:e1001251.

229. **Montero M., N. E. van Houten, X. Wang, and J. K. Scott**. 2008. The membrane-proximal external region of the human immunodeficiency virus type 1 envelope: dominant site of antibody neutralization and target for vaccine design. Microbiol. Mol. Biol. Rev. **72**:54–84.

230. **Moog C., H. J. Fleury, I. Pellegrin, A. Kirn, and A. M. Aubertin**. 1997. Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. J. Virol. **71**:3734–3741.

231. **Moore P. L., E. S. Gray, I. A. Choge, N. Ranchobe, R. Nthabeleng, K. Mlisana, S. S. A. Karim, C. Williamson, and L. Morris**. 2007. The C3-V4 Region Is a Major Target of Autologous Neutralizing Antibodies in Human Immunodeficiency Virus Type 1 Subtype C Infection. J. Virol. **82**:1860–1869.

232. **Moore P. L., E. S. Gray, D. Sheward, M. Madiga, N. Ranchobe, Z. Lai, W. J. Honnen, M. Nonyane, N. Tumba, T. Hermanus, S. Sibeko, K. Mlisana, A. S. S. Karim, C. Williamson, A. Pinter, and L. Morris**. 2011. Potent and broad neutralization of HIV-1 subtype C by plasma antibodies targeting a quaternary epitope including residues in the V2 loop. J. Virol. **85**:3128–3141.

233. **Moore P. L., E. S. Gray, C. K. Wibmer, J. N. Bhiman, M. Nonyane, D. J. Sheward, T. Hermanus, S. Bajimaya, N. L. Tumba, M.-R. Abrahams, B. E. Lambson, N. Ranchobe, L. Ping, N. Ngandu, Q. Abdool Karim, S. S. Abdool Karim, R. I. Swanstrom, M. S. Seaman, C. Williamson, and L. Morris**. 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. Nat. Med. **18**:1688–1692.

234. **Moore P. L., N. Ranchobe, B. E. Lambson, E. S. Gray, E. Cave, M.-R. Abrahams, G. Bandawe, K. Mlisana, S. S. Abdool Karim, C. Williamson, and L. Morris**. 2009. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. PLoS Pathog. **5**:e1000598.

235. **Moore P. L., D. Sheward, M. Nonyane, N. Ranchobe, T. Hermanus, E. S. Gray, S. S. Abdool Karim, C. Williamson, and L. Morris**. 2013. Multiple

pathways of escape from HIV broadly cross-neutralizing V2-dependent antibodies. J. Virol. **87**:4882–4894.

236. **Morris L., X. Chen, M. Alam, G. Tomaras, R. Zhang, D. J. Marshall, B. Chen, R. Parks, A. Foulger, F. Jaeger, M. Donathan, M. Bilska, E. S. Gray, S. S. Abdool Karim, T. B. Kepler, J. Whitesides, D. Montefiori, M. A. Moody, H.-X. Liao, and B. F. Haynes**. 2011. Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. PLoS One **6**:e23532.

237. **Mortimer E., I. Hitzeroth, A. Buys, S. Mbewana, and E. Rybicki**. 2013. An H5N1 influenza DNA vaccine for South Africa. S. Afr. J. Sci. **109**:1–4.

238. **Mortimer E., J. M. Maclean, S. Mbewana, A. Buys, A.-L. Williamson, I. I. Hitzeroth, and E. P. Rybicki**. 2012. Setting up a platform for plant-based influenza virus vaccine production in South Africa. BMC Biotechnol. **12**:14–24.

239. **Musiychuk K., N. Stephenson, H. Bi, C. E. Farrance, G. Orozovic, M. Brodelius, P. Brodelius, A. Horsey, N. Ugulava, A.-M. Shamloul, V. Mett, S. Rabindran, S. J. Streatfield, and V. Yusibov**. 2007. A launch vector for the production of vaccine antigens in plants. Influenza Other Respi. Viruses **1**:19–25.

240. **Muster T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Rüker, and H. Katinger**. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67**:6642–6647.

241. **Nabel G. J., P. D. Kwong, and J. R. Mascola**. 2011. Progress in the rational design of an AIDS vaccine. Philos. Trans. R. Soc. London. **366**:2759–2765.

242. **Ni M., D. Cui, J. Einstein, S. Narainmhulu, C. E. Vergara, and S. B. Gelvin**. 1995. Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. plant … **7**:661–676.

243. **Nimmerjahn F., R. M. Anthony, and J. V Ravetch**. 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. Proc. Natl. Acad. Sci. U. S. A. **104**:8433–8437.

244. **Nitayaphan S., P. Pitisuttithum, C. Karnasuta, C. Eamsila, M. de Souza, P. Morgan, V. Polonis, M. Benenson, T. VanCott, S. Ratto-Kim, J. Kim, D. Thapinta, R. Garner, V. Bussaratid, P. Singharaj, R. El-Habib, S. Gurunathan, W. Heyward, D. Birx, J. McNeil, and A. E. Brown**. 2004. Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults. J. Infect. Dis. **190**:702–706.

245. **Nuttall J., N. Vine, J. L. Hadlington, P. Drake, L. Frigerio, and J. K.-C. Ma**. 2002. ER-resident chaperone interactions with recombinant antibodies in transgenic plants. Eur. J. Biochem. **269**:6042–6051.

246. **Nwanegbo E., E. Vardas, W. Gao, H. Sun, D. Rowe, P. D. Robbins, H. Whittle, and A. Gambotto**. 2004. Prevalence of Neutralizing Antibodies to Adenoviral Serotypes 5 and 35 in the Adult Populations of The Gambia, South Africa and the United States. Clin. vaccine Immunol. **11**:2004.

247. **O'Brien K. L., J. Liu, S. L. King, Y.-H. Sun, J. E. Schmitz, M. A. Lifton, N. A. Hutnick, M. R. Betts, S. A. Dubey, J. Goudsmit, J. W. Shiver, M. N. Robertson, D. R. Casimiro, and D. H. Barouch**. 2009. Adenovirus-specific immunity after immunization with an Ad5 HIV-1 vaccine candidate in humans. Nat. Med. Nature Publishing Group **15**:873–875.

248. **Obembe O. O., J. O. Popoola, S. Leelavathi, and S. V Reddy**. 2011. Advances in plant molecular farming. Biotechnol. Adv. Elsevier Inc. **29**:210–222.

249. **Ofek G., M. Tang, A. Sambor, H. Katinger, J. R. Mascola, R. Wyatt, and P. D. Kwong**. 2004. Structure and Mechanistic Analysis of the Anti-Human Immunodeficiency Virus Type 1 Antibody 2F5 in Complex with Its gp41 Epitope. Structure **78**:10724–10737.

250. **Ohya K., N. Itchoda, K. Ohashi, M. Onuma, C. Sugimoto, and T. Matsumura**. 2004. Expression of biologically active human tumor necrosis factor-alpha in transgenic potato plant. J. Interf. cytokine Res. **22**:371–378.

251. **Opar A.** 2011. "Pharmers" hope for first plant drug harvest. Nat. Rev. Drug Discov. **10**:81–82.

252. **Padidam M.** 2003. Chemically regulated gene expression in plants. Curr. Opin. Plant Biol. **6**:169–177.

253. **Pancera M., Y. Yang, M. K. Louder, J. Gorman, G. Lu, J. S. McLellan, J. Stuckey, J. Zhu, D. R. Burton, W. C. Koff, J. R. Mascola, and P. D. Kwong**. 2013. N332-Directed broadly neutralizing antibodies use diverse modes of HIV-1 recognition: inferences from heavy-light chain complementation of function. PLoS One **8**:e55701.

254. **Pantaleo G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, L. K. Schrager, J. B. Margolick, S. Buchbinder, J. V Giorgi, and A. S. Fauci**. 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N. Engl. J. Med. **332**:209–216.

255. **Parmenter D. L., J. G. Boothe, G. J. van Rooijen, E. C. Yeung, and M. M. Moloney**. 1995. Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol. Biol. **29**:1167–1180.

256. **Paul M., and J. K.-C. Ma**. 2010. Plant-made immunogens and effective delivery strategies. Expert Rev. Vaccines **9**:821–833.

257. **Pegu P., M. Vaccari, S. Gordon, B. F. Keele, M. Doster, Y. Guan, G. Ferrari, R. Pal, M. G. Ferrari, S. Whitney, L. Hudacik, E. Billings, M. Rao, D. Montefiori, G. Tomaras, S. M. Alam, C. Fenizia, J. D. Lifson, D. Stablein, J. Tartaglia, N. Michael, J. Kim, D. Venzon, and G. Franchini**. 2013. Antibodies with high avidity to the gp120 envelope protein in protection from simian immunodeficiency virus SIV(mac251) acquisition in an immunization regimen that mimics the RV-144 Thai trial. J. Virol. **87**:1708–1719.

258. **Peiperl L., C. Morgan, Z. Moodie, H. Li, N. Russell, B. S. Graham, G. D. Tomaras, S. C. De Rosa, and M. J. McElrath**. 2010. Safety and immunogenicity of a replication-defective adenovirus type 5 HIV vaccine in Ad5-seronegative persons: a randomized clinical trial (HVTN 054). PLoS One **5**:e13579.

259. **Pejchal R., K. J. Doores, L. M. Walker, R. Khayat, P.-S. Huang, S.-K. Wang, R. L. Stanfield, J.-P. Julien, A. Ramos, M. Crispin, R. Depetris, U. Katpally, A. Marozsan, A. Cupo, S. Maloveste, Y. Liu, R. McBride, Y. Ito, R. W. Sanders, C. Ogohara, J. C. Paulson, T. Feizi, C. N. Scanlan, C.-H. Wong, J. P. Moore, W. C. Olson, A. B. Ward, P. Poignard, W. R. Schief, D. R. Burton, and I. A. Wilson**. 2011. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. Science (80-.). **334**:1097–1103.

260. **Pelham H. R.** 1990. The retention signal for soluble proteins of the endoplasmic reticulum. Trends Biochem. Sci. **15**:483–486.

261. **Perlak F., R. Fuchs, D. Dean, S. McPherson, and D. FIschoff**. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. **88**:3324–3328.

262. **Perreau M., G. Pantaleo, and E. J. Kremer**. 2008. Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. J. Exp. Med. **205**:2717–2725.

263. **Phogat S., and R. Wyatt**. 2007. Rational modifications of HIV-1 envelope glycoproteins for immunogen design. Curr. Pharm. Des. **13**:213–227.

264. **Pineo C. B., I. I. Hitzeroth, and E. P. Rybicki**. 2013. Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimaeras. Plant Biotechnol. J. **8**:964–975.

265. **Piot P., and T. C. Quinn**. 2013. Response to the AIDS pandemic-a global health model. N. Engl. J. Med. **368**:2210–2218.

266. **Pitisuttithum P., P. W. Berman, B. Phonrat, P. Suntharasami, S. Raktham, L.-O. Srisuwanvilai, K. Hirunras, D. Kitayaporn, J. Kaewkangwal, S. Migasena, H. W. Sheppard, E. Li, M. Chernow, M. L. Peterson, R. Shibata, W. Heyward, and D. P. Francis**. 2004. Phase I/II Study of a Candidate Vaccine Designed Against the B and E Subtypes of HIV-1. J. Acquir. Immunodefic. Syndr. **37**:1160–1165.

267. **Pitisuttithum P., P. Gilbert, M. Gurwith, W. Heyward, M. Martin, F. van Griensven, D. Hu, J. W. Tappero, and K. Choopanya**. 2006. Randomized, Double Blind, Placebo Controlled Efficacy Trial of a Bivalent Recombinant Glycoprotein 120 HIV-1 Vaccine among Injection Drug Users in Bangkok, Thailand. J. Infect. Dis. **194**:1661–1671.

268. **Pitisuttithum P., S. Nitayaphan, P. Thongcharoen, C. Khamboonruang, J. Kim, M. de Souza, T. Chuenchitra, R. P. Garner, D. Thapinta, V. Polonis, S. Ratto-Kim, P. Chanbancherd, J. Chiu, D. L. Birx, A.-M. Duliege, J. G. McNeil, and A. E. Brown**. 2003. Safety and immunogenicity of combinations of recombinant subtype E and B human immunodeficiency virus type 1 envelope glycoprotein 120 vaccines in healthy Thai adults. J. Infect. Dis. **188**:219–227.

269. **Plantier J.-C., M. Leoz, J. E. Dickerson, F. De Oliveira, F. Cordonnier, V. Lemée, F. Damond, D. L. Robertson, and F. Simon**. 2009. A new human immunodeficiency virus derived from gorillas. Nat. Med. **15**:871–872.

270. **Plotkin S. A.** 2008. Correlates of Vaccine-Induced Immunity. Clin. Infect. Dis. **47**:401–409.

271. **Popper S., A. Sarr, K. Travers, A. Gueye-Ndiaye, S. Mboup, M. Exxes, and P. Kanki**. 1999. Lower human immunodeficiency virus (HIV) type 2 viral load reflects the difference in pathogenicity of HIV-1 and HIV-2. J. Infect. **180**:1116–1121.

272. **Porta C., and G. Lomonossoff**. 2002. Viruses as vectors for the expression of foreign sequences in plants. Biotechnol. Genet. engenieering Rev. **19**:245–291.

273. **Poulson A., B. Kuinesdal, P. Aaby, K. Molbak, K. Frederikson, F. Kias, and E. Lauritzen**. 1989. Prevalence and mortality from Human Immunodeficiency Virus type 2 in Bissau, West Africa. Lancet **333**:827–831.

274. **Priddy F. H., D. Brown, J. Kublin, K. Monahan, D. P. Wright, J. Lalezari, S. Santiago, M. Marmor, M. Lally, R. M. Novak, S. J. Brown, P. Kulkarni, S. A. Dubey, L. S. Kierstead, D. R. Casimiro, R. Mogg, M. J. DiNubile, J. W. Shiver, R. Y. Leavitt, M. N. Robertson, D. V Mehrotra, and E. Quirk**. 2008. Safety and

immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clin. Infect. Dis. **46**:1769–1781.

275. **Pujol M., N. I. Ramírez, M. Ayala, J. V Gavilondo, R. Valdés, M. Rodríguez, J. Brito, S. Padilla, L. Gómez, B. Reyes, R. Peral, M. Pérez, J. L. Marcelo, L. Milá, R. F. Sánchez, R. Páez, J. A. Cremata, G. Enríquez, O. Mendoza, M. Ortega, and C. Borroto**. 2005. An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. Vaccine **23**:1833–1837.

276. **Quinn T., M. Wawer, N. Sewankambo, D. Serwadda, C. Li, F. Wabwire-Mangen, M. O. Meehan, T. Lutalo, and R. H. Gray**. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Engl. J. **342**:921–929.

277. **Ramessar K., T. Capell, and P. Christou**. 2008. Molecular pharming in cereal crops. Phytochem. Rev. **7**:579–592.

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

279. **Rerks-Ngarm S., P. Pitisuttithum, S. Nitiyaphan, J. Kaewkungwal, J. CHiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benensen, S. Gurunatha, J. Tartaglia, J. McNeil, D. Francis, D. Stablein, L. Deborah, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. Robb, N. Michael, P. Kunasol, and J. Kim**. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. **361**:2209–2220.

280. **Richman D. D., T. Wrin, S. J. Little, and C. J. Petropoulos**. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. U. S. A. **100**:4144–4149.

281. **Richter L. J., Y. Thanavala, C. J. Arntzen, and H. S. Mason**. 2000. Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat. Biotechnol. **18**:1167–1171.

282. **Ritola K., C. D. Pilcher, S. A. Fiscus, N. G. Hoffman, J. A. E. Nelson, K. M. Kitrinos, C. B. Hicks, J. J. E. Jr, and R. Swanstrom**. 2004. Multiple V1/V2 env Variants Are Frequently Present during Primary Infection with Human Immunodeficiency Virus Type 1. J. Virol. **78**:11208–11218.

283. **Robert-Guroff M., M. Brown, and R. C. Gallo**. 1985. HTLV-III-neutralizing antibodies in patients with AIDS and AIDS-related complex. Nature. Nature Publishing Group **316**:72–74.

284. **Robinson J. E., K. Franco, D. H. Elliott, M. J. Maher, A. Reyna, D. C. Montefiori, S. Zolla-Pazner, M. K. Gorny, Z. Kraft, and L. Stamatatos**. 2010. Quaternary epitope specificities of anti-HIV-1 neutralizing antibodies generated in rhesus macaques infected by the simian/human immunodeficiency virus SHIVSF162P4. J. Virol. **84**:3443–3453.

285. **Rolland M., P. T. Edlefsen, B. B. Larsen, S. Tovanabutra, E. Sanders-Buell, T. Hertz, A. C. DeCamp, C. Carrico, S. Menis, C. A. Magaret, H. Ahmed, M. Juraska, L. Chen, P. Konopa, S. Nariya, J. N. Stoddard, K. Wong, H. Zhao, W. Deng, B. S. Maust, M. Bose, S. Howell, A. Bates, M. Lazzaro, A. O'Sullivan, E. Lei, A. Bradfield, G. Ibitamuno, V. Assawadarachai, R. J. O'Connell, M. S. DeSouza, S. Nitayaphan, S. Rerks-Ngarm, M. L. Robb, J. S. McLellan, I. Georgiev, P. D. Kwong, J. M. Carlson, N. L. Michael, W. R. Schief, P. B. Gilbert, J. I. Mullins, and J. H. Kim**. 2012. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. Nature. Nature Publishing Group **490**:417–420.

286. **Rolland M., S. Tovanabutra, A. C. DeCamp, N. Frahm, P. B. Gilbert, E. Sanders-Buell, L. Heath, C. A. Magaret, M. Bose, A. Bradfield, A. O'Sullivan, J. Crossler, T. Jones, M. Nau, K. Wong, H. Zhao, D. N. Raugi, S. Sorensen, J. N. Stoddard, B. S. Maust, W. Deng, J. Hural, S. Dubey, N. L. Michael, J. Shiver, L. Corey, F. Li, S. G. Self, J. Kim, S. Buchbinder, D. R. Casimiro, M. N. Robertson, A. Duerr, M. J. McElrath, F. E. McCutchan, and J. I. Mullins**. 2011. Genetic impact of vaccination on breakthrough HIV-1 sequences from the STEP trial. Nat. Med. Nature Publishing Group **17**:366–371.

287. **Rong R., S. Gnanakaran, J. M. Decker, F. Bibollet-Ruche, J. Taylor, J. N. Sfakianos, J. L. Mokili, M. Muldoon, J. Mulenga, S. Allen, B. H. Hahn, G. M. Shaw, J. L. Blackwell, T. Korber, E. Hunter, and C. A. Derdeyn**. 2007. Unique Mutational Patterns in the Envelope α2 Amphipathic Helix and Acquisition of Length in gp120 Hypervariable Domains Are Associated with Resistance to Autologous Neutralization of Subtype C Human Immunodeficiency Virus Type 1 Unique Mutational Patter. J. Virol. **81**:5658–5668.

288. **Rong R., B. Li, R. M. Lynch, R. E. Haaland, M. K. Murphy, J. Mulenga, S. A. Allen, A. Pinter, G. M. Shaw, E. Hunter, J. E. Robinson, S. Gnanakaran, and C. A. Derdeyn**. 2009. Escape from autologous neutralizing antibodies in acute/early subtype C HIV-1 infection requires multiple pathways. PLoS Pathog. **5**:e1000594.

289. **Rosenberg Y., M. Sack, D. Montefiori, D. Forthal, L. Mao, S. Hernandez-Abanto, L. Urban, G. Landucci, R. Fischer, and X. Jiang**. 2013. Rapid high-level production of functional HIV broadly neutralizing monoclonal antibodies in transient plant expression systems. PLoS One **8**:e58724.

290. **Rouwendal G., O. Mendes, E. Wolbert, and A. de Boer**. 1997. Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol. Biol. **33**:989–999.

291. **Russell N., B. Graham, M. Keefer, J. M. McElrath, S. G. Self, K. J. Weinhold, D. Montefiori, G. Ferrari, H. Horton, G. D. Tomaras, S. Gurunathan, L. Baglyos, S. E. Frey, M. Mulligan, C. Harro, S. BUchbinder, L. Baden, W. Blattner, B. Koblin, and L. Corey**. 2007. Phase 2 study of an HIV-1 canarypox vaccine (vCP1452) alone and in combination with rgp120: negative results fail to trigger a phase 3 correlates trial. J. Acquir. Immunodefic. Syndr. **44**:203–212.

292. **Rybicki E. P.** 2010. Plant-made vaccines for humans and animals. Plant Biotechnol. J. **8**:620–637.

293. **Rybicki E. P.** 2009. Plant-produced vaccines: promise and reality. Drug Discov. Today **14**:16–24.

294. **Rybicki E. P., A.-L. Williamson, A. Meyers, and I. I. Hitzeroth**. 2011. Vaccine farming in Cape Town. Hum. Vaccin. **7**:339–348.

295. **Sala F., M. Manuela Rigano, A. Barbante, B. Basso, A. M. Walmsley, and S. Castiglione**. 2003. Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. Vaccine **21**:803–808.

296. **Salzwedel K., J. T. West, and E. Hunter**. 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. J. Virol. **73**:2469–2480.

297. **Sambrook J., and D. Rusell**. 1982. Molecular cloning: a laboratory manual.New York, 3rd ed. Cold Spring harbour Laboratory Press, New York.

298. **Sanders R. W., R. Derking, A. Cupo, J.-P. Julien, A. Yasmeen, N. de Val, H. J. Kim, C. Blattner, A. T. de la Peña, J. Korzun, M. Golabek, K. de Los Reyes, T. J. Ketas, M. J. van Gils, C. R. King, I. A. Wilson, A. B. Ward, P. J. Klasse, and J. P. Moore**. 2013. A next-generation cleaved, soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog. **9**:e1003618.

299. **Sanders R. W., M. Venturi, L. Schiffner, H. Katinger, K. O. Lloyd, D. Kwong, J. P. Moore, R. Kalyanaraman, and P. D. Kwong**. 2002. The Mannose-Dependent Epitope for Neutralizing Antibody 2G12 on Human Immunodeficiency Virus Type 1 The Mannose-Dependent Epitope for Neutralizing Antibody 2G12 on Human Immunodeficiency Virus Type 1 Glycoprotein gp120. J. Virol. **76**:7293–7305.

300. **Sandhu J. S., S. F. Krasnyanski, L. L. Domier, S. S. Korban, D. Mark, and D. E. Buetow**. 2000. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. Transgenic Res. **9**:127–135.

301. **Santi L., A. Giritch, C. J. Roy, S. Marillonnet, V. Klimyuk, Y. Gleba, R. Webb, C. J. Arntzen, and H. S. Mason**. 2006. Protection conferred by recombinant

Yersinia pestis antigens produced by a rapid and highly scalable plant expression system. Proc. Natl. Acad. Sci. U. S. A. **103**:861–866.

302. **Sather D. N., J. Armann, L. K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, X. Yu, B. Wood, S. Self, S. Kalams, and L. Stamatatos**. 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J. Virol. **83**:757–769.

303. **Scheid J. F., H. Mouquet, B. Ueberheide, R. Diskin, F. Klein, T. Y. K. Oliveira, J. Pietzsch, D. Fenyo, A. Abadir, K. Velinzon, A. Hurley, S. Myung, F. Boulad, P. Poignard, D. R. Burton, F. Pereyra, D. D. Ho, B. D. Walker, M. S. Seaman, P. J. Bjorkman, B. T. Chait, and M. C. Nussenzweig**. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. Science **333**:1633–1637.

304. **Schiffner T., Q. J. Sattentau, and L. Dorrell**. 2013. Development of prophylactic vaccines against HIV-1. Retrovirology. Retrovirology **10**:72–88.

305. **Schillberg S., R. M. Twyman, and R. Fischer**. 2005. Opportunities for recombinant antigen and antibody expression in transgenic plants-technology assessment. Vaccine **23**:1764–1769.

306. **Schmitz J., M. Kuroda, S. Santra, V. Sasseville, M. Simon, M. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. Scallon, J. Ghrayeb, M. Forman, D. Montefiori, E. Rieber, N. Letvin, and K. Reimann**. 1999. Control of Viremia in Simian Immunodeficiency Virus Infection by CD8+ Lymphocytes. Science (80-.). **283**:857–860.

307. **Schneider J., J. A. Langermans, S. C. Gilbert, T. J. Blanchard, S. Twigg, S. Naitza, C. M. Hannan, M. Aidoo, A. Crisanti, K. J. Robson, G. L. Smith, A. V. S. Hill, and A. W. Thomas**. 2001. A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the Plasmodium falciparum TRAP antigen in chimpanzees. Vaccine **19**:4595–4602.

308. **Schünmann P. H. D., G. Coia, and P. M. Waterhouse**. 2002. Biopharming the SimpliRED ™ HIV diagnostic reagent in barley, potato and tobacco. Mol. Breed. **9**:113–122.

309. **Schwartländer B., J. Stover, T. Hallett, R. Atun, C. Avila, E. Gouws, M. Bartos, P. D. Ghys, M. Opuni, D. Barr, R. Alsallaq, L. Bollinger, M. de Freitas, G. Garnett, C. Holmes, K. Legins, Y. Pillay, A. E. Stanciole, C. McClure, G. Hirnschall, M. Laga, and N. Padian**. 2011. Towards an improved investment approach for an effective response to HIV/AIDS. Lancet **377**:2031–2041.

310. **Scotti N., F. Alagna, E. Ferraiolo, G. Formisano, L. Sannino, L. Buonaguro, A. De Stradis, A. Vitale, L. Monti, S. Grillo, F. M. Buonaguro, and T. Cardi**. 2009. High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. Planta **229**:1109–1122.

311. **Scotti N., and E. Rybicki**. 2013. Virus-like particles produced in plants as potential vaccines. Expert Rev. Vaccines **12**:211–224.

312. **Seder R. A., P. A. Darrah, and M. Roederer**. 2008. T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol. **8**:247–258.

313. **Shaaltiel Y., D. Bartfeld, S. Hashmueli, G. Baum, E. Brill-Almon, G. Galili, O. Dym, S. A. Boldin-Adamsky, I. Silman, J. L. Sussman, A. H. Futerman, and D. Aviezer**. 2007. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol. J. **5**:579–590.

314. **Shao Y., and C. Williamson**. 2012. The HIV-1 Epidemic: Low- to Middle-Income Countries. Cold Spring Harb. Perspect. Med. **2**:a007187.

315. **Sharma A. K., and M. K. Sharma**. 2009. Plants as bioreactors: Recent developments and emerging opportunities. Biotechnol. Adv. Elsevier Inc. **27**:811–832.

316. **Shen W., and B. Forde**. 1989. Efficient transformation of Agrobacterium spp. by high voltage electroporation. Nucleic Acids Res. **17**:8385.

317. **Shephard E., W. A. Burgers, J. H. Van Harmelen, J. E. Monroe, T. Greenhalgh, C. Williamson, and A.-L. Williamson**. 2008. A multigene HIV type 1 subtype C modified vaccinia Ankara (MVA) vaccine efficiently boosts immune responses to a DNA vaccine in mice. AIDS Res. Hum. Retroviruses **24**:207–217.

318. **Shibata R., T. Igarashi, N. Haigwood, A. Buckler-White, O. R, W. Ross, R. Willey, M. Cho, and M. Martin**. 1999. Neutralizing antibody directed against the HIV–1 envelope glycoprotein can completely block HIV–1/SIV chimeric virus infections of macaque monkeys. Nat. Med. **5**:204–210.

319. **Shields R. L., J. Lai, R. Keck, L. Y. O'Connell, K. Hong, Y. G. Meng, S. H. A. Weikert, and L. G. Presta**. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J. Biol. Chem. **277**:26733–26740.

320. **Shin Y.-J., S.-Y. Hong, T.-H. Kwon, Y.-S. Jang, and M.-S. Yang**. 2003. High level of expression of recombinant human granulocyte-macrophage colony stimulating factor in transgenic rice cell suspension culture. Biotechnol. Bioeng. **82**:778–783.

321. **Shirasawa-Seo N., Y. Sano, S. Nakamura, T. Murakami, Y. Gotoh, Y. Naito, C. N. Hsia, S. Seo, I. Mitsuhara, S. Kosugi, and Y. Ohashi**. 2005. The promoter of Milk vetch dwarf virus component 8 confers effective gene expression in both dicot and monocot plants. Plant Cell Rep. **24**:155–63.

322. **Shiver J. W., T. Fu, L. Chen, D. R. Casimiro, M.-E. Davies, R. K. Evans, Z. Zhang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V Persaud, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Monte, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, N. L. Letvin, M. J. Caul, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini**. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti- immunode deficiency-virus immunity. Nature **415**:331–335.

323. **Shoji Y., H. Bi, K. Musiychuk, A. Rhee, A. Horsey, G. Roy, B. Green, M. Shamloul, C. E. Farrance, B. Taggart, N. Mytle, N. Ugulava, S. Rabindran, V. Mett, J. A. Chichester, and V. Yusibov**. 2009. Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. Vaccine **27**:1087–1092.

324. **Shoji Y., J. A. Chichester, H. Bi, K. Musiychuk, P. de la Rosa, L. Goldschmidt, A. Horsey, N. Ugulava, G. a Palmer, V. Mett, and V. Yusibov**. 2008. Plant-expressed HA as a seasonal influenza vaccine candidate. Vaccine **26**:2930–4.

325. **Shoji Y., C. E. Farrance, J. Bautista, H. Bi, K. Musiychuk, A. Horsey, H. Park, J. Jaje, B. J. Green, M. Shamloul, S. Sharma, J. A. Chichester, V. Mett, and V. Yusibov**. 2012. A plant-based system for rapid production of influenza vaccine antigens. Influenza Other Respi. Viruses **6**:204–210.

326. **Shoji Y., C. E. Farrance, H. Bi, M. Shamloul, B. Green, S. Manceva, A. Rhee, N. Ugulava, G. Roy, K. Musiychuk, J. A. Chichester, V. Mett, and V. Yusibov**. 2009. Immunogenicity of hemagglutinin from A/Bar-headed Goose/Qinghai/1A/05 and A/Anhui/1/05 strains of H5N1 influenza viruses produced in Nicotiana benthamiana plants. Vaccine **27**:3467–3470.

327. **Silva T. I. de, M. Cotten, and S. L. Rowland-Jones**. 2008. HIV-2: the forgotten AIDS virus. Trends Microbiol. **16**:588–595.

328. **Silva Z. J. da, I. Oliveira, A. Andersen, F. Dias, A. Rodrigues, B. Holmgren, S. Andersson, and P. Aaby**. 2008. Changes in prevalence and incidence of HIV-1, HIV-2 and dual infections in urban areas of Bissau, Guinea-Bissau: is HIV-2 disappearing? AIDS **22**:1195–202.

329. **Simon F., P. Mauclère, P. Roques, I. Loussert-Ajaka, M. C. Müller-Trutwin, S. Saragosti, M. C. Georges-Courbot, F. Barré-Sinoussi, and F. Brun-Vézinet**. 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat. Med. **4**:1032–1037.

330. **Sonnenberg P., A. Copas, J. R. Glynn, A. Bester, G. Nelson, S. Shearer, and J. Murray**. 2011. The effect of HIV infection on time off work in a large cohort of gold miners with known dates of seroconversion. Occup. Environ. Med. **68**:647–652.

331. **Soria-Guerra R. E., S. Rosales-Mendoza, C. Marquez-Mercado, R. Lopez-Revilla, R. Castillo-Collazo, and A. G. Alpuche-Solis**. 2007. Transgenic tomatoes express an antigenic polypeptide containing epitopes of the diphtheria, pertussis and tetanus exotoxins, encoded by a synthetic gene. Genet. Transform. Hybrid. **26**:961–968.

332. **Souza A. C., R. M. Vasques, A. K. Inoue-Nagata, C. Lacorte, F. R. Maldaner, E. F. Noronha, and T. Nagata**. 2013. Expression and assembly of Norwalk virus-like particles in plants using a viral RNA silencing suppressor gene. Appl. Microbiol. Biotechnol. **97**:9021–9027.

333. **Souza M. S. de, S. Ratto-Kim, W. Chuenarom, A. Schuetz, S. Chantakulkij, B. Nuntapinit, A. Valencia-Micolta, D. Thelian, S. Nitayaphan, P. Pitisuttithum, R. M. Paris, J. Kaewkungwal, N. L. Michael, S. Rerks-Ngarm, B. Mathieson, M. Marovich, J. R. Currier, and J. H. Kim**. 2012. The Thai phase III trial (RV144) vaccine regimen induces T cell responses that preferentially target epitopes within the V2 region of HIV-1 envelope. J. Immunol. **188**:5166–5176.

334. **Spetz A.-L., and F. Chiodi**. 2013. Reduction of HIV-1 load in semen during follow-up study of RV144 vaccine trial boosts interest for novel correlates of immune protection in genital mucosa. J. Infect. Dis. **207**:1189–1192.

335. **Spiker S., and W. F. Thompson**. 1996. Nuclear Matrix Attachment Regions and Transgene Expression in Plants. Plant Physiol. **110**:15–21.

336. **Spitsin S., V. Andrianov, N. Pogrebnyak, Y. Smirnov, N. Borisjuk, C. Portocarrero, V. Veguilla, H. Koprowski, and M. Golovkin**. 2009. Immunological assessment of plant-derived avian flu H5/HA1 variants. Vaccine **27**:1289–1292.

337. **Stamatatos L., L. Morris, D. R. Burton, and J. R. Mascola**. 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? Nat. Med. Nature Publishing Group **15**:866–870.

338. **Starcich B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal**. 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45**:637–648.

339. **Staub J. M., B. Garcia, J. Graves, P. T. Hajdukiewicz, P. Hunter, N. Nehra, V. Paradkar, M. Schlittler, J. A. Carroll, L. Spatola, D. Ward, G. Ye, and D. A. Russell**. 2000. High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat. Biotechnol. **18**:333–338.

340. **Stein B., and E. Englemansgll**. 1990. Intracellular Processing of the gp160 HIV- 1 Envelope Precursor. J. Biol. Chem. **265**:2640–2649.

341. **Stephenson K. E., and D. H. Barouch**. 2013. A global approach to HIV-1 vaccine development. Immunol. Rev. **254**:295–304.

342. **Stoger E., M. Sack, Y. Perrin, and C. Vaquero**. 2002. Practical considerations for pharmaceutical antibody production in different crop systems. Mol. Breed. **9**:149–158.

343. **Strasser R., F. Altmann, L. Mach, J. Glössl, and H. Steinkellner**. 2004. Generation of Arabidopsis thaliana plants with complex N-glycans lacking beta1,2-linked xylose and core alpha1,3-linked fucose. FEBS Lett. **561**:132–136.

344. **Strasser R., A. Castilho, J. Stadlmann, R. Kunert, H. Quendler, P. Gattinger, J. Jez, T. Rademacher, F. Altmann, L. Mach, and H. Steinkellner**. 2009. Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous beta1,4-galactosylated N-glycan profile. J. Biol. Chem. **284**:20479–20485.

345. **Strasser R., J. Stadlmann, M. Schähs, G. Stiegler, H. Quendler, L. Mach, J. Glössl, K. Weterings, M. Pabst, and H. Steinkellner**. 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.

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

347. **Streatfield S. J., J. R. Lane, C. A. Brooks, D. K. Barker, M. L. Poage, J. M. Mayor, B. J. Lamphear, C. F. Drees, J. M. Jilka, E. E. Hood, and J. A. Howard**. 2003. Corn as a production system for human and animal vaccines. Vaccine **21**:812–815.

348. **Sullivan N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel**. 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408**:605–609.

349. **Sullivan N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel**. 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. **424**:681–684.

350. **Tacket C., H. Mason, G. Losonsky, J. Clements, M. Levine, and C. Arntzen**. 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat. Med. **4**:607–609.

351. **Takeda A., K. Sugiyama, H. Nagano, and M. Mori**. 2002. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS Lett. **532**:75–79.

352. **Tan K., J.-H. Liu, J.-H. Wang, S. Shen, and M. Lu**. 1997. Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc. Natl. Acad. Sci. U. S. A. **94**:12303–12308.

353. **Tang D. C., M. DeVit, and S. A. Johnston**. 1992. Genetic immunization is a simple method for eliciting an immune response. Nature **356**:152–154.

354. **Tanzer F. L., E. G. Shephard, K. E. Palmer, M. Burger, A.-L. Williamson, and E. P. Rybicki**. 2011. The porcine circovirus type 1 capsid gene promoter improves antigen expression and immunogenicity in a HIV-1 plasmid vaccine. Virol. J. **8**:51–61.

355. **Tebit D. M., and E. J. Arts**. 2011. Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease. Lancet Infect. Dis. Elsevier Ltd **11**:45–56.

356. **Thanavala Y., and Y. Yang**. 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc. … **92**:3358–3361.

357. **Thanavala Y., M. Mahoney, S. Pal, A. Scott, L. Richter, N. Natarajan, P. Goodwin, C. J. Arntzen, and H. S. Mason**. 2005. Immunogenicity in humans of an edible vaccine for hepatitis B. Proc. Natl. Acad. Sci. U. S. A. **102**:3378–3382.

358. **Tomaras G. D., N. L. Yates, P. Liu, L. Qin, G. G. Fouda, L. L. Chavez, A. C. Decamp, R. J. Parks, V. C. Ashley, J. T. Lucas, M. Cohen, J. Eron, C. B. Hicks, H.-X. Liao, S. G. Self, G. Landucci, D. N. Forthal, K. J. Weinhold, B. F. Keele, B. H. Hahn, M. L. Greenberg, L. Morris, S. S. A. Karim, W. a Blattner, D. C. Montefiori, G. M. Shaw, A. S. Perelson, and B. F. Haynes**. 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. J. Virol. **82**:12449–12463.

359. **Trkola A., M. Putscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. Moore, and H. Katinger**. 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70**:1100–1108.

360. **Trower M. K.** 2004. Methods in Molecular Biology: In vitro mutagenesis protocolsVasa, 3rd ed. Humana Press, Glaxo Research and Development Ltd, Glaxo-Wellcome Medicines Research Centre, Stevenage, Hertfordshire, UK.

361. **Ülker B., G. Allen, W. Thompson, S. Spiker, and A. Weissinger**. 1999. A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants. plant J. **18**:253–263.

362. **Ulmer J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, and A. Friedman**. 1993.

Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259**:1745–1749.

363. **UNAIDS**. World AIDS Day Report 2011 - How to get to zero: Faster. Smarter. Better.

364. **UNAIDS W.** 2010. UNAIDS Report on the global AIDS epidemic 2010.

365. **Vaine M., S. Wang, E. T. Crooks, P. Jiang, D. C. Montefiori, J. Binley, and S. Lu**. 2008. Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. J. Virol. **82**:7369–7378.

366. **Vallari A., P. Bodelle, C. Ngansop, F. Makamche, N. Ndembi, D. Mbanya, L. Kaptue, L. Gurtler, C. McArthur, S. Devare, and C. Brennan**. 2010. Four new HIV-1 group N isolates from Cameroon: prevalence continues to be low. AIDS Res. Hum. Retroviruses **26**:109–115.

367. **Vallari A., V. Holzmayer, B. Harris, J. Yamaguchi, C. Ngansop, F. Makamche, D. Mbanya, L. Kaptué, N. Ndembi, L. Gürtler, S. Devare, and C. A. Brennan**. 2011. Confirmation of putative HIV-1 group P in Cameroon. J. Virol. **85**:1403–1407.

368. **Vaucheret H., C. Béclin, and M. Fagard**. 2001. Post-transcriptional gene silencing in plants. J. Cell Sci. **114**:3083–3091.

369. **Verdaguer B., A. de Kochko, R. N. Beachy, and C. Fauquet**. 1996. Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. Plant Mol. Biol. **31**:1129–1139.

370. **Verrier F., S. Burda, R. Belshe, A. Duliege, J.-L. Excler, M. Klein, and S. Zolla-Pazner**. 2000. human immunodeficiency virus prime-boost immunization regimen in humans induces antibodies that show interclade cross-reactivity and neutralize several X4-, R5-, and dualtropic clade B and C primary isolates. J. **74**:10023–10033.

371. **Vidal N., M. Peeters, C. Mulanga-Kabeya, N. Nzilambi, D. Robertson, W. Ilunga, H. Sema, K. Tshimanga, B. Bongi, and E. Delaporte**. 2000. Unprecendented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. J. **74**:10498–10507.

372. **Vitale A., and E. Pedrazzini**. 2005. Recombinant pharmaceuticals from plants: the plant endomembrane system as bioreactor. Mol. Interv. 216–225.

373. **Voinnet O., S. Rivas, P. Mestre, and D. B. Ã**. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. plant J. **33**:949–956.

374. **Vzorov A., D. Lea-Fox, and R. Compans**. 1999. Immunogenicity of full length and truncated SIV envelope proteins. Viral Immunol. **12**:205–215.

375. **Wagner R., V. J. Teeuwsen, L. Deml, F. Notka, A. G. Haaksma, S. S. Jhagjhoorsingh, H. Niphuis, H. Wolf, and J. L. Heeney**. 1998. Cytotoxic T cells and neutralizing antibodies induced in rhesus monkeys by virus-like particle HIV vaccines in the absence of protection from SHIV infection. Virology **245**:65–74.

376. **Walker B. D., R. Ahmed, and S. Plotkin**. 2011. Moving ahead an HIV vaccine: use both arms to beat HIV. Nat. Med. Nature Publishing Group **17**:1194–1195.

377. **Walker L. M., M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J.-P. Julien, S.-K. Wang, A. Ramos, P.-Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A. Olsen, P. Phung, S. Fling, C.-H. Wong, S. Phogat, T. Wrin, M. D. Simek, W. C. Koff, I. a Wilson, D. R. Burton, and P. Poignard**. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature. Nature Publishing Group **477**:466–70.

378. **Walker L. M. L., and D. R. D. Burton**. 2010. Rational antibody-based HIV-1 vaccine design: current approaches and future directions. Curr. Opin. Immunol. Elsevier Ltd **22**:358–366.

379. **Walker L. M., S. K. Phogat, P.-Y. Chan-Hui, D. Wagner, P. Phung, J. L. Goss, T. Wrin, M. D. Simek, S. Fling, J. L. Mitcham, J. K. Lehrman, F. H. Priddy, O. A. Olsen, S. M. Frey, P. W. Hammond, S. Kaminsky, T. Zamb, M. Moyle, W. C. Koff, P. Poignard, and D. R. Burton**. 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science (80-.). **326**:285–289.

380. **Walker L. M., M. D. Simek, F. Priddy, J. S. Gach, D. Wagner, M. B. Zwick, S. K. Phogat, P. Poignard, and D. R. Burton**. 2010. A limited number of antibody specificities mediate broad and potent serum neutralization in selected HIV-1 infected individuals. PLoS Pathog. **6**:e1001028.

381. **Walker L. M., D. Sok, Y. Nishimura, O. Donau, R. Sadjadpour, R. Gautam, M. Shingai, R. Pejchal, A. Ramos, M. D. Simek, Y. Geng, I. A. Wilson, P. Poignard, M. A. Martin, and D. R. Burton**. 2011. Rapid development of glycan-specific, broad, and potent anti-HIV-1 gp120 neutralizing antibodies in an R5 SIV/HIV chimeric virus infected macaque. Proc. Natl. Acad. Sci. U. S. A. **108**:20125–20129.

382. **Wang B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, a I. Sato, J. Boyer, W. V Williams, and D. B. Weiner**. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U. S. A. **90**:4156–60.

383. **Wang B.-Z., W. Liu, S.-M. Kang, M. Alam, C. Huang, L. Ye, Y. Sun, Y. Li, D. L. Kothe, P. Pushko, T. Dokland, B. F. Haynes, G. Smith, B. H. Hahn, and R. W. Compans**. 2007. Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles. J. Virol. **81**:10869–10878.

384. **Wang R., D. Doolan, T. Le, R. Hedstrom, K. Coonan, Y. Charoenvit, T. Jones, P. Hobart, M. Margalith, J. Ng, W. Weiss, M. Sedegah, C. de Taisne, J. Norman, and S. Hoffman**. 1998. Induction of Antigen-Specific Cytotoxic T Lymphocytes in Humans by a Malaria DNA Vaccine. Science (80-.). **282**:476–480.

385. **Wang S., J. Arthos, J. M. Lawrence, D. Van Ryk, I. Mboudjeka, S. Shen, T. W. Chou, D. C. Montefiori, and S. Lu**. 2005. Enhanced Immunogenicity of gp120 Protein When Combined with Recombinant DNA Priming To Generate Antibodies That Neutralize the JR-FL Primary Isolate of Human Immunodeficiency Virus Type 1. J. Virol. **79**:7933–7937.

386. **Wang Y., A. Davies, and I. Jones**. 1995. Expression and purification of glutathione S-transferase-tagged HIV-1 gp120: no evidence of an interaction with CD26. Virology **208**:142–146.

387. **Waterhouse P. M., M. Wang, and E. J. Finnegan**. 2001. Role of short RNAs in gene silencing. TRENDS pl **6**:297–301.

388. **Watkins D. I., D. R. Burton, E. G. Kallas, J. P. Moore, and W. C. Koff**. 2008. Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. Nat. Med. **14**:617–621.

389. **Webster D. E., M. L. Cooney, Z. Huang, D. R. Drew, I. A. Ramshaw, I. B. Dry, A. Richard, J. L. Martin, and S. L. Wesselingh**. 2002. Successful Boosting of a DNA Measles Immunization with an Oral Plant-Derived Measles Virus Vaccine. J. Virol. **76**:7910–7912.

390. **Wei X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. a Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw**. 2003. Antibody neutralization and escape by HIV-1. Nature **422**:307–312.

391. **Weiss C. D., J. A. Y. A. Levy, and J. M. White**. 1990. Oligomeric Organization of gp120 on Infectious Human Immunodefciency virus type 1 particles. J. Virol. **64**:5674–5677.

392. **Weissenhorn W., A. Dessen, S. Harrison, J. Skehel, and D. Wiley**. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nat. Lett. **387**:426–430.

393. **White T. A., A. Bartesaghi, M. J. Borgnia, J. R. Meyerson, M. J. V de la Cruz, J. W. Bess, R. Nandwani, J. A. Hoxie, J. D. Lifson, J. L. S. Milne, and S.**

Subramaniam. 2010. Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses: strain-dependent variation in quaternary structure. PLoS Pathog. **6**:e1001249.

394. **Wilde C. De, H. Van Houdt, S. De Buck, G. Angenon, G. De Jaeger, and A. Depicker**. 2000. Plants as bioreactors for protein production: avoiding the problem of transgene silencing. Plant Mol. Biol. **43**:347–359.

395. **Wilde C. De, K. Peeters, A. Jacobs, I. Peck, and A. Depicker**. 2002. Expression of antibodies and Fab fragments in transgenic potato plants: a case study for bulk production in crop plants. Mol. bree **9**:271–282.

396. **Willey R. L., R. A. Rutledge, S. Dias, T. Folks, T. Theodore, C. E. Buckler, and M. A. Martin**. 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proc. Natl. Acad. Sci. U. S. A. **83**:5038–5042.

397. **Williamson A.-L., E. Rybiki, E. Shephard, G. Gray, L.-G. Bekker, K. Downing, and C. Williamson**. 2012. South African HIV-1 vaccine candidates - the journey from the bench to clinical trials. South African Med. J. **102**:452–455.

398. **Wilson N. A., J. Reed, G. S. Napoe, S. Piaskowski, A. Szymanski, J. Furlott, E. J. Gonzalez, L. J. Yant, N. J. Maness, G. E. May, T. Soma, M. R. Reynolds, E. Rakasz, R. Rudersdorf, A. B. McDermott, D. H. O'Connor, T. C. Friedrich, D. B. Allison, A. Patki, L. J. Picker, D. R. Burton, J. Lin, L. Huang, D. Patel, G. Heindecker, J. Fan, M. Citron, M. Horton, F. Wang, X. Liang, J. W. Shiver, D. R. Casimiro, and D. I. Watkins**. 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. **80**:5875–5785.

399. **Wirth S., G. Calamante, A. Mentaberry, L. Bussmann, M. Lattanzi, L. Baranao, and F. Bravo-Almonacid**. 2004. Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems. Mol. Breed. **13**:23–35.

400. **Witcher D. R., E. E. Hood, D. Peterson, M. Bailey, D. Bond, R. Evangelista, Z. Nikolov, C. Wooge, R. Mehigh, J. Register, and J. A. Howard**. 1998. Commercial production of β -glucuronidase (GUS): a model system for the production of proteins in plants. Mol. Breed. **4**:301–312.

401. **Woodland D. L.** 2004. Jump-starting the immune system: prime-boosting comes of age. Trends Immunol. **25**:98–104.

402. **Worobey M., M. Gemmel, D. E. Teuwen, T. Haselkorn, K. Kunstman, M. Bunce, J.-J. Muyembe, J.-M. M. Kabongo, R. M. Kalengayi, E. Van Marck, M. T. P. Gilbert, and S. M. Wolinsky**. 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature **455**:661–664.

403. **Wu X., A. Changela, S. O'Dell, S. D. Schmidt, M. Pancera, Y. Yang, B. Zhang, M. K. Gorny, S. Phogat, J. E. Robinson, L. Stamatatos, S. Zolla-Pazner, P. D. Kwong, and J. R. Mascola**. 2011. Immunotypes of a quaternary site of HIV-1 vulnerability and their recognition by antibodies. J. Virol. **85**:4578–4785.

404. **Wu X., Z.-Y. Yang, Y. Li, C.-M. Hogerkorp, W. R. Schief, M. S. Seaman, T. Zhou, S. D. Schmidt, L. Wu, L. Xu, N. S. Longo, K. McKee, S. O'Dell, M. K. Louder, D. L. Wycuff, Y. Feng, M. Nason, N. Doria-Rose, M. Connors, P. D. Kwong, M. Roederer, R. T. Wyatt, G. J. Nabel, and J. R. Mascola**. 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science (80-.). **329**:856–861.

405. **Wu X., T. Zhou, J. Zhu, B. Zhang, I. Georgiev, C. Wang, X. Chen, N. S. Longo, M. Louder, K. McKee, S. O'Dell, S. Perfetto, S. D. Schmidt, W. Shi, L. Wu, Y. Yang, Z.-Y. Yang, Z. Yang, Z. Zhang, M. Bonsignori, J. A. Crump, S. H. Kapiga, N. E. Sam, B. F. Haynes, M. Simek, D. R. Burton, W. C. Koff, N. a Doria-Rose, M. Connors, J. C. Mullikin, G. J. Nabel, M. Roederer, L. Shapiro, P. D. Kwong, and J. R. Mascola**. 2011. Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. Science **333**:1593–1602.

406. **Wu Y.-Z., J.-T. Li, Z.-R. Mou, L. Fei, B. Ni, M. Geng, Z.-C. Jia, W. Zhou, L.-Y. Zou, and Y. Tang**. 2003. Oral immunization with rotavirus VP7 expressed in

transgenic potatoes induced high titers of mucosal neutralizing IgA. Virology **313**:337–342.

407. **Wyatt R., P. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. Hendrickson, and J. Sodroski**. 1998. The antigenic structure of the HIVgp120 envelope glycoprotein. Nat. Lett. **393**:705–711.

408. **Wyatt R., and J. Sodroski**. 2012. The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens. Science (80-.). **280**:1884–1888.

409. **Xie Y., Y. Liu, M. Meng, L. Chen, and Z. Zhu**. 2003. Isolation and identification of a super strong plant promoter from cotton leaf curl Multan virus. Plant Mol. Biol. **53**:1–14.

410. **Xu J., M. C. Dolan, G. Medrano, C. L. Cramer, and P. J. Weathers**. 2012. Green factory: plants as bioproduction platforms for recombinant proteins. Biotechnol. Adv. Elsevier Inc. **30**:1171–1184.

411. **Xu-Gang L., Z. Qian-Chun, C. Song-Biao, X. Jun-Wang, C. Tuan-Jie, and Z. Zhen**. 2002. Influence of Matrix Attachment Regions from Maize on Transgene Expression Level in Tobacco. Acta Bot. Sin. **44**:804–808.

412. **Ye L., Y. Sun, J. Lin, Z. Bu, Q. Wu, S. Jiang, D. A. Steinhauer, R. W. Compans, and C. Yang**. 2006. Antigenic properties of a transport-competent influenza HA/HIV Env chimeric protein. Virology **352**:74–85.

413. **Ye L., Z. Wen, K. Dong, X. Wang, Z. Bu, H. Zhang, R. W. Compans, and C. Yang**. 2011. Induction of HIV neutralizing antibodies against the MPER of the HIV envelope protein by HA/gp41 chimeric protein-based DNA and VLP vaccines. PLoS One **6**:e14813.

414. **Yu J., and W. Langridge**. 2003. Expression of rotavirus capsid protein VP6 in transgenic potato and its oral immunogenicity in mice. Transgenic Res. **12**:163–169.

415. **Yusibov V., and S. Rabindran**. 2008. Recent progress in the development of plant derived vaccines. Expert Rev. Vaccines **7**:1173–1183.

416. **Yusibov V., S. Rabindran, U. Commandeur, R. M. Twyman, and R. Fischer**. 2006. The Potential of Plant Virus Vectors for Vaccine. Drugs R D **7**:203–217.

417. **Zhang X., N. A. Buehner, A. M. Hutson, M. K. Estes, and H. S. Mason**. 2006. Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. Plant Biotechnol. J. **4**:419–432.

418. **Zhou T., I. Georgiev, X. Wu, Z.-Y. Yang, K. Dai, A. Finzi, Y. Do Kwon, J. F. Scheid, W. Shi, L. Xu, Y. Yang, J. Zhu, M. C. Nussenzweig, J. Sodroski, L. Shapiro, G. J. Nabel, J. R. Mascola, and P. D. Kwong**. 2010. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science (80-.). **329**:811–817.

419. **Zhu P., J. Liu, J. Bess, E. Chertova, J. D. Lifson, H. Grisé, G. A. Ofek, K. A. Taylor, and K. H. Roux**. 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature **441**:847–852.

420. **Zhu T., B. T. Korber, A. J. Nahmias, E. Hooper, P. M. SHarp, and D. D. Ho**. 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature **391**:594–597.

421. **Zhu Z., H. R. Qin, W. Chen, Q. Zhao, X. Shen, R. Schutte, Y. Wang, G. Ofek, E. Streaker, P. Prabakaran, G. G. Fouda, H.-X. Liao, J. Owens, M. Louder, Y. Yang, K.-A. Klaric, M. A. Moody, J. R. Mascola, J. K. Scott, P. D. Kwong, D. Montefiori, B. F. Haynes, G. D. Tomaras, and D. S. Dimitrov**. 2011. Cross-reactive HIV-1-neutralizing human monoclonal antibodies identified from a patient with 2F5-like antibodies. J. Virol. **85**:11401–11408.

422. **Zwick M. B., A. F. Labrijn, M. Wang, C. Spenlehauer, E. O. Saphire, J. M. Binley, P. Moore, G. Stiegler, H. Katinger, D. R. Burton, and P. W. Parren**. 2001. Broadly Neutralizing Antibodies Targeted to the Membrane-Proximal External Region of Human Immunodeficiency Virus Type 1. J. Virol. **75**:10892–10905.

423. 2013. GLOBAL REPORT: UNAIDS report on the global AIDS epidemic 2013.

424. 2001. HIV-1 Group O Infection in Cameroon, 1986-1998. Emerg. Infect. Dis. **7**:466–467.

APPENDIX A:
ADDITIONAL PROTOCOLS

A.1. Protein quantification of transfected cell lysate

The protein concentrations of the transfected cell lysate samples were quantified using the Bio-Rad DC Protein Assay in accordance with the manufacturer's instructions. A serial dilution series of Bovine Serum Albumen (BSA) standards were prepared in 1× Glo Lysis Buffer (Promega) with concentrations ranging from 0.1 mg/ml to 1.6 mg/ml. Aliquots of 5 µl of each standard were transferred, in triplicate, to a 96 well microtiter plate. The cell lysate samples were diluted 2-fold in sterile dPBS and 5 µl transferred to a 96 well microtiter plate, in triplicate. Triplicate samples of dPBS were loaded alongside the experimental samples as a blank. Aliquots of 25 µl of reagent A (Bio-Rad) and 200 µl of reagent B (Bio-Rad) were added to each well. The microtiter plate was gently agitated to mix the contents of each well, before being incubated for 15 minutes at room temperature. The OD_{750} values were then determined for each well using the VERSAmax Microplate Reader with the DC750pr template. The values derived from the BSA standards were used for the construction of a standard curve. The lysate samples were corrected for the dilution factor and their protein concentrations extrapolated from the standard curve.

A.2. Luciferase assay to measure the transfection efficiency

A serial dilution series of QuantiLum® Recombinant Luciferase (Promega) was prepared in 1× Glo Lysis Buffer (Promega); with concentrations ranging from 7.8125 pg/ml to 500 pg/ml. Aliquots of 0.2 µg/ml of total protein, derived from transfected cells, were prepared in a 350 µl volume of 1× Glo Lysis Buffer (Promega). Control samples, comprising of lysate derived from untransfected cells, were prepared in

parallal with the experimental samples. Aliquots of 100 µl of the luciferase standards and the experimental samples were dispensed, in triplicate, into a Nunc-immuno™ Microwell™ 96 well polystyrene plate (Sigma-Aldrich). Lastly, 100 µl aliquots of Glo lysis buffer were loaded, in triplicate, alongside the experimental samples as a blank. An aliquot of 100 µl of Bright-Glo™ substrate (Promega) was dispensed into each well using a multichannel pipette. Luminescence was measured immediately, using a luminometer, and a standard curve generated from the luciferase standards.

The average triplicate relative luciferase value was extrapolated from the standard curve and the transfection considered to be successful if the average triplicate relative luciferase value was greater than the lower limit of quantification,

APPENDIX B:

BUFFERS, MEDIA AND SOLUTIONS

Luria Bertani liquid broth (100 ml)

Tryptone	1 g
Yeast Extract	0.5 g
NaCl	0.5 g
Water	adjust to 100 ml

** Autoclave

Luria Bertani agar (100 ml)

Tryptone	1 g
Yeast Extract	0.5 g
NaCl	0.5 g
Agar	1.5 g
Water	adjust to 100 ml

** Autoclave

Transfer and storage (TSB) buffer (100ml):

Peptone	1.6 g
Yeast extract	1.0 g
NaCl	0.5 g
PEG	10.0 g
DMSO	5 ml
1 M $MgCl_2$	1 ml
1 M $MgSO_4$	1 ml
Water	adjust to 100 ml

TSGB media (100ml):

20% w/v glucose	2 ml
TSB buffer	98 ml

5 × Tris-Borate-EDTA (TBE) buffer (1 liter)

Trizma base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Water	adjust to 100 ml

Induction media (100 ml)

Tryptone powder	1 g
Yeast Extract	0.5 g
NaCl	0.5 g
MES	0.1952 g
Water	adjust to 100 ml
*Adjust pH to 5.6	
**Autoclave	

Infiltration media (1 litre)

MES	1.952 g
$MgCl_2$	2.03 g
Sucrose	30 g
Water	adjust to 1 l
*Adjust pH to 5.6	
200 mM Acetosyringone	1ml

Solution I: (100 ml)

1 M Tris-HCl (pH 8.0)	2.5 ml
0.5 M EDTA (pH 8.0)	10.0 ml
20% w/v glucose	5 ml
Water	82.5 ml

Solution II: (10 ml)

10 M NaOH	0.2 ml
10% SDS	1.0 ml
Water	8.8 ml

Solution III: (10 ml)

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

Block/wash buffer (100 ml)

1 × PBS	100 ml
Instant milk powder	5 g
10% Tween 20	1 ml

1 × Transfer buffer (1 litre)

1 × Tris base	5.82 g
Glycine	2.93 g
Methanol	200 ml
Water	adjust to 1 litre

10 × Ponceau S solution (1 litre)

Ponceau S	2.0 g
Trichloroacetic acid	30 g
5-Sulfosalicylic acid	30 g
Water	adjust to 1 litre

Complete Growth Media (GM)

Dulbecco's Modified Eagle Medium	450 ml
Heat inactivated Fetal Calf Serum	50 ml
Penecillin-Strepromycin	5 ml
Fungin	500 µl

4x Resolving Buffer

Tris base	36.3g
adjust pH to 8.8 with HCl	
water	Adjust to 200 ml

4x Stacking Buffer

Tris base	3.0g
Adjust pH to 6.8 with HCl	
Water	Adjust to 50 ml

10x Electrophoresis Running Buffer

Tris base	30.3 g
Glycine	144.2g
SDS	10g
Adjust pH to 8.5 with HCl	

Water	Adjust to 1 liter

5x Sample Loading Buffer

10% SDS	940 µl
1M TrisCl pH 7.5	470 µl
100mM EDTA	95 µl
Glycerol	2.45 ml
Distilled water	545 µl
Mercaptoethanol	205 µl
1% Bromophenol blue	10 µl