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Karen Rowlandson
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

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Production and Evaluation of Plant-Derived Vaccines for Cytomegalovirus Using Guinea Pig as an Animal Model

TITRE DE LA THÈSE / TITLE OF THESIS

Eilleen Tackaberry
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Wangxue Chen (National Research Council)

Lionel Filion

Sean Li

Martin Pelchat

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Production and evaluation of plant-derived vaccines for cytomegalovirus using guinea pig as an animal model

Karen Rowlandson

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the PhD degree in Microbiology & Immunology

Department of Biochemistry, Microbiology & Immunology
Faculty of Medicine
University of Ottawa

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Abstract

Human cytomegalovirus (HCMV) infection is usually asymptomatic in healthy individuals. However, severe disease and/or death can occur in immunocompromised patients. The overall objective of this project was to develop a plant-derived CMV subunit vaccine and determine its effectiveness. Since CMV is species-specific, guinea pig CMV (GPCMV) was used as a model for HCMV. Four gene constructs were engineered to contain either the rice glutelin-1 promoter (Gt1) or the maize ubiquitin promoter, and the gene for either glycoprotein B (gB) or phosphoprotein 65 (pp65) of GPCMV. *Agrobacterium tumefaciens* were transformed with the Gt1-gB construct and subsequently used to transform *Oryza sativa* (rice) or *Arabidopsis thaliana*. Due to technical difficulties associated with rice transformations, experimental work was continued with only *A. thaliana*. PCR analysis of plant tissue confirmed maintenance of the gB transgene for seven generations, and Western blots of selected seed extracts revealed a gB-specific band not found in non-transformed *A. thaliana* extracts. Guinea pigs (4 per group) were subcutaneously immunized with 20 mg of seed protein extract from GPCMV gB *A. thaliana* plants (estimated to contain approximately 50 μg of gB), an equal amount of total protein from non-transformed *A. thaliana* seeds (negative control), or baculovirus-derived GPCMV gB (positive control). Two animals immunized with seed-derived gB demonstrated GPCMV-specific antibody responses when analysed by ELISA. Western blot analysis confirmed that this response was specific for GPCMV gB. Neutralization assays were performed using serum obtained 14 days after the last immunization. Three of the 4 animals immunized with the gB seed extract showed viral neutralizing responses, with titres ranging from 40 to 3240. These results indicate that GPCMV gB expressed in the seeds of *A.*

thaliana can generate a GPCMV-specific immune response. Based on data from GPCMV-specific ELISAs, Western blots, and viral neutralization assays using serum from immunized animals, I concluded that gB was authentically expressed in *A. thaliana* seeds with at least some epitopes similar to those found on native GPCMV gB that were able to neutralize viral infectivity.

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List of Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AIDS	autoimmune deficiency syndrome
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
bp	base pairs
BCA	bicinchonic acid
CD4+/8+	cluster of differentiation 4+/8+
CFA	complete Freund's adjuvant
CHO	Chinese hamster ovary
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
F-12K	Kaighn's Modification of Ham's F-12 Medium
F-12K/FBS	F-12K medium supplemented with FBS
FBS	fetal bovine serum
gB	glycoprotein B
GPCMV	guinea pig cytomegalovirus
GFP	green fluorescent protein
Gt1	glutelin 1
GUS	β-glucuronidase
HAART	highly active anti-retroviral therapy
HBsAg	hepatitis B surface antigen
HCMV	human cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIG	hyperimmune globulin
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IFA	incomplete Freund's adjuvant

Ig	immunoglobulin
kb	kilobase pairs
kDa	kilodaltons
LB	Luria Burtani
LT-B	lymphotoxin B
mAb	monoclonal antibody
MHC	major histocompatibility
MS	Murashige and Skoog
NK	natural killer
NOS	nopaline synthase
NT	non-transformed/non-transgenic
NVCP	Norwalk virus capsid protein
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pp65	phosphoprotein 65
RhCMV	rhesus cytomegalovirus
SDS	sodium dodecyl sulfate
sIgA	secretory IgA
ss	signal sequence
TAE	tris-acetate-EDTA
T-DNA	transfer DNA
Ubi	ubiquitin
U_L	unique long
U_S	unique short
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
YEP	yeast extract peptone

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1.0 Introduction

1.1. General overview of project

Human cytomegalovirus (HCMV) is a virus that infects the majority of the population. While healthy individuals are usually asymptomatic, HCMV can cause morbidity and mortality in immunocompromised individuals. As a result of the species-specificity of CMV, concerns with viral reactivation with live vaccines and consequences of latent infection, there is currently no approved vaccine for HCMV. The study described in this thesis used guinea pig CMV (GPCMV) as a model for HCMV to evaluate the immunogenicity of a plant-derived CMV subunit vaccine. In this introduction, the importance of HCMV as a human health concern is first discussed, and a general overview of CMV molecular biology and pathogenesis is presented. Second, the current state of vaccine development for HCMV and associated difficulties are presented. Third, plants as expression systems for recombinant proteins used as biotherapeutics, including vaccines, will be reviewed. Last, the project rationale will be presented.

1.2 Human cytomegalovirus

1.2.1 A major health concern

HCMV is a herpesvirus with an infectivity rate of 40 to 90% of the world's adult population (reviewed in (Pass, 2001)). Although not as well known as some other members of the herpesvirus family, HCMV can cause serious disease and potentially death in vulnerable populations. For example, transplant recipients and AIDS patients are highly susceptible to HCMV infection because of their immunosuppressed status. HCMV

infection in transplant recipients and AIDS patients can result in a number of clinical manifestations including hepatitis, pneumonia, retinitis, and organ/graft rejection (reviewed in (Mocarski and Courcelle, 2001)). In infants infected with HCMV *in utero*, HCMV can cause blindness, hearing loss, jaundice, and neurodevelopmental deficiencies (reviewed in (Bale et al., 2002)). HCMV is now emerging as a possible link to diseases such as atherosclerosis, cancer, and inflammatory bowel disease (Streblow et al., 2008; Michaelis et al., 2009; Nakase et al., 2008), indicating that a number of common pathologies may also be linked to HCMV infection. In addition, research has revealed that elderly individuals infected with HCMV have up to 25% of their CD8+ memory T cells dedicated to HCMV (Khan et al., 2004), showing that HCMV infection can be a significant burden on the immune system, and may potentially reduce the ability of the immune system to respond to other challenges. For these reasons, the Institute of Medicine in the United States issued a report indicating that the production of a vaccine against HCMV infection is a high priority for the 21st century (Arvin et al., 2004).

1.2.2 Molecular biology

HCMV is a member of the herpesvirus family, which also includes Epstein-Barr virus, herpes simplex virus 1 and 2, varicella zoster virus, and human herpesvirus 8, responsible for Kaposi's sarcoma. All cytomegaloviruses have large, linear double-stranded DNA genomes ranging from 200 to 240 kb (Mocarski and Courcelle, 2001). The 230 kb DNA genome of HCMV is estimated to contain between 200 and 250 open reading frames (Murphy et al., 2003). Many of these are still hypothetical coding regions and have yet to be

confirmed as encoding viral proteins (Murphy et al., 2003; Cha et al., 1996). The coding regions are organized into unique short (U_S) and unique long (U_L) sequences, which are flanked by terminal repeats and separated by internal repeat sequences (Britt and Boppana, 2004). The U_L and U_S can invert during replication, creating four isoforms of HCMV DNA (Bankier et al., 1991). The genetic material of HCMV is encapsulated within an icosahedral protein capsid (Britt and Boppana, 2004). Surrounding the nucleocapsid is an outer envelope consisting of a lipid bilayer, viral glycoproteins, and proteins derived from the infected cell (Mocarski and Courcelle, 2001). A somewhat unstructured region, known as the tegument, lies in between the envelope and nucleocapsid. This region contains a number of phosphorylated viral proteins along with host-cell proteins (Varnum et al., 2004). The viral proteins found within the tegument are involved in processes such as viral gene expression and viral envelopment, although the mechanisms for how the proteins are targeted to the tegument region during virion assembly are not yet known (Shen et al., 2008; Scheller et al., 2008; Kalejta, 2008).

1.2.3 HCMV strains

Although there is a high degree of homology between different strains of HCMV, polymorphisms exist within regions of the viral genome (Chantaraarphonkun and Bhattarakosol, 2007; Pignatelli et al., 2004). To date, five low-passage clinical isolates and two extensively passaged laboratory strains of HCMV, Towne and AD169, have been sequenced (reviewed in (Murphy and Shenk, 2008)). Studies have shown that it is possible for both immunocompetent and immunocompromised individuals to be infected with several

different strains of HCMV (Chandler et al., 1987; Boppana et al., 2001; Novak et al., 2008), indicating that despite the high degree of homology, exposure to one strain does not necessarily produce an immune response sufficient to protect against another strain. Amongst all HCMV strains, the genetic sequence encoding most of the viral envelope glycoprotein B (gB) is highly conserved except for certain regions that display high degrees of polymorphism between different HCMV strains. This is especially true for a hypervariable site surrounding the protease cleavage site of gB (Chou and Dennison, 1991). Grouping of HCMV clinical isolates based on the sequence of this hypervariable region revealed that there are four common genotypes of HCMV (gB1 to 4) (Chou and Dennison, 1991), and three rare genotypes (gB5 to 7) (Trincado et al., 2000). Many studies have evaluated the association of a specific genotype with a certain HCMV-infected population (Fries et al., 1994; Barbi et al., 2001; Gilbert et al., 1999; Arista et al., 2003; Yamamoto et al., 2007), but it has not been definitively confirmed whether a specific genotype is related to the severity of an infection in immunocompromised individuals (Pignatelli et al., 2004).

1.2.4 Viral life cycle, replication, and expression of proteins

The exact mechanism of HCMV cellular entry has not been completely elucidated. Analysis of tissue samples taken from infected individuals indicate that HCMV is able to infect a number of different cell types, including macrophages, endothelial cells, epithelial cells, fibroblasts, stromal cells, neuronal cells, neutrophils, and hepatocytes (reviewed in (Mocarski and Courcelle, 2001)). Because of the ability of the virus to infect a variety of cell types, it would appear that either the HCMV receptors are proteins commonly found on

the surface of many cell types, and/or HCMV uses a number of different receptors for cellular entry (reviewed in (Compton, 2004)). The current model for CMV cell entry involves the binding of viral envelope-associated glycoproteins, such as gB, and a glycoprotein M/N complex (gM/N), with cell surface heparin sulphate proteoglycans present on many different cell types (Compton et al., 1993; Isaacson et al., 2007). Other studies have shown that in conjunction with binding to heparin sulphate proteoglycans, co-receptors such as cellular integrins are required for HCMV cellular entry (Wang et al., 2005; Feire et al., 2004).

Once HCMV binds to a cell, the viral envelope fuses with the cellular membrane in a pH-dependent manner with the aid of a glycoprotein H/glycoprotein L (gH/gL) complex to release the tegument and nucleocapsid (including the viral genome) into the cell cytoplasm (Compton et al., 1992; Ryckman et al., 2008). Within 30 minutes, the nucleocapsids have moved towards the nucleus via nuclear localization signals and the viral DNA is released into the nucleus of the infected cell (Wood et al., 1997; Mocarski and Courcelle, 2001; Nguyen et al., 2008). How the viral DNA enters into the nucleus of the cell is not yet known (Tang and Maul, 2006).

Expression of the viral DNA occurs in a temporally regulated cascade beginning with immediate early (IE) genes (reviewed in (Britt and Mach, 1996)). The expression of the IE genes is under the control of a very strong regulatory element, which itself is activated by host cell proteins and viral tegument proteins (reviewed in (Moss and Khan, 2004)). Two of

the most abundant IE proteins are IE1 and IE2 which are heavily involved in initiating and regulating subsequent expression of the early viral genes (Sourvinos et al., 2007; Kalejta, 2008). The early gene products are proteins involved in viral DNA replication, such as the viral DNA polymerase (Sweet, 1999; Bain and Sinclair, 2007). Finally, late viral gene expression is initiated with the aid of IE and early gene products. The late viral gene products are mainly structural viral proteins, such as phosphoproteins 65 and 28 (pp65 and pp28, respectively) which are found within the viral tegument, and glycoproteins such as gB,gM, and gN which are found on the viral envelope and are involved in viral cellular attachment and entry (Iwayama et al., 1994; Bain and Sinclair, 2007).

Maturation of the nucleocapsids, containing the DNA genome, begins within the nucleus. As reviewed by Eickmann et al. (Eickmann et al., 2006), the nucleocapsids fuse with the inner nuclear membrane, generating a temporary viral envelope. As with most herpesviruses, this primary envelope then fuses with the outer nuclear membrane and the naked nucleocapsid is subsequently released into the cytoplasm (Mettenleiter et al., 2006). The nucleocapsid then gains a secondary envelope, containing viral glycoproteins and host-cell proteins, after fusing with vesicles of the Golgi apparatus (Jiang et al., 2008). Vesicles transport the virion to the cell surface where it is exocytosed into the extracellular space (Mettenleiter et al., 2006; Eickmann et al., 2006; Kalejta, 2008).

1.2.5 CMV latency and immune evasion

A characteristic of all herpesviruses is their capacity to cause both lytic and latent infections. The lytic cycle is the main mode of viral replication, and results in destruction of host cells, whereas latency results in the maintenance of the viral genome without production of new viruses until reactivation (Sinclair, 2008). Initially, HCMV enters into a lytic infection, with viral shedding found in bodily secretions such as urine, saliva, breast milk and semen. Viral shedding occurs for up to 6 months in immunocompetent adults, and may occur for years in children until their immune systems reach maturity (reviewed in (Mocarski and Courcelle, 2001)). After primary infection, HCMV enters into a latent infection in CD34+ myeloid progenitor cells of the myeloid lineage (Sinclair, 2008).

The cell-mediated immune response is important for controlling an active lytic HCMV infection (reviewed in (Moss and Khan, 2004)). For example, a week after the peak of HCMV replication, viral-specific T helper cell type 1 CD4+ T cells can be found in circulation (van de Berg et al., 2008), indicating their involvement in the immune response to active HCMV infection. Similarly, CD8+ T cells are detected during HCMV infection, the majority of which are specific for tegument proteins (Wills et al., 1996; Khan et al., 2007; Khan et al., 2005). Further support for a role for CD8+ T cells in controlling active HCMV infection was shown by the adoptive transfer of HCMV-specific CD8+ T cells to stem cell transplant recipients with active CMV infections, which was able to control CMV viremia in all patients (Cobbold et al., 2005). Natural killer (NK) cells are also important for controlling HCMV infection through the use of cell surface receptors that detect a decrease

in the expression of the major histocompatibility complex (MHC) class I molecules on CMV-infected cells and that release cytokines to lyse cells infected with intracellular pathogens (Biron et al., 1989). These actions typically would prevent the virus from maintaining a lytic infection in immunocompetent individuals, but for CMV, result instead in latent infection.

CMV has evolved various mechanisms to maintain latency and evade the immune response. For example, the genome of the virus is maintained within the cells as a very low copy extrachromosomal plasmid, which may aid in its evasion from the host immune response (Bolovan-Fritts et al., 1999). The virus can also control production of specific cellular proteins that downregulate the expression of MHC molecules on the surface of infected cells. An example of such a viral protein is a glycoprotein that is the product of the U_S6 gene (Bego and St.Jeor, 2006). This glycoprotein binds to the transporter of the antigen complex, which ultimately prevents the translocation of peptides to MHC class I molecules (Jun et al., 2000; Mocarski and Courcelle, 2001). By disrupting the expression of antigens by MHC class I molecules, infected cells are not readily detected by CD8+ T cells. HCMV can also interrupt the CD4+ T cell response by decreasing the cell surface presention of MHC class II molecules in latent monocytes (Cebulla et al., 2002; Slobedman et al., 2002). HCMV is able to escape detection by NK cells by sequestering ligands for NK cell receptors, expression of decoy MHC class I molecules and by the direct inhibition of NK cell cytotoxicity by the interaction of a viral protein with a NK cell activating receptor (Chalupny et al., 2006; Prod'homme et al., 2007; Arnon et al., 2005).

1.2.6 Transmission of HCMV

The main sites of primary HCMV infection are mucosal surfaces such as the genitourinary, gastrointestinal, and respiratory tracts. HCMV spreads throughout a host to a number of different organs, including the kidneys, liver, spleen, heart, brain, retina, lungs, colon, and salivary glands, causing either symptomatic or asymptomatic infection, dependent at least in part on the host's immunological status (Toorkey and Carrigan, 1989; Sweet, 1999; Revello and Gerna, 2002; Bentz et al., 2006). It is not yet clear as to how HCMV is able to spread throughout an infected individual, but endothelial and myeloid-lineage cells are thought to be the two major types of cells involved in disseminating HCMV *in vivo* (reviewed in (Reeves and Sinclair, 2008)). For example, it has been shown that the virus can spread via vascular endothelial cells, which can become detached and enter the bloodstream (Percivalle et al., 1993). And, removal of leukocytes from blood transfusion products prevents the transmission of HCMV to recipients of these products (de Graan-Hentzen et al., 1989). Transmission between individuals generally occurs through the exchange of bodily fluids, including saliva, semen, urine, and breast milk, but can also occur through blood products and stem cell and organ transplants (reviewed in (Hamprecht et al., 2008; Eastlund, 1995)). Because of the close contact of the children, child day-care facilities are often a source of initial HCMV infection, with infection rates of 50-70% (Ornoy and Diav-Citrin, 2006).

1.2.7 HCMV infection in vulnerable populations

In contrast to the generally asymptomatic latent infection in healthy individuals, CMV can cause serious disease and death in immunocompromised individuals. These include transplant recipients, HIV patients, and neonates. In patients receiving solid-organ or stem cell transplants, HCMV is a major cause of disease, graft rejection and death, with approximately 75% of these patients acquiring either a primary or secondary HCMV infection after transplantation (reviewed in (Bueno et al., 2002; Hodson et al., 2005; Fishman et al., 2007; Sun et al., 2008)). In stem cell transplant recipients, gastrointestinal disease and pneumonia are the most common clinical manifestations of HCMV, and are associated with high rates of mortality (Einsele and Hebart, 1999; Boeckh et al., 2003). HCMV can be reactivated in latently infected transplant recipients due to a regimen of immunosuppressive drugs, or they may become infected with a different HCMV strain from the donor organ (reviewed in (Reeves and Sinclair, 2008)). Although clinical symptoms of HCMV infection in solid organ transplant recipients can be as mild as fever and malaise, severe consequences such as pneumonia, liver disease, fungal infection, renal failure, retinitis, and graft rejection can occur (Pass, 2001; Baldanti et al., 2008; Einsele and Hebart, 1999). HCMV seronegative recipients are at greatest risk for serious disease as a consequence of HCMV infection if they receive an organ from a seropositive donor, resulting in a primary infection (Fishman et al., 2007; Pass, 2001).

HCMV is one of the most prevalent opportunistic viruses found in HIV infected individuals and is considered a cofactor in the advancement of HIV infection to AIDS

(Biancotto et al., 2008; Griffiths, 2006). Nearly 100% of HIV positive adults are also seropositive for HCMV (Pass, 2001). Due to the loss of immune function associated with HIV infection, primary HCMV infection or reactivation of latent virus can cause end-organ disease in individuals infected with HIV. Consequences of an active HCMV infection in HIV/AIDS patients include retinitis, esophagitis, encephalitis, hepatitis, gastritis, and neuropathy (reviewed in (Steininger, 2007)). Before the recent introduction of highly active anti-retroviral therapy (HAART), HCMV was the leading cause of morbidity in HIV infected individuals, and up to 40% of patients with CD4+ T cell counts less than 50 cells/μL developed HCMV-associated retinitis (Hoover et al., 1996; Kedhar and Jabs, 2007; Jacobson et al., 2008). However, since the advent of this therapy, the incidence of CMV end-organ diseases and retinitis has dramatically decreased by 75% in AIDS patients (Kedhar and Jabs, 2007; Wohl et al., 2005).

Congenital HCMV infection, or infection via viral transmission *in utero*, occurs in approximately 1.0 to 1.5% of all live births in the United States, which translates to about 40 000 births each year (Alford et al., 1990; Demmler, 1996). Congenital HCMV infection is believed to occur due to infection of the placenta during pregnancy, which spreads to the developing fetus (Ornoy and Diav-Citrin, 2006). The virus is able to cross the placental barrier by neonatal F_c receptor-mediated transcytosis of IgG-CMV virion complexes (Maidji et al., 2007). The most severe manifestations of HCMV infection in neonates are observed following primary infection of the mother during the first trimester of pregnancy (Kenneson and Cannon, 2007). However, non-primary infections due to viral reactivation from latency

or re-infection with a different HCMV strain, can also have serious consequences (Lazzarotto et al., 2007; Gaytant et al., 2003). While most children congenitally infected with HCMV do not show outward signs of the infection at birth (Foulon et al., 2008; Michaels, 2007), symptomatic congenital HCMV infection occurs in 10% of seropositive newborns and is usually marked by low birth weight, jaundice, hepatosplenomegaly, microcephaly, and the presence of petechiae or purpura (red or purple discolouration of the skin) (Boppana et al., 1992; Demmler, 1999; Malm and Engman, 2007). Both symptomatic and asymptomatic infants are at risk for long-term effects from HCMV infection; these include hearing loss, neuro-developmental deficiencies, and visual impairment (Boppana et al., 1992; Demmler, 1999; Malm and Engman, 2007; Michaels, 2007). Congenital HCMV infection is thought to be the leading infectious cause of hearing loss in children, with up to 15% of infected children having some level of hearing impairment (Pass, 2001; Schleiss, 2007; Dahle et al., 2000). Children with congenital HCMV can continue to shed the virus for years after primary infection before the virus enters into a latent state, and as a result can transmit the virus to uninfected family members or others who are exposed (Malm and Engman, 2007).

In short, HCMV is a major health concern. The prevalence of adverse health effects associated with HCMV infection in immunocompromised individuals, and the continued risk of congenital transmission of the virus, have made development of an effective therapeutic or prophylactic vaccine for HCMV a major focus of research.

1.3 Treatments for HCMV

1.3.1 Antiviral drugs

The primary method for treating CMV disease at the present time is the use of antiviral drugs. There are several antiviral drugs available for the treatment of HCMV; these include ganciclovir, cidofovir, foscarnet, acyclovir, and fomivirsen (Baldanti et al., 2004; Mercorelli et al., 2007). All but the last of these drugs target the viral DNA polymerase to prevent HCMV replication within host cells. Fomivirsen, in contrast, targets an immediate-early gene of HCMV using an anti-sense oligonucleotide (Lalonde et al., 2004). The most commonly administered of these drugs is ganciclovir (or the oral pro-drug form, valganciclovir) (Singh, 2006; Sungkanuparph et al., 2008; Adler and Marshall, 2007; Sun et al., 2008). For HIV patients receiving HAART, some immune recovery occurs and the incidence of severe CMV disease has greatly decreased (O'Sullivan et al., 1999; Weinberg et al., 2006). However, HCMV disease does continue to be a serious problem for many HIV patients (e.g. in developing countries) for reasons related to delays in immune recovery after initiation of HAART, cost or availability of antiretroviral drugs, compliance issues, and emerging resistance to HAART (Holland, 2008). As a result, in developed countries, anti-CMV drugs, mainly ganciclovir, are still administered to HIV patients in the event of CMV disease (Lalonde et al., 2004; Wohl et al., 2005). Anti-CMV drugs are also given to organ transplant recipients prophylactically for a period of time after transplantation to, which has greatly decreased the incidence of HCMV-associated disease in these patients (Singh, 2006; Torres-Madriz and Boucher, 2008). Late-onset HCMV infection (greater than 3 months post-transplantation) has been reported to occur in up to 26% of donor positive/recipient

negative solid-organ transplant patients after ganciclovir treatment has stopped, and so further antiviral treatments are required for these individuals (Limaye et al., 2004).

None of these antiviral treatments are approved for use during pregnancy; however, clinical trials have been carried out to assess the effectiveness of ganciclovir in the treatment of symptomatic congenital HCMV infection in neonates (reviewed in (Schleiss, 2008b)). Although these trials showed that intravenous ganciclovir or oral valganciclovir were able to protect against hearing loss in congenitally infected children, the drugs only decreased viral shedding during the actual treatment (Kimberlin et al., 2008). To date, studies using antiviral therapy have not indicated any improvement, in addition to protecting against hearing loss, in neurodevelopmental consequences of infection (Ornoy and Diav-Citrin, 2006; Kimberlin et al., 2008).

Although anti-CMV drugs have helped control the severity of disease associated with HCMV infection in immunocompromised individuals, they can produce unpleasant and sometimes toxic side-effects, such as severe gastrointestinal discomfort, neutropenia, and renal toxicity (Torres-Madriz and Boucher, 2008; Pass, 2001; Eid et al., 2008; Mercorelli et al., 2007). These can result in decreased compliance or the complete discontinuation of antiviral drugs in patients with severe toxicity, leading to increased viral replication (Torres-Madriz and Boucher, 2008). As with long-term administration of many antiviral drugs, the emergence of viral resistance has become a serious problem in the treatment of HCMV disease (reviewed in (Avery, 2008)). Prior to the development of HAART, resistance to

ganciclovir in HIV patients approached nearly 30% in individuals receiving the drug for a period of nine months or greater (Jabs et al., 1998). In transplant recipients, most cases of antiviral resistance appear to occur in seronegative recipients that receive an organ from a seropositive donor (Limaye et al., 2002; Lurain et al., 2002). In one study, up to 7% of seronegative recipients of a seropositive organ developed drug resistance after oral ganciclovir prophylaxis (Limaye et al., 2000). It was thought that valganciclovir, because of its higher levels of bioavailability, would overcome the problems associated with ganciclovir resistance (Avery, 2008; Sun et al., 2008; Boivin et al., 2004). However, a recent study has shown that resistance to valganciclovir does develop in solid organ transplant patients receiving the treatment (Eid et al., 2008), illustrating the importance of HCMV antiviral drug resistance as a barrier to treatment.

1.3.2 Immunoprophylaxis

In addition to the use of antiviral drugs for prophylaxis, transfer of high-titre anti-HCMV antibodies derived from human serum is an approach used to treat some HCMV infected patients or those at risk of becoming infected (Snydman, 2001; Puius and Snydman, 2007). It is theorized that high-titre HCMV antibodies will bind the virus and decrease viral replication, thereby decreasing the severity of CMV disease. Studies have shown that using HCMV hyperimmune globulin (HIG), in conjunction with antiviral drugs, to treat persistent CMV disease in solid organ transplant recipients decreased CMV disease to a greater extent, as compared to treatment with antiviral drugs alone (Ruttmann et al., 2006; Solidoro et al., 2008). A meta-analysis of 11 randomized studies showed that HCMV HIG administered

after transplantation, decreased the frequency and severity of CMV disease among heart, lung, liver, and kidney transplant patients (Bonaros et al., 2008). However, neither HIG alone nor in combination with antiviral drugs completely prevented HCMV infection in previously seronegative transplant recipients (Bonaros et al., 2008). The effectiveness of HCMV HIG in decreasing the incidence of severe disease associated with congenital HCMV infection has also been evaluated in women infected with HCMV for the first time during pregnancy (Nigro et al., 2005; La et al., 2006). Both studies showed that administration of HIG to these women significantly reduced the risk of HCMV disease in newborn infants, but did not eliminate infection nor completely prevent CMV disease (Nigro et al., 2005; La et al., 2006). These findings indicate that fully protective immunity to HCMV may require an active immune response in the host, and that the T-cell response may be important.

1.3.3 Live viral vaccines

Antiviral drugs and immunoprophylaxis are used to treat CMV disease after infection has occurred. Prophylactic vaccines, on the other hand, are given to individuals prior to contact with the pathogen with the goal of generating a pathogen-specific immune response sufficient to protect against the pathogen. Most viral vaccines currently licensed for human use are either whole live attenuated or inactivated viruses (reviewed in (Sheppard, 2005)). Live attenuated vaccines are generated from live viruses by altering their virulence, usually by sequential passage *in vitro* cell culture (reviewed in (Plotkin, 2003)). Ideally, live viral vaccines are able to replicate within an immunized host without producing negative consequences. A major advantage of live vaccines is their ability to induce both humoral

and cell-mediated immune responses, similar to natural viral infections. Whole inactivated vaccines are also developed from live viruses but are treated with chemicals, such as formalin, to remove their ability to replicate within a host (reviewed in (Plotkin, 2003)).

There is currently no vaccine available for HCMV, although research into the development of a vaccine against the virus began in the 1970s with the development of a live, attenuated vaccine. Two separate laboratory attenuated strains of HCMV were developed: AD169 (Elek and Stern, 1974) and Towne (Plotkin et al., 1975). AD169 was generated by passaging the virus isolated from the adenoid tissue of a child over 50 times in human fibroblasts *in vitro* (Elek and Stern, 1974) . Similarly, the Towne strain of HCMV was isolated from a congenitally infected infant and passaged over 128 times in fibroblasts (Plotkin et al., 1975). Due to inability to test HCMV vaccines in animals (see section 1.4) much of the testing of live, attenuated HCMV vaccines has taken place in humans. Only two clinical trials in humans have been performed using AD169 (Neff et al., 1979; Elek and Stern, 1974). Although most of the individuals immunized in these two studies developed HCMV-specific antibodies, several developed mild symptoms of CMV disease and many developed injection site reactions (reviewed in (Schleiss, 2005)). A follow-up eight years after the first study indicated that only half of these individuals that were immunized had detectable HCMV antibodies as a result of vaccination (Stern, 1984). Further immunization studies using AD169 have not been conducted and attention has instead been focussed on the Towne strain.

The most studied candidate vaccine for HCMV has been the attenuated Towne strain. Many analyses have been performed to test the safety and efficacy of the potential vaccine in healthy individuals (Just et al., 1975; Plotkin et al., 1976; Adler et al., 1995; Adler et al., 1998) and renal-transplant recipients (Plotkin et al., 1984; Plotkin et al., 1991; Plotkin et al., 1994). Results from these immunization studies revealed that the Towne virus was not shed from immunized individuals and did not invoke clinical signs of infection, that it induced both humoral and cell-mediated immunity, and reduced the severity of CMV-disease in seronegative renal transplant recipients receiving a seropositive kidney; however, the Towne strain did not prevent HCMV infection (reviewed in (Zhong and Khanna, 2007; Plotkin, 2001; Schleiss and Heineman, 2005)). Prior to regulations limiting live viral challenges in humans in the United States, seronegative male volunteers were immunized with the Towne strain and subsequently challenged with a low-passage strain of HCMV known as Toledo (Plotkin et al., 1989). Although the Towne vaccine was able to prevent HCMV infection when volunteers were challenged with a low dose (10 plaque forming units) of Toledo HCMV as compared to non-immunized individuals, immunized volunteers showed signs of infection after receiving a 10-fold higher dose of Toledo HCMV. This differed from results presented in the same study, wherein it was observed that naturally seropositive volunteers were protected against infection after a similar dose of Toledo HCMV, indicating that the Towne strain does not provide complete protection against wild-type HCMV.

It has been hypothesized that extensive attenuation of the Towne strain *in vitro* has resulted in genetic deletions that have sufficiently altered the virus in such a way that an

immune response against Towne virus no longer provides effective immunity against the native virus (reviewed in (Zhong and Khanna, 2007)). The molecular relationship between reduced immunogenicity of the Towne strain and attenuation is not yet fully understood. Regions of the Towne strain have been deleted, with at least 19 genes missing as compared to HCMV clinical isolates (Cha et al., 1996). For example, studies have shown that Towne virus is unable to replicate within endothelial cells (Sinzger et al., 1999) and that the Towne strain elicits lower endothelial-neutralizing ability as compared to natural HCMV infection (Cui et al., 2008). These studies suggest that one possible explanation for the reduced protection provided by the Towne vaccine is due to a change in the cellular tropism of this laboratory strain during passaging in fibroblast cells. The neutralization of viral attachment to endothelial cells may therefore be an important factor to consider for vaccine design.

In an attempt to improve upon the protection conferred by the Towne strain of HCMV, four chimeric viruses with coding sequences from both the Towne and Toledo strains were developed (Heineman et al., 2006). These chimeric viruses were generated with the hope that the resulting Towne/Toledo chimeras would encode the regions of Towne that provided a safe, non-pathogenic infection, and in addition contain the regions of Toledo hypothesized to confer greater protection than Towne alone. These Towne/Toledo chimeras were then used to immunize HCMV seropositive volunteers in a phase I study in order to evaluate their safety and efficacy. Although these chimeric strains were shown to be safe in HCMV seropositive individuals (minimal non-significant clinical signs of infection and lack of viral shedding), and both humoral and cell-mediated immune responses were detected in

the immunized individuals (Heineman et al., 2006), additional larger-scale studies have not been reported, but do need to be conducted to further evaluate their safety in humans.

1.3.4 Dense bodies as vaccines

In the laboratory, HCMV- infected fibroblasts not only release infectious virions, but also non-infectious subviral particles. These are termed dense bodies and contain the viral envelope and tegument region but lack the capsid and viral DNA (Stinski, 1976; Baldick, Jr. and Shenk, 1996; Sarov and Abady, 1975). Although dense bodies are not pathogenic, they do contain major immune targets such as gB, pp65, and gH (Roby and Gibson, 1986; Stinski, 1976). An advantage of using dense bodies as a vaccine *in vivo* is their ability to enter into cells and subsequently release their contents into the cell cytoplasm (Topilko and Michelson, 1994), thus potentially initiating a protective immune response against wild-type HCMV upon natural infection. Based on these observations, the immunogenicity of dense bodies was assessed *in vivo*. Subcutaneous immunization of mice with gradient-purified HCMV dense bodies was shown to induce viral-neutralizing antibodies and a viral-specific CD8+ T cell response in the absence of viral replication (Pepperl et al., 2000; Pepperl-Klindworth et al., 2003). Dense bodies may therefore have the potential to act as effective HCMV vaccines based on their ability to induce strong humoral and cell-mediated immune response. Studies are currently underway to incorporate other antigenic proteins into dense bodies to further enhance the immunogenic properties of these viral particles (Mersseman et al., 2008a; Mersseman et al., 2008b).

1.3.5 Subunit vaccines

Subunit vaccines comprise immunogenic components of a certain pathogen, such as a viral protein, and are therefore not able to cause infection. As a result, the safety concerns surrounding the possible reversion to virulence, genetic integration, and the generation of unwanted immune responses associated with live attenuated vaccines is eliminated (reviewed in (Purcell et al., 2007)). Subunit vaccines are based on the theory that immunization with a single antigen or combination of antigens will induce an immune response sufficient to protect against the actual pathogen. For HCMV, the major types of subunit vaccines that are currently being studied are purified recombinant proteins, viral vectors, and DNA plasmids engineered to express subunits.

It has been recognized that both humoral and cell-mediated immune responses are important for protecting against HCMV infection (see section 1.2.5 for brief review). Subunit HCMV vaccines therefore usually comprise pathogenic proteins that are major targets of either the humoral or cell-mediated immune responses. For generation of the humoral antibody response, antibodies can be generated against any region of an immunogen. However, to produce a neutralizing antibody response, antibodies must be able to prevent the virus from binding to a host cell, and thus inhibit infection. The neutralizing antibody response is therefore an important *in vitro* measure for assessment of vaccine efficacy. Although a number of HCMV proteins are considered good candidates for subunit vaccines, the two proteins that have been most widely used are the viral envelope glycoprotein, gB, and the tegument protein, pp65 (reviewed in (Plotkin, 2001; Zhong and

Khanna, 2007; Schleiss, 2008c)). gB is the major target of the neutralizing antibody response in an infected individual (Britt et al., 1990; Britt and Mach, 1996), making it an excellent candidate for a subunit vaccine. It has also been the most studied HCMV protein as a subunit vaccine (reviewed in (Schleiss, 2008c)). Although HCMV gB-specific CD8+ T cells are found in naturally infected individuals, pp65 is the major target of the CD8+ T cell response (Laughlin-Taylor et al., 1994; Wills et al., 1996) and adoptive transfer of CD8+ T cells specific for pp65 to transplant recipients has been shown to decrease CMV disease (Walter et al., 1995). As a result, pp65 is considered a good candidate for a HCMV subunit vaccine for induction of the cell-mediated immune response.

1.3.5.1 Purified recombinant gB

As detailed in section 1.2.4, gB is a transmembrane glycoprotein involved in entry of the virus into cells. The glycoprotein is 907 amino acids in the Towne strain and is highly glycosylated with numerous sites possessing either N-linked or O-linked glycosyl groups (Britt and Vugler, 1989). gB is translated as a single polypeptide of approximately 120 kDa. This is then post-translationally cleaved between animo acid number 460 and 461, into two disulfide-linked subunits, an amino-terminal fragment of 116 kDa, and a carboxy-terminal fragment of 55 kDa (Britt and Auger, 1986; Spaete et al., 1988; Spaete et al., 1990). Both fragments consist conformational and continuous epitopes , as well as neutralizing epitopes (Banks et al., 1989; Meyer et al., 1990; Qadri et al., 1992; Spaete et al., 1994).

To produce a recombinant subunit HCMV vaccine consisting of gB, HCMV was stably expressed in Chinese hamster ovary (CHO) cells (Spaete et al., 1990). The cleavage site was deleted from the protein and, in order to aid purification of gB from cell culture, the transmembrane region was removed prior to expression in CHO cells (Spaete et al., 1990; Spaete, 1991). Several clinical trials have been performed to evaluate the safety and immunogenicity of purified CHO-derived gB in humans (reviewed in (Schleiss, 2008c)). The results of two phase I trials indicated that the vaccine generated minimal local and adverse systemic reactions in healthy seronegative adults immunized with CHO-derived HCMV gB in conjunction with the adjuvant MF59 (Pass et al., 1999; Frey et al., 1999). Individuals immunized three times with recombinant gB and MF59 generated higher titres of gB-specific antibodies and viral neutralizing activity greater than those observed in non-immunized HCMV seropositive control adults (Pass et al., 1999; Frey et al., 1999). During the same study by Pass *et al*, the ability of the non-replicating recombinant gB subunit vaccine to induce a humoral memory response was evaluated by immunizing a subset of the individuals for a fourth time, six months after the third immunization (Pass et al., 1999). Antibody and neutralizing titres increased quickly within a two week period. A subsequent phase I trial involved the immunization of seronegative children aged one to three years with the same purified recombinant gB subunit vaccine in conjunction with MF59 (Mitchell et al., 2002). Again, there were minimal local and adverse systemic reactions in all of the children after immunization, indicating that the vaccine and adjuvant were well-tolerated. After three immunizations, the children had six-fold higher antibody titres than the adults from the previous study (Mitchell et al., 2002). These high antibody titres in children relative to

adults are consistent with results observed for another herpesvirus vaccine, varicella zoster (Gershon et al., 1992). These studies suggest that CHO-derived gB is safe and induces a strong, humoral immune response that persists over a period of at least 12 months.

Although the results from these trials have confirmed the immunogenicity and safety of this vaccine in both adults and children, it has been difficult to test the ability of the vaccine to prevent viral infection. However, a recent phase II clinical trial was conducted using a natural viral challenge population of HCMV seronegative young women with children in daycare (Zhang et al., 2006). These women, who are highly susceptible to primary HCMV infection (Marshall and Adler, 2009), were immunized three times with purified recombinant gB. A follow-up study indicated that rates of HCMV infection were reduced by 50% as compared to those in the placebo group (Pass et al., 2009), demonstrating that this purified recombinant gB is able to generate protective immune responses in at least vaccine recipients. In addition to this study, a second phase II clinical trial is currently underway in renal transplant recipients, another highly HCMV-susceptible population (reviewed in (Schleiss, 2008c)). Overall, the results from these trials indicate that a purified recombinant gB subunit vaccine shows promise in inducing immune responses capable of preventing HCMV infection.

1.3.5.2 Viral vectors

To increase cell-mediated immunity against pathogens such as viruses, viral vectors have been engineered for intracellular delivery of specific viral antigens in order to promote

antigen presentation by the class I MHC for the induction of CD8+ cytotoxic T lymphocytes (reviewed in (Robert-Guroff, 2007)). A key requirement of these vectors is their ability to stimulate an immune response while remaining non-pathogenic in the immunized host. Several viral vectors have been developed as vaccine delivery systems, including adenovirus, poxvirus, alphavirus, measles virus, and vesicular stomatitis virus (reviewed in (Robert-Guroff, 2007)).

One viral vector used for the expression of HCMV proteins is the canarypox viral vector known as ALVAC. ALVAC is naturally replication-deficient in mammalian cells (reviewed in (Plotkin, 2001)) and has been developed for the delivery of recombinant proteins for measles (Taylor et al., 1992), HIV (Pialoux et al., 1995), and rabies (Fries et al., 1996). An ALVAC vector with the gB gene was engineered and preclinical testing of the ALVAC vector-gB vaccine in mice indicated that the vaccine was able to induce cell-mediated and neutralizing antibody responses (Gonczol et al., 1995). However, these responses were not observed in subsequent trials in humans and as a result, ALVAC-gB vectors were used in "prime-boost" immunization series in HCMV seronegative humans with live Towne vaccine (Adler et al., 1999) or purified recombinant gB/MF59 (Bernstein et al., 2002). Such a strategy has been successful in HIV canarypox vector vaccine studies (Pialoux et al., 1995). In the study using the Towne vaccine as a boost, neutralizing antibody titres higher than those obtained from the Towne vaccine alone were observed (Adler et al., 1999). The trial using purified recombinant gB as a boost did not reveal the same response; in fact, priming with the ALVAC-gB vaccine did not induce higher

neutralizing antibody titres than those observed in individuals receiving receiving gB/MF59 alone (Bernstein et al., 2002), indicating there was no apparent advantage to priming with the ALVAC-gB vaccine. HCMV pp65 has also been expressed using ALVAC. Phase I clinical trials revealed that this vaccine induced pp65-specific CD8+ T cells in all immunized volunteers at a frequency comparable to that observed in HCMV seropositive individuals (Berencsi et al., 2001).

In addition to canarypox viral vectors, other viral vectors, such as adenovirus and alphavirus replicon systems, have been used as delivery systems for HCMV subunit vaccines (reviewed in (Zhong and Khanna, 2007)). Both HCMV gB and pp65 have been expressed using adenovirus and alphavirus replicon systems, along with the IE1 protein which has also been shown to be an important CD8+ T cell target in infected individuals (Slezak et al., 2007; Reap et al., 2007a; Reap et al., 2007b). Although these vaccines have not yet been tested in humans, both show potential after preclinical testing in animal models (reviewed in (Schleiss, 2008c)). While these results from studies using viral vectors to express HCMV proteins as subunit vaccines are promising, future studies will need to be performed to determine if the observed immune responses will be able to prevent HCMV infection.

1.3.5.3 DNA plasmids

The viral antigens pp65, gB, and the IE1 gene product have also all been used as potential DNA vaccines for HCMV. DNA vaccines are usually administered by intramuscular delivery, where they subsequently transfect important antigen presenting cells

such as dendritic cells and monocytes (Chattergoon et al., 2000). Because the genetic material is transcribed and translated within these cells, both the humoral and cell-mediated responses are usually activated (reviewed in (Abdulhaqq and Weiner, 2008)). DNA vaccines have been used to successfully induce immune responses to antigens from a number of different viruses, including hepatitis C (Lang et al., 2008), avian influenza A (Laddy et al., 2008), and HIV (Boyer et al., 1997).

The first DNA vaccines engineered against HCMV encoded pp65 as the viral antigen. Mice immunized with plasmids encoding HCMV pp65 generated pp65-specific CD8+ T-cell and antibody responses (Endresz et al., 1999). In another study, approximately 60% of mice immunized with plasmids carrying the pp65 gene under the control of the human β-actin promoter were found to have anti-pp65 antibodies (Pande et al., 1995). A DNA vaccine carrying the gene for HCMV gB has also been evaluated for immunogenicity. In mice, both full-length and truncated gB lacking the transmembrane region were evaluated for their ability to induce both antibody and T-cell responses (Endresz et al., 1999; Endresz et al., 2001). Interestingly, in this study, truncated gB induced a greater antibody response than full-length gB. This suggests that the transmembrane region interferes with induction of an immune response due to processing of the recombinant protein.

To engage both the antibody and T-cell mediated immune responses, bivalent plasmids encoding pp65 and gB have been tested in both animal models and humans.

Preclinical studies using the bivalent vaccine indicated that the vaccine did not induce serious toxic responses and elicited high antibody and T-cell responses in mice (Selinsky et al., 2005). A phase 1 clinical trial in HCMV seronegative human volunteers using a bivalent DNA vaccine encoding HCMV pp65 and gB found no serious adverse effects, and both antibody and CD8+ T cell responses were induced (Wloch et al., 2008). Examination of the biodistribution and persistence of a trivalent DNA vaccine encoding pp65, gB, and IE1, in mice and rabbits did not find any evidence of incorporation of the plasmid DNA into the genome (Vilalta et al., 2005). In general, the studies have not found significant toxicity to the DNA vaccines, and have shown that DNA vaccines against CMV are immunogenic in animal models and in humans. Because of these promising results, development of DNA vaccines against CMV continues to be an active area of research (reviewed in (Schleiss and Heineman, 2005)).

1.4 Animal models of CMV

Development of any vaccine requires a number of steps prior to assessment in humans. Ideally, after *in vitro* characterization, preclinical testing is performed in order to evaluate the safety and efficacy of a vaccine in an animal model. A major challenge in the development of a vaccine for HCMV is the species specificity of the virus, a trait characteristic of all herpesviruses (reviewed in (Pass, 2001)). Viruses have evolved along with their hosts, resulting, in some cases, in restricted host-ranges (Jurak and Brune, 2006). The biological mechanisms governing species specificity in CMV are not well understood. For example, despite the fact that primate CMVs (rhesus and baboon) are more closely

related to HCMV than CMVs in other species (such as mouse, rat, and guinea pig), infection of primates with HCMV does not result in a productive infection (reviewed in (Yue and Barry, 2008)). Therefore, although immunogenicity of prospective HCMV vaccines can be tested in animals, the ability to induce protective immunity cannot be assessed. Instead, vaccines must be developed against the specific animal CMVs to allow for *in vivo* assessment of safety and efficacy as a surrogate for HCMV vaccines. Vaccines assessed as safe and effective in the animal model would provide evidence supporting development and testing of similar vaccines against HCMV in humans. Nonetheless, animal models of CMV infection and disease have been useful in understanding the genetics, molecular mechanisms of disease, and vaccine development, as described below.

1.4.1 Primate models

Several animal models of CMV have been studied. Rhesus macaques CMV (RhCMV), is the most closely related animal model to HCMV in terms of molecular biology, pathogenesis, and epidemiology (reviewed in (Yue and Barry, 2008)). RhCMV is a good model for testing the protective immunity of vaccines developed against RhCMV for the purpose of developing similar vaccines against HCMV. First, the structure and size of RhCMV is similar to HCMV (Hansen et al., 2003). The genome of RhCMV is predicted to contain 230 to 250 open reading frames, with 135 of these homologous to HCMV proteins (Rivailler et al., 2006). One of these proteins is the homologue of the HCMV immunodominant protein, gB. RhCMV gB is similarly processed into two subunits and certain epitopes of these proteins are cross-reactive with HCMV gB monoclonal antibodies,

further demonstrating the immunogenic similarity between these two gB homologues (Kravitz et al., 1997; Kropff and Mach, 1997; Yue et al., 2003). Although naturally occurring congenital RhCMV infection is difficult to detect, the direct infection of a fetal macaque *in utero* leads to neurodeficiencies similar to those observed in human congenitally infected infants (Barry et al., 2006; Powers and Fruh, 2008; Vogel et al., 1994). Co-infection of rhesus monkeys with RhCMV and simian immunodeficiency virus produces a similar clinical profile as that observed in HIV patients infected with HCMV (Sequar et al., 2002), indicating that the rhesus model may also have applicability to the study of CMV infection in susceptible populations. These observations, coupled with the anatomical and immunological similarities between humans and primates, indicate that RhCMV is an excellent model for studying congenital HCMV (Powers and Fruh, 2008). RhCMV could therefore potentially act as a model for the evaluation of anti-CMV vaccines for the purpose of preventing congenital CMV infection. However, ethical considerations, cost, and lack of seronegative rhesus monkeys are major drawbacks for studying the pathogenesis, treatment, and prevention of RhCMV as a model for HCMV (Schleiss, 2002; Powers and Fruh, 2008).

1.4.2 Guinea pig model

Due to the issues associated with use of primate models for CMV vaccine research, small animal models have been considered as alternatives. There has been considerable characterization of mouse CMV in the murine model, as well as testing of immunogenicity of HCMV vaccines in mice. However, the mouse model does not permit the study of congenital CMV infection, or the testing of vaccines designed to prevent such infection, as

murine CMV does not cross the placenta to produce CMV infection *in utero* (reviewed in (Schleiss, 2002)), as occurs in humans. In contrast, guinea pig CMV (GPCMV) is able to cross the placenta in pregnant dams to produce an infection *in utero* that more closely resembles the human experience. GPCMV was first described as a possible model for congenital CMV infection in the late 1970s and early 1980s (Kumar and Nankervis, 1978; Griffith et al., 1986). The gestational period of 65 to 70 days in guinea pigs is relatively long for a small animal, and can be divided into distinct trimesters (reviewed in (Schleiss, 2002)). Moreover, the single trophoblast layer of the guinea pig placenta separating maternal and fetal circulation is histologically similar to that in humans (Schleiss, 2006). These characteristics allow the study of effects of CMV infection at different time points during pregnancy and the subsequent consequences of infection on the newborn pups, analogous to events in HCMV infection. These same properties also enable the study of vaccines and their effectiveness in preventing infection or modulating disease outcomes.

In vivo testing of the efficacy of GPCMV vaccines has shown that a number of the vaccine strategies designed for HCMV (see sections 1.3.3 and 1.3.5) are able to minimize CMV disease, and in some cases prevent infection in guinea pigs (reviewed in (Schleiss, 2008a)). For example, immunization of dams with a gB DNA vaccine prior to conception decreased overall pup mortality (Schleiss et al., 2003). Moreover, in dams with high antibody titres the pup mortality was negligible, clearly indicating a protective effect of the vaccine (Schleiss et al., 2003). Similarly, immunization of female guinea pigs with purified GPCMV gB protein expressed in baculovirus cell culture resulted in high titres of

neutralizing antibodies (Schleiss and Jensen, 2003), decreased viral load, and significant decreases in pup mortality (Schleiss et al., 2004). Expression of a GPCMV pp65 in an alphavirus replicon system induced both humoral and cellular immune responses in female guinea pigs, and challenge of pregnant immunized females with GPCMV revealed a decrease in pup mortality from 57% to 13% (Schleiss et al., 2007). The positive results from these immunization studies suggested that guinea pigs would provide an appropriate model for the testing of CMV vaccines produced using alternative expression systems.

1.5 Plants as expression systems

A variety of different expression systems are currently used for the production of recombinant proteins for therapeutic or other use. These systems include mammalian cells, bacteria, and yeast. More recently, insect cells, transgenic animals, and transgenic plants have been utilized as alternatives, with the intent of overcoming certain disadvantages associated with mammalian, bacterial, and yeast systems. Disadvantages of using bacterial systems include their inability to perform complex post-translational modifications such as glycosylation, which potentially affect the biological activity of some therapeutic proteins and may result in the accumulation of recombinant proteins in inclusion bodies (reviewed in (Boehm, 2007)). In addition, although mammalian systems may be effective for production of therapeutic proteins due to their ability to perform complex modifications, these systems are costly and potential contamination with viral pathogens is a major regulatory concern (reviewed in (Ma et al., 2003)). Consequently, there has been tremendous interest in the development of other expression systems for production of therapeutic proteins that would

not only be more cost effective, but would be less likely to contain human pathogens and might also provide alternative methods of drug delivery.

1.5.1 Why use plants?

As reviewed in a number of recent articles (Sala et al., 2003; Ma et al., 2003; Thanavala et al., 2006; Mett et al., 2008) plants have several potential advantages over traditional expression systems, some of which are particularly relevant when considering the production of therapeutic proteins such as subunit vaccines. First, plants have the capacity to perform complex post-translational modifications. An example of such a modification is the glycosylation of proteins, an important factor since immunogenic proteins derived from viruses and parasites are often glycoproteins, and the glycosyl groups in some cases have been shown to be essential for the immunogenicity of proteins (Bolt et al., 1999; Hudrisier et al., 2001). Second, plant-derived products are relatively safe when compared to bacterial and mammalian systems, in which end-products may be contaminated with bacterial endotoxins and human pathogens (reviewed in (Mett et al., 2008)). Third, plants may offer certain economical advantages, the greatest of which is the ability to grow on an agricultural scale with minimal inputs such as sunlight, water, and fertilizer, all of which are inexpensive compared to the cost of media and large fermentation processes (reviewed in (Ma et al., 2003; Mett et al., 2008)). A fourth benefit of expressing recombinant proteins in plants is the ability to specifically target recombinant protein expression to edible plant parts, allowing for the possibility of oral delivery of the proteins with minimal requirements for

downstream processing (reviewed in (Mitragotri, 2005)). This is particularly advantageous for the delivery of subunit vaccines for mucosal immunization.

1.5.2 Expression of therapeutic proteins in plants

The concept and development of plant-derived biopharmaceuticals has evolved over the past decade, with expression of close to 200 therapeutic proteins in a wide range of plant species (reviewed in (Twyman et al., 2005)). Examples of therapeutic proteins that have been expressed in plants include human growth hormone in tobacco (Barta et al., 1986), human serum albumin in potatoes (Sijmons et al., 1990), granulocyte macrophage-colony stimulating factor in tobacco and rice (Sardana et al., 2002; Sardana et al., 2007)), human interleukin-13 in tobacco (Wang et al., 2008), hepatitis B surface antigen in lettuce (Marcondes and Hansen, 2008), and cholera toxin B subunit in tomatoes (Jani et al., 2002). Recombinant antibodies derived from transgenic plants have been studied mainly as therapeutic agents for cancer, but also for autoimmune diseases, cardiovascular disease, and as passive vaccines for the prevention of infectious diseases (reviewed in (Fischer et al., 2003; Ko et al., 2009)). The overall structure of an antibody is somewhat complex in that two heavy and two light polypeptide chains fold into the typical antibody Y-shape, and glycosylation of antibody product is critical not only for proper folding, but also for overall function. Although parts of recombinant antibodies have been successfully produced in bacterial systems, the lack of glycosylation machinery in bacteria makes full-length antibody production difficult and even partial antibodies may be folded incorrectly (Chadd and Chamow, 2001). The first plant-derived recombinant antibody was engineered in 1989

(Hiatt et al., 1989). In this study, tobacco plants were initially transformed with either the light or heavy chains of an IgG_1 monoclonal antibody and then sexually crossed to produce transgenic tobacco plants that expressed both chains. These tobacco-derived IgG_1 recombinant antibodies were correctly assembled, folded, and maintained functionality (Hiatt *et al.* 1989). Plant cells, in a manner similar to animal cells, possess a protein secretory pathway that involves trafficking of proteins through the endoplasmic reticulum to the cell surface. It is during this process that proteins are glycosylated (Lerouge et al., 1998). Although there are minor differences between the glycosylation patterns produced in plants as compared to animals, these slight differences did not appear to affect the folding, function, or immunogenicity of tobacco-derived IgG_1 (Cabanes-Macheteau et al., 1999). Since 1989, monoclonal antibodies have been produced in other plant species such as rice and wheat; several plant-derived antibodies have been produced as possible treatments for human diseases and several of these are currently in human clinical trials (reviewed in (Ko et al., 2009)).

1.5.3 Expression of subunit vaccines in plants

In the past, tobacco was used as an initial host for expressing recombinant proteins, including subunit vaccines, because of its relative ease of transformation, rapid growth, and high regeneration levels (Ma et al., 2003). The first plant-derived subunit vaccine produced was the hepatitis B surface antigen (HBsAg) expressed in tobacco plants (Mason et al., 1992). While this highly immunogenic protein is currently expressed in yeast, the large fermentation facilities involved in generating this vaccine make it costly to produce for both

developed and developing countries (Kong et al., 2001). Mice injected intraperitoneally with the crude plant extracts were found to produce HBsAg-specific serum IgG and IgM antibodies at levels comparable to those derived from immunization by injection with recombinant yeast-derived HBsAg (Mason et al., 1992), indicating for the first time the immunogenicity of plant-derived recombinant subunit vaccines.

Since this study, numerous proteins have been expressed in many different plant systems and have been shown to provide protective immunity in pathogen challenge studies. These include subunit vaccines for foot-and-mouth disease virus in alfalfa leaves and potatoes (Dus Santos et al., 2002; Carrillo et al., 2001), transmissible gastroenteritis coronavirus in maize (Lamphear et al., 2004), bovine herpes virus in tobacco (Perez Filgueira et al., 2003), and cholera toxin in potatoes (Arakawa et al., 1998).

1.5.4 Plant-derived vaccines for mucosal immunization

The mucosal immune system is consists of a number of different mucosal lymphoid tissues, which include the intestines, bronchial tract, nasal passages, and genitourinary tract (reviewed in (Brandtzaeg, 2007)). In the gut-associated lymphoid tissues, the immune response inductive sites are specialized regions known as Peyer's patches which are found within the lamina propria of the small intestine (Mestecky et al., 2008). Peyer's patches are sites that are enriched with CD4+ T cells, macrophages, and secretory IgA (sIgA)-designated B cells. These areas are overlaid with specialized phagocytic cells called M cells which recognize particulate antigens and transport them to the mucosal B and T cells below

(Florence and Jani, 2003). Once activated, these antigen-specific sIgA B cells and T cells migrate to other mucosal immune sites throughout the body and may also spill over into the systemic immune system via the lymphatic system (reviewed in (Neutra et al., 1996)). In this way, an immune response induced at one specific mucosal surface may provide protective immunity at other mucosal immune sites, such as the bronchial associated lymphoid tissue, the nasal associated lymphoid tissue, and the urogenital tract (Butterworth et al., 2001; Zuercher et al., 2002; Mitragotri, 2005).

The first studies to demonstrate actual mucosal immune responses to plant-derived subunit vaccines were conducted with Norwalk virus capsid protein (NVCP) and the B subunit of *Escherichia coli* heat-labile enterotoxin (LT-B) expressed in potatoes (Haq et al., 1995; Mason et al., 1996). Since both pathogens gain entry into human hosts via the oral route and subsequently infect the gastrointestinal tract, oral vaccines for protection against Norwalk virus and enterotoxigenic *E. coli* have been major targets for vaccine development. In both studies, referred to above, the majority of mice fed the transgenic raw potatoes developed both serum IgG and mucosal sIgA antibodies (Haq et al., 1995; Mason et al., 1996). These results indicate that oral immunization with plant-derived vaccines induces mucosal-specific immune responses and also induces immune responses within the circulatory system. Since these original studies, a number of groups have shown that transgenic plants carrying subunit vaccines can induce an immune response via mucosal immunization (Yusibov et al., 2002; Gomez et al., 2008; Guetard et al., 2008; Matsumoto et al., 2008).

Immunization studies in human volunteers have been carried out to further test the feasibility of orally delivered plant-derived vaccines. In the case of LT-B transgenic potatoes (Tacket et al., 1998), eleven healthy volunteers were fed either 50 g or 100 g of raw transgenic potato and an immune response was measured via LT-B specific IgG in serum and LT-B specific sIgA in stool samples. Ten out of eleven people showed a four-fold increase in LT-B specific IgG in serum, indicating a systemic immune response; and five out of ten people displayed more than a four-fold increase in LT-B specific fecal sIgA. In addition to measuring antibody responses in the volunteers, LT-B neutralization titers of greater than 1:100 were detected in most volunteers, signifying the presence of neutralizing serum antibodies. In a separate study, twenty healthy volunteers were fed transgenic potatoes expressing NVCP (Tacket et al., 2000). Four out of twenty individuals produced NVCP specific serum IgG and IgM responses and six out of twenty individuals produced NVCP specific fecal IgA. The lower response observed with transgenic potatoes expressing NVCP as compared to those expressing LT-B is likely due to the difference between the immunogenic potential of each of the proteins; LT-B is known to be highly immunogenic and in fact is currently used as an adjuvant to enhance immune responses in many animal immunization studies (Tacket, 2007).

1.5.5 Challenges with plant-derived vaccines

Numerous studies have now verified that plant expression systems can be used to produce recombinant proteins which retain structural features required for antigenicity and

immunogenicity. Furthermore, data gathered from published studies have shown that oral and parenteral immunizations with plant-derived subunit vaccines can stimulate the production of serum and in some cases, mucosal antibodies. However, various issues remain outstanding regarding safety and efficacy, and these must be addressed before plant-derived vaccines can be employed for human use. One challenge relates to transgene expression levels, which are often low. In the case of oral vaccines, low expression levels would require the consumption of large quantities of plant-derived materials in order to achieve proper vaccine dosages, which may be impractical. In order to increase yields and expression levels, various strategies have been developed, including codon optimization, targeted expression, plant virus-based transient expression, and chloroplast transformation, with the intent of increasing expression of the vaccine product (reviewed in (Yusibov and Rabindran, 2008)). Plant virus-based expression of recombinant proteins by plant viruses such as tobacco mosaic virus and cowpea mosaic virus, have shown that high levels of expression can be obtained within a few weeks (Wigdorovitz et al., 1999; Gleba et al., 2007). One drawback of transient expression is the instability of recombinant protein expression over multiple generations of a plant, which reduces the advantage of cost-effective large-scale production of transgenic plants (Thanavala et al., 2006). Chloroplast transformation has more recently been utilized for the expression of proteins, including subunit vaccines, and has been associated with high recombinant protein expression levels (Kang et al., 2004; Molina et al., 2004; Daniell et al., 2005; Arlen et al., 2008). Chloroplast expression has the added advantage of maternal inheritance, so outcrossing from transgenic plants to wild-type plants is minimal (Daniell et al., 2002).

Another issue surrounding plant-derived vaccines is the glycosylation of recombinant proteins within plants. It is known that although plants are able to post-translationally glycosylate proteins, plants use different glycosylation patterns as compared to animals, specifically xylose and fucose residues (Brooks, 2006). In particular, these different glycosyl groups on plant recombinant glycoproteins may cause the production of immune responses that do not reflect those observed after a natural infection. Concern regarding immune responses to altered glycosylated epitopes remains a barrier in the development of plant-derived vaccines, due to the possibility of these epitopes causing allergic reactions when delivered parenterally (Rybicki, 2009). However, at least one study has shown that glycosylation of a plant-derived subunit vaccine does not necessarily affect its antigenicity (Bouche et al., 2003). In this study, the hemagglutinin protein of the measles virus was expressed in carrots and different monoclonal antibodies were used to determine if the protein was properly glycosylated and folded. Measles virus hemagglutinin protein is a glycoprotein expressed on the surface of the measles virus; glycosylation of this protein, and many other viral proteins, is important not only for proper folding, but also for the induction of immune responses. Results showed that two conformation-dependent monoclonal antibodies demonstrated high binding to carrot-derived hemagglutinin, suggesting that abnormal glycosylation did not affect the folding of the protein. In addition, human serum from naturally infected individuals showed high reactivity to carrot-derived hemagglutinin (Bouche et al., 2003). More extensive protein characterization studies of plant-derived recombinant proteins are required before this question can be adequately resolved.

Questions also surround the dose and ultimate form in which plant-derived vaccines will be delivered. Although beyond the scope of this thesis, the induction of oral tolerance remains a considerable concern for oral plant-derive vaccines. Previously, plant-derived vaccines had been referred to as "edible vaccines" because it was originally believed that individuals could be immunized by simply ingesting the original plant material. It is now recognized that a certain amount of down-stream processing may be required to ensure product stability and standardization of dose. In some cases, and depending on the plant product, it may be possible to administer plant-derived vaccines as a lyophilized powder, which is encapsulated prior to oral delivery. Thus, from a cost-benefit analysis, it will be necessary to balance costs of down-stream processing with the benefits associated with production of a plant-derived vaccine (reviewed in (Yusibov and Rabindran, 2008)).

Despite these uncertainties regarding plant-derived vaccines, these products show promise for the prevention of human and animal diseases. Studies are currently being undertaken to address many of the above issues, with the intention of developing plant-derived vaccines as potential alternatives to traditional vaccines, and/or to use this technology platform to develop effective vaccines where other approaches have failed.

2.0 Rationale, Hypothesis, and Objectives

2.1 Rationale

HCMV is a major health concern due to its significant impact on morbidity and mortality in immunocompromised individuals and infants. Currently, there is no commercially available vaccine for HCMV, and treatment options are limited. The species specificity of CMV makes testing of a vaccine for HCMV in animal models impossible. However, GPCMV infection of guinea pigs most closely models the human situation because like HCMV it can cross the placenta and cause congenital infection. Plant-derived vaccines may offer certain advantages over traditional vaccines, such as increased stability, the potential for direct exposure of mucosal sites by oral delivery, and improved immune responses against those pathogens which initiate infection at mucosal surfaces. Expression of immunogenic proteins from CMV in plants offers a novel approach for the development of a vaccine.

2.2 Hypothesis

The hypothesis of this study was that expression of the immunodominant GPCMV proteins gB and pp65 expressed in rice or *A. thaliana* evokes a biologically relevant immune response in immunized guinea pigs.

2.3 Objectives

To address this hypothesis, the following specific objectives were formulated:

1. Engineer genetic constructs suitable for expression of guinea pig CMV proteins in plants using the rice glutelin-1 (Gt1) promoter for seed-specific expression or the maize ubiquitin (Ubi) promoter for plant-wide expression.

2. Transform and regenerate rice and *A. thaliana* plants via *Agrobacterium tumefaciens*-mediated transformation to express recombinant gB and pp65.

3. Verify expression and stability of transgene in transformed plants by PCR and Western blots.

4. Immunize guinea pigs by subcutaneous injection with plant-derived subunit vaccines and analyse immune responses.

5. Verify neutralizing ability of antibodies generated against plant-derived subunit vaccine.

3.0 Materials and Methods

3.1 Engineering of gene constructs

3.1.1 Plasmids

Plasmid vectors were obtained from commercial sources or as gifts from colleagues (see Table 1).

3.1.2 Polymerase chain reaction (PCR)

Primers for the generation of new restriction enzyme sites were designed based on either published sequences or in-house sequencing (Centre for Biologics Research, Health Canada). New restriction enzyme sites were incorporated during primer design. For PCR products requiring adenosine residue overhangs, PCR was performed using the Advantage High Fidelity 2 (HF 2) PCR kit (Clontech, Mountainview CA, US), 1 μM final concentration of each primer, and 50 ng of plasmid DNA. All customs primers are from Invitrogen (Burlington ON, Canada). Primer sequences are listed in Table 2, with new restriction sites indicated in bold. Thirty cycles of PCR with the Gene Amp PCR System 2400 thermocycler (Perkin Elmer, Watham MA, US) were conducted using with a 1 minute denaturation at 95°C, 30 seconds at the appropriate annealing temperature (Table 2), and elongation at 72°C for a specified time dependent on length of amplicon (Table 2). PCR products were generated using Vent DNA polymerase (New England Biolabs, Pickering ON, Canada) during the screening for the presence of specific genes or if blunt-ended PCR products were required with 2 μM final concentration of each primer, 200 μM final dNTP concentration (New England Biolabs), and similar thermocycler programs (Table 2).

Plasmid	Source
pGEM4Z/Gt1/ss	(Sardana et al., 2002)
pCaMVCN	GE Healthcare (Pittsburgh PA, USA)
pGEM4Z	Promega (distributed by Fisher Scientific, Ottawa ON, Canada)
pCR2.1	Invitrogen (Burlington ON, Canada)
pKTS507	(Schleiss et al., 2000)
pKTS437	(Schleiss et al., 2000)
pCAMBIA1301	CAMBIA (Canberra, Australia)
pAHC25	(Christensen and Quail, 1996)

Table 1: Sources of plasmids.

Table 2: Primer sequences, annealing temperatures (T_A), and elongation times used during the engineering of the gene constructs and screening of transformed *A. tumefaciens* and *A. thaliana*. New restriction enzyme sites incorporated into the final PCR product are shown in bold.

Primer Names	Primer sequences	T_A (°C)	Elongation time
5'-Gt1*Sac*I	5'TATAGAATAC**GAGCTC**CTCTGTTTTG	50°C	2.5 minutes
3'-Gt1ss*Nhe*I	5'GCTTGCAT**GCTAGC**AGGTCGACTT		
5'-*Sph*INOS	5'TGGCAGAAATTCG**GCATGC**AGATCCGCA	60°C	45 seconds
3'-*Sac*INOS	5'TATCATCGATAAGCTTCTA**GAGCTC**TAGTAC		
5'-gBGt1	5'TTGACGATCTTGTACCTCGGTGTCTACTG	68°C	3 minutes
3'-gBGt1	5'TGGTACCGA**GCTAGC**ATCCACTAGGATC		
5'-pp65Gt1	5'ATGGAGCGGTACGTGGGTCTCGG	65°C	2 minutes
3'-pp65Gt1	5'AGTACAGGGGG**TCTAGA**CGCGCG		
5'-gBUbi	5'AGGACTTCTTTCGA**GCATGC**GACCCGTACG	70°C	2.5 minutes
3'-gBUbi	5'TCGATAAGCTTGGTA**GCATGC**TCGGATCCAC		
5'-pp65Ubi	5'ATGGAGCGGTACGTGGGTCTCGG	55°C	2 minutes
3'-pp65Ubi	5'TTATTTCGGG**GAGCTC**AGGGGGAC		
5'-gBscreen1	5'GATTGCCGCGAATGAATCTGC	53°C	45 seconds
3'-gBscreen1	5'AGATCCGGTAATCCTGCGTCT		
5'-gBscreen2	5'CTTGCCGTCCGGTGTTATAGT	53°C	45 seconds
3'-gBscreen2	5'CACCACGCCTCTGCAATATGA		
5'-gBscreen3	5'CTAACGTGACGTGCGAGATGA	53°C	45 seconds
3'-gBscreen3	5'CATCCTGTGCGTTCCGAGTAA		
5'-gBscreen4	5'CGCGAATCGTACCGAAGATGG	53°C	45 seconds
3'-gBscreen4	5'CCACGCCTCTGCAATATGACC		

3.1.3 Quantification and purification of DNA by agarose gel electrophoresis

Agarose gel electrophoresis was used to verify the generation of appropriately sized PCR products, plasmids, and integrity of genomic plant DNA. Ethidium bromide-stained DNA was visualized at a wavelength of 300 nm. When required, purification of PCR products from agarose gels was performed using either the QIAEX II kit (Qiagen, Mississauga ON, Canada) or the GENECLEAN kit (Qbiogene, Carlsbad CA, US) using the supplied protocols, and products were eluted using a buffer of 10 mM Tris, 1 mM EDTA at a pH of 8.0.

3.1.4 Ligation reactions

For TA cloning ligation reactions, plasmid DNA and DNA inserts were ligated together using the TA Cloning Kit and the supplied protocol. Ligation reactions not involving TA cloning were performed using T4 DNA ligase (New England Biolabs) according to manufacturer's instructions. When required, plasmids were dephosphorylated at 50°C for 5 minutes using calf intestinal alkaline phosphatase (Invitrogen). All ligation reactions were incubated overnight at 14°C.

3.1.5 Bacterial transformations

For transformations not involving TA cloning, Subcloning DH5α Chemically Competent *E. coli* (Invitrogen) were used. Briefly, 2 μL of the ligation reaction was added to 50 μL of DH5α cells and incubated on ice for 30 minutes. The tubes were then placed in a 42°C water bath for 30 seconds and subsequently incubated on ice for 2 minutes. Luria

Bertani (LB) liquid medium (Fisher Scientific, Ottawa ON, Canada) was added to bring the final volume to 1 mL and the tubes were incubated at 37°C for 1 hour with shaking. The cells were grown overnight at 37°C on LB agar plates containing either 100 μg/mL ampicillin (Sigma-Aldrich Canada Ltd.) or 50 μg/mL kanamycin (Sigma-Aldrich Canada Ltd.), depending on the antibiotic resistance gene encoded within the plasmid. When required, 40 μL of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Invitrogen) was spread onto the surface on LB agar (Fisher Scientific) plates prior to inoculation with transformed bacteria to enable blue-white screening. Single white antibiotic resistant colonies were randomly chosen and grown overnight at 37°C in LB liquid medium with the appropriate antibiotic. Transformation of INVαF' chemically competent *E. coli* cells (Invitrogen) was carried out according to manufacturer's instructions. For long-term storage of transformed bacteria, glycerol stocks were prepared. An aliquot of overnight bacterial cultures (see section 3.1.5) were frozen at -85°C with a final concentration of 15% glycerol (Fisher Scientific).

3.1.6 Extraction of plasmid DNA from bacterial cultures

Plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions, and eluted using TE buffer (pH 8.0). The presence and size of plasmid DNA was confirmed using agarose gel electrophoresis.

3.1.7 Restriction enzyme digests

Restriction enzymes were purchased from New England Biolabs, and digests were performed according to supplied protocols. Agarose gel electrophoresis was used to verify digestion, and products were purified (QIAEX II kit) and eluted using TE buffer (pH 8.0).

3.1.8 Enzymatic generation of blunt ends

For generation of blunt ends following restriction enzyme digestion, restriction enzymes were heat inactivated at 65°C. Mung Bean Nuclease (New England Biolabs) was added at a concentration of 1 unit/μg DNA and the reaction was incubated at 30°C for 30 minutes. The enzymes were removed using 25:24:1 (v/v) phenol: chloroform: isoamyl alcohol (phenol chloroform) (Invitrogen) extraction, to which an equivalent volume of phenol/chloroform was added to the reaction mixture. Centrifugation at 13 000 g was used to separate the resulting two phases. The top layer, containing the DNA, was extracted and ethanol precipitation of DNA was carried out. Briefly, two volumes of 100% ethanol were added, along with sodium acetate to a final concentration of 0.3 M, and the solution frozen at -85°C for two hours. The tubes were centrifuged at 13 000 g and the resulting DNA pellet was washed with 80% ethanol, centrifuged a second time at 13 000 g, and finally resuspended in a small volume of TE buffer (pH 8.0).

3.2 Transformation of *Agrobacterium tumefaciens*

Prior to transformation of *A. tumefaciens*, the cells were made chemically competent for transformation. This was done by inoculating yeast extract/peptone (YEP) medium (Phytotechnology Laboratories, Shawnee Mission KS, US) with a single colony of *A.*

tumefaciens strain EHA105 (a gift from Dr. Hans Schernthaner, Agriculture and Agri-Food Canada), and overnight incubation at 28°C. The following day, 2 mL of this culture was added to 50 mL of YEP medium and incubated overnight at 28°C. The optical density (OD) of the culture at 600 nm after overnight incubation was measured to confirm an OD_{600} between 0.5 and 1. The culture was centrifuged at 3210 g for 10 minutes at 4°C. The bacteria were resuspended in 1 mL of 20 mM $CaCl_2$ and 100 µL aliquots were prepared and stored at -85°C until use.

Competent *A. tumefaciens* were transformed with the desired plasmid purified from recombinant DH5α cells. Five µL of plasmid DNA was used to inoculate a tube of competent *A. tumefaciens*. This mixture was then incubated on ice for 30 minutes and placed into liquid nitrogen for 30 seconds. The cells were thawed at 37°C for 90 seconds. One mL of YEP medium without antibiotics was added to the bacterial-plasmid suspension. The tube was incubated at 28°C for 2 hours, followed by brief centrifugation. Five hundred µL of the supernatant was removed to increase the bacterial cell concentration. The cells were gently resuspended in the remaining media and plated onto YEP agar plates containing 50 µg/mL kanamycin, and incubated at 28°C for 48 hours. Single colonies were then used to inoculate YEP liquid medium with 50 µg/mL kanamycin as indicated above, grown at 28°C for 48 hours, and plasmids were purified using the Qiagen Miniprep Kit as per manufacturer's instructions. Plasmids isolated from the cultures were screened by digesting by restriction enzyme digests to verify the presence of the construct. Colonies containing the approximately 4 kb insert were made into glycerol stocks and stored at -85°C until use.

3.3 Transformation, selection, and regeneration of rice

Please see Appendix, section A1.0 on page 159.

3.4 Transformation, selection, and regeneration of *Arabidopsis thaliana*

A flow diagram (Figure 1) is included to provide an overview of the *A. thaliana* transformation process.

3.4.1 General cultivation of A. thaliana

Wild-type Columbia Col-0 ecotype *A. thaliana* seeds were obtained (gift Dr. Hans Schernthaner, Agriculture & Agri-Foods Canada). The seeds were cold-treated by placing a seed volume of approximately 100 μL in 30 mL of 0.5% agarose and incubating overnight at 4°C. The resuspended seeds were then pipetted onto soil (ProMix BX, Ritchie Feed & Seed, Ottawa ON, Canada) contained within 4" plastic pots (Ritchie Feed & Seed). The pots were placed in a propagation tray (Ritchie Feed & Seed) with a clear cover (Ritchie Feed & Seed) to keep a high level of humidity within the try. Approximately 2 cm of water was added to the bottom of the tray. The plants were grown in a growth chamber with a cycle of 16 hours of light/9 hours of dark and a temperature of 22°C. Once germination of the seeds was observed, the domes were opened half way for 3 to 4 days, at which point the domes were completely removed. The plants started bolting 2 weeks after germination; flowers generally appeared at the 3 to 4 week period with seed pods appearing soon thereafter. Mature seeds were harvested 6 to 8 weeks after germination.

3.4.2 Nomenclature for generations of transformed A. thaliana

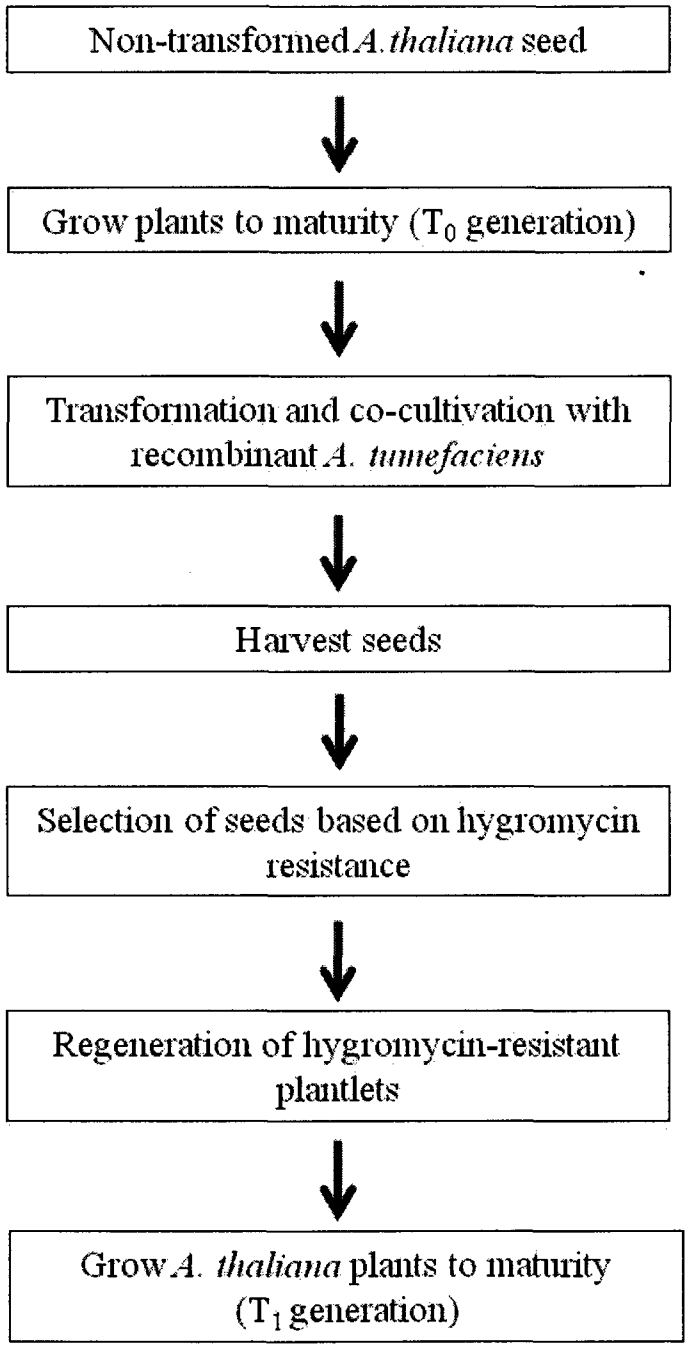
The plants that were initially transformed are called the parent plants, or the T_0 generation. The seeds produced by the T_0 plants, and subsequently screened by the expression of hygromycin resistance, are the T_1 generation. The plants derived from the T_1 seeds are also designated the T_1 generation. The seeds produced by the T_1 plants are the T_2 generation and will produce T_2 plants, and so on.

3.4.3 Floral dip method of transformation

Four days prior to the transformation, YEP plates containing 50 µg/mL hygromycin and 50 µg/mL kanamycin were inoculated with *A. tumefaciens* containing the desired construct and incubated at 28°C for 48 hours. A single colony was then used to inoculate 4 mL of 16 g/L tryptone (Fisher Scientific), 5 g/L yeast extract (Fisher Scientific), and 5 g/L sodium chloride (Sigma-Aldrich Canada Ltd.) liquid medium (2YT) containing 50 µg/mL hygromycin and 50 µg/mL kanamycin and incubated at 28°C overnight. Early the next morning, the 4 mL culture was used to inoculate 500 mL of 2YT medium. The 500 mL culture was grown overnight at 28°C with shaking. The next afternoon, the OD_{600} of the culture was tested and the *A. tumfaciens* were used when the OD_{600} value was between 1.2 and 1.5. The culture was then centrifuged at 3210 g and the supernatant was removed. The cells were then resuspended in 300 mL of transformation medium (½ strength MS medium, 5% D-sucrose, and 150 µL Silwet-77 (Lehle Seeds, Round Rock TX, US).

Meanwhile, non-transgenic *A. thaliana* seeds were cold-treated and planted as indicated in section 3.4.1, except the top of the soil was wrapped with insect meshing (Home

Figure 1: **Flow diagram of the transformation, selection, and regeneration of *A. thaliana*.**


Non-transformed *A. thaliana* seed
Grow plants to maturity (T_0 generation)
Transformation and co-cultivation with recombinant *A. tumefaciens*
Harvest seeds
Selection of seeds based on hygromycin resistance
Regeneration of hygromycin-resistant plantlets
Grow *A. thaliana* plants to maturity (T_1 generation)

Depot, Ottawa ON, Canada). The plants were dipped upside down in resuspended *A. tumefaciens* and held for 45 seconds. Excess medium was shaken off and the plants were laid on their sides in the propagation trays with the clear covers. After 48 hours, the clear covers were removed. After 4 days, the pots were placed upright and transferred back to the growth chamber. All pots were wrapped with Arasystem acetate sheets (Lehle Seeds). The plants were allowed to reach maturity as indicated in section 3.4.1 and the seeds were harvested.

3.4.4 Selection of transformed seeds by antibiotic resistance

Prior to selection with antibiotics the seeds were surface sterilized. A volume of 100 µL of seeds in a 1.5 mL tube were washed briefly with 70% (v/v) ethanol, followed by 50% (v/v) household bleach, and lastly, rinsed 5 times with sterilized water. One mL of 0.1% agarose was added to the tubes and the seeds were incubated at 4°C overnight. The next day, the content of each tube was transferred to a 15 mL plastic tube and the volume was completed to 9 mL with 0.1% agarose. This slurry of seeds was spread onto 4 petri plates (100 mm diameter) of selection medium (1/2 strength of MS salts, 10 g/L D-sucrose, 0.8% agarose) containing 50 µg/mL hygromycin and 250 mg/mL timentin (SmithKline Beecham, Oakville ON, Canada). The plates were incubated for 2 weeks at 22°C in a growth chamber with 16 hours of light and 9 hours of darkness.

3.4.5 Regeneration of antibiotic-resistant plantlets

Antibiotic resistant plantlets were transferred after 2 weeks to recovery medium (½ strength MS salts, 10 g/L D-sucrose, and 0.8% agarose) in sterile Petri plates. Plantlets on

recovery plates were incubated in the same conditions as those for the seeds on selection medium in section 3.4.4. After 2 weeks, the individual plants were transferred to soil and wrapped with Arasystem acetate sheets. A few leaves of the plants were removed and frozen at -85°C for later screening. The plants were grown to maturity over a period of 6 to 8 weeks and the seeds were harvested.

3.5 Testing for expression of β-glucuronidase (GUS) in transformed tissues

A solution of 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc) (Sigma-Aldrich Canada Ltd.) was prepared and plant tissue was placed into the solution. As a negative control, a tube containing non-transformed plant tissue was placed into a separate tube of X-gluc. Mixtures were incubated overnight at 37°C and the following day the development of a blue precipitate was visually recorded.

3.6 Screening for presence of transgene in transformed plants

3.6.1 Extraction and quantitation of plant genomic DNA

One hundred mg of plant material (leaves and stems) was frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted from plant material using the DNeasy Plant Mini Kit (Qiagen), as per manufacturer's instructions. Genomic DNA was eluted using TE buffer (pH 8.0). The integrity of the DNA was determined by agarose gel electrophoresis (see section 3.1.3)

3.6.2 Screening for the presence of the transgene by PCR

Four sets of PCR primers designated gBscreen1 to gBscreen4, as specified in Table 2, were designed to amplify the specific regions along the gene for gB. For PCR reactions with either non-transgenic (negative control) or test samples, 500 to 1000 ng of genomic DNA was used. Twenty-five ng of the plasmid pKTS507 was used as the positive control. A 30 cycle PCR program was used with a 1 minute denaturation at 95°C, 30 seconds of annealing, and elongation at 72°C (see Table 2 for annealing temperature and elongation times). This program was used for all primer sets. Agarose electrophoresis of the PCR products was carried out as indicated in section 3.1.3. PCR screening was used to test all generations of transgenic plants for the presence of the gB transgene.

3.7 Protein extraction from seeds

Proteins were extracted from the seeds of the plants using an extraction buffer which consisted of cold 1X phosphate buffered saline (PBS; Fisher Scientific) supplemented with 1/100 Sigma Plant Protease Inhibitor (Sigma-Aldrich Canada). Two hundred mg of seeds were ground to a fine powder using a mortar, pestle, and liquid nitrogen. The ground seeds were placed into a 1.5 mL microfuge tube and 600 µL of extraction buffer (1:3 (v/v) ratio of seeds to buffer) was added. The ground seeds were vortexed briefly with the buffer and the tube was then incubated at 4°C for 30 minutes with continual mixing. The solids were removed by centrifugation at 10 000 g, 4°C for 30 minutes.

3.8 Quantitation of proteins

Protein concentration was determined by the Pierce bicinchonic acid (BCA) assay kit (Fisher Scientific), as per manufacturer's instructions. Extracted proteins were either used immediately or frozen at -85°C.

3.9 Enzyme-linked immunosorbant assay (ELISA)

Ninety-six well ELISA plates (VWR International, Mississauga ON, Canada) were coated overnight at room temperature with proteins diluted in carbonate buffer (0.1 M sodium carbonate (Sigma-Aldrich Canada Ltd.), 0.1 M sodium bicarbonate (Sigma-Aldrich Canada Ltd.), pH 9.6). Plates were washed in triplicate using ELISA wash buffer (1X PBS with 0.5% Tween-20 (Sigma-Aldrich Canada Ltd.), pH 7.2) after each of the following steps. All incubations were for 1 hour at 37°C. Antibody dilutions and blocking were done using 2% skim milk (bulk purchase, Herb & Spice, Ottawa ON, Canada) in 1X PBS. Primary antibodies were diluted as specified and HRP-labelled secondary antibodies were diluted 1/1 000. Colourmetric determination of protein concentration or antibody titre was done using TMB One Component HRP Microwell Substrate (BioFX, Owings Mills MD, US). Reactions were stopped after 15 minutes with 0.6 N H_2SO_4 and plates were read at 450 nm using the μ-Quant standard microplate reader (Bio-Tek Instruments Inc., Winooski VT, US). All ELISAs were performed twice in duplicate.

3.10 Western blots

Proteins were mixed 1:1 with Laemmli buffer (Sigma-Aldrich Canada Ltd.) and loaded onto pre-cast polyacrylamide gels (either Fisher Scientific or Invitrogen). Proteins

were electrophoresed in a Tris-HEPES-SDS denaturing running buffer (Fisher Scientific) at 150 to 200 V and transferred to 0.45 μm nitrocellulose membranes (BioRad, Hercules CA, US) overnight at 20 V, 4°C. Membranes were blocked in 3% Amersham ECL Blocking Reagent (GE Life Sciences, Piscataway NJ, US) overnight at 4°C. The initial wash after blocking was for 15 minutes using Tris-buffered saline (Fisher Scientific) containing 0.05% Tween-20 (Sigma-Aldrich Canada Ltd.). All subsequent washes were done three times for 10 minutes each. Membranes were probed with a primary antibody for 3 hours at room temperature. All antibodies were appropriately diluted (see section 3.9) in 3% ECL Advance Blocking Reagent (GE LifeSciences). After washing, the membranes were probed with a 1/10 000 dilution of a secondary antibody labelled with horseradish peroxidise, as specified, and incubated with the membranes for 1 hour at room temperature. ECL Western Blotting Detection Reagents (GE LifeSciences) were used as per manufacturer's instructions. Membranes were exposed to Kodak XL X-ray film (VWR International) and the film was developed using an X-OMAT machine (KODAK, Fisher Scientific). All Western blots were repeated at least three times.

3.11 Immunization of guinea pigs

3.11.1 Experimental animals

Twenty female Hartley guinea pigs were obtained from Elm Hill Breeding Labs (Chelmsford MA, US). Approval for the study was obtained from the University of Minnesota. Assistance was provided by staff from the laboratory of Dr. Mark Schleiss, University of Minnesota.

3.11.2 Immunizations

Total soluble proteins were extracted from 2 g of *A. thaliana* seeds expressing GPCMV gB and quantitated as indicated in sections 3.7 and 3.8. As a negative control, proteins were also extracted from non-transformed *A. thaliana* seeds. Twenty-two mg of total soluble seed proteins was emulsified with an equal volume of Complete Freund's adjuvant (Sigma Aldrich, St. Louis MO, US) for the first immunization and Incomplete Freund's adjuvant (Sigma Aldrich, US) for subsequent doses. Fifty μg of purified baculovirus-derived GPCMV gB (Schleiss and Jensen, 2003) was used as a positive control. All doses were delivered subcutaneously into the necks of animals at several sites on days 0, 14, and 45.

3.11.3 Collection of serum

Whole blood was collected from guinea pigs by toe nail-clipping directly into BD Vacutainer tubes (BD Biosciences, Franklin Lakes NJ, US). The tubes were placed on ice until all samples were collected. Tubes were then centrifuged as per manufacturer's instructions. Serum was carefully removed and stored at -20°C. Serum was collected on days 0, 13, 35, and 60.

3.12 Neutralization of GPCMV infectivity

3.12.1 Culturing of guinea pig lung fibroblasts

Guinea pig lung (GPL) fibroblasts (ATCC # CCL-158) were obtained from ATCC (Manassas VA, US). Cells were grown in Kaighn's Modification of Ham's F-12 Medium (F-12K, Cedarlane, Burlington ON, Canada) supplemented with 10% heat-inactivated fetal

bovine serum (FBS, Invitrogen), henceforth referred to as F-12K/FBS. Tissue culture was carried out at 37°C, with 5% CO_2 in 75 cm^2 culture flasks (T75, Fisher Scientific) until confluent. Cells were subcultivated in a 1:4 ratio using 0.25% trypsin/0.53 mM EDTA (ATCC).

3.12.2 Generation of viral stock

A green fluorescent protein (GFP)-tagged isolate of GPCMV was a kind gift from Dr. Mark Schleiss (McGregor and Schleiss, 2001). A viral stock of this isolate was prepared by infecting confluent GPL fibroblasts at 0.001 multiplicity of infection. This was accomplished by removing the medium from the cells and adding 1.5 mL of diluted virus in F-12K/FBS to the T75 flask. The flasks were incubated for 2 hours with gentle tilting every 30 minutes. The medium was then removed and the cells were washed with 1X PBS, after which 20 mL of F-12K/FBS was added and the flasks were incubated for 10 days. The medium was collected and replenished on days 5, 7, and 9. It was centrifuged at 10 000 g for 10 minutes and the supernatant containing the virus was frozen in 1 mL aliquots at -85°C.

3.12.3 Determination of viral titre

Twenty-four well tissue culture plates (Fisher Scientific) were seeded with 1.0×10^4 GPL fibroblasts per well in F-12K/FBS. The plates were incubated until cells were confluent. Viral dilutions as specified were made in F-12K/FBS using supernatant harvested from day 7 of the viral preparation (see section 3.12.2). The medium was removed from the cells and 150 µL of diluted viral stock was used to inoculate the cells. The plates were then incubated for 1.5 hours with intermittent gentle tilting. The unabsorbed virus was removed

and the cells washed 2 times with 1X PBS. F-12K/FBS was added to each well and the plates were again incubated for 72 hours, whereupon the plaques in each well were enumerated using fluorescent microscopy with an inverted microscope equipped with a GFP-specific filter (Leica, Richmond Hill ON, Canada). Assays were done in duplicate. The viral stock dilution producing 60 to 80 plaques per well was used for all subsequent experiments.

3.12.4 Viral neutralization assay

GPL fibroblasts at an initial density of 1.0×10^4 cells/well were grown to confluence in 24 well plates. The viral aliquot was diluted appropriately in F-12K medium supplemented with 20% FBS and 10% normal rabbit serum as a source of complement (Sigma-Aldrich Canada Ltd.). A 3-fold dilution series of the test sera, as specified, was prepared (starting at 1/20, ending at 1/4 860). The dilutions were prepared in F-12K medium supplemented with 10% normal rabbit serum. As a positive control, 3 samples (1/1 250, 1/2500, 1/5000) were prepared using serum from a guinea pig immunized in a previous study with baculovirus-derived GPCMV gB (Schleiss et al., 2004). As a negative control, a pre-immune pool of guinea pig serum was included at a 1/20 dilution. Virus as prepared above was added to the test and control sera at a 1:1 ratio, and incubated at 37°C with intermittent gentle tilting of the plate. After 1 hour 150 µL from each of these sample mixtures was applied to each well of confluent cells, from which media had been removed. The plates were then incubated for 1.5 hours at 37°C, 5% CO_2. The supernatants were removed from each well and the cells were gently washed 2 times with 1X PBS. F-12K supplemented with 10% FBS was added to each well and the plates were incubated at 37°C,

5% CO_2 for 72 hours. At this time, the plaques were enumerated by fluorescent microscopy, as described in section 3.12.3. Neutralization assays were performed twice, in duplicate. The titre was defined as the reciprocal of serum dilution that resulted in 50% reduction of plaques.

4.0 Results

4.1 Engineering of plant expression vectors

4.1.1 Construction of pCAMBIA1301/Gt1/ss/gB/NOS

To enable expression of GPCMV gB in plant seeds, constructs were engineered to allow expression of the gB gene to be under the control of the rice seed glutelin Gt1 promoter. Briefly, the steps involved in this were:

- PCR generation and isolation of the G1 promoter and signal sequence
- Insertion of Gt1 promoter and signal sequence into pGEM4Z
- PCR amplification and insertion of NOS into pGEM4Z/Gt1/ss
- PCR amplification and insertion of the gene for gB into pGEM4Z/Gt1/ss/NOS
- Cloning of Gt1/ss/gB/NOS construct into pCAMBIA1301

Schematic diagrams (Figures 2 and 3) further illustrate the cloning strategy and results are described in the text, with reference to specific figures as indicated.

PCR generation and isolation of the G1 promoter and signal sequence: The 1.8 kb Gt1 promoter and 72 bp signal sequence were previously cloned into the multiple cloning site of pGEM4Z to generate pPH3 (Table 1). The 5' and 3' ends of the Gt1 promoter and signal sequence were sequenced using pUC/M13 forward and reverse sequencing primers (Promega, data not shown). Based on these sequences, the 5'-Gt1*Sac*I and 3'-Gt1ss*Nhe*I PCR primers were designed to generate a *Sac*I restriction enzyme site at the 5' end of the Gt1 promoter and a *Nhe*I site at the 3' end of the Gt1 signal sequence (Table 2). The promoter and signal sequence were PCR amplified using these primers, generating a PCR

Figure 2: **Schematic diagram of the engineering of pGEM4Z/Gt1/ss/NOS.**

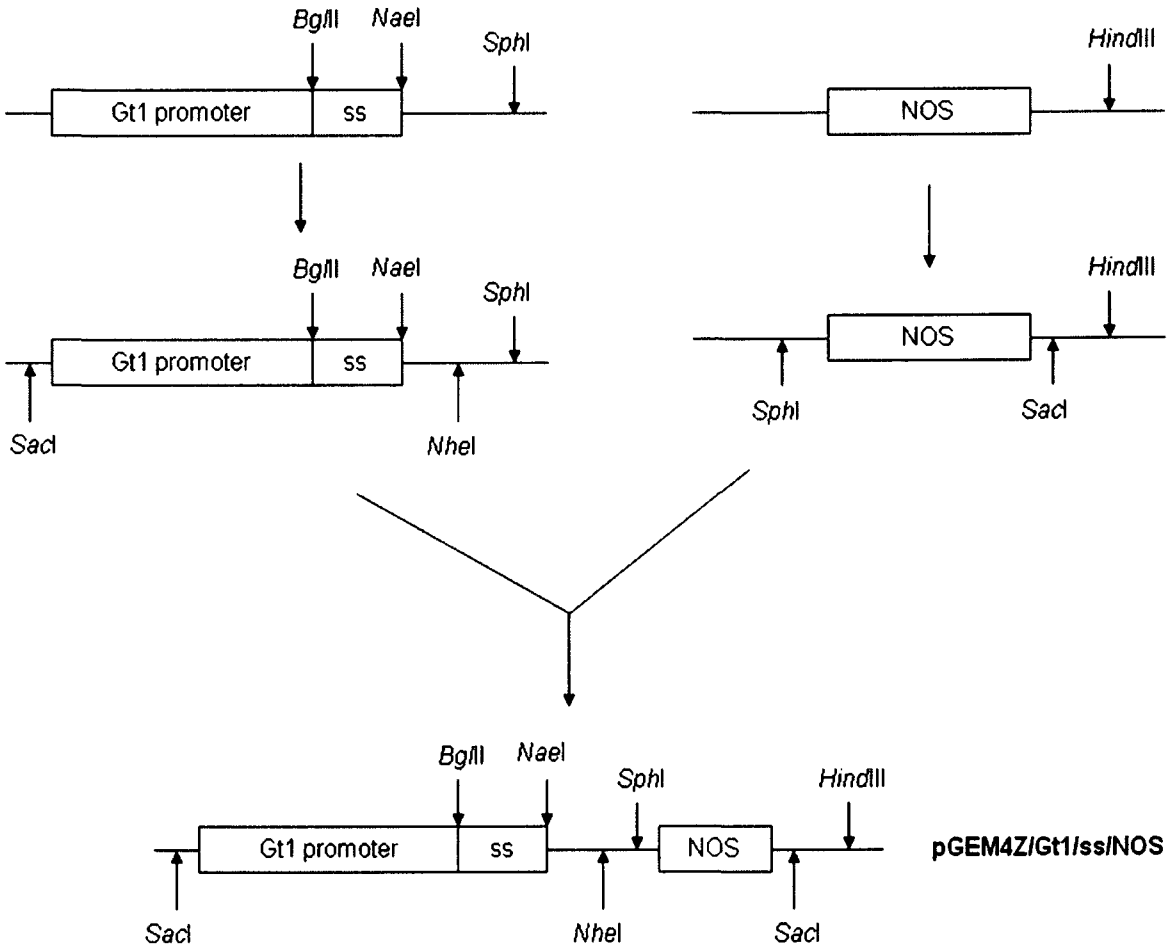
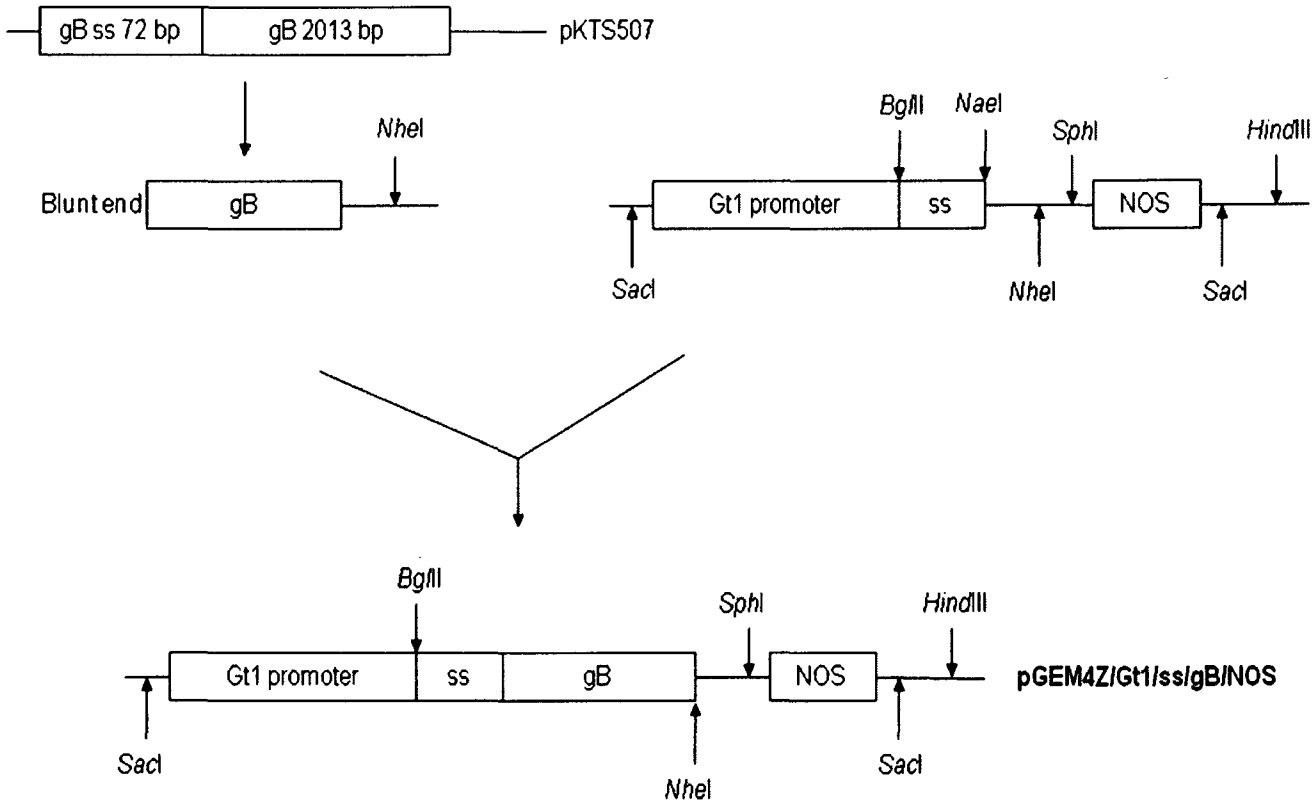

BglII
NaeI
SphI
Gt1 promoter
ss
HindIII
NOS
SacI
NheI
pGEM4Z/Gt1/ss/NOS

Figure 3: **Schematic diagram of the engineering of pGEM4Z/Gt1/ss/gB/NOS.**


gB ss 72 bp
gB 2013 bp
pKTS507
NheI
Blunt end
gB
BglII
NaeI
SphI
HindIII
Gt1 promoter
ss
NOS
SacI
NheI
SacI
BglII
SphI
HindIII
Gt1 promoter
ss
gB
NOS
pGEM4Z/Gt1/ss/gB/NOS
SacI
NheI
SacI

product of 2 kb, as seen in Figure 4A, lanes 2 to 6. The 3'-A overhangs generated by the polymerase allowed TA cloning of the Gt1/ss PCR product into the subcloning vector, pCR2.1. The resulting plasmid was named pCR2.1/Gt1/ss.

Insertion of Gt1 promoter and signal sequence into pGEM4Z: To insert the Gt1 promoter and signal sequence with the new *Sac*I and *Nhe*I sites into pGEM4Z, *Sac*I and *Hind*III restriction enzyme digests of the pCR2.1/Gt1/ss plasmid were performed. As can be observed in Figure 4B, the resulting 2 kb Gt1/ss insert was visualized by agarose gel electrophoresis and subsequently cut from the gel and purified using the GENECLEAN kit. The Gt1 promoter and signal sequence were then inserted into *Sac*I and *Hind*III-digested pGEM4Z, and *E. coli* DH5α cells were transformed. Plasmid DNA extracted from ampicillin-resistant colonies was digested with *Nhe*I to verify creation of the *Nhe*I site during PCR (data not shown). The resulting plasmid was termed pGEM4Z/Gt1/ss.

PCR amplification and insertion of NOS into pGEM4Z/Gt1/ss: A termination sequence, NOS, was inserted into the plasmid to ensure transcription termination. PCR amplification of NOS from pCaMVCN using primers designed to incorporate new *Sph*I (5'-*Sph*INOS) and *Sac*I (3'-*Sac*INOS) sites at the 5' and 3' ends respectively, generated an approximately 300 bp PCR product (Figure 4C; lanes 2 to 6). This was inserted into pCR2.1 and thus termed pCR2.1/NOS. Restriction enzyme digestion of pCR2.1/NOS with *Sph*I and *Hind*III confirmed that the *Sph*I site was successfully generated by the appearance of an approximate 300 bp fragment (Figure 4D; lanes 2 to 4). The 300 bp DNA fragment corresponding to NOS was then inserted into pGEM4Z/Gt1/ss as a *Sph*I-*Hind*III fragment.

Figure 4: **The cloning and generation of pCAMBIA1301/Gt1/ss/gB/NOS.** Initially, a construct consisting of the Gt1 promoter and signal sequence, and the NOS termination sequence was generated. The gene for gB was then inserted. The following are agarose gels stained with ethidium bromide showing the stages of construction.

A: PCR was used to amplify the Gt1 promoter and signal sequence from a plasmid, pPH3. Lanes 1, 8: 1 kb Plus DNA Ladder (Invitrogen); Lanes 2, 3: 54 ng and 108 ng respectively of double PCR product using pPH3 as template; Lanes 4, 5, 6: 160 ng, 320 ng, and 640 ng respectively of pPH3 as PCR template; Lane 7: no DNA template.

B: pCR2.1/Gt1/ss digested with *Sph*I and *Sac*I. Lanes 1, 13: 1 kb Plus DNA Ladder; Lanes 2, 12: undigested pCR2.1/Gt1/ss; Lanes 3 to 11: SacI/HindIII restriction enzyme digests from plasmids isolated from DH5α cells transformed with pCR2.1/Gt1/ss ligation product.

C: PCR product resulting from the use of primers designed to amplify NOS from a commercial vector, pCaMVN. Lanes 1, 8: 1 kb Plus DNA Ladder; Lanes 2 to 6: PCR products using varying amounts of $MgSO_4$ (2 mM increments, starting with 2 mM) in the PCR reaction.

D: Restriction enzyme digests of pCR2.1/NOS. Lanes 1, 7: 100bp DNA Ladder (New England Biolabs); Lanes 2 to 4: plasmids isolated from DH5α cells transformed with pCR2.1/NOS ligation reaction and subsequently digested with *Sph*I and *Hind*III; Lane 5: pCR2.1 digested with *Sph*I and *Hind*III; Lane 6: identical plasmid DNA as lane 2 but digested with *Eco*RI.

E: *Sph*I and *Hind*III digestion of pGEM4Z/Gt1/ss/NOS to verify presence of NOS insert. Lanes 1, 15: 100 bp DNA Ladder; Lanes 2, 14: undigested plasmid DNA isolated from DH5α cells transformed with pGEM4Z/Gt1/ss/NOS ligation reaction; Lanes 3 to 13: *Sph*I, *Hind*III digested plasmid DNA originally isolated from separate colonies resulting from DH5α cells transformed with pGEM4Z/Gt1/ss/NOS ligation reaction

F: PCR products using pKTS507 as template. Lanes 1, 7: 1 kb Plus DNA Ladder; Lanes 2 to 5: PCR products using varying amounts of $MgSO_4$ (2 mM increments, starting with 2 mM) in the PCR reaction; Lane 6: no DNA template.

G: Verification of presence of Gt1/ss/gB/NOS in pCAMBIA1301. Lanes 1, 9: 1 kb Plus DNA Ladder; Lanes 2 to 7: *Sac*I digested plasmid DNA isolated from DH5α cells transformed with pCAMBIA1301/Gt1/ss/gB/NOS ligation reaction; Lane 8: pCAMBIA1301 digested with *Sac*I.

A
1 2 3 4 5 6 7 8
2 kb
B
1 2 3 4 5 6 7 8 9 10 11 12 13
2 kb
C
1 2 3 4 5 6 7 8
300 bp
D
1 2 3 4 5 6 7
300 bp
E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
300 bp
F
1 2 3 4 5 6 7
2 kb
G
1 2 3 4 5 6 7 8 9
2 kb

Successful ligation was confirmed by re-digesting the plasmid with *Sph*I and *Hind*III and the presence of a 300 bp band as visualized by agarose gel electrophoresis (Figure 4E; lanes 3 to 13). All ligation junctions were sequenced and data confirmed that correct ligation had occurred, without any loss of base pairs. This plasmid was designated pGEM4Z/Gt1/ss/NOS.

PCR amplification and insertion of the gene for gB into pGEM4Z/Gt1/ss/NOS: To insert the gene for gB into pGEM4Z/Gt1/ss/NOS, a 2 kb amplicon with blunt ends and a *Nhe*I site at the 3' end of the gene was generated by PCR amplification of the gB gene from pKTS507 using the primers (5'-gBGt1, 3'-gBGt1) (Figure 4F; lanes 2 to 5). The PCR product was digested with *Nhe*I and inserted into pGEM4Z/Gt1/ss/NOS in-frame with the signal sequence. Sequencing of the gB gene confirmed correct insertion into pGEM4Z/Gt1/ss/NOS. The new plasmid was designated pGEM4Z/Gt1/ss/gB/NOS.

Cloning of Gt1/ss/gB/NOS construct into pCAMBIA1301: The Gt1/ss/gB/NOS construct was excised from this final pGEM4Z/Gt1/ss/gB/NOS plasmid and inserted into the plant expression vector pCAMBIA1301 as a *Sac*I fragment, in order to allow protein expression of gB in plants. *Sac*I digestion of pCAMBIA1301/Gt1/ss/gB/NOS produced a 4 kb fragment, confirming insertion of the construct (Figure 4G; lanes 2 to 7).

4.1.2 Construction pCAMBIA1301/Gt1/pp65/NOS

As for the expression of GPCMV gB in seeds, it was necessary to place the pp65 gene under the control of the rice Gt1 promoter. Briefly, the steps involved in this were:

- Removal of the signal sequence from pGEM4Z/Gt1/ss/NOS
- PCR amplification and insertion of the gene for pp65 into pGEM4Z/Gt1/NOS

A schematic diagram (Figure 5) further illustrates the cloning strategy and results are described in the text, with reference to specific figures as indicated.

Removal of the signal sequence from pGEM4Z/Gt1/ss/NOS: To remove the signal sequence, the plasmid was digested with *Bgl*II, a restriction enzyme that cut just upstream from the 5' end of the signal sequence. Next, the plasmid was treated with Mung Bean nuclease to create blunt ends. Finally, the plasmid was digested with *Nhe*I which completely removed the signal sequence from the plasmid by cutting downstream of the 3' end of the signal sequence. Removal of the signal sequence was confirmed by the presence of a band of 72 bp as visualized by agarose electrophoresis (Figure 6A; lane 2).

PCR amplification and insertion of the gene for pp65 into pGEM4Z/Gt1/NOS: PCR was used to amplify the pp65 gene from pKTS437 and generate a new *Xba*I site at the 3' end of the gene, using the primers 5'-pp65Gt1 and 3'-pp65Gt1. The resulting 1.5 kb PCR product (Figure 6B; lanes 3 to 5) was digested with *Xba*I and inserted into the *Nhe*I site of pGEM4Z/Gt1/NOS. Transformation of *E. coli* cells did not result in the generation of any colonies on agar plates containing ampicillin. Subsequent work was therefore directed at engineering other plasmids.

Figure 5: **Schematic diagram of the engineering of pGEM4Z/Gt1/pp65/NOS.**

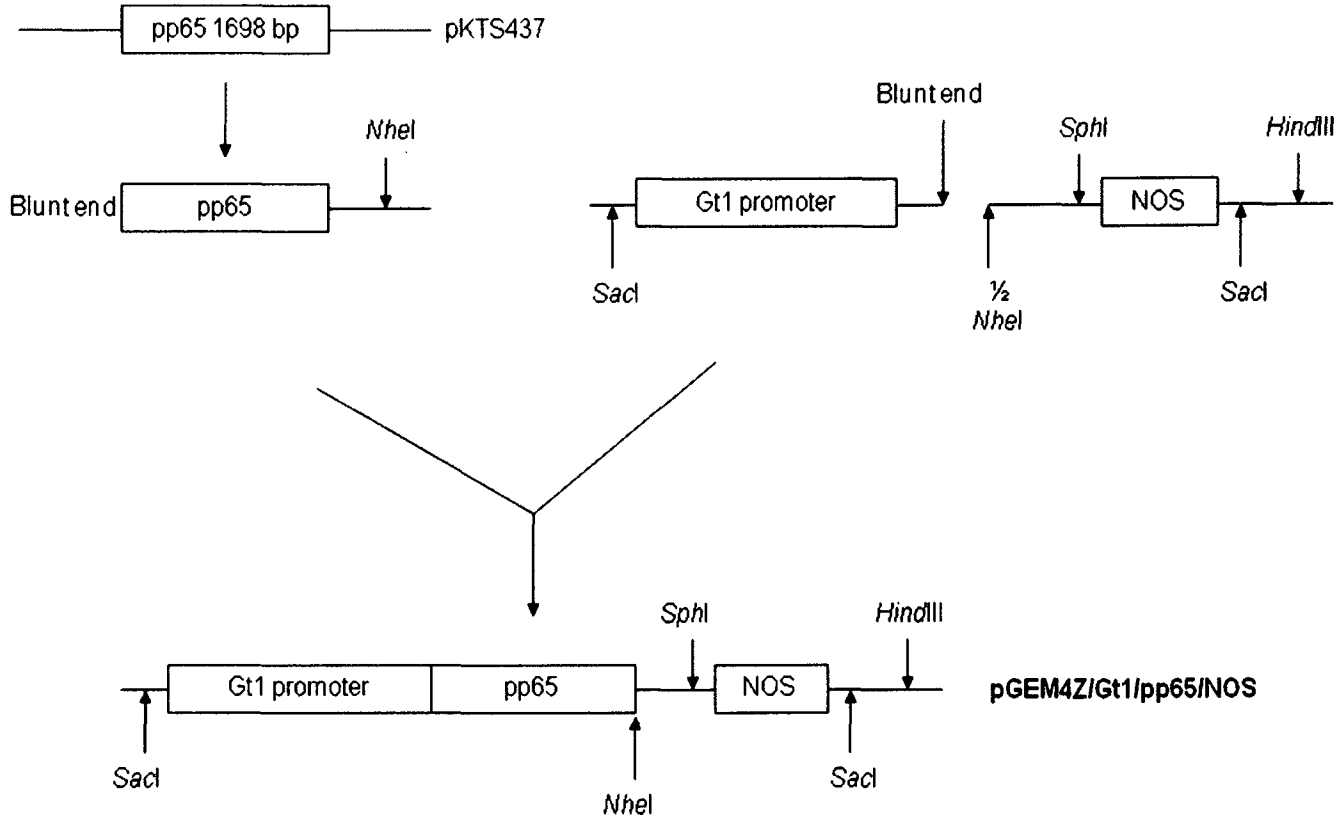
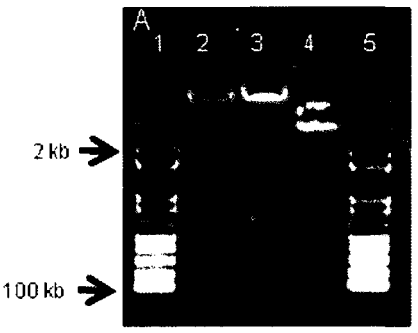
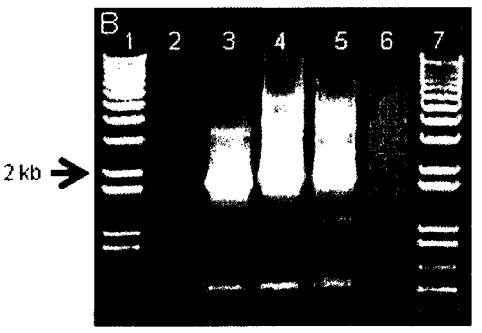

pp65 1698 bp
pKTS437
NheI
Blunt end
pp65
Blunt end
Gt1 promoter
SphI
HindIII
NOS
SacI
½
NheI
SacI
SphI
HindIII
Gt1 promoter
pp65
NOS
pGEM4Z/Gt1/pp65/NOS
SacI
NheI
SacI

Figure 6: The cloning and generation of pCAMBIA1301/Gt1/pp65 /NOS. The initial backbone of the construct consisting of the Gt1 promoter and signal sequence, and the nopaline synthetase termination sequence generated for the pGEM4Z/Gt1/ss/gB/NOS construct was used. The Gt1 signal sequence was removed and the gene for pp65 was inserted. The following are agarose gels stained with ethidium bromide showing the stages of construction.

A: Removal of the Gt1 signal sequence was done using the restriction enzymes, *Bgl*II and *Nhe*I, and Mung bean nuclease. Lanes 1, 5: 1 kb Plus DNA Ladder; Lane 2: pGEM4Z/Gt1/ss/NOS digested with *Bgl*II, treated with Mung Bean nuclease, and digestion with *Nhe*I; Lane 3: pGEM4Z/Gt1/ss/NOS digested with BglII and treated with mung bean nuclease; Lane 4: undigested pGEM4Z/Gt1/ss/NOS.

B: PCR amplification of pp65 from pKTS437. Lanes 1, 7: 1 kb Plus DNA Ladder; Lanes 2 to 5: PCR products using varying amounts of $MgSO_4$ (2 mM increments, starting with 2 mM) in the PCR reaction; Lane 6: no DNA template.


A
1 2 3 4 5
2 kb
100 kb


B
1 2 3 4 5 6 7
2 kb

4.1.3 Construction of pCAMBIA1301/Ubi/gB/NOS

To enable expression of GPCMV gB in all plant constituents rather than just seeds, constructs were generated to express gB under the control of the maize Ubi promoter. Briefly, the steps involved in this were:

- Isolation and insertion of Ubi promoter into pGEM4Z
- Isolation of NOS from pGEM4Z and subsequent insertion into pGEM4Z/Ubi
- PCR amplification of the gene for gB and insertion into pGEM4Z/Ubi/NOS
- Cloning of Ubi/gB/NOS into pCAMBIA1301

A schematic diagram (Figure 7) further illustrates the cloning strategy and results are described in the text, with reference to specific figures as indicated.

Isolation and insertion of Ubi promoter into pGEM4Z: *Pst*I digestion of the pAHC25 plasmid resulted in the removal of the 2 kb maize Ubi promoter (Figure 8A; lanes 3 to 6). The promoter was then inserted into pGEM4Z at the *Pst*I site and the resulting plasmid was designated pGEM4Z/Ubi. To verify that the ubiquitin promoter inserted into pGEM4Z in the correct orientation, the plasmid was re-digested with *Sal*I. Correct orientation of the promoter was confirmed by the generation of fragments of approximately 600 bp and 4100 bp sizes (Figure 8B, lanes 3 and 4).

Isolation of NOS from pGEM4Z and subsequent insertion into pGEM4Z/Ubi: To accomplish this, NOS was released from pCR2.1/NOS (previously described in section 4.1.1) as a *Sph*I-*Hind*III fragment and inserted into pGEM4Z/Ubi at the *Sph*I and *Hind*III

Figure 7: **Schematic diagram of the engineering of pGEM4Z/Ubi/gB/NOS**

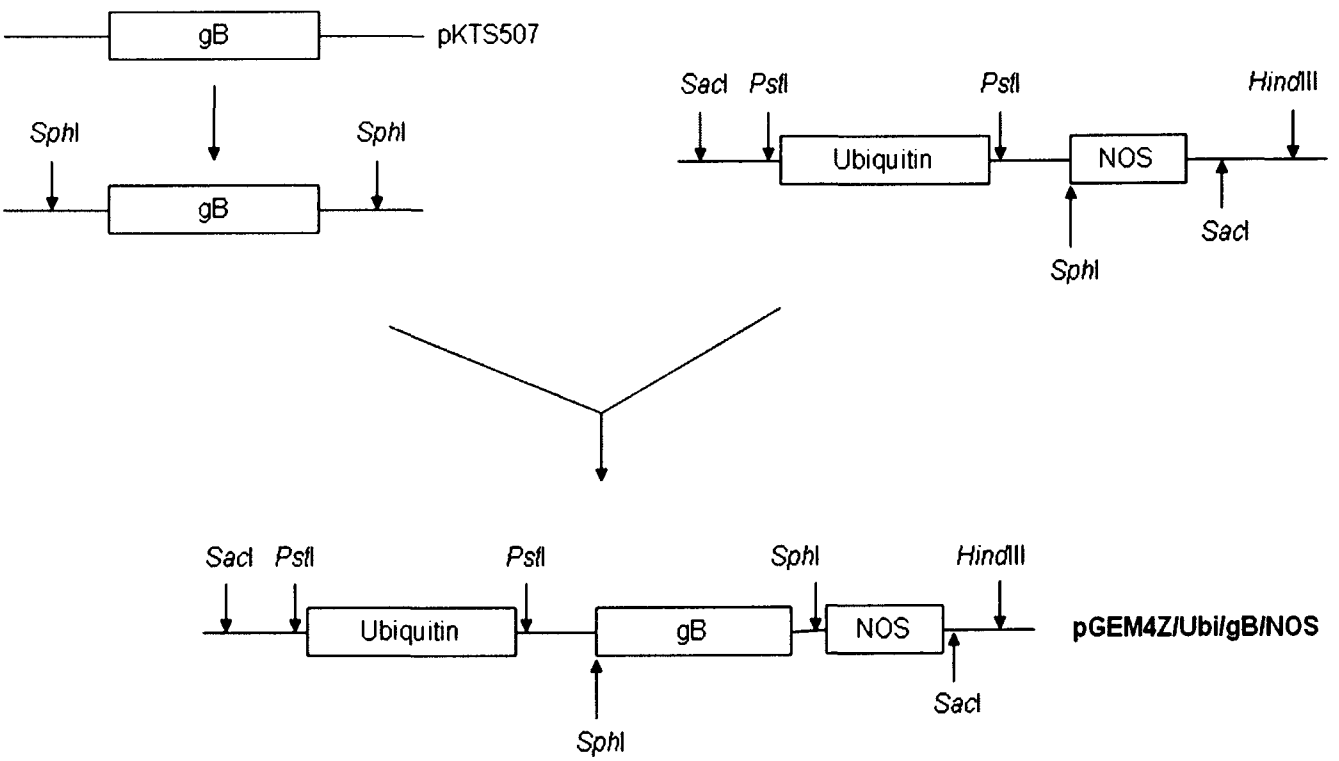

gB
pKTS507
SphI
SphI
gB
SacI
PstI
PstI
HindIII
Ubiquitin
NOS
SphI
SacI
SacI
PstI
PstI
SphI
HindIII
Ubiquitin
gB
NOS
SacI
SphI
pGEM4Z/Ubi/gB/NOS

Figure 8: The cloning and generation of pCAMBIA1301/Ubi/gB/NOS. The maize Ubi promoter and the NOS termination sequence were first ligated together. The gene for gB was then inserted. The following are agarose gels stained with ethidium bromide showing the stages of construction.

A: The maize Ubi promoter was removed from pAHC25 by digesting the plasmid with *Pst*I. Lane 1: 1 kb DNA ladder (New England Biolabs); Lane 2: undigested pAHC25; Lanes 3 to 6: products of *Pst*I digestion of pAHC25.

B: Screening of pGEM4Z/Ubi by *Sal*I digestion to verify presence and correct orientation of the promoter within the plasmid. Lane 1: 1 kb DNA ladder; Lane 2: pGEM4Z digested with *Sal*I; Lanes 3, 4, 5: pGEM4Z/Ubi DNA from colonies digested with *Sal*I.

C: NOS was extracted from pGEM4Z/NOS as a SphI-HindIII fragment and inserted into pGEM4Z/Ubi. Colonies were screened by SphI-HindIII restriction enzyme digestion to verify the insertion of NOS. Lane 1: 1 kb DNA ladder; Lanes 2 to 9: *Sph*I/*Hind*III digestion of plasmid DNA extracted from DH5α cells transformed with pGEM4Z/Ubi/NOS ligation reaction; Lanes 10, 11: *Sph*I/*Hind*III digestion of pGEM4Z/Ubi.

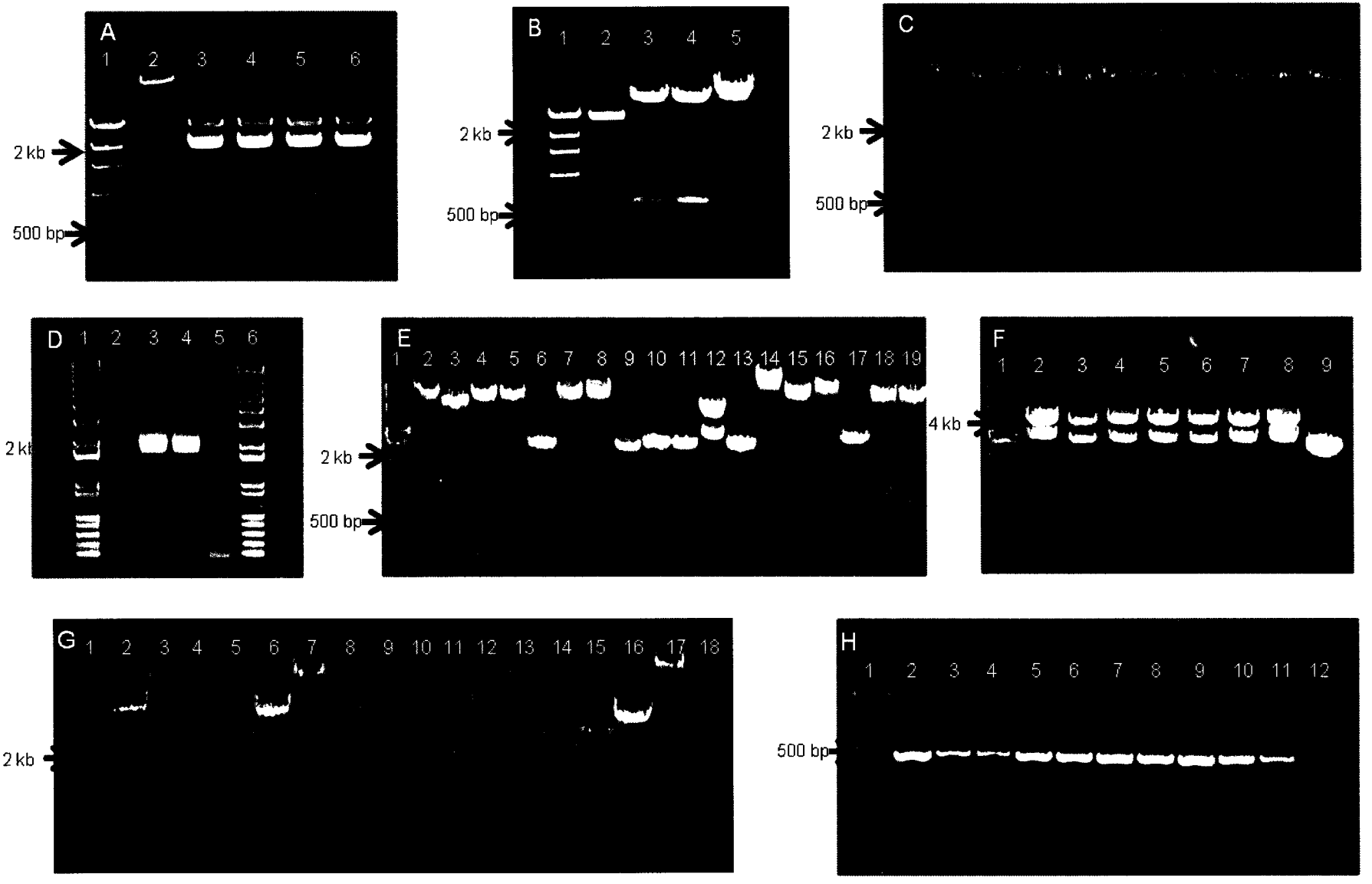
D: The gene for gB was PCR-amplified from pKTS507 using primers to include the gB 72 bp signal sequence and generate a *Sph*I at the 5' and 3' end of the gene. Lanes 1, 6: 1 kb Plus DNA ladder; Lanes 2 to 4: PCR products resulting from the PCR amplication of pKTS507; Lane 5: no DNA template in PCR reaction.

E: pGEM4Z/Ubi/gB/NOS plasmid DNA was screened using *Hind*III to verify orientation of gB within the plasmid after ligation. Lane 1: 1 kb DNA ladder; Lane 2: undigested pGEM4Z/Ubi/gB/NOS; lanes 3 to 19: *Hind*III digested plasmid DNA isolated from DH5α transformed with pGEM4Z/Ubi/gB/NOS ligation reaction.

F: Colonies that tested positive for the correct orientation of gB within pGEM4Z/Ubi/gB/NOS were digested with *Sac*I to remove the Ubi/gB/NOS construct. Lane 1: 1 kb DNA ladder; Lanes 2 to 8: *Sac*I digestion of pGEM4Z/Ubi/gB/NOS colonies; Lane 9: undigested pGEM4Z/Ubi/NOS.

G: Ubi/gB/NOS was inserted into the *Sac*I site of pCAMBIA1301. DH5α cells were transformed with this ligation reaction and the presence of Ubi/gB/NOS was verified by *Sac*I digestion. Lane 1, 18: 1 kb DNA ladder. Lanes 2, 4, 6, 8, 10, 12: undigested plasmids; Lanes 3, 5, 7, 9, 11. 13: corresponding plasmids digested with *Sac*I; Lanes 14, 16: undigested pCAMBIA1301; Lanes 15, 17: pCAMBIA1301 digested with *Sac*I.

H: To further verify the presence of the gB gene within pCAMBIA1301/Ubi/gB/NOS, primers specific for a region of gB. Lane 1: 100 bp DNA ladder; Lanes 2 to 10: PCR products using pCAMBIA1301/Ubi/gB/NOS plasmid DNA extracted from transformed DHα cells. Lane 11: pKTS507 as template DNA; Lane 12: no DNA template.


A
1 2 3 4 5 6
2 kb
500 bp
B
1 2 3 4 5
2 kb
500 bp
C
2 kb
500 bp
D 1 2 3 4 5 6
2 kb
E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
2 kb
500 bp
F
1 2 3 4 5 6 7 8 9
4 kb
G 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
2 kb
H
1 2 3 4 5 6 7 8 9 10 11 12
500 bp

sites to create pGEM4Z/Ubi/NOS. To verify insertion, pGEM4Z/Ubi/NOS was re-digested with *Sph*I and *Hind*III, releasing the 300 bp terminator sequence (Figure 8C; lanes 2 to 9).

PCR amplification of the gene for gB and insertion into pGEM4Z/Ubi/NOS: To this end, primers (5'-gBUbi, 3'-gBUbi) were designed to incorporate the gB signal sequence and gene and also generate new *Sph*I sites at the 5' end of the signal sequence and the 3' end of the gB gene. A PCR reaction with these primers amplified the gB signal sequence and gene from pKTS507, producing a 2 kb PCR product (Figure 8D; lanes 3 and 4). The orientation of the gB gene was confirmed within pGEM4Z/Ubi/gB/NOS by digesting the plasmid with *Hind*III. Correct orientation was verified by the presence of DNA fragments of the size approximately 700 bp and 8000 bp (Figure 8E; lanes 3 to 19).

Cloning of Ubi/gB/NOS into pCAMBIA1301: To enable expression of the gB protein in plants, the Ubi/gB/NOS construct was inserted into the plant transformation vector, pCAMBIA1301. The entire 4200 bp Ubi/gB/NOS construct was removed from pGEM4Z/Ubi/gB/NOS by restriction enzyme digestion with *Sac*I (Figure 8F; lanes 2 to 8) and inserted into the *Sac*I site of pCAMBIA1301. To verify insertion, the pCAMBIA1301/Ubi/gB/NOS plasmid was re-digested with *Sac*I. As shown in Figure 8G (lanes 3, 9, 11, and 13), the 4200 bp fragment resulting from this digestion confirmed that the insert was present. As well, a PCR reaction was carried out using gB-specific primers (5'-gBscreen1, 3'-gBscreen1) and the pCAMBIA1301/Ubi/gB/NOS plasmid as a template. A PCR product of approximately 2 kB confirmed that gB was present within pCAMBIA1301/Ubi/gB/NOS (Figure 8H; lanes 2 to 10). Sequencing of the 5' and 3' ends

of the gB gene within the pCAMBIA1301/Ubi/gB/NOS plasmid indicated that the full gB gene was present within the plasmid.

4.1.4 Construction of pCAMBIA1301/Ubi/pp65/NOS

To create an expression vector for ubiquitous expression of pp65 in plants, the gene for pp65 was ligated to the maize Ubi promoter within the plasmid pAHC25. Briefly, the steps involved in this were:

- Removal of gene for GUS from pAHC25
- PCR amplification of the gene for pp65 and insertion into pAHC25
- Cloning of Ubi/pp65/NOS into pCAMBIA1301

A schematic diagram (Figure 9) further illustrates the cloning strategy and results are described in the text, with reference to specific figures as indicated.

Removal of gene for GUS from pAHC25: This was accomplished by digesting pACH25 with *Sac*I and *Sma*I. This released the gene for GUS, which was confirmed by the generation of a 2 kb fragment, as visualized by agarose gel electrophoresis (Figure 10A; lanes 3 to 9). This permitted the exchange of GUS for pp65.

PCR amplification and insertion of gene for pp65: The 1700 bp gene for pp65 was amplified from pKTS437 by PCR, using a 3' primer (3'-pp65Ubi) that generated a new *Sac*I site at the 3' end of the gene and a 5' primer (5'-pp65Ubi) that incorporated the ATG codon (Figure 10B; lanes 3 and 4). This allowed pp65 to be cloned into pAHC25 as a blunt-end/*Sac*I fragment, resulting in the generation of the plasmid referred to as

Figure 9: **Schematic diagram of the engineering of pAHC/Ubi/pp65/NOS.**

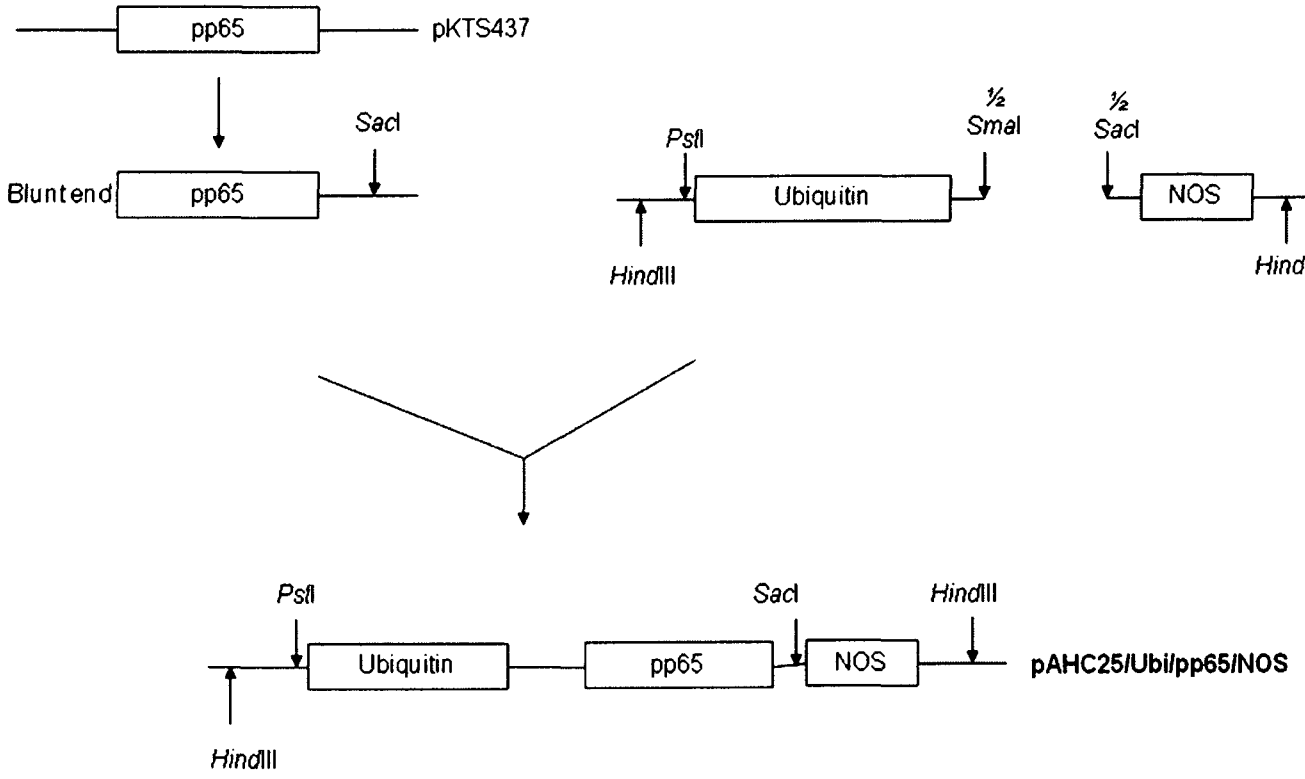

pp65
pKTS437
SacI
Blunt end
pp65
PstI
½
SmaI
Ubiquitin
HindIII
½
SacI
NOS
HindIII
PstI
Ubiquitin
pp65
SacI
NOS
HindIII
HindIII
pAHC25/Ubi/pp65/NOS

Figure 10: The cloning and generation of pCAMBIA1301/Ubi/pp65/NOS. The gene for GUS was exchanged for pp65 to generate pAHC25/pp65. The following are agarose gels stained with ethidium bromide showing different stages of construction.

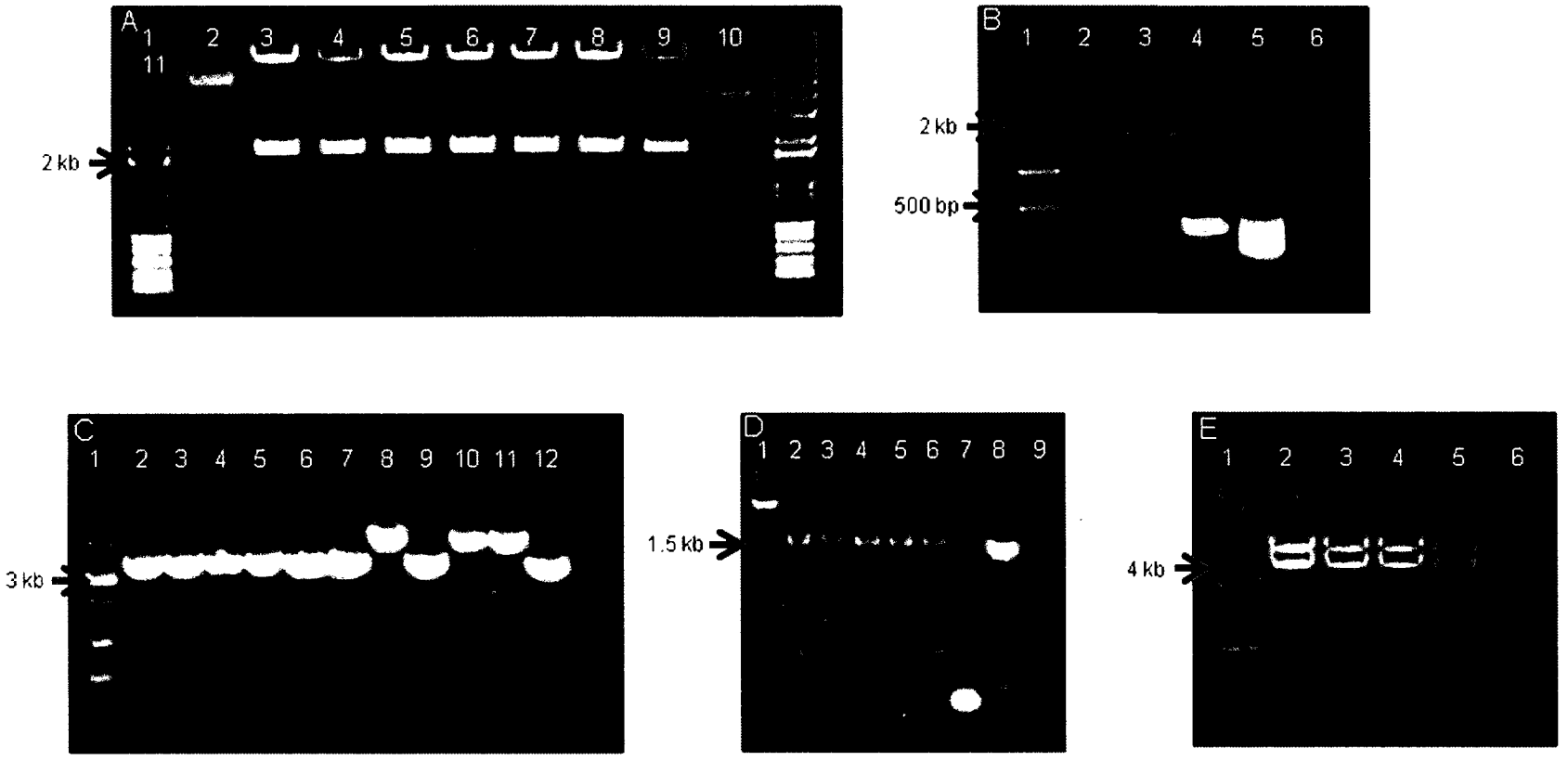
A: The gene for GUS was removed from pAHC25 by digesting the plasmid with *Sma*I and *Sac*I. Lanes 1, 11: 1 kb Plus DNA ladder; Lane 2: undigested pAHC25; Lanes 3 to 9: pAHC25 digested with *Sma*I and *Sac*I; Lane 10: undigested pKTS437.

B: The gene for pp65 was amplified by PCR from pKTS437. The primers incorporated a new *Sac*I site at the 3' end of the gene. Lane 1: 1 kb DNA ladder; Lanes 2 to 4: PCR products using varying amounts of $MgSO_4$ (2 mM increments, starting at 2 mM); Lane 5: no DNA template.

C: The PCR product for pp65 was digested with *Sac*I and inserted into pAHC25 as a blunt-end/*Sac*I fragment. Lane 1: 1 kb DNA ladder; Lanes 2 to 12: plasmid DNA extracted from DHα cells transformed with pAHC25/pp65 ligation product.

D: To verify presence of pp65 in the plasmid, pAHC25/pp65, PCR was performed using primers specific for pp65. Lane 1: 1 kb DNA ladder; Lanes 2 to 6: plasmids extracted from DH5α cells transformed with pAHC25/pp65; Lane 7: no DNA template; Lane 8: pKTS437 as the template.

E: Ubi/pp65/NOS was extracted from pAHC25 by restriction enzyme digestion with *Hind*III. This would enable insertion into the *Hind*III site of pCAMBIA1301. Lane 1: 1 kb DNA ladder; Lanes 2 to 4: *Hind*III digestion of pAHC25/pp65 extracted from DH5α cells; Lane 5: pAHC25 digested with *Hind*III. Lane 6: blank.


A
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11
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4
5
6
7
8
9
10
2 kb
B
1
2
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5
6
2 kb
500 bp
C
1
2
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4
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7
8
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10
11
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3 kb
D
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2
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4
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6
7
8
9
1.5 kb
E
1
2
3
4
5
6
4 kb

pAHC25/Ubi/pp65/NOS. Plasmid DNA isolated from *E. coli* cells was screened by the visualization of plasmids containing the pp65 gene (as opposed to pAHC25 self ligation) of approximately 7 kb in size in an agarose gel (Figure 10C; lanes 8, 10, and 11). Successful insertion of pp65 into pAHC25 was further confirmed by the PCR amplification of a 1500 bp product using pp65-specific primers (Figure 10D; lanes 2 to 6).

Cloning of Ubi/pp65/NOS into pCAMBIA1301: The 4000 bp Ubi/pp65/NOS construct was removed from pAHC25 as a *Hind*III-*Hind*III fragment (Figure 10E; lanes 2 to 5) and finally inserted into the *Hind*III site of pCAMBIA1301, generating pCAMBIA1301/Ubi/pp65/NOS.

4.2 Screening of transformed *A. tumefaciens*

A. tumefaciens-mediated transformation was used to insert the genetic constructs described in section 4.1 into the genomes of both rice and *A. thaliana*. To do this, *A. tumefaciens* was first transformed with either the plant transformation vector pCAMBIA1301/Gt1/ss/gB/NOS or pCAMBIA1301/Ubi/gB/NOS. For the pCAMBIA1301/Gt1/ss/gB/NOS plasmid, verification of successful transformation of *A. tumefaciens* was carried out by extracting plasmid DNA from the bacteria and digesting these plasmids with the restriction enzyme *Sac*I. As shown in Figure 11A, an approximate 4 kb DNA fragment was produced by the *Sac*I digestion, corresponding to the size of the Gt1/ss/gB/NOS construct. Successful transformation with pCAMBIA1301/Ubi/gB/NOS

was confirmed by an 800 bp PCR amplicon using gB-specific primers (5'-gBscreen3, 3'-gBscreen3) and extracted plasmid from *A. tumefaciens* as a DNA template (Figure 11B).

Please note that only the gene construct Gt1/ss/gB/NOS was used in the work further described in this thesis.

4.3 Selection and regeneration of rice transformed with pCAMBIA1301/Gt1/ss/gB/NOS

Please see section A2.0, page 164 in the Appendix for the results regarding the selection and regeneration of transformed rice callus tissue.

4.4 Selection, regeneration, and screening of *A. thaliana* transformed with pCAMBIA1301/Gt1/ss/gB/NOS

4.4.1 Selection based on expression of hygromycin phosphotransferase

Since regeneration of transformed rice callus tissue was problematic, I decided to express the GPCMV proteins in the dicotyledonous plant, *A. thaliana* in parallel studies. The rationale for this was that *A. thaliana* is relatively easy to transform and transformed plants reach maturity within 6 to 8 weeks. To transform *A. thaliana*, unopened flowers on non-transgenic plants were transformed as described in section 3.4.3, using *A. tumefaciens* containing the engineered pCAMBIA1301/Gt1/gB/NOS vector (Figure 12A). These plants were grown to maturity and seeds were harvest. Seeds from these T_0 transformed plants were then selected based on resistance to hygromycin. After 2 weeks, seeds not carrying the hygromycin resistance gene did not grow past the initial stage of germination. Those resistant to

Figure 11: ***A. tumefaciens*** **strain, EHA105, was transformed with pCAMBIA1301/Gt1/ss/gB/NOS and pCAMBIA1301/Ubi/gB/NOS.** Prior to the transformation of rice callus tissue or *A. thaliana*, plasmid DNA was extracted and digested with *Sac*I. As well, PCR was performed using gB-specific primers with plasmids extracted from *A. tumefaciens* EHA105 as the template DNA. Both of these procedures were performed in order to verify the presence of the constructs within transformed EHA105.

A: *Sac*I digestion of pCAMBIA1301/Gt1/ss/gB/NOS. Lanes 1, 6: 1 kb DNA ladder. Lanes 2 to 4: separate colonies of EHA105 digested with *Sac*I; Lane 5: undigested pCAMBIA1301/Gt1/ss/gB/NOS.

B: PCR products using gB-specific primers. Lane 5: 1 kb DNA ladder; Lanes 1 to 4, 6 to 9: PCR products resulting from a PCR reaction using pCAMBIA1301/Ubi/gB/NOS extracted from separate colonies of EHA105 as the template DNA; Lane 10: pKTS507 as template DNA; Lane 11: pCAMBIA1301/Gt1/ss/gB/NOS extracted from EHA105 as template DNA; Lane 12: no DNA template.

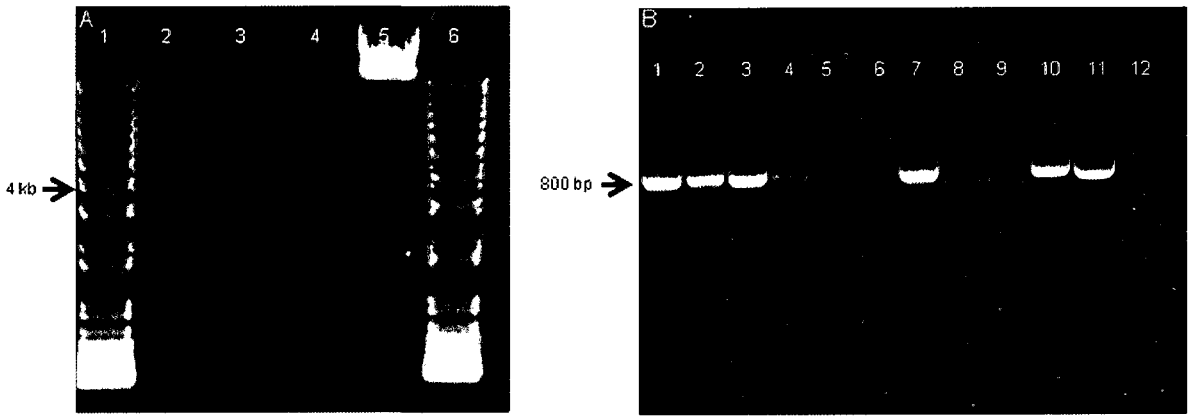

A
1
2
3
4
5
6
4 kb
B
1
2
3
4
5
6
7
8
9
10
11
12
800 bp

Figure 12: Overall transformation procedure for *A. thaliana*. Non-transgenic, wild-type *A. thalianas* plants were transformed using the floral-dip method described in section 3.4.3. These plants were transformed with *A. tumefaciens* carrying the pCAMBIA1301/Gt1/ss/gB/NOS plasmid.

A: Unopened flowers are the target for *A. tumefaciens*-mediated transformation.

B: Selection of the seeds produced by the transformed plants. This was done by placing the seeds on selection media containing hygromycin and timentin for 2 weeks.

C: Hygromycin-resistant plantlets were transferred to regeneration media for 2 weeks.

D: After 2 weeks, the plantlets were transferred to soil and wrapped with Arasystem acetate sheets to prevent cross-pollination.

E: Seeds were collected from the plants and grown in soil. Several generations of plants were produced.

B

C

hygromycin were able to grow into tiny T_1 plantlets approximately 1 cm in height (Figure 12B). Independent transformations were carried out twice, generating a total of over 18 T_1 plantlets. Based on an estimated 2500 seeds per plate,
and 1 to 2 hygromycin resistant plantlets per plate, the transformation frequency was calculated as 0.04 to 0.08%.

4.4.2 Regeneration of hygromycin-resistance A. thaliana

The selected T_1 plantlets were transferred to recovery medium without antibiotics, on which they grew into bushy masses with multiple leaves and stems (Figure 12C). The plantlets were kept on recovery medium for a period of 2 weeks and then transferred to soil, where they were allowed to grow to maturity (Figure 12D). Ten out of the approximately 18 plantlets survived in soil.

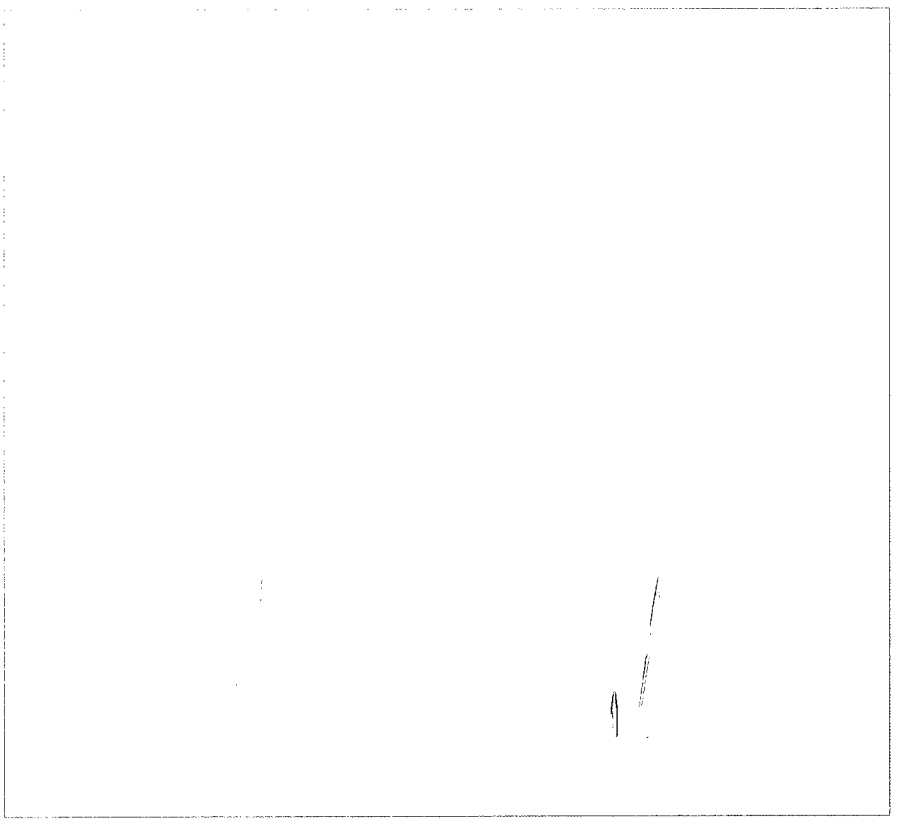
4.4.3 Screening based on expression of β-glucuronidase

When the plants were large enough and there was enough plant material available, some leaves were removed for screening. To further verify that hygromycin-resistant plants carried the T-DNA region of pCAMBIA1301, an assay for GUS was performed. The gene for GUS is found within the T-DNA region of pCAMBIA1301, along with the gene for hygromycin resistance. Approximately equal amounts of leaf tissue, from both transformed and non-transformed plants, were immersed in a solution of X-gluc and incubated overnight at 37°C. All 10 of the 18 surviving hygromycin-resistant plants grown in soil produced a blue precipitate, indicating they were GUS positive, compared to the non-transformed leaf material which remained clear and colourless (Figure 13A, B).

Figure 13: Testing of transformed *A. thaliana* for expression of GUS. Leaves and stems from plants transformed with pCAMBIA1301/Gt1/ss/gB/NOS were placed into a solution containing the X-gluc substate for GUS. The tubes were incubated overnight at 37°C and it was noted if a blue precipitate formed.

A: Leaves from non-transformed plants.

B: Leaves from transformed plants.



4.4.4 Screening based on PCR analysis of genomic DNA for gB transgene

Once it was determined that the plants contained at least portions of the T-DNA region of pCAMBIA1301, the plants were screened for the presence of the GPCMV gB transgene within the plant genomic DNA. Genomic DNA was extracted from the leaves and stems from each of the 10 hygromycin-resistant and GUS positive plants. PCR was carried out using four sets of PCR primers designed to span regions along the gB transgene (Figure 14A). As shown in Figure 14B with representative data from T_1 plants #1 and #2, the expected PCR products of 515 bp (primer set #1: 5'-gBscreen1, 3'-gBscreen1), 699 bp (primer set #2: 5'-gBscreen2, 3'-gBscreen2), 800 bp (primer set #3: 5'-gBscreen3, 3'-gBscreen3), and 557 bp (primer set #4: 5'-gBscreen4, 3'-gBscreen4) were observed in all cases.

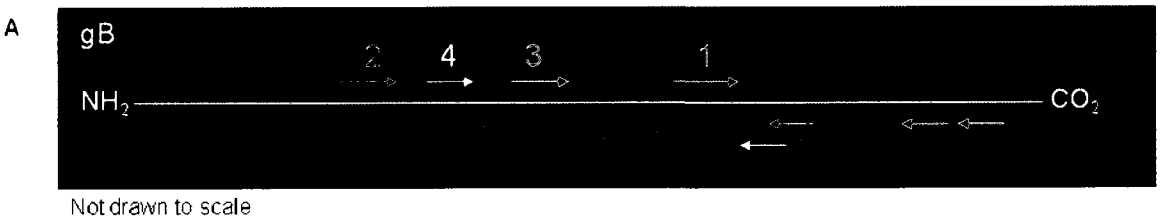
4.5 Stability of gB transgene in *A. thaliana* over multiple generations

The seeds from each generation of pCAMBIA1301/Gt1/ss/gB/NOS transformed *A. thaliana* plants were planted to produce a stock of seeds, for the purpose of generating enough plant material for further genetic and protein analyses. Using primer set #2 (see Figure 14A), PCR was used to determine the stability of the gB transgene over several generations. As an example, Figure 15 demonstrates that the expected PCR product of 699 bp was present in plant lines #5 and #10 in the T_2, T_3, and T_4 generations. Since plant line #5 was used in the scale-up for the animal immunizations (see Section 4.8 below), the presence of the gB transgene was verified throughout all generations derived from plant line #5. Using two of the four primer sets, the expected PCR products of 515 bp and 699 bp were observed by agarose gel electrophoresis in the T_{11} generation (Figure 16), confirming

Figure 14: PCR screening of genomic DNA for presence of gB transgene. Genomic DNA was isolated from the leaves and stems of the *A. thaliana* plants using the Qiagen DNeasy Plant Mini Kit. Four different sets of PCR primers spanning different regions of the gB gene were designed.

A: Schematic diagram of primer placement along length of gB gene.

B: Lanes 1, 12, 23: 100 bp DNA ladder (Invitrogen); Lanes 2, 7, 13, 18: positive control plasmid, pKTS507; Lanes 3, 8, 14, 19: non-transgenic genomic DNA as template; Lanes 4, 9, 15, 20: genomic DNA from pGt1/ss/gB/NOS plant #1 (T_1); Lanes 5, 10, 16, 21: genomic DNA from pGt1/ss/gB/NOS plant #2 (T_1); Lanes 6, 11, 17, 22: no DNA template control.



Not drawn to scale

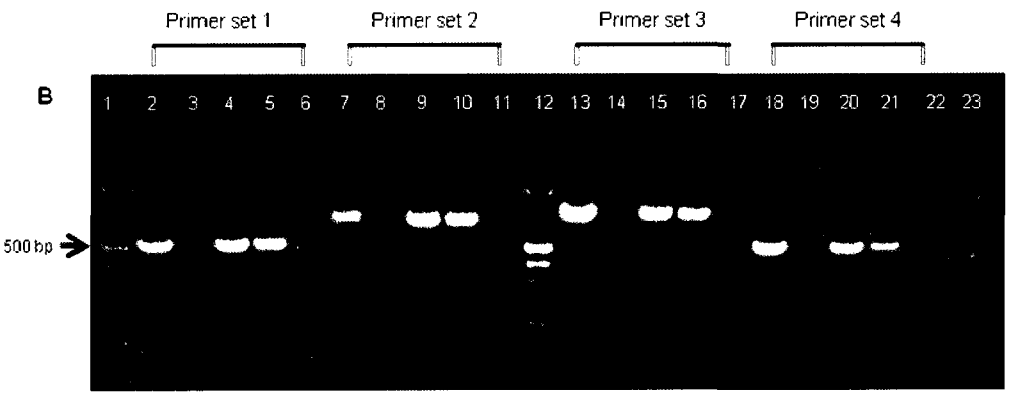


Figure 15: gB transgene present in 3 generations (T_2, T_3, T_4) of *A. thaliana* plants originally transformed with the pCAMBIA1301/Gt1/ss/gB/NOS construct. Genomic DNA from pCAMBIA1301/Gt1/ss/gB/NOS plants #5 and #10 was isolated and PCR primer set #2 were used to detect the gB gene in 3 generations. Lanes 1, 11: 100bp DNA ladder (Invitrogen); Lane 2: positive plasmid control, pKTS507; Lane 3: non-transgenic genomic DNA as template; Lanes 4 to 6: genomic DNA from plant #5 T_2, T_3, T_4, respectively; Lanes 7 to 9: genomic DNA from plant #10 T_2, T_3, T_4, respectively; Lane 10: no DNA template control.

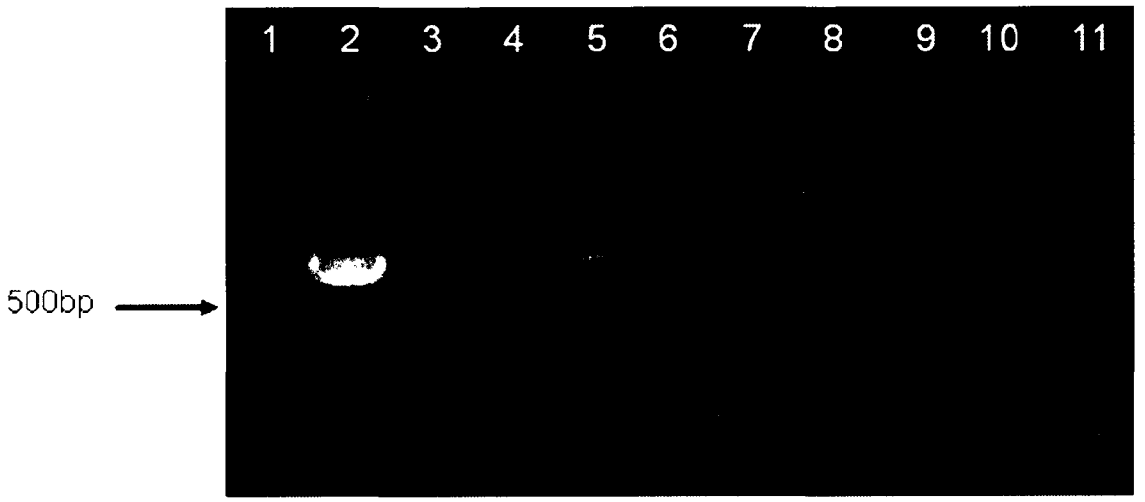
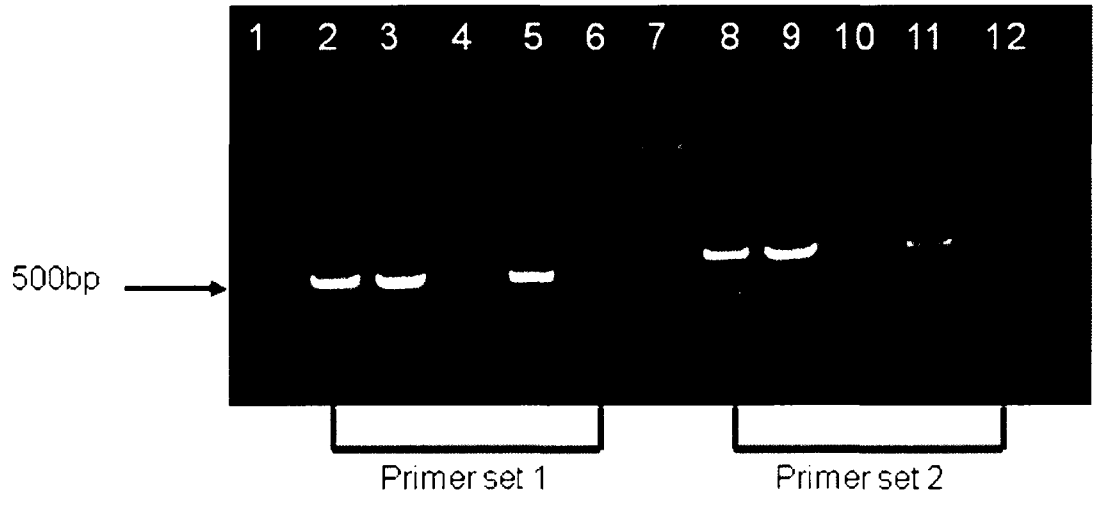

1 2 3 4 5 6 7 8 9 10 11
500bp

Figure 16: gB transgene present in *A. thaliana* T_{11} originally transformed with the pCAMBIA1301/Gt1/ss/gB/NOS construct. pCAMBIA1301/Gt1/ss/gB/NOS plant #5 genomic DNA was isolated and PCR primers were used to detect the gB gene in the generations. Lanes 1, 7: 1 kb ladder (Invitrogen); Lanes 2, 8: positive plasmid control, pKTS507; Lanes 3, 9: positive plasmid control, pCAMBIA1301/Gt1/ss/gB/NOS; Lanes 4, 10: non-transgenic genomic DNA; Lanes 5, 11: T_{11} *A. thaliana* from pCAMBIA1301/Gt1/ss/gB/NOS transformation; Lanes 6, 12: no DNA template control.


1 2 3 4 5 6 7 8 9 10 11 12
500bp
Primer set 1
Primer set 2

the presence of the gene for gB in the 11th generation of transgenic *A. thaliana*. Please note that although the bands appear higher for the T2, T3, and T4 generations, the second DNA ladder (lane 11) also appears to be running at a higher level.

4.6 Expression of GPCMV gB protein in *A. thaliana seeds*

Once I had demonstrated that the gB transgene was present within the transformed *A. thaliana*, the next step was to determine if gB protein was being expressed. For this, ELISAs and Western blots were used to evaluate if gB protein was expressed, and then for quantitating the level of expression of the gB protein within seeds of the pCAMBIA1301/Gt1/ss/gB/NOS plants. Soluble proteins were extracted from seeds of PCR positive plants as described in section 3.7 and protein concentrations ranged from less than 3 μg/μL to 12 μg/μL. Note that for all protein analyses, by Western blot or ELISA, the amount of total seed protein used from the transformed plants was always either equal to or less than that used from non-transformed plants used as negative controls.

4.6.1 ELISAs for detecting GPCMV gB

Several different ELISAs were designed to detect and quantitate the gB protein within *A. thaliana* seeds; however, none was able to detect any gB, relative to values of the negative control (seeds from non-transformed *A. thaliana*). A sandwich ELISA was first designed to measure levels of GPCMV gB within total soluble seed protein extracts. Briefly, the wells of a 96-well ELISA plate were coated with a murine monoclonal antibody specific for GPCMV gB. The seed protein extracts were then applied, followed by either a GPCMV gB-specific guinea pig polyclonal antibody or serum from a GPCMV-infected

guinea pig. A dilution series of recombinant baculovirus-derived gB as a positive control did not generate an absorbance value above background, indicating this ELISA configuration was not able to detect GPCMV gB.

A direct ELISA was therefore developed to try to measure the expression of GPCMV gB in the *Arabidopsis* seeds. This involved using the seed protein extracts, from both gB and non-transformed seeds, and baculovirus-derived GPCMV gB (positive control) to coat the wells of the ELISA plate. A GPCMV gB-specific murine monoclonal antibody, a guinea pig GPCMV gB polyclonal antibody, or serum from GPCMV-infected guinea pigs was used as the primary antibody. In this case, the baculovirus-derived GPCMV gB generated a signal with the polyclonal antibody and serum from GPCMV seropositive animals; however, it was not possible to distinguish between the non-transformed and gB seed protein extracts (data not shown). The minimum amount of baculovirus–derived GPCMV that produced a signal above background at 450 nm was less than 1 ng.

4.6.2 Western blots for detecting GPCMV gB

Even though the ELISA failed to detect seed-derived gB, Western blots were performed. As described in sections 3.10, 100 μg of protein extracts from seeds of both gB and non-transformed seeds were analysed by Western blot. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and the blots were probed with a 1/1000 dilution of a polyclonal antibody against baculovirus-derived GPCMV produced in guinea pigs. A 1/10 000 dilution of donkey anti-guinea pig labelled with HRP (Santa Cruz, Santa Cruz CA, US) was used as the secondary antibody. As a positive control, baculovirus-derived

GPCMV gB was included. Although many bands are observed for the positive control by Western blot, the expected molecular weight proteins are 90 kDa and 30 kDa (Schleiss and Jensen, 2003). A protein band of 90 kDa (Figure 17, lane 2) was observed with the positive control, GPCMV baculovirus-derived gB. As can be seen in Figure 17, numerous bands are observed for both the gB (lane 5) and non-transformed (lane 4) seed protein extracts. However, there is a distinct band at approximately 70 kDa in the gB seed protein (lane 5) extract not observed in the non-transformed sample (lane 4).

4.7 Scale-up of pGt1/UL55 plants for immunizations

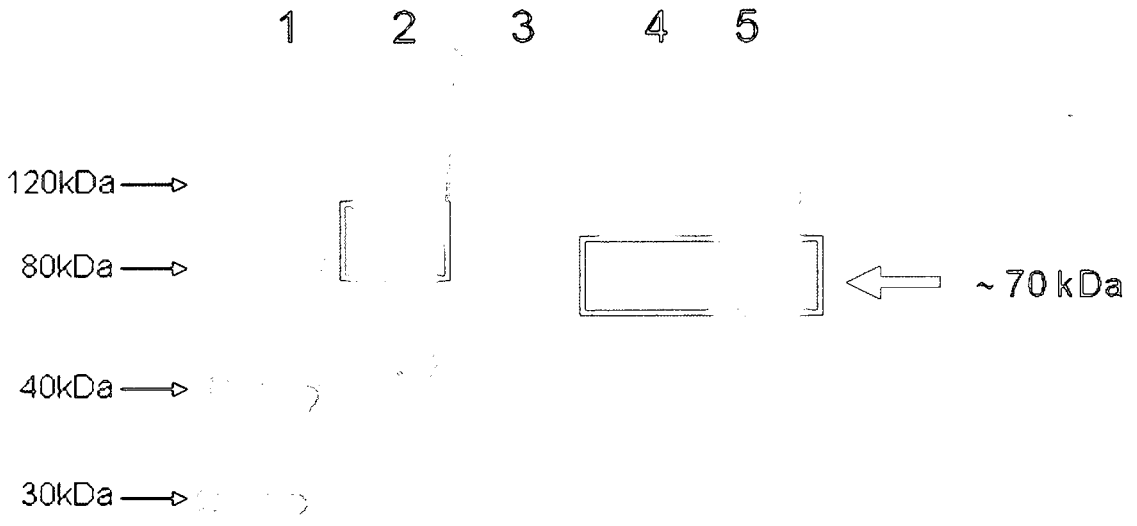
Several generations of *A. thaliana* plants (plant line #5) were grown as described earlier (section 3.4.1), in order to generate enough seeds for the immunization studies. To this end, 400 pots of Arabidopsis plants (15-20 plants per pot) were grown over a period of 5 to 6 months, yielding about 6 g of T_7 seeds. Space restrictions in the plant growth room limited the number of pots at any one time, i.e. two growing cycles were carried out, with 200 pots per cycle.

4.8 Detection of immune response in guinea pigs immunized with GPCMV gB derived from *A. thaliana* seeds

4.8.1 Immunization of guinea pigs with seed-derived GPCMV gB

Since Western blots identified a transformation-specific band in *A. thaliana* seed protein extracts, the next step was to determine the immunogenicity of gB derived from these seeds. I carried out these experiments at the University of Minnesota, in collaboration with Dr. Mark Schleiss. Female Hartley guinea pigs were subcutaneously immunized with

Figure 17: Western blot analysis of protein extracts from seeds of non-transgenic plants and gB plant #5. Seeds were ground in a mortar and pestle and resuspended in 1X PBS and plant protease inhibitors (Sigma). One hundred μg of seed protein extracts from both non-transformed and plant #5 were loaded onto a 10% denaturing polyacrylamide gel and subsequently transferred to nitrocellulose. The blot was probed with a guinea pig polyclonal antibody specific for baculovirus-derived GPCMV gB. An HRP-labelled anti-guinea pig antibody was used as the secondary antibody. The red box highlights a band of 90 kDa, the blue box highlights a band of 70 kDa. Lane 1: MagicMark XP Protein Ladder (Invitrogen); Lane 2: approximately 45 μg of baculovirus-derived gB; Lane 3: empty; Lane 4: protein extract from non-transformed *A. thaliana*; Lane 5: protein extract from plant #5. This is a representative of one out of three Western blots.


1
2
3
4
5
120kDa
80kDa
40kDa
30kDa
~70 kDa

soluble proteins extracted from gB or from non-transformed seeds as negative controls. As a positive control for the immunizations, some animals were also immunized with baculovirus-derived GPCMV gB. All animals were immunized three times on days 0, 14, and 45 (initial immunization followed by two boosts), using Freund's Complete Adjuvant for the initial immunization and Freund's Incomplete Adjuvant for subsequent boosts (Figure 18). Note that 8 of the 20 guinea pigs ordered were found to seropositive by ELISA for antibodies to GPCMV. Unfortunately, this is not uncommon, but it resulted in fewer guinea pigs per group than was originally intended. The following is a summary of the three groups of guinea pigs included in the immunization experiments:

Group 1: Guinea pigs (A1, A3, A6, A7) receiving non-transformed seed protein extract

Group 2: Guinea pigs (A10, A11, A13, A15) receiving gB seed protein extract

Group 3: Guinea pigs (A16, A17, A18, A20) receiving baculovirus-derived GPCMV gB

4.8.2 Detection of antibodies by ELISA

Once the final trial bleed was obtained (day 60) from all animals, the antibody response was evaluated by ELISA. To this end, the antibody titres of the final trial bleeds were evaluated relative to the pre-bleed titres. As shown in Figure 19, three out of four guinea pigs from Group 2, immunized with gB seed protein, generated a GPCMV-specific IgG antibody response as determined by ELISA. Animal A11 generated the greatest antibody response with a titre of 5120. A13 and A15 had antibody titres of 40 and 640 respectively. An antibody response was not detected for animal A10. The IgG antibody titre

Figure 18: **Overview of guinea pig immunization schedule.**

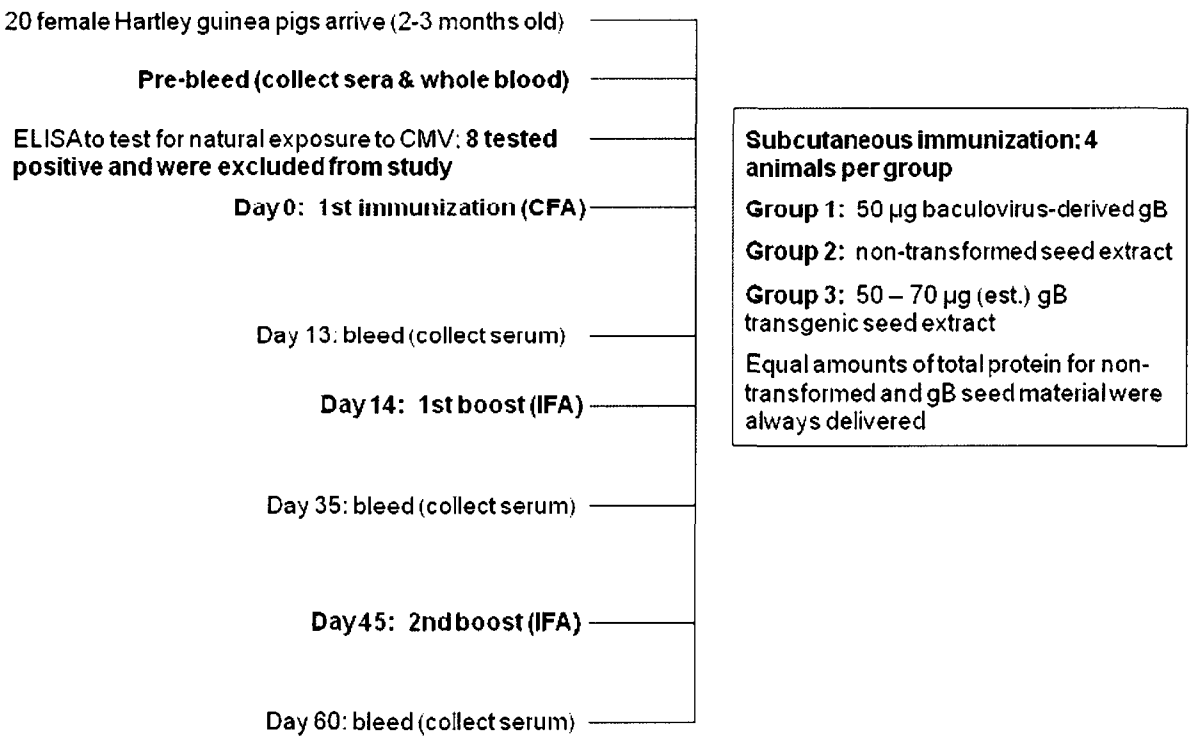
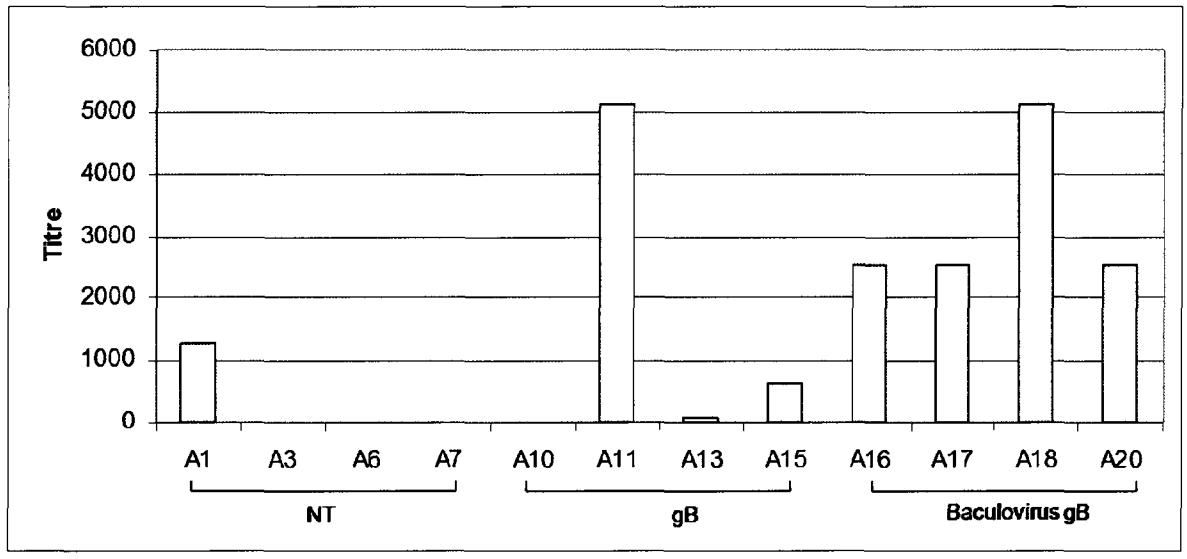

20 female Hartley guinea pigs arrive (2-3 months old)
Pre-bleed (collect sera & whole blood)
ELISA to test for natural exposure to CMV; 8 tested positive and were excluded from study
Day 0: 1st immunization (CFA)
Day 13: bleed (collect serum)
Day 14: 1st boost (IFA)
Day 35: bleed (collect serum)
Day 45: 2nd boost (IFA)
Day 60: bleed (collect serum)
Subcutaneous immunization: 4 animals per group
Group 1: 50 µg baculovirus-derived gB
Group 2: non-transformed seed extract
Group 3: 50 – 70 µg (est.) gB transgenic seed extract
Equal amounts of total protein for non-transformed and gB seed material were always delivered

Figure 19: IgG anti-GPCMV gB antibody titres produced in female Hartley guinea pigs subcutaneously immunized with seed-derived gB protein extracts, non-transgenic seed protein extracts, or baculovirus-derived gB. The titres were measured by ELISA, using trial bleeds obtained 14 days after the 2nd boost. Ninety-six-well plates were coated with an antigen preparation derived from GPCMV-infected guinea pig fibroblasts. The guinea pig test serum was diluted in a 2-fold series, starting at 1/40. The titre was defined as the highest dilution producing an absorbance of at least 0.1 and two times that obtained from the same dilution of pre-immune serum. Assays were carried out in duplicate and performed twice.


6000
5000
4000
3000
2000
1000
0
Titre
A1
A3
A6
A7
A10
A11
A13
A15
A16
A17
A18
A20
NT
gB
Baculovirus gB

was defined as the inverse of the dilution that gave a minimum absorbance of 0.1 and at least twice the absorbance of the pre-immunization serum (day 0). Pre-immunization (day 0) serum from each individual animal was also tested by ELISA. Absorbance readings for the pre-immunization sera were evaluated for each dilution and were subtracted from the absorbances obtained for the trial bleeds. An antibody response was surprisingly detected for animal A1, in the group immunized with non-transformed seed extract. Other than the response from A1, all the negative and positive control samples gave results as expected.

4.8.3 Development of antibody response over course of immunization experiment

To clarify the kinetics of the antibody response to gB, the ELISA titre of each of the trials bleeds over the course of immunization was determined for all animals (see Table 2). These data provide insight into the development of the immune response over the 60 days of the study. For animals immunized with seed-derived GPCMV (Group 2), 3 out of 4 animals generated antibody responses over the immunization course of 60 days. Specifically, animal A11, the highest responder in Group 2, generated an antibody titre of 640 at day 14 which increased greatly to 10 240 by day 35. This response however decreased by 2-fold to 5120 by day 60. Both animals A13 and 15 (Group 2) were negative at day 14 but both displayed antibody titres of 40 on day 35. This titre remained the same for A13 by day 60 but increased to 640 by day 60. Animal A10 did not produce an antibody response throughout the study. Aside from animal A1, whose antibody titre increased over the 60 days, all other

Table 3: IgG antibody responses produced in guinea pigs immunized with seed-derived gB protein extract, non-transgenic seed protein extract, or baculovirus-derived GPCMV gB. The IgG response was measured approximately 14 days after each immunization. As well, due to the discrepancy with animal A1, an ELISA was set up to measure if an immune response to GUS could be detected in each animal. Only the final bleed was tested for the GUS ELISA. All ELISAs were performed in duplicate.

		Antibody titre			
	Animal	Day 14	Day 35	Day 60	GUS ELISA*
Group 1 (negative control)	A1	0	5120	1280	+
	A3	0	0	0	-
	A6	ND	0	0	-
	A7	ND	0	0	-
Group 2	A10	0	0	0	-
	A11	640	10240	5120	-
	A13	0	40	40	+
	A15	0	40	640	+
Group 3 (positive control)	A16	0	1280	2560	-
	A17	0	640	2560	-
	A18	ND	40	5120	-
	A20	ND	80	2560	-

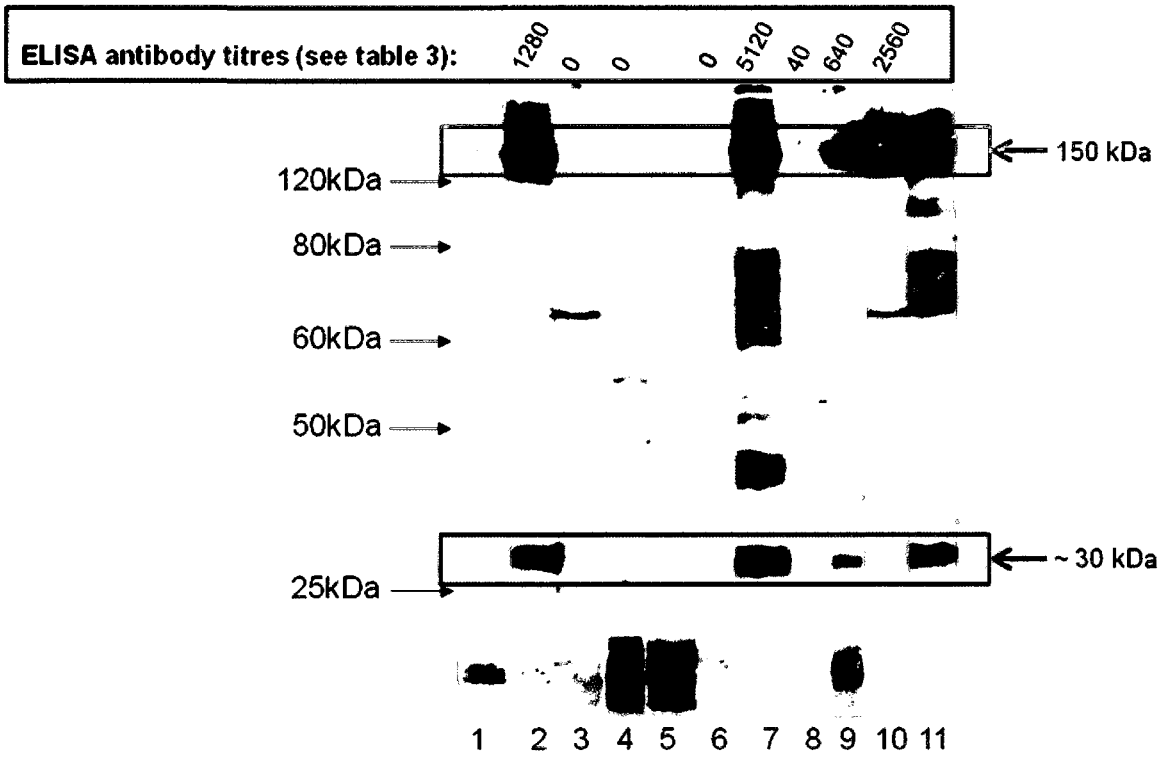
ND = not done; *using day 60 trial bleeds

negative control animals (Group 1, A3, A6, A7) remained negative over the course of the study. As expected, all positive control animals (Group 3), immunized with baculovirus-derived GPCMV, had positive antibody titres at day 35 which increased at day 60.

4.8.4 Detection of antibodies by Western blots

In order to further understand the antibody response detected by ELISA, whole GPCMV viral particles were used as the antigen in Western blot analysis, with the serum of immunized guinea pigs used as the primary antibody. Prior to probing with the primary antibodies, the membrane was cut into individual strips and each strip was placed into a separate container. In preliminary experiments, dilutions of the final trial bleed serum, obtained at day 60, 14 days after the second boost, were optimized for each animal for ability to distinguish between bands as observed by Western blotting. These optimized dilutions of trial serum were subsequently used for the Western blot analysis. As a negative control, a pre-immunization serum pool from the animals was used. This was negative by Western blot, as indicated by the lack of proteins in Figure 20, lane 1, except for a low molecular weight band which can be ignored. As positive controls, serum from guinea pigs previously immunized with baculovirus-derived GPCMV gB was used (lane 11), along with another polyclonal antibody (9881) produced in the laboratory of Dr. Mark Schleiss (lane 5). Both positive controls recognized a high molecular weight band of approximately 150 kDa (Schleiss and Jensen, 2003), corresponding to full-length glycosylated GPCMV gB. Similarly, animal A16 immunized with baculovirus-derived GPCMV gB (Group 3), produced a band of 150 kDa (lane 10). Negative control animals (A6 and A7) immunized

Figure 20: The antibody response generated by immunization with seed-derived gB recognized GPCMV viral proteins. Whole viral GPCMV particles were used as the target antigen and transferred to nitrocellulose. Dilutions of day 60 serum (14 days post 2nd boost) from 7 of the immunized guinea pigs were prepared. The dilutions were optimized for each animal. The red box highlights bands at 150 kDa, and the blue box highlights a band at approximately 30 kDa. Lane 1: 1/200 preimmune serum; Lane 2: 1/1000 A1; Lane 3: 1/500 A6; Lane 4: 1/500 A7; Lane 5: 1/500 pAb 9881; Lane 6: 1/500 A10; Lane 7: 1/2000 A11; Lane 8: 1/500 A13; Lane 9: 1/1000 A15; Lane 10: 1/1000 A16; Lane 11: 1/2000 polyclonal antibody produced in guinea pigs immunized with baculovirus-derived GPCMV gB. This Western blot is a representation of 3 blots.


ELISA antibody titres (see table 3):
1280
0
0
0
5120
40
640
2560
120kDa
80kDa
60kDa
50kDa
25kDa
150 kDa
~ 30 kDa
1 2 3 4 5 6 7 8 9 10 11

with non-transformed seed extracts (Group 1), did not generate antibodies recognizing this high molecular weight protein. As revealed in lanes 7 and 9, two of the four animals immunized with seed-derived GPCMV gB (Group 2, A11 and A15) generated an antibody response to a band of approximately 150 kDa, similar to that observed for the positive controls. Serum from animal A10 (Group 2), which did not generate an antibody response as measured by ELISA, correspondingly did not recognize this 150 kDa GPCMV protein. A very faint band is also observed at this molecular weight for animal A13 (lane 8). Not surprisingly based on antibody titres observed by ELISA, animal A1 (lane 2) also produced an antibody response to this protein. These Western blot results also correspond to the ELISA titres for the immunized animals, including A1. To highlight this, the antibody titres are provided across the top of the Western blot (Figure 20). There is also a band at approximately 30 kDa that is recognized by serum from animals A1 (lane 2), A11 (lane 7), A15 (lane 9), and the positive control polyclonal antibody (lane 11). It is not known what this protein band corresponds to, but may be due to a degradation product.

4.8.5 Immune response to GUS

In view of the apparent presence of anti-gB antibodies in animal A1, an ELISA for antibodies to GUS was carried out. Interestingly, an antibody response was observed for animals A1, in addition to A13, and A15 (Table 3), right-hand column.

4.9 Viral neutralizing activity of antibodies to seed-derived gB

Since the ELISA and Western blot data indicated that the seed-derived gB was immunogenic, it was of great interest to determine if the antibodies would neutralize viral

infectivity. GPCMV viral stock was prepared and titred as described in section 3.12.3 (see Figure 21 for example of a viral plaque). Table 4 shows the average number of counts per well. The right-hand column shows the final neutralization titre for all animals tested. Titre was defined as the reciprocal of serum dilution that resulted in 50% reduction of plaques. As expected, animal A16 (Group 3, immunized with baculovirus-derived GPCMV) generated a high neutralization titre of 3240. Animal A3 (Group 1, immunized with non-transformed seed extract) did not exhibit viral neutralization, corresponding to ELISA antibody titres. Not surprisingly, A11 (Group 2, immunized with seed-derived GPCMV gB) produced a high neutralization titre of 3240, similar to that observed for A16. Animals A13 and 15 (Group 2) had viral neutralizing titres of 40; however, both also showed slight neutralizing capability at a serum dilution of 1/120, although the number of plaques at this dilution did not fit within the defined parameters of a neutralization titre. Animal A1 (Group 1, immunized with non-transformed seed extract) also displayed neutralizing ability with a titre of 360. This corresponds to the ELISA and Western blots results obtained for this animal.

Figure 21: Example of a viral plaque from neutralization experiments. GFP-labelled GPCMV was used for all neutralization assays to aid in visualization of plaques formed after infection of guinea pig fibroblasts. Red arrows indicate two individual viral plaques. Picture shown is of 10X magnification.

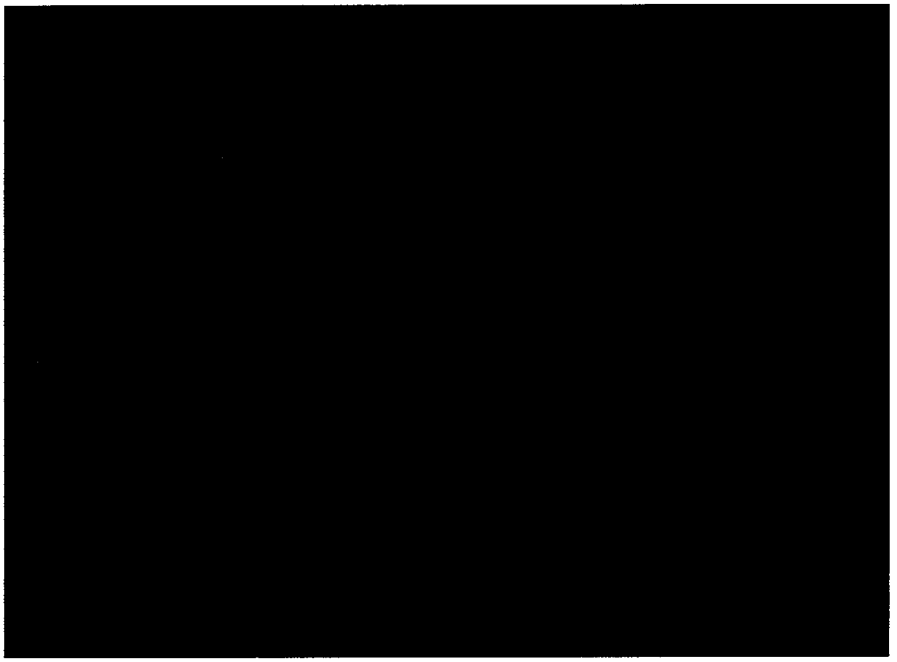


Table 4: Viral neutralization titres. The viral neutralizing ability of serum (day 60) was tested using animals A1, A3, A11, A13, A15, and A16. The assay was carried out twice, each in duplicate. Data in table shows the average number of plaques counted per well (each well was counted twice) and is representative of one neutralization assay. Pre-immune serum at a dilution of 1/40 was included in all neutralization assays as a negative control, and produced between 60 and 80 plaque forming units. Final neutralization titres are in right-hand column, as indicated.

	Plaque forming units per duplicate well, with different dilutions of guinea pig serum						Final Neutralization Titre
Animal	**1/40**	**1/120**	**1/360**	**1/1080**	**1/3240**	**1/9720**	
A1	0,0	0,0	17,18	43,48	63,64	75,>90	360
A3	58,60	76,75	ND	ND	ND	ND	0
A11	0,0	0,0	0,0	12,8	32,24	55,42	3240
A13	35,27	57,46	76,64	78,70	ND	ND	40
A15	34,30	56,46	64,67	81,69	ND	ND	40
A16	0,0	0,0	4,5	17,21	38,32	68,82	3240

ND = not done

5.0 Discussion

5.1 Overview

In recognition of the impact of HCMV on the population, development of a vaccine has been identified by the United States Institute of Medicine of the National Academy of Sciences as a top priority (Stratton et al., 1999). Much progress has been made in understanding the molecular biology of HCMV, and a number of potential vaccines have been developed and tested (reviewed in (Schleiss and Heineman, 2005)). For example, in human trials testing the safety and efficacy of live attenuated HCMV vaccines, it was demonstrated that the vaccine provided protection against severe manifestations of CMV disease, but in the end did not prevent CMV infection (reviewed in (Schleiss and Heineman, 2005)). For a variety of reasons, including the inability of live attenuated vaccines to prevent CMV infection, and safety issues associated with the use of these vaccines, attention has now shifted to other types of vaccines, including subunit vaccines (Zhong and Khanna, 2007). Subunit vaccines can be produced in a number of expression systems including bacteria, yeast, mammalian cells, and plants. Of these, plants offer several advantages, namely their ability to glycosylate proteins (unlike bacteria), their minimal risk of carrying human pathogens (unlike mammalian cells), and the capacity to be grown on a large scale (reviewed in (Yusibov and Rabindran, 2008)). The work in this thesis describes the development of a novel plant-derived vaccine for CMV, using GPCMV infection in guinea pigs as an animal model for HCMV infection in humans. In this thesis it is shown that immunodominant CMV protein can be expressed in the seeds of the plant *A. thaliana*. The transgene was found to be stable over 11 generations. Immunization of guinea pigs with plant protein extracts containing the GPCMV protein gB, in this preliminary immunization

study, generated an antibody response specific to viral gB. In addition, this antibody response to plant-derived GPCMV gB was found to have neutralizing properties against live GPCMV. Based on these results, it is reasonable to propose that plant-derived GPCMV gB might act as an effective vaccine against live GPCMV challenges in guinea pigs. Furthermore, this work provides a proof-of-concept for the development of a plant-derived vaccine against HCMV.

5.2 Generation of constructs

5.2.1 Selection of promoters

Expression of foreign proteins in plants requires promoters recognized by the plant transcriptional machinery. The promoters chosen should allow sufficiently high levels of expression for effective recovery of the protein of interest, while ensuring that recombinant protein levels remain below any threshold of toxicity. In addition, selection of promoters can allow for protein expression throughout the plant, or for directed expression in specific plant organs. In the present work, two promoters were chosen to drive expression of the GPCMV recombinant proteins gB and pp65 in plant expression systems. The first was the developmentally-regulated rice Gt1 promoter. The family of glutelin promoters are responsible for controlling expression of the major storage protein, glutelin, in rice seeds (Okita et al., 1989), and have been shown previously to direct recombinant protein expression to seeds (Wright et al., 2001; Zhao et al., 1994). For example, a number of studies have used the glutelin promoters to successfully express recombinant pharmaceuticals such as granulocyte macrophage colony stimulating factor in tobacco and rice (Sardana et al., 2002; Sardana et al., 2007), HCMV gB in tobacco and rice (Tackaberry

et al., 2003; Tackaberry et al., 2008), cholera toxin B subunit in rice (Nochi et al., 2007), and Newcastle disease virus fusion protein in rice (Yang et al., 2007). Seeds were targeted for expression of recombinant proteins because they are considered good storage vessels due to their ability to offer a dry and stable environment (Rybicki, 2008) and recombinant proteins have been shown to be expressed at relatively high levels in seeds (Lamphear et al., 2002; Takaiwa, 2007). However, using a seed-specific promoter requires waiting until a plant reaches a stage of maturity when seeds can be harvested, which can take many months in certain plants. In contrast to the glutelin promoter, the maize ubiquitin promoter is constitutively active throughout the plant, and consequently allows expression of recombinant proteins, in this case gB and pp65, in all plant parts. Use of this promoter therefore permits assessment of recombinant protein expression at much earlier stages in a plant life cycle. The generation of constructs containing Gt1 or ubiquitin promoters was undertaken to allow the targeting of transgene expression to either seeds or throughout the plant.

5.2.2 Selection of CMV proteins for expression

Viral proteins that are major targets of the immune response to a given virus are good candidates for use as potential subunit vaccines. Previous studies have shown that protective immunity against HCMV involves both the antibody response and the T-cell response (reviewed in (Schleiss and Heineman, 2005; Plotkin, 2002)). Because gB and pp65 are the major targets of the humoral and cell-mediated immune responses, respectively (Britt and Mach, 1996; Wills et al., 1996), genetic constructs were designed and generated to allow expression of either gB or pp65 under the control of the Gt1 or Ubi plant-specific promoters.

While an effective vaccine may require both antibody and T-cell responses, the development of a single plant line expressing both recombinant proteins is technically challenging. We therefore utilized a step-wise approach to evaluating an effective plant-derived vaccine for GPCMV and focussed initially on a single recombinant protein, gB. gB was chosen for initial trials because it is the dominant target of the antibody response in HCMV-infected individuals and is consequently the most well-characterized protein in GPCMV. Furthermore, it has been used in many studies as a subunit vaccine (Pass et al., 1999; Endresz et al., 1999; Spaete, 1991). For example, the approach of delivering gB as a single immunogen has been shown to decrease viral load and protect against severe CMV disease in guinea pigs (Schleiss et al., 2004), showing that even immunization with a single viral protein displays some efficacy as a CMV vaccine, in this animal model. In line with this animal work, immunization of humans with purified recombinant Chinese hamster ovary cell-derived HCMV gB was shown to induce a gB-specific antibody response that possessed viral neutralizing activity greater than that observed in HCMV seropositive individuals, indicating the potential for protection against HCMV (Pass et al., 1999). Successful expression of gB in a plant, and generation of an antibody immune response in animals immunized with the transgenic material, would serve as an important proof-of-principle that could later be expanded to include additional plant lines expressing other recombinant proteins (such as pp65). Immunization with both recombinant proteins could then be conducted.

Although gB is the major target of the neutralizing antibody response in naturally infected individuals, the full-length protein may not be required for a subunit vaccine, and

indeed may be less immunogenic than truncated versions. For example, intramuscular immunization of guinea pigs with a full-length gB DNA vaccine was poorly immunogenic (Schleiss et al., 2000), whereas a gB protein lacking the transmembrane region that anchors gB to the viral envelope (Reschke et al., 1995) was shown to be immunogenic in both guinea pigs and humans (Pass et al., 1999; Schleiss et al., 2004). The N-terminal 700 amino acid GPCMV gB lacking the transmembrane region still retains the major antigenic domain to which the majority of antibodies are directed (Schleiss and Jensen, 2003), consistent with its ability to induce an immune response. Although it is unknown why removal of the transmembrane region improves immunogenicity, the results suggest that this approach may be advantageous in the development of an effective subunit vaccine. Moreover, recombinant gB proteins lacking the transmembrane region may be easier to purify. Previous studies in our laboratory indicated that full-length recombinant HCMVgB is difficult to purify when expressed in tobacco seeds (manuscript in preparation). Studies have also shown that full-length HCMV gB expressed in tobacco seeds accumulated in protein storage vesicles (Wright et al., 2001), which could require extensive protein purification. In contrast, truncated GPCMV gB expressed in baculovirus and truncated HCMV gB expressed in Chinese hamster ovary cells, both lacking the hydrophobic transmembrane region, are secreted into cell culture supernatants, facilitating their purification (Spaete et al., 1988; Schleiss and Jensen, 2003; Pass et al., 1999). Therefore, in the present work an N-terminal 700 amino acid (of the full-length 901 amino acid protein) truncated form of gB was used with the intention of producing an easily-extractable protein that retains its immunogenic properties.

5.2.3 Appropriate presentation of epitopes: the role of glycosylation

There are two major types of epitopes found on antigens: linear epitopes and conformational epitopes. Antibody responses are produced against specific epitopes; therefore, integrity of the epitopes is critical for the generation of an immune specific to the authentic viral protein. Linear epitopes are based on the primary amino acid sequence and any post-translational modifications. Conformational epitopes result from the specific manner in which a protein is folded. Therefore, factors that influence protein folding must be considered in the generation of recombinant protein antigens. Glycosylation is an important post-translational modification that can impact both linear and conformational epitopes by impacting on folding and blocking access of antibodies to a specific epitope. This may be particularly relevant for gB, which is a highly glycosylated protein (Britt and Vugler, 1989; Qadri et al., 1992; Meyer et al., 1990). Although it is known that GPCMV gB contains 16 potential N-glycosylation sites (Schleiss, 1994), the extent of glycosylation and the structure of these sugar groups is not yet known. Nevertheless, maintenance of GPCMV gB glycosylation may influence the immune response. Glycosylation of proteins occurs to a large extent within the endoplasmic reticulum and Golgi apparatus via the secretory pathway, and requires the presence of a signal sequence directing the protein to this organelle (reviewed in (Marth and Grewal, 2008)). Constructs that are described in this thesis were therefore designed to include a signal sequence. Previous studies have shown that removal of the native endoplasmic reticulum signal sequence and replacement with a plant-specific signal sequence may increase recombinant protein expression (Richter et al., 2000; Franconi et al., 2006; Lou et al., 2007). Therefore, for the Gt1/gB construct, the signal

sequence for gB was removed, and gB was ligated to the Gt1 promoter and Gt1 signal sequence.

Four constructs for the expression of GPCMV gB and pp65 in plants were designed and generated. These constructs should allow either seed-specific or ubiquitous expression of two recombinant proteins that are major targets of the immune response to GPCMV. During the generation of the Gt1/gB construct, an intermediate construct comprising the Gt1 promoter, the signal sequence, and the NOS termination sequence, was also produced. This construct was designed to permit insertion of any gene of interest with relative ease. Therefore, it may be of use for future studies involving seed-specific expression of other recombinant proteins.

5.3 Transformation of *A. tumefaciens*

A. tumefaciens-mediated transformation of plants requires the use of a Ti plasmid. Typically, any foreign genetic material enclosed within the T-DNA region of this plasmid is incorporated into the plant genome (reviewed in (Gelvin, 2003)). Ti plasmids have been developed specifically for *A. tumefaciens*-mediated transformation of plants. The rice-specific Ti plasmid pCAMBIA1301 contains an appropriate antibiotic resistance gene that permits screening of transformants in the presence of hygromycin, as well as the gene for β-glucuronidase which allows screening using the GUS test. In the current studies, insertion of Gt1/ss/gB/NOS and Ubi/gB/NOS constructs into pCAMBIA1301 was confirmed by restriction enzyme digests and PCR using primers spanning varying regions of the gene for gB. Successful transformation of *A. tumefaciens* with pCAMBIA1301/Gt1/ss/gB/NOS or

pCAMBIA1301/Ubi/gB/NOS was similarly confirmed using restriction enzyme digests of plasmid DNA recovered from the transformed bacteria. These results supported the conclusion that the constructs in both cases were successfully transferred to the bacteria.

5.4 Transformation and regeneration of rice with pCAMBIA1301/Gt1/ss/gB/NOS

In addition to the advantages of using plants for the expression of recombinant vaccines, as discussed above, plants offer the added advantage of allowing expression of recombinant proteins in plant tissues amenable to long-term storage with minimal requirement for purification and refrigeration, such as seeds. Initial work involving production of recombinant pharmaceuticals in plants primarily used the tobacco plant as a model due to the ease of transformation and regeneration (Barta et al., 1986; Hiatt et al., 1989; Ma et al., 2003). However, substantial purification of the recombinant protein is required due to the presence of toxins in tobacco plants (Richter and Kipp, 1999). Use of edible plant parts for the expression of subunit vaccines circumvents the need for extensive purification, and may permit oral delivery. Rice is one of the world's most important food crops, and previous studies have confirmed the utility of using rice as an expression system for recombinant proteins (Yang et al., 2007; Matsumoto et al., 2008; Takagi et al., 2005; Oszvald et al., 2007). Moreover, our laboratory had previously demonstrated the expression of human granulocyte macrophage cell stimulating factor (Sardana et al., 2007) and HCMV gB in rice (Tackaberry et al., 2008). Rice was therefore chosen to express GPCMV gB for the current studies. Please see section A3.0, page 166 of the Appendix for a further discussion on rice transformation and regeneration that were carried out during this research.

5.5 Transformation and regeneration of *A. thaliana* with pCAMBIA1301/ Gt1/ss/gB/NOS

A. thaliana is commonly used for expression of recombinant proteins (Wu et al., 2004b; Aerts et al., 2007; Kohl et al., 2007; Mazorra-Manzano and Yada, 2008). Although it is not considered a food product, as rice is, *A. thaliana* offers several advantages for the expression of subunit vaccines for research purposes. For example, it is readily transformed by *A. tumefaciens*, reaches maturity within eight weeks, and can be grown in a laboratory setting. A drawback of *A. thaliana* is that it has less biomass than rice, important for later studies requiring the generation of large quantities of plant biomass (specifically seeds for the work described in this thesis) for immunization studies. Nonetheless, *A. thaliana* was a good candidate for a proof-of-concept study showing that GPCMV gB expressed in plants could serve as an effective plant-derived subunit vaccine. Constructs designed and generated for expression of gB in rice were employed for expression in *A. thaliana* without further manipulation. Although both the Gt and Ubi family of promoters are derived from monocot genes, several studies have used these promoters to express recombinant proteins in dicots (Tackaberry et al., 1999; Assem et al., 2002; Tackaberry et al., 2003; Sardana et al., 2002). In line with these studies, the present work demonstrated expression of gB in the dicot *A. thaliana*, as revealed by Western blot analysis and immunization studies.

5.5.1 Transformation and regeneration of A. thaliana

The process for transforming *A. thaliana* is markedly different from that of rice. Rather than infection of undifferentiated callus tissue with *A. tumefaciens*, as was performed for rice, the unfertilized ovules of unopened *A. thaliana* flowers are the target for *A.*

tumefaciens infection. The treated plants are allowed to reach maturity, and seeds are harvested. It is these seeds that are subsequently screened for transformation, as indicated by their ability to grow in the presence of hygromycin. Note here that not all seeds from a treated plant will contain the construct. This is because only those seeds generated from an unopened *A. thaliana* flower that was successfully infected during transformation will carry the T-DNA region of the transformation vector. Indeed, transformation rates from these experiments ranged from 0.04 to 0.08%. This is consistent of the reported ranges for the transformation *A. thaliana* using the floral-dip method (Clough and Bent, 1998). Hygromycin-resistant plantlets were successfully regenerated and subsequently transferred to soil. PCR analysis using primers spanning four different regions of the gB transgene was carried out and confirmed the presence of the intact gB gene. Furthermore, GUS testing of a subset of gB PCR positive plants confirmed in all cases the presence of active β-glucuronidase. The ability to grow in the presence of hygromycin, the presence of the gB transgene, and the positive GUS test indicate that all critical components of the T-DNA region of pCAMBIA1301/Gt1/ss/gB/NOS were incorporated into the plant genome. The ability of transformed plants to reach maturity indicates that, at least for *A. thaliana*, expression of gB was not toxic.

5.5.2 Stability of gB transgene

To enable production of the GPCMV gB protein in sufficient quantities to permit protein analysis and immunization studies, multiple generations derived from successfully transformed plants were grown. In a previous study in our laboratory, it was found that after several generations, one line of transgenic tobacco expressing HCMV gB eliminated the

antibiotic resistance gene used for initial screening (personal communication with Dr. Eilleen Tackaberry). I therefore sought to confirm stable maintenance of the GPCMV gB transgene across generations of *A. thaliana*. Through PCR analysis for the presence of the gB gene, stability of the transgene in these studies was confirmed for 11 generations. This indicates that the transgene was stably integrated into the plant genome.

5.6 Expression of GPCMV gB in *A. thaliana* seeds

5.6.1 Western blot analysis

Although transgene stability is an important property, it is not the only factor to consider for the successful expression of recombinant proteins. In order for the recombinant protein to serve as an effective subunit vaccine, it must also be produced in a manner that contains critical epitopes similar to those found on the native viral protein. Western blot analysis of protein extracts from transformed *A. thaliana* seeds probed with a polyclonal antibody specific to baculovirus-derived GPCMV gB, resulted in the presence of non-specific bands indicating that there is some cross-reactivity of the polyclonal antibody generated against recombinant gB and normal seed proteins (Figure 17). However, the presence of a unique band at 70 kDa was not observed in untransformed *A. thaliana* seeds. It was not known what molecular weight of proteins were expected; however based on the expression of truncated GPCMV gB in insect cells, the 700 amino acid protein of approximately 120 kDa is cleaved into two subunits of approximately 30 kDa and 90 kDa (Schleiss et al., 2000). Several possible explanations may be postulated to explain the 70 kDa size of the detected gB protein. First, it is possible that transcription of the GPCMV gB gene resulted in codon biases towards those used by *A. thaliana* and may have resulted in

transcript truncation (reviewed in (Ma et al., 2003)). This could ultimately lead to the expression of a protein of random size.

A second possibility is that the gB protein undergoes processing either pre- or post-translationally in *A. thaliana* seeds. In insect cells infected with baculovirus carrying the same truncated gB gene as used here, the GPCMV gB protein is cleaved by a homologue of the mammalian furin enzyme, resulting in the two subunits (Lopper and Compton, 2002; Schleiss and Jensen, 2003). Although homologues of this enzyme have been found in plants, it is unknown whether they cleave the gB protein in the same manner (Matsuoka, 2003). However, multiple smaller fragments resulting from cleavage of the 120 kDa gB protein, were not detected. The presence of a number of non-specific bands detected in both the transformed and untransformed material may have masked the presence of additional fragments.

A third possible explanation for the size of the seed-derived GPCMV gB is that the 70 kDa protein is the result of enzymatic degradation during protein extraction, as was observed for recombinant HCMV gB recovered from rice seeds (Tackaberry et al., 2008). Proteins from plants are notoriously difficult to isolate, and preparations may contain proteases that could partially degrade isolated proteins (reviewed in (Conlon and Salter, 2007)), resulting in degradation of GPCMV gB.

A fourth possible explanation for the size of the detected band may be altered glycosylation. Although plants glycosylate proteins, they possess enzymes that produce core

α-1,3-fucose and xylose glycans on proteins, which are not found on proteins expressed in mammals (reviewed in (Sethuraman and Stadheim, 2006)). It is therefore possible that recombinant gB expressed in a plant system will possess altered glycosylation patterns as compared to viral gB expressed in mammalian or insect cells. If glycosylation of recombinant GPCMV gB is altered in *A. thaliana*, the seed-derived protein may be larger or smaller than the native form. Characterization of recombinant protein glycosylation in plants is not yet routinely performed. However, the potential effect of altered glycosylation patterns on both immunogenicity and allergenicity of the recombinant glycoprotein is recognized as a major issue for plant-made biotherapeutics designed for human use. *Physcomitrella patens* (moss) (Koprivova et al., 2004), *A. thaliana* (Schahs et al., 2007; Strasser et al., 2004), and *Medicago sativa* (alfalfa) (Sourrouille et al., 2008) have been generated in which the genes for fucosyltransferase and xylosyltransferase enzymes responsible for addition of these glycosyl groups have been knocked out. Nonetheless, although it cannot be excluded that the *A. thaliana*-expressed gB is differentially glycosylated, binding of the monoclonal antibody in the Western blot suggests that any altered glycosylation does not affect this particular target epitope. Based on the Western blot results, it appears that at least one epitope of the native viral gB was authentically expressed in seed-derived gB.

The presence of non-specific bands observed by Western blots was a problem in this study. Additional Western blots conducted with serum from GPCMV infected guinea pigs yielded so much cross-reactivity with normal seed proteins that identification of any unique bands was not possible. Despite the presence of non-specific bands, identification of a

unique band in the transgenic *A. thaliana* seeds, relative to the untransformed *A. thaliana* seeds, indicates that the epitope recognized by the polyclonal antibody to gB was present in the seed-derived gB. Consequently, even in altered form, seed-derived gB warranted assessment as a subunit vaccine for GPCMV.

5.6.2 Quantification of gB expression in seeds

Previous studies involving the subcutaneous immunization of guinea pigs with baculovirus-derived gB used 50 μg of recombinant protein per injection (Schleiss et al., 2004). Although HCMV gB antibodies are commercially available, and have been characterized to some extent (for summary table see (Tackaberry et al., 2008)), little is known about GPCMV gB antibodies, and no commercial antibody is available. To quantify levels of GPCMV gB expression in *A. thaliana* seeds, a direct ELISA was adapted for use with seed protein extracts. The wells of ELISA plates were coated directly with protein extracts from non-transformed or transformed *A. thaliana* seeds, or with baculovirus-derived gB as a positive control. However, elevated background levels against protein extracts from non-transformed seeds indicated cross-reactivity with normal seed proteins, and precluded further use of this approach. In an attempt to reduce these high background levels, several in-house sandwich ELISA protocols were developed. In one configuration, the ELISA plate wells were coated with an in-house murine monoclonal antibody to GPCMV gB, and after application of gB samples, serum from GPCMV-infected guinea pigs was applied to the wells as a secondary antibody. In a second configuration, GPCMV-infected guinea pig serum was first applied to coat the wells, followed by the in-house monoclonal antibody to GPCMV gB as a secondary antibody. Third and fourth configurations involved serum from

guinea pigs immunized with baculovirus-derived gB and the monoclonal antibody as primary or secondary antibodies, respectively. As a fifth strategy, commercial HCMV gB monoclonal antibodies were used to determine if there was sufficient cross-reactivity with GPCMV gB to detect baculovirus-derived GPCMV gB.

Unfortunately, none of these configurations was successful in detecting even the positive control, the baculovirus-expressed gB above background. There are several possible explanations for the lack of detectable signal using a sandwich ELISA but the most likely reason is interference between the two antibodies, possibly as a result of overlapping epitopes. Because of the inability to detect and thus quantify gB in my transgenic seed extracts, the amount of recombinant gB was estimated based on the following rationale: an average level of expression of transgenes expressed in *A. thaliana* leaves is considered to be 1% of total soluble protein (Wu et al., 2004a; Kohl et al., 2007; Rigano et al., 2004). Based on this, a conservative estimate of 0.25% of total soluble protein was used to calculate the quantity of seed protein extract required for immunization.

To summarize, based on Western blot experiments, *A. thaliana*-derived GPCMV gB possessed at least one epitope found on native GPCMV gB. Using antibodies currently available for detecting GPCMV gB, it was not possible to detect gB expression in *A. thaliana* seeds by ELISA, despite repeated attempts to optimize the procedure. GPCMV gB produced in *A. thaliana* seeds had a different molecular weight than expected, suggesting altered processing or glycosylation. Nevertheless, I postulated that *A. thaliana* GPCMV gB might have immunogenic properties similar to native GPCMV gB, and could act as an

effective subunit vaccine. I therefore proceeded to evaluate the immunogenic properties of plant-derived gB *in vivo* since the immune system is highly sensitive to recognizing foreign antigens.

5.7 Immunogenicity of plant-derived GPCMV gB

A major impediment to the testing of vaccines developed against HCMV is the inability to test these vaccines in an animal model due to the species specificity of the virus. The guinea pig is recognized as a good small animal model for HCMV infection, largely because the GPCMV virus can cross the placenta (Griffith et al., 1985), as occurs in humans. Recombinant GPCMV gB produced in baculovirus has been shown to prevent congenital GPCMV infection in pups after subcutaneous immunization of females prior to conception (Schleiss et al., 2004). It was therefore chosen as a suitable model for the testing of a plant-derived subunit vaccine against CMV.

Previous work indicated that a strong immune response was generated following subcutaneous immunization of guinea pigs with baculovirus-derived gB in conjunction with Freund's adjuvant (Schleiss et al., 2004). This immune response was also shown to be protective against live GPCMV infection, and reduced the clinical manifestations of congenital infection (Schleiss et al., 2004). Therefore, to test the immunogenicity of plant-derived gB, this same approach was used, and subcutaneous immunization with Freund's adjuvant was performed using a similar dosing schedule. Of the four animals immunized with seed-derived gB, three produced anti-gB IgG antibody responses, as determined by specific ELISA. One of these three animals had a substantially higher antibody titre than the

other two (A11 versus A13 and A15). Differences in the magnitude of response may relate to these animals being an outbred strain. This strain of guinea pig has been shown previously to have varying responses to immunization (Schleiss et al., 2000).

Unexpectedly, one of the four animals immunized with non-transformed seed protein extracts, i.e. a negative control animal, displayed a positive antibody titre. Although it is not known how this occurred, one possible explanation is that the animal was accidentally immunized with transgenic material. These studies were carried out at the University of Minnesota animal care facility. Animals were not ear-tagged, but instead were identified only by cage labelling. This approach is subject to error when removing animals for cage cleaning and immunization. It is therefore feasible that this animal was actually a member of the group of guinea pigs immunized with seed-derived gB. To test for this possibility, an ELISA was designed to detect antibodies specific for *E. coli* β-glucuronidase, encoded within the T-DNA region of the plant transformation vector, pCAMBIA1301. The rationale was that an animal immunized with transgenic material might also generate antibodies to *E. coli* β-glucuronidase. Data showed that this animal did in fact exhibit similar antibody titre levels to β-glucuronidase as seen in animals in the gB immunized group, whereas the other animals in the negative control group or baculovirus-gB-immunized group did not. This further strengthened the suspicion that the animal with the positive response in the negative control group was actually immunized with transgenic material. If so, the response of this animal is consistent with the three animals exhibiting IgG responses to seed-derived gB.

Further analysis of the antibody response was conducted by Western blot to substantiate the ELISA results. All four animals that were ELISA positive, including the animal in the negative control group, possessed antibodies that recognized a large protein of greater than 120 kDa derived from whole viral particles. Full length GPCMV gB has a molecular weight of approximately 150 kDa, which is cleaved into two subunits of 60 and 90 kDa. The large molecular weight band I observed could therefore correspond to unprocessed, full-length gB within the viral particles. The major antigenic domain of GPCMV gB has been mapped to the 58 kDa subunit of the full-length gB and the 30 kDa of subunit of baculovirus-derived gB (Schleiss and Jensen, 2003). Although the presence of this major antigenic domain on seed-derived gB cannot be confirmed from the present work, it is plausible that the antibody response observed in the guinea pigs after immunization may be directed towards this or a similar domain. These results further substantiate the contention that seed-derived GPCMV gB possesses epitopes similar to those found on baculovirus-derived GPCMV gB, and native viral GPCMV gB. Despite differential processing of the protein and the potential for altered glycosylation, the data indicate that some critical epitopes are authentically produced relative to native GPCMV gB and are immunogenic.

5.8 Viral neutralizing activity of antibodies produced against seed-derived gB

An ideal effective viral vaccine should generate an immune response that will subsequently prevent viral replication. One method for preventing replication is to block viral entry into the host cell. Specifically, antibodies generated against the vaccine will bind to the virus when it enters the host and prevent the virus from binding to cell-surface

receptors. In the case of GPCMV, the major target of antibodies in naturally occurring infection is the gB protein on the surface of the viral envelope (reviewed in (Britt and Mach, 1996)). Immunization of guinea pigs with baculovirus-derived gB and DNA vaccines carrying the gene for gB have been shown to induce an antibody response capable of neutralizing GPCMV *in vitro* in guinea pig fibroblasts (Schleiss et al., 2004). A similar assay was used in the present work to evaluate the neutralizing capacity of guinea pig antibodies generated against seed-derived GPCMV gB after immunization. Results showed that all animals displaying a gB-specific antibody response, including the negative control animal with a positive antibody titre, possessed antibodies that were able to neutralize infectivity of GPCMV. Neutralizing titres varied in a manner corresponding to those observed for ELISA titres, i.e. serum samples with greatest ELISA titres had the greatest neutralizing ability. The neutralizing ability of the sera from immunized animals confirmed that gB expressed in the seeds of *A. thaliana* displays viral neutralizing epitopes. These data indicate that although possible differences in glycosylation and processing of *A. thaliana* seed-derived GPCMV may have occurred, certain epitopes were expressed that induced an immune response able to neutralize GPCMV infectivity *in vitro*. This was an important result from this study, as it has not been previously demonstrated that a plant-derived CMV vaccine could induce a viral neutralizing immune response. Overall, these data indicate that seed-derived gB has the potential to act as a vaccine for the prevention of GPCMV transmission *in vivo*.

5.9 Implications of study findings and future directions

The present study describes the generation of a plant-derived vaccine for GPCMV. First, the design and generation of gene constructs for expression of a truncated and soluble GPCMV gB in seeds or throughout the plant provided the basic tools needed for expression of the transgene in plants. Second, the successful transformation of the plant *A. thaliana* provided a model for expression of the recombinant protein in plants. And third, immunization of guinea pigs with *A.thaliana* seed protein extract containing GPCMV gB showed the potential for use of plant-derived GPCMV gB as a vaccine for HCMV. Confirmation of the neutralizing capacity of antibodies generated against seed-derived gB supported the notion that plant-derived recombinant proteins could be effective against CMV. In short, this work validates the concept of using plant-derived vaccines for immunization against HCMV.

5.9.1 Technical implications

The significant findings of this work were achieved despite certain limitations, which warrant discussion. First, monocot promoters were used in the design of constructs, as they were originally intended for the expression of recombinant proteins in rice. Although monocot promoters have been used in previous studies to express foreign proteins in dicots, dicot promoters may provide higher expression levels (Christensen et al., 1992). Although relatively small quantities of the plant-derived vaccine are required for immunization, higher levels of expression would permit delivery of the vaccine using less plant material. This would be particularly important for oral vaccines, in which the plant material could be ground into a powder, for example, and fed to animals. Note that despite the use of a

monocot promoter for driving expression of GPCMV gB in the dicot *A. thaliana*, recombinant gB was expressed and did provoke a specific neutralizing antibody response in immunized guinea pigs. This indicates that the Gt1 monocot promoter was functional in the dicot plant model, *A. thaliana.*

Secondly, in the present work, transformation of rice with the Gt1/ss/gB/NOS gene construct was successful, but rice plants failed to regenerate. There are many possible reasons for this lack of regeneration, as discussed above. In particular, this may have been due either to problems associated with the transformation procedure or to toxicity resulting from expression of gB. As presently designed, the experiments do not allow one to distinguish between these two possibilities. Most studies investigating the use of plants for the expression of plant-derived pharmaceuticals use non-transformed (wild-type) plants as the negative control, as was done in the present studies for both rice and *A. thaliana.* However, it could be argued that a more appropriate negative control would involve the use of plants transformed with the plant expression vector alone, i.e. pCAMBIA1301 without the gB transgene. Use of a negative control that involved transformation with the pCAMBIA1301 vector would permit selection of negative control callus tissue in the presence of hygromycin, and would therefore clarify whether the lack of regeneration was influenced by the vector itself, the gB gene construct within the vector, or some element of the transformation procedure. Based on the challenges experienced in transforming rice in the present study, use of a vector negative control warrants consideration as a best-practice for future transformation experiments.

Third, the small size of *A. thaliana* plants and seeds means that recovery of sufficient material to carry out immunizations requires substantial scale-up of production. This is challenging in a research laboratory environment, requiring considerable space and time. For increased production of the subunit vaccine, larger plants would serve as more appropriate plant expression systems. Potatoes, tobacco, tomatoes, alfalfa, lettuce, and rice have all been used previously to express subunit vaccines (reviewed in (Yusibov and Rabindran, 2008)). The present work further demonstrates the feasibility of expressing recombinant CMV proteins in a plant system.

Fourth, data from Western blot and ELISA experiments alone could not prove that the transformed *A. thaliana* seeds expressed the GPCMV gB protein. However, given that the immune system is highly sensitive to foreign antigens, immunization studies were nonetheless carried out. Data from these studies indicate that *A. thaliana*-derived GPCMV gB possessed at least one epitope found on native GPCMV gB. In addition, antibodies from guinea pigs immunized with seed-derived gB were able to neutralize viral infection *in vitro*. These data indicate that not only was seed-derived gB able to stimulate antibodies in immunized animals, but that these antibodies were able to bind to authentic gB found on the surface of live GPCMV and effect its neutralization in an *in vitro* situation. Collectively, these data support the conclusion that GPCMV gB expressed in *A. thaliana* seeds was immunogenic and elicited the generation of viral neutralizing antibodies.

5.9.2 Efficacy of seed-derived gB in vivo

The ability of the plant-derived vaccine to induce an antibody response against gB that is effective at neutralizing GPCMV *in vitro* demonstrates that an epitope is present on plant-derived GPCMV gB that is immunogenic and neutralizing. Clearly, the efficacy of the vaccine *in vivo* must be tested. Due to the presence of an antibody response in one of the animals of the negative control group, the immunization studies are currently being repeated. After these immunizations are conducted, and ELISA and viral neutralization assays have been completed, guinea pigs will be challenged with live GPCMV. Female guinea pigs immunized with seed-derived gB will also be mated, and the ability of the vaccine to prevent congenital CMV infection will be evaluated. Work in the Schleiss laboratory has previously shown that subcutaneous immunization of guinea pigs with baculovirus-derived gB significantly reduced pup mortality due to congenital CMV infection (Schleiss et al., 2004), and also decreased viral load in the surviving guinea pig pups. It is expected that the plant-derived gB, based on its ability to induce viral neutralization *in vitro*, may support the results of this previous study. If so, this would further substantiate the notion that plant-derived gB could act as an effective component of a vaccine strategy.

While recombinant gB may serve as a potent inducer of a humoral immune response against CMV, it is recognized that immunity to CMV involves both the antibody response and the T-cell response (reviewed in (Plotkin, 2002)). Protective immunity to CMV may therefore require a vaccine strategy that addresses both the humoral and cell-mediated immune responses. For this reason, constructs in the present work were also generated to

express pp65, the major target of the cell-mediated response. The demonstration in this thesis of successful expression of gB in *A. thaliana* seeds under the control of the Gt1 promoter indicates that CMV proteins can be expressed in plants, and suggests that pp65 could also be authentically expressed in this model system. Once expressed, the immunogenic properties of plant-derived pp65 could be tested by immunizing guinea pigs with protein extracts in the same manner as described here for GPCMV gB. Should it be demonstrated that plant-derived pp65 is effective at generating antibody and T-cell responses, then a vaccine strategy involving co-immunization with plant-derived gB and pp65 would be possible. Co-immunization with both proteins has yet to be investigated in the GPCMV model, but may be a critical experiment to demonstrate the efficacy of a vaccine strategy against CMV that may be applicable to HCMV.

5.9.3 Role of glycosylation

A. thaliana has been used to express a number of recombinant proteins, as it is well-characterized, relatively easy to transform, and reaches maturity quickly. However, it was unknown whether expression of large glycoproteins in this plant model would retain important, viral neutralizing epitopes. Surface glycoproteins are often the main target of the immune response against enveloped viruses, and so maintenance of authentic epitopes in any expression model is critical to their utility as subunit vaccines. An important finding of the present work is that expression of a large envelope viral glycoprotein in plants resulted in an antibody response specific to GPCMV. This finding is of interest because despite the fact that plants differ in their glycosylation of proteins (reviewed in (Brooks, 2006)), our data suggest that this protein still maintains some epitopes critical for the induction of a

neutralizing immune response. A number of studies have now described the expression of immunogenic viral glycoproteins in plants. For example, two studies have shown that expression of recombinant glycoproteins (HIV glycoprotein gp41 and smallpox B5) in *Nicotiana benthamiana* each invoked mucosal and serum antibodies in mice (Matoba et al., 2008; Portocarrero et al., 2008). Rabies virus glycoprotein produced in tobacco was immunogenic and protective in mice (Yusibov et al., 2002). Hepatitis B surface antigen expressed in lettuce leaves was immunogenic in humans (Kapusta et al., 1999). These studies and others (reviewed in (Yusibov and Rabindran, 2008)) indicate that glycoproteins can be successfully expressed in plants and elicit virus-specific immune responses. Enveloped viruses include all herpesviruses, influenza, rabies, rubella, hepatitis B, hantavirus, and HIV. Based on the successful use of plant-derived recombinant glycoproteins, plants may be an attractive expression system for the development of other subunit vaccines using glycoproteins from these viruses. There is, however, concern regarding the immunoreactivity of different glycoforms in humans, as non-human glycosylation patterns may provoke an allergic response (Brooks, 2006; Bardor et al., 2003; Gomord et al., 2005; LaTemple et al., 1999).

5.9.4 Implications for development of a plant-derived vaccine for HCMV

Although a vaccine for HCMV is not yet available, considerable effort has been invested for the development of a vaccine against it (Plotkin, 2002; Schleiss, 2005; Zhong and Khanna, 2007; Schleiss, 2008c). As discussed, a major issue surrounding the development of a HCMV vaccine is the difficulty of pre-clinical testing of vaccines in an animal model due to the species-specificity of the virus. GPCMV has been used as an

animal model for studying the prevention of HCMV infection, specifically congenital CMV infection. Immunization at mucosal sites, such as the gastrointestinal and respiratory tracts, has been studied and used as an effective method to prevent infection by mucosal pathogens (reviewed in (Brandtzaeg, 2007; Wang and Coppel, 2008)), as systemic immunization does not always spill over into the mucosal immune system (Mestecky et al., 2008). Plant-derived vaccines have the potential to directly target the mucosal immune system through oral delivery, and several studies have shown the induction of the IgA antibody response after their oral delivery (Berinstein et al., 2005; Alvarez et al., 2006; Wen et al., 2006; Gomez et al., 2008; Portocarrero et al., 2008). Plant-derived vaccines are also thought to provide better protection for oral immunization, as some plant components may provide protection against the harsh environment of the gut (Yusibov et al., 2006). Although *A. thaliana* is not considered an edible plant, it has been used as an expression system for oral delivery of a vaccine for bursal disease virus in chickens (Wu et al., 2004b). Therefore, oral delivery of *A. thaliana* gB is a possible route of immunization that may warrant future consideration.

5.10 Summary

Stability of recombinant proteins is a major benefit of expression in seeds. Recombinant proteins expressed in seeds stored in dry conditions at room temperature have been shown to retain immunogenic properties years after harvest (Tackaberry et al., 2008). This contrasts with the requirement for refrigeration of conventional vaccines. Indeed, the seeds used in the present work were stored at room temperature for up to two years and were able to elicit GPCMV-specific immune responses in guinea pigs. Long-term stability of a

CMV vaccine in seeds would permit easy transport and storage in environments where access to refrigeration is limited.

Despite considerable efforts in the field, there are currently no licensed vaccines for HCMV. The main goal of the present work was to develop a plant-derived subunit vaccine for CMV, using GPCMV as a model for HCMV. Constructs generated for the expression of two major targets of the immune system in infected individuals, gB and pp65, were designed for seed-specific or ubiquitous expression in plant systems. Two different plant expression systems, namely rice and *A. thaliana*, were used to express the gB transgene. Crude protein preparations from *A. thaliana* seeds provoked a gB-specific neutralizing antibody response in immunized guinea pigs, confirming expression of an immunogenic form of the gB protein in the seeds. A major impediment to the development of a vaccine against HCMV is the inability to perform preclinical assessment of efficacy of these vaccines in animals. The GPCMV vaccine developed as a result of the present work provides a mechanism for testing the efficacy of plant-derived subunit vaccines against CMV in an animal model. Future work will require *in vivo* assessment of vaccine efficacy through viral challenges of immunized guinea pigs. Although more work is needed to validate the use of the *A. thaliana* seed-derived gB subunit vaccine described here, the present research supports the use of the GPCMV small animal model for assessing the efficacy of CMV vaccines. Work described in this thesis represents a critical first step towards production of a plant-derived vaccine for HCMV.

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Appendix

As described in the main text of this thesis, the original goal involved expressing GPCMV proteins in rice. Although much effort was placed into the generation of transgenic rice plants, regeneration of transformed rice callus tissue was not successful. This component of the thesis is outlined and discussed below.

A1.0 Materials and Methods

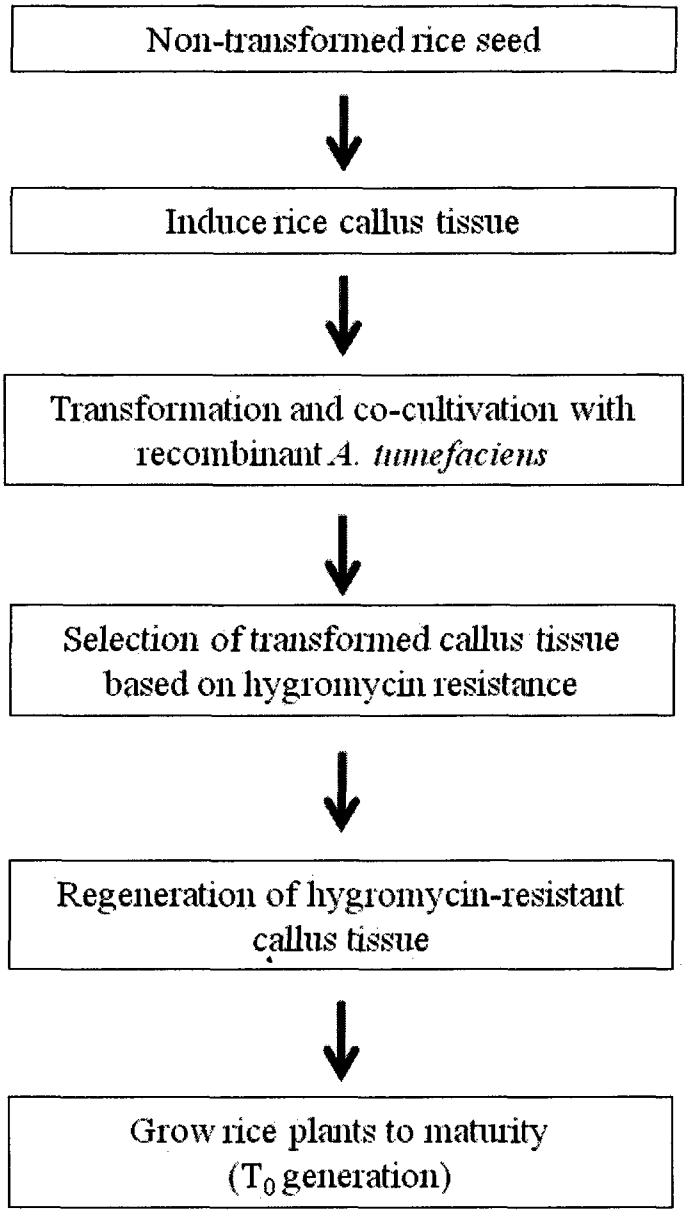
Two methods of rice transformation are described below. As there are many steps involved in each, a flow diagram is included as Figure A1.

A.1.1 First method

A1.1.1 Rice callus induction

The initial rice transformation procedure was a synthesis of two published methods. The first was developed in the laboratory of Dr. Seiichi Toki (Toki, 1997) and the second in the laboratory of Dr. Xiongying Cheng (Cheng et al., 1997). Prior to callus induction, non-transgenic *Japonica* cultivar Xiushui 11 seeds (a gift from Dr. Xiongying Cheng, University of Ottawa) were dehusked and surface sterilized for 2 minutes in 50% household bleach with 20 μL of Tween-20 and shaking at 40 rpm for 30 minutes. The seeds were then quickly rinsed 3 times with sterile distilled water. The seeds were placed endosperm side-down into petri plates containing N6D callus induction medium (N6 salts and vitamins (Phytotechnology Laboratories), 30 g/L D-sucrose (Sigma-Aldrich Canada Ltd.), 0.3 g/L casamino acids (Phytotechnolgoy Laboratories), 2.8 g/L proline (Phytotechnology Laboratories), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich Canada Ltd.),

Figure A1: Flow diagram of the transformation, selection, and regeneration of rice.


Non-transformed rice seed
Induce rice callus tissue
Transformation and co-cultivation with recombinant A. tumefaciens
Selection of transformed callus tissue based on hygromycin resistance
Regeneration of hygromycin-resistant callus tissue
Grow rice plants to maturity (T_0 generation)

2 g/L gelrite (Sigma-Aldrich Canada Ltd.), pH of 5.7). The plates were incubated at 30°C for 2 to 3 weeks with continuous dim light. Three days prior to transformation, the pieces of callus tissue were transferred to fresh N6D medium.

A1.1.2 Preparation for A. tumefaciens transformation

Approximately 10 days prior to the transformation, YEP plates with 50 μg/mL kanamycin and 50 μg/mL hygromycin (Sigma-Aldrich Canada Ltd.) were inoculated with *A. tumefaciens* containing the pCAMBIA1301/Gt1/ss/gB/NOS plasmid (see section 3.2). The plates were incubated at 28°C for 48 hours. A single colony was grown in 5 mL of liquid AB medium (Cheng et al., 1997) and incubated at 28°C with shaking overnight. The next day, the 5 mL culture was used to inoculate 30 mL of AB medium supplemented with 0.1 mM acetosyringone (Sigma-Aldrich Canada Ltd.), 50 μg/mL kanamycin, and 50 μg/mL hygromycin. The bacteria were incubated at 28°C for 48 hours with shaking at 120 rpm.

A1.1.3 Transformation of rice callus tissue

The bacterial culture was centrifuged at 2060 g for 10 minutes at 4°C. The supernatant was removed and the bacteria were resuspended in Murishige and Skoog (MS) callus induction medium (MS basal medium (Sigma-Aldrich Canada Ltd.), 30 g/L D-sucrose, 2 mg/L 2,4-D, pH 5.8) supplemented with 0.1 mM acetosyringone. Wounds were created in previously induced callus tissue by breaking the callus tissue with forceps into small pieces. These pieces of callus were then incubated with the bacterial suspension at room temperature. After 30 minutes, excess medium was removed from the callus tissue by blotting onto filter paper. The pieces of callus tissue were transferred to 2N6-AS medium

(Chu's basal salts and vitamins (Phytotechnology Laboratories), 1 g/L casamino acids, 30 g/L D-sucrose, 10 g/L D-glucose (Sigma-Aldrich Canada Ltd.), 100 μM acetosyringone, 2 mg/L 2,4-D, 4.5 g/L gelrite, pH 5.2) and incubated in the dark at 30°C for 72 hours.

A1.1.4 Selection of transformed rice callus tissue

The pieces of callus tissue were transferred to fresh plates of N6D medium with 50 μg/mL hygromycin and 500 μg/mL carbenicillin (Sigma-Aldrich Canada Ltd.). The plates were incubated at 30°C with 11 hours of bright light/13 hours of dark for 2 weeks.

A1.1.5 Regeneration of transformed rice callus tissue

Healthy, white pieces of callus tissue were transferred to regeneration medium (MS salts and vitamins (Phytotechnology Laboratories), 30 g/L D-sucrose, 30 g/L sorbitol (Sigma-Aldrich Canada Ltd.), 2 g/L casamino acids, 2 mg/L kinetin (Sigma-Aldrich Canada Ltd.), 0.02 mg/L naphthaleneacetic acid (Sigma-Aldrich Canada Ltd.), 0.5 g/L carbenicillin, 50 μg/mL hygromycin, 4 g/L gelrite, pH 5.7). They were incubated at 30°C with the same light cycle as in section 3.3.1.4. Black pieces of dead callus tissue were discarded.

A1.2 Second method

A1.2.1 Rice callus induction

The second transformation method was identical to the method developed by Dr. Seichii Toki (Toki, 1997). Briefly, Xiushui 11 seeds were surface sterilized exactly as described in section 3.3.1.1 except the callus tissue was not transferred to fresh induction medium prior to transformation.

A1.2.2 Preparation of A. tumefaciens for transformation

The pCAMBIA1301/Gt1/ss/gB/NOS recombinant *A. tumefaciens* were prepared for rice callus transformation as in section 3.3.1.3 except the cells were resuspended in AAM medium (Hiei et al., 1994).

A1.2.3 Transformation of rice callus tissue

Pieces of callus tissue were incubated with the transformed bacteria for 2 minutes and blotted dry, transferred to 2N6-AS medium, and incubated in the dark at 28°C for 72 hours.

A1.2.4 Selection of transformed rice callus tissue

After co-cultivation, the transformed callus tissue was washed 5 times with N6D medium containing 500 μg/mL carbenicillin. The pieces of callus tissue were then transferred to N6D medium containing 50 μg/mL hygromycin and 500 μg/mL carbenicillin. The callus tissue was incubated at 30°C for 2 weeks with constant light.

A1.2.5 Regeneration of transformed rice callus tissue

Regeneration of selected callus tissue was carried out exactly as in section 3.3.1.5.

A1.2.6 Regeneration of transformed rice callus tissue with N6-benzylaminopurine

Regeneration of selected callus tissue was carried out exactly as in section 3.3.1.5 except varying concentrations (1 mg/L, 2 mg/L, and 4 mg/L) of N6-benzylaminopurine (Sigma-Aldrich Canada Ltd.) were incorporated into the regeneration medium.

A1.2.7 Nipponbare and Kitaake seeds

Nipponbare and Kitaake seeds were obtained from (National Small Grains Collection, Aberdeen IO, US). Both of these rice cultivars were transformed with pCAMBIA1301/Gt1/ss/gB/NOS exactly as described in section 3.3.2.2 to 3.3.2.5.

A1.3 Commercial rice transformation

Rice transformations using the pCAMBIA1301/Ubi/gB/NOS vector were contracted out to the Plant Transformation Facility at the University of California, Davis, California. The facility was supplied with the pCAMBIA1301/Ubi/gB/NOS plasmid.

A2.0 Results

A2.1 Selection, regeneration, and screening of rice callus tissue transformed with pCAMBIA1301/Gt1/ss/gB/NOS

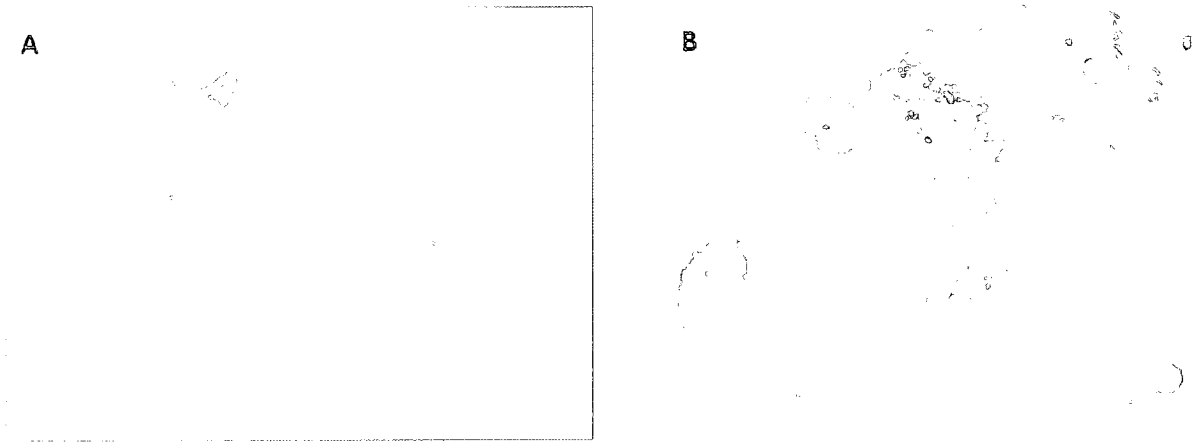
A2.1.1 Selection based on expression of hygromycin phosphotransferase

Once transformation of *A. tumefaciens* was carried out, the next step was to use the recombinant *A. tumefaciens* to transform rice callus tissue. Rice callus tissue was transformed using the two different procedures as outlined in section 3.3 of Materials and Methods. The gene for hygromycin resistance (hygromycin phosphotransferase) is encoded within the pCAMBIA1301 T-DNA region, permitting selection of successfully transformed rice callus tissue based on hygromycin resistance. Selection of transformed rice callus tissue with the plasmid pCAMBIA1301/Gt1/ss/gB/NOS was confirmed based on ability to grow in the presence of hygromycin (Figure A2A, B). Untransformed callus tissue became dark brown or black in the presence of hygromycin (Figure A2B). In sharp contrast, transformed

Figure A2: Induction of rice callus tissue and selection of rice callus transformed with *A. tumefaciens* containing the plasmid, pCAMBIA1301/Gt1/ss/gB/NOS. After co-incubation with *A. tumefaciens*, pieces of callus tissue were transferred to selection medium containing hygromycin and carbenicillin. Since the pCAMBIA1301 vector contains a gene for hygromycin resistance within the T-DNA region, calli successfully transformed appeared to remain white and healthy on the media after 2 weeks. Those pieces of calli unsuccessfully transformed turned black and died.

A: Healthy, untransformed rice callus tissue on callus induction medium.

B: Pieces of transformed callus tissue on selection medium.


A
B

callus tissue remained white in the presence of hygromycin, indicating healthy tissue (Figure A2A). As a negative control, untransformed callus tissue was placed on selection medium, resulting in the production of only dark brown callus tissue. This indicated that selection based on antibiotic resistance was indeed working. Seven independent transformations were carried out using the transformation method described in section 3.3.1 and 10 independent transformations were done using the method described in section 3.3.2. Both methods appeared to generate live (white) and dead (dark brown) callus tissue in approximately the same proportions.

A2.1.2 Regeneration of hygromycin-resistant rice callus tissue

To generate mature rice plants expressing the gB protein, transformed callus tissue was transferred from selection medium to regeneration media after a 2 week period. Initially, transformed callus tissue was kept on regeneration media for 1 month. From published reports and personal communication with other researchers, it was expected that after this time period some of the callus tissue would turn green in colour, indicating that regeneration was occurring. However, callus tissue remained white during this time period. To test whether regeneration would occur over a longer time period, the regeneration period was extended up to 4 months by transferring the healthy, white callus tissue to fresh regeneration medium approximately every 3 to 4 weeks. However, there was no outward change in the appearance of the callus tissue. As described in section 4.3.1, 10 additional independent transformations were performed, for example, incorporating N6-benzylaminopurine in the regeneration medium, and with different rice cultivars

(Nipponbare and Kitaake). Most of these transformations were successful in generating transformed callus, as indicated by their ability to survive in the presence of antibiotics. However, none of these modifications resulted in regenerated callus tissue. In order to ascertain whether the lack of regeneration was due to a factor used in our transformation methods, rice transformations were contracted to a facility at University of California at Davis (Sacramento CA, US). After several months, this facility reported that callus tissue transformed with the pCAMBIA1301/Ubi/gB/NOS vector did not regenerate and became necrotic on regeneration medium. Importantly, the facility was able to regenerate many rice plants from callus tissue transformed with the pCAMBIA1301 vector alone.

A2.1.3 GUS screening of hygromycin-resistant callus tissue

Further confirmation of successful transformation of callus tissue with the GUS-containing plasmid pCAMBIA1301/Gt1/ss/gB/NOS was done through the determination of GUS expression in a subset of hygromycin-resistant calli. All transformed calli tested were positive for GUS, as indicated by the production of a blue precipitate in the presence of X-gluc (Figure A3A). In contrast, immersion of non-transformed calli in the substrate did not produce a blue precipitate (Figure A3B). This confirmed that transformation had occurred.

A3.0 Discussion

A3.1 Transformation of rice callus tissue

Rice transformation is a multi-step process, involving transformation of *A. tumefaciens*, induction of embryogenic rice callus tissue, co-culture of rice callus tissue with transformed *A. tumefaciens*, selection of transformed callus tissue based on antibiotic resistance, and

finally regeneration of rice plantlets from the transformed embryogenic callus tissue. This process was successfully used in the past to express both human and viral proteins in our laboratory (Sardana et al., 2007; Tackaberry et al., 2008). However, despite many attempts at regenerating callus tissue transformed with the seed-specific Gt1/gB construct, no regeneration was ever observed. Thus, it was not possible to recover recombinant GPCMV gB from rice seeds. To understand the possible steps in the transformation process which may have been responsible for the lack of callus regeneration, each step was scrutinized in turn.

A. tumefaciens-mediated transformation of dicots has been used for the expression of recombinant proteins for many years (reviewed in (Gelvin, 2003; Veluthambi et al., 2003)). In contrast, *A. tumefaciens*-mediated transformation of rice, a monocotyledonous plant (monocot) was only first successfully accomplished in the early 1990s (Raineri et al., 1990; Hiei et al., 1994), and transformed rice tissue is still notoriously recalcitrant to regeneration as compared to dicots, such as tobacco (Shrawat and Lorz, 2006). Transformation of rice is carried out using callus tissue, which is a mass of undifferentiated rice cells susceptible to *A. tumefaciens* infection in the presence of the hormone acetosyringone (Hiei et al., 1994; Rashid et al., 1996; Cheng et al., 1997; Toki, 1997). In my experiments, transformation of callus tissue was confirmed by its ability to survive in the presence of hygromycin. As a second test to confirm successful transformation, hygromycin-resistant callus tissue was assayed for the presence of β-glucuronidase using the GUS test. All hygromycin-resistant callus tissue samples tested for β-glucuronidase activity were GUS positive. These two tests confirm successful transformation of rice callus tissue with the

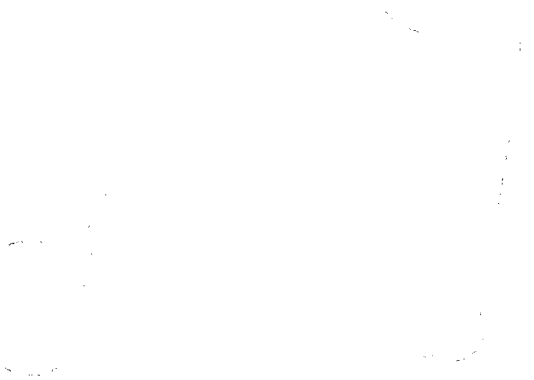
Figure A3: Testing of transformed rice callus tissue for expression of GUS. Pieces of callus tissue transformed with pCAMBIA1301/Gt1/ss/gB/NOS were placed into a solution containing the substate for GUS, 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc). When GUS breaks down X-gluc, a blue precipitate forms, allowing for colourimetric determination of the expression of GUS. The tubes were incubated overnight at 37oC and it was noted if a blue precipitate formed.

A: Transformed callus tissue.

B: Untransformed callus tissue.

A

B



pCAMBIA1301/Gt1/ss/gB/NOS vector, and indicate that at least these portions of the T-DNA region were transferred to the rice genome, as indicated by the presence of functional hygromycin resistance and β-glucuronidase genes.

A3.2 Regeneration of transformed rice callus tissue

Given the successful incorporation of the construct into the pCAMBIA1301 vector, and confirmation of callus tissue transformation, I considered the possibility that the lack of regeneration was due to regeneration conditions. Two distinct protocols were used for the rice transformations, differing primarily in the composition of the induction media (Toki, 1997; Cheng et al., 1997). Both protocols have been successfully used for rice transformation (for example, (Cheng et al., 1998; Sardana et al., 2007; Tackaberry et al., 2008; Ku et al., 1999; Toki, 2006)), but remarkably, neither protocol resulted in successful regeneration of transformed rice callus tissue in my experiments. I therefore sought to boost regeneration rates. While not used in either published method, incorporation of 6-benzylaminopurine in the regeneration medium has been shown by several groups to increase regeneration rates (Rashid et al., 2001; Noor et al., 2005; Lee et al., 2002). However, attempts at regenerating hygromycin-resistant rice callus tissue using four different concentrations of this compound were all unsuccessful.

To verify whether lack of regeneration was attributable to the specific cultivar of rice (Xuishui 11), two additional varieties specified by Toki (1997) were obtained. These two varieties, Nipponbare and Kitaake, have both previously been used in successful rice transformations (Toki, 1997). Rice calli were again transformed, selected by hygromycin

resistance, and placed on regeneration medium. As was observed for the initial variety, no regeneration occurred even after several months. In contrast, untransformed rice callus tissue placed on regeneration media was successfully regenerated into mature rice plants, indicating that the composition of the regeneration medium was not inherently toxic.

The lack of success at regenerating transformed rice callus tissue suggests that at least one of the following may have occurred. First, it is possible that the induced callus tissue used in transformation studies was not embryogenic, and therefore did not have the potential to regenerate (Rueb et al., 1994; Rachmawati et al., 2004; Rachmawati and Anzai, 2006). Secondly, if a component of the regeneration medium was lacking, or present in too great or too little abundance, regeneration may have been inhibited. However, the same protocols have been applied routinely to other studies involving rice transformation, including previous ones in our laboratory (Sardana et al., 2007; Tackaberry et al., 2008), suggesting that this is not a likely explanation. Third, it may be that expression of GPCMV gB in rice, in this particular construct, is toxic to the plant tissue. Contracting of rice transformations to a commercial facility did not result in regeneration of mature rice plants, suggesting that the problems may not have been specific to our laboratory or to the approaches used, but rather to the construct itself. For example, it is possible that the transgene itself was in some way toxic to the callus tissue, preventing regeneration. One way to evaluate gB transgene toxicity would be to transform rice with the pCAMBIA1301 vector alone: if the rice was successfully regenerated, regeneration problems could be attributed to toxicity resulting from transgene expression. Unfortunately, plant material transformed with the pCAMBIA1301 vector alone was not available at the time. In short, despite successful transformation of rice

callus tissue with GPCMV gB, no transgenic plants were produced. Callus induction, transformation, selection, and initial regeneration of rice is a lengthy process (approximately four to six months under optimal circumstances), making numerous sequential tests difficult within a finite period of time. Thus, an alternative plant expression system (*A. thaliana*) was used for subsequent work, as described in the main body of this thesis.

Karen Rowlandson

EDUCATION

Doctor of Philosophy Candidate
Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine
University of Ottawa (2001 - present)

- Submitted final copy of thesis May 2009
- Transferred from MSc to PhD program in 2003
- Advanced courses in immunochemistry, immunology, virology, and molecular mechanisms of human diseases

Bachelor of Science
Honours Biochemistry, Co-operative Education, University of Waterloo (1995 - 2000)

- Courses in analytical chemistry, organic chemistry, immunology, statistics, natural products, genetics, and biotechnology

CURRENT PROJECT

PhD Research Project
Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine
University of Ottawa (2001 - present)

- Thesis entitled "Production and evaluation of plant-derived vaccines for cytomegalovirus using guinea pig as an animal model". The goal of this project was to develop a cytomegalovirus plant-derived vaccine suitable for oral delivery.
- Designed and engineered four genetic constructs for the purpose of expressing viral proteins in rice plants and *Arabidopsis thaliana*
- Generated transgenic plants using plant transformation techniques
- Immunized guinea pigs with plant material
- Evaluated immune responses to the plant-derived vaccine by ELISAs, Western blots and viral neutralization assays

WORK EXPERIENCE

Scientific Support Specialist
Centre for Biologics Research, Health Canada, Ottawa, Ontario (June 2009 to Sept. 2009)

- Extraction and purification of recombinant human interferon from *E. coli*
- FPLC affinity-purification of proteins

Scientific Support Specialist
Centre for Biologics Research, Health Canada, Ottawa, Ontario (Nov. 2008 - March 2009)

- Large-scale extraction and purification of recombinant human prion peptide from *E. coli*
- FPLC affinity-purification of proteins
- HPLC purification and analysis of recombinant proteins

Research Assistant/Contractor
Research Services Division, Health Canada, Ottawa, Ontario (Sept. - Dec. 2000)
- Isolation of genomic DNA from rice plants
- Developed and optimized protocols for analysis of transgenic rice plants by PCR and Southern blots

Laboratory Technician
Grace Bioremediation, Mississauga, Ontario (May - Aug. 2000)
- Extraction of environmental pollutants from soil and water samples using a soxhlet apparatus, followed by HPLC analysis

Research Assistant
Research Services Division, Health Canada, Ottawa, Ontario (May - Sept. 1999)
- Co-op placement (University of Waterloo co-op program)
- Engineering of genetic constructs for the expression of subunit vaccines in rice plants
- Used standard molecular biology techniques such as polymerase chain reaction, ligation reactions, restriction enzyme digests, and bacterial transformations

Research Assistant
Forest Pathology, Natural Resources Canada, Sault Ste. Marie, Ontario (Jan. - May 1999)
- Co-op placement (University of Waterloo co-op program)
- Investigation of the involvement of *Agrobacterium tumefaciens* in tumour formation in black spruce
- Extracted genomic DNA from black spruce callus tissue
- Tested callus tissue for *A. tumefaciens* infection using PCR

Research Assistant
Department of Biochemistry, Laurentian University, Sudbury, Ontario (May - Sept. 1998)
- Co-op placement (University of Waterloo co-op program)
- Study of apoptotic pathway activation during prolonged cell culture
- Used SDS-PAGE and Western blotting to detect apoptosis-related proteins

Laboratory Technician
Inco Ltd., Sudbury, Ontario (Sept. - Dec. 1997)
- Co-op placement (University of Waterloo co-op program)
- Preparation and analysis of soil and water samples by graphite furnace atomic adsorption

TEACHING EXPERIENCE

Teaching Assistant
Molecular Biology Laboratory, Biochemistry Department, University of Ottawa, Ottawa, Ontario

- Teaching assistant for three years (Fall 2002 to Fall 2004)
- Demonstrated molecular biology techniques to third-year undergraduate students
- Responsibilities included supervising a six hour laboratory period once per week, marking of reports, and answering student questions

Teaching Assistant
Biochemistry Laboratory, Biochemistry Department, University of Ottawa, Ottawa, Ontario

- Teaching assistant for three years (Winter 2003 to Winter 2005)
- Demonstration of lipid analysis to second-year undergraduate students

Student Supervision
Centre for Biologics Research, Health Canada, Ottawa, Ontario

- Trained and supervised a first-year summer student in rice callus tissue culturing and various molecular biology techniques
- Trained a fourth-year honours student to design and engineer genetic constructs for the expression of recombinant proteins in rice plants
- Trained and supervised an undergraduate student in the analysis of recombinant protein production in plants and the evaluation of immune responses

AWARDS

Awards for Poster Presentations

- Awarded silver at Canadian Institute of Health Research (CIHR) National Student Poster Competition (May 2003)
- Awarded first place in the Microbiology and Immunology PhD category at the University of Ottawa, Biochemistry, Microbiology, and Immunology Graduate Student Poster Day (April 2003)

Awards for Oral Presentations

- Awarded first prize, as voted by peers, in the Graduate Student Biochemistry, Microbiology, and Immunology Presentation Forum (February 2006)

Scholarships

- Doctoral Research Award from the University of Ottawa
- Undergraduate Student Research Award from NSERC for employment at Grace Bioremediation (May to August 2000)

PUBLICATIONS AND POSTERS

Publications

- **Karen Rowlandson,** Eilleen Tackaberry, and Mark Schleiss (2009). Cloning and expression of guinea pig cytomegalovirus glycoprotein B in *Arabidopsis thaliana.* Manuscript in preparation.
- Eilleen Tackaberry, Fiona Prior, **Karen Rowlandson**, Monika Tocchi, Jelica Mehic, Suzanne Porter, Mike Walsh, Mark Schleiss, Peter Ganz, Ravinder Sardana, Illimar Altosaar, and Anil Dudani (2008). *Sustained expression of human cytomegalovirus glycoprotein B (UL55) in the seeds of homozygous rice plants.* Molecular Biotechnology 40(1): 1-12.
- Ravinder Sardana, Anil Dudani, Eilleen Tackaberry, Suzanne Porter, **Karen Rowlandson**, Peter Ganz, and Illimar Altosaar (2007). *Biologically active human GM-CSF produced in the seeds of transgenic rice plants.* Transgenic Research 16(6): 713-721.
- **Karen Rowlandson** and Eilleen Tackaberry (2003). *Edible vaccines: alternatives to conventional immunization.* AgBiotechNet 5:1-7 (ABN 115). Invited review article.

Poster and Oral Presentations

- **Karen Rowlandson**, Mark Schleiss, and Eilleen Tackaberry. *Plant-derived glycoprotein B of guinea pig cytomegalovirus as a model oral vaccine.* Presented at the Plant-Based Vaccines and Antibodies Conference, Verona, Italy (June 2007, poster)
- **Karen Rowlandson.** *Expression of a plant-derived vaccine for cytomegalovirus.* Presented at the University of Ottawa BMI Student Forum, Ottawa, Ontario (February 2006, oral presentation).
- **Karen Rowlandson**, Mark Schleiss, and Eilleen Tackaberry. *Development of cytomegalovirus plant-derived subunit vaccine.* Presented at the University of Ottawa BMI Graduate Student Poster Day, Ottawa, Ontario (April 2005, poster)
- **Karen Rowlandson,** Mark Schleiss, and Eilleen Tackaberry. *Expression of plant-derived guinea pig cytomegalovirus subunit vaccines.* Presented at the 10th International Cytomegalovirus/Betaherpesvirus Workshop, Williamsburg, Virginia (April 2005, poster).
- **Karen Rowlandson,** Mark Schleiss, and Eilleen Tackaberry. *Arabidopsis thaliana as an expression system for a cytomegalovirus subunit vaccine.* Presented at the Health Canada Forum, Ottawa, Ontario (October 2004, poster).
- **Karen Rowlandson,** Mark Schleiss, and Eilleen Tackaberry. *A plant-made oral vaccine for cytomegalovirus using guinea pigs as animal models for congenital infection.* Presented at the Rational Design of Vaccines and Immunotherapeutics, Keystone, Colorado USA (January 2004, poster).
- **Karen Rowlandson,** Mark Schleiss, Anil Dudani, Ken Dimock, Peter Ganz, and Eilleen Tackaberry. *Production of transgenic rice expressing a potential guinea pig cytomegalovirus edible subunit vaccine.* Presented at the Health Canada Forum, Ottawa, Ontario (November 2003, poster).

- **Karen Rowlandson,** Ken Dimock, Anil Dudani, Mark Schleiss, and Eilleen Tackaberry. *Generation of an edible cytomegalovirus subunit vaccine in rice plants.* Presented at the University of Ottawa BMI Graduate Student Poster Day, Ottawa, Ontario (April 2003, poster); and the CIHR National Student Poster Competition, Winnipeg, Manitoba (May 2003, poster).
- **Karen Rowlandson.** *An oral plant-derived vaccine for cytomegalovirus.* Presented at the University of Ottawa BMI Student Form (February 2003, oral presentation)
- **Karen Rowlandson,** Ken Dimock, Anil Dudani, Mark Schleiss, Peter Ganz, and Eilleen Tackaberry. *Towards an edible vaccine: expression of guinea pig cytomegalovirus gB and pp65 in rice plants.* Presented at the Agricultural Biotechnology International Conference, Saskatoon, Saskatchewan (September 2002, poster).