

EVALUATION OF THE EFFICACY OF CHLOROPLAST-DERIVED ANTIGENS  
AGAINST MALARIA

by

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## ABSTRACT

Malaria is the most prevalent vector-borne parasitic disease worldwide and a major cause of death from infections. There is a great need to develop a low cost vaccine for malaria to control transmission of infection and impact of disease, due to the emergence of anti-malarial resistance. Two leading blood stage malarial vaccine candidates are the apical membrane antigen-1 (AMA-1) and the merozoite surface protein-1 (MSP-1). The aim of this project is to express malarial antigens in tobacco plants *via* plastid transformation and deliver them by subcutaneous or oral gavage of minimally processed transplastomic tissue to evaluate their efficacy to elicit an immune response and protect against malarial infection. Transplastomic lines expressing the malarial antigens fused to the transmucosal carrier Cholera toxin B subunit (CTB-AMA-1) and CTB-MSP-1 were generated. CTB-AMA-1 and CTB-MSP-1 accumulated up to 9.5% and 2% of the total soluble protein, respectively. Chloroplast-derived CTB-AMA-1, CTB-MSP-1, or both antigens were administered to BALB/c mice orally or by subcutaneous injections. The immune response in the experimental animals compared to the control animals was found to be significant. Using an immunofluorescence assay (IFA) and immunoblot, anti-AMA-1 and anti-MSP-1 found in sera of immunized mice recognized the native parasite and the native parasite protein, respectively. Anti-malarial antibodies inhibited parasite invasion into erythrocytes by utilizing an *in vitro* parasite inhibition assay. Results of these investigations may lead to a cost-effective malarial vaccine, much needed in developing nations.

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## LIST OF ACRONYMS/ABBREVIATIONS

*aadA* – Aminoglycoside 3' adenosyltransferase

Ab – Antibody

APS – Ammonium Persulfate

Arg – Arginine

BAP – Benzylaminopurin

BME – Beta-mercaptopropanol

BSA – Bovine Serum Albumin

CSP – Circumsporozoite Protein

CT – Cholera Toxin

CTB – Cholera Toxin B Subunit

CTAB – Cetyltrimethylammonium Bromide

DABCO – 1,4-diazabicyclo[2.2.2]octane

EDTA – Ethylene Diamine Tetra-Acetic Acid

ELISA – Enzyme Linked ImmunoSorbent Assay

FC – Furin Cleavage Site

GFP – Green Fluorescent Protein

GPI – Glycosylphosphatidylinositol

IFA – Immunofluorescence Assay

LSA – Liver Stage Antigen

Lys – Lysine

MR4 - Malaria Research and Reference Reagent Resource Center

NaBH<sub>4</sub> – Sodium Borohydride

NaCl – Sodium Chloride

NAA –  $\alpha$ -Naphthalene Acetic Acid

PBS – Phosphate Buffered Saline (T – Tween 20)

pBSK+ – pBlueScript SK+

PA – Protective Antigen

PCR – Polymerase Chain Reaction

PEI - Polyethylenimine

Pf – *Plasmodium falciparum*

PfAMA1 – *Plasmodium falciparum* Apical Membrane Antigen 1

PfMSP1<sub>19</sub> – *Plasmodium falciparum* Merozoite Surface Antigen 1<sub>19</sub>

psbA – Photosystem b/A

PTM – Phosphate Buffered Saline – Tween – Milk

RBC – Red Blood Cells

RESA – Ring-Infected Erythrocyte Surface Antigen

RT-PCR – Reverse Transcriptase – Polymerase Chain Reaction

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

TEMED – N,N,N,N'-Tetra-Methyl-Ethylene Diamine

TMB – 3,3', 5,5' Tetramethyl Benzidine

TRAP – Thrombospondin-Related Anonymous Protein

TSP – Total Soluble Protein

UTR – Untranslated Region

UV – Ultraviolet

## INTRODUCTION

Malaria is a vector-borne protozoan disease and four different species of the genus *Plasmodium* affects humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*) with *P. falciparum* the most virulent species causing the majority of morbidity and mortality across the world. More than 2 billion people are at risk for malaria with approximately 500 million cases and 1 million deaths annually, mainly in children in sub-Saharan Africa (Greenwood, Fidock et al. 2008; Langhorne, Ndungu et al. 2008). Malaria has become a prominent public health issue for the international health community and an effective malaria control program is imperative (Greenwood, Bojang et al. 2005).

### *Plasmodium* Life Cycle

Malaria parasites multiply in female *Anopheles* mosquitoes and are transmitted to humans when a mosquito takes a blood meal. *Plasmodium* sporozoites, alongside saliva, enter the bloodstream, migrate to the liver, and then enter hepatocytes where amplification lasts for 2 to 9 days, hence the exo-erythrocytic cycle (Langhorne, Ndungu et al. 2008). Malaria parasites differentiate into thousands of merozoites, following rupture of liver cells, where they invade RBCs and initiate the asexual erythrocytic stage of the life cycle. The ring stage of parasite develops into a trophozoite and matures into schizonts, which ruptures and releases merozoites approximately 48 and 72 hours, depending on the species of *Plasmodium* (Langhorne, Ndungu et al. 2008). The blood stage parasites are responsible for the clinical manifestations such as fever and severe malaria. Some parasites develop into sexual

erythrocytic male (microgametocytes) and female (macrogametocytes) gametocytes, which fuse to form an oocyst, after a mosquito takes a blood meal and ingests the gametocytes. The sporogonic cycle begins when the parasites multiply in the mosquito gut. Oocysts develop into sporozoites. Once the sporozoites migrate to the mosquito's salivary glands, they are ready to infect a new human host to continue the malaria life cycle.

### Clinical Manifestations of Malaria

The most common symptoms of malaria include flu-like illness such as fever, shivering, vomiting, nausea, joint pain, muscle aches, and headaches. The classical symptom of malaria is the sudden coldness with shivering followed by fever and then sweating persisting six to ten hours that occurs in cycles due to the asexual erythrocytic stage of the *Plasmodium spp.* life cycle. Other symptoms experienced by malaria patients include dizziness, malaise, myalgia, abdominal pain, mild diarrhea, and dry cough. The causative organism of severe malaria is *P. falciparum* and consequences include coma and death if untreated. Other complications of severe malaria may occur and include splenomegaly, cerebral ischemia, hepatomegaly, hypoglycemia, hemoglobinuria, renal failure, pulmonary edema, and acidosis. Young children and pregnant women are most vulnerable to severe malaria along with individuals with no or decreased immunity. Severe malaria is considered a medical emergency and should be treated urgently because it can rapidly progress to death within hours or days (Trampuz, Jereb et al. 2003).

## Diagnosis and Treatment of Malaria

The number of cases of malaria is increasing and drug resistance is common so prompt diagnosis is essential to reduce morbidity and mortality (Yamey 2004). Clinical diagnosis of patients involve examination of symptoms but the “gold standard” from a laboratory perspective is examining a blood smear stained with Geimsa stain *via* microscopic examination (Icke, Davis et al. 2005). If a microscope and staining reagents are not available and lack of quality microscopy, modern antigen detection kits such as a “dipstick” and molecular techniques can be used as alternative in diagnosis (Greenwood, Bojang et al. 2005; Icke, Davis et al. 2005). Several issues have evolved with using antigen detection kits and molecular practices such as cost-benefit ratio, accuracy of results, and adequate performance in field conditions.

Malaria needs to be recognized without delay in order to treat the patient and prevent further disease transmission. Treatment of malaria can be conducted without hospitalization but if severe malaria persists hospitalization should be advised if possible. Several antimalarial drugs are available for treatment such as chloroquine, sulfadoxine-pyrimethamine, mefloquine, quinine, artemisinin and doxycycline but combination treatment is ideal. The combination of drugs is preferred because different modes of action are included to hopefully inhibit the emergence of drug resistant parasites (Greenwood, Bojang et al. 2005). Many factors should be considered when treating a patient with malaria such as species of infecting parasite, demographic region, cost, pregnancy, pre-existing conditions, and drug allergies.

### Need for an Antimalarial Vaccine

Preventing mosquito bites with mosquito nets or insect repellents, spraying insecticides, can reduce malaria transmission or expensive prophylactic drugs but resurgence of the parasite continues. The causes of resurgence includes drug resistance to common antimalarials such as chloroquine, antifolates, sulfadoxine, and artemisin; mosquito's resistance to widely used insecticides, pharmaceutical's lack of interest in developing new drugs, implementing effective control measures, increase of tourism, and migration of non-immune populations to malaria endemic areas (Aide, Bassat et al. 2007; Hyde 2007). A traditional public health tool to effectively reduce the tremendous disease burden would be to develop an efficacious antimalarial vaccine (Doolan and Stewart 2007). Vaccination is one of the most effective means of preventing disease transmission, cost-effective in reducing new infections, and is easily administered. Many concerns arise when developing an effective vaccine such as complexity of antigens *Plasmodium* presents throughout the different stages of its life cycle, high polymorphism among parasitic proteins, no appropriate animal model to test the efficacy of a vaccine, high cost of designing a vaccine, and length of vaccine development before it can be marketed by pharmaceutical companies (Aide, Bassat et al. 2007). Currently, there is no licensed effective vaccine for the prevention of malaria. The desirable vaccine to prevent malaria progression would consist of multi-antigens from different phases of the life cycle.

### Malaria Vaccines are Feasible

There are four main arguments supporting the belief that a malaria vaccine is feasible (Aide, Bassat et al. 2007). Individuals living in endemic areas progressively exhibit naturally acquired immunity by developing partial immunity against severe malaria (Gupta, Snow et al. 1999). Individuals may still become infected with malaria but clinical manifestations and symptoms may be non-existent due to suppression of parasitemia to undetectable levels (Webster and Hill 2003). Passive transfer of antibodies from either immune malaria patients or maternal transmission during pregnancy has protected patients exposed to the parasite (Sabchareon, Burnouf et al. 1991) or newborn infants (Ballou, Arevalo-Herrera et al. 2004), respectively. In the 1970s, experiments were carried out on non-immune volunteers that were exposed to UV irradiated-weakened sporozoites and re-challenged by normal sporozoites with 90% of cases exhibiting short-lived immunity (Rieckmann, Beaudoin et al. 1979). Several studies have reported the efficacy of recent development of protective malaria vaccine candidates in humans (Greenwood, Fidock et al. 2008; Maher 2008).

### Candidates for Malaria Vaccine

Due to the complexity of the malaria life cycle, vaccines can be targeted to the different stages beginning with the initial stage, exo-erythrocytic. The ultimate goal in vaccine development targeting the exo-erythrocytic stage are the sporozoites and liver stage parasites to completely prevent infection (Greenwood, Fidock et al. 2008) by protecting against invasion of hepatocytes or inhibiting parasite development in hepatocytes. Antibodies elicited at this stage

would either kill the sporozoite or block hepatocyte invasion. Disrupting parasite development in infected hepatocytes would involve cytotoxic T-lymphocyte mediated lysis. The earliest and now the most advanced pre-erythrocytic studied vaccine candidate utilizes CSP as a target because it is the most abundant surface antigen at this stage (Greenwood, Bojang et al. 2005). Current vaccine trials utilizing CSP consists of a hybrid with the hepatitis-B surface antigen and a three-component adjuvant, AS02, known as RTS,S/AS02A but has provided only short-term protection (Greenwood, Bojang et al. 2005). Other antigens in clinical trials include TRAP and LSA but with disappointing results (Maher 2008).

Another strategy in vaccine development could target the second phase of the life cycle, erythrocytic, also known as the asexual blood phase. Vaccines targeted at this stage are designed to prevent disease not infection by reducing the number of blood stage parasites (Greenwood, Fidock et al. 2008). The vaccine could either prevent merozoite multiplication or invasion of RBCs, with current research mainly focusing on antigens involved in erythrocyte invasion (Greenwood, Bojang et al. 2005). Antibodies can be elicited to agglutinate merozoites before schizont rupture or blocking invasion of RBCs. Current clinical trials are under way looking at several blood stage candidates such as AMA-1, MSP-1, and RESA (Greenwood, Bojang et al. 2005; Maher 2008).

A final approach in vaccine development is to target the last stage in development, referred as the sexual phase. Vaccines, also known as transmission-blocking, targeted at this stage are important in reducing parasite transmission to others by preventing feeding mosquitoes from becoming infected

or sexual fusion of gametocytes in the midgut of the mosquito (Greenwood, Bojang et al. 2005; Saxena, Wu et al. 2007). This is an indirect way of providing protection but it helps in reducing disease transmission in the community (Greenwood, Fidock et al. 2008). Antibodies can be induced to kill gametocytes, interfere with fertilization of gametocytes, transformation of the zygote into ookinete, or egress of ookinetes into viable sporozoites. The approach of using a transmission-blocking vaccine is usually combined with other vaccines targeting other stages (Greenwood, Fidock et al. 2008). Current research on transmission-blocking vaccine candidates include *Pfs* 25/28, *Pfs* 48/45, and *Pfs* 230 (Greenwood, Bojang et al. 2005; Saxena, Wu et al. 2007) and could play a role in reducing transmission in the population.

#### Apical Membrane Antigen-1 (AMA-1)

AMA-1 is a leading asexual blood-stage vaccine candidate (Good, Kaslow et al. 1998) because it plays a crucial role in invasion of *Plasmodium* parasites. AMA-1 is a type I integral membrane protein (Remarque, Faber et al. 2008) and initially trafficked to micronemes as an 83 kDa precursor protein and proteolytically processed to *PfAMA-1<sub>66</sub>* before exportation to the merozoite surface. AMA-1 has been implicated as playing a function in reorienting with the merozoite as the apical organelles and RBC membrane align during invasion (Mitchell, Thomas et al. 2004). Animal and *in vitro* studies support the crucial role for AMA-1 during invasion of RBCs such as anti-AMA-1 antibodies inhibiting invasion *via* growth inhibition assays (Hodder, Crewther et al. 2001), antibody-mediated inhibition of antigen processing (Dutta, Haynes et al. 2003), anti-AMA-1

antibodies found in exposed individuals via sero-epidemiological surveys (Thomas, Trape et al. 1994), and AMA-1 has conferred protection in immunization studies (Narum, Ogun et al. 2000). A critical issue with the using AMA-1 as a vaccine candidate is it is highly polymorphic (Healer, Murphy et al. 2004) and this reduces susceptibility to the action of inhibitory antibodies. Even though AMA-1 exhibits high polymorphism the C-terminal region is highly conserved and can be blocked by inhibitory antibodies. AMA-1 is not only found in asexual blood stage merozoites but also expressed by sporozoites and liver stage merozoites (Remarque, Faber et al. 2008). Targeting AMA-1 as a vaccine candidate not only can reduce the risk of malaria infection causing clinical disease but also the possibilities of cellular immunity may be stimulated and reduction in exo-erythrocytic viability. The current literature definitely supports the idea of AMA-1 and its potential as a vaccine candidate.

#### Merozoite Surface Antigen-1 (MSP-1)

MSP-1 is another leading asexual blood stage vaccine candidate (Siddiqui, Tam et al. 1987) and is proposed to play a role in parasite invasion of RBCs (Blackman, Heidrich et al. 1990). MSP-1 is a 195-kDa glycoprotein (Mehrizi, Zakeri et al. 2008) found on the merozoite surface, which undergoes two proteolytic cleavages for entry into RBCs. The first cleavage occurs when the merozoite is released from an infected RBC resulting in four polypeptide fragments (83, 30, 38, and 42 kDa) and the second cleavage occurs during invasion of a RBC and involves the C-terminal 42 kDa fragment that is cleaved into 33 and 19 kDa polypeptides (Mehrizi, Zakeri et al. 2008). MSP-1<sub>19</sub> stays

anchored to the merozoite surface *via* a GPI tail when RBC invasion takes place (Chenet, Branch et al. 2008). The C-terminal portion of MSP-1<sub>19</sub> is a target of some monoclonal antibodies because they inhibit the growth of parasites *in vitro* (Uthaipibull, Aufiero et al. 2001) and has shown to provide protective immunity (O'Donnell, de Koning-Ward et al. 2001). Vaccines based on the C-terminal region of MSP-1 including MSP-1<sub>42</sub> and MSP-1<sub>19</sub> have provided protection after parasite challenge in Aotus monkeys (Kumar, Yadava et al. 1995; Chang, Case et al. 1996), antibodies have been shown to inhibit RBC invasion and parasite growth (Blackman, Heidrich et al. 1990; Chang, Case et al. 1996), and anti-MSP-1<sub>19</sub> has been correlated to clinical immunity with reduced parasite numbers and febrile illness (Branch, Udhayakumar et al. 1998). A limiting factor in asexual stage vaccine development is that the C-terminal fragments of MSP-1 parasites isolated in different geographical areas have displayed sequence variation (Mehrizi, Zakeri et al. 2008). Previous studies have provided the insight of using MSP-1 as a potential malaria vaccine candidate.

#### The Use of Plants for an Anti-Malarial Vaccine

There needs to be alternative approach in preparing an effective vaccine to enhance expression levels and potentially protection against malaria infection. Commonly used expression systems such as yeast, bacteria, mammalian cells, and baculovirus have expressed several malarial antigens but with the era of biotechnology other expression systems are being developed. Plants could be considered as an optimal expression system because they can reduce cost due to the expense of fermentation, purification, processing, cold storage,

transportation, and delivery (Ruhlman, Ahangari et al. 2007; Arlen, Singleton et al. 2008). The genetic manipulation of tobacco is highly achievable and yields large biomass and the success could be extended to other edible crops such as carrot, tomato, or lettuce. Oral delivery of plant-derived vaccines has been shown to induce both mucosal and systemic immunity (Nochi, Takagi et al. 2007; Verma and Daniell 2007). The genetic engineering of plants could establish a cost-effective approach in vaccine development to poor, developing countries where malaria infection is most severe.

#### Advantages of Chloroplast Genetic Engineering

Many crop species have been genetically modified to express human therapeutic proteins *via* the nuclear genome but expression levels of the foreign protein are inefficient to purify or oral deliver. Chloroplast genetic engineering has been a targeted approach to overcome the concerns of using nuclear transformation such as high level expression of foreign proteins due to thousands of genomes per cell (De Cosa, Moar et al. 2001; Daniell, Khan et al. 2002), gene containment (Daniell 2002; Daniell 2007), gene silencing and position effect (De Cosa, Moar et al. 2001), pleiotropic effects (Daniell, Lee et al. 2001), and multi-gene expression (De Cosa, Moar et al. 2001; Quesada-Vargas, Ruiz et al. 2005) in a single transformation event. Chloroplast transformation technology has contributed in the medical advancement such as expressing proteins in disease resistance (DeGray, Rajasekaran et al. 2001), biopharmaceuticals (Staub, Garcia et al. 2000; Fernandez-San Millan, Mingo-Castel et al. 2003; Arlen, Falconer et al. 2007; Ruhlman, Ahangari et al. 2007), and vaccines (Daniell, Streatfield et al.

2001; Kamarajugadda and Daniell 2006; Arlen, Singleton et al. 2008); and in agriculture such as herbicide (Daniell, Datta et al. 1998) and insect resistance (Kota, Daniell et al. 1999), and phytoremediation of toxic metals (Ruiz, Hussein et al. 2003; Hussein, Ruiz et al. 2007) in transgenic plants. Genetic engineering is achieved by stably integrating the flanking sequences of the foreign gene through homologous recombination with the intergenic regions of the chloroplast genome (Kumar and Daniell 2004). The use of plastid transformation and chloroplast genetic engineering has allowed the expression of foreign genes at a level that is optimal for the oral delivery of vaccines.

#### Vaccine Antigens Expressed via the Chloroplast Genome

Several vaccine antigens have been expressed using the approach of chloroplast genetic engineering (Kamarajugadda and Daniell 2006). The many advantages of expressing antigens in the chloroplast listed above supports the current rationale of producing transgenic lines expressing the vaccine antigen of interest. Numerous vaccine antigens expressed *via* the chloroplast are targeted against bacterial, viral, and protozoan pathogens such as the plague F1-V fusion antigen (Arlen, Singleton et al. 2008), *Entamoeba histolytica* (Chebolu and Daniell 2007) anthrax protective antigen of *Bacillus anthracis* (Watson, Koya et al. 2004; Koya, Moayeri et al. 2005), VP6 protein of rotavirus (Birch-Machin, Newell et al. 2004), 2L21 peptide from the virulent canine parvovirus (CPV) (Molina, Hervas-Stubbs et al. 2004), and CTB for cholera (Daniell, Lee et al. 2001). The expression levels of the PA of anthrax was up to 14.2% of TSP and subcutaneous immunization of mice with partially purified chloroplast-derived PA

resulted in 100% survival after challenge with lethal doses of anthrax toxin (Koya, Moayeri et al. 2005). The expression levels of the F1-V fusion antigen accumulated up to 14.8% of TSP and after challenge with aerosolized *Yersinia pestis* all control animals died in three days and 33% of animals receiving boosts of subcutaneous injections of F1-V and 88% of mice receiving oral boosts of F1-V were protected (Arlen, Singleton et al. 2008). This finding brings hope of utilizing the approach of chloroplast technology and oral-deliverable, cost-effective vaccines closer to reality.

#### Cholera Toxin B Subunit (CTB)

The Gram-negative bacteria *Vibrio cholerae* secretes an enterotoxin known as cholera toxin. CT is oligomer made up of six proteins AB<sub>5</sub> consisting of one toxic 27 kDa A subunit and five non-toxic B subunits each weighing 11.6 kDa (Daniell, Lee et al. 2001). This hexameric complex facilitates entry into the mucosal epithelia of the intestine via CTB and the GM<sub>1</sub> ganglioside receptors (Daniell, Lee et al. 2001). GM<sub>1</sub> gangliosides are found on the gut epithelial surface and it is known CTB to have a high affinity to these glycosphingolipids (Mor, Gomez-Lim et al. 1998). CTB is known when given orally to be a safe, potent, mucosal immunogen and adjuvant (Holmgren, Lycke et al. 1993). CTB has the potential to enhance the immune response when coupled to other pathogenic antigens (Daniell, Lee et al. 2001). A previous study used CTB-GFP plants and oral administered the transgenic leaf material to mice and observed CTB in the intestinal wall and GFP fluorescence in mouse intestinal mucosa, liver, and spleen (Limaye, Koya et al. 2006) This opens the possibility of oral

deliverable human therapeutic proteins via receptor-mediated oral delivery (Ruhlman, Ahangari et al. 2007).

#### Bioencapsulation for Oral Delivery

A critical issue arises with the oral delivery of vaccine antigens into the body. The antigen delivered needs to be intact and retain its biological activity and withstand the digestive enzymes present in the stomach. Bioencapsulation is the process of plant cells' ability to protect an oral-delivered protein from acid digestion (Walmsley and Arntzen 2000). Oral delivery of vaccine antigens needs to cross the mucosal barrier effectively to provide protection in the event of the immune system encountering the pathogen. Previous reports show that GFP fused with transmucosal carrier CTB was bioencapsulated because following oral administration of CTB-GFP leaf material both CTB and GFP was found in mouse intestinal cells (Limaye, Koya et al. 2006). GFP but not CTB was present in the liver of mice after oral delivery indicating it was protected from the digestive enzymes of the stomach. The role of receptor-mediated oral delivery and bioencapsulation provides insight in producing low-cost vaccines that deliver vaccine antigens effectively.

## RATIONALE AND APPROACH

The primary objective of this project is to express and characterize the malarial antigens CTB-AMA-1 and CTB-MSP-1 *via* the chloroplast genome and evaluate the immunogenicity of the chloroplast derived antigens. The novelty of this project is the approach of delivering the chloroplast-derived CTB-malarial antigens *via* oral delivery and comparing the immunogenicity to the typical route of administration of vaccines, through injection. To achieve these objectives, malarial gene products need to be cloned into pLD-UTR for expression in plants. Using particle bombardment, transgenic lines need to be obtained to express the chloroplast-derived CTB-malarial antigens. The chloroplast-derived proteins need to be quantified for enrichment for subcutaneous injection and delivery to rodents *via* oral gavage. The immunogenicity of the mouse titers needs to be determined *via* ELISAs for the comparison of subcutaneous injection versus oral delivery. The antibodies present in the immunized mouse sera will be tested with immunoblot and IFA analysis to determine recognition of native parasite protein and native parasite, respectively. If the antigens are immunogenic, they will be tested to determine their ability inhibit parasite invasion into RBCs.

## MATERIALS AND METHODS

### Amplification of AMA-1 and MSP-1 in Asexual Stages and Cloning

Based on the C-terminal regions of AMA-1 and MSP-1 (Pan, Huang et al. 2004), forward and reverse primers were designed to amplify the gene. The total RNA used for RT-PCR was isolated prior (stored at -80°C) by harvesting an asynchronous 3D7 *P. falciparum* parasite culture with 0.05% saponin lysis and isolating total RNA by using the RNaGents Total RNA Isolation System (Promega). The forward primers designed were (5'-CCCGGGGCCAAGAAAA-AGAAGAGCTTGTCCCATCCCAT-3') with a SmaI site and Furin Cleavage site (Arg-Lys-Arg-Arg) and (5'-CCCGGGGCCAATTTCACAACACCAATG-3') with a SmaI site for AMA-1 and MSP-1, respectively. The reverse primers designed were (5'-CGGC GGCCGCTTCATGTTATCATAAGTTG-3') and (5'-ATAAGAA-TGCGGCCGCTTAGTTAGAGGAAGTGCAGAAAA-TAC-3') with a NotI site for AMA-1 and MSP-1, respectively. The StrataScript one-tube RT-PCR system (Stratagene) with Easy-ATM High-Fidelity PCR Cloning Enzyme was used to reverse transcribe and amplify the genes using 200 ng of total RNA and the gene-specific primers. RT-PCR cycling conditions were as follows: 1 cycle of reverse transcription at 42°C for 30 minutes followed by transcriptase enzyme inactivation at 95°C for 30 seconds; 5 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 68°C for 6 minutes; 35 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 65°C for 6 minutes and 1 cycle at 65°C for 10 minutes. 5 µL of RT-PCR products were analyzed by electrophoresis on a 0.8% agarose gel and visualized by the Gel Doc 2000 (Bio

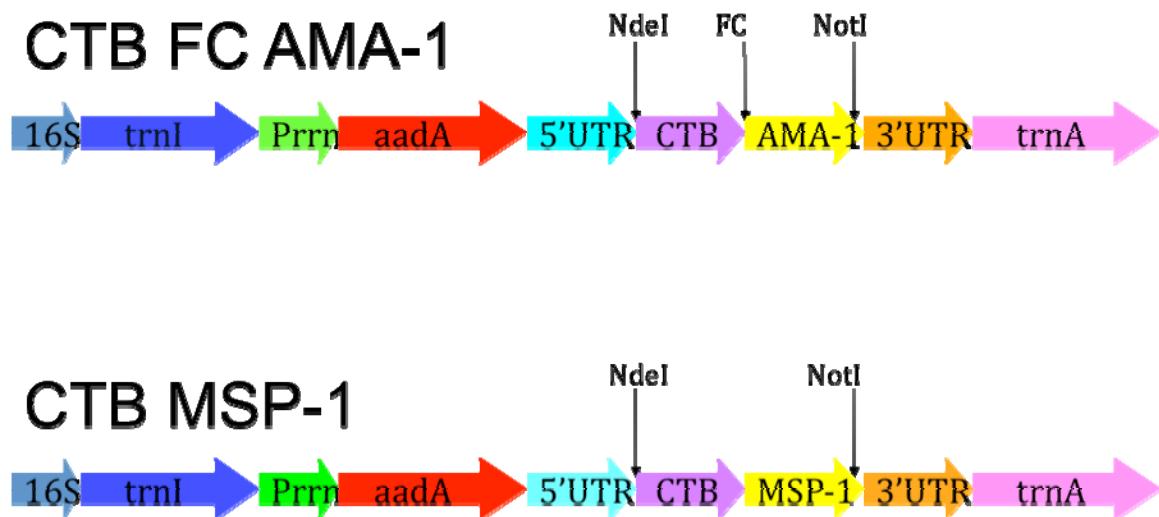
Rad). The remainder of the RT-PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and confirmed by a 0.8% agarose gel. The genes were cloned into the pGEMT Easy Vector (Promega) and the presence of the insert was confirmed by digestion with EcoRI and gel electrophoresis. The DNA was sent to the University of Florida: DNA Sequencing (ICBR: The Biotechnology Program) facility for sequencing. Accuracy of the insert sequence was confirmed by using the DNA STAR SeqMan program. The FC AMA-1 and MSP-1 genes were subcloned into the pBSK+ (Stratagene) vector by using the SmaI and NotI restriction sites.

#### Amplification and Cloning of CTB

Template DNA containing the CTB gene was provided by Dr. Daniell's lab. The CTB gene was amplified *via* PCR with a forward primer (5'-CCGCTCGAGCATATGACACCTCAAAATATTACT-3') with Xhol and NdeI sites and a reverse primer (5'-CCCGGGCCATTGCCATACTAATTGC-3') with a SmaI site. The CTB gene was cloned into the pGEMT Easy Vector (Promega) and the sequence was confirmed. CTB was subcloned into the pBSK+ vector using the Xhol and SmaI restriction sites. The pBSK+ CTB, pBSK+ FC AMA-1, and pBSK+ MSP-1 were digested with SmaI and NotI restriction enzymes and the FC AMA-1 and MSP-1 genes were ligated into the pBSK+ CTB plasmid to complete the fusion genes: CTB FC AMA-1 and CTB MSP-1.

### Chloroplast Plasmid Construction

The CTB FC AMA-1 and CTB MSP-1 transgenes in the pBSK+ vector were cut with NdeI and NotI restriction enzymes and ligated into the pLD-UTR vector with T4 ligase (New England BioLabs) and transformed into supercompetent *E. coli* XL-10 Gold cells. The figure below demonstrates the proposed orientation of the transgene into the chloroplast vector.



**Figure 1:** Chloroplast pLD-UTR CTB-Malarial Antigens (not drawn to scale)

Following transformation of chloroplast vector constructs into *E. coli*, colonies were selected and grown overnight in 5 mL of LB broth and 5 µL of ampicillin (100 mg/mL). The DNA was isolated by using the QIAprep Spin Miniprep Kit (Qiagen), digested with NdeI and NotI restriction enzymes, and analyzed by a 0.8% agarose gel. A positive clone containing the chloroplast vector construct was obtained from the glycerol stock and grown overnight. The chloroplast plasmid DNA was purified with the QIAprep Plasmid Maxi Kit (Qiagen) and the DNA was analyzed by gel electrophoresis on a 0.8% agarose gel.

## Generation of Transgenic Plants

### **Preparation of Gold Particles**

A mixture of 50 mg of gold particles and 1 mL of 100% ethanol was vortexed for two minutes and centrifuged at 10,000 x g for three minutes in a 1.5 mL eppendorf tube. The supernatant was discarded and the gold particles were resuspended in 1 mL of 70% ethanol for one minute. The suspension was left at room temperature for fifteen minutes with mixing intermittently. Centrifuging at 5,000 x g for two minutes pelleted the gold particles and the supernatant was discarded. The gold particles were vortexed with 1 mL of sterile distilled water and incubated at room temperature for one minute and centrifuged at 5,000 x g for two minutes. The steps to wash the gold particles with sterile water were repeated three times (Kumar and Daniell 2004). The gold particles were resuspended in 1 mL of sterile 50% glycerol and stored on ice until use.

50 µL of gold particles was removed from the stock and transferred into a 1.5 mL microcentrifuge tube along with 10 µg of plasmid DNA. To ensure proper binding of DNA to the gold particles 50 µL of 2.5 M CaCl<sub>2</sub> and 20 µL of 0.1 M spermidine-free base was added. The mixture was vortexed for twenty minutes at 4°C and the DNA-coated gold particles were centrifuged at 10,000 x g for one minute. The supernatant was removed and the pellet was washed four times in 200 µL of absolute alcohol. The DNA-coated gold particles were resuspended in 50 µL of 100% ethanol. 10 µL of vortexed DNA-coated gold particles were loaded onto sterile macrocarriers and allowed to air dry in the laminar air flow hood.

### **Bombardment of Leaf Tissue**

The bombardment was performed under sterile conditions with all equipment including the gene gun (Bio-Rad PDS-1000/He) sterilized with 95% ethanol. Green healthy leaves from the *in vitro* tobacco plant *Nicotiana tabacum* variety Petit Havana were cut from young plants and placed with the adaxial side facing up on autoclaved Whatman filter paper on the top of solidified RMOP medium. The macrocarrier with the DNA-gold coated particles were placed on the macrocarrier holders. The bombardment was performed at 1,100 psi and 28 Hg. After the bombardment, the transformed leaves were covered with aluminum foil and kept in the dark for 48 hours at room temperature (Kumar and Daniell 2004).

### **Regeneration and Selection of Transplastomic Shoots**

After 48 hours, the leaves were cut into 5x5 mm<sup>2</sup> pieces and transferred to RMOP media (one pack of MS basal salt mixture, 30 g of sucrose, 100 mg myoinositol, 1 mL of 1 mg/mL BAP, 100 µL of 1 mg/mL NAA, 1 mL of thiamine hydrochloride to 1 L of sterile distilled water and adjusted to pH 5.8 using 1N KOH; 6g of phytagar was added to media to solidification; autoclaved and cooled before pouring into plates) containing 500 µg/mL of spectinomycin with the bombarded side in contact with medium. The Petri dish was sealed with parafilm and kept in the culture room until putative transgenic shoots appear. Confirmed positive transgenic lines by PCR analysis were subjected to second round of selection to achieve homoplasmy. After four weeks on secondary selection, the shoots were transferred to MSO media (one packet of MS basal salt mixture and

30 g of sucrose to 1 L of sterile distilled water; prepared to pH of 5.8 using 1N KOH; 6 g of phytagar) with 500 µg/mL spectinomycin. This accounts for the third round of selection and rooting.

#### Isolation of Plant DNA and Confirmation of Transgene Integration

Before proceeding to the next round of selection, 100 mg of leaf material was harvested from putative transplastomic shoots. The QIAprep DNeasy Plant Mini Kit (Qiagen) was used to isolate plant genomic DNA, following the manufacturer's protocol. The procedure yields approximately 20-30 µg of DNA and the isolated DNA was used for PCR analysis. PCR was used to confirm transgene cassette integration into the chloroplast genome by the primer pair 3P (5'AAAACCCGT CCTCEGTT CGGATT GC-3') and 3M (5'CCGCGTT GTT CAT-CAAGCCTTACG-3') (Daniell, Ruiz et al. 2005). The integration of the gene of interest was confirmed by PCR using the primer pair 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-TGACTGCCACCTGAG-AGCGGACA-3') (Daniell, Ruiz et al. 2005). DNA isolated from wild type Petit Havana was used as the negative control and DNA isolated from known transgenic plant material was used as the positive control. For PCR analysis, 50 µL of reaction volume was prepared in a 0.2 mL PCR tube: 1µL of 100 ng/µL genomic DNA, 5 µL of 10X PCR reaction buffer, 4 µL of 2.5 mM dNTP, 1 µL of 3P and 3M primers (or 5P and 2M primers), 1 µL of *Taq* DNA polymerase, and sterile distilled water to make up the total volume. The initial denaturation was set at 94° for 5 minutes and amplification was carried out for thirty cycles of the following program: 94°C for 1 minute (denaturation), 60°C for one minute

(annealing), and 72°C for 2 minutes (extension). Final extension of ten minutes at 72°C was carried at the end of PCR. To examine the PCR product via agarose gel electrophoresis, 5 µL of was loaded, along with controls, into a 0.8% agarose gel and visualized by the gel doc.

### Southern Blot Analysis to Identify Homoplasmic Plants

#### **Isolation of Plant Genomic DNA**

Leaf material from transgenic and non-transgenic plants was removed with aseptic technique from the *in vitro* greenhouse and ground with liquid nitrogen into 100 mg of a fine powder. Previously made and stored at 65°C, 1 mL of DNA extraction buffer (Tris-HCl pH8, EDTA, NaCl, CTAB, BME up to 10 mL of water) was added to the leaf material and incubated at 65°C for thirty minutes with gentle mixing every five to eight minutes. 667 µL of (48:2) chloroform:isoamyl alcohol was added to the homogenate with gentle inverting for one minute followed by centrifugation at 10,000 rpm for ten minutes. The supernatant was collected and placed into new tubes and 667 µL of ice, cold isopropyl alcohol was added with gentle mixing to precipitate nucleic acid. A visible, dense clump was seen and centrifuged for ten minutes at 10,000 rpm. The nucleic acid was washed twice with 70% ethanol and allowed to air dry at room temperature followed by further drying with the DNA 110 speed vac (Savant Instruments, Inc.) for five minutes. The pellet was dissolved in 500 µL of 0.1X TE (1mM Tris-HCl (pH 8) + 0.1mM EDTA (pH 8)) containing 0.1 µg/µL RNase and incubated at 37°C for thirty minutes. 500 µL of (24:25:1) of

chloroform:phenol:isoamyl alcohol was added and mixed thoroughly to visualize three layers and centrifuged for fifteen minutes at 12,000 rpm. The upper layer containing the DNA was removed and placed into new tubes and the same volume of chloroform was added to remove phenol. The tube was mixed thoroughly and centrifuged for fifteen minutes at 14,000 rpm. Two layers were visualized and the top layer containing DNA was transferred to new tubes. 1 mL of 100% chilled ethanol and 33  $\mu$ L (1/10<sup>th</sup> volume of DNA) of 3M Na Acetate, pH 5.2 was added to allow precipitation of DNA. The reaction was kept at -20°C for one hour and centrifuged for ten minutes at 14,000 rpm. The supernatant was decanted very slowly with a visible white pellet remaining. The pellet was washed with 70% ethanol and centrifuged for ten minutes at 14,000 rpm. The pellet was dried with a DNA 110 speed vac (Savant Instruments, Inc.) for five minutes to remove remaining ethanol and dissolved in 100 or 200  $\mu$ L 0.1X TE (1mM Tris-HCl (pH 8) + 0.1mM EDTA (pH 8)), depending on the pellet size. The genomic samples were loaded on a 0.8% agarose gel to visualize the bands. The concentration of the DNA was determined by a spectrophotometer.

### **Restriction Digestion of Genomic DNA**

The transgenic and untransformed samples containing equal amounts of DNA were digested with Apal in a reaction containing: 1.5  $\mu$ g of DNA, 4  $\mu$ L of 10X BSA, 4  $\mu$ L of 10X Buffer 4 (New England Biolabs), 2  $\mu$ L of Apal (New England Biolabs), and sterile distilled H<sub>2</sub>O to make up the volume of 40  $\mu$ L. The reaction was incubated overnight at 37°C.

## **Agarose Electrophoresis and DNA Transfer**

Each of the 40  $\mu\text{L}$  reaction volume samples was loaded on a 0.8% agarose gel and electrophoresis was run for 3 hours at 100 volts. The gel was visualized with a fluorescent ruler. The gel was depurinated with 0.3N HCl for ten minutes (until the color of the dye became yellow) and removed. This was followed by immersing the gel into Hybridization Denaturing Solution (5 PRIME – 3PRIME) for thirty minutes followed by a wash with sterile  $\text{H}_2\text{O}$ . The gel was immersed with Hybridization Neutralization Solution (5 PRIME – 3 PRIME) for thirty minutes followed by a wash in transfer buffer (2X SSC, Eppendorf) for 5 minutes. The membrane (S & S Nytran: SuperCharge, Schleicher & Schuell) was immersed in sterile  $\text{H}_2\text{O}$  for fifteen minutes and left in transfer buffer until use. The transfer of DNA from the agarose gel to membrane utilized the Turboblotter (Rapid Downward Transfer System/Buffer Tray, Schleicher & Schuell) protocol. Twenty sheets of dry GB004 paper, followed by four sheets of dry GB002, and one sheet of prewet, in transfer buffer, GB002 was placed in the stack tray. The prewet transfer membrane was placed on the stack followed by the agarose gel on top. A rolling pin was used to remove any air bubbles that were present. The top of the gel was wet with the application of transfer buffer and three sheets of prewet GB002 paper was placed on top. The buffer tray was attached to the stack tray and filled with 125 mL of transfer buffer. The wick was placed on top of the stack so the ends would drape into the buffer tray. The wick cover was placed on top to prevent evaporation and the transfer continued overnight at room temperature. The following day, the membrane was marked with a pencil

and rinsed with transfer buffer for five minutes. The membrane was placed on chromatography paper and allowed to air dry. The membrane was cross-linked using the UV Stratalinker 2400 (Stratagene) at the setting autocrosslink and stored at a dry place until further use.

### **Generation of Probes**

The following PCR reaction was set up for the amplification of the 2P2M flanking sequence probe with the 2P primer (5'-GCGCCTGACCCTGAGATGTG-GATCAT-3') and 2M primer (5'-TGACTGCCAACCTGAGAGCGGACA -3'): 1  $\mu$ L of DNA, 5  $\mu$ L of 10X Buffer, 2.5  $\mu$ L of MgCl<sub>2</sub>, 1  $\mu$ L of dNTP, 1  $\mu$ L of 2P primer, 1  $\mu$ L of 2M primer, 1  $\mu$ L *Taq* polymerase, and 37.5  $\mu$ L of sterile H<sub>2</sub>O. The initial denaturation was set at 94° for 5 minutes and amplification was carried out for twenty-nine cycles of the following program: 94°C for 30 seconds (denaturation), 61°C for 30 seconds (annealing), and 72°C for 1 minute and 30 seconds (extension). Final extension of seven minutes at 72°C was carried at the end of PCR. The 50  $\mu$ L PCR reaction was loaded into a 0.8% agarose gel and visualized with the Bio Rad Gel Doc 2000. The bands were excised and gel extracted with the QIAquick Gel Extraction Kit (Qiagen) by following the manufacturer's protocol and eluting with 35  $\mu$ L of Buffer EB.

### **Prehybridization of Membrane**

Into the hybridization bottle, the membrane was placed with the side exposed to DNA facing inwards and 20 mL of prehybridization solution (36.5 mL of sterile water, 10 mL of 20X SSC, 2.5 mL of 100X Denhardt's (6% Ficoll, 6%

polyvinylpyrrolidone, 6% BSA), 500 µL of 10% NaPPi, and 500 µL of 10% SDS) was added and incubated at 65°C with the Hybridiser HB-1D (Techne). Sonicated salmon sperm DNA (10 mg/mL) (Stratagene) was boiled for minutes and 500 µL was added into the hybridization bottle. The pre hybridization of the membrane incubated at 65°C for four hours.

### **Probe Labeling and Purification**

The PRIME-It Random Primer Labeling Kit (Stratagene) protocol was followed to label the radioactive probe. In a clean microcentrifuge tube: 20 ng of the flanking probe, 10 µL of random oligonucleotide primers, and up to 23 µL of sterile H<sub>2</sub>O was added and boiled for five minutes. The mixture was briefly centrifuged at room temperature. The following was added in order: 10 µL of 5X ATP buffer, 5 µL of Redivue (Alpha-32P) DATP, and 1 µL of Exo (-) Klenow (5U/µL) and mixed by stirring. The mixture was heated for fifteen minutes at 37°C followed by adding 2 µL of stop mix. A NucTrap probe purification column (Stratagene) was placed in a push column device and a clean microcentrifuge tube containing 100 µL of salmon sperm DNA was placed below. 80 µL of 1X STE was injected into the column with a syringe and collected into the microcentrifuge tube. The radiolabeled probe was added to the column and pushed through with a syringe followed by 80 µL of 1X STE. The syringe was applied to the column once more and 300 µL of 1X STE was added to the purified probe.

### **Hybridization and Washing of Membrane**

The purified probe was boiled for five minutes and added to the hybridization bottle with a sterile transfer pipette and allowed to hybridize with the membrane overnight at 65°C. The following day, the membrane was washed twice, five minutes each with wash buffer #1 (2X SSC, 0.1% SDS, and 0.1% NaPPi) followed by washing with wash buffer #2 (0.2X SSC, 0.1% SDS, and 0.1% NaPPi) four times, fifteen minutes each. The wash buffer was discarded into the P32 waste and the membrane was allowed to air dry behind the plastic shield. The radioactive membrane was wrapped with saran wrap and a ladder (Stratagene) was placed on front.

### **Autoradiography**

The film cassette along with the hybridized blot was taken to the dark room and under safe, red light the blot was placed faced down onto the intensifier screen and the BIOMAX MS Film: Maximum Sensitivity (Kodak) was placed in between the screen. The cassette with the hybridized blot and the film was incubated at -80°C. The next day the cassette was taken out of the -80°C freezer to thaw and the film was developed with the X-ray film processor.

### Characterization of Expressed Chloroplast-Derived Proteins

### **Extraction of Protein from Transformed Tobacco Leaves**

Mature leaf material was harvested around four to five o'clock in the early evening hours. Leaves were washed in the lab and allowed to air dry and stored at -80°C until further use. Chloroplast-derived CTB-malarial proteins from

transgenic lines was extracted by grinding 100 mg of plant tissue with a mortar and pestle in liquid nitrogen and fine powdered leaf material was placed in a 1.5 mL microcentrifuge tube with a hole poked through the top. The microcentrifuge tube was immediately placed in liquid nitrogen until further use. 200  $\mu$ L of plant extraction buffer (100mM NaCl, 10mM EDTA, 200mM Tris-HCl pH8, 0.05% Tween 20, 0.1% SDS, 14mM BME, 200mM sucrose, 3.18 mL of sterile H<sub>2</sub>O, and 1 tablet of Roche complete mini EDTA-free protease inhibitor cocktail) was added to the leaf material. The samples were placed on ice and mixed for two minutes using a mechanical pestle and centrifuged at 14,000 rpm for fifteen minutes at 4°C to obtain the supernatant (soluble fraction). The pellet (insoluble fraction) was resuspended with equal volume of protein extraction buffer and sonicated for thirty seconds. The supernatant and pellet was subjected to Bradford analysis to determine total protein concentration and stored at -20°C until further use.

### **SDS-Page and Immunoblot Analysis**

Clean Bio-Rad glass plates and casting chamber was set up for SDS-Page analysis. 12.5% separating gel (4.15 mL of 30% Acrylamide/Bis solution (BioRad), 2.5 mL of 4X Separating Buffer: 5M Tris-HCl, pH 8.8, 3.2 mL of H<sub>2</sub>O, 0.1 mL of 10% SDS, 0.1 mL of 10% APS, and 10  $\mu$ L of TEMED) was mixed in a 50 mL beaker and by using a syringe it was added in between the two glass plates leaving about 1.5 cm empty for the stacking gel. Immediately water was added to the top of the separating gel and allowed to polymerize for thirty minutes. The water was removed with a Kim wiper and 4% stacking gel (665  $\mu$ L

of 30% Bio Rad Acrylamide/Bis solution, 1.25 mL of 4X Stacking Buffer: 0.5M Tris-HCl, pH 6.8, 3.0 mL of H<sub>2</sub>O, 50 µL of 10% SDS, 50 µL of 10% APS, and 5 µL of TEMED) was prepared. The 4% stacking gel mixture was layered on top of the resolving gel and a comb was inserted for the formation of wells. After polymerization for thirty minutes, the gel was put vertically into the PAGE apparatus with 1X Protein Buffer (10X Protein Buffer: 0.25M Tris Base, 1.92M Glycine, and 1% SDS). The protein samples (wild type plants, transgenic plants, monomeric CTB (Sigma C9903), and *E.coli*-derived CTB MSP-1) were prepared by the following: 12 µL of protein extract and 12 µL of 2X gel loading buffer (2.5 mL of 4X Stacking Buffer, 4 mL of 10% SDS, 2 mL of Glycerol, 40 µL of 5% Bromophenol Blue, 0.31 g of DTT in a total of 10 mL of distilled H<sub>2</sub>O). The protein samples were boiled for 5 minutes and loaded into the wells along with 7 µL of Precision Plus Protein Standard (BioRad). The gel was run at 85 Volts until the protein samples entered into the Separating Buffer and the voltage was increased to 150 Volts until the dye front reached the bottom of the gel. The proteins from the SDS-PAGE were transferred overnight at 4°C to a HyBond nitrocellulose membrane via the Bio Rad Transfer Cassette using Transfer Buffer (200 mL of methanol, 100 mL of 10X Protein Buffer, and 700 mL of H<sub>2</sub>O) and 20 Volts.

For immunoblot analysis, the membrane was washed three times for five minutes each with PBS-T and blocked for one hour in 5% PTM at room temperature. Primary antibody, Anti-Cholera Toxin produced in rabbit (Sigma), was diluted 1:4,000 in 5% PTM and incubated at room temperature for two

hours. The membrane was washed three times for ten minutes each with 5% PTM. Secondary antibody, Stabilized Goat Anti-Rabbit Horseradish Peroxidase-Conjugated (PIERCE), was diluted 1:5,000 in 5% PTM and incubated at room temperature for one hour. Blots were washed with PBS-T two times for ten minutes each and a final wash of PBS for ten minutes. The membrane was incubated for five minutes in the dark using SuperSignal West Femto Maximum Sensitivity Substrate (PIERCE). The membrane was exposed to Classic Blue Autoradiography Film (MIDSCI) in the dark room and the films were developed via the film processor to visualize the bands.

#### Protein Quantification of Expressed Proteins

ELISA was performed to quantify CTB-FC-AMA1 and CTB-MSP-1 in plant crude extract versus using densitometric analysis because preliminary ELISA results provided a dilution effect in concentration of protein with increasing dilutions of the plant extract. Transgenic leaf samples of mature stages along with wild type were quantified. Total soluble protein was extracted using the protocol from the section, extraction of protein from transformed tobacco leaves (p.33-34). CTB (Sigma C9903) was used as the standard and diluted in coating buffer (1.59 g of Na<sub>2</sub>CO<sub>3</sub>, 2.93 g of NaHCO<sub>3</sub>, and 0.2 g of NaN<sub>3</sub> in 1 L of water; adjusted to pH 9.6 using HCl) ranging from 500-25 pg. Total soluble protein extracted from wild type non-transgenic plants, CTB-FC-AMA-1 plants, and CTB-MSP-1 plants was diluted 1:10, from 1:50,000 – 1:200,000, and from 1:25,000 – 1:150,000 in coating buffer, respectively. A 96 well plate (CoStar EIA/RIA plate, flat bottom without lid ELISA plate) was coated with 100 µL of CTB standards

and test samples and incubated overnight at 4°C. The next day, the plate was washed three times with PBS-T and three times with water. The plate was blocked with 300 µL of 3% PTM and incubated for one hour at 37°C. The plate was washed and 100 µL of primary antibody, Anti-Cholera Toxin produced in rabbit (Sigma), was diluted 1:4,000 in 3% PTM and incubated for one hour at 37°C. Following primary antibody, the plate was washed and secondary antibody, Horseradish Peroxidase-Conjugated Donkey Anti-Rabbit (BioMedia), was diluted 1:12,500 in 3% PTM and incubated for one hour at 37°C. The plate was washed and 100 µL of the substrate, TMB (American Qualex Antibodies), was added and incubated at room temperature for 5 minutes. The reaction was stopped with 50 µL of 2N sulfuric acid and the plates were read at 450nm with the BioRad microplate reader, Model 680. A Bradford assay using the Bradford reagent (Bio-Rad Protein Assay), BSA standards ranging from 0-8 µg/µL, absorbance of 595 nm, and the SmartSpecPlus Spectrophotometer (BioRad) was used to determine total soluble protein extracted from the wild type and transgenic plants.

To determine the quantity of chloroplast-derived CTB-FC-AMA1 and CTB-MSP1 the following equation was used. The concentration (pg) derived from the ELISA multiplied by dilution factor multiplied by 100,000 resulted in concentration of transgenic protein in µg. The concentration of transgenic protein was then divided by the volume of sample (100 µL) placed in the well of the ELISA plate. The number derived after dividing by the volume plated then was divided by the concentration of total soluble protein (provided by Bradford Analysis) and

multiplied by 100. The calculated percentage provides an estimate of the chloroplast-derived protein accumulation among all proteins expressed by the plant.

### Enrichment of Chloroplast-Derived Proteins

#### **Immobilized Metal Affinity Chromatography**

Chloroplast-derived CTB-malarial proteins from transgenic lines was extracted by grinding 10 g of plant tissue with a mortar and pestle in liquid nitrogen and fine powdered leaf material was placed in a 50 mL tube with a hole poked through the top and immediately placed in liquid nitrogen until further use. 20 mL of plant extraction buffer (100mM NaCl, 200mM Tris-HCl pH8, 0.05% Tween 20, 0.1% SDS, 200mM sucrose, 12 mL of sterile H<sub>2</sub>O, and 1 tablet of Roche complete mini EDTA-free protease inhibitor cocktail) was added to the leaf material. The samples were placed on ice and homogenized for five minutes with an OMNI International (GLH-2596) probe and centrifuged at 14,000 rpm for fifteen minutes at 4°C to obtain the supernatant (soluble fraction).

The supernatant (lysate) was subjected to TALON Superflow Metal Affinity Resin (Clontech) to enrich the chloroplast-derived CTB-malarial proteins. The manufacturer's protocol, BATCH/Gravity-Flow Column Purification, was followed exactly. The TALON Resin was resuspended thoroughly and 4 mL was placed in a sterile 50 mL tube and centrifuged at 700 x g for two minutes to pellet the resin. The pellet was pre-equilibrated twice with ten bed volumes of 1X Wash Buffer (2.5 mL of 4X Wash Buffer: 0.12M dibasic Na<sub>2</sub>HPO<sub>4</sub>, 0.08M monobasic

NaH<sub>2</sub>PO<sub>4</sub>, 1.2M NaCl, 4% Tween-20, made up to 100 mL of sterile H<sub>2</sub>O, pH8; 20mM Imidazole, sterile H<sub>2</sub>O was added to make up the volume to 10 mL, and 1 tablet of Roche complete mini EDTA-free protease inhibitor cocktail). The plant extract was added to the resin and agitated at 4°C for two hours. The mixture was centrifuged at 700 x g for five minutes and the supernatant (flow through) was removed carefully without disturbing the resin. The mixture was washed twice with ten bed volumes of 1X Wash Buffer and the supernatant was discarded. 2 mL of 1X Wash Buffer was added to the resin and transferred to a 2 mL gravity-flow column with an end-cap in place. The end cap was removed and the buffer was allowed to drain. The column was washed once with five bed volumes of 1X Wash Buffer. To elute the chloroplast-derived CTB-malarial proteins, five bed volumes of Elution Buffer (2.5 mL of 4X Wash Buffer, 20 mM Imidazole, 100 mM EDTA, volume made up to 10 mL of sterile H<sub>2</sub>O, and 1 tablet of Roche complete mini EDTA-free protease inhibitor cocktail, pH8) was added and the elute fraction was collected. The eluted fraction collected along with wild type material, lysate, flow through, and wash was analyzed with a Bradford and the Bio-Rad RC-DC Protein Assay to determine protein concentration. The eluted fractions were dialyzed with 1X sterile PBS and the Slide-A-Lyzer Dialysis Cassette 10,000 MW (PIERCE).

#### **Analysis of Chloroplast-Derived CTB-Malarial Protein Enrichment**

The eluted, wild type material, lysate, flow through, and wash fractions were subjected to a gradient gel and immunoblot to determine the efficiency of enrichment. 7 µg of the CTB FC AMA-1 fractions under reduced and non-

reduced conditions were heated at 70°C for ten minutes and loaded into a NuPAGE Novex Bis Tris Gel (Invitrogen) and electrophoresed at 200 Volts until the dye front reached the bottom of the gel. The gel was rinsed in water and stained overnight with the GelCode Blue Stain Reagent (PIERCE). 5 µg of the CTB FC AMA-1 and CTB MSP-1 fractions (eluted, wild type, lysate, flow through, wash) were electrophoresed and analyzed by an immunoblot. The primary antibody, Anti-Cholera Toxin produced in rabbit (Sigma), diluted 1:4,000 in 5% PTM, was incubated at room temperature for two hours. Secondary antibody, Stabilized Goat Anti-Rabbit Horseradish Peroxidase-Conjugated (PIERCE), diluted 1:5,000 in 5% PTM, was incubated at room temperature for one hour. Following incubation with substrate, membranes were exposed to X-ray film and developed via the film processor to visualize the bands.

### **Densitometry**

An immunoblot of the eluted chloroplast-derived CTB-malarial proteins and known quantities of CTB protein was analyzed by using spot densitometric analysis. 1000, 500, 250, 125 ng of CTB protein (Sigma, C9903) and 1.5, 0.75, 0.375, 0.1875 µg of eluted CTB FC AMA-1 and 1.5, 0.75, 0.375 µg of eluted CTB MSP-1 was electrophoresed and analyzed by an immunoblot. The primary antibody added was rabbit anti-CTB and the secondary antibody was goat anti-rabbit. Following exposure to film, the blots with known CTB concentrations and eluted fractions were analyzed by using the AlphaImager and AlphaEase FC Software (Alpha Innotech). The concentration of the enriched fraction the program calculated was divided by the known concentration of the enriched

fraction loaded and multiplied by one hundred to determine efficiency of the talon enrichment.

#### Adsorption of Enriched Protein to Adjuvant

Chloroplast-enriched proteins (~2.5 mg) derived from transgenic tobacco crude extract was mixed with 1:4 diluted Alhydrogel in PBS (Aluminum Hydroxide Gel, Sigma) and incubated overnight with gentle rocking at 4°C. The samples were centrifuged at 2,000 x g for five minutes at 4°C. The RC-DC Protein Assay (BioRad) was used to determine the adsorption efficiency by comparing the total amount of protein added to the adjuvant and the protein remaining in the supernatant after binding to adjuvant. The protein-adsorbed pellet was resuspended in sterile PBS to a final concentration of 1 µg/µL.

#### Immunizations

For subcutaneous injections 100 µL of Alhydrogel or chloroplast-derived protein adsorbed to Alhydrogel was injected into the scruff of the neck using a tuberculin syringe fitted with a 27-G needle. The leaf material for the doses for oral delivery was previously ground in liquid nitrogen with mortars and pestles and stored at -80°C until the day of immunization. Oral doses (500 mg each) of either wild type or transgenic leaf material was resuspended in 200 µL of sterile PBS and homogenized on ice for five minutes with an OMNI International (GLH-2596) probe. The plant cell suspension was stored on ice until oral delivery. 200 µL of the plant cell suspension was delivered via oral gavage by using a tuberculin syringe and a 20-G bulb-tipped gastric gavage needle.

Ninety female BALB/c mice were purchased at 7 weeks from Charles River Laboratories and were immunized by the following schedule listed below.

**Table 1:** Schedule of Immunization of Mice

Group	Immunization Type	Immunization Sample	Route of Administration	Schedule	Amount of Material or Antigen Delivered Per Dose
1	Boost	WT PH	Oral	Days 10, 17, 24, 31, 37, 45, 52, 59, 150, 157, 189	0 µg
2	Boost	AIH	s.c.	Days 13, 27, 43, 55, 150, 189	0 µg
3	Prime	enCTB FC AMA-1	s.c.	Day 0	25 µg
	Boost	enCTB FC AMA-1	s.c.	Days 13, 27, 43, 55, 150, 189	25 µg
4	Prime	enCTB FC AMA-1	s.c.	Day 0	25 µg
	Boost	CTB FC AMA-1	Oral	Days 10, 17, 24, 31, 37, 45, 52, 59, 150, 157, 189, 220	500 mg***
5	Prime	enCTB MSP-1	s.c.	Day 0	25 µg
	Boost	enCTB MSP-1	s.c.	Days 13, 27, 43, 55, 150, 189	25 µg
6	Prime	enCTB MSP-1	s.c.	Day 0	25 µg
	Boost	CTB MSP-1	Oral	Days 10, 17, 24, 31, 37, 45, 52, 59, 150, 157, 189	500 mg***
7	Prime	enCTB FC AMA-1 enCTB MSP-1	s.c.	Day 0	25 µg
	Boost	enCTB FC AMA-1 enCTB MSP-1	s.c.	Days 13, 27, 43, 55, 150, 189	25 µg
8	Prime	enCTB FC AMA-1 enCTB MSP-1	s.c.	Day 0	25 µg
	Boost	CTB FC AMA-1 CTB MSP-1	Oral	Days 10, 17, 24, 31, 37, 45, 52, 59, 150, 157, 189	500 mg***
9	Nothing	Nothing	Nothing	Nothing	0 µg
<b>10 Mice Per Group: 90 mice Total</b>					
WT PH: Non-transgenic tobacco plants					
AIH: Alhydrogel (Adjuvant)					
Oral: Oral gavage					
s.c: Subcutaneous injection					
enCTB FC AMA-1: Enriched CTB FC AMA-1					
CTB FC AMA-1: Transgenic tobacco plants expressing CTB FC AMA-1					
enCTB MSP-1: Enriched CTB MSP-1					
CTB MSP-1: Transgenic tobacco plants expressing CTB MSP-1					
***ORAL DELIVERY: Leaf material not antigen					

## Determination of Antibody Titers from Serum Samples

### **Blood Samples Collection**

Blood samples were obtained on days 21, 35, 63, 163, and 197-post immunization. The mouse was restrained and blood was collected by inserting a golden rod animal lancet 4mm, 5mm, or 5.5mm, depending on the age and size of the mouse, in the submandibular vein. The blood was collected in Microtainer serum separation tubes (Becton-Dickinson) and allowed to clot for a minimum of 30 minutes at room temperature. The blood samples were centrifuged at 15,000 rpm for 5 minutes and the serum was transferred to new microcentrifuge tubes and stored at -80°C.

### **ELISA to Determine Antibody Titers**

96 well plates (CoStar EIA/RIA plate, flat bottom without lid ELISA plate) were coated with 100 ng of CTB (Sigma C9903) or 50 ng of MRA-49 *PfMSP1<sub>19</sub>* protein in coating buffer (1.59 g of Na<sub>2</sub>CO<sub>3</sub>, 2.93 g of NaHCO<sub>3</sub>, and 0.2 g of NaN<sub>3</sub> in 1 L of water; adjusted to pH 9.6 using HCl) per well. The plates were incubated overnight at 4°C. The following day, the plates were washed three times with PBS-T and water and blocked with PBS containing 0.1% Tween and 3% skim milk powder for one hour at 37°C. The following dilutions were prepared in PTM to determine CTB titers for all control and experimental mice: bleed #1 ranging from 1:1,000 to 1:50,000; bleed #2 ranging from 1:25,000 to 1:150,000; bleed #3 ranging from 1:50,000 to 1:400,000; and bleeds #4 and #5 ranging from 1:100,000 to 1:800,000. For quantification of MSP-1 titers, the

serum samples from groups 5, 6, and 9 (unimmunized) were diluted in PTM with the following dilutions: bleed #1 and #2 ranging from 1:100 to 1:1000; bleed #3 from 1:250 to 1:25,000; bleed #4 from 1:500 to 1:25,000; and bleed #5 from 1:1000 to 1:50,000. The plates were washed and incubated with 100 µL of diluted serum samples (in duplicate) and incubated for one hour at 37°C. The plates were washed and incubated with 100 µL of 1:5,000 diluted goat anti mouse IgG1 (American Qualex) conjugated with horseradish peroxidase enzyme in PTM for one hour at 37°C. The plates were washed and 100 µL of TMB substrate (American Qualex Antibodies) was added and incubated at 37°C for thirty minutes. The reaction was stopped with 50 µL of 2N sulfuric acid and the plates were read at 450nm with the plate reader (BioRad microplate reader, Model 680). The titer values were determined by using the O.D. (optical density) of the negative control (unimmunized mice, group 9) + 0.1.

#### Immunoblot for Detection of Anti-Malarial Antibodies from Serum Samples

The immunoblot protocol followed was mentioned above in the characterization of chloroplast-derived proteins (p.34-35). The protein samples loaded into the SDS-PAGE gel were isolated prior and stored in the -80° freezer. 36.8 µg of ring, trophozoite, and schizont stage proteins were electrophoresed and analyzed by an immunoblot. The primary antibody, sera collected from an immunized mouse from groups 3 and 5, used was diluted 1:100 in 5% PTM. Secondary antibody, Stabilized Goat Anti-Mouse Horseradish Peroxidase-Conjugated (PIERCE), was diluted 1:5,000 in 5% PTM. Following incubation

with substrate, membranes were exposed to X-ray film and developed via the film processor to visualize the bands.

#### IFA for Detection of Anti-Malarial Antibodies from Serum Samples

A revised protocol by Tonkin et al. was followed for the preparation and fixation of cells (Tonkin, van Dooren et al. 2004) for IFA analysis. Cells were washed once with PBS and fixed in 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min at room temperature. Following fixation, cells were washed once with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for ten minutes and to reduce free aldehydes cells were treated with 0.1 mg/mL NaBH<sub>4</sub>/PBS for ten minutes at room temperature. Cells were washed once with PBS and finally blocked with 3% BSA/PBS for one hour at room temperature. Cells were probed with antibodies, (sera obtained from a immunized mouse in group 3 and 5) diluted 1:500 in 3% BSA/PBS for two hours at room temperature followed by three washes with PBS. The secondary antibodies were Alexa Fluor 555 goat anti-mouse diluted 1:1000 in 3% PBS containing BSA and inverted in the dark for one hour at room temperature. Cells were washed three times with PBS and allowed to settle on previously coated coverslips with 1% PEI for thirty minutes at room temperature. The mounting solution, 50% glycerol with 0.1 mg/mL DABCO (Sigma) was added to the coverslips and then inverted on microscope slides. Fluorescence images were observed and captured by using an LSM 510 confocal laser scanning microscope (Carl Zeiss).

### In Vitro Parasite Inhibition Assay

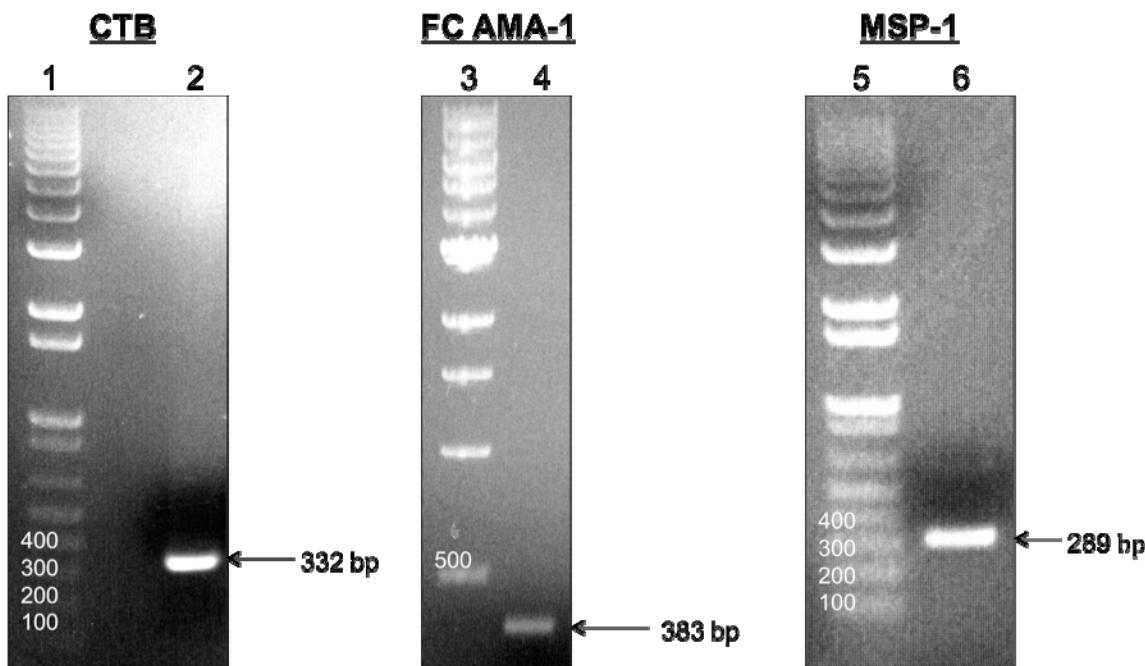
The 3D7 *P. falciparum* culture was synchronized with ring stage parasites and sorbitol lysis. The parasite completed one cycle and was allowed to mature to the trophozoite-schizont stage. The hematocrit and parasitemia were adjusted to 2% (2.5% parasitemia for the MRA-35 *PfMSP1<sub>19</sub>* *in vitro* parasite inhibition assay). Mouse sera and MRA-35 *PfMSP1<sub>19</sub>* (positive control) were heat inactivated at 56°C for 30 minutes and absorbed with human RBCs overnight at 4°C (Sachdeva, Mohmmmed et al. 2006). The mouse serum was added to the parasite culture in 96 well plates at a final concentration of 20% (for the MRA-35 *PfMSP1<sub>19</sub>* *in vitro* parasite inhibition assay 5 µL of antibody was added and diluted 1:5 – 1:625 to 25 µL of parasite culture). To serve as a negative control, no serum was added to wells and replaced with culture media. The cultures were incubated for 48 hours to allow for schizont rupture and merozoite invasion. These assays were preformed in duplicate. For microscopic analysis using the 100X oil immersion lens, blood smears were made and stained with Giemsa and the numbers of parasites per 900-1,100 RBCs were determined for each well. Parasitemia was measured using the following formula (infected RBCs / infected+uninfected RBCs) x 100. Percent of inhibition was determined by the following formula (% parasitemia of no sera added - % parasitemia of experimental mouse sera / % parasitemia of no sera added) x 100. Relative percent of inhibition was determined by the following formula (% of inhibition from experimental mouse sera / % inhibition of MRA-35 *PfMSP1<sub>19</sub>* (positive control)) x 100 and the percent of inhibition for the positive control was set at 100%.

Pictures were taken from the slides to show the extent of parasitemia and inhibition under the light microscope.

## RESULTS

### Amplification of AMA-1 and MSP-1 in Asexual Stages and CTB

To verify AMA1 and MSP-1 expression in 3D7 *P. falciparum* parasites, transcripts were detected by RT-PCR by using total RNA from mixed stages of parasites and gene-specific primers (**Figure 2**). By using gene-specific primers, CTB template DNA, and PCR; CTB was amplified with the expected size as shown in **Figure 2**.

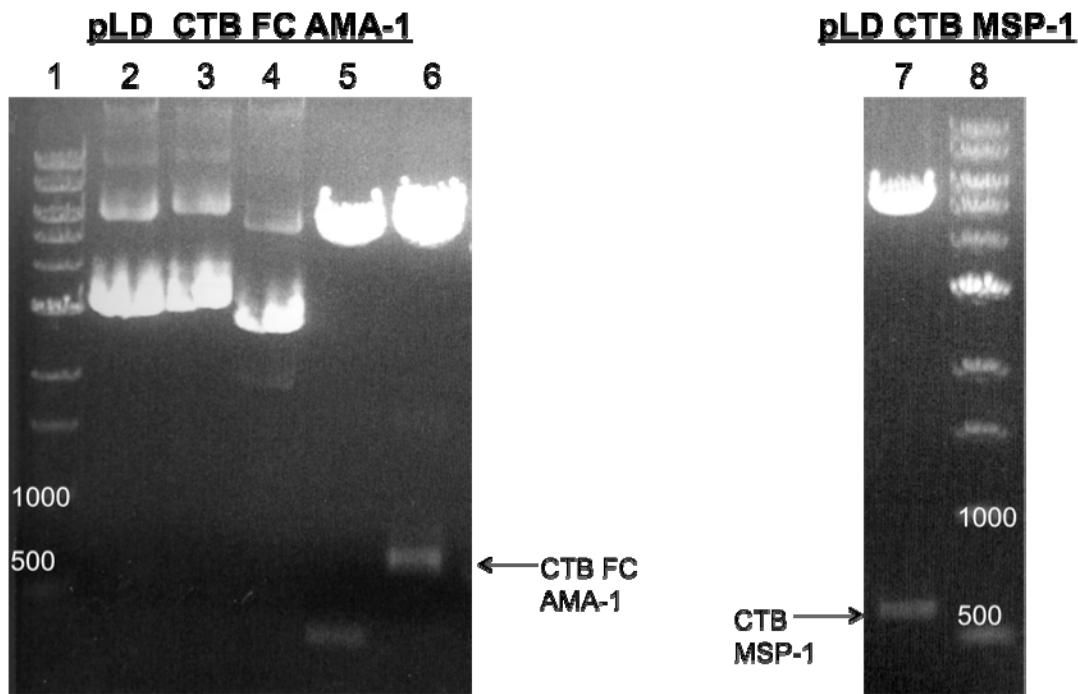


**Figure 2:** PCR and RT-PCR Analysis of CTB, FC AMA-1, and MSP-1

DNA was amplified using a single tube PCR and RT-PCR approach and gene specific primers as discussed under “Materials and Methods” resulting in Lane 2: (CTB, 332 bp), Lane 4: (FC AMA-1, 383 bp), and Lane 6: (MSP-1, 289 bp). Molecular size standards are indicated in Lanes 1, 5: 1 kb + ladder and Lane 3: 1 kb ladder.

### Cloning Analysis into Chloroplast Vector

The inserts, FC AMA-1, MSP-1, and CTB, were successfully cloned into the pGMET easy and pBSK+ vectors successfully (data not shown). The DNA sequences were confirmed after successful ligation into the pGEMT easy vector. Once the transgene fusions, CTB FC AMA-1 and CTB MSP-1, were constructed in the pBSK+ vector, the constructs were excised and ligated into the chloroplast pLD-UTR vector with expected sizes as shown in **Figure 3**. The constructs were then bombarded into the tobacco plant *Nicotiana tabacum* variety Petit Havana by the protocol listed in the “Materials and Methods” section.



**Figure 3:** Analysis of Cloning CTB FC AMA-1 and CTB MSP-1 Into the pLD-UTR Chloroplast Vector

After purifying plasmid DNA with the Qiagen Plasmid Maxi Kit, the DNA was analyzed by gel electrophoresis. CTB FC AMA-1 is depicted in **Lane 3:**

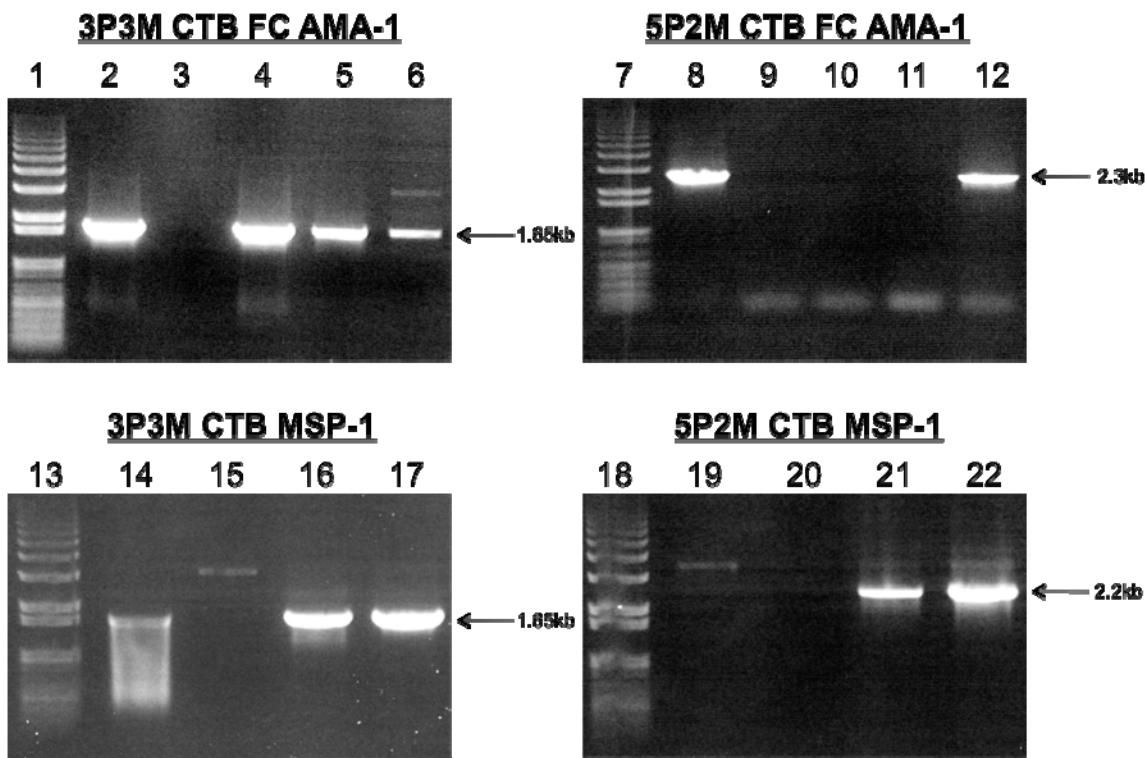
undigested and **Lane 6:** digested with NotI and NdeI resulting in a 715 bp fragment. CTB MSP-1 is illustrated in **Lane 7:** after digestion with NotI and NdeI (621 bp fragment). In **Lanes 2, 4, 5:** genes were inserted into the pLD-UTR vector but were not further studied. Molecular size standards are indicated in **Lanes 1, 8:** 1 kb ladder.

### Confirmation of Chloroplast Integration of Transgenes

The gene cassette (*aadA* and CTB FC AMA-1/CTB MSP-1) was introduced into the tobacco chloroplast genome through homologous recombination of the flanking sequences of the pLD-UTR vector (*trnI* and *trnA*) and the native plastid genome. The Prrn downstream of the *trnI* gene is for transcription of *aada* that confers resistance to spectinomycin and streptomycin and the transgene that encodes the CTB-malarial gene. The 3' UTR upstream of the *trnA* gene provides stability for the transcript and may be involved in ribosome recruitment.

Five to six resistant shoots appeared on the regeneration media (RMOP) containing spectinomycin. Using specific primers and PCR analysis, mutant and nuclear integrated shoots were differentiated from shoots where the transgene integrated into the chloroplast genome. By using 3P/3M primers, site-specific integration of the gene cassette was confirmed. To eliminate all nuclear transformants, the 3P primer was used because it annealed to the native chloroplast genome. The 3M primer was used because it landed on the *aadA* gene and eliminated all mutant transformants. The 3P/3M primers, resistant shoots, and PCR analysis yielded a 1.65 kb band as shown in **Figure 4** (left panels). Another set of primers 5P/2M were used to confirm the integration of the *aadA* gene and the CTB-malarial gene. The 5P primer annealed to the *aadA* gene and the 2M primer annealed to the *trnA* gene. The amplified PCR product size was 2.3 kb for CTB FC AMA-1 and 2.2 kb for CTB MSP-1 as shown in **Figure 4** (right panels). The 5P/2M PCR analysis eliminated all mutants and

only positive transformants with transgene integration were visualized. The positive transformants were subjected to second and third round of selection to advance towards homoplasmy.



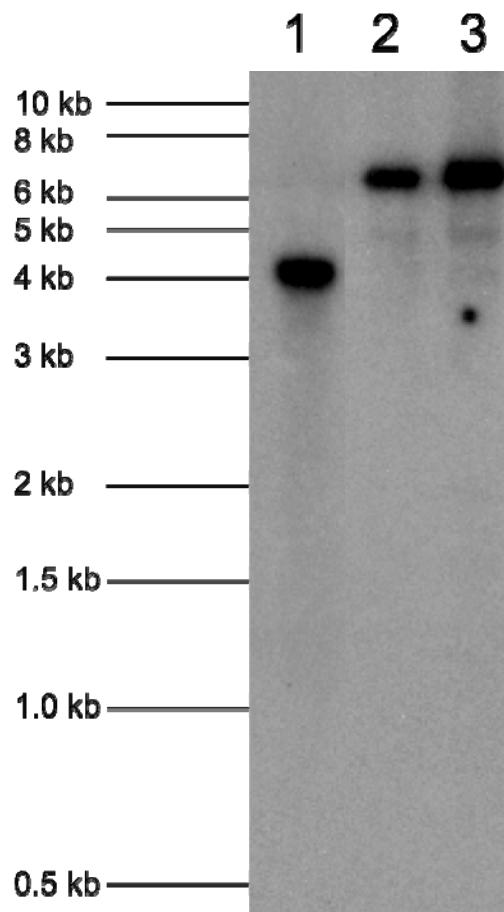
**Figure 4:** PCR Analysis of Wild Type and Positive Transformants

PCR using specific primers land within the native chloroplast genome and the *aadA* gene (3P3M) to yield a 1.65 kb product **Lanes 2, 14:** positive control, **Lanes 3, 15:** wild type, **Lanes 4-6:** transgenic lines pLD-UTR CTB FC AMA-1, and **Lanes 16, 17:** transgenic lines pLD-UTR CTB-MSP-1. Using the specific 5P/2M primers, landing on the *aadA* gene and *trnA* gene, respectively yields a 2.3 kb and 2.2 kb product **Lanes 8, 19:** positive control, **Lanes 9, 20:** wild type, **Lanes 10, 11:** negative transformants pLD-UTR CTB FC AMA-1, **Lane 12:** transgenic line pLD-UTR CTB FC AMA-1, and **Lanes 21, 22:** transgenic lines

pLD-UTR CTB-MSP-1. Molecular size standards are indicated in **Lanes 1, 7, 13**,  
**18**: 1 kb + ladder.

### Southern Analysis of Transgenic Plants

To confirm site-specific integration and homoplasmy plants, untransformed and transgenic plant genomic DNA was probed with the 2P/2M flanking sequence probe of 1.3 kb in size. The transgenic and non-transgenic genomic DNA was digested with the restriction enzyme Apal. After digestion, the untransformed chloroplast genome resulted in a 4.5 kb fragment and the transformed chloroplast genome resulted in 6.5 and 6.6 kb fragments for CTB MSP-1 and CTB FC AMA-1, respectively, as depicted in **Figure 5**. The absence of the 4.5 kb fragment in the transplastomic lines confirms homoplasmy (**Figure 5**).

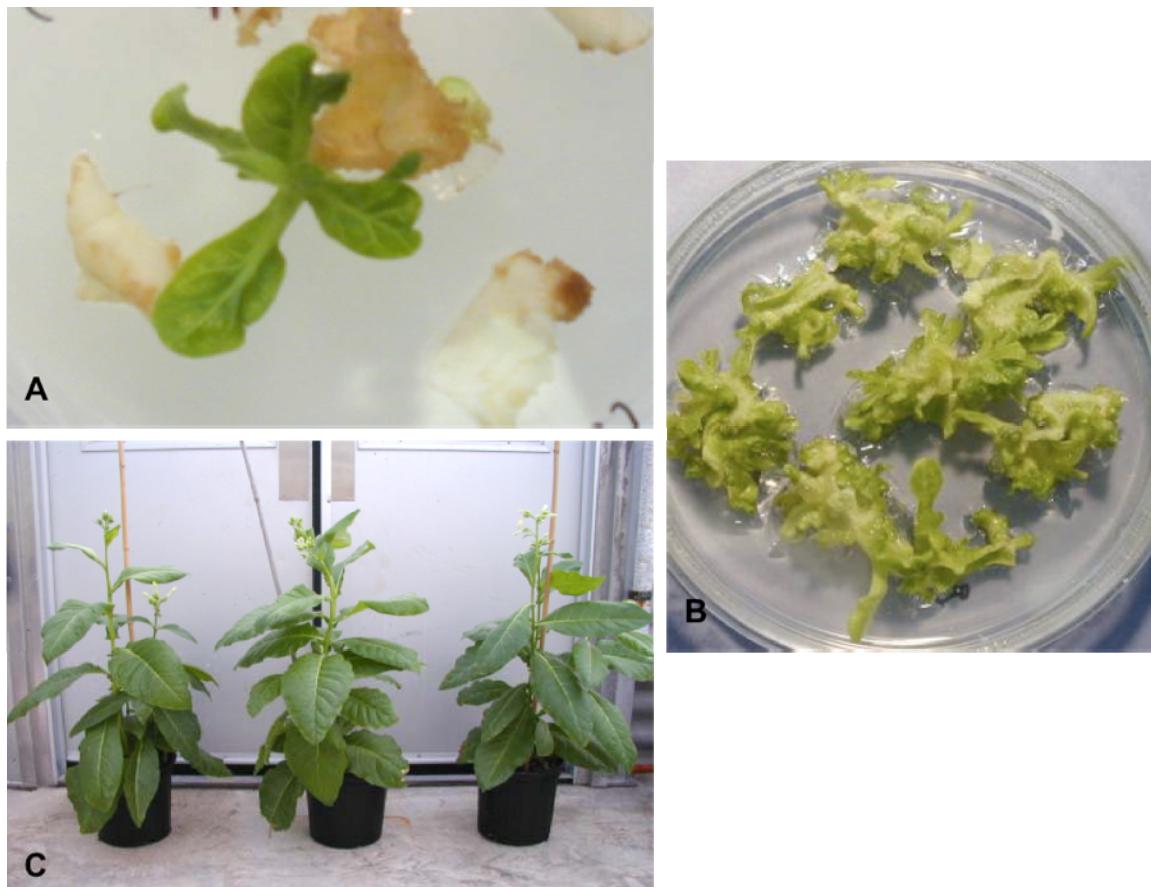


**Figure 5:** Evaluation of Transgene Integration into the Chloroplast Genome of Homoplasmic Plants by Southern Blot

Southern blot probed with the 2P/2M flanking sequence probe to determine homoplasmy **Lane 1:** wild type, **Lane 2:** homoplasmic CTB MSP-1 (6.5 kb), and **Lane 3:** homoplasmic CTB FC AMA-1 (6.6 kb).

### Selection and Generation of Transgenic Plants

Transgenic plants were selected and generated by the use of RMOP media and spectinomycin (**Figure 6A**). After positive transformants were determined by PCR analysis, further selection and generation of transgenic shoots was maintained (**Figure 6B**). MSO selection media containing spectinomycin was used for rooting. Once the *in vitro* plants were confirmed homoplasmic by Southern Blot analysis the plants were transferred to the greenhouse for optimal growth and maximal protein expression (**Figure 6C**). No pleiotropic effects were observed with the transgenic plants (CTB FC AMA-1, middle plant and CTB MSP-1, right plant) in comparison with untransformed control (left plant) (**Figure 6C**).

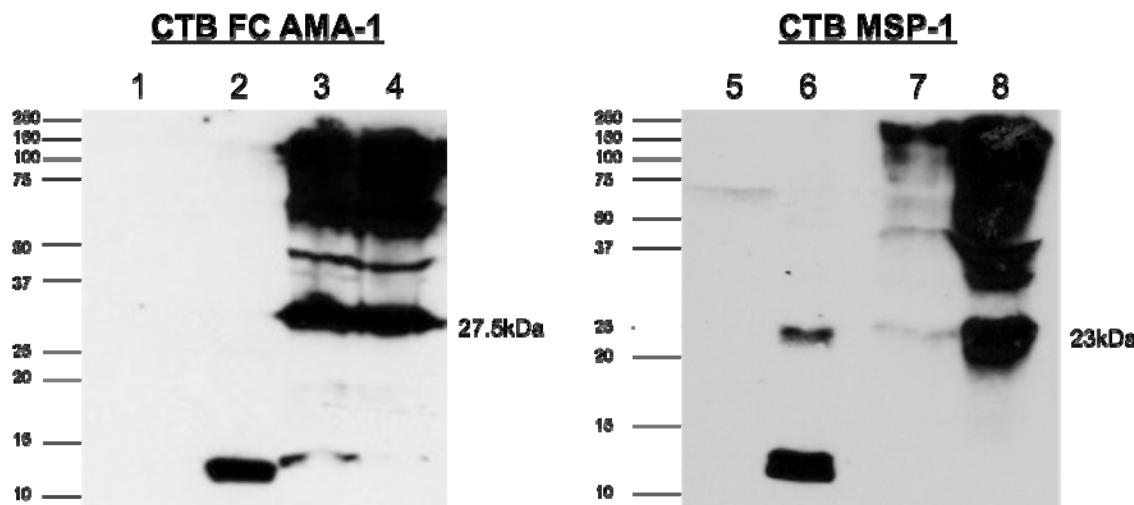


**Figure 6:** Generation of Transgenic Plants

**(A)** Four to five weeks after particle bombardment, transplastomic shoots appear on RMOP selection medium. For second round of selection, leaves from PCR positive transformants are transferred to RMOP selection medium. **(B)** Several shoots appear within two to three weeks. For third round of selection, regenerated shoots are transferred to MSO selection medium and roots appear in about 10 days. **(C)** Plants were transferred to the greenhouse after confirmation of homoplasmic plants *via* Southern blot analysis. In the picture, from left to right the following plants are visualized: untransformed control, CTB FC AMA-1, and CTB MSP-1.

### Characterization of the Chloroplast-Derived CTB Malarial Proteins

Crude extracts of approximately 50 µg of wild type and transgenic plants along with monomeric CTB protein and an *E.coli* expressed CTB MSP-1 extract were loaded in the wells of a SDS-PAGE gel. The blots were probed with the polyclonal anti-CTB primary antibody and the monomeric forms of CTB FC AMA-1 showed a 27.5 kDa protein and CTB MSP-1 depicted a 23 kDa protein in the insoluble (pellet) and soluble (supernatant) fractions (**Figure 7**). The immunoblot displayed others forms of the CTB-malaria proteins such dimers, trimers, tetramers, and pentamers (**Figure 7**).

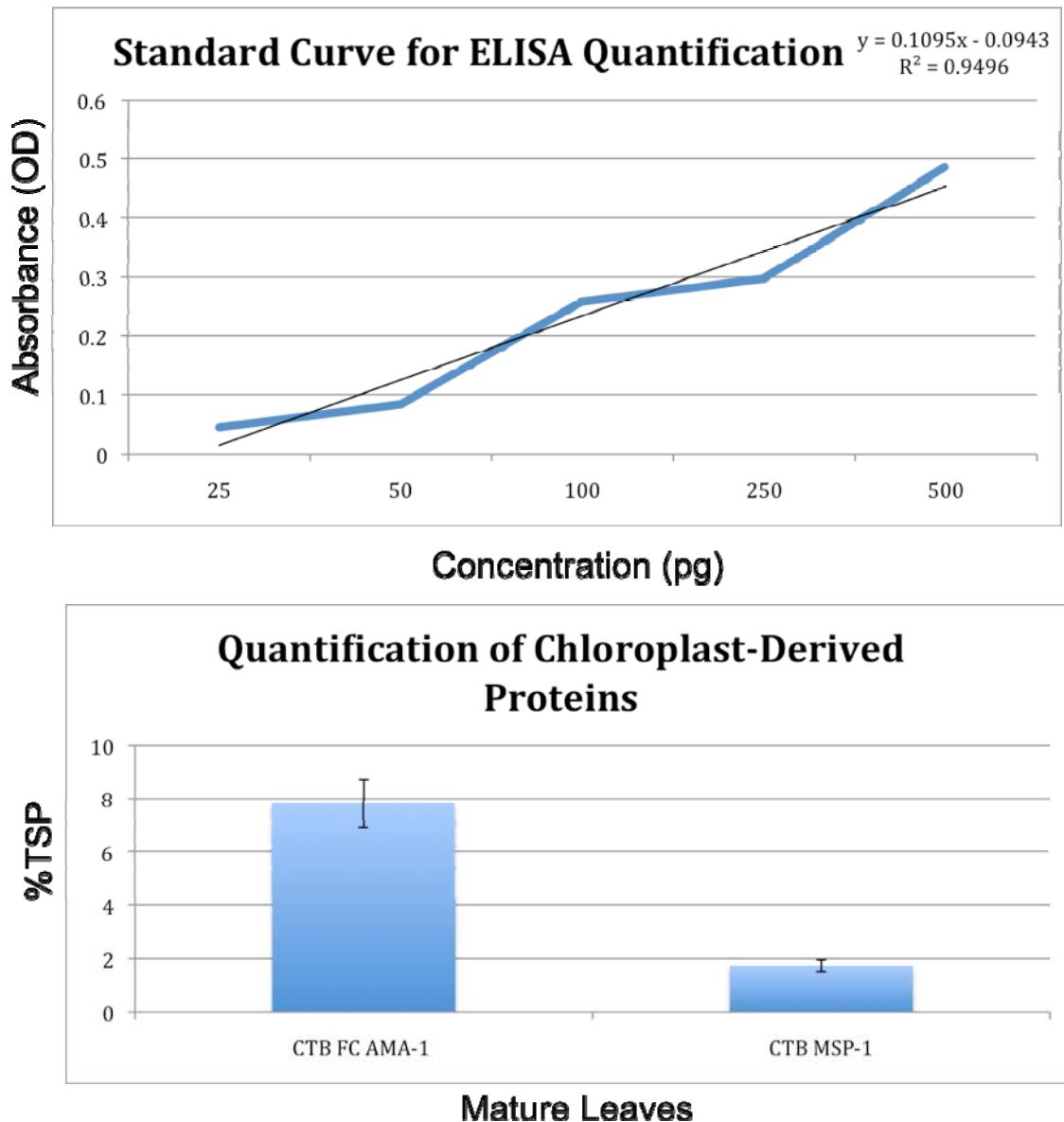


**Figure 7:** Immunoblot Analysis to Confirm Expression of CTB-Malarial Antigens in *Nicotiana tabacum* Crude Extracts

Immunoblot with anti-CTB polyclonal antibody showed full-length protein. **Lanes 1, 5:** negative control wild type extract, **Lane 2:** positive control monomeric 11.6 kDa CTB protein, **Lane 6:** positive control CTB MSP-1 *E. coli* expressed protein, **Lane 3:** CTB FC AMA-1 pellet, **Lane 4:** CTB FC AMA-1 supernatant, **Lane 7:** CTB MSP-1 pellet, and **Lane 8:** CTB MSP-1 supernatant.

### Quantification of Chloroplast-Derived CTB Malarial Proteins

To determine the concentration of the chloroplast derived CTB-malarial proteins, a standard ELISA was performed. Using CTB protein of known concentrations, a standard curve was generated as depicted in **Figure 8** (top panel). The ELISA displayed a dilution effect with the diluted crude plant extracts and the standard curve (data not shown). The CTB FC AMA-1 and CTB MSP-1 protein expression levels of mature leaves reached 6.3-9.5% and 1.4-2%, respectively as shown in **Figure 8** (bottom panel). The accumulation of the CTB-malarial proteins in high level of expression is due to the presence of high number of chloroplasts and chloroplast genomes (up to 10,000 copies per cell).



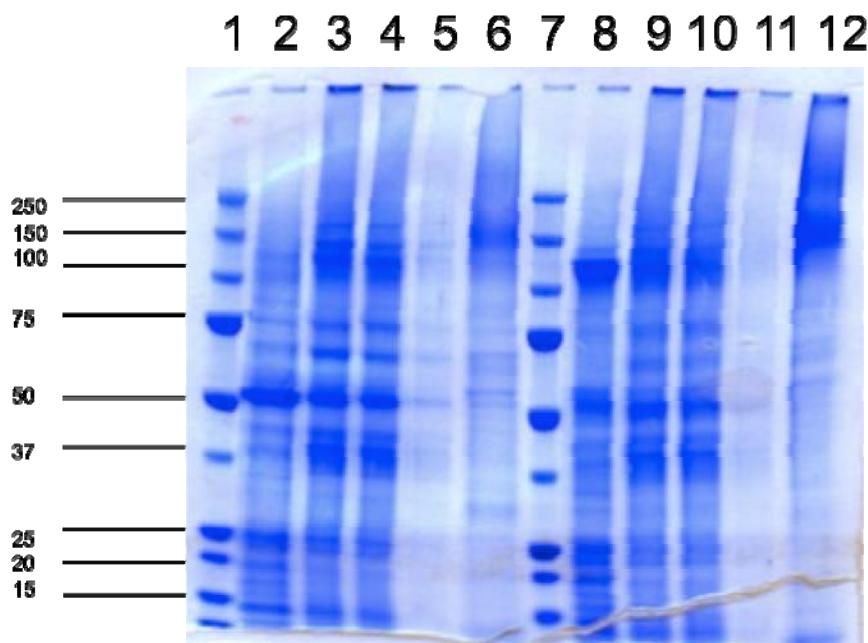
**Figure 8:** Quantification of Chloroplast-Derived CTB-Malarial Expression

An ELISA with the following expression levels determined quantification: 6.3-9.5% of CTB FC AMA-1 and 1.4-2% of CTB MSP-1 of total soluble protein of mature leaves.

## Enrichment of Chloroplast-Derived CTB-Malarial Proteins

### Resolution of Enriched Proteins

A crude extract of chloroplast-derived proteins was subjected to immobilized metal affinity chromatography by using the TALON Superflow Metal Affinity Resin and analysis followed. A NuPAGE Novex Bis-Tris gradient gel was used to increase the resolution of the enriched CTB FC AMA-1 protein. The gel was performed under reduced and non-reduced conditions. The large subunit of rubisco (55 kDa) is apparent in the wild type, lysate, and flow through fractions under reduced and non-reduced conditions (**Figure 9**). In the wash fractions there are minimal bands present. In the eluted CTB FC AMA-1 fraction, the monomer of 27.5 kDa in size is present under reduced conditions (**Lane 6**) and the pentameric form is present under both reduced (**Lane 6**) and non-reduced (**Lane 13**) conditions (**Figure 9**).



**Figure 9:** Increased Resolution of Chloroplast-Derived CTB FC AMA-1 Protein

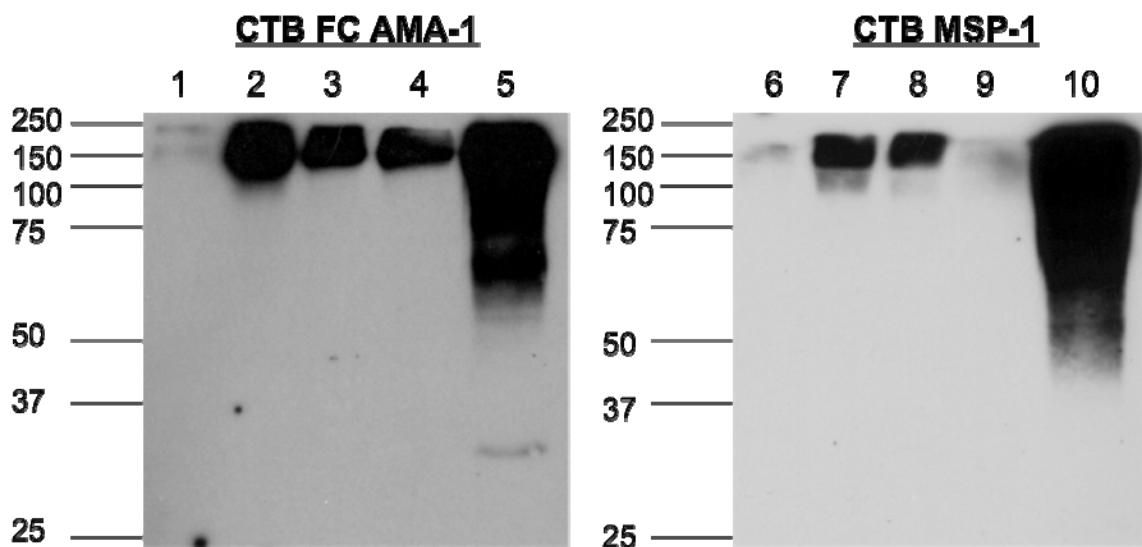
After Immobilized Metal Affinity Chromatography

CTB FC AMA-1 protein was extracted from transformed leaves and the crude extract was subjected to Talon Superflow Metal Affinity Resin and analyzed.

**Lanes 2-6:** reduced and **Lanes 8-12:** non-reduced conditions of CTB FC AMA-1 protein enrichment was observed by using a gradient gel (4-12%) and gel electrophoresis. The following fragments were visualized **Lanes 2, 8:** wild type, **Lanes 3, 9:** lysate, **Lanes 4, 10:** flow through, **Lanes 5, 11:** wash, and **Lanes 6, 12:** enriched protein. Molecular size standards are indicated in **Lanes 1, 7:** 1 kb ladder.

### Immunoblot Analysis of Enriched Proteins

An immunoblot probed with anti-CTB antibody was conducted to confirm the presence of the CTB-malarial proteins after talon enrichment. Equal amounts of protein from wild type, lysate, flow through, wash, and enrichment was loaded into the gel and visualized *via* an immunoblot. In the fractions containing protein harvested from wild type leaves resulted in no apparent band in the immunoblot analysis (**Figure 10**). Lower levels of CTB-malarial proteins were found in the flow through and wash fractions compared to the lysate and in the enriched fractions significant protein expression were found confirming enrichment of the CTB-malarial proteins (**Figure 10**).



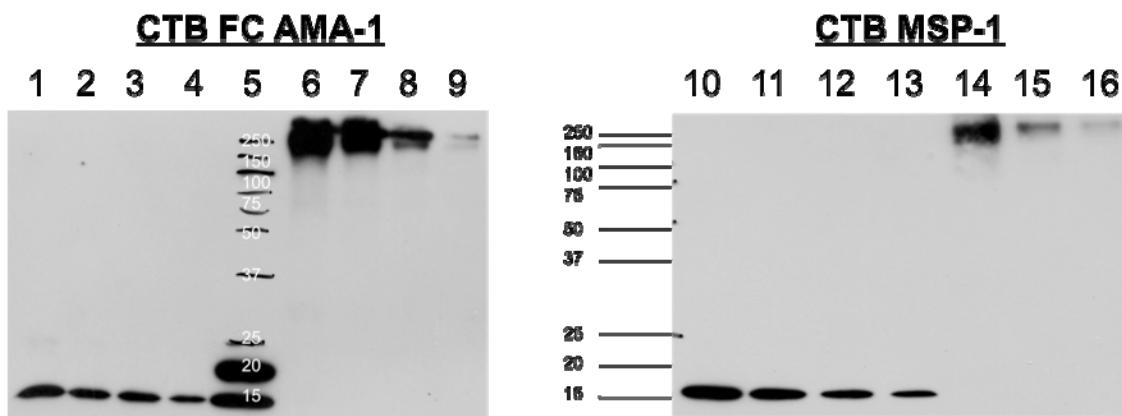
**Figure 10:** Immunoblot Analysis of Enrichment of Malarial Antigens from *Nicotiana tabacum* Extracts

Malaria protein was extracted from transformed leaves and the crude extract was subjected to Talon Superflow Metal Affinity Resin and the following fractions were collected and analyzed using an immunoblot with polyclonal anti-CTB

antibody: **Lanes 1, 6:** wild type, **Lanes 2, 7:** lysate, **Lanes 3, 8:** flow through, **Lanes 4, 9:** wash, and **Lanes 5, 10:** eluted CTB FC AMA-1 and CTB MSP-1, respectively.

### Densitometric Analysis

An immunoblot with known concentrations of CTB protein and different concentrations of the enriched fractions were probed with anti-CTB antibody. Quantification of the enriched CTB-malarial proteins on immunoblots was analyzed by spot densitometry. Linearity of the standard curve was achieved by using 1000, 500, 250, and 125 ng of CTB (data not shown) and assisted in the estimation of the enriched samples in the same blot (**Figure 11**). The standard curve provided the concentration of the enriched fractions and the efficiency of the talon enrichment was determined to be 90% and 73% in CTB FC AMA-1 and CTB MSP-1, respectively.



**Figure 11:** Immunoblot of the Eluted Protein Fractions were Analyzed and

Compared to Known Quantities of CTB Protein

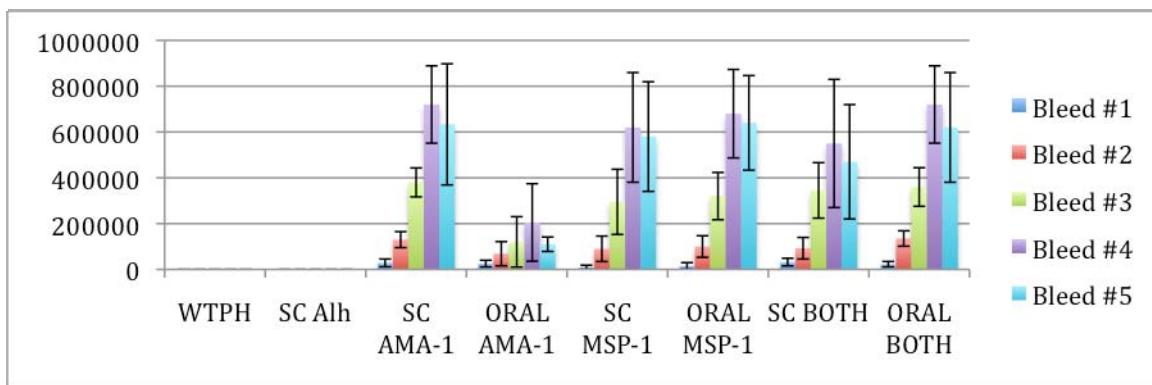
Immunoblot analysis of **Lanes 1-4 and 10-13**: CTB protein (1000, 500, 250, 125 ng, respectively), **Lanes 6-9**: eluted CTB FC AMA-1 (1.5, 0.75, 0.375, 0.1875 µg, respectively), and **Lanes 14-16**: eluted CTB MSP-1 (1.5, 0.75, 0.375 µg, respectively). Eluted proteins and CTB were subjected to densitometry to

determine the enrichment of CTB FC AMA-1 and CTB MSP-1 to be administered to mice for subcutaneous injection.

## Immunogenicity of the Chloroplast-Derived CTB Malarial Antigens

### Quantitation of CTB Titers from Immunized Mice

The serum collected from the five bleeds following immunization were on days 21, 35, 63, 163, and 197-post immunization. The serum was tested for anti-CTB antibodies by a capture ELISA with CTB protein. Minimal mice titers were detected from bleeds #1 and #2 ranging from 1:1,000 – 1:150,000 and titers were present ranging from 1:50,000 – 1:800,000 in bleeds #3, #4, and #5 (**Figure 12**). Titters for anti-CTB were found to be higher in groups 3, 5, 6, 7, and 8 versus group 4 (oral delivery CTB FC AMA-1) (**Figure 12**). No CTB titers were found for group 1 (oral delivery non-transgenic leaf material) or group 2 (subcutaneous injection alhydrogel alone).



**Figure 12:** Immunogenicity of CTB Titers Using a Capture ELISA

ELISA detection of anti-CTB antibody titers from groups 1 – 8 serum samples collected from five bleeds.

### **Quantitation of MSP-1<sub>19</sub> Titers from Groups 5 and 6**

The serum collected from the five bleeds following immunization was tested for anti-*PfMSP1<sub>19</sub>* antibodies by a capture ELISA with MRA-49 *PfMSP1<sub>19</sub>*. Minimal mice titers were detected from bleeds #1 and #2 and titers were present ranging from 1:100 – 1:50,000 in bleeds #3, #4, and #5 (**Table 2**). Titers for anti-*PfMSP1<sub>19</sub>* were found to be higher in group 5 (subcutaneous injection) than in group 6 (oral delivery) (**Table 2**). Four mice in groups 5 and 6 (5A1, 5B3, 6A1, 6B4) showed undetectable titers with MRA-49 *PfMSP1<sub>19</sub>* protein (**Table 2**) but showed similar CTB titers with the other mice in the group.

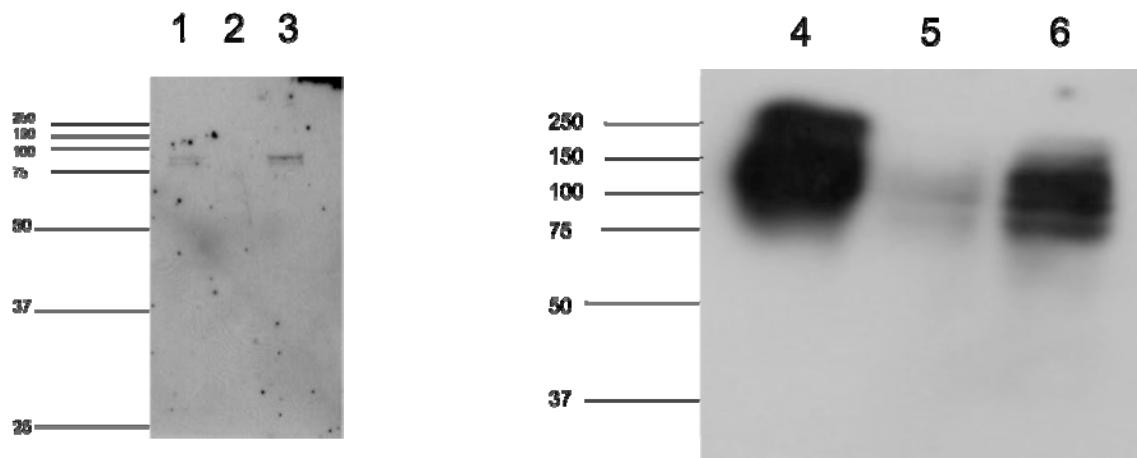
**Table 2:** Immunogenicity of a Malarial Antigen Using MSP-1 Protein

Mouse #	MSP-1 IgG1 Titers Bleed #1	MSP-1 IgG1 Titers Bleed #2	MSP-1 IgG1 Titers Bleed #3	MSP-1 IgG1 Titers Bleed #4	MSP-1 IgG1 Titers Bleed #5
5A1	0	0	0	0	0
5A2	100	1000	25000	25000	50000
5A3	1000	1000	25000	25000	25000
5A4	100	1000	12500	25000	50000
5A5	0	1000	1000	1000	1000
5B1	0	0	250	1000	12500
5B2	100	1000	12500	25000	25000
5B3	0	0	0	0	0
5B4	0	1000	25000	25000	50000
5B5	0	0	1000	1000	12500
6A1	0	0	0	0	0
6A2	0	500	1000	12500	12500
6A3	100	250	1000	12500	12500
6A4	100	1000	1000	1000	1000
6A5	0	0	250	1000	1000
6B1	0	0	250	1000	1000
6B2	500	1000	12500	12500	12500
6B3	0	100	1000	1000	1000
6B4	0	0	0	0	0
6B5	0	250	1000	12500	12500

ELISA detection of anti-MSP-1 antibody titers from groups 5 and 6 serum samples collected from five bleeds.

### Recognition of Parasite Protein by Anti-Malarial Antibodies

An immunoblot confirmed anti-malarial antibodies from immunized mice recognized parasite protein from *P. falciparum* infected erythrocytes. Anti-AMA-1 antibodies, sera collected from a mouse immunized with CTB FC AMA-1 antigen, recognized the ring and schizont stages with the presence of an 83 kDa band (**Figure 13**). The serum from an immunized mouse from group 5 contained anti-MSP-1 antibodies, which recognized ring and schizont stages with an apparent 190 kDa band (**Figure 13**).



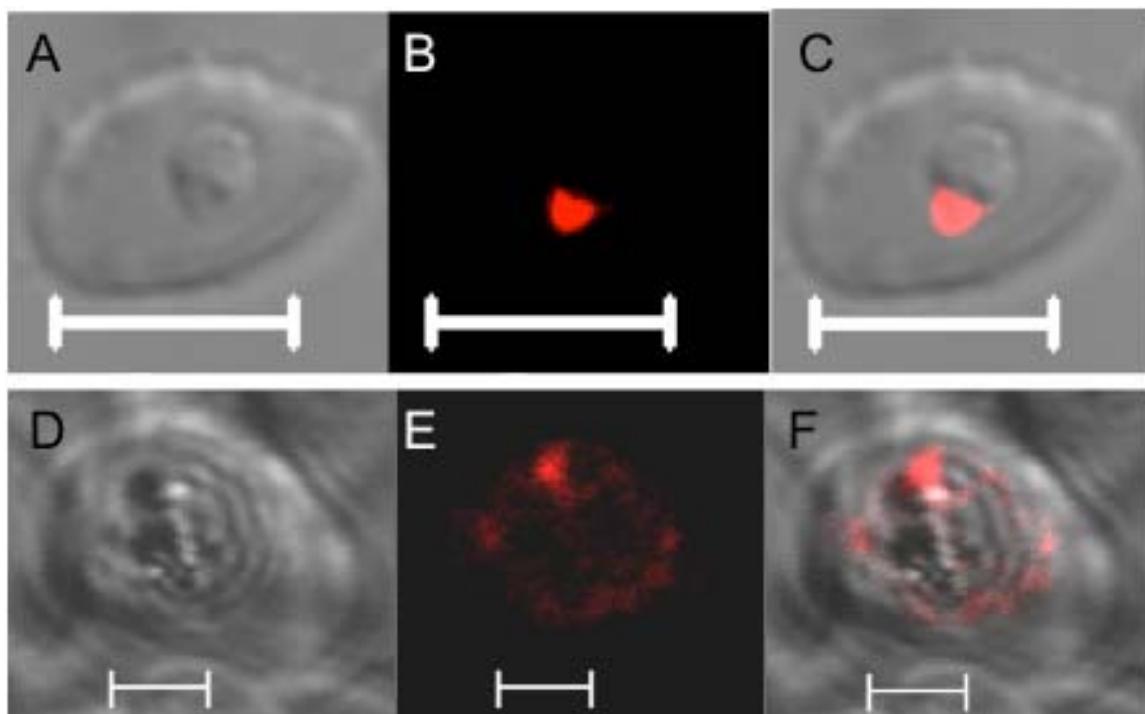
**Figure 13:** Recognition of Parasite Protein by Anti-AMA-1 and Anti-MSP-1 Antibodies

36.8 µg of ring, trophozoite, and schizont parasite protein was loaded into the SDS PAGE gel and analyzed by an immunoblot with 1:1,000 diluted sera from immunized mice. Immunoblot displaying **Lanes 1-3:** anti-AMA-1 collected from an immunized mouse recognizes 83 kDa AMA-1 protein and **Lanes 4-6:** anti-MSP-1 recognizes 190 kDa MSP-1 protein. The parasite stages analyzed from

the 3D7 *P. falciparum* culture include **Lanes 1, 4:** ring, **Lanes 2, 5:** trophozoite, and **Lanes 3, 6:** schizont.

### Recognition of Parasite by Anti-Malarial Antibodies

Sera collected from immunized mice with chloroplast-derived CTB-malarial antigens resulted in the recognition of parasite localization. Anti-AMA-1 antibodies were found in the immunized sera because parasites were stained in the apical end of the ring stage parasite (**Figure 14 B, C**). A mouse immunized with the chloroplast-derived CTB-MSP-1 antigen resulted in stained schizonts indicating the presence of anti-MSP-1 antibodies (**Figure 14 E, F**).



**Figure 14:** Recognition of Parasite by Anti-AMA-1 and Anti-MSP-1 Antibodies

Visible and immunofluorescence (IFA) images of 3D7 *P. falciparum* parasite immunostained with (**A, B, C**) anti-AMA-1 raised in mice displaying recognition of the apical end of the parasite and (**D, E, F**) anti-MSP-1 collected from immunized mouse sera recognizing the MSP-1 of a schizont parasite.

### In Vitro Parasite Inhibition Assay

#### **Anti-AMA-1 and Anti-MSP-1 Antibodies Prevent Invasion**

An *in vitro* parasite inhibition assay was performed to test the ability of anti-AMA-1 and anti-MSP-1 antibodies in inhibiting parasite entry into erythrocytes. Synchronized trophozoite-schizont stage parasites (2% parasitemia and hematocrit, **Figure 15 A**) were incubated with control and test sera for forty-eight hours and blood smears were made. The slides were stained with Giemsa and the number of parasites and total number of RBCs were counted. The parasitemia was determined and the percent inhibition was calculated. The predominant stage found under microscopic examination was the ring stage. The average parasitemia for the blank control (no serum added) was determined to be 6.6% (**Figure 15 B**) while the lowest parasitemia found was from group 5, subcutaneous CTB MSP-1 (2.5%, **Table 3**) and in mouse 5A4 with the highest MSP- $1_{19}$  titer of 50,000 (2.4%, **Figure 15 C**) representing the group and the mouse with highest percent of relative inhibition to the positive control (117.2%, **Table 3**) and percent of inhibition (63.6%, **Table 4**), respectively, of parasite invasion. The remaining experimental groups displayed 85.8 – 105.8% relative inhibition to the positive control (**Table 3**). The control groups who did not receive chloroplast-derived CTB-malarial antigen resulted in 14.3 – 25.7% relative inhibition to the positive control (**Table 3**). The immunized mouse, 6B3, with the lowest MSP- $1_{19}$  titer of 1,000 was found to exhibit the highest parasitemia (3.5%, **Figure 15 D**) after the *in vitro* parasite inhibition assay. The serum from the positive control (MRA-35 rabbit antiserum against

purified recombinant yeast secreted *PfMSP1-19*, 3D7) resulted in only 53% inhibition (**Table 4**). The control groups (groups 1, 2, 9) resulted in 7.6 – 13.6% inhibition and mice 5B5 and 6B5 with a MSP- $1_19$  titer of 12,500 resulted in 59.1% and 54.5%, respectively (**Table 4**).

**Table 3:** Calculation of Average Parasitemia and Relative Inhibition of Experimental Mice Sera After an *in Vitro* Parasite Inhibition Assay

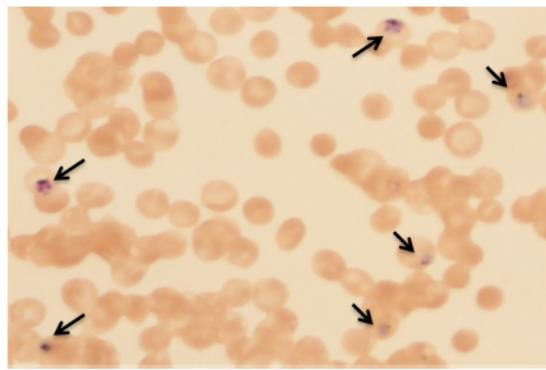
Group	Parasitemia	Mean Parasitemia	Relative Inhibition
No Ab	6.6 - 6.7%	6.6%	-
MRA-35 <i>PfMSP1-19</i>	2.5 - 3.5%	3.1%	100.0%
Group 1	5.9 - 6.6%	6.1%	14.3%
Group 2	5.5 - 6.2%	5.8%	22.8%
Group 3	2.8 - 3%	2.9%	105.8%
Group 4	2.4 - 3.3%	3.0%	102.8%
Group 5	2.4 - 2.6%	2.5%	117.2%
Group 6	2.6 - 3.6%	3.2%	97.2%
Group 7	2.7 - 3.9%	3.3%	94.3%
Group 8	3.3 - 3.8%	3.6%	85.8%
Group 9	5.6 - 5.8%	5.7%	25.7%

Synchronized 3D7 *P. falciparum* trophozoite-schizont stage culture (2% parasitemia and hematocrit) and no sera, MRA-35 *PfMSP1-19* sera, and immunized mouse sera was incubated for 48 hours; the parasitemia was estimated and relative percent of inhibition was determined in comparison to the positive control.

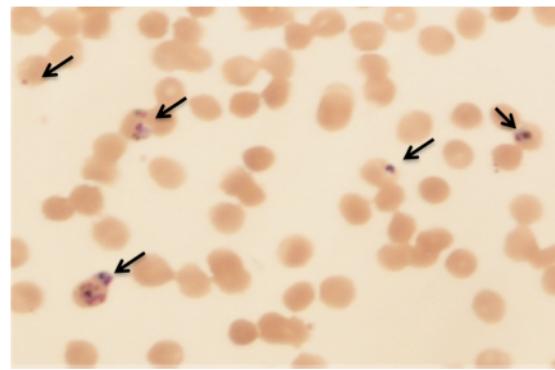
**Table 4:** Calculation of Average Parasitemia and Percent Inhibition of Selected Mice After an *in Vitro* Parasite Inhibition Assay

Group	MSP-1 <sub>19</sub> Titer	Parasitemia	Inhibition
No Ab (Control)	–	6.6%	–
MRA-35 PfMSP1-19	–	3.1%	53%
Group 1	0	6.1%	7.6%
Group 2	0	5.8%	12.1%
Mouse 5A4 (s.c.)	50000	2.4%	63.6%
Mouse 5B5 (s.c.)	12500	2.7%	59.1%
Mouse 6B3 (oral)	1000	3.5%	47%
Mouse 6B5 (oral)	12500	3.0%	54.5%
Group 9	0	5.7%	13.6%

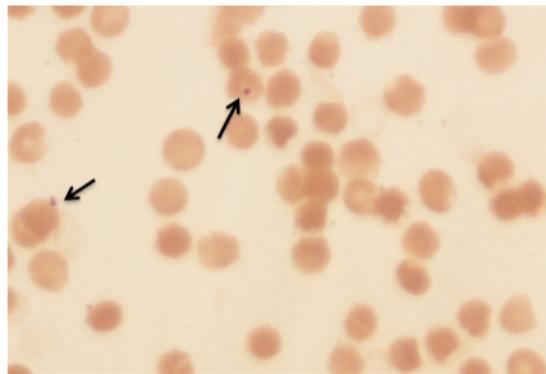
Synchronized 3D7 *P. falciparum* trophozoite-schizont stage culture (2% parasitemia and hematocrit) and no sera, MRA-35 PfMSP1-19 sera, and immunized mouse sera was incubated for 48 hours; the parasitemia was estimated and percent of inhibition was determined.



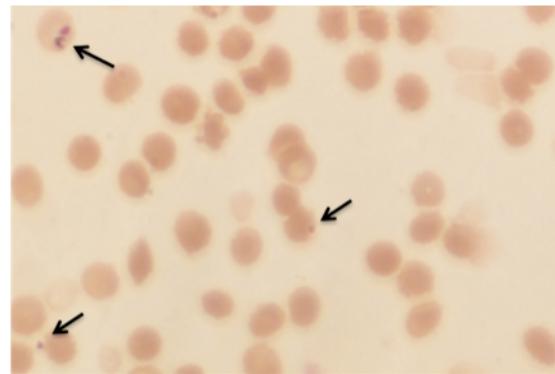
(A) Pre-Treatment



(B) Blank – RPMI Treated



(C) Mouse 5A4



(D) Mouse 6B3

**Figure 15:** Microscopic Examination of the *in Vitro* Parasite Inhibition Assay

Synchronized 3D7 *P. falciparum* trophozoite-schizont stage culture 2% parasitemia and hematocrit (A), and no sera (B), sera from mouse 5A4 (C), and sera from mouse 6B3 (D) was incubated for 48 hours; the parasitemia was estimated by counting infected and uninfected RBCs.

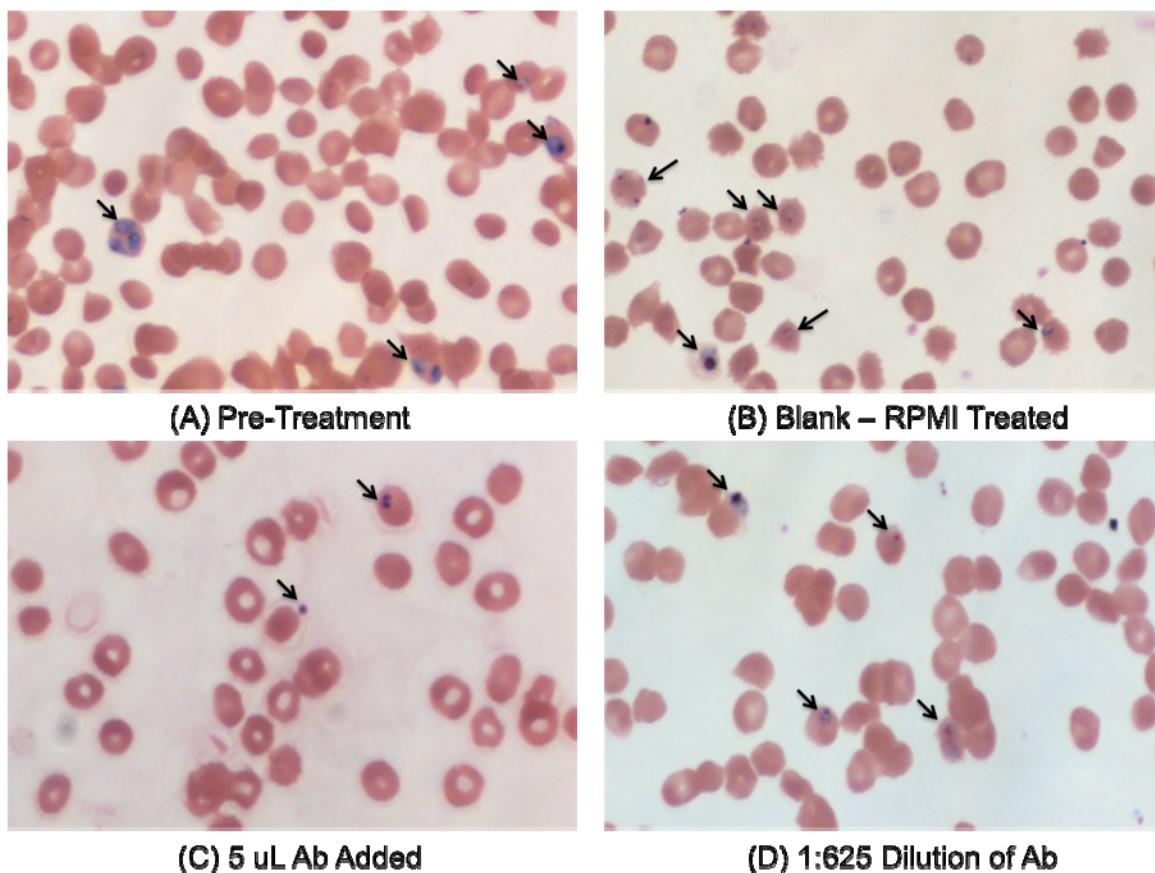
### **Anti-MRA-35 *PfMSP1-19* Antibodies Prevent Invasion**

An *in vitro* parasite inhibition assay was performed to test the ability of anti-MRA-35 *PfMSP1-19* antibodies inhibiting parasite entry into erythrocytes in a dose-dependent manner. Synchronized trophozoite-schizont stage parasites (2.5% parasitemia and 2% hematocrit, **Figure 16 A**) were incubated with 5 µL and diluted 1:5-1:625 MRA-35 *PfMSP1-19* antibodies for forty-eight hours and blood smears were made. The slides were stained with Giemsa and the number of parasites and total number of RBCs were counted. The parasitemia was determined and the percent inhibition was calculated. The predominant stage found under microscopic examination was the ring stage. The average parasitemia for the blank control (no serum added) was determined to be 8.0% (**Table 5, Figure 16 B**) while parasitemia ranged from 3-5.5% and the percent of inhibition ranged from 62.5-31.3% from least to most diluted sera, respectively (**Table 5, Figure C, D**).

**Table 5:** Calculation of Average Parasitemia and Percent Inhibition After *in Vitro* Parasite Inhibition Assay Using Different Dilutions of MRA-35 *PfMSP-1-19* Antibody

Group	Parasitemia	Average Parasitemia	Inhibition
<b>Blank (Control)</b>	<b>6.9-8.9%</b>	<b>8.0%</b>	—
<b>5 µL</b>	<b>2.7-3.3%</b>	<b>3.0%</b>	<b>62.5%</b>
<b>1:5 Dilution</b>	<b>3.4-3.8%</b>	<b>3.6%</b>	<b>55%</b>
<b>1:25 Dilution</b>	<b>4-4.4%</b>	<b>4.2%</b>	<b>47.5%</b>
<b>1:125 Dilution</b>	<b>4.3-5.1%</b>	<b>4.8%</b>	<b>40.0%</b>
<b>1:625 Dilution</b>	<b>5.4-5.7%</b>	<b>5.5%</b>	<b>31.3%</b>

Synchronized 3D7 *P. falciparum* trophozoite-schizont stage culture (2.5% parasitemia and 2% hematocrit); no sera or MRA-35 *PfMSP1-19* sera was incubated for 48 hours; the parasitemia was estimated and percent of inhibition was determined.



**Figure 16:** Microscopic Examination of the *in Vitro* Parasite Inhibition Assay After Adding Different Dilutions of MRA-35 *PfMSP-1-19* Antibody

Synchronized 3D7 *P. falciparum* trophozoite-schizont stage culture 2.5% parasitemia and 2% hematocrit (A), and no sera (B), 5  $\mu$ L of MRA-35 *PfMSP1-19* antibody (C), and 1:625 diluted MRA-35 *PfMSP1-19* antibody (D) was incubated for 48 hours; the parasitemia was estimated by counting infected and uninfected RBCs.

## DISCUSSIONS

Currently, there is no licensed vaccine for the prevention of malarial disease despite the vast knowledge of genomics and proteomics of the malaria parasite (Sharma and Pathak 2008). The need for a malarial vaccine is imperative because the global burden of the disease is increasing due to drug resistance, mosquito's resistance to insecticides, ineffective control measures, re-emergence of the disease, and increased tourism. Malaria vaccine research has investigated several vaccine candidates in clinical trials but with disappointing results in low titers and poor efficacy. Previous studies have lead to the discovery of AMA-1 and MSP-1, which are two of the leading asexual blood-stage malarial vaccine candidates. The malarial genes, AMA-1 and MSP-1, were successfully amplified *via* PCR along with the transmucosal carrier, CTB. CTB was included in the present study because of it's potential adjuvant activity on the immune system and possibility of performing as an oral immunogen (Li and Fox 1996). The fusion CTB-malarial gene cassettes were constructed in the pBSK+ vector.

Malaria antigens have been expressed in several expression systems such as in *E. coli* (Sachdeva, Mohammed et al. 2006), yeast (Gozalo, Lucas et al. 1998), and mammalian cells (Burghaus, Gerold et al. 1999) but plants could be considered as an alternative in vaccine development because they can reduce cost due to the expense of fermentation, purification, processing, cold storage, and delivery. The purpose of expressing the CTB-malarial antigens in the chloroplast system was to develop a safer and more effective malarial vaccine.

By using the flanking sequences *trnI* and *trnA*, the chloroplast pLD-UTR vector with the CTB-malarial cassette was integrated into the chloroplast genome via homologous recombination. The pLD-UTR vector contains the *aadA* gene, which codes for the enzyme aminoglycoside 3' adenyltransferase and transgenic shoots were allowed to overcome the selection of spectinomycin (Svab and Maliga 1993; Verma and Daniell 2007). 3P/3M and 5P/2M primers along with PCR confirmed the integration of the gene cassette into the chloroplast genome. The positive transformants were subjected to second and third round of selection to remove any remaining wild type cells. The transgenic plants were confirmed to be homoplasmic (presence of only transformed genomes) after performing southern blot analysis with a flanking sequence probe.

The expression of CTB FC AMA-1 and CTB MSP-1 was driven by the *psbA* 5'UTR in transgenic lines and confirmed by an immunoblot. The proteins were observed at 27.5 kDa in CTB FC AMA-1 and 23 kDa in CTB MSP-1 in both the insoluble (pellet) and soluble (supernatant) fractions as seen in **Figure 7**. Along with the expression of the CTB-malarial monomer, the immunoblot displayed other forms such as dimers, trimers, tetramers, and pentamers. The expression levels in mature leaves of CTB FC AMA-1 and CTB MSP-1 protein reached 6.3-9.5% and 1.4-2%, respectively as shown in **Figure 8**. The expression level of CTB FC AMA-1 and CTB MSP-1 is comparable to CTB-Pins in lettuce of 1.8% TSP (Ruhlman, Ahangari et al. 2007) but higher levels were seen with 14.8% accumulation of F1-V (Arlen, Singleton et al. 2008), 14% of anthrax protective antigen (Koya, Moayeri et al. 2005), and 16% of CTB-Pins in tobacco (Ruhlman,

Ahangari et al. 2007). Although lower levels of expression were observed with the chloroplast-derived CTB-malarial proteins in tobacco it still provides a sufficient level of expression to proceed with animal or preclinical studies (Ruhlman, Ahangari et al. 2007).

The antigen administered to the mice *via* a subcutaneous injection was enriched versus purified because the CTB-malarial protein did not consist of an epitope tag for the facilitation of purification. CTB is known to bind to immobilized nickel ions (Dertzbaugh and Cox 1998) and this lead to enriching the CTB-malarial antigen in plant extracts using nickel beads. The CTB-malarial antigens were successfully enriched with nickel beads and to ensure adequate amounts for immunization studies, immobilized metal affinity chromatography using Talon Superflow Metal Affinity Resin was used. The oral-deliverable plant material administered to mice was done *via* oral gavage.

Majority of vaccines use the needle-based subcutaneous injection method but delivering the antigen *via* the oral route is a possibility. Following immunization, the immune titers of the mice were determined for mice receiving the antigen *via* the injectable route versus oral boosts. Immunization studies confirmed the chloroplast-derived malarial antigens were immunogenic with respect to anti-CTB and anti-MSP-1<sub>19</sub> antibodies (**Figure 12** and **Table 2**). The titers of groups 3 and 4 in regards to anti-AMA-1 titers were not completed because of the lack of AMA-1 protein. Also, the MSP-1 titers of groups 7 and 8 were not completed because of the lack of adequate amounts of MSP-1 protein. CTB was fused with the malarial antigens because of it's potential of acting as an

oral-mucosal adjuvant (Holmgren, Lycke et al. 1993) and also possibly enhancing the immune response (Daniell, Lee et al. 2001). The mice were found to be immunogenic in regards to CTB and the IgG1 titers ranged from 0 – 1:800,000 with higher titers in bleeds #3, #4, and #5. High titers were observed in experimental groups besides group 4 (oral delivery CTB FC AMA-1) and a likely reason for low IgG1 titers is because CTB IgA sera titers were significantly higher possibly suppressing the synthesis of IgG1 in these mice (data not shown). Mice titers for IgG1 MSP-1<sub>19</sub> ranged from 0 – 1:50,000 with higher titers in bleeds #3, #4, and #5 and higher titers in group 5 CTB MSP-1 (subcutaneous injection) versus group 6 (oral delivery). Several mice showed no detectable MSP-1<sub>19</sub> titers (two mice each in group 5 and 6) with ELISA analysis (**Table 2**) and a likely explanation is not every mouse responds to the same antigen in the same manner but these mice showed similar CTB titers in comparison with the other mice in the group. Higher titers were observed in group 5 compared to group 6 because of the challenge to deliver the exact amount of antigen in oral gavage to every mouse and titers are expected to be higher when antigen is directly introduced into the immune system *via* an injection. A reason why the titers for MSP-1 were significantly lower than CTB titers may be because malarial antigens generally display poor immunogenicity even with the use of an adjuvant (Sachdeva, Mohmmed et al. 2006). Several strategies for optimizing immunogenicity of malarial antigens include the use of different adjuvants, optimizing immunization protocols, using rabbits or monkeys for animal model testing, fusing exogenous viral or bacterial antigens, or constructing multivalent

antigen chimeras (Pan, Huang et al. 2004; Qian, Wu et al. 2007; Greenwood, Fidock et al. 2008). The *Pf* chimeric protein 2.9 (PfCP-2.9) consisting of domain III of AMA-1 and 19-kDa C-terminal fragment of MSP-1 was found to be highly immunogenic in rabbits and rhesus monkeys by inducing 11 to 18 fold higher titers than the individual antigens alone (Pan, Huang et al. 2004). IFA and immunoblots confirmed the presence of anti-AMA-1 and MSP-1 antibodies in the sera of immunized mice because of the recognition of parasite and parasite protein, as shown in **Figures 13, 14**. In the immunoblot analysis, both anti-AMA-1 and anti-MSP-1 antibodies recognized the ring and schizont stages and this follows the expression profiles of AMA-1 and MSP-1 provided by PlasmoDB. The same volume of the sera was used for both immunoblots but the intensity was greater with using anti-MSP-1 antibodies and this may be due to the titer of the mouse immunized with MSP-1 was higher than the mouse immunized with AMA-1. AMA-1 titers were not determined because of the lack of AMA-1 protein for ELISA analysis.

To investigate if the AMA-1 and MSP-1 antibodies mice generate after immunization prevents parasite invasion into RBCS, an *in vitro* parasite inhibition assay was performed. If anti-AMA-1 and anti-MSP-1 antibodies are present in the sera collected from immunized mice there should be a decrease in parasitemia indicating the inhibition of the parasites from invading RBCs. In the control groups, sera from unimmunized mice and mice immunized with wild type leaf material or adjuvant only, similar levels of parasitemia were observed compared to control wells incubated with no sera (**Table 3**). The relative percent

of inhibition in comparison to the positive control (MRA-35 *PfMSP1-19*) was determined for all control and experimental groups because the highest percent of inhibition observed for the positive control was 53%. In comparison to the positive control, relative percent of inhibition was found to be the highest in group five, subcutaneous CTB MSP-1, (117.2%, **Table 3**). Direct correlation of mouse titers and percent of parasite inhibition of invasion into RBCs was observed with a mouse with a high MSP-1<sub>19</sub> titer (1:50,000, **Table 2**) resulting in the greatest percent of inhibition (63.6%) in comparison with a mouse with lower titer (1:1,000, **Table 2**) resulting in only 47% inhibition (**Table 4**). Other studies have found higher percentages of inhibition (Pan, Huang et al. 2004; Arnot, Cavanagh et al. 2008) but they incubated parasite culture with purified IgG or isolated specific antibodies and did not use whole sera. A positive control, antibody from MR4 (MRA-35 *PfMSP1-19*), was included in the assay to achieve the greatest possible percent of inhibition but only resulted in 53%. The positive control has no known published antibody titer and since it was produced in rabbit the titer was not determined because the titer cannot be directly correlated with the titer of the immunized mice because the lack of a comparative secondary antibody. The results provided from the positive control inefficiently prevented the maximal percent of invasion, which proceeded to the experimentation of using different dilutions of the antibody and examining a direct comparison of parasitemia levels. The most diluted concentration of antibody resulted in about half of the percent of inhibition compared to the antibody incubated with the parasite culture at 20% (**Table 5**). Both *in vitro* parasite inhibition assays displayed greater inhibition with

higher immune titers or antibody concentration compared to lower titers and concentration of antibody with less inhibition. Although the *in vitro* inhibition assay provided evidence that the antibodies generated from mice were effective in preventing parasite invasion of RBCs, a more accurate quantification needs to be assessed by using an appropriate animal model and a lethal parasite challenge (Sachdeva, Mohmmed et al. 2006) because the mice were challenged with a rodent malaria strain (*Plasmodium berghei*) that was generated to express the *P. falciparum* form of MSP-1<sub>19</sub> (de Koning-Ward, O'Donnell et al. 2003) and the challenge was unsuccessful. One report was found to be successful in protecting the mice after the parasite challenge with the *P. berghei/P. falciparum* chimeric line (Pb-PfM19) but they passively immunized the mice with anti-*PfMSP-1*<sub>42</sub> IgG purified from rabbit sera before parasite challenge instead of using active immunization (Sachdeva, Mohmmed et al. 2006).

## CONCLUSIONS

Malaria is a prominent vector-borne parasitic disease and severe public health problem globally, especially prevalent in poor-developing countries. There is a great need to create a low cost human malarial vaccine with the elimination of laborious purification techniques and technical skills. Two leading blood stage malarial vaccine candidates AMA-1 and MSP-1 were constructed in a fusion cassette with CTB. The CTB-malarial antigens were expressed in tobacco plants *via* plastid transformation and accumulated to high to moderate levels in CTB FC AMA-1 and CTB-MSP-1 to 9.5% and 2% of the total soluble protein, respectively. The chloroplast-derived CTB-malarial proteins were administered to mice by subcutaneous injection and oral gavage. The immunogenicities of the CTB and MSP-1<sub>19</sub> antigens were determined to be in the range of 1:1000 – 1:800,000 and 1:100 – 1:50,000 in mice titers, respectively. To maximize the MSP-1 titers in oral delivery of CTB MSP-1, there needs to a better approach on delivering the same quantity of antigen to every mouse. Sera collected from mice immunized with both CTB FC AMA-1 and CTB MSP-1 was found to recognize native parasite and native parasite protein *via* IFA and immunoblot analysis, indicating anti-AMA-1 and anti-MSP-1 antibodies were generated in immunized mice. Anti-malarial antibodies were found to inhibit parasite invasion into erythrocytes with sera collected from immunized mice. The lack of an appropriate animal model needs to be established before *in vivo* challenge and protection can be investigated. Results of these investigations may lead to further experimentation in malarial vaccine development with other malarial antigens and in edible crops.

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