Biological and Immunological Characterization of Plant-Produced HIV-1 Gag/dgp41 Virus-Like Particles

by

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A Dissertation Presented in Partial Fulfillment
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Doctor of Philosophy

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ABSTRACT

Anti-retroviral drugs and AIDS prevention programs have helped to decrease the rate of new HIV-1 infections in some communities, however, a prophylactic vaccine is still needed to control the epidemic world-wide. Despite over two decades of research, a vaccine against HIV-1 remains elusive, although recent clinical trials have shown promising results. Recent successes have focused on highly conserved, mucosally-targeted antigens within HIV-1 such as the membrane proximal external region (MPER) of the envelope protein, gp41. MPER has been shown to play critical roles in the viral mucosal transmission, though this peptide is not immunogenic on its own. Gag is a structural protein configuring the enveloped virus particles, and has been suggested to constitute a target of the cellular immunity potentially controlling the viral load. It was hypothesized that HIV-1 enveloped virus-like particles (VLPs) consisting of Gag and a deconstructed form of gp41 comprising the MPER, transmembrane, and cytoplasmic domains (dgp41) could be expressed in plants. Plant-optimized HIV-1 genes were constructed and expressed in *Nicotiana benthamiana* by stable transformation, or transiently using a tobacco mosaic virus-based expression system or a combination of both. Results of biophysical, biochemical and electron microscopy characterization demonstrated that plant cells could support not only the formation of HIV-1 Gag VLPs, but also the accumulation of VLPs that incorporated dgp41. These particles were purified and utilized in mice immunization experiments. Prime-boost strategies combining systemic and mucosal priming with systemic boosting using two different vaccine

candidates (VLPs and CTB-MPR – a fusion of MPER and the B-subunit of cholera toxin) were administered to BALB/c mice. Serum antibody responses against both the Gag and gp41 antigens could be elicited in mice systemically primed with VLPs and these responses could be recalled following systemic boosting with VLPs. In addition, mucosal priming with VLPs allowed for a robust boosting response against Gag and gp41 when boosted with either candidate. Functional assays of these antibodies are in progress to test the antibodies' effectiveness in neutralizing and preventing mucosal transmission of HIV-1. This immunogenicity of plant-based Gag/dgp41 VLPs represents an important milestone on the road towards a broadly-efficacious and inexpensive subunit vaccine against HIV-1.

DEDICATION

I dedicate this dissertation to the many people who have helped me to get to this point in my pursuit of knowledge: To my mom for instilling in me a love of learning, science, and curiosity from a very young age; To my dad for instilling in me a love for the beauty of the natural world and the knowledge that could be gained by spending time with Mother Nature; To my sister for sharing and learning beside me for all of our lives; To all of my extended family for the backbone they have provided – I could not have done this without their support; To Ms. McElroy and all of my past science teachers for nurturing my sense of curiosity and enlightening me to the vast horizons that stretched before me if I chose to pursue a career in science; To my friends both inside and outside of the lab for keeping me sane in the midst of the stress of graduate school; To Norman Bourlaug, Peter Raven, and Charles Arntzen for their incredible inspiration in life and research – I hope to one day emulate these heroes in using my research to help solve global challenges and make the world a better place in which to live; To Tim for his unfaltering love and support through everything, for his incredible patience during my long days in the lab and his innate understanding of when I needed to be dragged back to the outside world for perspective.

"The destiny of world civilization depends upon providing a decent standard of living for all mankind." –Norman Bourlaug

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LIST OF ABBREVIATIONS

Ab	antibody
AIDS	aquired immune deficiency syndrome
AIM	Agrobacterium infiltration media
BA	6-benzylaminopurine
BALB/c	inbred mouse strain for use in immunology experiments
CA	capsid
CD4bs	CD4+ cell binding site
CTA2	cholera toxin subunit A2
CTB	cholera toxin subunit B
CTB-MPR	cholera toxin subunit B – membrane proximal region
CTL	cytotoxic T-lymphocyte
DTT	dithiothreitol
dgp41	deconstructed gp41
EDTA	ethylenediaminetetraacetic acid
ELISPOT	enzyme-linked immunosorbent spot assay
Env	envelope protein of HIV-1
ESCRT	endosomal sorting complex required for transport
F1-V	fusion of fraction 1 capsular antigen/V antigen of *Yersinia pestis*
GalCer	galactosylceramide
HBc	Hepatitis B virus capsid protein
HEPS	Highly Exposed Persistantly Seronegative
HIV	Human Immunodeficiency Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
i.n.	intranasal
IN	integrase
i.p.	intraperitoneal
MA	matrix
MAb	monoclonal antibody
MES	N-morpholino enthanesulfonic acid
MPER	membrane proximal external region
NAA	1-naphthaleneacetic acid
NC	nucleocapsid
NMT	n-myristol transferase
ORF	open reading frame
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PBSTM	phosphate buffered saline with Tween-20 and 5% dry milk
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PR	protease
$TCID_{50}$	50% tissue culture infective dose
TEM	transmission electron microscopy
RT	(in introduction) reverse transcriptase
RT	(in materials and methods) room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VLP	virus-like particle

LIST OF BUFFERS

AIM - 2.15 g/L Murashige and Skoog (MS) salts, 0.5 mg/L pyridozine, 0.5 mg/L nicotinic acid, 2 mg/L thiamine HCl, 100 mg/L myoinositol, 0.9 g/L glucose, 1.95 g/L N-morpholino enthanesulfonic acid (MES), pH 5.2

Agroinfiltration buffer - 10mM $MgSO_4$, 10 mM MES, pH 5.5

PBS – 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4

PBST - 0.5% (v/v) Tween-20 in PBS

PBSTM - 0.5% (v/v) Tween-20, 5% (w/v) dry milk in PBS

Plant extraction buffer – 25 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8

Protoplast isolation buffer - , 0.625 M sucrose, 25 mM MES, pH 5.7

6x SDS loading buffer – 30% (v/v) glycerol, 35 mM SDS, 60 mM DTT, 18 mM bromophenol blue, 350 mM Tris-HCl, pH 8.0

TEM primary fixation buffer - 2% (v/v) gluteraldehyde, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.8

TEM secondary fixation buffer - 2% (v/v) osmium tetroxide, 0.1 M sodium phosphate (51% monobasic, 49% dibasic), pH 6.8

Chapter 1

INTRODUCTION

Challenges for Development of an HIV-1 Vaccine

Since the initial description of Acquired Immune Deficiency Syndrome (AIDS) and discovery of its causal agent, Human Immunodeficiency Virus-1 (HIV-1), in the early 1980s, HIV/AIDS has become a global epidemic with more than 33 million people currently infected worldwide (Brown et al., 2010). Antiretroviral therapy and AIDS prevention programs have helped to curb the infection rate in developed countries, but an estimated 2.6 million new infections in 2009 alone prove that the epidemic is not contained worldwide (UNAIDS/WHO, 2009). Despite over two decades of research, an effective prophylactic vaccine against HIV remains elusive (Johnston and Fauci, 2008).

Although some countries continue to experience significant rates of new HIV-1 infections due to unsanitary reuse of syringe needles or blood transfusions, the majority of new infections worldwide are established mucosally, in particular the mucosal tissues of the vagina and lower gastrointestinal tract (Pope and Haase, 2003). HIV-1 can breach both pluristratified (multilayered) or simple epithelial through a mucosal breach caused by epithelial damage or an underlying sexually transmitted disease (illustration of mucosal transmission shown in Figure 1). In addition, HIV-1 can disseminate pluristratified epithelium by attaching to dendritic cells. In simple epithelium with tight junctions, transcytosis of HIV-1 can occur through the binding of the membrane proximal external region (MPER) of HIV's envelope protein subunit gp41 to the galacosyl ceramide coreceptor (Alfsen and Bomsel, 2002; Alfsen et al., 2005). Transcytosis

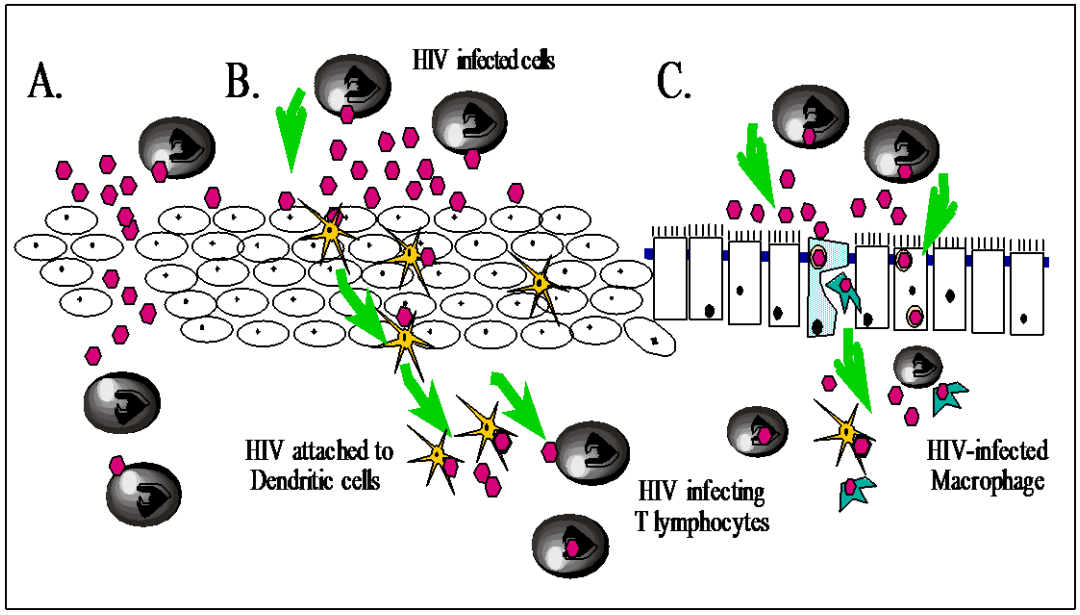


Figure 1. Mucosal transmission of HIV. HIV-1 can gain access through the mucosal barrier by several ways: A. mucosal breach (caused, for example, by an underlying sexually transmitted disease). B. In pluristratified epithelium, HIV-1 can disseminate by attaching to dendritic cells. C. In simple epithelium with tight junctions, transcytosis through the cells (mediated by binding of the MPER region to galacosyl ceramide) predominates. Transcytosis through M-cells (light blue) is also possible.

through M-cells of simple epithelium is also possible. Regardless of the initial infection route, the CD4+ cells of the gut-associated lymphoid tissue are the first cells to be depleted during the acute phase of HIV infection (Brenchley et al., 2004; Mehandru et al., 2004). A vaccine targeted to the mucosal system could be one of the most effective methods of stopping the initial infection of the virus.

The hypervariability of HIV is one of the leading challenges to vaccine development. The high rates of replication, mutation, and recombination all contribute to this vast genetic variability of the virus, both globally and within an individual (Taylor et al., 2008). Globally, five different major clades of virus are responsible for the majority of infections in different regions around the world, with several other clades contributing to the epidemic in regions where the virus is most prevalent (Geretti, 2006) (Table 1). On an individual level, the immune system is dealing with rapidly mutating viral antigens outpacing the elicitation of effective immune responses against them (Kwong et al., 2002; Leslie et al., 2004; Kawashima et al., 2009). This creates a “moving target” for potential vaccines candidates. By focusing vaccine targets on highly conserved regions of the viral proteome, the vaccine could be more effective at targeting a wider range of clades and perhaps offer longer term protection (Johnston and Fauci, 2011). In addition to the challenges regarding the hypervariablility of HIV, the lack of correlates of immune protection against the virus (Sullivan, 2009; Corey et al., 2011), the lack of standardized, bona fide animal models for preclinical vaccine trials (Girard et al., 2006), and the logistical challenges and costs of clinical trials also contribute to the challenges facing researchers striving to develop a vaccine candidate (Girard et al., 2006; Corey et al., 2011).

Table 1. Global predominance and geographical prevalence of HIV-1 subtypes.

(Adapted from (Geretti, 2006))

Subtype	Global Predominance	Main Geographical Prevalence
A	High	Eastern Africa, Eastern Europe, Central Asia
B	High	Americas, Western Europe, Australia, Japan
C	High	South and Eastern Africa, India, China, Nepal
D	High	Eastern Africa, Eastern Europe, Central Asia
F	Low	South America, Central Africa, Eastern Europe
G	Low	Central Africa
H	Low	Central Africa
J	Low	Central Africa
K	Low	Central Africa

HIV-1 Organization and Virion Structure

The viral genome contains nine genes that are responsible for the production of the virus' structural and regulatory proteins, several of which have become important targets for protein vaccines against HIV-1 (Figure 2). Three viral genes, *gag*, *pol*, and *env*, give rise to three precursor polyproteins (gag, pol, and env) that are responsible for the production of the main structural proteins and enzymes of HIV-1. These polyproteins are then further processed to create mature viral proteins. The structural proteins, Env and Gag, will be further discussed below. In addition to these structural proteins and enzymes, six other low molecular mass proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef) are also produced. Tat and Rev play regulatory roles in viral transcription, while Vif, Vpr, Vpu, and Nef have been found to be important in viral replication and infectivity (Knipe and Howley, 2007).

On the outermost surface of the virus, HIV-1 expresses the envelope (Env) protein consisting of two subunits: gp120 on the surface of the virus and the membrane-embedded gp41. The gp120 subunit is important in initiating viral infection by its binding to the primary viral receptor, CD4, present on T_H cells, dendritic cells, and macrophages (Bour et al., 1995). This process exposes binding sites for the chemokine receptors CCR5 and CXCR4. Once the virus binds to these coreceptors, further conformational changes occur, facilitating the fusion of the viral membrane to that of the host and allowing the nucleocapsid to enter the cell (Hladik and McElrath, 2008). In addition to these binding mechanisms, the binding of gp41 residues 649-684 encompassing the membrane proximal external region (MPER)) to the glycosphingolipid galactosylceramide (GalCer) on the apical surface of epithelial cells triggers the

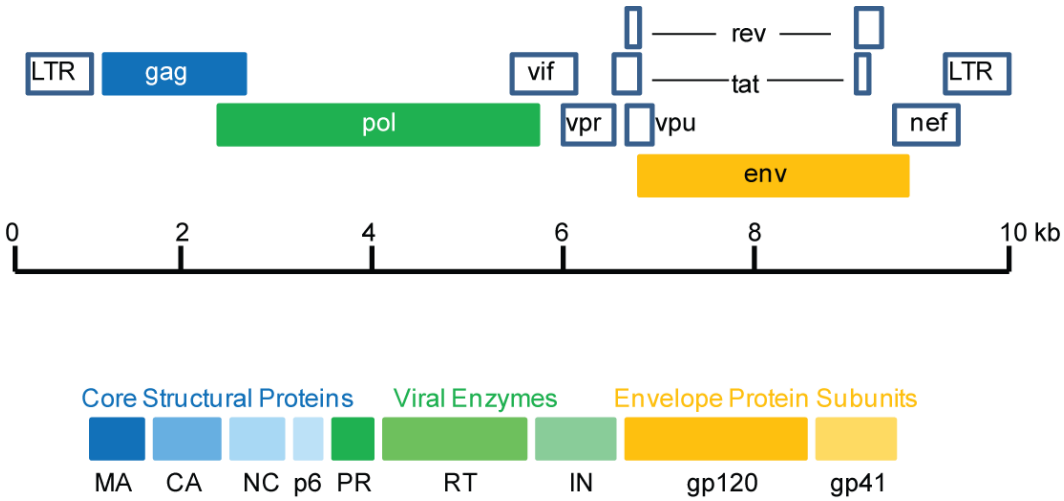


Figure 2. Organization of HIV-1 genome. The HIV-1 genome consists of several overlapping open reading frames (ORFs). The three primary translation products for the structural proteins and viral enzymes are synthesized as polyprotein precursors, which are then cleaved into mature, particle associated proteins. The gag precursor is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. The pol precursor is cleaved into protease (PR), reverse transcriptase (RT), and integrase (IN) proteins. The env precursor is cleaved into gp120 and gp41. Six other viral proteins (Vif, Vpr, Vpu, Tat, Rev, and Nef) are also produced.

transcytosis of the virus towards the serosal side of the cell (Alfsen and Bomsel, 2002).

The surface subunit of Env, gp120, is highly immunogenic and some of the antibodies generated from this protein can mediate viral neutralization during natural infection, but unfortunately these neutralizing antibodies are not broadly reacting, and are unable to completely eliminate virus from the patient due to the rapid mutation of the virus which evade the specific antibodies (Montero et al., 2008). In addition, gp120 is heavily glycosylated, with over 50% of the protein surface covered with carbohydrates, which obstruct the binding of neutralizing antibodies (Pantophlet and Burton, 2006). Despite the variability and glycosylation of gp120, much research has focused on the CD4 binding site (CD4bs) and the adjacent V3 loop of gp120, due to the broadly-neutralizing antibody epitopes found within these regions of gp120 (Nandi et al., 2010).

In contrast to the variability of gp120, the transmembrane glycoprotein gp41 contains a highly conserved membrane proximal external region (MPER) corresponding to amino acids 649-683 of the protein (Montero et al., 2008). The MPER resides just outside the lipid envelope, above the transmembrane domain (TM) of gp41 (Figure 3).

The MPER of gp41 is important in the viral transcytosis across mucosal membranes (Bomsel and David, 2002). The cell receptor glycosphingolipid galactosyl ceramide (GalCer) on the luminal surface of epithelial cells binds to the MPER of gp41, mediating the transcytosis of the virus to the serosal side of the cell. This binding can be inhibited by peptides corresponding to the MPER, and both mucosal and systemic antibodies against

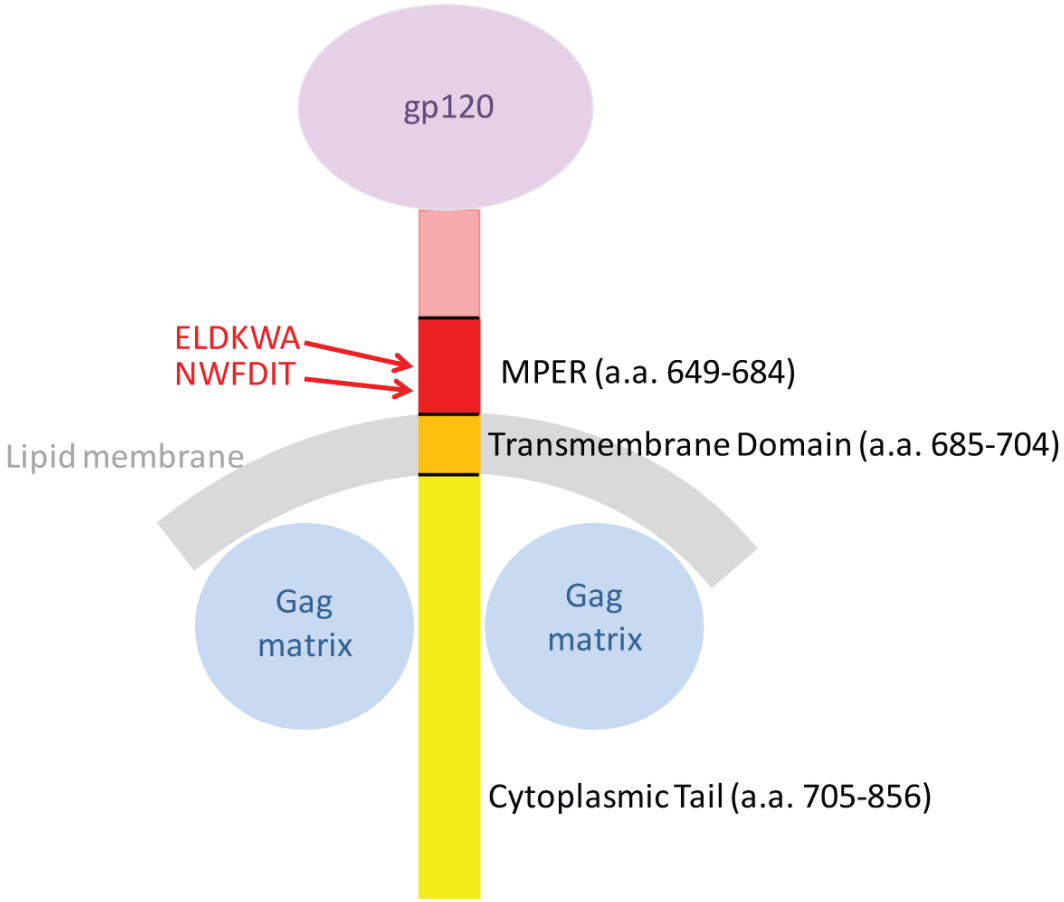


Figure 3. HIV-1 gp41 structure. The membrane-embedded gp41 subunit of HIV-1 Env consists of a region that interacts with the gp120 subunit, the membrane proximal external region (MPER, a.a. 649-684), the transmembrane domain (a.a. 685-704), and the cytoplasmic tail (a.a. 705-856). Two epitopes (ELDKWA and NWFDIT) within the MPER recognized by broadly neutralizing antibodies, 2F5 and 4E10, respectively, are also shown.

MPER can block the transcytosis of HIV across the epithelial barrier (Matoba et al., 2004; Matoba et al., 2006).

In addition, the broadly neutralizing human monoclonal antibody 2F5 (whose core epitope is the ELDWKA sequence within the MPER) and polyclonal secretory IgAs (sIgAs) found in the mucosal secretions of highly exposed persistently seronegative (HEPS) individuals have been shown to block transcytosis (Devito et al., 2000; Broliden et al., 2001; Kaul et al., 2001). These results show potential for mucosal immunity through a vaccine utilizing the MPER region of gp41 (Alfsen et al., 2001).

Since MPER is not immunogenic on its own (Zolla-Pazner et al., 2011), a platform with which to introduce the protein as a vaccine candidate is needed for successful mucosal immunization. Past studies from the Mor lab have utilized fusion proteins containing the cholera toxin B (CTB) subunit and plague antigens F1 and V for mucosal immunizations along with MPER with varying degrees of success (Matoba et al., 2004; Matoba et al., 2006; Matoba et al., 2008; Shah, 2008; Matoba et al., 2011), and these studies will be discussed in more detail in Chapter 4.

Gag, the main structural protein of HIV-1, which plays a key role in virus budding, is a potential candidate for a platform with which to introduce the MPER peptide in a vaccine candidate. During viral assembly, the immature p55 Gag provides structure for the budding virus, localizing to membrane rafts within the plasma membrane where HIV-1 envelope proteins are also localizing (Bhattacharya et al., 2006). The accumulating Gag oligomers bud from the cell (Figure 4), taking the host membrane and associated membrane-proteins with it,

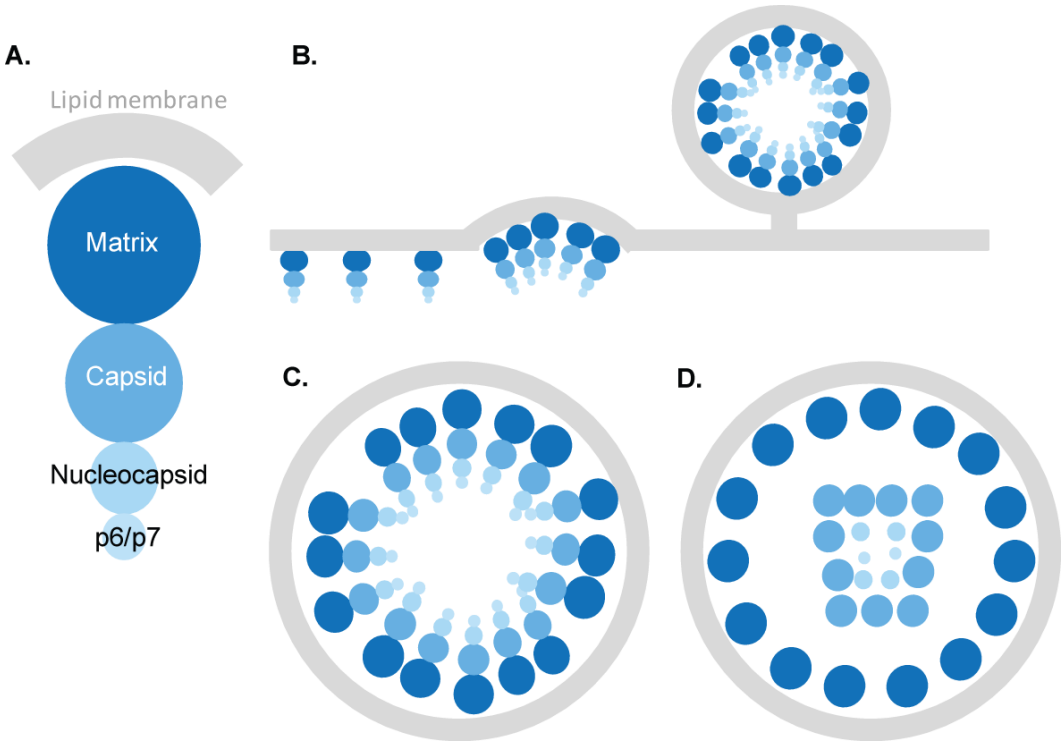


Figure 4. HIV-1 Gag protein. A. Schematic representation of the components of the p55 Gag polyprotein as comprised in the immature virion, before cleavage by viral protease. B. The budding of HIV Gag from a lipid membrane. From the left to right, Gag proteins line up along the membrane, start to protrude from the cell, and then pinch off from the host membrane as an immature virion. C. Gag structures within an immature virus. The matrix, capsid, nucleocapsid and p6/p7 are in their immature conformation. D. Gag protein after cleavage by viral protease, with the matrix protein forming the outer structure of the virus and the capsid protein encapsulating the viral RNA (not pictured) and the nucelocapsid and p7/p6 proteins.

and eventually pinch off from the host cell. After budding, the immature p55 Gag protein is then cleaved by viral protease in the maturing virus into the N-terminal matrix domain (MA, p17), the capsid domain (CA, p24) encapsulating the RNA molecules and retrotranscriptase, the nucleocapsid domain (NC, p7), and the C-terminal domain, p6, with two spacer peptides (p2 and p1 respectively) separating p24/p7 and p7/p6. The N-terminal p17 domain interacts with the membrane and cytoplasmic tail of the gp41 envelope protein and is important for viral assembly and release (Freed, 1998). The C-terminal p6 domain is important for the budding of viruses from the host cell (Strack et al., 2003).

Gag is a promising vaccine candidate against HIV-1, as the combination of the p17 and p24 domains contains the highest density of cytotoxic T-lymphocyte (CTL) epitopes of any HIV protein (Novitsky et al., 2002; Addo et al., 2003). In addition, the immature Gag polyprotein (p55 Gag) is both necessary and sufficient for forming enveloped virus-like particles (VLPs) of HIV (Garoff et al., 1998).

VLPs as Vaccine Candidates

VLPs consist of structural protein shells of viruses, and do not contain genetic material. The protein shell allows the presentation of itself as particulate matter to the immune system. In addition to providing this immunogenicity of the VLP proteins itself, VLPs can be used to display other antigens.

VLPs are promising new candidates for viral vaccines, and the success of prophylactic VLP-based vaccines such as the human papillomavirus L1 VLP vaccines (GlaxoSmithKline's Cervarix and Merck's Gardasil) provide the motivation for continued research on VLP vaccines (Schiller et al., 2008; Stanley, 2009). Devoid of nucleic acids, unlike conventional "Jennerian" live-attenuated

virus vaccines, VLP-based vaccines are able to initiate protective immune responses without the risk of viral infection or other major harmful side effects. Gag-based VLPs are capable of inducing strong CTL responses without adjuvant (Doan et al., 2005). The Gag protein of HIV has been shown to produce VLPs in various eukaryotic expression systems including mammalian (Rovinski et al., 1992), insect (Deml et al., 2004), and yeast cell cultures (Sakuragi et al., 2002) and shown to be immunogenic (Deml et al., 2005). Gag is highly conserved within clades, relatively conserved across diverse HIV clades, and spans multiple CD4+ and CD8+ T-cell epitopes (McAdam et al., 1998; Ferrari et al., 2000). In addition, Gag VLPs can display HIV Env proteins on their surface in their native conformation (Hicar et al., 2010), and have been shown to induce both Env- and Gag-specific antibodies and CTLs (Rovinski et al., 1992; Montefiori et al., 2001), making Gag VLPs attractive candidates for an HIV vaccine platform.

Expression Host Considerations for HIV-1 Vaccines

Regardless of the antigen used as a vaccine candidate, the expression system with which it is produced is an important factor to consider when developing an HIV vaccine. One of the difficulties in developing an HIV vaccine lies in the fact that HIV is most prevalent in sub-Saharan Africa and other developing countries, where resources and funding are not readily available for distribution of expensive vaccines (International AIDS Vaccine Initiatve, 2006). Therefore, in order for the vaccine candidate to truly be effective in reaching at risk populations in developing countries, the vaccine must be manufactured, stored, and distributed inexpensively. Production of vaccines in mammalian, insect, and yeast cell cultures is expensive and difficult to scale up for production on a large scale. In addition, pathogens and toxins in these cultures present

additional hurdles for protein purification and use in human vaccines (Daniell et al., 2001; Thanavala et al., 2006).

Plants, on the other hand, offer simple and inexpensive means of recombinant protein production which can be readily scaled up according to production needs in greenhouse or field conditions (Streatfield and Howard, 2003a, b), while still providing the cellular machinery and eukaryote-common post-translational modifications necessary for producing VLPs utilizing the Gag and Env proteins. Plants have been shown to be effective in expressing VLPs of Hepatitis B (Kapusta et al., 1999; Kapusta et al., 2001; Huang et al., 2005; Thanavala et al., 2005; Huang et al., 2006; Huang et al., 2009), Norwalk virus (Tacket et al., 2000; Zhang et al., 2006; Santi et al., 2008), influenza (D'Aoust et al., 2008), and even HIV (Meyers et al., 2008; Scotti et al., 2009), and several of these VLP-based vaccine candidates have been successful in clinical trials (Tacket et al., 2000; Mason et al., 2002).

Aims of Research and Review of Dissertation

Despite the many biological and economical challenges related to the development of an HIV-1 vaccine, by addressing these challenges in the context of a new vaccine candidate that utilizes a mucosally targeted subunit vaccine component focused on highly conserved regions of HIV-1 and expressed in highly scalable, inexpensively grown *Nicotiana benthamiana*, I hoped to create a novel immunogen for use as an effective HIV-1 vaccine constituent. In this project, a vaccine candidate based on a Clade C Gag VLP with deconstructed gp41 (dgp41, consisting of the MPER, transmembrane domain, and cytoplasmic tail region) proteins embedded in its membrane envelope (Figure 5) was

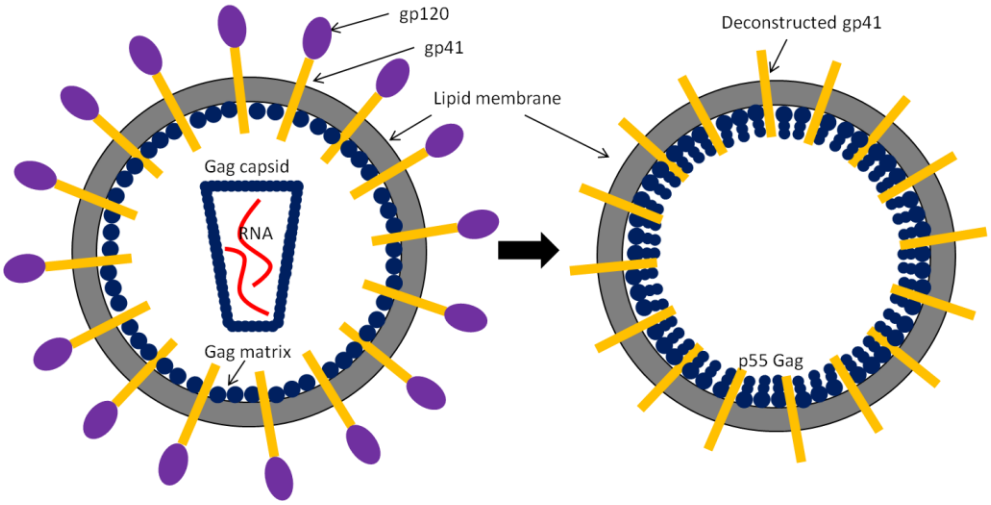


Figure 5. Comparison of structures within mature, infectious native HIV-1 virus (left) and VLP consisting of p55 Gag and dgp41 (right).

expressed in plants and tested for immunogenicity in mice. The goal of the project was to first express p55 Gag in *Nicotiana benthamiana* in both transient and transgenic expression systems. Then dgp41 was transiently expressed first in wild type plants and then co-expressed with Gag in Gag transgenic plants. The expression of these proteins is discussed in Chapter 2. In Chapter 3, these expressed proteins were characterized to determine whether they were developing into enveloped VLPs. Once it was established that Gag and dgp41 were co-localizing into VLPs, the VLPs were purified from plant extract and utilized in two separate immunization trials. The first trial tested the immunogenicity of the VLPs in comparison to the fusion peptide CTB-MPR when injected into mice intraperitonially (i.p.). This immunization trial and the results of antibody production against p24 and MPER and functional assays are discussed in Chapter 4. A second trial in mice was conducted to test the mucosal response of a heterologous prime-boost strategy utilizing both plant-produced VLPs and CTB-MPR with intranasal (i.n.) priming and i.p. boosting. This trial and the results of antibody production against p24 and MPER, functional assays, and cytotoxic T-cell lymphocyte responses against Gag are discussed in Chapter 5.

Chapter 2

PLANT OPTIMIZATION AND EXPRESSION OF GAG AND DGP41 IN NICOTIANA BENTHAMIANA

ABSTRACT

The HIV-1 proteins Gag and gp41 have had limited expression in plants in both transient and transgenic expression systems due to a number of factors. Through plant codon-optimization and the use of both transgenic expression and the MagnICON transient expression system, the expression of both HIV-1 Gag and deconstructed gp41 (dgp41) proteins were tested for expression in *Nicotiana benthamiana*. It was determined that HIV-1 Gag could be expressed by both a transient and transgenic expression system, and it was also determined that dgp41 proteins could be expressed by a transient expression system. In addition, it was demonstrated that Gag and a deconstructed gp41 (dgp41) can be co-expressed in *N. benthamiana*.

INTRODUCTION

HIV-1 Gag, gp41, and their derivatives have been expressed in plants in only a handful of studies, and the results of these studies have been mixed. Although recombinant Gag has been expressed in other cell culture systems for more than 20 years (Gheysen et al., 1989; Shioda and Shibuta, 1990; Vzorov et al., 1991; Wagner et al., 1994b; Wagner et al., 1994a; Sakuragi et al., 2002), Gag has only been expressed in plants in two studies (Table 2), by Edward Rybicki's group at the University of Cape Town (Meyers et al., 2008), and Teodoro Cardi's group at Italy's National Research Council Institute of Plant

Table 2. Plant-based expression of HIV-1 p55 Gag protein.

Group	Species	Expression System	Subcellular Localization	Protein (p24) Yield
Meyers et al.	N. benthamiana	TMV viral vector (transient)	Cytosol (WT gag gene)	<0.01 µg/kg FW
Meyers et al.	N. benthamiana	TMV viral vector (transient)	Cytosol (codon optimized)	2 µg/kg FW
Meyers et al.	N. benthamiana	Agroinfiltration (transient)	Cytosol	7 - 44 µg/kg FW
Meyers et al.	N. benthamiana	Agroinfiltration (transient)	Endoplasmic Reticulum	8 - 13 µg/kg FW
Meyers et al.	N. benthamiana	Agroinfiltration (transient)	Chloroplast	1 - 29 µg/kg FW
Meyers et al.	N. tabacum	Nuclear transformation (stable)	Cytosol	Died
Meyers et al.	N. tabacum	Nuclear transformation (stable)	Endoplasmic Reticulum	0.13 - 7
Meyers et al.	N. tabacum	Nuclear transformation (stable)	Chloroplast	0.03 - 48 µg/kg FW
Scotti et al.	N. benthamiana	Agroinfiltration (transient)	Cytosol	None
Scotti et al.	N. benthamiana	Agroinfiltration (transient)	Apoplast	None
Scotti et al.	N. benthamiana	Agroinfiltration (transient)	Endoplasmic Reticulum	None
Scotti et al.	N. benthamiana	Agroinfiltration (transient)	Chloroplast	Positive on Western
Scotti et al.	N. benthamiana	Agroinfiltration (transient)	Mitochondrion	None
Scotti et al.	N. tabacum	Nuclear transformation (stable)	Chloroplast	28 mg/kg FW
Scotti et al.	N. tabacum	Plastid transformation (stable)	Chloroplast	38.6 mg/kg FW
Scotti et al.	N. tabacum	Plastid transformation (stable)	Chloroplast	338 mg/kg FW

Genetics (Scotti et al., 2009). Both groups had difficulty in expressing full-length p55 Gag in the cytoplasm of *N. benthamiana* and *N. tabacum* in either transient or transgenic systems. Both groups had better success with expression of p55 Gag in chloroplasts, but neither group confirmed the production of enveloped VLPs in their study. Scotti and colleagues (2009) were able to produce transmission electron microscopy (TEM) images of their VLPs, but did not confirm that these were enveloped (Scotti et al., 2009). No immunogenic studies were performed with either of the p55 Gag products, though Meyers et al. did conduct successful mice immunization trials with a protein containing both the matrix (p17) and capsid (p24) portions of Gag (Meyers et al., 2008).

Several groups have attempted expression of parts of the HIV envelope proteins in plants with varying degrees of success (Table 3). The main candidates for plant-expression have been some of the main targets of vaccine and microbiocide production: the V3 loop of gp120 (Sugiyama et al., 1995; Joelson et al., 1997; Yusibov et al., 1997; Kim et al., 2004), the b12 epitope of gp120 (Sexton et al., 2009), the membrane proximal region of gp41 (Marusic et al., 2001; Matoba et al., 2004; Matoba et al., 2006), and the cytoplasmic tail of gp41 (McLain et al., 1995) (Table 3). All of these proteins or peptides were expressed on a virus platform or as fusion partners with other proteins/peptides, but the expression levels of the proteins were fairly low.

There are several important factors that affect the success of high-level expression of recombinant proteins in plants. The host in which to express the protein of interest can be determined by the end goal of the vaccine, but a wide variety of hosts ranging from edible fruits and tubers such as tomatoes and potatoes to edible leafy crops such as alfalfa, spinach, and lettuce to non-food

Table 3. Plant-based expression of HIV-1 Env proteins and derivatives. Color is representative of Env subunit derivative. Green - gp120, Yellow - gp41, Blue - both gp120 and gp41.

Group	Antigen (Displayed on)	Species	Expression System	Subcellular Localization	Protein Yield
Yusibov et al.	gp120 V3 Loop (TMV)	*N. benthamiana*	TMV plant viral vector	Cytosol	N/A
Sugiyama et al.	gp120 V3 Loop (TMV)	*N. tabacum*	TMV plant viral vector	Cytosol	N/A
Joelson et al.	gp120 V3 Loop (TBSV)	*N. benthamiana*	TBSV plant viral vector	Cytosol	N/A
Kim et al.	CTB-gp120 V3 Loop	Potato	Nuclear transformation	Cytosol	0.0021-0.0041% TSP
Marusic et al.	gp41 ELDKWA 662-667 (PVX)	*N. benthamiana*	PVX plant viral vector	Cytosol	15 mg/kg FW
Matoba et al.	CTB-MPR 649-684	*N. benthamiana*	Agroinfiltration	Cytosol	N/A
Matoba et al.	CTB-MPR 649-684	*N. benthamiana*	Nuclear transformation	Cytosol	0.01 - 0.2% TSP
Porta et al.	Peptide 731-752 (CPMV)	Cowpea	CPMV plant viral vector	Cytosol	N/A
Shchelkunov et al.	HBsAg-HIV polyepitope	Tomato	Nuclear transformation	Cytosol	7 µg/g FW

crop species such as tobacco and *Arabidopsis thaliana* have been utilized in the past for antigen production (Rybicki, 2010). In addition to whole plant expression systems, plant cell cultures such *Nicotiana tabacum* –derived cell suspension cultures (Fischer et al., 1999a; Fischer et al., 1999b), carrot-cell suspension cultures (Shaaltiel et al., 2007; Aviezer et al., 2009), and hairy root cell cultures (Skarjinskaia et al., 2008; Woods et al., 2008) have been used in the past, with one therapeutic protein from plant cell cultures currently in human trials (Rybicki, 2010). Although many factors such as antigen delivery and global vaccine quantity requirements need to be taken into consideration in choosing a plant expression host at the level of providing proof of principle, many researchers have opted to express vaccine antigens in high-biomass model systems such as *N. benthamiana* and *tabacum* for both proof of concept and production of tested vaccine candidates (Sharma and Sharma, 2009; Tiwari et al., 2009).

In addition to choosing the expression host, there are several methods to choose from in which the recombinant proteins can be expressed within the plant. The main choices for expression systems are based on either stable transgenic expression or transient expression. Of the various methods to stably transform the plant genome, the one employing the ubiquitous soil bacterium and plant pathogen *Agrobacterium tumefaciens* has been most widely applied to both dicotyledonous plants and to monocotyledonous plants (Rybicki, 2009). Alternative methods, like the biolistic (“gene gun”) method, can be used to transform other species which have proven to be refractory to Agrobacterium-mediated transformation (Ye et al., 1990). Stable transformation techniques produce transgenic and transplastomic plants that stably express the proteins of interest through subsequent generations (Komari et al., 2006; Komori et al.,

2007). Once the genes are introduced into nuclear genomes or plastid genomes of small leaf samples, cotyledons, hypocotyls, and other explants, these explants are then grown on selective growth media to select for transformed cells, which grow as a non-differentiated cell mass or callus. Regeneration of calli into whole plants is achieved through the use of several plant hormones. Since the gametes of such plants will contain the transformed gene of interest, the gene of interest will be stably inherited in future generations, allowing for protein expression in these generations without the need for successive transformations.

Stable transgenic expression is beneficial for the stable expression of proteins, as the transgenes are stably inherited and allow for seed production and high biomass production. Unfortunately, the time to develop stable transgenic plants can be limiting. The need to screen many different kinds of constructs would require a faster production system. In addition, if the multiple genes are needed for co-expression, multiple transgenic expression can be performed but with an even larger time constraint. Therefore transient expression of foreign proteins can be applied.

Viral vectors based on the genomes of various plant virus families have been proposed and examined including those based on tobacco mosaic virus (TMV) (Sugiyama et al., 1995) and Gemini virus (GV) (Mor et al., 2003). For a recent review about plant based viral vectors see (Canizares et al., 2005). In particular, vectors based on TMV have proven to be successful. In this work, I have used a TMV-based transient expression system. Although expression with viral-vectors by themselves has been successful, the delivery of these viral vectors with *Agrobacterium tumefaciens* has increased relative levels of expression and ease by which constructs can be transported into plant cells

(Rybicki, 2010). In this method, termed Agrobacterium-mediated transient expression, viral vectors are incorporated into bacterial cells, which are then infiltrated into plant cells by needless-syringe or vacuum pressure, resulting in the bacterial suspension infiltrating intercellular spaces of infiltrated cells. The combination of agroinfiltration and viral vectors was revolutionized with the 'Magnifection' system of ICON Genetics in 2005 (Gleba et al., 2005). Magnification incorporates a "deconstructed" TMV-based vector, and allows researchers to choose between several subcellular targeting modules in order to target their protein of interest to different parts of the plant cell without having to redesign their own construct. In contrast to stable transgenic expression systems, transient expression systems are beneficial for the rapid production of antigens in plants, but they are limited in that the expression is contingent on continual infiltration of vectors/agrobacterium for generation of recombinant proteins.

In addition to choosing an expression host and expression system, optimizing the gene for expression in plants has also been shown to play a role in how well foreign proteins express in plants (Rybicki, 2010). Codon optimization, or replacing infrequently used codons in the gene of interest with codons that are more frequently used in the host plant, can increase the expression of the protein of interest (Geyer et al., 2007; Geyer et al., 2010). Codon usage bias is species dependent, and these species-specific differences in codon usage have been shown to regulate the levels of gene expression, with the primary factors affecting expression often cited to be mRNA processing and accumulation (Geyer et al., 2007). Moreover, the tRNA levels that serve these codons is not uniform and can be rate limiting affecting overall accumulation levels of certain

proteins. Therefore, by changing the codons of transgenes to conform wiith that of highly expressed proteins in a specific host, the expression of that transgene can be increased. In addition, expression of foreign proteins in plants can be increased by removing mRNA destabilizing sequences and potential methylation sites in the gene of interest through silent mutations (Perlak et al., 1991; Mason et al., 1998). Although various optimization processes can be successful, it should be noted that the process is empirical and can be different for each protein of interest, host plant, and expression system (Rybicki, 2010).

The expression of Env and Gag in plants was previously attempted in several systems (see Tables 2 and 3), but thus far the plant expression of these proteins has proved to be difficult. In this study, plant codon optimization was utilized for both proteins. Transient TMV-based expression (MagnICON) for both proteins was utilized, and transgenic expression was also utilized for Gag. I hypothesized that increased accumulation levels of both recombinant proteins could be achieved by applying three strategic innovations. First I applied a newly developed algorithm to redesign full length Gag and deconstructed variant of gp41. Second I employed one of the most robust expression systems available, MagnICON. Third, I combined stable and transient transformation approaches to achieve co-expression of the two transgenens. Here I report on experiments aimed at testing my hypotheses and demonstrate success of my approach.

MATERIALS AND METHODS

Plant Optimization and de novo Construction of the gag and dgp41 Genes.

The gag gene (from subtype C R5 HIV-1 isolate, 1084i, GenBank accession no. AY805330) containing the coding sequences for the entire p55 Gag protein (Figure 6) was optimized for expression in *N. benthamiana* using methods described in (Geyer et al., 2010). Codon usage bias in genes encoding highly expressed proteins of dicotyledonous plants was assessed according to Sharp and Li (1987). The reference set included cDNAs encoding the small subunit 1B of RuBisCO, chlorophyll A/B binding protein 2, ribosomal protein L1 and L2, 40S ribosomal protein S2, S3 and S4 (accession nos. NM_123204, NM_102733, NM_202757, NM_201956 NM_115247, NM_115247 and NM_125228, respectively) of the model plant *Arabidopsis thaliana*. The relative synonymous codon usage (RSCU = observed frequency/unbiased frequency), the relative adaptiveness of a codon (*w*, RSCU normalized to the most abundant synonymous codon for an amino acid) and the codon adaptation index (CAI, geometric mean of w values over the entire length of the gene) values were calculated according to Sharp and Li (1987). Unfavorable (or infrequently used) codons were defined here as codons with $w < 0.5$. This codon bias analysis corresponds very well with a recently published analysis (Mukhopadhyay et al., 2008). Putative RNA-destabilizing sequences were analyzed through a genome-wide *in silico* study by Narsai et al. (2007), but only few such sequences were positively identified *in planta*, including the AU-rich elements, AUUUA, AUAGAU and UUUUUU. Briefly, *in silico* evaluation of the native gag gene was performed by codon bias analysis in order to determine the presumed expression levels of the gene in plants. Cryptic sequences such as putative RNA-destabilizing sequences, spurious polyadenalation signals, and untoward splicing signals were

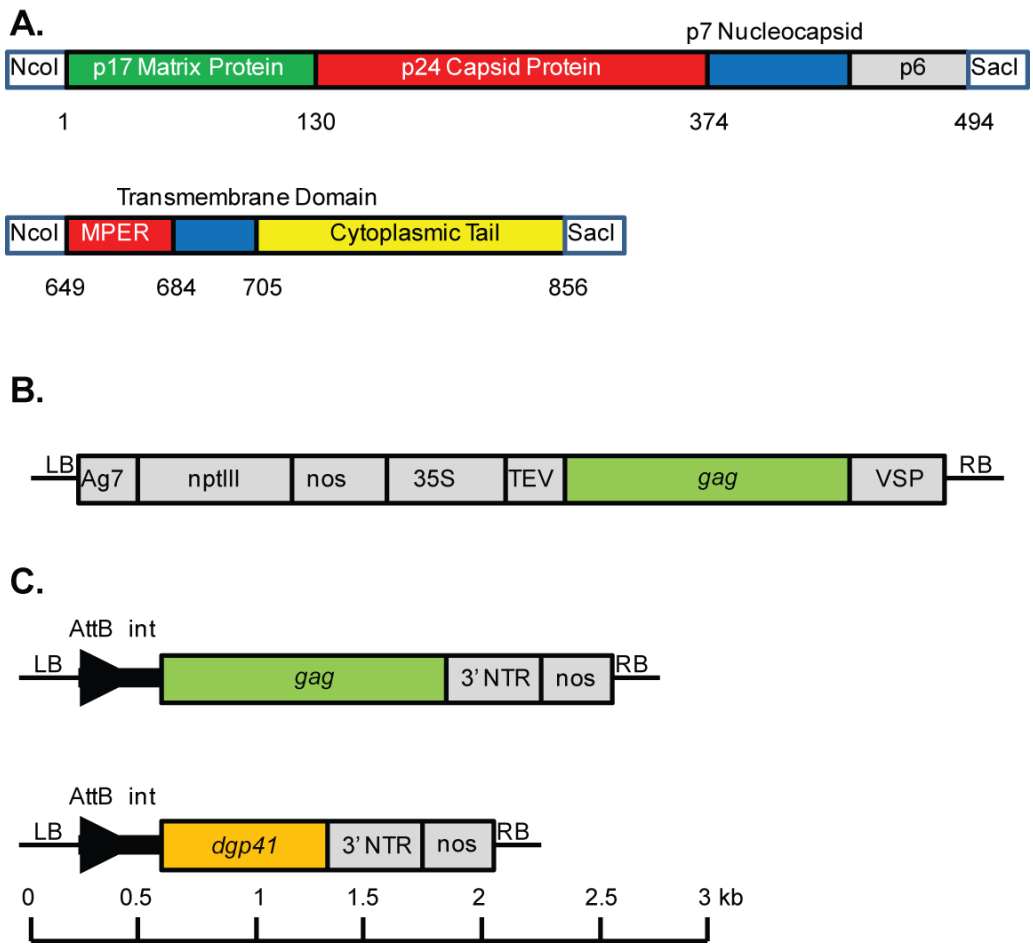


Figure 6. DNA constructs of gag and dgp41. A. Gag (top) and dgp41 (bottom) constructs, showing domain regions of the corresponding protein, as well as codon number of native HIV-1 gene. B. T-DNA construct of *gag* in pGPTV-Kan. LB - left T-DNA border, Ag7 - Agrobacterium gene 7 poly-A signal, nptIII – kanamycin resistance gene, nos - nos terminator, 35S – cauliflower mosaic virus 35S promoter, TEV - tobacco etch virus 5' untranslated region, VSP - polyadenylation signal of soybean vegetative storage protein gene, RB - right T-DNA border. C. T-DNA constructs of *gag* (top) and *dgp41* (bottom) in the 5' module of the MagnICON system. AttB - recombination site, int - intron, NTR - non-translated region. Scale for A. and B. at the bottom of the figure.

identified according to (Loke et al., 2005). The optimized gag gene was constructed *de novo* using the assembly PCR method (Figure 7A) using complimentary overlapping oligonucleotides (Stemmer et al., 1995). A total of 29 forward and 29 reverse overlapping primers (Table 4, 26-62 bp in length, keeping melting temperatures at 60+/-2°C, ordered from Integrated DNA Technologies) were assembled via assembly PCR using the Expand High Fidelity PCR kit (Roche). The primers at the 5' and 3' end of the gene (GagF1 and GagR1, Table 5) were then used to amplify the entire gene. The deconstructed gp41 gene comprises a chimera consisting of the gp41 MPER derived from the B-clade MN isolate (GenBank accession number AF075722) and the transmembrane domain and cytoplasmic tail region of the C clade 1084i isolate (GenBank accession number AY805330). The gene was plant optimized as above and ordered from Integrated DNA Technologies.

The constructed genes were cloned into the PCR-cloning vector, pTOPO-TA (Invitrogen) to create pTM 445 and pTM 601 for gag and dgp41, respectively. The restriction sites NcoI and SacI were added to the 5' and 3' ends of the genes (Figure 6), respectively, for aid in further cloning and the DNA sequences were verified.

Gag Stable Expression

The *gag* construct from pTM 445 was cloned as per standard procedures according to Current Protocols in Molecular Biology (Wiley) into the binary vector, pGPTV-Kan (pTM036) (Mor et al., 2001), for stable expression, as previously described (Richter et al., 2000) to create pTM 535.

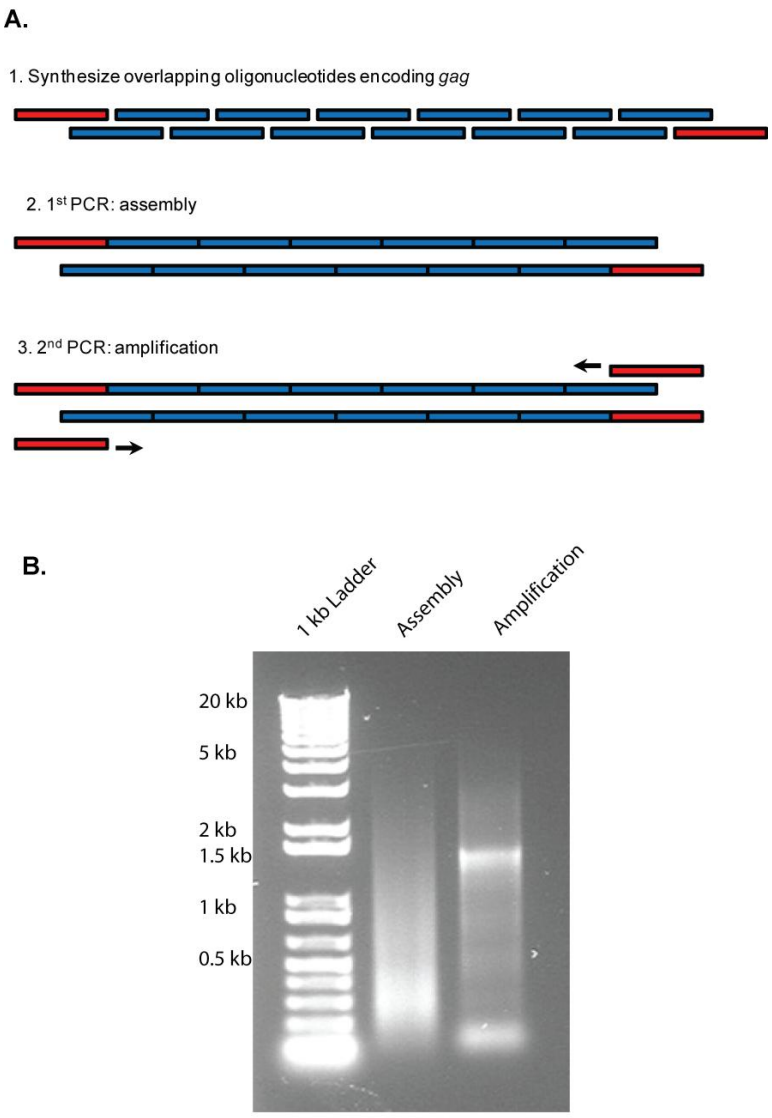


Figure 7. De-novo construction of Gag gene. A. 1. A total of 58 forward and reverse overlapping primers (26-62 bp in length) were designed so each half of any forward oligonucleotides would be complementary to two adjacent reverse oligonucleotides and vice versa. 2. During the first round of PCR, low levels of correctly assembled oligonucleotides form a template for a second round of PCR. 3. Durin that second round, the entire gene was amplified. B. DNA gel showing both rounds of PCR. The amplified band (1.6 kb) was purified and cloned into a TOPO cloning vector.

Table 4. Forward (top) and reverse (bottom) overlapping oligonucleotides used in de novo synthesis of *gag*.

Name	5' Sequence 3'
R1	GAGCTCTTATTGAGAGGAAGGGTCACTTCCAAAGAGAGACTTGAGGGAAGTCAAAG
R2	GCTCCCTATCATTTGGCTCTTGCTTTGGAGCAGGGGTTGTCTCTTCAAAC
R3	CTAAAGCTCTCAGCTGGTGGGGCTGTTGGCTCTGGCCTGTTTTGGAG
R4	GAAATTTCCTGGCCTTCCCTTGTGGGAAGGCCAAATCTTTCCCAAGAA
R5	GTTAGCTTGCCTCTCAGTACAATCCTTGATTTGGTGTCCCTCCTTTCCACACTT
R6	CCAACACCCCTTCTTCCTAGGGGCCCTACAATTCCTAGCAATGTGTCCCT
R7	CCTTGCCACAATTGAAACACTTAATGATCCTTCTAGGGCCCTTGAAGTTGCTC
R8	CTTTGCATCATAATGTTTGTATTGCCAGCTTGGCTCATAGCCTCAGCCAAAA
R9	CCCTAGCCTTGTGGCTAGGTCCTCCCACTCCTTGACAAGCTGTCATCAT
R10	CTCCTCCAATGTAGCTCCTGTGCCCAAAGCCCTCAAGATGGTCTTACAATCTG
R11	GGTTGGCATTTTGAACCAACAAGGTGTCTGTCATCCAATTCTTCACCTCTTGTGTA
R12	GATTGCTCAGCCCTCAAAGTCTTGAAGAACCTATCCACATAGTCCCTAAAAGGCTC
R13	CTTTGGTCCTTGCTTAATGTCCAAGATGCTAACAGGGCTATACATCCTCACAATCTTGTT
R14	CAATCCAAGAATAATCCATCTCTTGTAGATATCTCCCACTGGGATAGGTGGGTTGTTTGT
R15	CATCCAAGCAATTTGCTCTTGGAGGGTGCTAGTAGTTCCAGCAATGTCACTTCC
R16	CCTTGGCTCCCTAATTTGGCCTGGAGCAATAGGTCCAGCATGCACTG
R17	GGTGCAACCTATCCCACTCAGCAGCCTCCTCATTGATGGTGTCCTTCAA
R18	CATTTGCATGGCAGCTTGGTGTCCTCCCACTGTGTTCAACATGGTGTT
R19	CAAGTCTTGTGGGGTGGCTCCCT CACTCAAAGCTGTGAACATAGGGATCAC
R20	CTCTGGGCTAAAAGCCTTCTCCTCAATCACCTTCACCCAAGCATTGAGAGTC
R21	CTTGGAGAGATGGCTTGGTGCACCATTTGTCCTTGGAGGTTTTGAACAATAGG
R22	GTAGTTTTGGCTAACCTTTCCCTTGTCAGCAGCCTCAGCTTGTTGTGTCTTTT
R23	GTTGGCTCTTGTTTTGCTCCTCCTCCATCTTGTCCAAGGCCTCCTTG
R24	GTGTCCCTCACCTCAATCTTCTCATGCACACAACAAAGAGTAGCCACTGTGTTGTA
R25	CAAAGACCTAAGCTCCTCTGTTCCTGTTTGGAGAGCTGGTTGAAGTTGCTTCAT
R26	GATTTGCTTACAGCCCTCACTTGTCTCTAAGAGGCCAGGGTTGAGAGCAAAC
R27	CTCTCAAGCTCCCTACTAGCCCACACCAAGTGCTTGAGCATGTAGTGCTTCTTG
R28	CCTCCTGGCCTCAACCTAATCTTCTCCCACTTGTCCAACTTCTCTCCCCT
R29	CAAGATAGAGGCTCTAGCTCCCATGG

Name	5' Sequence 3'
F1	CCATGGGAGCTAGAGCCTCTATCTTGAGGGGAGAGAAGTTGGACAAGTGGG
F2	AGAAGATTAGGTTGAGGCCAGGAGGCAAGAAGCACTACATGCTCAAGCACTTG
F3	GTGTGGGCTAGTAGGGAGCTTGAGAGGTTTGCTCTCAACCCTGGCCTC
F4	TTAGAGACAAGTGAGGGCTGTAAGCAAATCATGAAGCAACTTCAACCAGCTCTCC
F5	AAACAGGAACAGAGGAGCTTAGGTCTTTGTACAACACAGTGGCTACTCTTTGTTGTGTG
F6	CATGAGAAGATTGAGGTGAGGGACACCAAGGAGGCCTTGGACAAGATGG
F7	AGGAGGAGCAAAACAAGAGCCAACAAAAGACACAACAAGCTGAGGCTGC
F8	TGACAAGGGAAAGGTTAGCCAAAACTACCCTATTGTTCAAAACCTCCAAGGACAAATG
F9	GTGCACCAAGCCATCTCTCCAAGGACTCTCAATGCTTGGGTGAAGGTG
F10	ATTGAGGAGAAGGCTTTTAGCCCAGAGGTGATCCCTATGTTCACAGCTTTGAGTG
F11	AGGGAGCCACCCCACAAGACTTGAACACCATGTTGAACACAGTGGGAG
F12	GACACCAAGCTGCCATGCAAATGTTGAAGGACACCATCAATGAGGAGG
F12	CTGCTGAGTGGGATAGGTTGCACCCAGTGCATGCTGGACCTATTGCTC
F14	CAGGCCAAATTAGGGAGCCAAGGGGAAGTGACATTGCTGGAACTACTAGCAC
F15	CCTCCAAGAGCAAATTGCTTGGATGACAAACAACCCACCTATCCCAGTGG
F16	GAGATATCTACAAGAGATGGATTATTCTTGGATTGAACAAGATTGTGAGGATGTATAGCCCTGTTAG
F17	CATCTTGGACATTAAGCAAGGACCAAAGGAGCCTTTTAGGGACTATGTGGATAGGTTC
F18	TTCAAGACTTTGAGGGCTGAGCAATCTACACAAGAGGTGAAGAATTGGATGACAGAC
F19	ACCTTGTTGGTTCAAAATGCCAACCCAGATTGTAAGACCATCTTGAGGGCTTTG
F20	GGCACAGGAGCTACATTGGAGGAGATGATGACAGCTTGTCAAGGAGTGGG
F21	AGGACCTAGCCACAAGGCTAGGGTTTTGGCTGAGGCTATGAGCCAAG
F22	CTGGCAATACAAACATTATGATGCAAAGGAGCAACTTCAAGGGCCCTAGAAG
F23	GATCATTAAGTGTTTCAATTGTGGCAAGGAGGGACACATTGCTAGGAATTGTAGGG
F24	CCCCTAGGAAGAAGGGGTGTTGGAAGTGTGGAAAGGAGGGACACCAAAT
F25	CAAGGATTGTACTGAGAGGCAAGCTAACTTCTTGGGAAAGATTTGGCCTTCC
F26	CACAAGGGAAGGCCAGGAAATTTCCTCCAAAACAGGCCAGAGCCAAC
F27	AGCCCCACCAGCTGAGAGCTTTAGGTTTGAAGAGACAACCCCTGCTCC
F28	AAAGCAAGAGCCAAATGATAGGGAGCCTTTGACTTCCCTCAAGTCTCTCTTTGG
F29	AAGTGACCCTTCCTCTCAATAAGAGCTC

Table 5. Oligonucleotides used as primers in this study.

#	Name	5' Sequence 3'
1	GagF1	CCATGGGAGCTAGAGCCTCTATCTTGAGGGGAGAGAAGTTGGACAAGTGGG
2	GagR1	GAGCTCTTATTGAGAGGAAGGGTCACTTCCAAAGAGAGACTTGAGGGAAGTCAAAG
3	oTM515	CCATGGGATCTCAAACTCAACAAG
4	oTM516	GAGCTCTTATTGCAAAGCAGCCT
5	oTM374	ATGGCGCGCCCCAAGGACTCTCAATGCTTG
6	oTM375	ATGGCCGGCCCCACTCCTTGACAAGCTG

Stable transgenic *N. benthamiana* expressing Gag were established by transforming *A. tumefaciens* LBA4404 with pTM 535 (pGPTV-Kan vector harboring the plant-optimized gag gene, for construct, see Figure 7). Sterile leaf explants (>300) were transformed with these cultures using the *Agrobacterium*-mediated method, as previously described (Figure 8) (Geyer et al., 2007). Agrobacterium cultures, grown to logarithmic phase, were harvested by centrifugation and resuspended in Agrobacterium-induction media (AIM, 2.15 g/L Murashige and Skoog (MS) salts, 0.5 mg/L pyridozine, 0.5 mg/L Nicotinic acid, 2 mg/L thiamine HCl, 100 mg/L Myoinositol, 0.9 g/L glucose, 1.95 g/L N-morpholino enthanesulfonic acid (MES), pH 5.2) to an optical density at 600 nm of 0.4. Sterile leaf explants excised from axenic 4 wk old tissue-culture grown *N. benthamiana* were co-cultivated with these cultures using the *Agrobacterium*-mediated method, as previously described.

Explants were transferred to non-selective plant-growth solid media (4.3 g/L MS salts, 30 g/L sucrose, 7.5 g/L agar) and allowed to recover for two days before being transferred to selective plant-growth solid media (same as above, but containing 300 mg/L kanamycin). Transformed cells were allowed to form calli, and surviving calli were transferred to selective plant-growth solid media containing 0.1 mg/L 1-naphthaleneacetic acid (NAA) and 1 mg/L 6-benzylaminopurine (BA) that allowed shoot formation. Shoots were excised from calli and transferred to selective plant-growth solid media containing 0.1 mg/L NAA to allow for root formation. Gag expression in regenerated plants was confirmed using immunoblots, and regenerated Gag-expressing plants were transferred to soil for seed generation.

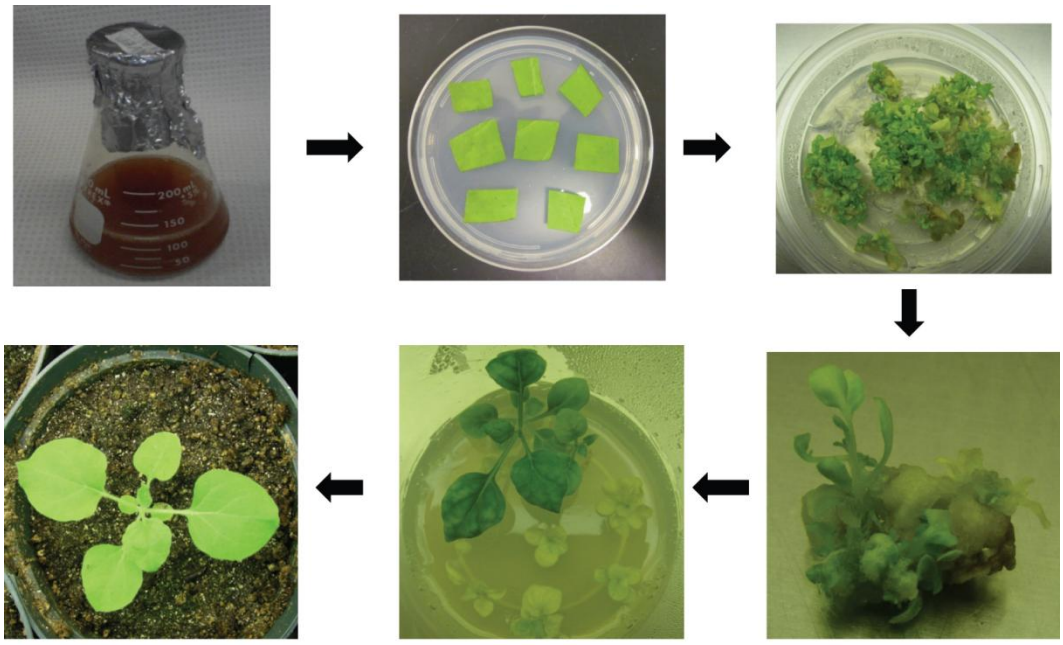


Figure 8. Development of stable transgenic Gag-expressing plants. Binary vectors (pGPTV-Kan) containing the gag gene were introduced into *A. tumefaciens*, which was then incubated with sterile leaf explants. Transformed cells from the explants were allowed to develop into an undifferentiated cell mass (callus) on selective media containing kanamycin. Callus cells were transferred to selective media containing hormones specific for shoot formation. Shoots were transferred to rooting media to allow root formation. Regenerated whole plants were tested for Gag protein and transferred to soil.

Transient Gag and Dgp41 Expression

A deconstructed TMV Vector system (MagnICON, ICON Genetics, Bayer CropScience) was used for transient expression of gag and dgp41 in *N. benthamiana* (Marillonnet et al., 2005). To this end, the NcoI-SacI restriction fragrments from pTM 445 and pTM 601 were cloned into the 3' module of the ICON system (pTM 202) to yield, respectively, pTM488 and pTM602, which were introduced into competenet *A. tumefaciens* LBA4404 (Figure 9). For transient expression (illustrated in Figure 9), *A. tumefaciens* LBA4404 cell lines harboring the modules of the viral vector and either pTM 488 or pTM 602 were grown to logarithmic phase, resuspended in infiltration buffer (10 mM MES, 10 mM $MgSO_4$, pH 5.5) at a final optical density at 600 nm of 0.01, and inoculated into 6-week old *N. benthamiana* plants using a vacuum infiltration method. Plants were inverted into a vacuum container containing 2 L of *A. tumefaciens* and a vacuum of 600 mm Hg was applied to the container for 2 min. The vacuum was quickly released, allowing the *A. tumefaciens* to infiltrate the leaf cells. Infiltrated plants were kept in a controlled environmental growth chamber at 25°C until harvest, and expression of Gag and dgp41 proteins were confirmed with SDS-PAGE and immunoblotting of the leaf extract.

Immunoblotting

For immunoblotting, leaf extract was homogenized with plant extraction buffer in a ratio of 1 mg leaf tissue: 3 µl extraction buffer (25 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8) and a ceramic bead in a Fast Prep-24 (MB Biomedicals) machine for 40 s. Extract was clarified at 12,000 g for 10 min and the supernatant was collected. Extract (200 µl) was added to 5x SDS loading buffer

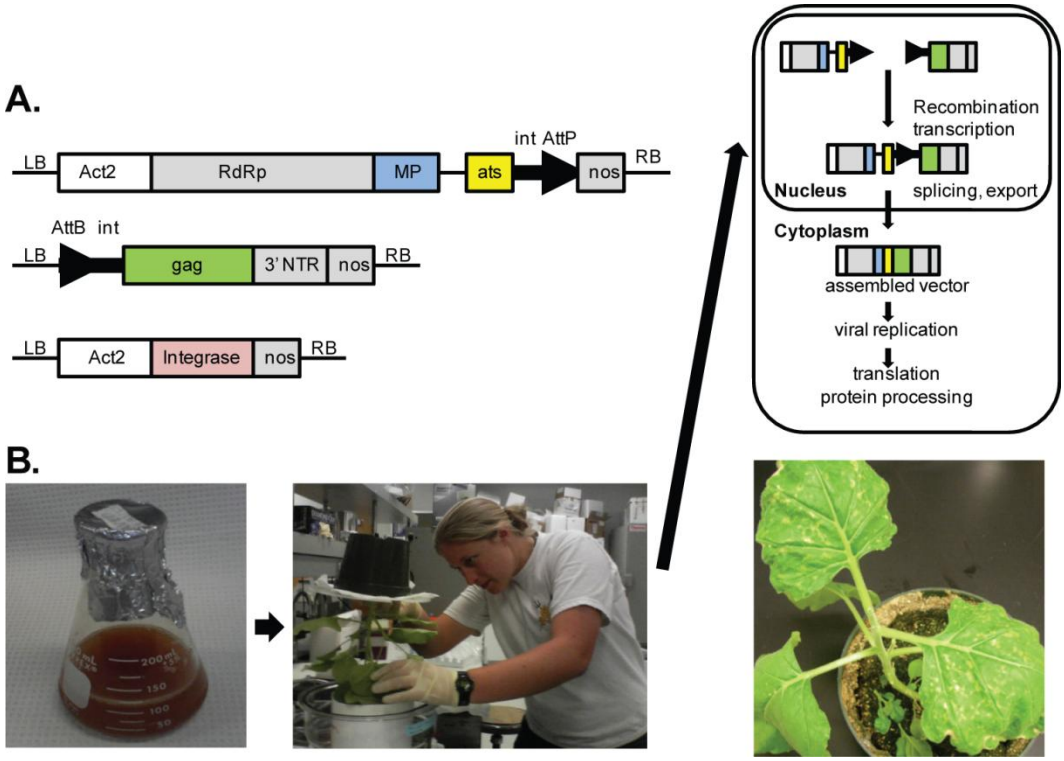


Figure 9. Transient expression of gag using the MagnICON system. A. Representation of T-DNA modules used in ICON expression. Top: 5' module. Middle: 3' modlue. Bottom: Integrase module. LB - left T-DNA border, Act2 - Arabidopsis Actin 2 promoter, RdRp - RNA-dependent RNA polymerase, MP - movement protein, ats - barley alpha-amylase apoplastic targeting sequence, int - intron, AttP and AttB - srecombination sites, nos - nos terminator, NTR - non-translated region, RB - right T-DNA border. B. Flowchart of transient expression. Vectors are introduced into Agrobacterium and cultured to logarithmic phase. Plants are then suspended in Agrobacterium culture and subjected to vaccum infiltration. Once Agrobacterium is inoculated into cells, the vectors of the ICON system work together to recombine in the nucleus. The recombineed modules are spliced and exported to the cytoplasm where the assembled vector can replicate and be translated into the dgp41 protein.

(40 µl, 30% glycerol, 35 mM SDS, 60 mM DTT, 18 mM bromophenol blue, 350 mM Tris-HCl, pH 8.0) and incubated for five min at 100°C to denature the proteins. 12% polyacrylamide gels were prepared and each sample (20 µl) was loaded into its respective well. Gels were run at 40 mV until proteins were separated. Proteins were then transferred to nitrocellulose membrane and blocked with PBST containing 5% dry milk (PBST-M) for 1 h at 25°C. Membranes were then incubated for 1 h in primary antibody solution containing blocking buffer and either a 1:5,000 dilution of a polyclonal anti-p24 rabbit serum (for detection of Gag protein) or a 1:10,000 dilution of a monoclonal 2F5 antibody, a kind gift from Morgane Bomsel (for detection of dgp41). Membranes were triple-washed for 30 min and then incubated with a 1:10,000 dilution of a secondary antibody conjugated to HRP (anti-rabbit IgG for p24 and anti-human IgG for 2F5) for 1 h at 25°C. Membranes were then triple washed for 30 min, developed with ECL reagent (Santa Cruz), and exposed to film.

The quantification of proteins was performed with quantitative immunoblotting (Figure 10). Pure samples of either p24-CTA2 or CTB-MPR were used as standards, and 10 µl samples containing 50, 40, 30, 20, or 10 µg of p24 (80% mass of p24-CTA2) or the MPER peptide (33% mass of CTB-MPR) were loaded onto a SDS-PAGE gel. Unknown samples were also loaded onto the gel in several dilutions (dependent upon sample, ie. crude extract or partially enriched fraction). The gel was run and processed as above. The resulting developed blot was scanned as a color image using an HP4050 (Helwett-Packard) scanner and the raw image was imported into ImageJ software. The background of the image was removed, and the lanes were separated using the

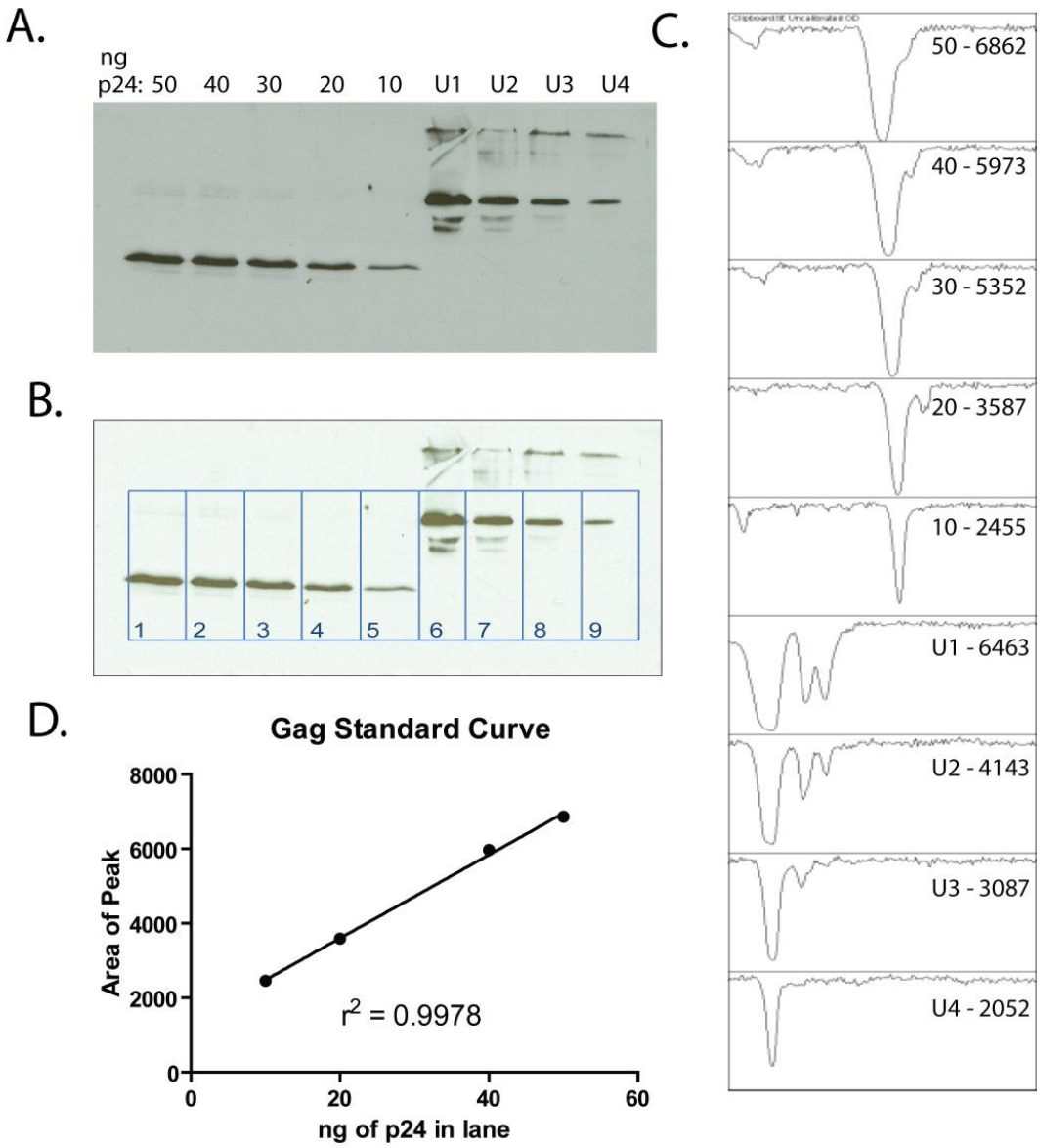


Figure 10. Quantitative immunoblot quantification. A. Raw image of immunoblot showing lanes corresponding to both known (50-10 ng/well) and unknown (U) quantities of p24. B. Immunoblot in ImageJ with background removed and lanes separated. C. Pixel area as calculated by ImageJ. D. Standard curve generated from the pixel area results.

gel analyzer tool. The pixel area of each lane was calculated using the software, and a standard curve was generated based on these pixel areas. The unknown protein concentration of p24 or MPER in the samples was generated from these standard curves.

Generation of Anti-p24 Antibodies

In order to develop anti-p24 antibodies, a fusion construct consisting of the plant-optimized p24 gene and the cholera toxin A2 subunit (CTA2) was first developed. The gene for p24 was PCR amplified from the plant-optimized *gag* gene with primers oTM374 and oTM375 (Table 5), which added the FacsI and FseI restriction sites to the 5' and 3' ends of the gene, respectively (Table 5). The amplified gene product was cloned into pTM319, a pET-22b(+) *E. coli* expression vector containing the gene for CTA2, to create pTM 460. The p24 gene was cloned in-frame upstream of the CTA2 gene. The p24-CTA2 fusion construct was expressed in *E. coli* BL21 (DE3) cells and purified as previously described (Matoba et al., 2008). Briefly, *E. coli* cultures containing pTM 460 were grown from single colonies overnight at 37°C. Logarithmic phase cultures were induced with 100 μM isopropyl β- D -1-thiogalactopyranoside (IPTG) and allowed to grow for 2 h before centrifugation of the cells. Cell pellets were resuspended in PBS (20 ml) and lysed by microfluidization. Lysed cells were pelleted by centrifugation, and the insoluble pellet was solublized in 8M urea buffer (50 mM Tris, 500 mM NaCl, 8 M urea, pH 8.0), rocked at 4°C for 60 min, centrifuged to remove the remains of the insoluble cell fractions, and dialyzed in PBS with a 10 kD-cutoff membrane for 24 h to remove urea. The soluble supernatant was then added to a 3 ml bed volume Talon gravity flow column and purified by metal affinity chromatography. The final column eluate (6 ml) was

dialyzed in PBS with a 10 kD-cutoff membrane for 24 h to remove imidizole. Protein remained souble throughout the dialysis. Purity and quantification of the pure p24-CTA2 was determined by Coomassie stained gels, immunoblots, and by determining the absorbance at 280 nm using ($\varepsilon = 1.22\ mM^{-1}\ cm^{-1}$). Two New Zealand white rabbits were inoculated with 4 subcutaneous injections of 200 µg p24-CTA2 emulsified with Freund's complete adjuvant (100 µl at 4 sites per rabbit, 0.5 µg/µl p24-CTA2 in sterile PBS). Rabbits were boosted with 4 subcutaneous injections of 200 µg p24-CTA2 emulsified with Freund's incomplete adjuvant (100 µl at 4 sites per rabbit, 0.5 µg/µl p24-CTA2 in sterile PBS) two weeks after the initial inoculation. The level of antibody response was determined by p24 ELISA (explained in detail in Chapter 4), and the rabbits were exsanguinated upon peak response observed (two weeks following boost). More than 120 ml of blood was collected from each rabbit, and the serum (>90 ml/rabbit) was separated from the red blood cells by centrifugation at 4,000 g for 10 min. Serum was allocated into 1 ml fractions and stored at -80°C until use.

RESULTS

The de novo Construction of Plant-expression Optimized gag and dgp41 Genes.

To increase the level of transcription of the two HIV-1 genes under the two expression modalities explored here, stable transgenic expression was driven by the strong constitutive cauliflower mosaic virus 35S promoter and by the vigorous activity of TMV's RNA dependent RNA polymerase (RdRp) of the MagnICON transient expression system. In addition, to reduce the extent of DNA methylation of the *gag* transgene, associated in plants with transcriptional gene silencing, 49 of the 85 potential methylation sites present in the native gene were

abolished by silent mutations introduced into the plant-optimized version of the gene.

Beyond transcription rates, the accumulation of recombinant proteins in plants can be greatly affected by post-transcriptional, translational and post-translational events. To this end, I have removed from the plant-expression optimized *gag gene* all 30 spurious splicing signals (Hebsgaard et al., 1996), cryptic polyadenylation sites (Loke et al., 2005) and mRNA destabilizing sequences with which the native sequence was ridden (Figure 11). Similarly, 14 such deleterious sequences were removed from the sequence of the plant-optimized dgp41 sequence (Figure 12).

Translatability of foreign gene transcripts can be increased by replacing unfavorable codons with synonymous codons that are more widely used in plant genes of highly expressed proteins. My analyses demonstrated that approximately a third of the codons in the native *gag* and *dgp41* genes were unfavorable for expression in plants (33% and 32% of the codons, respectively, had $w<0.5$, for definition see the Materials and Methods section above). In the plant-expression optimized versions of the *gag* and *dgp41* genes, the majority of the unfavorable codons have been eliminated reducing their occurrence, respectively, to 7% and 2% (Figures 11 and 12). In accordance with these results, the calculated codon adaptiveness (CAI) increased from 0.5 to 0.8 (*gag*) and from 0.6 to 0.9 (dgp41) similar to the CAI value of the small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO, CAI value = 0.8), a nuclear encoded protein that accumulates to very high levels (Figure 13).

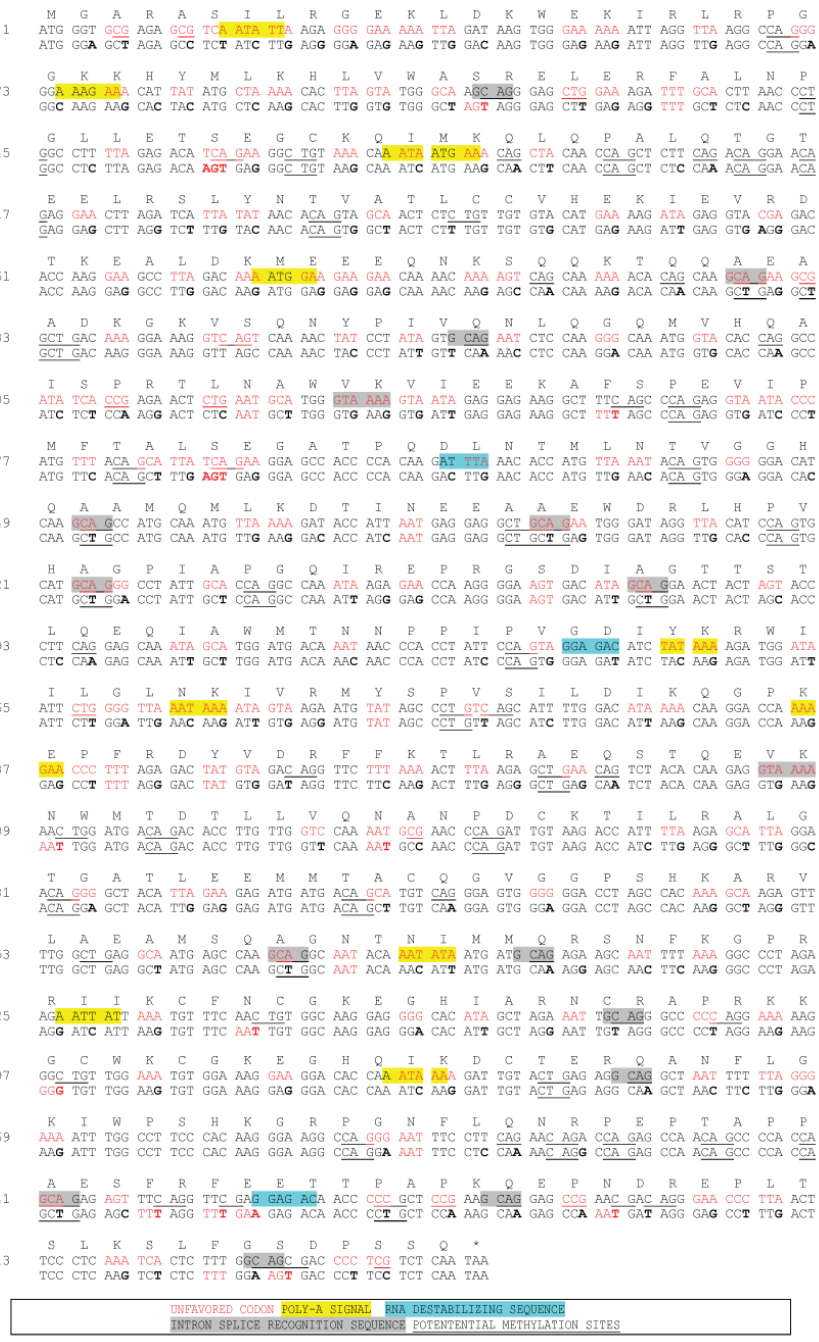


Figure 11. Sequence alignment of native and plant-optimized *gag* genes and their expected translation product. Both genes encode for an identical protein (top line), but the nucleotide sequence of the native *gag* was adapted to allow higher levels of expression in designing the plant-optimized *gag* gene (bottom line). Various molecular features that were targeted in optimizing the gene are labeled as shown on the figure.

Figure 12. Sequence alignment of native and plant-optimized *dgp41* genes and their expected translation product. Both genes encode for an identical protein (top line), but the nucleotide sequence of the native *dgp41* was adapted to allow higher levels of expression in designing the plant-optimized *dgp41* gene (bottom line). Various molecular features that were targeted in optimizing the gene are labeled as shown on the figure.

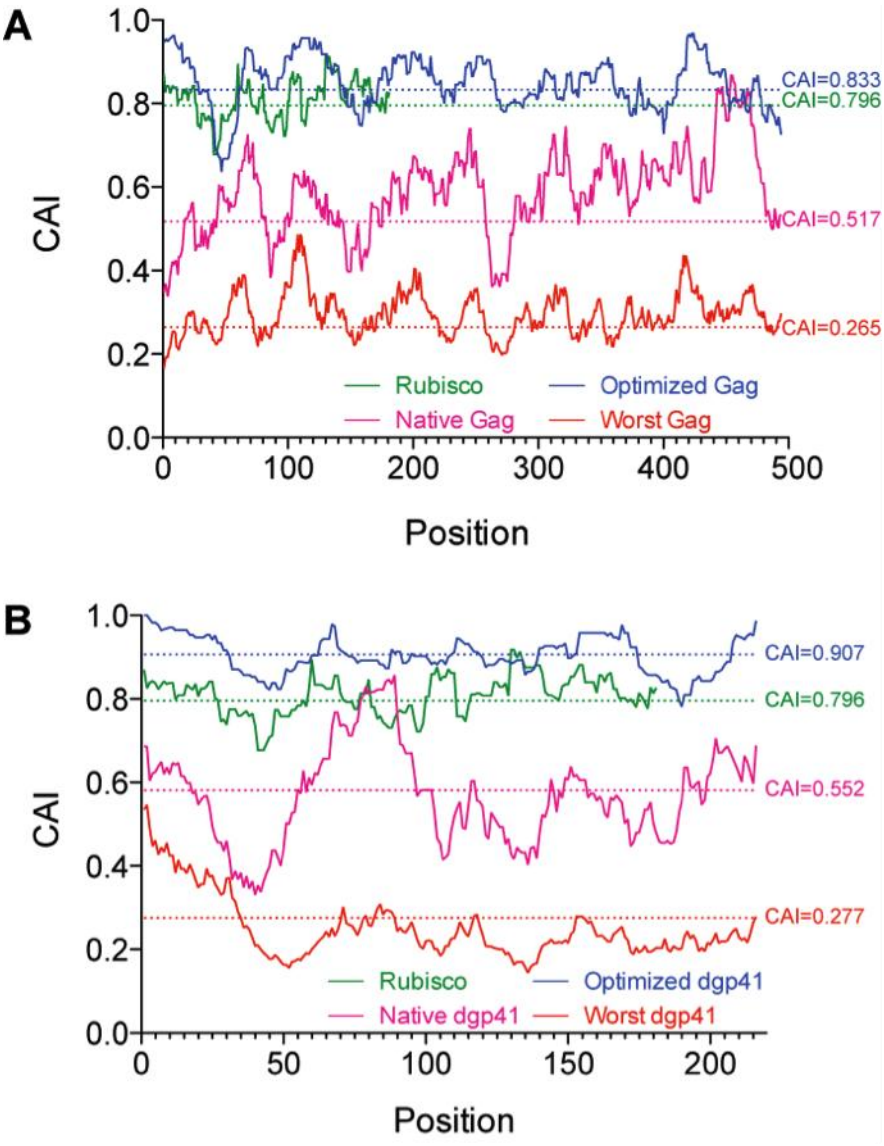


Figure 13. Codon usage of plant-optimized *gag* and dgp41 is comparable to that of a highly expressed plant gene. Plotted values of the relative adaptiveness, w, of each codon represent a moving average (geometric mean, window size 51) centering on each codon for the coding regions of rbcS1B (encoding the small subunit of the enzyme RuBisCO) and both native and plant-optimized gag (A) and dgp41 (B). The codon adaptation index (CAI, relative adaptiveness averaged over the length of a sequence) for each gene is shown as a broken line.

Gag Transient and Stable Expression

Transient virus-assisted expression of the plant-optimized Gag gene in *N. benthamiana* was confirmed by immunoblotting. The results (Figure 14) showed a prominent band at 55 kD, corresponding to the intact p55 Gag polyprotein, as well as two smaller truncated products containing p24 (corresponding to ~48 and 41 kD). Similar HIV-1 Gag protein degradation products have been detected in HIV-1 Gag produced in mammalian cells (Fouchier et al., 1997; Mullner et al., 2008). Expression of gag protein peaked at 10 days post-infiltration (dpi), after which point the leaves became yellow and necrotic. Two lines of stably transformed *N. benthamiana* which expressed the Gag protein as determined by SDS-PAGE followed by immunoblot analysis were generated. Immunoblot results (Figure 15) again showed a prominent band at 55 kD, and lower expression of the degradation products as compared to the transiently expressed Gag protein. It is interesting to note that out of more than 300 explants, only two *gag*-positive plants had been regenerated, however these two plant lines were phenotypically indistinguishable from wild type plants, at least under normal greenhouse growth conditions. Quantification of the stably-expressed Gag was determined by quantitative immunoblot to be approximately 22 mg/kg fresh weight.

Dgp41 Transient Expression

The deconstructed gp41 was first expressed in wild type *N. benthamiana* using an ICON apoplastic targeting module containing the barley alpha-amylase signal peptide. Expression was confirmed using immunoblotting (Figure 16), showing a prominent band at 24 kD, the expected size of the dgp41 protein. As

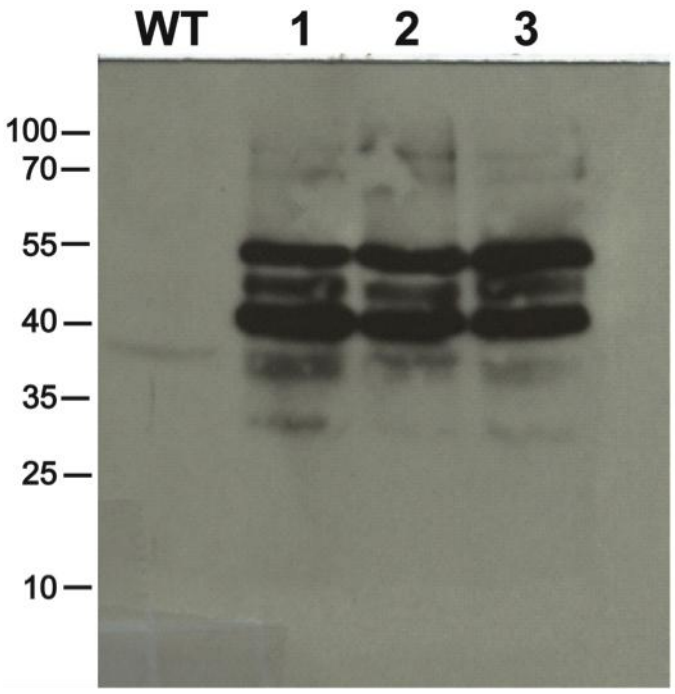


Figure 14. Transient expression of Gag. WT – Non-infiltrated control plant extract. 1, 2, and 3 are three samples of three independently infiltrated plants that were harvested 9 days post-infiltration. Gag protein was detected using rabbit α-p24 antiserum.

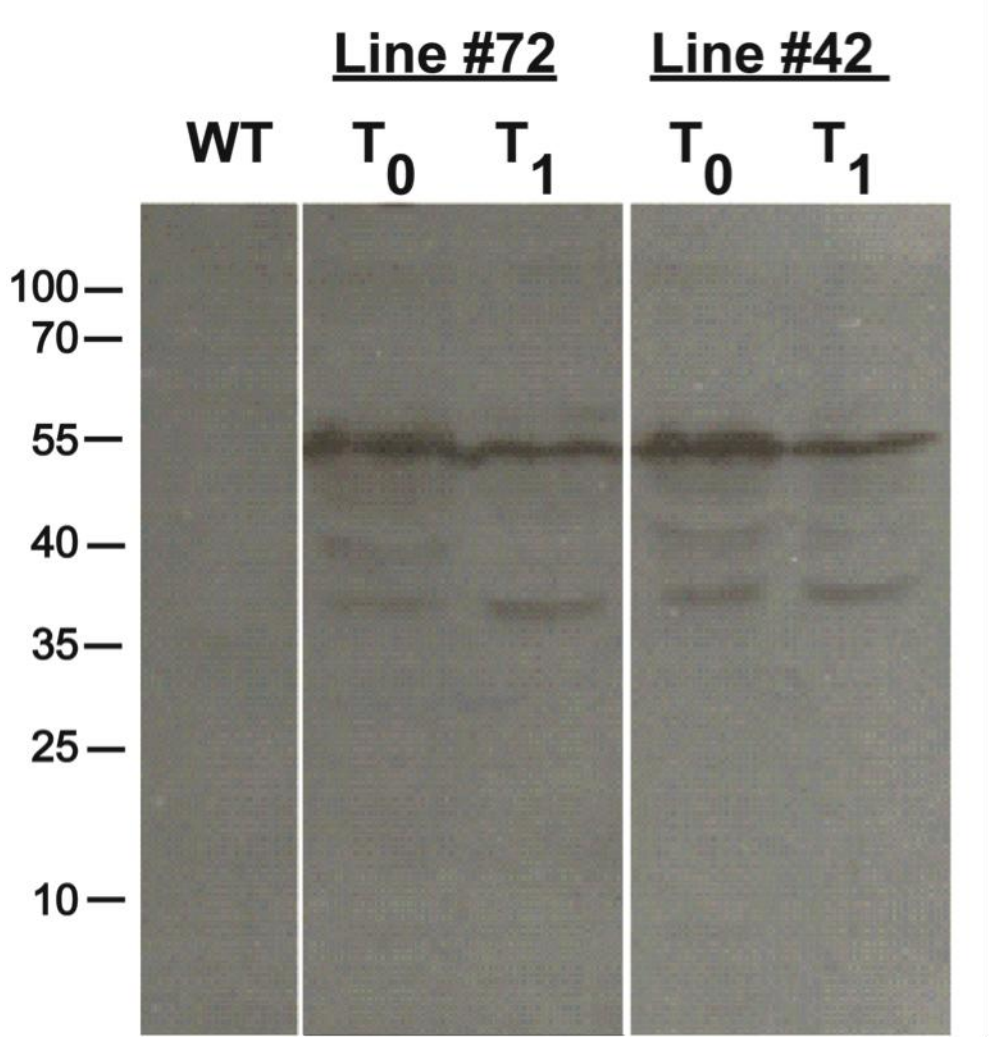


Figure 15. Expression of Gag in transgenic plants. WT – Wild type plant extract. Two lines (#72 and 42) were created. T_0 is the first generation, T_1 is the second generation for both of those lines. Immunoblot developed with α-p24 antibodies.

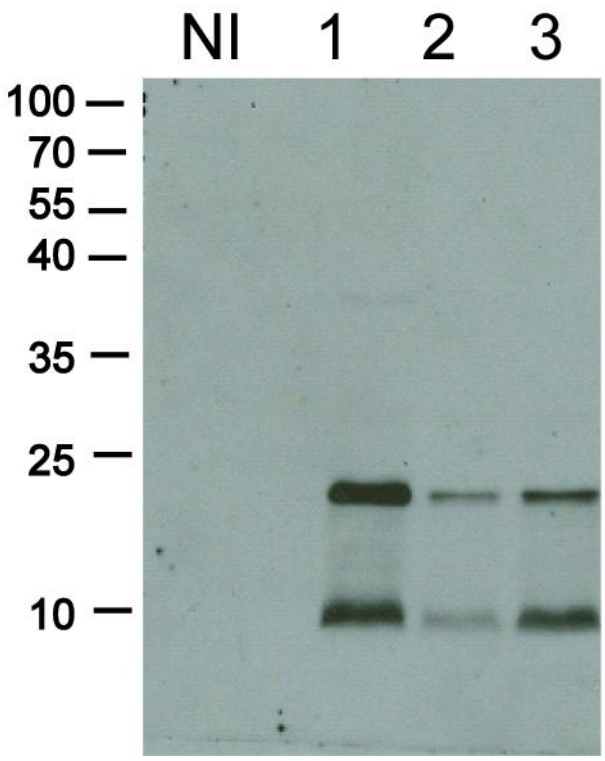


Figure 16. Transient expression of dgp41. NI – Non-infiltrated wild type. 1, 2, and 3 are three samples of three independently infiltrated plants that were harvested 5 days post-infiltration. Immunoblot developed with 2F5 antibodies.

expected, targeting the recombinant protein to the cytoplasm did not allow its accumulation to detectible levels (data not shown). Interestingly, using alternative 5'-modules equipped with different signal peptides, including those of apple pectinase, rice alpha-amylase, and *N. plumbagenifolia* calreticulin, resulted in expression which was below the detection level of my immunoblot assay.

Once expression was confirmed in wild type plants, stable lines expressing the Gag protein were infiltrated with the dgp41 constructs. Co-expression of both proteins in these plants was confirmed by immunoblotting (Figure 17). Interestingly, the co-expression of the two proteins seemed to stabilize the proteins and allowed their accumulation to higher levels as compared to their expression on their own. Quantification of gp41 when co-expressed with stably-expressed Gag was determined by quantitative immunoblot to be approximately 9 mg/kg fresh leaf weight.

DISCUSSION

Although the expression of HIV-1 Gag and envelope proteins, as well as their derivatives, have been expressed extensively in mammalian (Krausslich et al., 1993; Wagner et al., 1994b; Hammonds et al., 2007), insect/baculovirus (Gheysen et al., 1989; Griffiths et al., 1993; Brand et al., 1995b; Deml et al., 1997b; Deml et al., 2004; Jaffray et al., 2004; Hammonds et al., 2007; Wang et al., 2007; Speth et al., 2008; Tagliamonte et al., 2010), and yeast (Sakuragi et al., 2002; Morikawa et al., 2007; Powilleit et al., 2007) cell cultures, data concerning their expression of in plants are scant. Only a few published studies described the expression of derivatives of Gag (p24 and/or p17) in plants, but

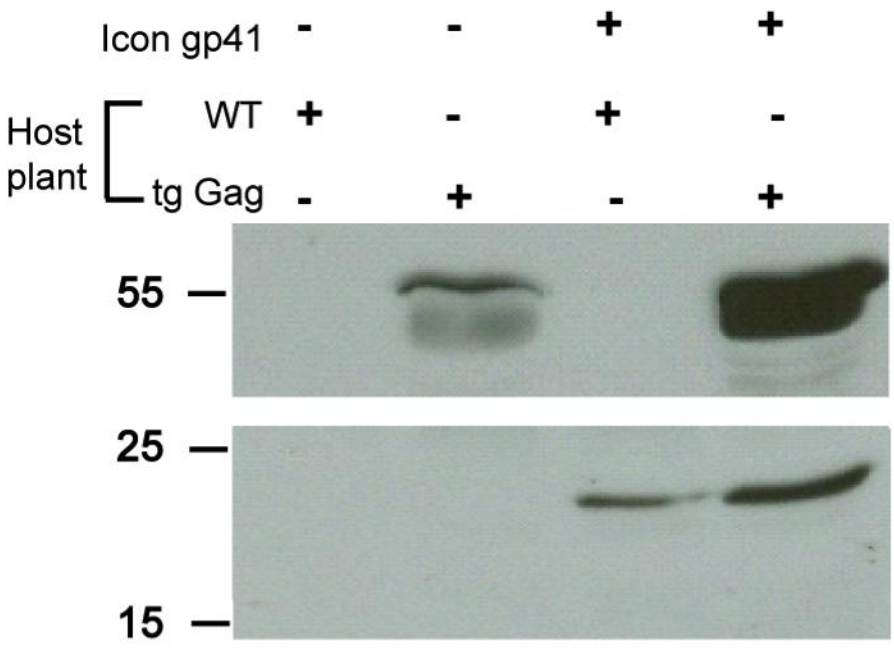


Figure 17. Coexpression of Gag and dgp41. The coexpression of Gag and dgp41 seemed to stabilize the expression of both proteins as compared to the expression of either protein by itself, suggesting that the two proteins might be interacting with each other, possibly in the context of a VLP.

only two have produced full length p55 Gag in plants (Meyers et al., 2008; Scotti et al., 2009) and both studies reported little or no success when the protein was directed to accumulate in its natural subcellular compartment, the cytoplasm. Better accumulation levels were reported when the protein was expressed in chloroplasts (i.e. transplastomic plants) or targeted to the organelle following transient or stable nuclear transformation (Meyers et al., 2008; Scotti et al., 2009). In contrast, my approach as was documented in this chapter, led to successful accumulation of the full-length Gag protein in the cytoplasm, when a plant-expression optimized version of the gene was either transiently expressed using a deconstructed TMV-based system (MagnICON) or integrated stably in the plant genome.

It is difficult to predict methods by which plant-expression of HIV-1 proteins can be enhanced, and according to some researchers, this process is largely empirical (Rybicki, 2010), and therefore it is suggested that a plethora of expression systems, expression hosts, subcellular targeting strategies, and codon optimization strategies should be employed for each protein of interest in order to determine its optimal method of plant expression. While this can be prohibitively expensive and time consuming for a single lab to investigate, by combining the knowledge obtained in all published studies related to plant-based expression of the protein of interest, the most favorable expression strategies can be deduced. In this case, two other published studies attempted the cytoplasmic expression of HIV-1 p55 Gag in addition to this study. Meyer et al. used a Clade C gag gene as the basis of their expression studies, while Scotti et al. utilized a Clade B gag gene as their expression target. Meyer et al. utilized a variety of codon-optimization strategies including native genes and both human-

and *Nicotiana*-optimized genes, while Scotti et al. opted to use only native genes (with no codon optimization) for their expression. In addition, great differences in expression systems were also utilized between groups. For transient expression, Meyer et al. utilized a TMV based system (TMV Geneware^{TM}, Large Scale Biology) as well as agroinfiltration with binary vectors while Scotti et al. utilized only agroinfiltration with binary vectors. In regard to amino acid sequence of the protein, final codon optimization strategy, and transient expression systems, this study was more similarly aligned with Meyer et al. in that a Clade C HIV-1 gag gene was used as a template, plant-codon optimization was utilized, and a TMV-based transient expression system was used. Interestingly, the transient cytoplasmic expression of p55 Gag in this study was similar to that of Meyer et al. who achieved 2-44 µg/kg p24 using transient expression of plant-optimized p55 Gag. In addition, their transient cytoplasmic expression of native (non-codon-optimized) p55 Gag yielded less than 0.01 µg/kg p24, results that aligned with Scotti et al's complete lack of transiently expressed cytoplasmic p55 Gag. These results suggest that plant-optimization of the *gag* gene could positively influence the expression of the protein in plant cytoplasm. Despite this fact, it is still difficult to compare plant-codon optimization strategies between this study and that of Meyer et al., as their optimization strategy was not published. In this project's strategy, employing the removal of mRNA destabilizing sequences, poly-A-like signals, potential methylation sites, intron splice recognition sequences, and unfavored codons could be responsible for the differences in transient expression of p55 Gag in the cytoplasm in comparison to Meyer et al. In addition, the use of the ICON system could also have played a role in this study's higher expression levels. Regarding nuclear transformation of p55 Gag,

Meyer et al. utilized pPAM-derived (Genebank Accession number AY027531) binary vectors, while Scotti et al. utilized pBIN plus (ImpactVector) binary vectors. It is difficult to compare the three different binary vector systems used in the three studies, but neither Meyer et al. or Scotti et al. developed stable transgenic lines expressing p55 Gag in the cytosol, suggesting that this study's binary vector system (pGPTV-kan) is more suitable for this expression, although the combined factors of plant-optimization and virus clade could also play a role.

The expression of envelope proteins and derivatives within plants has been varied (Table 3), but no more extensive than that of Gag expression. Due to the varied nature of the various constructs, it is difficult to compare them to each other, but to date, only two other groups have focused on the expression of MPER epitopes in plants. A deconstructed gp41 (dgp41), containing the cytoplasmic tail, transmembrane domain, and MPER domain of gp41, using a deconstructed TMV-based system (ICON) was successfully expressed in this study.

In addition to expressing both of these proteins separately, co-expression of the two proteins was successful. In all of more than 30 plants examined (more than 10 each of transgenic Gag plants without dgp41, dgp41 expressed in wild type plants, and transgenic Gag plants expressing dgp41), the co-expression of the two proteins seemed to stabilize the expression of both Gag and dgp41. This phenomenon promisingly suggests the interactions of the two proteins, possibly within the context of an enveloped virus-like particle (VLP). The characterization of how these proteins are interacting within the context of the plant cell will be discussed in Chapter 3.

Chapter 3

BIOCHEMICAL CHARACTERIZATION OF GAG/DGP41 EXPRESSION

ABSTRACT

Displaying the MPER peptide in its native conformation is vital to its potential role as a vaccine component. Enveloped virus-like particles (VLPs) are able to display HIV-1 Env proteins within the context of the envelope membrane, as has been proven in mammalian, insect, and yeast cells. Although plants have the eukaryotic cellular machinery necessary for the formation of HIV-1 Gag/dgp41 VLPs, this phenomenon has not been characterized to date. Through a series of biochemical, biophysical, and transmission electron microscopy characterization, the formation of HIV-1 Gag VLPs in *N. benthamiana* was confirmed, and it was also determined that the VLPs are incorporating dgp41.

INTRODUCTION

As discussed at some length in Chapter 1, the MPER domain of the HIV-1 envelope protein gp41 is considered to be a potentially important component in a subunit vaccine against the virus. But to be effective as an immunogen, it is likely that the MPER would need to mimic its conformation in the native HIV-1 virus, and consequently, it needs to be presented in the context of a membrane (Visciano et al., 2011). Enclosed membrane vesicles with viral membrane proteins construe one kind of virus-like particle (VLP) and have been demonstrated in the past to be of value as immunogens (e.g. hepatitis B surface antigen (Roldao et al., 2010)). However, HIV-1's gp41 cannot form VLPs on its

own and requires the vesicle-forming function of Gag. Therefore, it was hypothesized that enveloped VLPs consisting of Gag that incorporated dgp41 into the membrane of the VLPs may present the MPER in its natural state.

Several components of human cellular machinery are necessary for the assembly and budding of HIV-1 VLPs from the cell. In order for VLPs to form and bud from the cell, the Gag protein must be post-translationally modified by several enzymes. An N-terminal myristoyl group must be covalently attached (N-myristoylation) to the terminal glycine of the matrix portion of the Gag protein covalently by N-myristoyl-transferase (NMT) during translation to increase membrane affinity of Gag. Most eukaryotes have two NMT genes (Podell and Gribskov, 2004) which have been sufficient for VLP production in mammalian, insect, and yeast cell cultures.

In addition to the presence of an N-terminal myristoyl group, membrane targeting of Gag also requires the presence of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a phospholipid located at the plasma membrane in mammals (Campbell et al., 2001; Saad et al., 2007; Patil et al., 2010). The 2' fatty acid occupies hydrophobic cavity of the Gag matrix, and negatively charged phosphate groups on the inositol ring interact with basic residues of the matrix. This binding changes conformation of the matrix so that the N-terminal myristoyl group becomes exposed and therefore increases the affinity of Gag to the plasma membrane (Saad et al., 2007).

The N-myristoylation of the matrix protein and $PI(4,5)P_2$ are both important for targeting of Gag to the membrane, but in order for a VLP to bud out of the cell, additional cellular components are required. For VLP budding within mammalian cells, the endosomal sorting complex required for transport (ESCRT)

plays an important role (Jager et al., 2007; Pincetic and Leis, 2009). Two epitopes, PTAP and YPLTSL, on the C-terminus of Gag's p6 domain are involved in the ESCRT pathway (Strack et al., 2003). PTAP (which is dominant) binds the cellular ESCRT protein, Tsg101 (Stuchell et al., 2004), and YPLTSL binds ALIX/AIP1 (Strack et al., 2003). In addition, the monoubiquitination of the p6 L domain is required. It is not understood why this is important, although it is speculated that this monoubiquitination could possibly increase the binding of p6 to Tsg101 (Strack et al., 2003).

HIV-1 VLPs have been shown to assemble in mammalian (Krausslich et al., 1993; Wagner et al., 1994b; Hammonds et al., 2007), insect (Yamshchikov et al., 1995; Deml et al., 1997b; Deml et al., 2004; Jaffray et al., 2004; Wang et al., 2007; Tagliamonte et al., 2010, 2011), and yeast cells (Sakuragi et al., 2002; Morikawa et al., 2007), but despite several attempts, expression of HIV-1 VLPs in plants has never been successfully demonstrated in the cellular compartment equivalent to that of its natural assembly place – the cytoplasm. Two studies (Meyers et al., 2008; Scotti et al., 2009) were successful in expressing full-length p55 Gag within the chloroplasts of cells, but both had difficulty in expressing Gag within the plant cytoplasm. Chloroplast expression might present problems for creating an enveloped VLP due to the double-membrane nature of chloroplasts and the lack of cellular machinery within the plastids for budding of the VLPs outside of the plastid. Although two studies were able to express p55 Gag in plants, only Scotti et al. were able to visualize that the p55 Gag formed VLPs, and neither of the studies looked into the characterization of the VLPs. In this chapter, the characterization of plant-produced Gag/dgp41 VLPs will be examined.

MATERIALS AND METHODS

Sucrose Density Sedimentation

Initial characterization of VLPs was performed using sucrose density gradient sedimentation as well as 30% sucrose cushions. For sucrose density gradients, 14×89 mm Ultra Clear tubes (Beckman-Coulter) were layered from the bottom with 1.5 ml each of 70%, 60%, 50%, 40%, 30%, and 20% (w/v) sucrose in phosphate-buffered saline (PBS), pH 7.4. Clarified, water-soluble leaf extract (3 ml) was layered on the top of the sucrose gradient and the tube was spun at 35,000 rpm in a SW41Ti rotor (Beckman-Coulter) for 5 h at 4°C. For 30% sucrose cushions, 70% sucrose (1 ml) was layered on the bottom of 30% sucrose (2 ml), and an upper layer of 20% sucrose (2 ml). Clarified, water-soluble leaf extract (7 ml) was layered on top of the sucrose and gradient was processed as the sucrose gradients. Fractions (1 ml) were collected from the top of the gradients and cushions by pipetting from the gradients and cushions, analyzed as described below, and used in further experimentation.

Optiprep Density Sedimentation

Further characterization and purification of VLPs was performed using Optiprep (60% iodixanol in water, Sigma Aldrich) density gradient sedimentation as well as 30% Optiprep cushions. For Optiprep density gradients, 14×89 mm Ultra Clear tubes (Beckman-Coulter) were layered from the bottom with 1.5 mls each of 60%, 50%, 40%, 30%, 20%, and 10% iodixanol. Clarified, water-soluble leaf extract (3 ml) was layered on the top of the Optiprep gradient and the tube was spun at 35,000 rpm in a SW41Ti rotor (Beckman-Coulter) for 5 h at 4°C. For 30% Optiprep cushions, 50% iodixanol (1 ml) was layered on the bottom of 30% iodixanol (1 ml), and an upper layer of 20% iodixanol (1 ml). Clarified, water

soluble leaf extract (9 ml) was layered on top of the iodixanol and the tube was spun at 35,000 rpm in a SW41Ti rotor (Beckman-Coulter) for 5 h. Fractions (1 ml) were collected from the top of the gradients and cushions by pipetting from the gradients and cushions, analyzed as described below, and used in further experimentation.

Trypsin Digestion Assay

Enriched fractions containing VLPs were digested with trypsin in the presence or absence of 1% Triton X-100 as per Sakuragi et al (2002). VLP-enriched fractions, containing 200 µg/ml p24 obtained from Optiprep gradient centrifugation, were aliquoted (1 ml) to each of four 1.5 ml centrifuge tubes. Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF)) were added to tubes 1 and 2, while tube 3 and 4 contained no protease inhibitors. Tube 1 remained a negative control, while 1% Triton X-100 was added to tubes 2 and 4, and trypsin (final concentration 1 µg/ml) was added to tubes 3 and 4. Extract was incubated at 26°C for 30 min, and was analyzed for Gag protein by immunoblotting following digestion.

Transmission Electron Microscopy

For visualization of whole VLPs, clarified extract from transiently expressing Gag plants and Day 6 post-infiltration Gag/dgp41-expressing plants was incubated for 2 min on 200 mesh formvar coated grids and stained by incubating grids containing sample with 2% (w/v) uranyl acetate for 2 min. For visualization of VLPs *in situ*, leaf tissue (cut into 1 mm^2 sections) from Day 8 post-infiltration Gag-expressing plants was chemically fixed in primary fixation buffer (2% (v/v) gluteraldehyde, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.8) for 2 h at 25°C. Following primary fixation, tissue was washed with phosphate buffer (pH 6.8) and

incubated in secondary fixation buffer (2% (w/v) osmium tetroxide0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.8) for 2 hr at 25°C. Following fixation, samples were washed three times with phosphate buffer (pH 6.8) to remove osmium tetroxide, and then incubated with 0.5% (w/v)urynl acetate for 2 hr at 25°C to stain samples. Stained samples were completely dehydrated with ethanol (10 min incubations with five increasing concentrations of ethanol for a total of 60 min dehydration time) and then infiltrated with a 1:3 ratio of Spurr's resin: acetone. After a 4 h incubation in 1:3 Spurr's resin: acetone, samples were moved to a 1:1 Spurr's resin: acetone mixture for another 4 h incubation at 25°C. Additional 4 h incubations with 3:1 and 100% Spurr's resin were performed to completely embed the samples in resin. Fresh resin was then added to samples and the mixture was placed into molds and heated in a 60°C oven for 24 h. Leaf tissue from transgenic Gag plants as well as Gag plants expressing dgp41 were also fixed and immobilized in the same manner. Sections (70 nm) from these samples were cut on diamond knives, positioned on formvar coated grids and stained with 2% uranyl acetate and 2% lead acetate as above. Specimen grids were viewed on a Philips CM12S transmission electron microscope.

Protoplast Experiments

Leaves (10 g) of transgenic Gag-expressing plants were surfaced sterilized with 70% ethanol, rinsed in water, cut into ~1 cm^2 sections, and immersed in protoplast isolation buffer (20 ml, 0.625 M sucrose, 25 mM MES, pH 5.7) containing cellulase (2.5 mg/ml) and pectinase (4 mg/ml) for 1 h at 25°C. The protoplast isolation buffer was then carefully siphoned off and replaced with fresh isolation buffer and gently shaken (50 RPM) for 5 min. The solution containing released protoplasts was then centrifuged at 200 g for 5 min to pellet

any remaining cell debris and the supernatant containing live protoplasts was carefully transferred to a sterile petri dish and incubated for 6 h at 25°C with gentle shaking (50 RPM), with supernatant samples taken at the indicated time points for further analyses.

RESULTS

Co-sedimentation of Gag and dgp41.

Virion and VLPs have characteristic size and densities and can be separated from other cellular component by rate-zona centrifugation (commonly referred to as gradient centrifugation). I subjected clarified homogenates of leaf material to both sucrose gradient centrifugation (20-70%, step gradient) and optiprep® (iodixanol) gradient centrifugation (20-60%, steps). Results of sucrose (Figure 18) and optiprep (Figure 19) gradient sedimentation experiments demonstrated that a large portion of both the Gag and dgp41 proteins migrated well into the gradients and could be recovered in the 30-50% sucrose fractions or the corresponding the 20-30% optiprep fractions. Similar results were previously reported for HIV-1 VLPs from other sources such as insect cells (Buonaguro et al., 2001) and yeast (Sakuragi et al., 2002). For later pufication purposes I substituted density gradient fractionation with simpler 30% sucrose or optiprep cushions.

Plant-produced HIV-1 VLPs are Enveloped

The previous results suggested that Gag/dgp41 may co-localize to the same particles, which would further suggest that the particles may be enveloped. This hypothesis was tested by subjecting to proteolysis by trypsin the Gag/dgp41

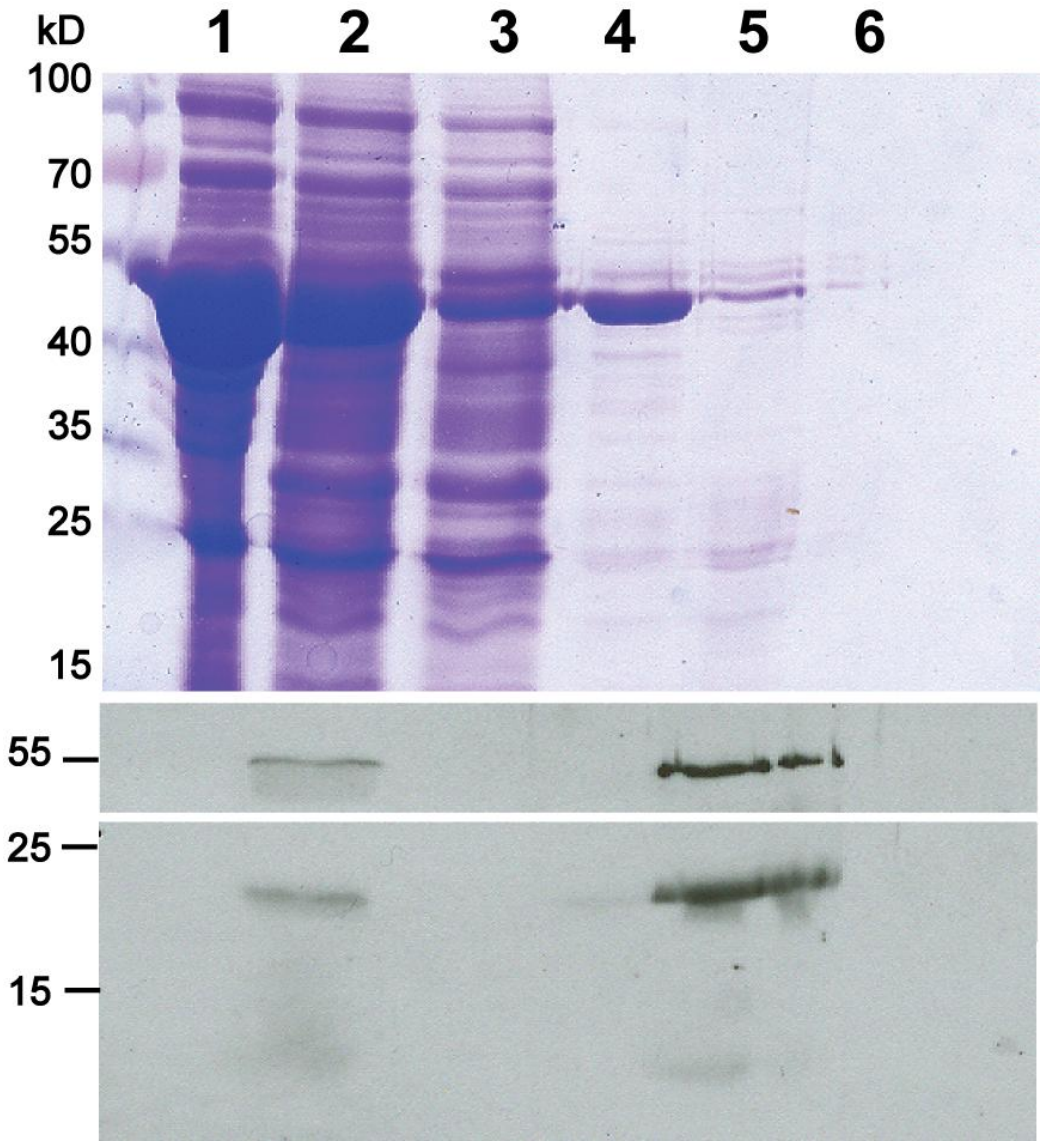


Figure 18. Sucrose Gradient Co-localization of Gag and dgp41. Lane 1: Wild type control. Lane 2: Whole extract Lane 3: Sample fraction. Lane 4: 20% Sucrose fraction. Lane 5: 30% Sucrose fraction. Lane 6: 70% Sucrose fraction. Upper panel is Coomassie stained gel. Middle panel is immunoblot showing Gag protein (anti-p24 antibodies). Bottom panel is immunoblot showing dgp41 protein (2F5 antibody).

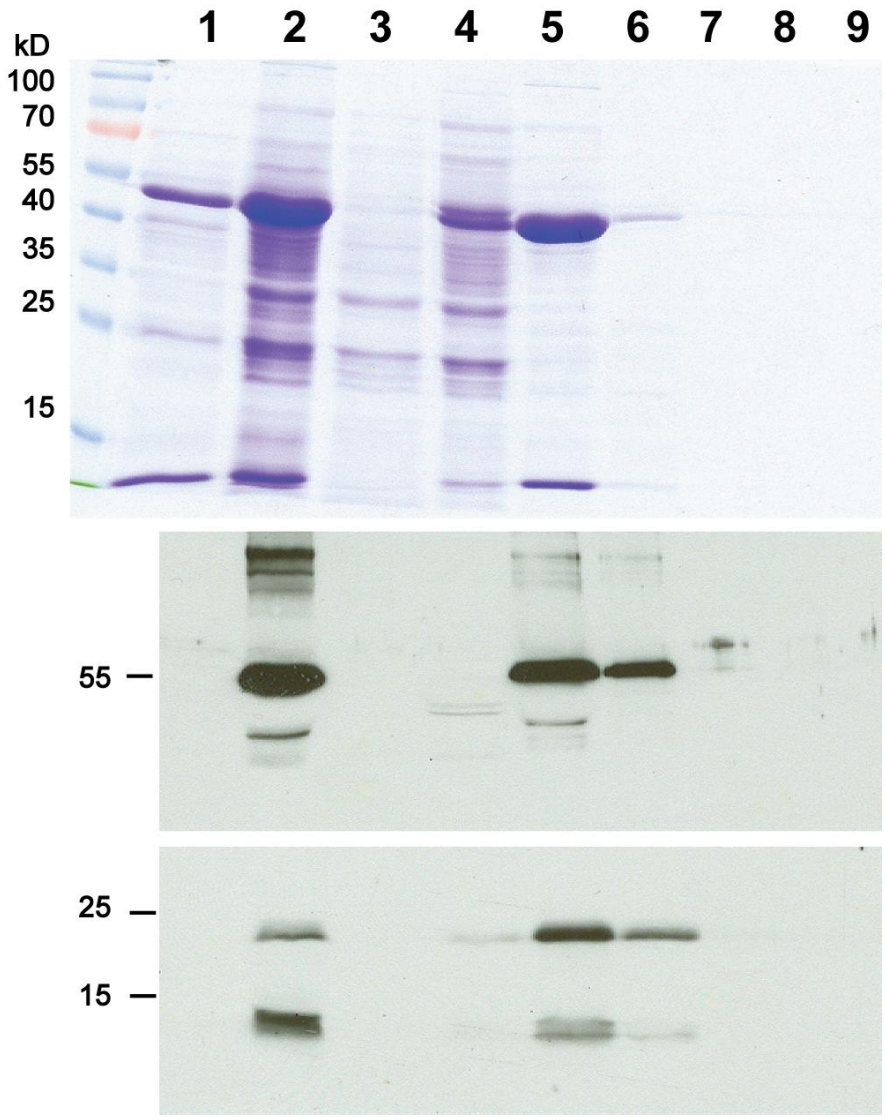


Figure 19. Optiprep Co-localization of Gag and dgp41. Lane 1: Wild type control. Lane 2: Whole extract Lane 3: Sample fraction. Lane 4: 10% iodixanol fraction. Lane 5: 20% iodixanol fraction. Lane 6: 30% iodixanol fraction. Lane 7: 40% iodixanol fraction. Lane 8: 50% iodixanol fraction. Lane 9: 60% iodixanol fraction. Upper panel is Coomassie stained gel. Middle panel is immunoblot showing Gag protein (anti-p24 antibodies). Bottom panel is immunoblot showing dgp41 protein (2F5 antibody).

optiprep gradient centrifugation-enriched preparations. Results (Figure 20) demonstrate that plant-derived Gag protein within VLPs is protected from trypsin digestion. When VLPs were treated with a detergent that solubilizes and removes the membrane, thereby exposing the membrane-enclosed Gag protein, the latter now became susceptible to proteolysis. Thus my results essentially mimic the case of HIV-1 VLPs produced in other expression systems (Spearman and Ratner, 1996; Sakuragi et al., 2002) and are supportive of the suggestion that plant-produced Gag/dgp41 VLPs are organized in a fashion not unlike their animal-cell derived counterparts.

HIV-1 VLPs Can Be Directly Observed in situ and in Preparation by TEM

Transmission electron microscopy was performed on both the leaf extract and intact leaf tissue of both stably and transiently transformed Gag-expressing plants. In leaf extracts, enveloped VLPs with diameters of approximately 100 nm were visualized (Figures 21C, D, Figure 22E, F). Similarly-sized particles could also be oveserved *in situ* in both transiently and stably expressed leaf tissue (Figure 21A, B, Figure 22A-D). VLPs were found in the apoplastic space between the cell wall and the plasma membrane (Figure 22C, D), but also within in intracellular compartments such as the vacuole (Figure 21A, B), in the lumen of cytoplasmic membrane vesicles (Figure 22B) and perhaps even in the cytoplasm itself (Figure 22A).

Moreover, in tissues of plants that express the Gag protein (Figure 21A, B), but not in tissues from untransformed plants (data not shown), I observed electrodense patches on various cellular membranes that I interpreted as congregating Gag protein molecules during during the process of VLP budding across the plasma membrane out into the apoplastic space and across the

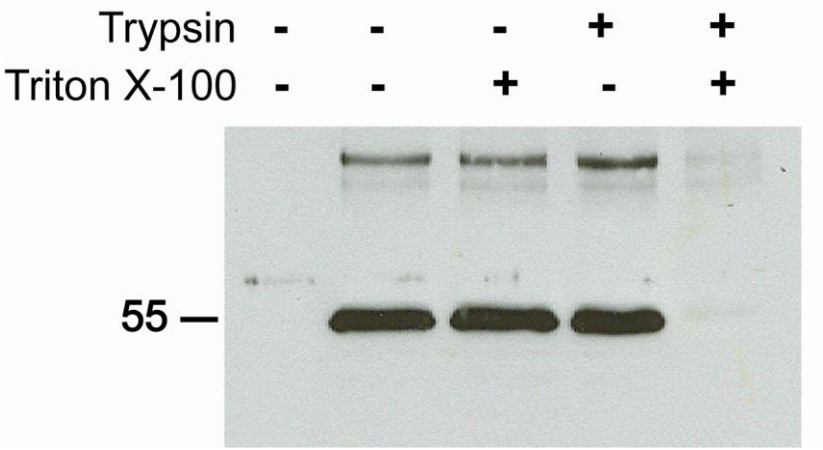


Figure 20. Trypsin digestion assay. The first lane (from left) contains proteins from non-infiltrated wild type plant extract. The remaining lanes contain VLP enriched samples from a transgenic Gag-expressing plant, incubated with or without Triton X-100 and/or trypsin. When incubated with trypsin alone, the Gag is protected from proteolytic digestion by the membrane envelope, but when this envelope is disrupted with the addition of detergent (Triton X-100), the Gag is digested by the enzyme, suggesting that the Gag VLPs are fully enveloped.

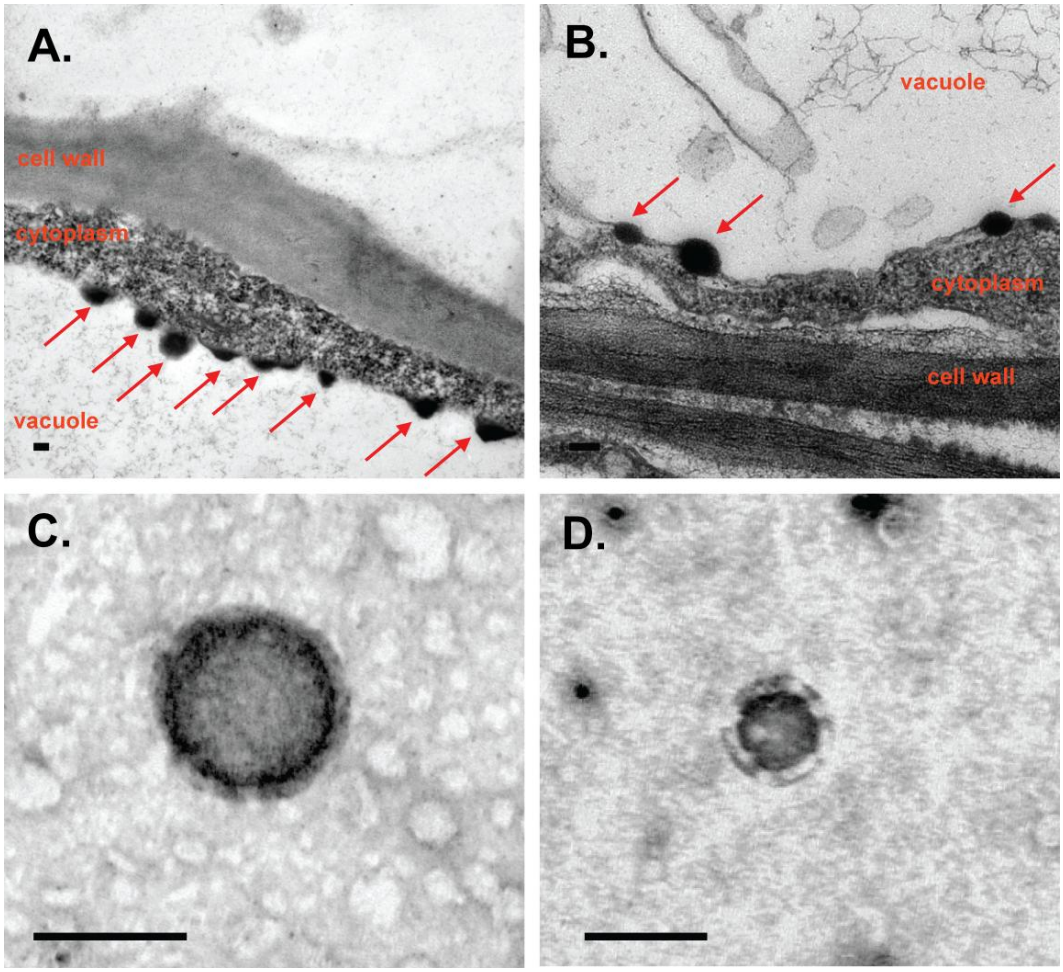


Figure 21. Results of TEM in plants transiently expressing gag. A and B. Representative sections from chemically fixed leaf tissue transiently expressing Gag VLPs, showing Gag VLPs (red arrows) budding from the vacuolar membrane into the vacuole. C and D. Representative negatively stained VLPs from leaf tissue extract of plants transiently expressing Gag protein. Bar represents 100 nm.

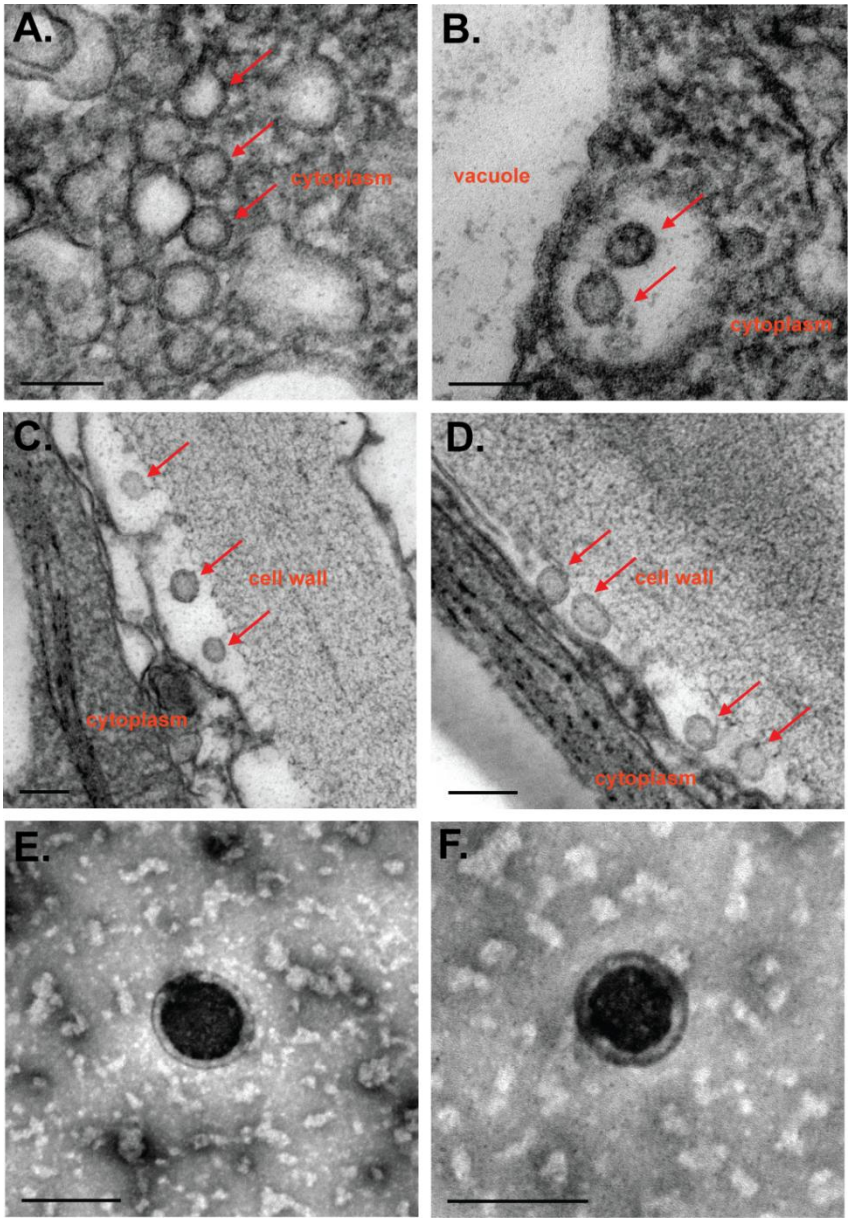


Figure 22. Results of TEM in transgenic gag plants. A - D. Representative sections from chemically fixed transgenic gag leaf tissue showing VLP production accumulation (red arrows) in cytoplasm (A.), in endosomes near the vacuole (B.) and within the apoplastic space (C. and D.). E and F. Representative negatively stained VLPs from VLP enriched extract of transgenic gag leaves. Bar represents 100 nm.

vacuolar membrane into the vacuole lumen. Similar structures were observed in mammalian, insect and yeast cells that express Gag from HIV-1 and other lentiviruses.

VLPs from leaf tissue of plants co-expressing Gag and dgp41 were also observed in both leaf tissue and enriched VLP containing extracts, and these VLPs were morphologically similar to those found in the Gag-only expressing plants (data not shown).

Protoplast Experiments

As described above, I observed by TEM the budding of HIV-1 Gag and Gag/dgp41 VLPs across cellular membranes. To further substantiate this observation, I wanted to actually collect such budded particles. However, due to the presence of cellulose cell walls surrounding plant cells, when VLPs bud from the plasma membrane of plant cells, I expected them to become trapped in the apoplastic space between the plasma membrane and the cell wall. I further hypothesized that such particles would be released if the constraint of the cell wall is removed. To test this hypothesis, protoplasts were generated from transgenic Gag plants by enzymatically removing the cell walls and the culture medium was sampled every hour up to 6 h after protoplast isolation to check for presence of Gag protein (Figure 23). Protoplasts remained intact for the duration of the 6 hours of incubation, and lysis was apparent only after longer incubation periods (data not shown). I observed time-dependent accumulation of Gag protein in the protoplast medium (separated from the protoplasts by centrifugation). The initial supernatant (fresh buffer with newly isolated protoplasts) did not contain any Gag protein. The presence of Gag was detected

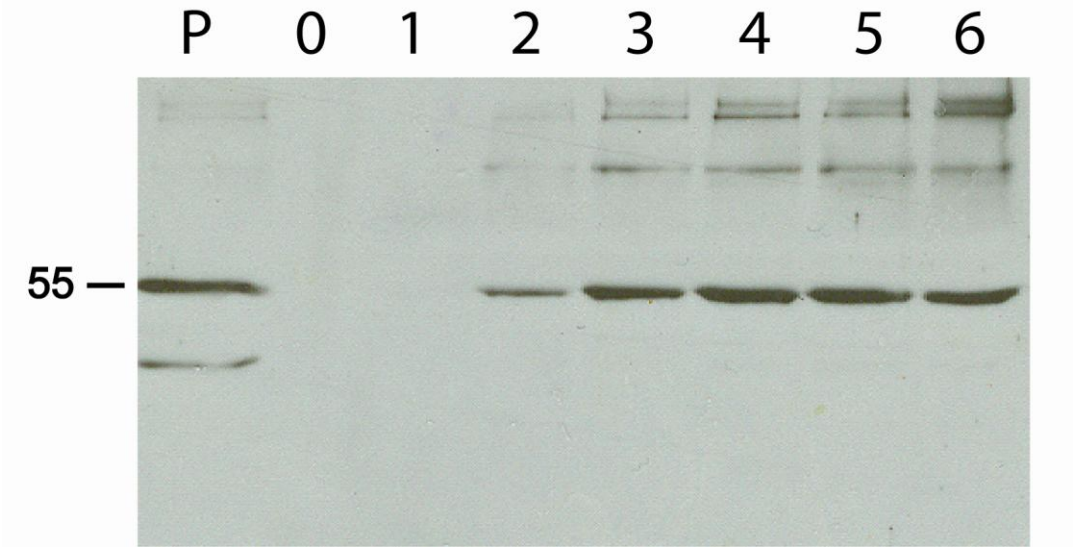


Figure 23. Protoplast Assay. P – Pelleted protoplast cells showing total amount of Gag in cells. 0 - initial protoplast buffer at time point 0. 1-6 – hourly samples taken from protoplast supernatant. Results show that Gag was able to bud from the cell after the cell wall was removed, possibly suggesting that the protein is forming VLPs that are able to bud from the plasma membrane.

at 2 h and their levels continued to increase as a function of the incubation time.

Importantly, only intact Gag protein was found in the medium, whereas intracellular accumulation of the protein was always accompanied by the accumulation of truncated forms/degradation products. Specifically a 44 kD band was found in the cells but not in the incubation medium. These results strongly suggest that Gag, not a secretory protein, is released from the cells, probably in the form of VLPs that bud out of the plant plasma membrane into the medium. Taken together with the TEM pictures showing VLPs budding into the apoplastic space *in vivo*, it can be concluded that plant cells can support the formation and budding of HIV-1 Gag and also Gag/dgp41 VLPs.

DISCUSSION

To date, the mechanisms behind VLP formation in plants has not been extensively studied. Only a handful of plant viruses form enveloped virus-like particles (Tas et al., 1977; Chu et al., 2001; Whitfield et al., 2004; Margaria et al., 2007) and researchers have only recently begun to express animal virus VLPs. The field of plant-based VLP production is still in its youth, and extensive characterization of plant-produced VLPs and their assembly processes are needed in order to better understand how these particles are formed in plant cells.

Despite the lack of *in planta* characterization of either naturally occurring enveloped viruses or VLPs expressed using recombinant techniques, there is ample, albeit circumstantial, evidence that plants have the machinery needed for enveloped VLP production, which is supported by the fact that the 19 species of the enveloped plant-virus genus *Tomspovirus* have one of the widest ranges of

hosts (over 800 plant species) of any plant-virus (Adkins et al., 2005; Pappu et al., 2009). In addition to the existence of enveloped plant-viruses, many of the genes (and subsequent gene products) involved in the formation of enveloped viruses in mammals and other animals have homologs in plants.

Most eukaryotes have two NMT genes, and the *Arabidopsis thaliana* NMT1 has close sequence homology to animal NMTs (Podell and Gribskov, 2004). Several important plant enzymes such as calcium dependent protein kinases require myristoylation for proper function (Benetka et al., 2008), and as such, plants express several myristoylated proteins (*A. thaliana* has over 300) (Podell and Gribskov, 2004). This evidence suggests that plants have the capability to myristoylate HIV-1 Gag protein and thus increase its membrane affinity and assist the formation of VLPs. Although no *Nicotiana* NMT sequences have been characterized to date, presence of NMT activity in tobacco cells (Podell and Gribskov, 2004) and the ubiquitous nature of NMT genes throughout the plant kingdom implies that the genes within the genus would be sufficient to myristylate HIV-1 Gag and help target the protein to the plasma membrane.

$PI(4,5)P_2$ in higher plants is found at levels 10 times lower than those found in animals and lower plants, but it seems to be important for vesicular trafficking and cytoskeleton regulation (Meijer and Munnik, 2003). It becomes concentrated at the plasma membrane during certain conditions such as phospholipase C inhibition, salt stress, during the final stage of BY-2 cell division, and at the tips of growing root hairs (van Leeuwen et al., 2007). When it is localized to the plasma membrane, it is mostly concentrated at membrane rafts (Furt et al., 2010). Despite the low amounts of $PI(4,5)P_2$ in plants, its existence in plants and the ability for it to concentrate at membrane rafts (where Gag and

other HIV-1 proteins localize during virus-formation) suggest that $PI(4,5)P_2$ is able to interact with HIV-1 Gag when expressed in plants and thus assisting the formation of VLPs.

Plants contain orthologs for all major ESCRT complex subunits, which have varied endogenous functions due to vacuolar trafficking (Otegui and Spitzer, 2008). Most of the research on the ESCRT pathway in plants has been performed in *A. thaliana,* which has orthologs of both Tsg101 and Alix (Spitzer et al., 2009). Orthologs in *N. benthamiana* have not yet been fully characterized (ie. not fully sequenced), although some dominant mutant studies inhibited replication of tomato bushy stunt tombusvirus in *N. benthamiana* (Barajas et al., 2009; Barajas and Nagy, 2010). The endogenous ESCRT pathways within plants could aid in the budding of VLPs from plant cells into the apoplastic space between the cell wall and plasma membrane.

All of this evidence, paired with the eukaryote-common post-transcriptional/translational features of plant cells, suggests that *N. benthamiana* is capable of expressing Gag-based VLPs that bud from the plasma membrane, but this had not been proven until this study. Results suggest that Gag and gp41 can be co-expressed together in plants where they assemble into VLPs that have the potential to be used as a mucosally-targeted HIV-1 subunit vaccine.

Immunogenic Gag VLPs have previously been produced in various eukaryotic expression systems including mammalian, insect, and yeast cells, but only two published studies have shown production of full-length Gag and Gag derivatives in *N. benthamiana* or *N. tabacum* {Meyers, 2008 #94; Scotti, 2009 #92} Both studies had very limited success with cytosolic expression, focusing instead on chloroplast expression of Gag. Although Meyers et. al showed TEM

micrographs of their Gag VLPs, neither of the two groups fully characterized their expression products to support the formation of enveloped VLPs, which would be essential in the co-expression of Gag VLPs with envelope anchored proteins such as gp41.

This study is the first to fully characterize VLPs in *N. benthamiana*. The results of density gradient sedimentation, trypsin digestion of delipidized Gag, and electron microscopy have all suggested that Gag is forming ~100 nm enveloped VLPs in *N. benthamiana*. In addition to expressing Gag in plants, the co-expression of dgp41 with the Gag VLPs was also observed. To my knowledge, this is the first study to show that Gag and dgp41 can be co-expressed in plants. In addition to co-expressing the two proteins, all of the above biochemical assays have suggested that the two proteins are assembling together into enveloped VLPs. Based on these results, these VLPs could hold great promise for future studies involving Gag/dgp41 VLPs as potential vaccine candidates.

Chapter 4

I.P. IMMUNIZATION OF MICE WITH GAG/DGP41 VLPS

ABSTRACT

Gag VLPs expressed in a number of systems have been shown to elicit substantial antibody and CTL responses in mice, rabbits, and non-human primates, but the immunogenicity of plant-expressed VLPs (with or without surface-displayed Env proteins) had not been tested until this study. An immunization trial involving systemic priming and boosting with plant-based HIV-1 Gag/dgp41 VLPs was conducted in mice. As will be presented, it was revealed that these plant-based HIV-1 Gag/dgp41 VLPs can elicit antibody responses against both Gag and MPER.

INTRODUCTION

Substantial evidence is available for the immunogenicity of Gag, gp41, and chimeric Gag/Env VLPs expressed in insect and mammalian cell cultures. The MPER peptide has been tested in numerous immunological studies (reviewed in (McGaughey et al., 2004; Montero et al., 2008; Nieva et al., 2011)), with the overarching theme of these studies illustrating that while peptides of the MPER region are not immunogenic on their own, when expressed as a fusion protein with other more immunogenic proteins (ie. keyhole limpet hemocyanin, viral coat proteins, VLPs of influenza, CTB, etc.), high titers of neutralizing antibodies against the ELDKWA and NWFDIT epitopes can be elicited.

The Mor lab has focused on studies using MPR fused to the B subunit of cholera toxin (CTB) (Matoba et al., 2004; Matoba et al., 2006; Matoba et al., 2008), plaque antigens F1 and V (Matoba et al., 2011), and hepatitis B core

antigen (HBc) (Cherni, 2008). Three different studies by Matoba et al. describe the immunogenicity of *E. coli*-derived CTB-MPR in both mice and rabbits (Matoba et al., 2004; Matoba et al., 2006; Matoba et al., 2008). Initially, the fusion peptide was tested by mucosal priming followed by both mucosal and systemic boosts in mice. This immunization scheme elicited strong serum IgG responses as well as vaginal and fecal IgA responses against the MPER peptide, although the mucosal response was relatively transient. The antibodies produced in the trial were able to block transcytosis of HIV-1 virus across an epithelial barrier (Matoba et al., 2004). The initial success with this immunogen lead to additional studies exploring the effectiveness of a heterologous prime-boost strategy in which the effect of immunization route (systemic or mucosal) in priming and boosting strategies was explored (Matoba et al., 2006). In congruence with the initial studies, significant transcytosis-blocking antibody titers against the MPER peptide were elicited from the immunizations. It was determined that mucosal priming with adjuvant followed by systemic boosting elicited the best induction of anti-MPER antibodies, although antibodies against the CTB portion of the immunogen appeared to be dominant over anti-MPER responses. In addition to the immunization studies conducted with *E. coli*-produced CTB-MPR, a further trial was conducted using CTB-MPR expressed in transgenic plants. In accordance with the previous two trials, serum and vaginal antibodies against MPER were elicited when mice were mucosally primed with plant-based CTB-MPR and systemically boosted with the same (Matoba et al., 2009). In congruence with the murine studies, transcytosis-blocking anti-MPER antibodies were elicited in rabbit serum. Once again, however, the CTB portion of the immunogen seemed to be immunodominant. Further characterization of

the antibody response revealed that although the antibodies could block HIV-1 transcytosis, the antibodies were not able to neutralize virus in CD4-dependent infection in either a PBMC neutralization assay or a pseudovirus neutralization assay.

In order to counteract the immunodominace of the CTB portion of the CTB-MPR fusion peptide, additional fusion partners, F1-V of *Yersina pestis* and the Hepatitis B core antigen (HBc) were fused to MPER and tested for their immunogenicity in mice. Similar to trials with CTB- MPR, F1-V-MPR induced a strong IgG response to the carrier molecule, but unfortunately only induced low titers of antibodies against the MPER moiety (Matoba et al., 2011). When a heterologous prime-boost strategy using a priming immunization of CTB- MPR and boosting immunization of F1-V- MPR was utilized, significant antibody titers were elicited which could not be elicited with either immunogen alone. These studies confirmed both the low immunogenicity of the MPR peptide and the effectiveness of a heterologous prime-boost strategy with more than one presentation of the MPER peptide (Matoba et al., 2011). Further studies were conducted using VLPs formed by the fusion of a truncated hepatitis capsid protein with MPR (Cherni, 2008). Unfortunately, only extremely low titers of serum IgGs were elicited from homologous prime-boost with the HBc- MPR VLPs, and the priming with HBc- MPR followed by a boost of CTB- MPR seemed no more effective than priming with control HBc protein. The lack of immunogenicity against MPER of the HBc- MPR VLPs could be due to a number of factors including the lack of presentation of MPER in a suitable manner for immune recognition, immunodominance of the HBc VLP, and/or lack of optimization of adjuvant (Cherni, 2008). All of these studies employing fusion

peptides with the MPER confirm the low immunogenicity of MPER on its own, the success of heterologous prime-boost strategies with multiple routes of immunization and more than one vaccine construct, and the promise of producing transcytosis-blocking antibodies from vaccine candidates utilizing the MPER of HIV-1 gp41. Despite the success of some of these constructs, additional platforms with which to display the MPER peptide (preferably in a native conformation) are needed to combat the immunodominance of the tested fusion partners and to increase the immune response against MPER through heterologous construct strategies.

In addition to the MPER region of gp41, the cytoplasmic tail domain (CTD) of gp41 has also been shown to play a key role in immunological studies. Despite the fact that the CTD resides below the surface of the virus membrane, there are still several mAbs that recognize the “Kennedy epitope” (amino acids 724-745, PRGPDRPEGIEEEGGERDRDRS), a hydrophilic region at the N terminus of the CTD (Buratti et al., 1998; Cleveland et al., 2000; Montero et al., 2008).

The immunogenicity of HIV-1 Gag VLPs has also been extensively studied. The Gag protein of HIV-1 has been shown to produce VLPs in various eukaryotic expression systems including mammalian (Rovinski et al., 1992), insect (Buonaguro et al., 2002; Deml et al., 2004; Buonaguro et al., 2005; Tagliamonte et al., 2010, 2011; Visciano et al., 2011), and yeast cell cultures (Sakuragi et al., 2002). Many of these particles were tested in immunogenicity studies in mice, rabbits, and non-human primates (Doan et al., 2005). Humoral immune responses induced by priming with Gag VLPs was shown in rabbits (Wagner et al., 1996a; Deml et al., 2005) and rhesus macaques (Montefiori et al.,

2001). In rabbits, intramuscular (i.m.) injection of baculovirus-derived VLPs elicited strong anti-Gag antibodies, but these antibodies were non-neutralizing. In a second animal model, rhesus macaques were inoculated with VLP in QS21 adjuvant with similar results to the rabbit trials (Montefiori et al., 2001). In contrast, long-lived CD8+ cytotoxic T-cell (CTL) responses against multiple epitopes of the Gag protein were stimulated in rhesus macaques when the VLPs were inoculated without adjuvant (Paliard et al., 2000).

In addition to the immunogenicity of Gag alone, Gag VLPs have been able to display HIV-1 Env proteins either as fusion proteins (Wagner et al., 1993; Wagner et al., 1994b; Brand et al., 1995b; Brand et al., 1995a; Truong et al., 1996) or on their surface in their native conformation (Haffar et al., 1990; Vzorov et al., 1991; Rovinski et al., 1992; Krausslich et al., 1993; Wagner et al., 1993; Rovinski et al., 1995; Deml et al., 1997b; Deml et al., 1997a; Hicar et al., 2010). When Gag was displayed as VLPs fused to Env proteins, the VLPs still elicited anti-Gag antibodies and CTL responses, but immune response against the fused Env proteins was fairly low (Deml et al., 2005). In contrast, when various Env peptides were displayed in their native conformation, successful humoral and cellular immune responses against Gag and the displayed Env protein were elicited in both rodents (Haffar et al., 1990; Rovinski et al., 1992; Rovinski et al., 1995; Wagner et al., 1996b; Deml et al., 1997b; Deml et al., 1997a; Buonaguro et al., 2002) and non-human primates (Wagner et al., 1998). Several types of Gag/Env VLPs have been able to elicit significant titers of Env-specific antibodies which were able to neutralize homologous (Rovinski et al., 1995; Deml et al., 1997b; Deml et al., 1997a) and heterologous virus isolates (Buonaguro et al., 2002), the activity of which largely corresponded to the quantity of Env particles

that were incorporated into the VLPs. In addition to eliciting antibodies against Gag and Env, significant Gag- and Env- specific CD8+ CTL activities were observed in mice (Deml et al., 1997b; Deml et al., 1997a) and non-human primates (Wagner et al., 1998).

Although all of this evidence of immunological success of Gag and Gag/Env VLPs is promising, very little immunological data is available for plant-based HIV-1 full-length Gag or gp41 vaccine antigens. Promisingly, the little information that has been garnered from the limited studies shows potential for success. Although the lack of substantial expression of HIV p55 Gag in plants precludes its use in immunological studies, many trials have been performed with derivatives of Gag (ie. p24, p17, and p41) with great success in the elicitation of strong anti-p24 and p17 antibodies as well as CTL response (Perez-Filgueira et al., 2004; Guetard et al., 2008; Iglesias et al., 2008; Mahdavi et al., 2010; Gonzalez-Rabade et al.). There have also been several studies showing immunological responses against plant-based gp41 constructs (McLain et al., 1995; McLain et al., 1996; Durrani et al., 1998; McInerney et al., 1999; Marusic et al., 2001; Matoba et al., 2009). Two different studies focused on epitopes in the MPER of gp41. As described earlier, a murine trial was conducted by Matoba et al. using CTB-MPR expressed in transgenic plants. This construct elicited serum and vaginal antibodies against MPER when mice were mucosally primed with plant-based CTB-MPR and systemically boosted with the same (Matoba et al., 2009). One other group has tested the immunogenicity of plant-based portions of the MPER. Marusic et al. fused the ELDKWA epitope (recognized by the 2F5 neutralizing antibody) to the coat protein of potato virus X, expressed the construct in *N. benthamiana*, and used it to immunize mice. The construct was

able to elicit HIV-1 neutralizing antibodies when administered mucosally and intraperitoneally (Marusic et al., 2001). Portions of the cytoplasmic tail of gp41 (a.a. 731-752) have also been expressed in plants via insertion into the S protein of cowpea mosaic virus (CPMV) and were shown to be immunogenic in several trials (McLain et al., 1995; McLain et al., 1996; Durrani et al., 1998; McInerney et al., 1999). Through a series of subsequent trials exploring the effects of immunization route and use of several different adjuvants, it was determined that the CPMV-HIV/1 particles utilizing gp41 a.a. 731-752 could elicit neutralizing antibody titers in mice. It was established that a nasal immunization route was more effective than oral immunization (Durrani et al., 1998), and that the adjuvant Quil A was most effective in eliciting immune responses in subcutaneous immunizations (McInerney et al., 1999).

The overwhelming evidence that Gag/Env VLPs can elicit both neutralizing antibodies and CTL responses is promising for the development of Gag/Env VLPs for use as vaccine candidates against HIV-1. The few plant-based vaccine candidates that have been tested for immunogenicity have showed promise equal to those produced in other cell culture systems, and as will be presented in this chapter, immunization trials employing plant-based HIV-1 Gag/dgp41 VLPs confirm these previous results.

MATERIALS AND METHODS

Plant-based VLP Purification

Purification of VLPs for immunization trials was performed in 20 gram batches. Twenty grams of leaves expressing Gag/dgp41, which had been stored at -80°C, were crushed with liquid nitrogen into powder and added to plant

extraction buffer (60 ml, 25 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8) and shaken on ice for 60 min. The mixture was then strained through miracloth to remove pulp and centrifuged at 14,000 g for 20 min to clarify the extract. Ammonium sulfate was added to clarified extract to 40% and the extract was shaken on ice for 60 min to precipitate VLPs. The extract was then centrifuged at 36,000 rpm for 30 min to pellet the VLPs. Pelleted VLPs were resuspended in PBS (4 ml) and layered on top of two 30% Optiprep cushions (from bottom, 1 ml 50% iodixanol, 2 ml 30% iodixanol, 7 ml 20% iodixanol, 2 ml VLP extract) and spun at 35,000 rpm in an SW41Ti rotor (Beckman-Coulter) for 5 h. The 30% iodixanol fraction (2 ml each) was collected and concentrated with a 300 kD-cutoff centricon. Quantifications of both Gag and gp41 were determined with quantification immunoblots as previously described.

E. coli-based CTB-MPR Expression and Purification

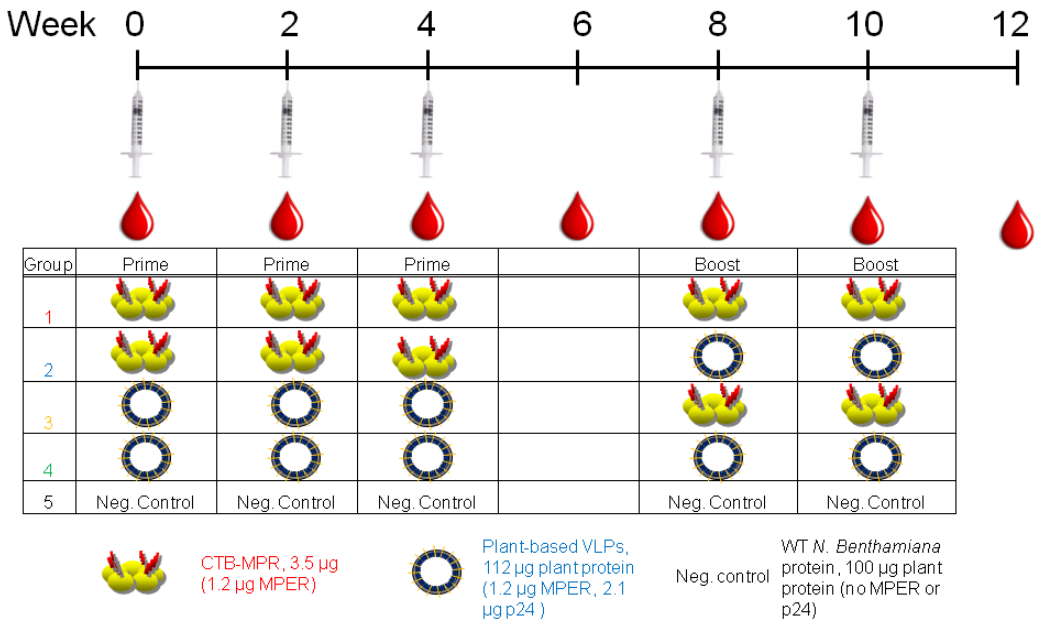
Expression of the CTB-MPR fusion protein in *E. coli* and its purification was done as previously described (Matoba et al., 2008). Briefly, *E. coli* cultures containing pTM 199 were grown from single colonies overnight at 37°C. Logarithmic phase cultures were induced with 100 μM isopropyl β- D -1-thiogalactopyranoside (IPTG) and allowed to grow for 2 h before centrifugation of the cells. Cell pellets were resuspended in PBS (20 ml) and lysed by microfluidization. Lysed cells were pelleted by centrifugation, and the insoluble pellet was solublized in CHAPS buffer (80 mM Tris, 2 M NaCl, 1.5% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 8.0), rocked at 4°C for 60 min, and then centrifuged to remove the remains of the insoluble cell fractions. The soluble supernatant was then added to a 25 ml

Talon gravity flow column and purified by metal affinity chromatography. The final column eluate (50 ml) was dialyzed in PBS with a 3.5 kD-cutoff membrane for 24 h to remove CHAPS and imidizole, and then centrifuged at 2,000 g for 20 min to pellet the CTB-MPR. Pelleted protein was collected, washed 2x with PBS, and resuspended in sterile PBS (500 µl). Purity and quantification of the pure CTB-MPR was determined by Coomassie stained gels, quantitative immunoblots (using a pure standard), and by determining the absorbance at 280 nm using (ε = 2.1 mM^{-1} cm^{-1}).

Immunizations

The experimental protocol involving animals was approved by the Institutional Animal Care and Use Committee of Arizona State University. Female BALB/c mice (6-wk old, Charles River) were immunized intraperitoneally (i.p.) with 200 µl of CTB-MPR (3.5 µg, including 1.2 µg MPER), purified VLPs (1.2 µg MPER, 2.1 µg p24, 111 µg total plant protein), or wild-type plant protein (100 µg prepared in the same manner as the VLP proteins). Each concentrated protein was mixed with PBS and ribi adjuvant (Sigma) to a final concentration of 2% oil as per manufacturer's instructions. Four experimental groups (n=8) were given either VLP or CTB-MPR during each of three priming immunizations, and were then given either VLP or CTB-MPR during each of two boosting immunizations (Table 6). As a negative control, a fifth group was immunized with wild-type plant protein for each of the five immunizations. Serum was collected from all mice in two week intervals through the end of the trial.

Table 6. Immunization schedule for i.p. immunizations.



Antibody Titer Assays

ELISA plates were coated with 20 µg of streptavidin (Sigma) and 2 µg of biotinylated MPER peptide (for detection of MPER antibodies (Matoba et al., 2006)) or 1 µg of p24-CTA2. Threefold serial dilutions of serum starting from 1:50 were applied on to the plates and incubated for 1 h at 37°C. Serum IgGs were detected by peroxidase conjugate of anti-mouse IgG (Calbiochem). Endpoint titers were determined by standard curve, using the reciprocal of the dilution factor of sample giving background levels of OD_{490}.

Transcytosis Assays

Transcytosis assays were performed by the lab of Morgane Bomsel at the Institute Cochin in Paris, France as described (Bomsel et al., 1998; Alfsen et al., 2001). Epithelial cells were grown as a confluent monolayer on permeable support (Figure 24). Samples of Ig were partially purified by ammonium sulfate (0-50% saturation fraction) and then dialyzed against RPMI medium 1640, 10% FCS. Cells (10^6/40µl) infected with HIV-1NDK were incubated at the apical pole of epithelial cells. Transcytosis was allowed to proceed for 110 min at 37°C. Viral load in the basolateral chamber was estimated from the p24 content (p24 kit, Coulter). Transcytosis was expressed as percentage of control.

Neutralization Assays

Neutralization assays were performed by the lab of Nobuyuki Matoba at the Owensboro Cancer Research Center in Owensboro, KY as previously described (Figure 25) (Montefiori, 2009; Matoba et al., 2010). Env-pseudotyped viruses were prepared by co-transfection of 293T/17 cells with various *env*-expressing plasmids and an *env*-deficient HIV-1 backbone vector (pSG3ΔEnv)

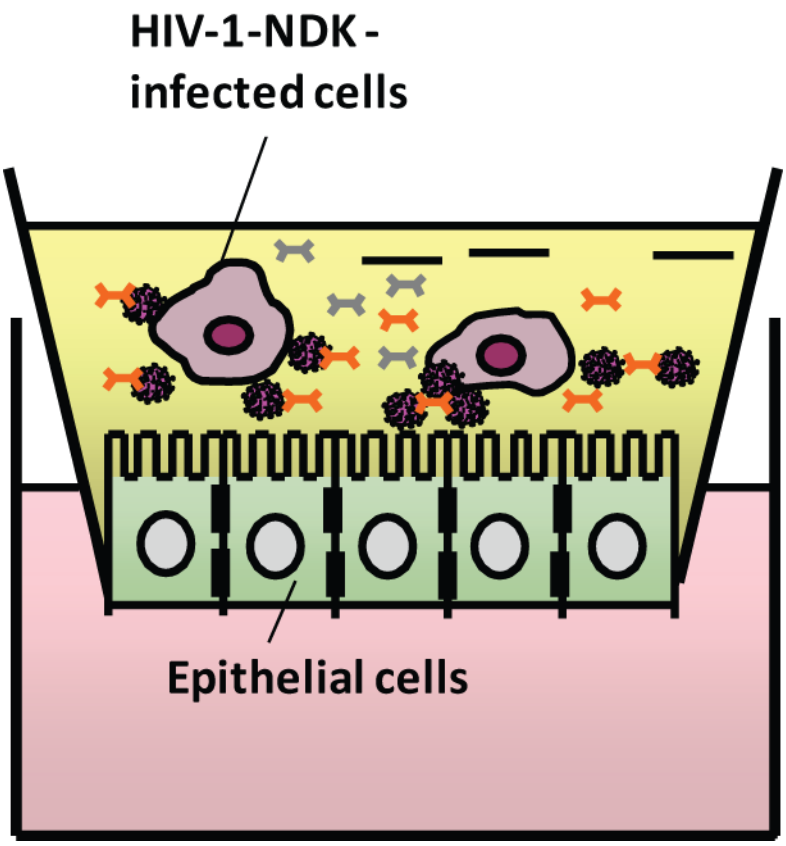


Figure 24. Model of epithelial transcytosis assay. Epithelial cells were grown as a confluent monolayer on permeable support. Partially purified samples of Ig were incubated with HIV-1 NDK-infected cells at the apical pole of epithelial cells. HIV-1 specific antibodies (in orange) bound to the virus prevented the transcytosis of virus across the epithelial cell layer. Amount of virus transcytosis was measured by p24 ELISAs.

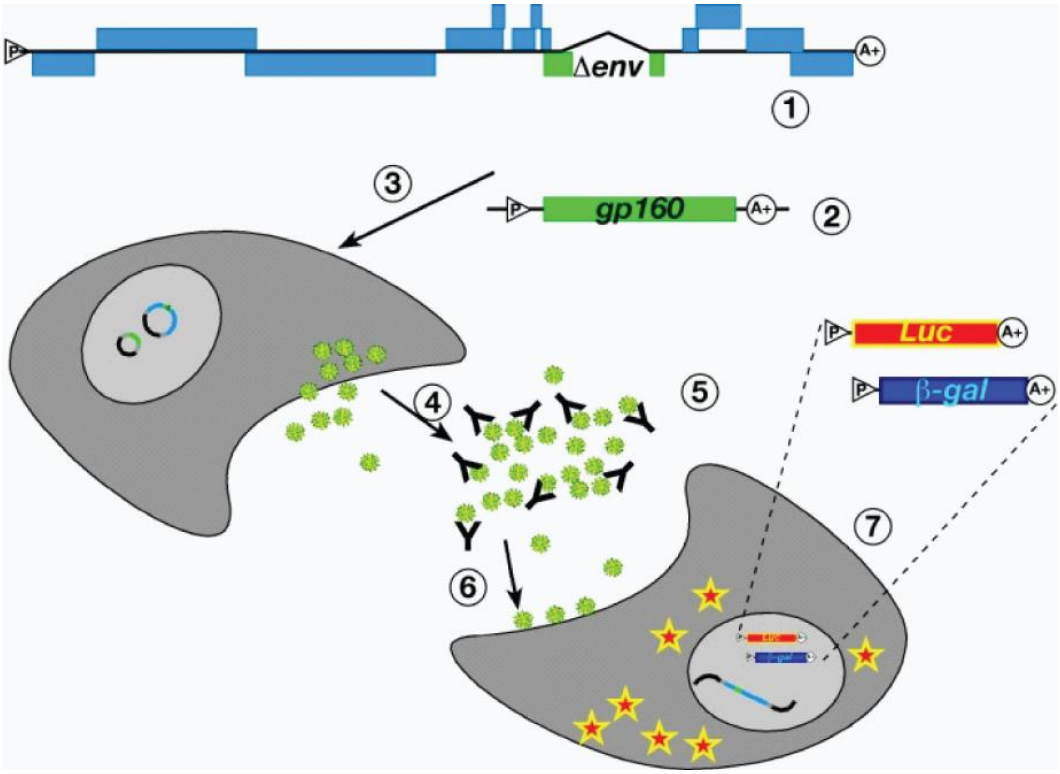


Figure 25. Pseudovirion neutralization assay. On top are schematic diagrams of expression vectors used in this assay. The envelope-defective, provirus clone (1) is shown above the vector that expresses an intact *env* gene of the virus of interest (2). Both vectors are used for co-transfection of 293T cells (3), which are allowed to produce pseudovirions that are then harvested (4). Neutralization is carried out by incubating the harvested pseudovirions for 1 hour in the presence of serial dilutions of Ab preparations (5). Finally, residual pseudovirions are allowed to infect target (CD4+/CCR5+) cells (6). Single round of infection results in cells, which express the luciferase reporter gene (and the β galactosidase) and the resultant luminescence is measured (7).

and were titrated in TZM-bl cells to determine the 50% tissue culture infective dose ($TCID_{50}$). Antiviral activity was expressed as an IC_{50} value, which is the sample concentration giving 50% of relative luminescence units (RLUs) compared with those of virus control after subtraction of background RLUs. The broadly neutralizing mAbs b12, 2G12, 2F5 and 4E10, as well as soluble CD4 were used as positive controls. Two-hundred $TCID_{50}$ of pseudoviruses were used for the neutralization assay. Samples and the virus were mixed and incubated for 1 h at 37°C, to which 10^4 cells/well of TZM-bl cells were added and incubated for 72 h. Luciferase activity was measured using the Britelite Plus Reagent (PerkinElmer, Waltham, MA).

RESULTS

Antibody Titers Against p24

The average OD_{490} (Figure 26) and endpoint titers (Figure 27) were calculated after the third priming immunization (Week 6), before the first boost (Week 8), after the first boost (Week 10), and after the second boost (Week 12) for antibodies against p24. In both groups primed with VLPs, very significant antibody titers ($p < 0.01$, in comparison to the negative control group) were elicited after priming and remained at those levels until the first boost. The VLP priming groups were then split into two groups, with one boosted with CTB-MPR and the other boosted with VLPs. The group primed with CTB-MPR retained significant titers of anti-p24 antibodies through the end of the trial, despite the fact that the mice did not receive any further Gag protein. Boosting with VLPs resulted in increased antibody titers, with final antibody titers reaching extremely

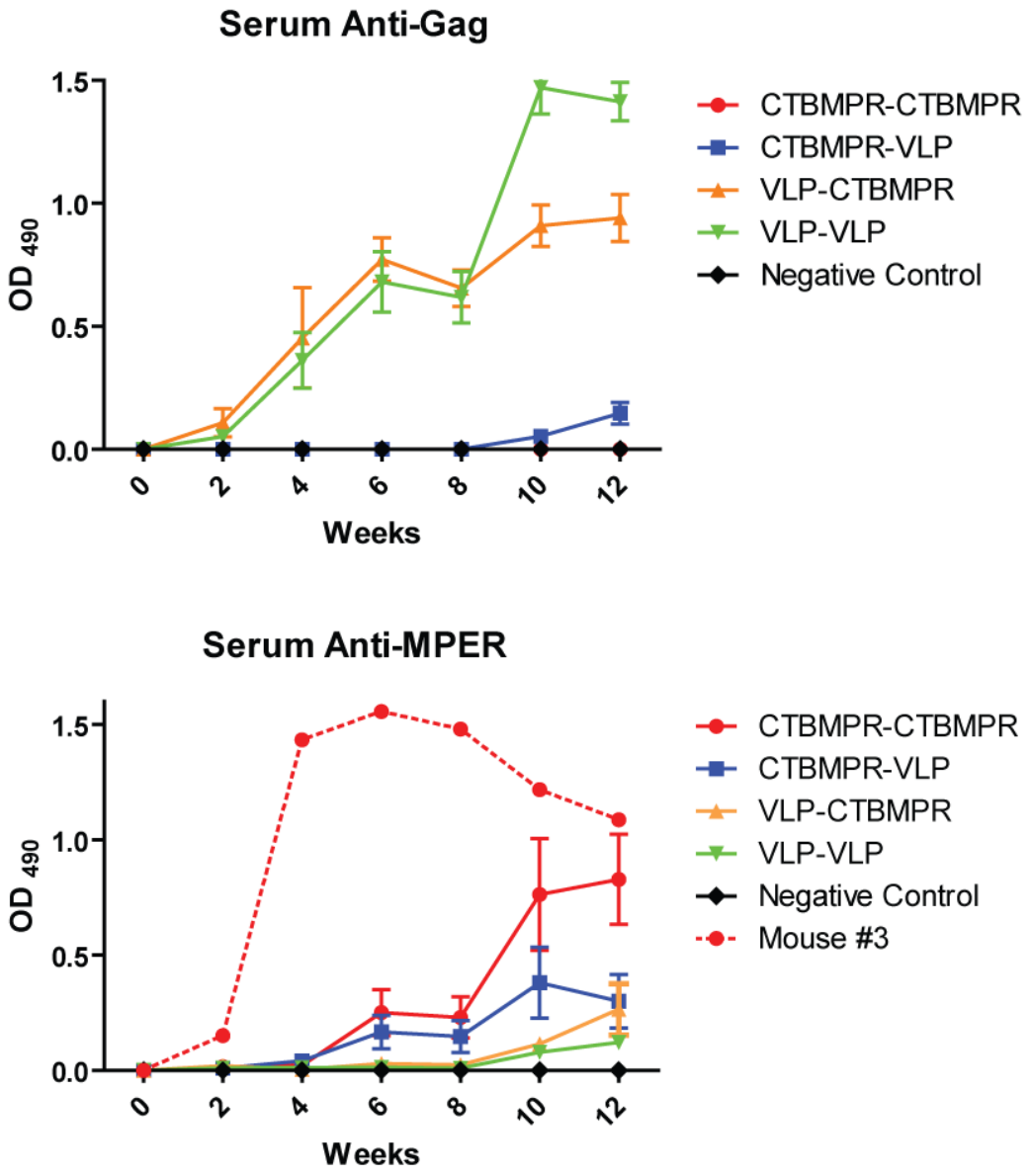


Figure 26. Serum anti-p24 and anti-MPER IgG levels. Mice were immunized as per Table 6. Serum samples were diluted 1:50 and IgGs were detected by direct ELISA on indicated weeks. Shown are average net OD_{490} values (mean +/- SEM). Mouse #3 (from CTBMPR-CTBMPR group) responded significantly higher than other mice from this group and was considered an outlier, and antibody response from this mouse is shown by itself.

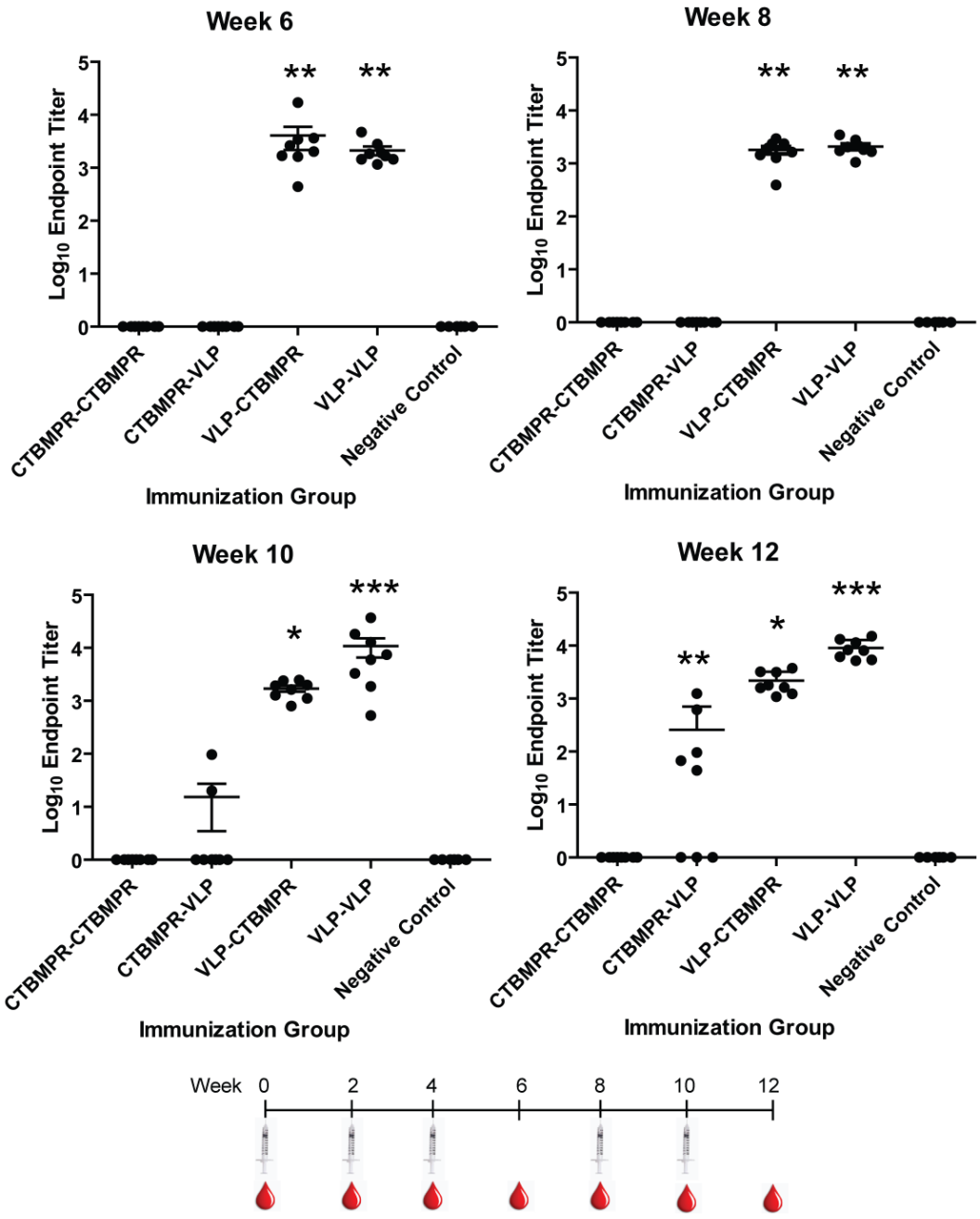


Figure 27. Serum anti-p24 IgG levels. Ab endpoint titers at response peaks after priming (Week 6), before first boost (Week 8), after first boost (Week 10), and after second boost (Week 12). Symbols indicate statistical significance as compared to week zero within the group evaluated by Kruscal-Wallis test and Dunn's Multiple Comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

significant ($p < 0.001$) values over the negative control mice. In mice primed with CTB-MPR and then boosted with VLPs, anti-p24 antibody levels corresponding to the first two priming immunizations of the VLP priming groups were elicited, and these titers were very significant ($p < 0.01$, in comparison to pre-boost levels) following the second boost. All of these data advocate that plant-produced VLPs can elicit robust, long-lived antibody responses against the Gag protein after only two immunizations.

Antibody Titers against MPER

The average OD_{490} (Figure 26) and endpoint titers (Figure 28) were calculated after the third priming immunization (Week 6), before the first boost (Week 8), after the first boost (Week 10), and after the second boost (Week 12) for antibodies against the MPER region of gp41. In mice primed with CTB-MPR, 11/16 mice responded to the MPER moiety before the second boost. One of these mice (#3 from the CTBMPR primed and boosted group) responded significantly more than the rest of the mice, and as such, the response of this mouse is shown on its own in Figure 24. In mice primed with VLPs, 7/16 mice responded to the MPER portion of dgp41. Boosting of the CTB-MPR primed group with CTB-MPR elicited very significant ($p < 0.01$) titers of anti-MPER antibodies after the second boost, which continued to increase after the second boost to extremely significant titers ($p < 0.001$) with 8/8 mice responding. Boosting the CTB-MPR primed group with VLPs elicited very significant titers as well with 8/8 mice responding, and though antibody titers did not reach the levels of the CTB-MPR boosted group, the difference between boosting groups was not significant. In mice primed with VLPs and boosted with CTB-MPR, extremely significant titers of anti-MPER antibodies were elicited compared to priming

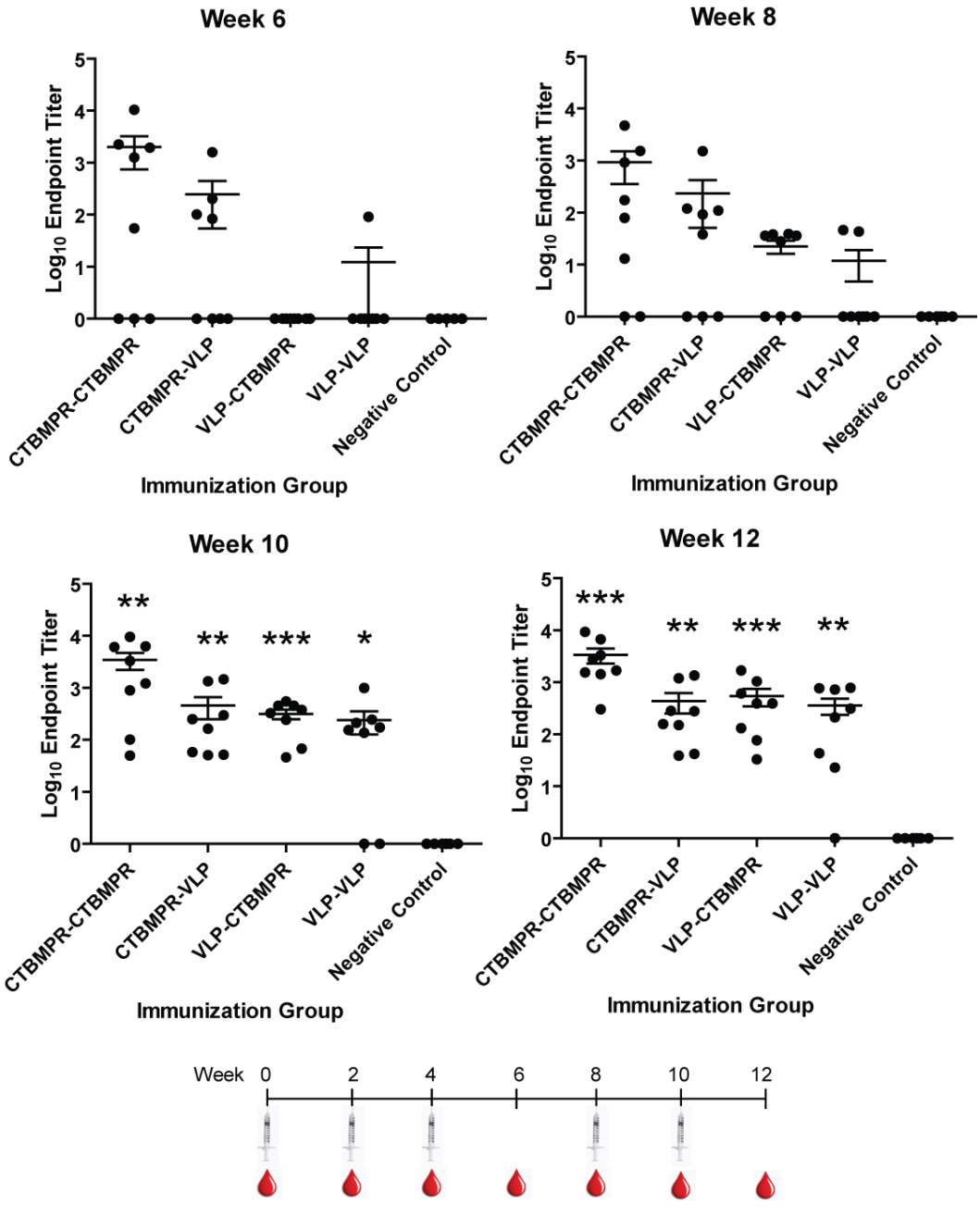


Figure 28. Serum anti-MPER IgG levels. Ab endpoint titers at response peaks after priming (Week 6), before first boost (Week 8), after first boost (Week 10), and after second boost (Week 12). Symbols indicate statistical significance as compared to week zero within the group evaluated by Kruscal-Wallis test and Dunn's Multiple Comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

immunizations, with 8/8 mice responding after the second boost. In mice primed with VLPs and boosted with VLPs, significant ($p < 0.05$) titers were raised after the first boost, which continued to increase to very significant ($p < 0.01$) titers following the second boost, with 7/8 mice responding. All four experimental groups elicited statistically significant antibody levels following the second boost, and while the group given CTB-MPR in both priming and boosting immunizations had slightly higher titers than the other groups, the remaining three groups utilizing either both CTB-MPR and VLPs or only VLPs elicited titers very similar to each other at the end of the second boost, and all four groups were not statistically different from each other.

Neutralization Assays

Mouse serum from the best responders was sent to collaborators for neutralization assays and I am currently awaiting results of these assays.

Transcytosis Assays

Mouse serum from each of the mice in the trial was sent to collaborators for performing transcytosis assays and I am currently awaiting results of these assays.

DISCUSSION

The immunogenicity of HIV-1 Gag/Env VLPs has been established in a number of animal trials in the past, but the immunogenicity of plant-expressed HIV VLPs (with or without surface-displayed Env proteins) had not been tested until this study. As presented above, HIV-1 Gag/dgp41 VLPs produced in *N. benthamiana* were able to elicit substantial titers of antibodies against both Gag and gp41 epitopes.

Much can be learned from this first trial with plant-based HIV-1 VLPs. The MPER peptide has been expressed in *E. coli* and plants before as a fusion peptide with CTB and shown to be immunogenic in mice in both cases (Matoba et al., 2004; Matoba et al., 2006; Matoba et al., 2009). Although only modest levels of anti-MPER antibodies were elicited through the trials, these antibodies possessed neutralization ability, suggesting that MPER could play an important role as a viable component of a vaccine candidate. Despite the relative success of the vaccine candidate, immunodominance of the CTB moiety presented an immunological challenge. It was hypothesized that this immunodominance could be combated by the development of additional fusion constructs including those utilizing peptides from *Yersinia pestis* (F1-V) or the Hepatitis B core antigen (HBc). Unfortunately, neither of these constructs elicited significant titers of anti-MPER antibodies, but instead further demonstrated the low immunogenicity of the MPER peptide. Once again, titers against the carrier peptide moiety of the fusion proteins continued to express immunodominance.

Since HIV-1 Gag had been shown to display HIV-1 Env proteins on their surface in native confirmation and been shown to be immunogenic against both the Gag and Env proteins, it was hypothesized that if enveloped particles made of Gag displaying a deconstructed gp41 consisting of the MPER region, transmembrane domain, and cytoplasmic tail could be expressed in plants, then they would also be immunogenic. If the particles were found to be immunogenic, then they could present possible solutions to the limitation of the CTB-MPR fusion candidate.

As was presented, plant-based VLPs did elicit an immune response when used as the vaccine antigen in either priming or boosting injections in either

homologous or heterologous platform prime-boosts in conjunction with CTB-MPR. Highly significant levels of antibodies were raised against the MPER region of gp41 after boosting with VLPs. These levels of antibodies were robust, continuing to rise throughout the course of the study without the taper evident in antibody levels raised by mice primed and boosted with CTB-MPR alone (confirming a phenomenon observed in previous studies (Matoba et al., 2006)), suggesting that antibodies produced by VLPs are long-lived. The levels of antibody titers after the second boost were not significantly different between groups primed or boosted with CTB-MPR, suggesting that VLPs can be as effective as CTB-MPR in eliciting an anti-MPER response.

In addition to the elicitation of anti-MPER antibodies, extremely significant titers of antibodies specific to the Gag protein were also elicited in groups that were either primed or boosted with the VLPs. Every mouse (16/16) that was primed with VLPs elicited a strong antibody response at the end of the three priming immunizations, with many responding as early as two weeks after the first priming immunization (Figure 26). These antibodies remained high throughout the remainder of the study even when mice were boosted with a construct that did not contain Gag, but two boosters with VLPs were successful in boosting the antibody titers of these mice. In addition, several (5/8) mice primed with CTB-MPR and boosted with VLPs elicited anti-p24 antibodies in levels that corresponded to the first two priming immunizations with VLPs, suggesting that if the trial had been extended, these titers would have continued to rise. Although the IgG subtypes of these antibody responses was not yet analyzed, the anti-Gag antibodies elicited in this trial represent an important

immune response against HIV-1, as the induction of a humoral response against the polyprotein could induce a protective response against the virus.

At the current time, results of functional assays assessing the quality of antibodies produced in this trial are not available, although these experiments are currently in progress by collaborators. Past results of trials utilizing VLPs and MPER peptides in other systems show that the antibodies raised against Gag and Env are able to block transcytosis of and neutralize HIV-1 virus. While results are not confirmed yet, the relatively high titers of antibodies raised against both of these proteins is promising, and could suggest that antibodies produced in this trial will also be successful in functional assays.

Overall, the results of this trial show that plant-produced VLPs can elicit significant titers of antibodies against both Gag and gp41 proteins. These results are promising, and inspire the use of these particles in additional immunization experiments with varied routes of immunization, which will be discussed in Chapter 5.

Chapter 5

MUCOSAL IMMUNIZATION OF MICE WITH GAG/DGP41 VLPS

ABSTRACT

Heterologous prime-boost immunizations, in which the route of immunization and/or the platform with which an antigen is displayed differ between the priming and boosting immunizations, have been shown to enhance the immune response versus a homologous immunization scheme. In this chapter, the success of a heterologous prime-boost strategy utilizing two different antigen displays of MPER as well as both mucosal and systemic routes of immunization will be explored.

INTRODUCTION

Decades worth of research have been conducted to no avail in the immense search for a prophylactic HIV-1 vaccine, but recent results of clinical trials have given researchers newfound hope in the development of an effective vaccine. In the most promising clinical trial to date, the RV 144 trial conducted in Thailand, a heterologous prime-boost strategy using priming injections of a recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]) expressing Env, Gag, and protease plus two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) was employed for modest protection against HIV-1 (Rerks-Ngarm et al., 2009). In clinical trials involving only one of the components of the vaccine, no significant protection was inferred (Pitisuttithum et al., 2006), which has lead researchers to investigate the reasons behind the success of the trial (Johnston and Fauci, 2011). Many researchers believe that

the protection could be due to the heterologous prime-boost administration of the vaccine components (Benmira et al., 2010).

The repeated use of the same vector or platform with which the vaccine is administered during priming and boosting (ie. same viral vector for DNA vaccines or fusion partner for peptide-based vaccines) can induce a strong immune response against the vector or fusion partner, and overpower immune response against the target antigen (Brown et al., 2010). The immunodominance of the cholera toxin B-subunit (CTB) moiety in Matoba et al.'s CTB-MPR candidate is an example of this challenge (Matoba et al., 2004; Matoba et al., 2006; Matoba et al., 2008; Matoba et al., 2011). The dominant immune response against adenovirus-based vaccine vectors (Sumida et al., 2005), might have contributed to the failure of Ad5-based HIV-1 vaccines large-scale clinical trials such as the Step Study (Buchbinder et al., 2008). Although such examples of the immunodominance of carrier proteins and vectors are widespread (Brown et al., 2010), this recognition and response to the vectors and carrier proteins can be combated with a prime-boost strategy. By utilizing multiple vectors and/or protein presentations, immune response can be targeted away from these supporting platforms and directed instead to the intended vaccine component.

Heterologous prime-boost immunizations, vaccine regimens that employ different target antigens, delivery vehicles, and immunization routes throughout the course of vaccination, have seen great success across the field of HIV vaccinology over the past decade (reviewed in (Lu, 2009; Radosevic et al., 2009; Brown et al., 2010; Paris et al., 2010)).

In addition to the use of multiple antigens and/or antigen presentation, it has been accepted that a wholly systemic immunization strategy that lacks a

mucosal component is not sufficient for inducing sustained mucosal responses, even though they are able to elicit systemic T-cell responses (Ranasinghe and Ramshaw, 2009). In previous studies in the Mor lab examining the immunogenicity of the MPER peptide, it was discovered that a heterologous prime-boost strategy in which one immunogen was given either mucosally or systemically during priming, and then given as either mucosal or systemic injections during boosting immunizations, was more successful in eliciting a mucosal response than a single route of immunization (Matoba et al., 2006). In previous studies, it was determined that mucosal priming with adjuvant followed by systemic boosting elicited the best induction of anti-MPER antibodies. These data, in which *E. coli*-derived CTB-MPR was utilized, were supported by a second heterologous prime-boost trial involving mice mucosally primed with plant-based CTB-MPR and systemically boosted with the same (Matoba et al., 2009). As with the case of multiple vaccine platforms, the multiple routes of immunization are gaining impetus through the success of recent trials exploiting this strategy (Ranasinghe and Ramshaw, 2009).

It was previously shown (Chapter 4) that plant-based Gag/dgp41 VLPs are effective in eliciting immune responses when delivered systemically. In order to engage the mucosal system, mucosal immunization was employed to test the efficacy of a heterologous prime-boost strategy comparing the mucosal priming of two separate antigen displays with systemic boosting of either a homologous or heterologous antigen display platform.

MATERIALS AND METHODS

Plant-based VLP Enrichment

Plant-based VLPs for immunization trials were purified from leaves of transgenic plants expressing Gag/dgp41 that were snap frozen in liquid nitrogen and kept at -80°C in 20 g batches. Each batch was separately crushed with liquid nitrogen into a powder using a mortar and pestle and then suspended in plant extraction buffer (60 ml, 25 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8). Following shaking the suspension for 60 min on ice, it was then strained through miracloth to remove solid particles and centrifuged at 14,000 g for 20 min. Ammonium sulfate was added to clarified extract to 40% and shaken on ice for 60 min to precipitate VLPs. The extract was then centrifuged at 36,000 rpm for 30 minutes to pellet the VLPs. Pelleted VLPs were resuspended in PBS (4 ml) and layered on top of two 30% Optiprep cushions (from bottom, 1 ml of 50% iodixanol, 2 mls 30% iodixanol, 7 mls of 20% iodixanol, 2 mls VLP extract) and spun at 35,000 rpm in an SW41Ti rotor (Beckman-Coulter) for 5 h. The 30% iodixanol fraction (2 mls each) was collected and concentrated with a 300 kD-cutoff centricon. Quantifications of both Gag and gp41 were determined with quantification immunoblots as previously described.

E. coli-based CTB-MPR Expression and Purification

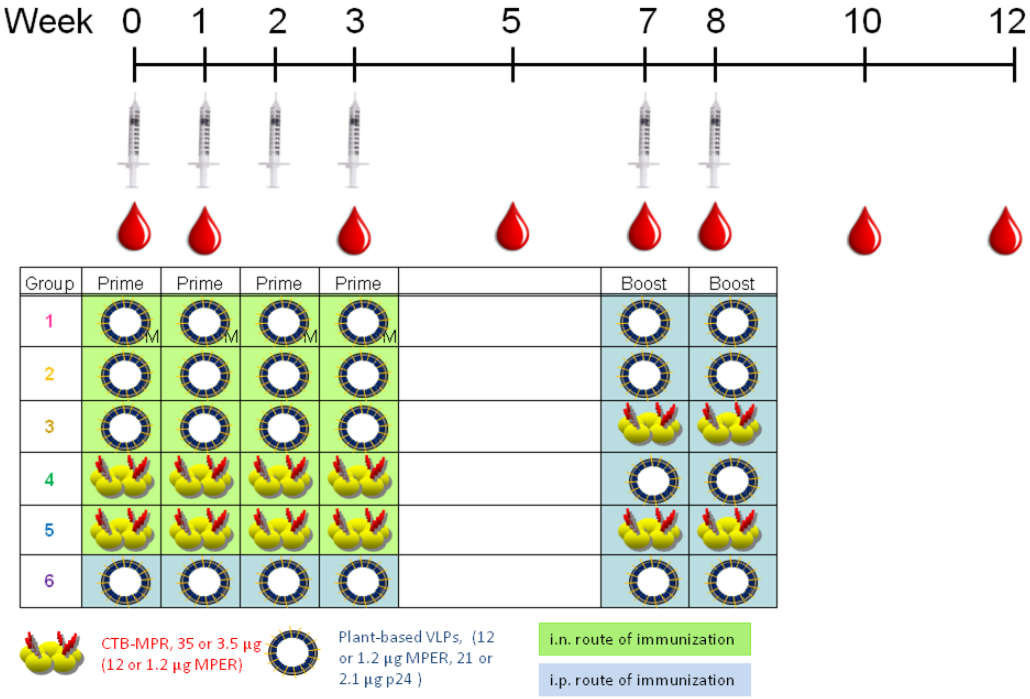
Expression of the CTB-MPR fusion protein in *E. coli* and its purification was performed as previously described (Matoba et al., 2008). Briefly, *E. coli* cultures containing pTM 199 were grown from single colonies overnight at 37°C. Logarithmic phase cultures were induced with 100 μM isopropyl β- D -1-thiogalactopyranoside (IPTG) and allowed to grow for 2 h before centrifugation of the cells. Cell pellets were resuspended in PBS (20 ml) and lysed by microfluidization. Lysed cells were pelleted by centrifugation, and the insoluble

pellet was solublized in CHAPS buffer (80 mM Tris, 2 M NaCl, 1.5% (w/v) CHAPS, pH 8.0), rocked at 4°C for 60 min, and then centrifuged to remove the remains of the insoluble cell fractions. The soluble supernatant was then added to a 25 ml Talon gravity flow column and purified by metal affinity chromatography. The final column eluate (50 ml) was dialyzed in PBS with a 3.5 kD-cutoff membrane for 24 h to remove CHAPS and imidizole, and then centrifuged at 2,000 g for 20 min to pellet the CTB-MPR. Pelleted protein was collected, washed 2x with PBS, and resuspended in sterile PBS (500 µl). Purity and quantification of the pure CTB-MPR was determined by Coomassie stained gels, quantitative immunoblots (using a pure standard), and by determining the absorbance at 280 nm using (ε = 2.1 mM^{-1} cm^{-1}).

Immunizations

The experimental protocol involving animals was approved by the Institutional Animal Care and Use Committee of Arizona State University. Female BALB/c mice (6-wk old, n=8 per group) were given four intranasal (i.n.) priming immunizations with CTB-MPR (35 µg, containing 12 µg MPER) with cholera toxin (CT) (1 µg), purified VLPs (containing 12 µg MPER, 21 µg p24) with CT (1 µg), or purified VLPs (containing 12 µg MPER, 21 µg p24) with murabutide (200 µg) (Table 7). One group was given four intraperitoneal (i.p.) priming immunizations with purified VLPs (1.2 µg MPER, 2.1 µg p24) with ribi adjuvant (Sigma-Aldrich). All mice were given two i.p. boosting immunizations with either CTB-MPR (3.5 µg, containing 1.2 µg MPER) or purified VLPs (1.2 µg MPER, 2.1 µg p24) (Table 7). Serum, vaginal secretions, and fecal pellets were collected from all mice on indicated days through the end of the trial. Fecal pellets were prepared by soaking five pellets (~50 mg) in PBS containing 0.02% Na-azide

Table 7. Mucosal immunization schedule.



(500 µl) for 30 min at 4°C with occasional vortex and clarification by centrifugation at 12,000 rpm for 10 min. Vaginal secretions were collected by lavaging using PBS (100 µl) with a blunt-tipped syringe needle.

Antibody Titer Assays

ELISA plates were coated with 20 µg of streptavidin (Sigma-Aldrich) and 2 µg of biotinylated MPR peptide (for detection of MPER antibodies) or 1 µg of p24-CTA2. Threefold serial dilutions starting from 1:50 for serum, 1:5 for vaginal secretions, and 1:2 for fecal extract in PBS containing 0.5%Tween 100 and 5% dry milk (PBSTM) were applied on to the plates and incubated for 1 h at 37°C. Serum IgGs and IgAs were detected by peroxidase conjugate of anti-mouse IgG (Calbiochem) and anti-mouse IgA (Sigma), respectively. Endpoint titers were determined as the reciprocal of the dilution factor of sample giving background levels of OD_{490}.

IFN-γ ELISPOT Assay

Splenocytes were prepared from spleens harvested on week 12 and pooled from 8 mice per group. Interferon-gamma (IFN-γ) Enzyme-Linked Immunosorbent Spot Assay (ELISPOT) responses were measured using a mouse IFN-γ set (BD Biosciences). Threefold serially diluted triplicates of splenocytes (starting at 1×10^6 splenocytes/well) were applied to the plates in a final volume of 200 µl RPMI 1640 culture medium (with 10% heat inactivated fecal bovine serum, 100 U/ml penicillin, 100 µg streptomycin). The peptides AAMQMLKDTINEEAA (corresponding to the GagCD8 epitope, from HIV-1 Consensus C Gag (15-mer) Peptides, Cat#8118, NIH AIDS Reagent Program) and SNPPVPVGDIYKRWI/VPVGDIYKRWIILGL (corresponding to the GagCD4 epitope, from HIV-1 Consensus C Gag (15-mer) Peptides, Cat#8118, NIH AIDS

Reagent Program) were used as stimuli in the assay at a final concentration of 5 µg/ml. Reactions without peptide served as background controls. Reactions were allowed to proceed for 30 h at 37°C in a humidified 5% CO_2 atmosphere. Spots were detected with the detection antibody, developed with 3-amino-9-ethyl-carbazole (AEC Substrate Set; BD Biosciences), and analyzed using the CTL ImmunoSpot plate reader and counting software (Cellular Technology Ltd.). For each group of mice, the number of background spots in the absence of peptide was subtracted from the average of the triplicate values in order to determine the number of peptide-relevant spots.

Transcytosis Assays

Transcytosis assays were performed by the lab of Morgane Bomsel at the Institute Cochin in Paris, France as described (Bomsel et al., 1998; Alfsen et al., 2001). Epithelial cells were grown as a confluent monolayer on permeable support (Figure 24). Samples of Ig were partially purified by ammonium sulfate (0-50% saturation fraction) and then dialyzed against RPMI medium 1640, 10% FCS. Cells (10^6/40µl) infected with HIV-1NDK were incubated at the apical pole of epithelial cells. Transcytosis was allowed to proceed for 110 min at 37°C. Viral load in the basolateral chamber was estimated from the p24 content (p24 kit, Coulter). Transcytosis was expressed as percentage of control.

Neutralization Assays

Neutralization assays were performed by the lab of Nobuyuki Matoba at the Owensboro Cancer Research Center in Owensboro, KY as previously described (Montefiori, 2009; Matoba et al., 2010). Env-pseudotyped viruses were prepared by co-transfection of 293T/17 cells with various *env*-expressing plasmids and an *env*-deficient HIV-1 backbone vector (pSG3ΔEnv) and were

titrated in TZM-bl cells to determine the 50% tissue culture infective dose ($TCID_{50}$) (Figure 25). Antiviral activity was expressed as an IC_{50} value, which is the sample concentration giving 50% of relative luminescence units (RLUs) compared with those of virus control after subtraction of background RLUs. The broadly neutralizing mAbs b12, 2G12, 2F5 and 4E10, as well as soluble CD4 were used as positive controls. Two-hundred $TCID_{50}$ of pseudoviruses were used for the neutralization assay. Samples and the virus were mixed and incubated for 1 h at 37°C, to which 10^4 cells/well of TZM-bl cells were added and incubated for 72 h. Luciferase activity was measured using the Britelite Plus Reagent (PerkinElmer, Waltham, MA).

RESULTS

Serum Antibody Titers Against p24

No serum antibody titers against p24 were elicited in any of the mucosally primed mice prior to boosting immunizations (Figure 29), although mice primed systemically displayed anti-p24 titers consistent with the first immunization trial (Chapter 4) with 6/8 mice responding to Gag by Week 3 of the trial. After boosting, mice in all groups boosted with VLPs responded to the Gag protein, with all but two mice (one in the group primed mucosally with VLPs and one in the group primed with CTB-MPR) in these groups expressing antibodies against p24 (Figure 30). Titers in Groups 1 and 6 were extremely significantly greater ($p < 0.001$) than mice naïve to Gag protein (Group 5), as well as very significantly ($p < 0.01$) greater than those in Group 3.

Serum Antibody Titers Against MPER

Similar to the anti-p24 priming response, no serum antibody titers against MPR were elicited in any of the mucosally primed mice prior to boosting immunizations (Figure 29). In accordance with the systemic trial, no significant antibody titers against MPR were raised in the systemically primed mice prior to boosting, either. Only one mouse, from Group 5 (mucosally primed with CTB-MPR and systemically boosted with the same) responded to MPER after the first boost. After the second boost, 6/8 mice in Group 1, 5/8 mice in Group 2, 3/8 mice in Group 3, 4/8 mice in Group 4, 8/8 mice in Group 5, and 8/8 mice in Group 6 were responding to MPER (Figure 30). Titers between Groups 1, 2, 3, 4, and 6 were all statistically similar, though antibody titers in Group 5 were significantly ($p < 0.05$) higher than the rest of the groups.

Fecal Antibody Titers Against p24

Anti-Gag fecal IgAs were detectible after the second boost. Two weeks after the second boost, 2/8 mice in Group 1, 2/8 mice in Group 2, 3/8 mice in Group 3, 1/8 mice in Group 4, 0/8 mice in Group 5, and 6/8 mice in Group 6 were responding to p24. Only the mice in Group 6 (primed and boosted systemically with VLPs) elicited significant ($p < 0.01$) anti-p24 titers compared to mice naïve to Gag.

Fecal Antibody Titers Against MPER

Similar to antibody titers against p24, no fecal antibody titers against MPER were elicited in any of the mice prior to boosting immunizations. Only

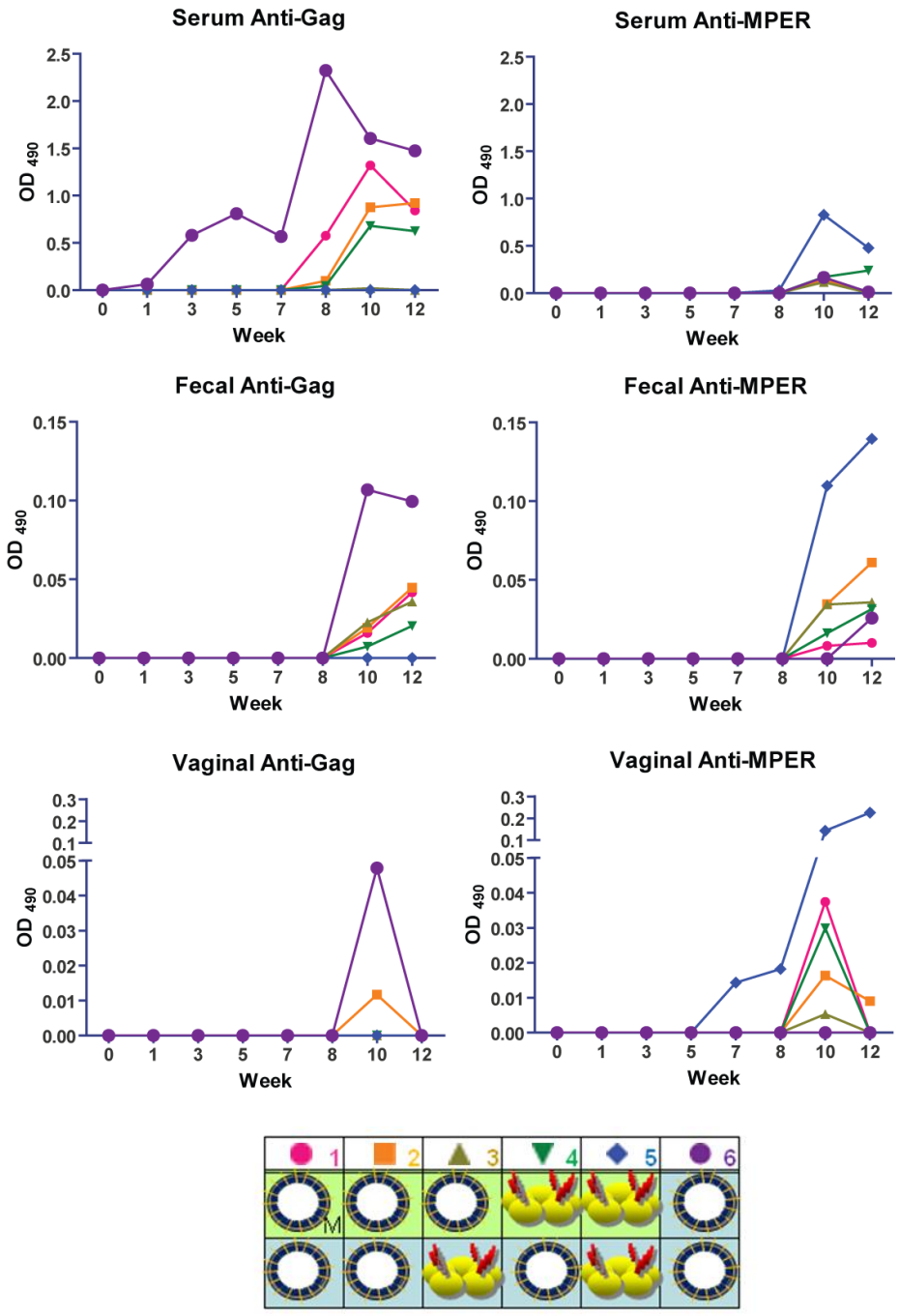


Figure 29. IgG and IgA response across all weeks of mucosal immunization. Serum, fecal, and vaginal secretion samples were taken on weeks 0,1, 3, 5, 7, 8, 10, and 12. Time points are averages of all mice in each group.

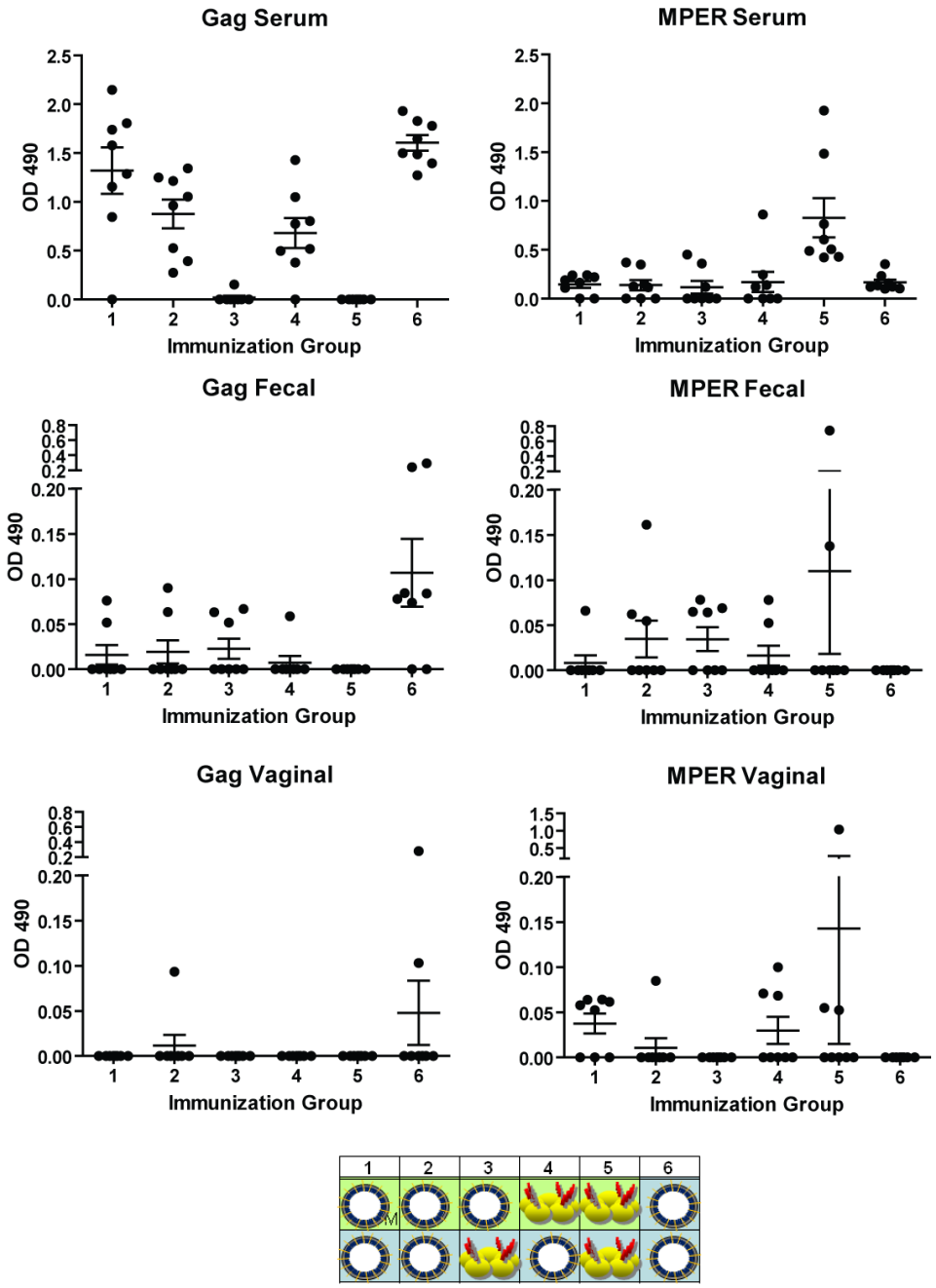


Figure 30. Post-boosting (Week 10) antibody titers against Gag or MPR in serum (IgG), fecal (IgA), and vaginal (IgA) samples. OD values are arithmetic means of all mice in each group +/- S.E.M.

after the second boost were fecal antibody titers seen in any of the groups. Two weeks after the second boost, 1/8 mice in Group 1, 3/8 mice in Group 2, 4/8 mice in Group 3, 2/8 mice in Group 4, 2/8 mice in Group 5, and 0/8 mice in Group 6 were responding to MPER. Although only two mice responded to MPER in Group 5, one mouse responded very strongly (OD_{490} = 0.74), though this high titer was not enough to make the average values significant over the other groups.

Vaginal Antibody Titers Against p24

No vaginal antibody titers against p24 were elicited in any of the mice prior to boosting immunizations, and only three mice (one from Group 2 and two from Group 6) responded two weeks after the second boost.

Vaginal Antibody Titers Against MPER

Unlike vaginal titers against p24, one mouse in Group 5 showed antibody titers against MPR in vaginal secretions at Week 7 prior to the boosts, and two mice in Group 5 were positive for anti-MPR antibodies at Week 8 after the second boost. Two weeks after the second boost, 5/8 mice in Group 1, 1/8 mice in Group 2, 0/8 mice in Group 3, 3/8 mice in Group 4, 3/8 mice in Group 5, and 0/8 mice in Group 6 were responding to MPER.

IFN-γ ELISPOT Assay

Cellular responses against the Gag protein were assayed by IFN-γ ELISPOT (Figure 31). The mice in Groups 1, 2, 4, and 6 responded to both Gag CD8+ and CD4+ peptides, while the mice in Group 5 had marginal response to the Gag CD4+ peptide. Mice in Group 5 did not respond to either peptide. Responses seemed to be generally higher against the GagCD8+ epitope, with Groups 2 and 6 showing the greatest response for either peptide.

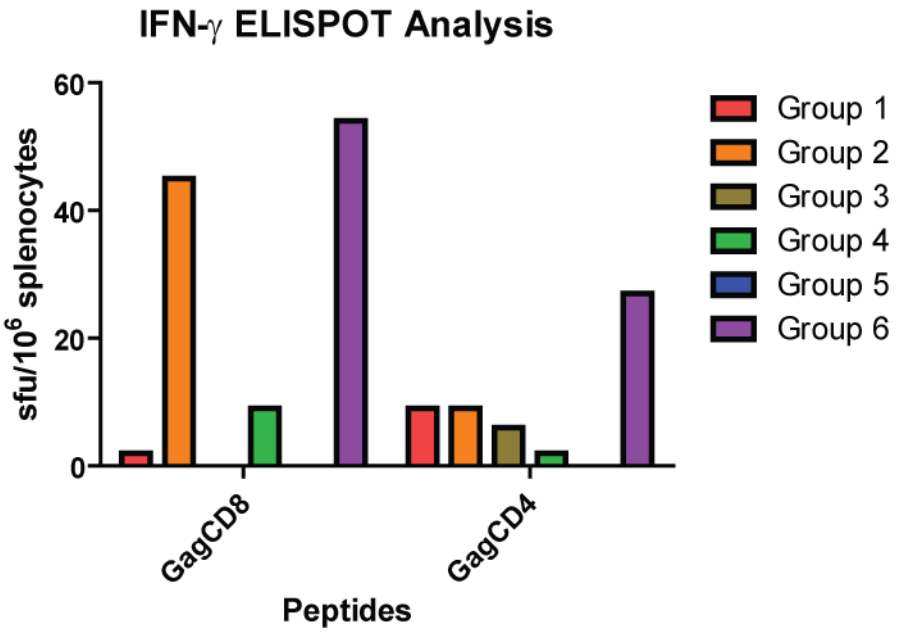


Figure 31. IFN-γ ELISPOT assay. Splenocytes were harvested, isolated, and pooled from mice in each group and incubated with or without peptides corresponding to the Gag CD8+ or GagCD4+ epitopes. Data are averages of triplicate values, with number of background spots from wells containing no peptide subtracted from this average.

Neutralization Assays

Mouse serum from the best responders was sent to collaborators for neutralization assays and I am currently awaiting results of these assays.

Transcytosis Assays

Mouse serum from each of the mice in the trial was sent to collaborators for performing transcytosis assays and I am currently awaiting results of these assays.

DISCUSSION

After a successful trial that resulted from VLPs used to prime and boost mice systemically, a mucosal trial utilizing a prime-boost strategy was employed to test the effectiveness of intranasal priming with systemic boosting of plant-produced VLPs, CTB-MPR, or a combination of both.

The mucosal priming of all groups: either with VLPs (with either murabutide or CT as adjuvants) or CTB-MPR was not able to elicit significant antibody titers in serum, fecal, or vaginal samples before boosting. Although this lack of mucosal priming response was discouraging, the results were fairly consistent with previous studies employing the mucosal priming with CTB-MPR, in which only marginal (or no) response was seen prior to boosting immunizations (Matoba et al., 2004; Matoba et al., 2006). In accord with the previous systemic trial, i.p. priming of VLPs (Group 6) was able to elicit significant serum titers of antibodies against Gag, although the lack of response against MPER in these mice prior to boosting was also confirmed in this trial.

Despite the lack of response during mucosal priming immunizations, systemic boosting was able to elicit significant antibody titers against Gag and

gp41 in serum, fecal, and vaginal samples in many of the groups. Systemic boosting with VLPs was able to elicit anti-p24 antibodies in all but two mice boosted with the VLPs. Although there was no significant difference in anti-p24 antibodies post-boost between the groups boosted with VLPs, only Groups 1 and 6 (primed systemically with VLPs or primed mucosally with the use of murabutide adjuvant, respectively) were able to elicit significant titers over mice naïve to the Gag protein. Once again, this confirms the effectiveness of VLP systemic priming and boosting to elicit a response against Gag, but it also suggests the effectiveness of mucosal priming with systemic boost to elicit this same response. Mucosal response against Gag was limited in all groups either primed or boosted with VLPs, although 6/8 mice were positive for IgAs in fecal samples in Group 6, with these levels being significant against those in Groups 4 and 5. Responses from Groups 1, 2, and 3 (mucosally primed with VLPs) were all statistically similar, and though their responses were not statistically different from Groups 4 and 5 (primed with CTB-MPR), antibody responses and number of responders were all greater in the VLP primed groups compared to those primed with CTB-MPR. While this evidence is not statistically significant, it does suggest that priming with VLPs might assist in eliciting a mucosal response against Gag, though this response is not as great as that elicited with systemic priming with VLPs. Unfortunately, very little response against Gag was discovered in vaginal secretions of mice in any group. Only three mice responded two weeks after the last boost (one in Group 2 and two in Group 3).

Serum antibodies against MPER were not seen until after boosting. Although one mouse from Group 5 responded after only one boost, the majority of serum anti-MPER antibody response did not occur until two weeks post-boost

in all groups. Systemic boosting with CTB-MPR following mucosal priming with the same (Group 5) elicited extremely significant ($p < 0.001$) levels of antibodies as compared to naïve mice, consistent with previous studies (Matoba et al., 2006). Unfortunately, mucosal priming with VLPs and boosted with either VLPs or CTB-MPR (Groups 1, 2, and 3) elicited only marginal levels of antibodies, and the same was true for mucosal priming with CTB-MPR and boosting with VLPs. Although 8/8 mice responded in Group 6 (systemically primed and boosted with VLPs), the levels of antibodies was lower in this group than in the CTB-MPR primed and boosted group (Group 5). However, it should be noted that these two groups were not statistically significantly different. Only low levels of antibodies and a total of 12/48 mice responded to MPER in fecal samples. The two responders in Group 5 had two of the highest responses, but no group reached statistically significant responses in comparison to naïve mice. Mice in Groups 2, 3, and 4 had similar responses, but only one mouse in Group 1 and zero mice in Group 6 responded to MPER in fecal samples. The results of MPER response in fecal samples is inconclusive, but it is suggested that mice mucosally primed with CTB-MPR and boosted systemically with the same can elicit a moderate IgA response in fecal samples, while systemic priming and boosting with VLPs was largely ineffective in eliciting an anti-MPR response in mice. In consistence with low levels of anti-MPER antibodies in fecal samples, only 12/48 mice from all groups responded to MPER in vaginal secretions, with no statistical significance. Once again, two mice in Group 5 (the same mice with higher anti-MPER response in fecal samples) had two of the highest overall responses, while vaginal anti-MPER responses from other groups were fairly insignificant. Consistent with anti-MPER fecal results, no anti-MPER antibodies were detected

in vaginal secretions from mice in Group 6. Overall, the results of anti-MPER response in vaginal secretions are congruent with results of anti-MPER response in fecal samples. All of these results suggest that while systemic priming and boosting with VLPs can be successful in eliciting antibody responses against Gag and gp41, mucosal priming may not be nearly as effective in this case.

In addition to the humoral and mucosal immue responses, the cellular responses against peptides corresponding to Gag CD4+ and CD8+ epitopes were also explored. Mice primed (either mucosally or systemically) and boosted with Gag/dgp41 VLPs had responses to both peptides, suggesting a cellular response against Gag in these groups. Mice primed and boosted systemically had higher responses than the mucosally primed groups. Mice receiving either only a priming immunization or a boosting immunization with the VLPs had lower responses than those groups that were both primed and boosted with the VLPs. Mice primed and boosted with CTB-MPR did not respond to either peptide. These results suggest that both CD8 and CD4 T-cell responses against Gag were elicited with the VLPs.

At the current time, results of functional assays assessing the quality of antibodies produced in this trial are not available, as terminal samples from mice were collected just prior to publication. As with the systemic trial, it is my hope that the modest levels of antibodies elicited by the mucosal trial are able to block transcytosis of and neutralize HIV-1 virus, and thus be consistent with previous studies using Gag/Env VLPs and MPER.

Overall, the results of this trial show that although antibodies against Gag or gp41 were not elicited during mucosal priming immunizations, plant-produced VLPs can elicit antibodies against both Gag and gp41 proteins in serum and

fecal samples after a systemic boost, offering further evidence that plant-based Gag/dgp41 VLPs could provide an effective component for future HIV-1 immunization trials.

Chapter 6

SUMMARY AND OUTLOOK

HIV/AIDS is one of the largest global epidemics with more than 33 million people currently infected worldwide and more than two million new infections occurring yearly (UNAIDS/WHO, 2009). Despite over two decades of research, an effective prophylactic vaccine against HIV remains elusive (Johnston and Fauci, 2008), but recent clinical trials have given researchers hope and direction in which to focus their efforts (Benmira et al., 2010).

Some of the recent successes both in clinical and animal trials have focused on utilizing mucosally-targeted, highly conserved HIV-1 antigens as vaccine candidates. Combating the challenges of the hypervariablity of HIV-1 and the high rates of mucosal transmission of the virus, these candidates provide hope in overcoming some of the substantial hurdles experienced by previous failed trials. In addition to these challenges, if a vaccine is to be effective in reaching the majority of the at-risk population, largely located in developing countries, it must be inexpensively manufactured, stored, and distributed. A vaccine candidate that addresses all of these components could be successful in accomplishing a decline in the rates of new infection worldwide, eventually leading to the eradication of the epidemic.

To this end, a vaccine candidate based on a Clade C Gag VLP with deconstructed gp41 (dgp41) proteins embedded in its membrane envelope was developed and expressed in *N. benthamiana*. By utilizing highly conserved, mucosally-targeted epitopes within gp41 in combination with clade-specific, CTL-inducing Gag VLPs as a platform with which to introduce the non-immunogenic gp41, I hoped to induce both mucosal and humoral responses against both

proteins. By expressing these VLPs in *Nicotiana benthamina*, I hoped to overcome the cost and scale limitations of other cell culture systems, which will prove beneficial for distributing the successful vaccine candidate to the at-risk populations around the globe.

The expression of HIV-1 Gag and envelope proteins in plants has not been studied extensively to date. Results from expression studies in plants with these proteins have varied tremendously, but only a few researchers experienced success with moderate levels of expression. Not only was I able to express p55 Gag and gp41 in transient expression systems, I was able to create stably transgenic plants expressing moderate levels of Gag. In addition to expressing both of these proteins separately, I was also successful in co-expressing the two proteins, which seemed to stabilize the expression of both Gag and dgp41.

Although simply expressing these two proteins together was a first in plants, I wanted to ensure that the Gag protein was successfully forming VLPs which incorporated the dgp41 into the envelope. One other group (Scotti et al., 2009) was able to express full length p55 Gag in plants that developed into VLPs, but these particles were not fully characterized and it was not determined if these particles were enveloped or not.

This study was the first to fully characterize enveloped VLPs in *N. benthamiana*. The results of the sucrose density sedimentation, Optiprep density sedimentation, trypsin digestion assay, and TEM all suggested that Gag is forming ~100 nm VLPs in *N. benthamiana*. In addition to expressing Gag in plants, I was able to co-express dgp41 with the Gag VLPs. In addition to co-

expressing the two proteins, all of my biochemical assays have suggested that the two proteins are assembling together into enveloped VLPs.

The expression and accumulation of HIV-1 Gag/dgp41 VLPs within plants presents a milestone in the field of plant biology, but the end goal of the project was to determine if these particles were immunogenic. The immunogenicity of HIV-1 Gag/Env VLPs (produced in other cell culture systems) has been established in a number of animal trials in the past, but the immunogenicity of plant-expressed HIV VLPs (with or without surface-displayed Env proteins) had not been tested until this study.

During the first trial, I demonstrated that plant-based VLPs can elicit immune responses against both Gag and dgp41 when used as the antigen in either priming or boosting injections in either homologous or heterologous antigen platform prime-boosts in conjunction with CTB-MPR. Highly significant levels of antibodies were raised against the MPER region of gp41 after boosting with VLPs, and these levels continued to rise throughout the course of the study, suggesting that the antibodies are robust and long-lived. Levels of antibodies after boosting suggested that VLPs can be as effective as CTB-MPR in eliciting an anti-MPER response.

In addition to the elicitation of anti-MPER antibodies, extremely significant titers of antibodies specific to the Gag protein were also elicited in groups that were either primed or boosted with the VLPs, with a 100% response rate in mice primed with the VLPs. Similar to anti-MPER titers, anti-Gag antibody titers remained high throughout the remainder of the study even when mice were boosted with a construct that did not contain Gag. It was also determined that mice which only received two boosters with VLPs (primed with CTB-MPR) were

still able to elicit significant levels of anti-Gag antibodies. All of these results were promising, and inspired the use of these particles in additional immunization experiments with varied routes of immunization.

A mucosal trial utilizing a prime-boost strategy was employed to test the effectiveness of intranasal priming with systemic boosting of plant-produced VLPs, CTB-MPR, or a combination of both. In congruence with previous studies (Matoba et al., 2004; Matoba et al., 2006), mucosal priming with CTB-MPR did not elicit significant titers of anti-MPER antibodies prior to boosting immunizations, and mucosal priming with VLPs mimicked this response. Promisingly, in accord with the previous systemic trial, i.p. priming of VLPs (Group 6) was able to elicit significant serum titers of antibodies against Gag, although the lack of response against MPER in these mice prior to boosting was also confirmed in this trial.

Systemic boosting following mucosal priming was able to elicit significant antibody titers against Gag and gp41 in serum, fecal, and vaginal samples in many of the groups. Although there was no significant difference in anti-p24 antibodies post-boost between the groups boosted with VLPs, only mice primed systemically with VLPs or primed mucosally with the use of murabutide adjuvant were able to elicit significant serum titers over mice naïve to the Gag protein. Once again, this confirms the effectiveness of VLP systemic priming and boosting to elicit a response against Gag, but it also suggests the effectiveness of mucosal priming with systemic boost to elicit this same response. Mucosal response against Gag was limited in all groups either primed or boosted with VLPs, although significant levels of fecal IgAs were elicited in mice systemically primed and boosted with VLPs, suggesting that mucosal priming with VLPs might

assist in eliciting a mucosal response against Gag. Unfortunately, very little response against Gag was discovered in vaginal secretions of mice in any group, but the little response that was elicited was manifested in groups mucosally primed with VLPs.

Systemic boosting with CTB-MPR following mucosal priming with the same elicited extremely significant ($p < 0.001$) levels of antibodies against MPER as compared to naïve mice, which was consistent with previous studies (Matoba et al., 2006). Unfortunately, mucosal priming with VLPs and boosted with either VLPs or CTB-MPR elicited only marginal levels of antibodies, and the same was true for mucosal priming with CTB-MPR and boosting with VLPs. A 100% response rate for anti-MPER serum antibodies was seen in mice systemically primed and boosted with VLPs, though the ODs of this group were lower than those in the CTB-MPR primed and boosted group. The results of MPER response in fecal samples are inconclusive, but it is suggested that mice mucosally primed with CTB-MPR and boosted systemically with the same can elicit a moderate IgA response in fecal samples, while systemic priming and boosting with VLPs was largely ineffective in eliciting an anti-MPR response in mice. In consistence with low levels of anti-MPR antibodies in fecal samples, only 12/48 mice from all groups responded to MPR in vaginal secretions, with no statistical significance. Overall, the results of anti-MPR response in vaginal secretions are congruent with results of anti-MPR response in fecal samples. All of these results suggest that while systemic priming and boosting with VLPs can be successful in eliciting antibody responses against Gag and gp41, mucosal priming may not be nearly as effective in this case.

At the current time, results of functional assays assessing the quality of antibodies produced in this trial are not available, as terminal samples from mice were collected just prior to publication. As with the systemic trial, it is my hope that the modest levels of antibodies elicited by the mucosal trial are able to block transcytosis of and neutralize HIV-1 virus, and thus be consistent with previous studies using Gag/Env VLPs and MPER.

Overall, the results of this trial show that although antibodies against Gag or gp41 were not elicited during mucosal priming immunizations, plant-produced VLPs can elicit antibodies against both Gag and gp41 proteins in serum and fecal samples after a systemic boost.

The promising results from both the systemic and heterologous prime-boost immunization trial suggest that plant-based Gag/dgp41 VLPs suggest that the VLPs might make an attractive candidate for an ant-HIV-1 vaccine. Additional animal studies utilizing different antigen levels, adjuvants, routes of inoculation, and other protein platforms can be performed to explore the best immunization protocol for eliciting strong mucosal and systemic immune responses. Successful protocols can then be used in additional animal models, such as rabbits and eventually non-human primates. Success in these animal models could lead to the use of the plant-produced VLPs in human clinical trials.

In addition to exploring other protein partners with which to immunize, the partnering of antigen vaccines with DNA vector vaccines is also an angle in which to explore. The moderately effective RV 144 trial utilized a recombinant canarypox DNA vector priming immunization regimen with gp120 boosts (Rerks-Ngarm et al., 2009). In addition to the success of this clinical trial, the use of DNA vector priming immunizations coupled with VLP-based boosting

immunizations have been successful in eliciting T_h responses and neutralizing antibody responses in non-human primates (Radaelli et al., 2003; Zanotto et al., 2003). This method of immunization might hold promise with the use of plant-based Gag/dgp41 VLPs. By priming with a DNA vector vaccine such as a pox-vector based vaccine (Pantaleo et al., 2010) and boosting with the plant-produced Gag/dgp41 VLPs, I might see an even greater immune response than that of either candidate alone.

All of these options will be explored in the future in hopes of one day formulating a successful vaccine regimen against HIV-1 and halting the spread of the devastating epidemic.

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BIOGRAPHICAL SKETCH

Sarah Adeline Kessans was born in Oceanside, California on July, 1, 1983. She received her secondary education at Eastern High School in Pekin, Indiana where she first became interested in scientific research, earning multiple accolades at the state, national, and international levels of various science research competitions, culminating with becoming a finalist in the Intel Science Talent Search in 2001. During the summer of 2001, Sarah earned a trip to Israel to participate in the International Summer Science Institute at the Weizmann Institute of Science, where she solidified her love for scientific research. Sarah entered Purdue University in 2001, where she majored in Plant Biology. While at Purdue, Sarah worked in the labs of Ron Coolbaugh and Nick Carpita on projects in plant hormone and cell wall biosynthesis, respectively. In addition, she participated in an internship at the University College Dublin's Plant Pathology Department with Fiona Doohan. During Sarah's undergraduate career, she was a top rower on the Purdue Crew team, winning several National Championships with her team and earning Purdue's Most Valuable Oarswoman award in 2004. After graduating from Purdue with honors, Sarah attempted to row across the Atlantic Ocean with teammate Emily Kohl as the only Americans in the 2005 Woodvale Events Atlantic Rowing Race. The pair capsized in a tropical storm after 46 days at sea and were rescued 16 hours later. Undaunted, the pair joined up with two additional rowers and went on to race in the 2007 Race, finishing the race in 51 days to break the Women's Fours Atlantic Rowing World Record. Sarah began her graduate work at Arizona State University in 2006, earning a one year fellowship from the Biological Design graduate program. During her graduate career, Sarah coached the ASU women's rowing team and was involved with several graduate student organizations such as the Graduate and Professional Student Association and SOLS Grads. After earning her PhD, Sarah plans to pursue post-doctoral research in biochemistry at the University of Otago in New Zealand. Following post-doctoral work, she hopes to pursue a career in scientific research, focusing on the development of plant-based pharmaceuticals.


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