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Characterization And Evaluation Of The Immunogenizity Of Chloroplast-Derived 19-Kilodalton C-Terminal Merozoite Surface Antigen 1 (MSP1) of Plasmodium Yoelii Yoelii

Sushamadevi Kamarajugadda
University of Central Florida

CHARACTERIZATION AND EVALUATION OF THE IMMUNOGENICITY OF CHLOROPLAST-DERIVED 19-KILODALTON C-TERMINAL MEROZOITE SURFACE ANTIGEN 1 (MSP1) OF *PLASMODIUM YOELII YOELII*

by
Sushama Kamarajugadda
B.Sc. (Ag.), A.P. Agriculture University, 2003.

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ABSTRACT

Malaria is a protozoan disease caused in humans by four different species of the genus *Plasmodium* (*P. falciparum, P. vivax, P. ovale, P. malarie*) and in rodents by *Plasmodium yoelii yoelii*. It has been reported that 1.5 to 3 million deaths occur worldwide due to malaria and the DALY (Daily affected life years) reports about 0.76% of world population affected by the disease in some of the major countries like Africa, Asia, Latin America etc., Due to the development of resistance to drugs by the parasite, there is an urgent need and prime importance for the development of an effective vaccine against malaria. During its entire life cycle, the *plasmodium sp.* expresses various stage-specific proteins that are considered potential candidates for vaccine development; the major ones belong to the (i) sporozoite, (ii) erythrocytic, (iii) gametocytic stages. Merozoite surface protein 1 (MSP1) is expressed on the surface of the parasite during the erythrocytic stage, which is considered as a potential vaccine candidate. The C-terminal portion of MSP1 is considered to be an effective vaccine candidate from inhibiting the parasite invasion into RBC.

PyMSP1$_{19}$ has been expressed in plants via the chloroplast transformation. The site-specific integration of PyMSP1$_{19}$ gene within chloroplast genome was confirmed by PCR using specific primers and the percentage of homoplasmy vs. heteroplasmy was confirmed by Southern blot. The expression of chloroplast-derived PyMSP1$_{19}$ plants was

confirmed by western blot using anti- PyMSP1$_{19}$ antibodies. These experiments showed a 17kDa protein under reducing conditions. The expression levels of PyMSP1$_{19}$ protein varied within transgenic plants were up to ~2% of total soluble protein (TSP) within mature leaves. To test the functionality of chloroplast-derived PyMSP1$_{19}$ protein, mice were immunized with the enriched chloroplast-derived PyMSP1$_{19}$ protein with Freund's adjuvant. The immune response of anti- PyMSP1$_{19}$ antibodies were tested against standard PyMSP1$_{19}$ protein and it yielded 1:7000 IgG titers. The immunized mice were challenged with *P.yoelii* infected red blood cells (35-40% parasitemia) and the percentage parasitemia suggested an inverse correlation with the immune titers. However, the concrete conclusions can be made when the study is extended to a larger animal group.

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LIST OF ACRONYMS/ABBREVIATIONS

aadA- Aminoglycoside 3' adenosyltransferase

DALY- Daily Adjusted Life Years

EDTA-Ethylene Diamine tetraacetic acid

ELISA- Enzyme Linked Immunosorbent Assay

FPLC- Fast Performance Liquid Chromatography

GST- Glutathione S- transferase

KMW- Kilo Molecular Weight

KDa- Kilo Dalton

PBS- Phosphate Buffered Saline

PMSF-Phenyl Methyl Sulphonyl Floride

Pf- *Plasmodium falciparum*

PyMSP1_{19}- *Plasmodium yoelii yoelii* Merozoite surface antigen 1_{19}

psbA- Photosystem b/A

SDS-PAGE – Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

TAE- Tris Acetate EDTA

TE- Tris EDTA

TSP- Total soluble protein

UTR- Untranslated region

INTRODUCTION

Malaria is a protozoan disease caused in humans by four different species of the genus *Plasmodium* (*P. falciparum, P. vivax, P. ovale, P. malarie*) and in rodents by *Plasmodium yoelii yoelii*. This disease is most widely prevalent in parts of Asia, Africa, central and south America, Oceania and certain parts of the Caribbean islands, causing 1.5 to 3 million deaths; it has also been recorded that annually $12 billion has been spent on this disease in Africa alone (Breman JG *et al*., 2004). According to DALY report, 0.76% of people are affected by malaria worldwide and the mortality rate is high among young children and pregnant women (http://www.who.int/en/).

Life cycle of Plasmodium sp. and its pathophysiology

The female Anopheles mosquito transmits malaria from person to person. It acts as a host for the multiplication of plasmodium sp. When a mosquito bites a human, sporozoites in the mosquito's saliva enter the blood circulation. *P. falciparum* is an obligatorily intracellular parasite within the human host. It first invades liver cells and then goes on to invade differentiated erythrocytes. The sporozoites migrate in the blood stream and reach the liver cells where they divide, multiply and mature in schizonts. Later the schizont ruptures to release the merozoites in the blood circulation. The merozoites then enter the RBC referred as the erythrocytic phase, where they undergo an asexual cycle of reproduction or develop into sexual forms known as gametocytes (Suh *et al.,* 2004). The gametocytes then enter the gut of female anopheles mosquito during

ingestion of blood from the humans. Within the gut of mosquito, the micro and macro gametocytes differentiate into micro and macrogametes respectively, which eventually fuse to form a zygote, which then becomes a motile and invasive ookinete. The ookinete pierces through the wall of the gut and forms oocyst at the basal lamina of the gut wall. Here it multiplies numerous times and forms thousands of sporozoites which are then released. Later they migrate to the salivary glands, from there they enter the human host while feeding on the blood (Lingelbach et al., 2004).

Clinical manifestations of Malaria

The most common symptoms experienced by malaria patients are fever, chills and headaches. Other common symptoms include dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, mild diarrhea, and dry cough. Physical signs include fever, tachycardia, jaundice, pallor, hypotension, hepatomegaly, splenomegaly and anemia due to destruction of red blood cells. The complications of severe malaria include cerebral malaria, pulmonary edema, acute renal failure, severe anemia, and/or bleeding. Acidosis and hypoglycemia are the most common metabolic complications (Trampuz et al., 2003). All these symptoms reoccur for every attack and eventually patient becomes weak. If untreated, the effect of *P.falciparum* might lead to cerebral malaria where the patient goes to prolonged coma. The clinical manifestations of malaria in rodents are very much similar to human malaria affecting lungs, liver and spleen; the symptoms of cerebral malaria have also been shown (Medana *et al*., 2001).

Need for malaria vaccines

Commonly used drugs against malaria, such as chloroquine, primaquine, and artemisinin, are becoming ineffective due to the development of resistance by parasite. This could be due to mutation in the drug target, as a result, the drug no longer binds or recognizes the target and the other mechanism is to increase the expression of target either by enhancing the transcription and translation processes or amplify the gene, which makes the availability of drug as a rate limiting factor to inhibit the target (http://www.tulane.edu/wiser/malaria/fv.html). Therefore, there is an urgent need for an effective vaccine development to eradicate this problem. However, some major concerns for designing an effective malaria vaccine include

(i) the expression of antigen that is developmentally regulated; (ii) variation in the antigens of different parasite isolates; (iii) Individuals are non-responsive to certain parasite antigens or epitopes (Tine JA *et al*., 1996).

Targets for Malaria vaccine

During invasion of RBC, the parasite exhibits various apical organelles like rhoptries, dense granules and micronemes (Preiser P *et al*., 2000, Sam-Yellowe, 1996). These help in proper penetration, adhering to the merozoite, re-orientating the apical end to get in contact with the cell, discriminating the host-cell, and ultimately forming a tight junction to penetrate into the cell (Barnwell *et al.,* 1998). Specific interaction between parasite proteins and host-cell receptors occurs at all stages (Shahid *et al*., 2001) and

these stage-specific proteins are the major targets for vaccination. They are divided into (i) the pre-erythrocytic-liver stage, (ii) the asexual blood stage, and (iii) transmission-blocking vaccines (Ya Ping Shi *et al.,* 1999). The elicited immune responses by all these stage-specific proteins are equally complex, and result in humoral and cell-mediated responses (Weidanz and Long, 1987).

Therefore, the major focus of vaccine development is to identify an antigen or protein of value especially from asexual stage. Such antigens such merozoite surface protein, rhoptries which are on the surface of the infected red blood cell are considered to be effective, efficacious vaccine candidates (Ander *et al*., 1993). Attacking such target antigens would effectively curb the host pathogen interactions. Proteins on the surface of the merozoites are considered to be targets of the immune response, which include merozoite surface protein 1 (MSP1; Cooper JA, 1993), MSP2 (Smythe *et al*., 1988) and Apical membrane antigen (AMA1; Peterson *et al*., 1989).

Merozoite surface protein (MSP1)

During schizogony stage, the precursor to the MSP1 protein is present on the surface of merozoites, which undergoes proteolytic processing (Holder, 1988). In *P.falciparum*, the molecular mass of MSP1 protein ranges from 185-250kDa and helps in erythrocytic invasion (Blackman *et al*., 1990). MSP1 in rodent malaria is about 230 kDa in size, and has been reported that the monoclonal antibody produced against the C-terminal portion of this protein, was able to protect mice from parasite challenge (Spencer Valero *et al*., 1998). It has also been reported that the amino acid sequence of the rodent

merozoite surface protein has signal peptide, GPI sequence to adhere to the membrane, tandem repeats of tetrapeptides (Gly-Ala-Val-Pro) and the C-terminal portion with a series of 10 cysteine residues (Burns *et al*., 1988). At the amino acid level, the 193 kDa *Plasmodium falciparum* analogue shows an overall homology of approximately 30%, with 60% areas highly conserved (Lewis, 1989). The 230 kDa MSP1 protein undergoes proteolytic processing to 83 kDa during erythrocyte invasion (Cooper, 1993), which eventually gets cleaved to 16- 19 kDa protein (Blackman *et al*., 1990). It has been reported that the C-terminal portion of the 42 kDa fragment and the 16 to 19 kDa polypeptide subfragment (MSP1-19) are highly immunogenic and that the MSP1-mAb reacts with the reduction sensitive epitopes present on malaria parasites (Lyon *et al.,* 1987). On the basis of the merozoite inhibition assays, the C-terminal 19kDa protein has been proved to be a leading vaccine candidate antigen against blood stages of malaria (Blackman *et al*., 1990, Chappel and Holder 1993, Daly and Long 1993, Kumar *et al*., 1995). The protective role for $MSP1_{19}$ – specific antibodies produced in response to infection or vaccination with recombinant proteins has been proved by conducting accurate quantification assays such as ELISA, IFA (O'Donnell *et al*., 2001, Saul *et al*., 2001, De-Koning- Ward *et al*., 2003). In order to remove the non-specific inhibitory factors and increase the sensitivity to use minimal volumes of test serum, high-throughput inhibition assays for anti-malaria antibodies are being developed (Persson et al., 2006)

The 19 kDa C-terminal portion of MSP1 protein was found to be highly conserved among different isolates and among different species, with slight variation at four different amino acid positions (Lalitha *et al.,* 1999). MSP1-19 has been expressed in *E.*

coli (Burghaus *et al.*, 1994, Burns *et al*., 1989), yeast (Kaslow *et al*., 1994) and its immunogenic property has been studied in mice (Burns *et al*., 1989, Daly and Long 1993) and monkeys (Kumar *et al*., 1995). It has also been shown in neutralization assays that anti-$MSP1_{19}$ antibodies inhibit the infection at blood-stage involving individuals naturally exposed to malaria, and they might correlate with the protection offered by MSP1-based vaccines in clinical trials (John *et al*., 2004). Oral immunization of mice with *E. coli* expressed C-terminal 19 kDa *Plasmodium yoelii* MSP1 induced immunoprotective levels of antibodies. However, immunization studies carried with the combination of two proteins (PyMSP1-19 and PyMSP4/5) conferred better protection as compared to that offered by either protein alone (Wang *et al*., 2004). Although the oral immunization studies have proven to be promising, the extensive purification and safety measures for *E. coli* or yeast- expressed proteins are always limiting factors. Therefore, an effective expression system that can provide a clean and safe vaccine is required.

Plants for Malaria vaccine production

The alternative system for the expression of safe, efficacious malaria vaccine antigen is considered to be plants. The plant expression system may be ideal for producing vaccines since, large amounts of vaccine can be produced at low cost. Plants can ensure proper post-translational modification as seen in higher organisms (Ma *et al*., 1995). Therefore, plants are used for expressing various vaccine antigens. Malaria epitopes have been expressed in plant viruses like TMV (tobacco mosaic virus) (Turpen *et al*., 1995) and the C-terminal portion of MSP1 of *Plasmodium falciparum* has also

been expressed via nuclear transformation in tobacco with expression levels up to 0.0035% of TSP (Ghosh *et al*., 2002). Such low expression levels of this protein prevented further functional studies. Therefore, an effective, alternative approach is needed to enhance the expression levels and also to ensure proper conformation of $MSP1_{19}$ protein to be functional.

Chloroplast genetic engineering

The chloroplast transformation technology unfolded in the 80s (Daniell and Dhingra, 2002). One of the very first chloroplast genome complementation was reported in the unicellular green alga *Chlamydomonas reinhardtii* having a single chloroplast (Boynton *et al*., 1988). The photosynthetic mutants lacking the *atpB* gene were complemented by targeting the *atp* gene into chloroplast genome via homologous recombination (Klein *et al*., 1987).

The integration of *uidA* gene encoding the beta glucoronidase flanked by chloroplast DNA sequences in the *C. reinhardtii* chloroplast genome was shown; although no protein expression was observed (Blowers *et al*., 1989). Hence, the chloroplast technology has been extended to higher plants by introducing foreign genes into isolated plastids (Daniell and McFadden, 1987).

Chloroplast transformation with the foreign genes was done transiently by culturing the plastid cells of tobacco using autonomously replicating chloroplast vectors (Daniell *et al*., 1990), which was further extended to wheat leaves, calli and somatic embryos (Daniell *et al*., 1991). However, the major breakthrough was the introduction of

spectinomycin or streptomycin resistant gene coded by *aadA* into the chloroplast genome of the *C. reinhardtii* (Goldschmidt-Clermont, 1991).

After the successful integration of selectable marker gene and isolating the transformants under selection, the focus was laid on optimizing the expression protocols. At the beginning, the foreign genes were targeted into the transcriptionally silent spacer regions of the chloroplast genome (Zoubenko *et al*, 1994). Later, the transgenes were inserted in transcriptionally active spacer regions (Daniell *et al*., 1998). The introduction of multigene operon (Quesada *et al.,* 2005) has facilitated the expression of several agronomic traits, biopharmaceutical proteins and vaccines (Chebolu *et al.,* 2005, Grevich *et al*., 2005). Majority of therapeutic proteins have been expressed in tobacco such as human interferon (Daniell *et al.,* 2004a, Leelavathi and Reddy *et al.,* 2003), human somatotropin (Staub *et al*., 2000) and human serum albumin (Fernandez *et al.,* 2003). Recently the technology has been extended to other commercial crop species such as carrot, cotton, tomato, potato, soybean and lettuce by transforming the plastids (Kumar *et al*., 2004a, Kumar *et al*., 2004b, Ruf *et al*., 2001.; Sidorov *et al*., 1999; Dufourmantel *et al*., 2004, Kanamoto *et al*., 2006, respectively).

The genetic manipulation is quite amenable in tobacco plant and also yields enormous biomass (40 tons fresh leaf weight/acre biomass per season). The tobacco plant is produces seeds in large numbers (up to one million seeds produced per plant). The chloroplast technology has been well exploited in tobacco by expressing various vaccine antigens and therapeutic proteins. A monoclonal antibody Guy's 13, against *Streptococcus mutans* has been expressed via the chloroplast transformation (Daniell *et al*., 2001b).

Some of the agronomic traits have been studied via chloroplast technology such as insect resistance (*Bacillus thuringiensis* (Bt), the Cry2Aa2 protein), which reported the highest protein expression of up to 46.1% tsp to date (DeCosa *et al*., 2001), herbicide resistance against Glyphosate(*EPSPS* gene; Daniell *et al*., 1998), the antimicrobial peptide MSI-99, an analog of magainin (De gray *et al*., 2001), which inhibited growth of *Pseudomonas aeruginosa*, multi-drug resistant bacteria and *Pseudomonas syringae*, a major plant pathogen (DeGray *et al*. 2001, Devine and Daniell, 2004, Daniell *et al*., 2004a), drought tolerance (the yeast trehalose phosphate synthase (TSP1); Lee *et al*., 2003), salt tolerance in carrot(BADH gene; Kumar *et al*., 2004a).Also, the cytoplasmic male sterility (Ruiz *et al*., 2005) eliminates the transfer of foreign gene via the chloroplast transformation.

Vaccine antigens Expressed Via the Chloroplast Genome

The advantages of expressing vaccine antigens in the chloroplast genome are: expression of subunit peptides that are non-toxic and do not multiply; due to the prokaryotic nature of chloroplast, the bacterial genes with AT content are expressed at high levels in the chloroplast; and oral delivery of vaccines induces high mucosal and high systemic responses, and helps the system to combat against germs at portals of entry. Some of the vaccines that have already been expressed in the chloroplast include C-terminus of *Clostridium tetani* (TetC) (Maliga P, 2003), the Cholera toxin B-subunit (CTB), which does not contain the toxic component that is in CTA (Daniell *et al*. 2001a), the 2L21 peptide from the Canine Parvovirus (CPV) (Molina *et al*., 2004), F1~V fusion

antigen for plague (Singleton *et al*., 2003), the protective antigen of *Bacillus anthracis* without lethal and edema toxins (Watson *et al* 2004, Koya *et al*., 2005), Lec A antigen for amebiasis (Chebolu *et al*., 2006), NSP4 protein for rotavirus vaccine (Kalluri *et al.,* 2005). The expression levels of CTB was up to 4 to 31% of Total Soluble Protein (TSP) and the G_{M1}-ganglioside binding assays have shown the proper folding and formation of disulfide bonds in chloroplast-derived CTB to form functional pentamers that recognized the GM1 receptors (Daniell *et al*. 2001a, Molina *et al*., 2004). The anthrax protective antigen (PA) was expressed at a maximum value of 14.2% TSP and at this rate, 360 million doses of anthrax vaccine could be obtained from an acre of land, which is sufficient for the entire U.S. population (Koya *et al*., 2005). The protective antigen of *Bacillus anthracis* expressed in tobacco chloroplast was shown to be immunoprotective in mice immunized with purified PA83 derived from tobacco chloroplasts along with adjuvant (Koya *et al*., 2005). The intranasal delivery of chloroplast-derived TetC produced both IgG and IgA and shown to be immunoprotective against toxin challenge (Tregoning *et al*., 2003). The expression levels of chloroplast-derived 2L21 peptide from Canine Parvovirus (CPV) fused to GFP showed up to 22% of TSP, and that fused to CTB showed up to 31% TSP (Molina *et al*., 2004)**.**

Bioencapsulation for the oral delivery of vaccine antigens and protection of immunogens in the alimentary tract

The oral delivery of pharmaceutical proteins into the body can be successful if the intact protein can be delivered without degradation by the digestive enzymes present

within the stomach. The ability to protect the protein against digestive juices is referred to as bioencapsulation (Walmsley and Arntzen, 2000; Yu and Langridge, 2001). The nuclear expression of Heat-labile enterotoxin B-subunit (LTB) from *E. coli* in tobacco and potato yielded <0.01% Total soluble protein and 0.19% TSP respectively. When administered orally, the LTB expressed in potato was found to be immunoprotective. Although expressed in lower amounts, the antigens expressed in tobacco were immunogenic (Haq *et al*., 1995, Mason *et al*., 1996, Tacket *et al*., 1998). Another vaccine antigen for Norwalk vaccine (the capsid protein) expressed in potato and tomato via the nuclear transformation was immunogenic, when delivered orally (Mason *et al*., 1996, and Richter *et al*., 2000, Tacket *et al*., 2000). The nuclear expression of the envelope surface protein of hepatitis B virus in tobacco, potato and lupin yielded less than 0.01% fresh weight but were immunogenic, though not protective when administered orally (Richer *et al*., 2000, Kapusta *et al*., 1999). These findings prove that the higher expression level of therapeutic proteins is required in plants to observe the therapeutic effects. When given orally, IFN-alpha showed biological activity in humans and other animals (Bocci 1999). Commercial and native animal trials have been done with plant-derived edible vaccines (Castanon *et al*., 2000, Tuboly *et al*., 2000). Therefore, the concept of bioencapsulation is important in protecting these vaccine antigens against digestive enzymes and ensures their biological activity. As a result, the chloroplast technology is currently being extended to other edible crop species such as potato, tomato, carrot and soybean (Sidorov *et al*., 1999, Ruf *et al*., 2001, Kumar *et al*, 2004a, Dufourmantel *et al*., 2004) for oral delivery.

The oral delivery of the foreign proteins expressed in chloroplast needs to be

delivered intact into the body for the complete therapeutic effect. Crossing the mucosal barrrier and resisting the acid digestion are the prime limiting steps for the oral delivered therapeutic proteins. For this a prototype molecule CTB-GFP molecule was designed and expressed in tobacco chloroplasts. Chloroplast-derived GFP was given orally to evaluate the transmucosal carrying ability and bioencapsulation of the chloroplast derived proteins. The Chloroplast-derived CTB acts as a transmucosal carrier by binding the GM1 ganglioside and also the protein molecule was protected from peptidases and/or acids by bioencapsulation within the plant cells. The rapid turnover of intestinal epithelial cells (Heath, 1996) for recycling GM_1 receptors makes this approach a reality (Limaye *et al*., 2006), thus creates an opportunity for the cheaper production and delivery of human therapeutic proteins. Recent reports on complete chloroplast genomic analysis of different crop species like grape (Jansen *et al*., 2006), cotton (Lee *et al*., 2006), potato & tomato (Daniell *et al*., 2006), legume crops like soybean (Saski *et al*., 2005), carrot (Kumar *et al*., 2004), lettuce (Kanamoto *et al*., 2006), etc., provide the basic understanding of chloroplast genome in different species. This information might help in designing appropriate vectors containing the plant regulatory sequences and also optimize the tissue regeneration on the growth media after the post bombardment (Tissue regeneration) for these crops, stepping towards the oral delivery.

Advantages of Chloroplast Expression versus Expression in E. coli

The major disadvantages with *E.coli* expression system over chloroplast expression system are as follows: 1) *E.coli* system involves expensive steps for the

production of recombinant proteins in microorganisms, demands stringent purification protocols and requires costly fermenters. All these problems can be overcome by vaccine production in plants, which involves a straightforward farming, fairly inexpensive, and can lower the cost in one season.

2) When compared with *E.coli* fermentation expenses, the production cost for recombinant proteins in tobacco is 10 to 50 times cheaper (with 20% expression levels in *E. coli*, Kusnadi *et al*., 1997).

3) The existing technologies for harvesting, storage, and purification of transgenic plant proteins, keeps the plant proteins within transgenic lines protected.

4) The risk of contamination with bacterial and animal pathogens is eliminated from plant-derived vaccines because the plants do not serve as host for humans pathogens unlike the bacterial and animal systems.

Furthermore, chloroplasts are meant to maintain the integrity of the expressed protein i.e., they ensure proper folding of proteins and maintain their natural conformation with correct disulfide bond formation (as seen in CTB; Daniell 2001a). The G_{M-1} ganglioside binding assays demonstrated that chloroplast-synthesized CTB is correctly folded with required disulfide bonds as it forms the pentameric stucture, which recognizes the G_{M-1} ganglioside receptors. The immunogenic properties of many chloroplast-derived vaccine antigens have been proven (Tregoning *et al*., 2003, Molina *et al*., 2004, Koya *et al*., 2005).

Also, the expensive purification process of recombinant protein produced in *E. coli* is the limiting factor. For example, the overall insulin production accounts for 30% of the production cost and 70% of the set-up cost (Petrides *et al*. 1995). Although, the

transformed tobacco plants require purification processes but the production of edible vaccine in carrot or tomato would eliminate this step and so are the expenses for delivery of vaccine. Therefore, extending the production of protective Antigen in carrot plastids for oral vaccination would be ideal.

Advantages of Chloroplast Transformation over Nuclear Transformation

Due to the maternal inheritance of transgenes in chloroplast transformation, the major concern of cross-pollination from genetically modified crops to wild type crops is eliminated. Thus, the transgene containment is ensured by the property of maternal inheritance (Daniell, 2002, Daniell and Parkinson, 2003).

For the commercial availability, the transgene expression in plants must be greater than 1% (Kunsnadi *et al*., 1997). Nuclear transformation of plants has usually produced lower expression levels of antigens (Daniell *et al*., 2001a, May *et al*., 1996, Richter *et al*., 2000, Tacket *et al*., 2000, Ramirez *et al*., 2003, Devine and Daniell, 2004, Daniell *et al*., 2004b, Daniell *et al*., 2004c). The oral delivery of vaccine demands high expression of the protein to elicit adequate immune response. Therefore, an alternative approach such as chloroplast transformation has been adopted, which yields high protein expression levels due to an increased number of chloroplast genomes per cell (up to 10,000). This results in high transcript and abundant translated product of the gene of interest (up to 47% of Cry2Aa2 ;DeCosa *et al*., 2001).

The site-specific integration of gene by homologous recombination in chloroplast eliminates position effect and gene silencing as opposed to nuclear transformation

(Grevich and Daniell, 2005).. In nuclear transformation, the random integration of transgene results in unstable and inconsistent gene expression or complete loss of transgenic activity due to insertion of regulatory sequences in a repetitive pattern. Also, the random recombination results in variable levels of transgene expression (position effect) and gene silencing (Fagard and Vaucheret, 2000). A very high accumulation of transcripts (169-fold higher than nuclear transgenic lines) has shown no gene silencing (Lee *et al*., 2003, Dhingra *et al*., 2004). Similarly, no gene silencing was seen at the translational level regardless of the accumulation of foreign protein up to 47% TSP (DeCosa *et al*., 2001). Another major advantage of chloroplast genetic engineering is the ability of chloroplast to transcribe operons and translate the polycistronic mRNA. (DeCosa *et al*., 2001, Ruiz *et al*., 2003, Daniell and Dhingra, 2002, Lossl *et al*., 2003, Quesada-Vargas *et al*., 2005).

Advantages of Producing the Malaria Vaccine in Plant Plastids

1. No human or animal pathogen contamination has been reported in plant expressed proteins due specific Pathogen-host specificity (Streatfield *et al*., 2001). Therefore, $PyMSP1_{19}$ protein in plants would yield a vaccine free of pathogens.
2. The $PyMSP1_{19}$ antigen genes have an A/T content of 65.14%, suitable for prokaryotic environment of chloroplast.
3. Chloroplast ensures proper folding of the protein with correct disulfide bonds as it has protein disulfide isomerases and thioredoxins (Kim et al., 2003). It has been shown in earlier studies such as CTB (DeCosa *et al*., 2001,) at 33% TSP (Daniell *et al*., 2001a),

the Human serum albumin (Fernandez-San Millan *et al.*, 2003), Interferons (Leelavathi and Reddy, 2003, Daniell et al., 200). Thus, the expression of $PyMSP1_{19}$ in chloroplast genome would ensure proper disulfide bond formations.

4. The $PyMSP1_{19}$ antigen is not glycosylated, which is good for our particular system because plastids do not glycosylate proteins.
5. Plants have up to 100 chloroplasts per cell, each containing about 100 chloroplast genomes. This provides up to 10,000 genomes per cell to efficiently produce the antigens.
6. Chloroplast eliminates cross-pollination of the transgene because of being maternally inherited (Daniell, 2002, Daniell and Parkinson, 2003).
7. Gene silencing and position effect is eliminated due to the integration of trangenes in the intergenic spacer sites of the chloroplast genome (Daniell *et al*., 2002; Grevich and Daniell, 2005).
8. Bioencapsulation has ensured protection of vaccine antigens in the stomach and has also proven to be immunogenic when administered orally in clinical trials (Tacket *et al*., 1998, Kapusta *et al*., 1999, Tacket *et al*., 2000, Castanon *et al*., 2000, Tuboly *et al*., 2000, Walmsley and Arntzen, 2000, Tacket *et al*., 2003 Tacket *et al*., 2004).
9. The success of producing vaccine antigens in tobacco could be extended to other edible crop species such as carrot and tomato. Delivery of plant-derived vaccine to mucosal tissues has been shown to induce both mucosal and systemic immune responses (Haq *et al*.1995., Mason *et al.*1996, Arakawa *et al*., 1998). Mucosal immunization may give higher protection from aerosolized spores of anthrax by producing s-IgA in the mucosal system.

RATIONALE AND APPROACH

The primary objective of the project is to express and characterize $PyMSP1_{19}$ protein in tobacco chloroplast followed by the evaluation of the immunogenicity of chloroplast-derived $PyMSP1_{19}$ protein in the mouse model. If the protein is immunogenic in nature, their ability to inhibit the parasite invasion of RBC will be tested. To achieve these goals, transgenic lines had to be obtained that express $PyMSP1_{19}$ protein with correct folding and proper disulfide bond formation required for its native conformation and formation of epitopes. Since the chloroplast cell has protein disulfide isomerases and thioredoxins (Kim *et al.,* 2002), which facilitated the disulfide bond formation in various vaccine antigens expressed earlier (CTB; Daniell *et al* 2001, Human interferon; Leelavathi and Reddy 2003, Human serum albumin; Fernandez *et al*., 2003, Human somatotropin; Staub *et al*., 2000) we decided to express $PyMSP1_{19}$ protein in the chloroplast genome under similar assumptions. The strategy for primer designing and performing polymerase chain reaction using primers to introduce $PyMSP1_{19}$ gene is as described in Materials and Methods section (page 43). The bacteriophage g10 5'UTR with no promoter but a ribosomal binding region ensures the translation of $PyMSP1_{19}$ protein, is introduced upstream the gene of interest. The $PyMSP1_{19}$ gene is transcribed under the control of prrn promoter present upstream the gene. The gene product will be confirmed and purified. The purified protein will then be evaluated for its immunogenic property in the mouse system. If successful, the mice will be challenged with the parasite

P.yoelii to observe the protection offered against the infection by chloroplast-derived PyMSP1$_{19}$- specific antibodies.

MATERIALS AND METHODS

General protocols

Preparation of Ultracompetent cells (Rubidium Chloride Method)

The ultracompetent cells were prepared by using rubidium chloride method (http://www.neb.com/nebecomm/tech_reference/protein_expression) from XL1 MRF Blue (Stratagene). From *E.coli* glycerol stock , some culture is taken and streaked on the LB agar plate containing 12.5 µg/ml tetracycline and incubated at 37°C overnight . The next day, a single colony is grown in 5 ml Psi broth with 12.5 µg/ml tetracycline and incubated at 37°C at 225rpm for 12-16 hr in a horizontal shaker. About 1 ml of the culture was inoculated in 100 ml of Psi broth and incubated in 37°C shaker for 2 hours at 225rpm. After two hours, the optical density (O.D) was checked at 550 nm for two hours and subsequently for every 30 to 60 minutes until it reached 0.48 O.D. Later, the culture was kept on ice for 15 minutes and centrifuged at 3000g/5000 rpm for 5 minutes in a sorvall centrifuge. The pellet was resuspended in 0.4 volume (40 ml) of ice cold TFB-I solution, discarding the supernatant. The centrifugation step was repeated, followed by resuspension of cells in 4 ml of TFB-II solution and kept on ice for 15

minutes. Finally, the suspension aliquoted into 100 µl and freezed in dry ice/liquid nitrogen to be stored at - 80°C.

Transformation of Competent E. coli XL1-Blue cells

Competent cells prepared as above (100 µl aliquots) were taken from -80°C and thawed on ice to transfer to a falcon tube. About one µl (100 ng) of plasmid DNA was added to the cells and the mixture was incubated on for 30 minutes on ice with a gentle mixing after first 15 min. Then heat shock treatment was given at 42°C in a water bath for 90 to 120 seconds and kept on ice for two minutes. Later, the cells were incubated with 900 µl of LB broth at 37^{o}C horizontal shaker for 45min at 225 rpm. The cells were centrifuged at 13,000 rpm for 30 seconds. The supernatant with 900 µl was discarded and remaining 100µl of cells were resuspended. The cell suspension was spread on agar plate with selection media in two different volumes i.e., 50µl and 100µl..

Isolation of Plasmid DNA by Alkaline lysis

The colonies were picked from the LB-agar plate and grown in LB-culture with selection media for 12-16hrs at 37^{o}C shaker. The grown culture is taken into an eppendorf tube at 1.5ml volume and centrifuged at 13,000 rpm for 1 minute. The pellet was resuspended in 150 µl of Solution I (GTE: 50 mM Glucose, 10 mM EDTA, 25 mM Tris, pH- 8) after discarding the supernatant. Each tube was added with 1 µl of RNase (100 mg/ml). Later, 150µl of Solution II (0.2N NaOH, 10% SDS) was added to each tube

and mixed gently by inverting the tube 6 times. After that, Solution III (60 ml of 5M Potassium Acetate, 11.5 M glacial acetic acid, 28.5 ml sterile dH_2O) was added in the same volume and mixed gently for 6 times. Then, the mixture was centrifuged at 13,000 rpm for 10 minutes at 4°C followed by, transfer of supernatant into a fresh eppendorf tube with care to exclude white debris (i.e., bacterial chromosomal DNA/SDS/membrane proteins). Then, the plasmid DNA was pelleted by adding 900 µl of ice cold, ethanol (95%) and centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant was removed and discarded, without disturbing the plasmid DNA pellet at the bottom. Further washing of the pelleted DNA was done using 400µl of 70 % chilled ethanol and (without mixing) centrifuged for 5 minutes. Subsequently, the pellet was dried in the speedvac on medium heat for 3-5 minutes and resuspended in TE (pH 8.0). The concentration and the quality of plasmid DNA was measured by spectrophotometer. The isolated plasmid DNA was run on a 0.8% agarose gel for 40 minutes at 80 volts to confirm the successful isolation of plasmid (Sambrook *et al*., 1989).

Construction for pLD-g10-PyMSP119 vector for transformation of tobacco Chloroplast

Nde1 and NotI restriction sites were introduced by polymerase chain reaction on 5' and 3'end of pRTL vector with $PyMSP1_{19}$ by using primers: Forward 5'-GCCCaTATGGATGGTATGGAT-3'(engineered Nde1 site) and Reverse 5'-TCAgCggccgCtCaGCTGGAGGA-3' (engineered stop codon and Not1 site) (Invitrogen). The plasmid pRTL with $PyMSP1_{19}$ was used as template to obtain the PCR product of

size 313 bp containing Nde I restriction site at the 5'end and NotI restriction site at the 3' end. For a 50 µl reaction volume, the PCR was set as follows: 100 ng of plasmid DNA, 5 µl of 10X pfu buffer, 5 µl of 2.5 mM dNTP, 1 µl of each 10 mM primer, 0.5 µl pfu DNA polymerase and H_2O to make up the final volume. The PCR cycle was set up for 25 cycles as follows: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The denaturation step was for 5 min at 94°C and followed by a final extension for 10 min at 72°C. The PCR product of 5 µl each including controls were loaded onto a 0.8% agarose gel. The amplified PCR product was purified using PCR purification kit (Qiagen). The phosphate groups were added to the PCR fragment to the 5' ends of both strands and cloned into EcoRV restriction site of digested and dephosphorylated p-Bluescript plasmid vector. The positive clone was isolated by alkaline lysis miniprep and was sequenced using M13 forward (5'-TGACCGGCAGCAAAATG-3') and M13 reverse (5'GGAAACAGCTATGACCATG-3') primers. The PCR product was digested from ligated p-Bluescript-PCR product with NdeI and NotI and cloned into pCR2.1 vector containing the g10-5'UTR with NdeI and Not1I (fragment size approximately 313 bp). Finally, the pCR2.1 containing the g10-5'UTR, $PyMSP1_{19}$ was digested with XbaI (fragment size approx. 345bp) and cloned into tobacco universal vector pLD-ctv.

Bombardment of the pLD-g10- PyMSP119

Preparation of gold particles

A mixture containing 50 mg of gold particles (0.6 μm in size) and 1 ml of 70% were vortexed for 3 to 5 minutes and then incubated for 15 minutes at room temperature. The gold particles were pelleted by centrifugation and the supernatant was discarded. Then the particles were vortexed with 1 ml of distilled water followed by a pulse centrifugation for 3 seconds; the supernatant was discarded. This step was repeated three times (Kumar and Daniell, 2004).

Preparation of tobacco tissue culture media

For regeneration and selction of transgenic plants, RMOP media containing MS basal salt mixture (one pack), 30 g of sucrose, 100 mg of myo-inositol, 1 ml of benzylaminopurine (BAP: 1 mg/ml), 100 μl of α-naphtalene acetic acid (NAA: 1 mg/ml), 1 ml of thiamine hydrochloride (1 mg/ml), and water (to 1 liter) was prepared and adjusted pH to 5.8 using 1N KOH. To solidify the media, 6g/ liter of phytagar was added to the media and autoclaved. The cooled media was poured into plates. For rooting, MSO media containing 30 g sucrose, 1 packet of MS basal salt mixture, and water to 1 liter was prepared with 5.8 pH using 1N KOH and 6 g per liter of phytagar (Kumar and Daniell, 2004).

Bombardment protocol for tobacco leaves

The bombardment was performed as described previously (Daniell, 1997). Under sterile conditions, the bombardment of tobacco leaves was done. All the equipment was sterilized either with 95% ethanol or autoclave. The gold particles (Fifty µl) were placed in a micro centrifuge tube followed by 10 µl of DNA (1 µg/µl). Fifty µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine-free base were added sequentially to the mixture to ensure proper binding of DNA to the gold particles. The mixture was then vortexed for 20 minutes at 4°C to ensure proper binding of the DNA with the gold particles. This was followed by washing four times in 200 µl of absolute ethanol at 3,000rpm for 30 seconds. The DNA with the gold particles was resuspended in 30 µl of 100% ethanol. Green healthy leaves from the aseptic tobacco plant *Nicotiana tabacum* variety Petit havana were cut from a young plant growing in jars containing MSO media and placed on a Petri dish *(100 x 15 mm)* containing RMOP media with no selection and a Whatman filter paper on the top of media. The leaves were placed with the abaxial side upwards. The gene gun (Bio-Rad PDS-1000/He) was sterilized with 70% ETOH prior to bombardment. The macrocarriers were placed on the macrocarriers holders. The gold particles lying on ice were vortexed and 5 µl of gold particles containing the DNA were placed on top of the macro carrier. Vortexing is an important step while placing the gold particles on the macrocarriers. The bombardment was carried on at 1,100 psi and 28 Hg. After the bombardment, the samples were covered with aluminum foil (to keep them in the dark) and incubated for 48 hours at room temperature. (Kumar and Daniell, 2004).

Tissue regeneration and selection

After 48 hrs of allowing the leaf tissue to recover, the leaves were cut into 5x5 mm^2 size and were transferred to RMOP media containing 500 μg/ml of spectinomycin (Daniell, 1997). After four to six weeks, confirmed positive transgenic lines by PCR analysis were subjected to second round of selection in a similar fashion. Finally, after 4 weeks on secondary selection, the shoots were transferred to a jar that contained MSO media with 500 μg/ml spectinomycin (Daniell, 1997). This accounts for the third round of selection. (Kumar and Daniell, 2004).

Plant genomic DNA extraction procedure

The Qiagen DNeasy Kit was used to isolate plant genomic DNA as described in the Qiagen manual. 100 mg of the leaf tissue was ground in a micro centrifuge tube, using a micro pestle in 400 μl of buffer AP1 and 4 μl of RNase A (stock solution 100mg/ml). The mixture was incubated for 10 minutes at 65°C and mixed 2-3 times. 130 μl of buffer AP2 were added to the lysate, vortexed and incubated for 5 minutes on ice. The mixture was centrifuged at maximum speed for 5 minutes and the supernatant was transferred to a Qiashredder spin column (lilac) sitting in a 2 ml collection tube. The centrifugation was performed at full speed for 2 minutes. The flow through was collected in a new tube and 1.5 volumes of buffer AP3/E were added to the lysate and mixed immediately. 650 μl of this mixture was applied to a DNeasy mini spin column (clear)

and centrifuged for 1 minute at 8000 rpm. The flow through was discarded and the step was repeated with the rest of the sample. The collection tube was discarded and the column was placed in a supplied 2 ml tube. Buffer AW (500µl) was added to the column and centrifuged for 1 minute at 8,000 rpm. The flow through was discarded, and the column was washed once again by using 500 µl of AW buffer followed by centrifuging for 2 minutes at maximum speed. The column was transferred to a clean 1.5 ml tube and 100 µl of preheated (65°C) buffer AE was added into the DNeasy membrane. The membrane was incubated for 5 minutes at room temperature and then centrifuged at 8,000 rpm for 1 minute to elute the DNA. The DNA was stored at -20°C.

Confirmation of transgene integration into the chloroplast genome

To confirm transgene cassette integration into the chloroplast genome, PCR was performed using the primer pair 3P (5'-AAAACCCGTCCTCEGTTCGGATTGC-3') and 3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3') (Daniell *et al*., 2001a). Also, the integration of gene of interest by PCR was performed using primer pair 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-TGACTGCCCACCTGAGAG-CGGACA-3') (Daniell *et al*., 2001a). Positive (known transgenic plant DNA sample) and negative controls (wild type petit havana DNA sample) were also used. For a 50 µl reaction volume, the PCR was set as follows: 150 ng of plant DNA, 5 µl of 10X buffer, 4 µl of 2.5 mM dNTP, 1 µl of each primer from the stock, 0.5 µl Taq DNA polymerase and H_2O to make up the total volume. The amplification was carried out for 25 cycles of the

following program: 94°C for 30 sec, 65°C for 30 sec, and 72°C for 2 min. Denaturation was done for 5 min at 94°C at the start and a final extension of 7 min at 72°C was carried on at the end. 5 ul of each PCR products including the controls were loaded into a 0.8% agarose gel to confirm the results.

Southern blot analysis of transgenic plants

Restriction Digestion of plant genomic DNA

Total plant genomic DNA was extracted from both transgenic T_0 as well as untransformed tobacco plants. The samples containing equal quantity of DNA were digested with BglII in a reaction containing: 2 ug of DNA, 3 µl of 10X buffer (New England Biolabs), 1.5 µL of BglII enzyme (New England Biolabs) and sterile dH_2O to make up the volume of 30µl. The reaction was incubated overnight at 37°C.

Agarose electrophoresis and DNA transfer

A total of 20 µl reaction volume was loaded on a 0.7% agarose gel for each of the transgenic plant DNA samples. The digested DNA of wild type plant (*Nicotiana tabacum* var. *Petit Havana*) acts as negative control and the unlabelled probe acts as the positive control. Electrophoresis was carried on for 2.5 hours at 50 volts following which, the DNA was transferred by capillary action to a nylon membrane. The parts of the gel that were not needed were removed, and the upper right corner was cut to help as a guide. The

gel was then depurinated by immersing it in 0.25 M HCl (depurination solution) for 15 minutes (until the color of the dye became yellow). This was followed by washing the gel twice in dH_2O for 5 minutes, and then equilibrated in transfer buffer (0.4N NaOH, 1M NaCl, filled to 1 liter with water) for 20 minutes. Whatman paper and the nylon membrane were cut to fit the size of the gel. The membrane was briefly washed in water. Later, the membrane was equilibrated by immersing it in the transfer buffer for 5 minutes. In a glass tray, a stack of two sponges was placed and enough transfer buffer was added to cover the sponge that is in touch with the tray. On top of the sponges two pieces of Whatman papers were placed and some transfer buffer was poured to soak the paper and to remove any air bubbles. The gel was placed facing down on the Whatman paper and then the nylon membrane was placed with the cut corner touching the cut corner of the gel taking care to ensure no air bubbles. Two Whatman papers and a stack of paper towels were placed on top of the membrane. A 500 g weight was placed on the paper towels to help overnight capillary transfer. Following day, the membrane was washed with 2X SSC (3 M NaCl, 0.3 M Na. citrate, the pH was adjusted with 1N HCl to 7, and water was added to 1L) for 5 minutes. The membrane was later allowed to dry on a Whatman paper for 5 minutes and then cross-linked using the Bio-Rad GS Gene Cross Linker at setting C3 (150 m joules). The membrane was wrapped in saran wrap and stored in a dry place until use.

Generation of probes

These steps were performed essentially as previously described (Daniell *et al*., 2004d). The flanking sequence probe was obtained from the plasmid pUC-ct vector that contains the chloroplast flanking sequences for the *trnI* and *trnA* genes. The digestion reaction was setup as follows: 15 µl of pUC-ct vector DNA, 2 µl of 10X buffer, 1 µl of BamHI (NEB), 1 µl of BglII and 1 µl of dH_2O. The reaction was performed overnight at 37°C and then run on an agarose gel to obtain the desired fragment of 0.8 kb. The band was cut out and eluted from the gel as previously explanied. For the final DNA elution, 50 µl of H_2O was used. The gene-specific probe was made by cutting out the 0.3 kb fragment from pBluescript-PyMSP1$_{19}$ in a reaction as follows: 2 µg of pBlue-PyMSP1-19, 2 µl of 10X buffer, 1 µl of EcoRV (NEB) and 12 µl of dH_2O. The reaction was incubated overnight at 37°C. The fragment of interest (0.3 kb) was eluted from the agarose gel and checked for the concentration on the spectrophotometer.

Probe labeling

The denatured probe (45 µl of the DNA) was made by incubating the tube at 94°C for 5 minutes, followed by placing the tube immediately on ice for 2 to 3 minutes. Following which, the probe was added to the ready mix and mixed well (Quant^{tm} G-50 Micro columns, Amersham). Five µl of α-^{32}P was added to the tube and the mixture was incubated for 1 hour at 37°C. Later, a G50 column was taken and the resin was

resuspended by vortexing. The column was placed in a microcentrifuge tube with the top cut off and centrifuged for 1 minute at 3,000 rpm. The collection tube was discarded and the column was transferred to a new 1.5 ml tube. The DNA probe was added in the center of the resin and spun at 3,000 rpm for 2 minutes and the column was discarded. From the labeled probe 1 μl was mixed with 98 μl of TE buffer. The mixture was aliquoted into 50 μl samples and to each sample 3 ml of Opti-Fluor was added. The activity of the radioactive probe was measured in a Beckman LS 5000TD. The two samples plus a blank containing 3 ml of Opti-Fluor were placed into the machine holder. The readings were taken by using the auto-read mode of the machine. The amount of probe to be used was determined by calculating the amount of probe needed to yield 2.5 x 10^6 cpm/2 ml. The amount of probe was calculated as follows: Reading value (502050) was equal to 0.50 x 10^6 cpm/μl, then multiplied by 50 μl of total volume of sample for a total of 35 x 10^6 cpm/μl. Because 5 ml of hybridization solution was used, we needed 6.25 x 10^6 cpm, therefore 6.25 x 10^6 cpm divided by 0.7 x 10^6 cpm is equal to 12.5 μl of labeled probe needed.

Prehybridization, hybridization and washing of membrane

The blot was incubated with 5 ml of Quick-Hyb solution from Stratagene in a hybridization bottle with the top facing in toward the solution for one hour at 68°C using Fisher Biotech Hybridization Incubator. Later, the probe was added with 100 μl of salmon sperm DNA and heated for 5 minutes at 94°C followed by addition of probe to

the bottle containing the membrane with pre-hybridized solution and incubated for 1 hour at 68°C. After that, the membrane was washed twice with 50 ml of wash solution 1 (2X SSC and 0.1% SDS) at room temperature for 15 minutes. A second round of washes were performed twice with 50ml of wash solution 2 (0.1X SSC and 0.1% SDS) for 15 minutes at 60°C to increase the stringency. Finally, the radioactive membrane was wrapped around with saran wrap and kept in a radioactive container in the radioactive hood.

Autoradiography

The film cassettes with the blots were taken to the dark room and under safe light (red light), the X-ray film was placed on the top of the blot with intensifier screen placed in between. The cassette with the blot and the film was incubated overnight at –80°C. The next day, the cassette was taken out from the –80°C to thaw, and then the film was developed.

Characterization of expressed proteins

Extraction of Protein from Transformed *E. coli C*ells

The *E.coli* XL1-blue cells with pLD-g10-PyMSP1$_{19}$ were grown in 5 ml of Terrific broth with ampicillin (100 μg/μl) and tetracycline (50 μg/μl) at 37^{o}C for 14 to 16 hrs. As a negative control, untransformed *E. coli* cells were used. After the boiling step (as described below), samples were immediately loaded into polyacrylamide gels. About

800 µl of cultured cells were centrifuged for 1 minute at 13,000 rpm and the pellet of *E. coli* cells was washed with 1 ml of 1x Phosphate-Buffered Saline (PBS: 140 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 1.8 mM KH2PO4, pH 7.2) followed by resuspension of the pellet in 1X PBS. The sample loading buffer (1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.0 ml of 10% (w/v) SDS, 0.2 ml of 0.5% (w/v) bromophenol blue, 2.5 ml of glycerol, add 9.5 ml of distilled water), and β-mercaptoethanol were mixed in 19:1 ratio to prepare 1x reducing SDS-loading buffer. Equal amounts of crude extract and 1X reducing-SDS sample loading buffer were mixed thoroughly and boiled for 5 minutes. Then, the denatured, reduced samples were immediately loaded onto gels. (Sambrook *et al*.,1989).

Extraction of Protein from Transformed Tobacco Leaves

The $PyMSP1_{19}$ protein from transgenic lines was extracted as previously described (Daniell *et al*., 2004d). The plant tissue (100 µg) was ground with a mortar and pestle in liquid nitrogen and 200 µl of extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl-pH8, 0.05% Tween-20, 0.1% SDS, 14 mM BME, 400 mM sucrose, 2 mM PMSF) was added. The samples were mixed for 3 minutes with a micropestle and centrifuged at 13,000 rpm for 10 min to obtain the supernatant containing the soluble proteins. Twenty µl of these extracts were mixed with 20 µl of sample loading buffer containing βME and boiled for 5 minutes to load it on gel for SDS-PAGE. For PAGE, the samples were extracted following the similar protocol but without SDS. To maintain the native conformation, the samples were not boiled or reduced with βME.

SDS-PAGE Buffers and Gels

The protein expression of PyMSP1$_{19}$ was detected by conducting SDS-PAGE using buffer solutions: Bio-Rad (cat#161-0158), 30% Acrylamide/Bis solution according to the ratio 37:5:1, the resolving buffer (5 M Tris-HCl, pH 8.8), the stacking buffer (0.5 M Tris-HCl, pH 6.8), 10x Electrode buffer: 30.3 g Tris base, 144.0 g glycine and 10.0 g SDS were added to 1000 ml double distilled water and stored at 4°C, 2x loading buffer, also called Sample buffer or SDS Reducing Buffer (see previous section), 10% (w/v) Sodium Dodecyl Sulfate (SDS), N,N,N,N'-Tetra-methyl-ethylene diamine (TEMED) from BIO-RAD (cat# 161-0800), 20% Ammonium Persulfate (APS)(dissolved 20 mg of APS into 1 ml dH_20 in a micro centrifuge tube and stored at 4°C for about a month). The 15% resolving gel was made by the following method: 5.0 ml of 30% Acrylamide/Bis, 2.5 ml of resolving buffer, 2.4 ml dH_20 and 100 µl of 10% SDS to a 50 ml flask were added. Later, 50 µl of 20% APS (#8 above), 10 µl of TEMED were added to cast the gel mixture between the two, vertical, glass plates (Mini-Protean 3 Cell gel system, Bio-Rad) leaving about 1.5 cm at the top of glass plates for the stacking gel. The gel was allowed to polymerize for 20 minutes. 4% stacking gel was made using 1.3 ml of 30% Acrylamide/Bis, 2.5 ml of the stacking buffer, 6.1 ml dH_20 and 100 µl of 10% SDS were taken together into a flask followed by 50 µl of 20% APS and 10 µl of TEMED. The 4% gel mixture was layered on top of resolving gel, and then the comb was inserted for the formation of wells. After polymerization for about 20 minutes, the gel was put vertically into PAGE apparatus containing 1x Electrode (running) buffer. 20 µl of protein extract along with the sample-loading buffer was loaded along with PyMSP1$_{19}$ protein standard,

and 10 µl protein marker. The gel was run at 50 V until samples stacked onto the top of the resolving gel, then run at 80 V for 2 to 3 hours so that the protein marker bands could spread out sufficiently.

Transfer of protein and analysis of Western Blot

The proteins from SDS-PAGE to trans-Blot nitrocellulose membrane (Bio-Rad) was done by electroblotting in a Mini-Transfer Blot Module at 80 V for 45 minutes using transfer buffer (360 ml of 10x Electrode buffer, 360 ml of methanol, 0.18 grams of SDS, 1080 ml distilled H_20). For Western blotting, nitrocellulose membrane with proteins was blocked for one hour in P-T-M (PBS [12 mM Na_2HPO_4, 3.0 mM NaH_2PO_4-H2O, 145 mM NaCl, pH 7.2], 0.5% Tween 20, and 3% Dry Milk) followed by incubation with P-T-M containing rabbit raised anti-PyMSP1-19 polyclonal antibody (1:1000). Later, Membranes were incubated with P-T-M containing goat-derived anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO). Blots were washed three times with PBST for 15 minutes each time followed by 10 min wash in 1X PBS. Finally, the membrane was incubated with Lumiphos WB (Pierce, Rockford, IL) as a substrate for AP at room temp for 5 min for the chemiluminescence. Then, X-ray films were exposed to chemiluminescence and the films were developed in the film processor to visualize the bands.

Coomassie Staining of the protein gel

Coomassie staining was performed using the Coomassie dye-250 Brilliant blue (Bio-Rad). The concentrated dye was diluted 5 times with distilled water. The SDS-PAGE protein gel was incubated in the stain for 30 minutes at room temperature followed by destaining overnight with destain solution (7% acetic acid, 5% methanol, 88% water) at room temperature.

Enzyme Linked Immunosorbent assay (ELISA)

ELISA was done to quantify PyMSP1$_{19}$ protein in the plant crude extract. The transgenic leaf samples (100 mg) of young, mature, old stages along with wild type leaf samples (young, mature, old) were collected.The leaf samples were finely ground in liquid nitrogen and extracted in a plant extraction buffer. The protein concentration for standards, test samples and antibody were diluted in 1x PBS. Diluting in 1x PBS buffer made purified PyMSP1$_{19}$ protein standard ranging from 1 to 25ng/ml. The 96 well ELISA plate was coated with standards and protein samples (100 µl) for 1 h at 37^{O}C followed by 3 washes with PBST and 2 washes with water. Blocking was done with 3% milk in PBS and 0.1% Tween and incubated for 1 hour followed by washing. The primary anti-PyMSP1$_{19}$ antibody (Immuno Chemicals) diluted (1:500) in PBST was added and the plate was incubated for 1 hour followed by washing steps. Again, 100 µl of anti-rabbit IgG-HRP conjugated antibody raised in goat (American Qualex) (1:5000) diluted in PBST was added and the plate was then incubated for 1 hour at 37 °C. After the

incubation the plate was washed thrice with PBST and twice with water. The wells were then loaded with 100 µl of 3,3,5,5-tetramethyl benzidine (TMB from American Qualex) substrate and incubated for 10–15 min at room temperature. The reaction was terminated by adding 50 µl of 2 N sulfuric acid per well and the plate was read on a plate reader (Dynex Technologies) at 450 nm (Modified form of protocol from Ausubel *et al.,* 4th edition).

Bradford assay for protein quantification (Bio-rad manual)

Extraction buffer was used to make Bovine Serum Albumin (BSA) standards ranging from 0.05 to 0.5 µg/µl. Plant extracts were diluted 1:5, 1:10 and 1:20 with extraction buffer. Ten µl of each standard and 10 µl of each plant dilution were added to the wells of a 96 well microtiter plate (Costar) in duplicate. Bradford reagent (BioRad protein assay) was diluted 1:4 with distilled water as specified and 200 µl was added to each well. Absorbance was read at 595 nm. Comparison of the absorbance to known amounts of BSA to that of the samples was used to estimate the amount of total protein.

Protein Purification

The protein expressed in the transgenic plant was purified using Mono-Q columns. 10g of plant leaves were finely ground in liquid nitrogen ant crude extract was obtained using 20 ml of plant protein extraction buffer (100 mM NaCl, 200 mM Tris-HCl-pH8, 0.05% Tween-20, 400 mM sucrose, 2 mM PMSF adjusted to pH 9.6). The

extract was transferred to a Sorvall tube and centrifuged at 12,500 rpm (20,000 x g) for 30 minutes and the supernatant was transferred into a new falcon tube .The buffers used for purification were as follows: Running buffer (Buffer A): 50 mM Tris-Hcl, 20 mM NaCl adjusted to pH 7.5. Elution Buffer (Buffer B): 50 mM Tris HCl and 1 M NaCl. Purification operations were performed on the FPLC machine. The running buffer was passed into the purification set up through A port, Elution buffer through Port B. The purification steps were carried out as follows: Washing the column with running buffer, followed by application of sample, followed by washing and gradient elution of sample with buffer B. The samples were collected in 1000 ul volume. The samples corresponding to the peak were aliquoted for further analysis. The flow through was collected to analyze the loss of $PyMSP1_{19}$. The protein aliquots, flow through samples were analyzed for the presence/absence of $PyMSP1_{19}$ using western blot. The fractions containing the purified $PyMSP1_{19}$ were pooled together and concentrated five fold using 10K MW concentrator columns (Millipore) by centrifuging at 3000 rpm to concentrate the protein and ran through FPLC using superdex columns using similar buffers as mentioned above. The fractions were collected in 1ml volume and the samples were tested for $PyMSP1_{19}$ by western blot.

Enrichment of chloroplast-derived PyMSP1-19

The plant leaves of 150 grams were finely ground in liquid nitrogen and the crude extract was prepared using 300ml of plant extraction buffer (100 mM NaCl, 10mM EDTA, 200 mM Tris-HCl-pH8, 0.05% Tween-20, 400 mM sucrose, 2 mM PMSF

adjusted to pH 9.6). The extract was transferred to a Sorvall tube and centrifuged at 12,500 rpm for 30 minutes and the supernatant was transferred into a new falcon tube. The total crude extract was lyophilized using a lyophilizer to ten-fold concentrated protein. The concentrated crude extract was run on big SDS-PAGE as mentioned earlier with all components added in the similar procedure but with three times its actual volume. A strip of the gel is tested for protein expression by western blot as mentioned earlier and the entire gel was stained with Coomassie dye- 250 Brilliant blue. The desired band of PyMSP1-19 protein was excised and eluted using an Electro-eluter (Bio-Rad)

Elution of PyMSP1$_{19}$ protein from SDS PAGE

The PyMSP1$_{19}$ protein was eluted by using Electro-eluter (Bio-Rad protocol). The dialysis membrane caps were soaked in 1X elution buffer (Electrode buffer used for SDS-PAGE running) for 1 hour at 65ºC before using them for elution. The glass tubes were taken and Frit was placed in the bottom of each glass tube. Glass tube with Frit was pushed into the electro-eluter module and was positioned evenly with the top of the grommet. All empty holes were filled with grommet stoppers. A pre-wetted membrane cap was placed in the bottom of each Silicone Adaptor and the adaptor was filled with elution buffer(electrode buffer protocol). Then, the Silicone adaptor was slided with the membrane cap onto the bottom of the Glass Tube with the Frit and all air bubbles in between Frit were removed for proper elution of the protein. The Glass Tube was filled with the elution buffer and the sliced gel was placed in the tube. The entire module was placed in buffer chamber. The lower buffer chamber was filled with ~600ml of elution

buffer and upper buffer chamber with ~100ml. A stir bar was placed in the bottom buffer tank for constant stirring to prevent air bubbles from sticking to the bottom of the dialysis membrane. Attach lid with cables. Elution was done at 8-10mA/glass.

Immunization studies in mice

Two Balb/c mice, 6 to 7 weeks old were immunized with enriched chloroplast-derived $PyMSP1_{19}$ with freund's adjuvant to raise anti-$PyMSP1_{19}$ antibodies and one control mouse (negative) is injected with the wild type crude extract commercially by Harlon Bioscience products. The proposed dosage by the company for polyclonal antibody production in mice was mentioned in the table below:

Days	**Dosage per mouse**	**Bleeds obtained after immunization**
0	50μg of enriched $PyMSP1_{19}$	
28	25μg of enriched $PyMSP1_{19}$ **(**first boost**)**	
35		Test bleed-0.1ml sera of 1:10 dilution
56	25μg of enriched $PyMSP1_{19}$ (second boost)	
70		Final bleed-0.3ml sera and ship the animals to us

ELISA to detect anti-PyMSP119 antibodies in the serum samples

96 well ELISA plates were coated with purified *Plasmodium yoelii* standard protein at a concentration of 1μg/ml in phosphate buffer saline solution i.e., 100ng of protein per well. The plates were incubated overnight at 4°C. The next day, the plates were washed and blocked with PBS containing 0.1%tween and 3% skim milk powder. Since the sera obtained were in 1:10 dilution, the serum samples were serially diluted ranging from 1:25 to 1:500 for the Bleed-1 and 1:25 to 1:1000 for Bleed-2. Plates were incubated with 100 μl of diluted serum samples for one hour at 37°C. Later, the plates were washed with PBS-tween(P-T) and water. Subsequently, the plates were incubated with 100 μl of 1:5000 diluted goat raised anti-mouse IgG conjugated with horseradish peroxidase enzyme for one hour at 37°C followed by washing with P-T. Addition of 100 μl TMB substrate and incubation of the plate at room temperature until the blue color is developed (approximately 10-15 min) was done. The reaction was stopped with 50 μl of 2M sulphuric acid and the plate was read at 450nm on the plate reader (Dynex technologies). The titer values were determined by using the formula O.D (optical density) of the negative control (unimmunized mice) + 0.1.

In vitro parasite inhibition assay

The plasmodium falciparum was used as test parasite for the assay instead of *Plasomodium Yeolli* due the availability of *P. falciparum* in Dr. Chakrabarti's lab. A

synchronized *plasmodium falciparum* culture consisting of trophozoite-schizont stage parasites with 10% parasitemia and 5% hematocrit was obtained from Dr.Debopam Chakrabarti's lab. The culture was diluted five-fold with RPMI media containing 0.05% albumax to obtain 2% parasitemia and 1% hematocrit. Later, 1% fresh human RBC was added to the culture. In a 96 well tissue culture plate, duplicates of different samples were added as mentioned in the below table:

S.No.	Sample in duplicates	Composition per well
1.	Blank	40µlofculture (2%parasitemia+2%hematocrit) + 10 µl of RPMI media
2.	Negative control	40µl of culture (2%parasitemia+2%hematocrit) + 10µl of sera obtained from mice immunized with wild type crude extract.
3.	$PyMSP1_{19}$	40µl of culture (2%parasitemia+2%hematocrit) +10µl of sera obtained from mice immunized with chloroplast-derived $PyMSP1_{19}$
4.	Positive control	47.5µl of culture (2%parasitemia+2% hem-atocrit) +2.5µl of purified anti-$PfMSP1_{19}$ antibodies

The plate was incubated at 37◦C for 48 hours as the parasite completes one cycle and invasion of the RBC. After incubation, the plate was taken out and thin blood smears were prepared on slides. The slide was stained using hemastain kit (from sigma Aldrich protocol) and observed under normal light microscope (X100 objective). Up to 200-300 red blood cells were counted manually and average parasitemia was measured using the formula (infected red blood cells/ infected+uninfected red blood cells) x 100. Later, the pictures were taken from all the slides to show the extent of parasitemia under light microscope.

ELISA of Plasmodium falciparum MSP119 coated plates using sera obtained from mice immunized with chloroplast-derived PyMSP119

96 well plate was coated with 1 µg/ml of purified $PfMSP1_{19}$ antigen overnight at 4◦C. The next day, the plate was washed with 1x PBS and 0.1% tween followed by blocking in 3% P-T-M at 37◦C for an hour. Then, after similar washing as mentioned earlier, the plate was incubated with 1:10 diluted anti- $PyMSP1_{19}$ antibodies using three different dilutions 1:25, 1:50, 1:100. The remaining steps were all similar as mentioned in earlier ELISA steps.

In vivo parasite challenge in immunized mice

a. *Plasmodium yoelii* infection to BALB/c mice

The frozen stock of 17XL (Lethal) strain of *P.yoelii* (a kind gift from Drexel Company) was used in parasite challenge studies. The infected RBC with 17XL strain was thawed by adding1ml Hank's Balanced Salt solution buffered with 0.02M HEPES pH 7.2-7.4 and shaking quickly with hand. Within 15min, the stabilite was drawn into a 1.0ml tuberculin syringe and injected into the normal 6-8 weeks old Balb/C mice with 100 µl each at two different sites via intraperitoneal route. The parasitemia was monitored every alternate day by collecting few drops of blood by snipping the mouse tail off and making smears on the slides. The smear was stained with hema-stain and the number of infected red blood cells was counted under a light microscope.

b. Parasite challenge into immunized mice

When the parasitemia in the infected mice reached 70-80%, the blood was collected from the heart of the mouse by performing a cardiac puncture. About 400-500 µl of blood was drawn and diluted in 1:1 with 20% glycerol, 20mM HEPES pH 7.4 in Hank's balanced salt solution containing 50units/ml Heparin. Immediately after diluting, 100 µl of 35-40% parasitemia was injected via i.p. into the two immunized mice and one control inimmunized mouse. The infection was monitored in all three mice as discussed above.

The remaining stabilite was aliquoted into 0.1ml in freezing vials and stored in Styrofoam rack and placed at-80C overnight.

RESULTS

The pLD vector contained the flanking sequences, which ensured the homologous recombination of the gene cassette (*aadA*, g10-PyMSP1$_{19}$) between the *trnI* and *trnA* genes of the chloroplast genome (Daniell *et al*., 2001a). Downstream of *trnI,* the vector provided the constitutive 16S rRNA promoter, which regulates the expression of *aadA* gene (aminoglycoside 3' adenyltransferase) that confers resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991), and the PyMSP1$_{19}$ gene encoding the merozoite surface protein. On the other hand, the vector contains the 3'UTR downstream of *trnA* gene, which terminates and stabilizes the transcripts derived from *psbA* gene.

PCR analysis of Confirmation of Chloroplast integration of transgenes

We obtained about 10 resistant shoot after a period of 3 to 5 weeks on regeneration media (RMOP) containing spectinomycin. To differentiate the chloroplast integrated shoots from nuclear integrated and mutant shoots, specific primers were used to perform PCR. A spontaneous mutation in the 16s RNA gene confers resistance to spectinomycin and could allow the shoots to grow without integration of the gene cassette and the *aadA* gene in the gene cassette confers resistance to spectinomycin (Daniell *et. al*., 2001a). The site-specific integration of the gene cassette with chloroplast genome was confirmed using 3P/3M primers (Daniell *et.al.,* 2001a). The 3P primer annealed to the native chloroplast genome, eliminating all nuclear transformants, and 3M

landed on the *aadA* gene, eliminating all mutant transformants (**fig 1a**).The amplified PCR product with 3P/3M primers yielded ~1.65Kb band (**fig 1b**).

The integration of *aadA* and g10-PyMSP1$_{19}$ were tested with a another set of primers for PCR analysis, 5P/2M. 5P annealed to the *aadA* gene and 2M annealed to the integral part of *trnA* region (**fig 1a)** (Daniell *et al* 2001a). From 5P/2M primers, the amplified PCR product was ~1.9kb size (**fig 1c**), confirming the integration of gene within the chloroplast genome and eliminating the mutants as they would amplify at all. The positive transgenic plants were subjected different rounds of selection until they attained homoplasmy.

Fig 1a

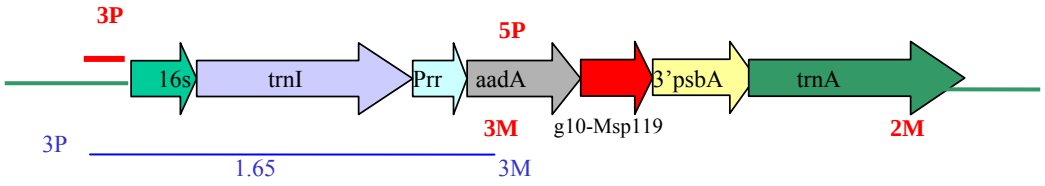
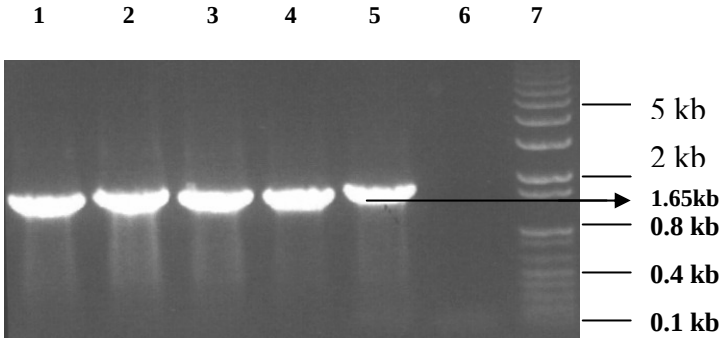


Fig 1b



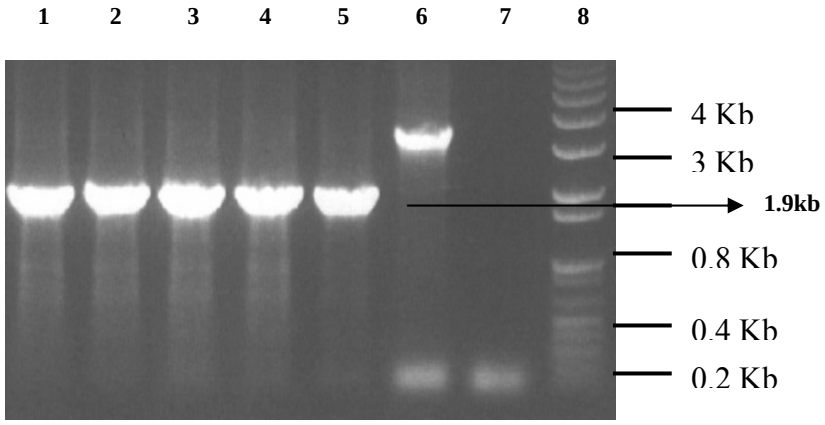


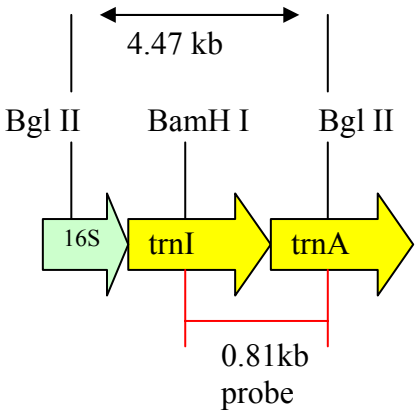
Figure 1: PCR analysis of Wild type and putative transformants of pLD-g10- PyMSP1$_{19}$ **1A)** PCR using specific primers land within the native chloroplast genome (3P/3M) to yield a 1.65 kb product and 5P/2M primers landing on *aadA* and *trnA* respectively to yield 1.9 kb product **1B(3P/3M)**: **Lane 1-4**: Trangenic lines pLD-g10- PyMSP1$_{19}$, **Lane 5**: Positive control (transgenic line pLD-g10-Lec A), **Lane 6**: Wild type, **Lane 7**: 1 kb DNA ladder **1C(5P/2M)**: **Lane 1-5**:Transgenic lines pLD-g10- PyMSP1$_{19}$, **Lane 6**: Positive control (transgenic line pLD-g10-Lec A), **Lane7:** Wild type, **Lane 8**: 1kd DNA ladder

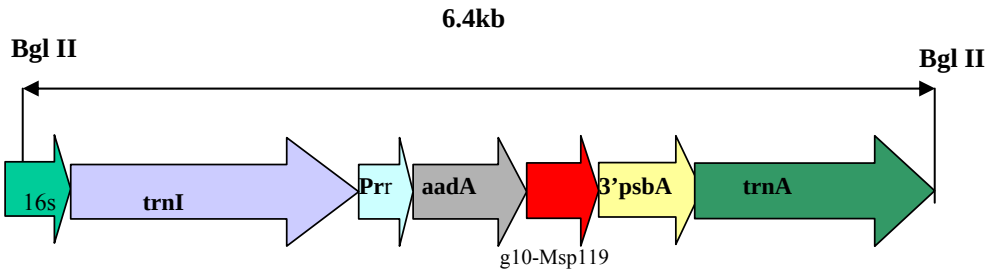
Southern Analysis of transgenic plants

To confirm the site-specific integration, the transgenic plant DNA was probed with the flanking sequence probe of 0.81 kb in size (**fig. 2a**) with BamHI and BglII (Daniell *et al*., 2001a). The transformed chloroplast genome digested with BglII produced a fragment of 6.4 kb, while the untransformed chloroplast genome gave a 4.47 kb fragment (**fig. 2b**). Some of the transgenic PyMSP1$_{19}$ lines showed slight degrees of heteroplasmy as few of the wild type genomes were not transformed. The gene-specific

probe of about 0.34 kb was used to show the integration of gene within the chloroplast and eliminated nuclear transformants as they would give a different size band. (**fig. 2c**)

2a





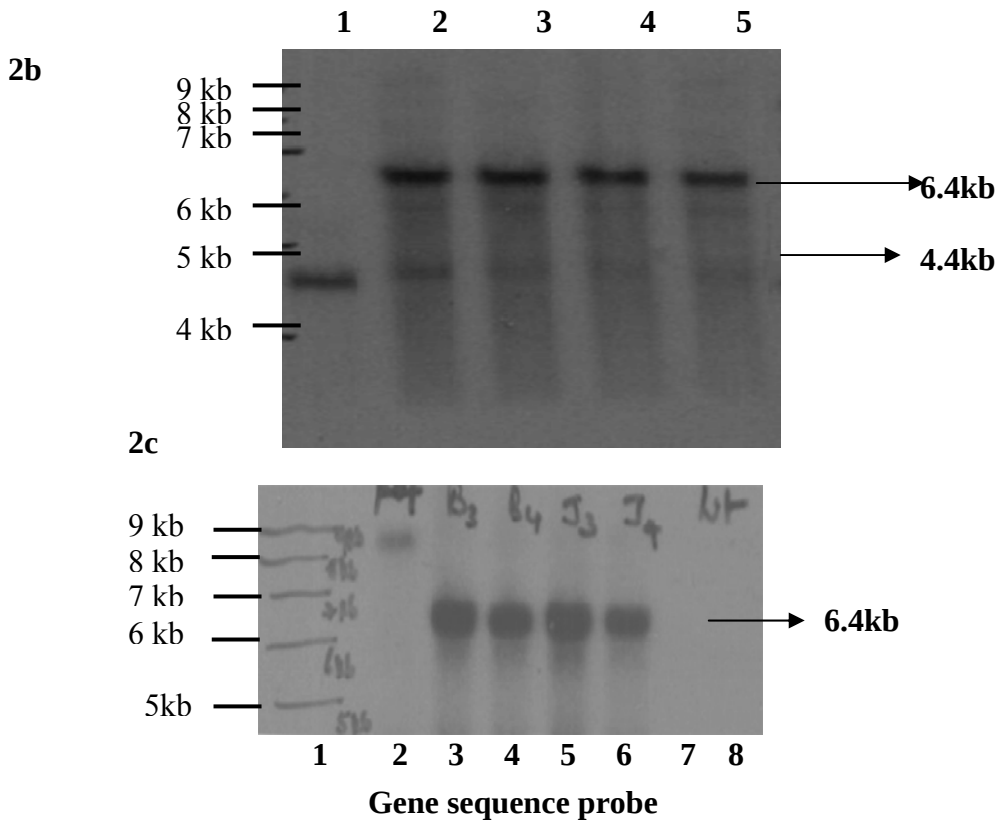


Figure 2: Southern blot analysis of pLD-g10- PyMSP1$_{19}$

Schematic diagram of expected products from digestion of (2a) wild type untransformed plant and Plants transformed with pLD-g10- PyMSP1$_{19}$ (2b) Southern with flanking sequence probe in pLD-g10- PyMSP1$_{19}$ transgenic lines showing slight heteroplasmy: Lane1: wild type, Lanes 2-5: Transgenic lines 2c) Southern with PyMSP1$_{19}$ gene specific probe showing the presence of PyMSP1$_{19}$ in the transgenic plants: Lane 1: Ladder, Lanes 2-6: pLD-g10- PyMSP1$_{19}$ transgenic lines, 7-Blank, 8-Wild type.

Immunoblot Analysis

Crude extracts of approximately 40 μg was loaded in each well to perform SDS-PAGE. The western blot with anti-PyMSP1$_{19}$ antibodies detected the PyMSP1$_{19}$ protein that showed a 17 kDa size protein under denaturing conditions (**fig.3**)

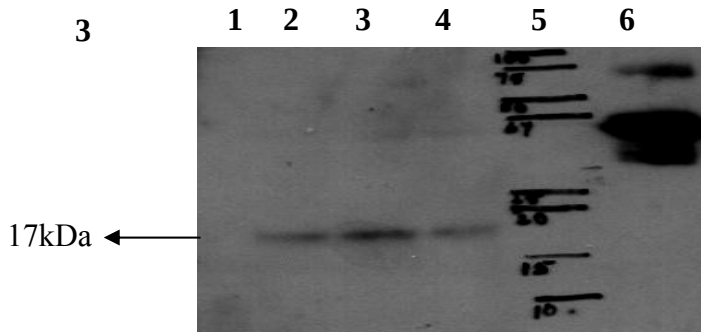
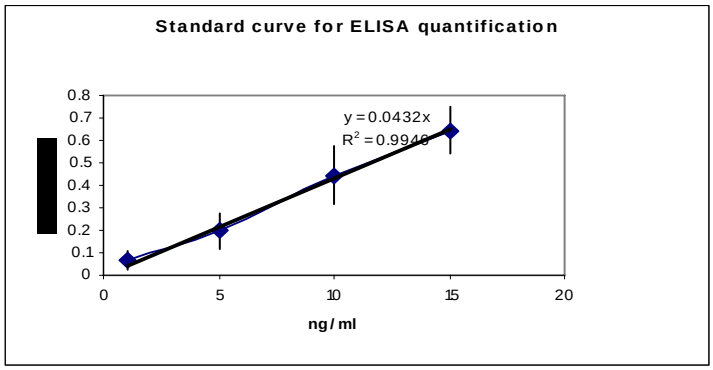


Figure 3: Western blotting demonstrating the expression of PyMSP1$_{19}$ in transgenic plant crude extracts under reducing conditions(SDS-PAGE) : Lane1: Wildtype, Lanes 2-4: 40ug crude extract of transgenic lines Lane 5: ladder, Lane 6: 250ng of GST- PyMSP1$_{19}$ as positive control(36kDa).

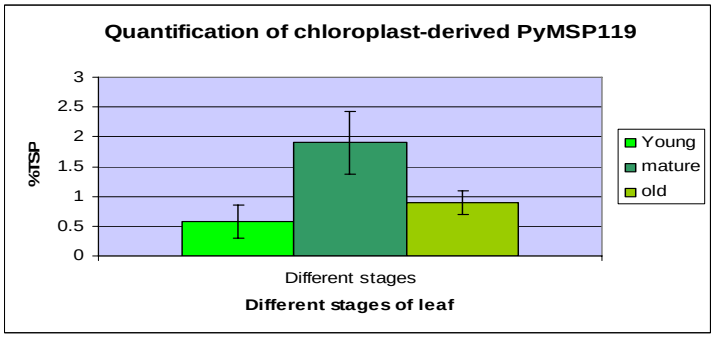
Protein quantification using ELISA

To quantify the protein levels, the standard protein (PyMSP1$_{19}$) of known concentration were optimized (**Fig 4a**). The PyMSP1$_{19}$ protein expression levels of pLD-g10- PyMSP1$_{19}$ plant of T_0 generation reached up to 2% of total soluble protein (TSP) in mature leaves, 1% of TSP in old leaves and 0.54% of TSP in young leaves (**fig. 4b**).The large contribution of PyMSP1$_{19}$ was from the mature leaves, due to the presence of high number of chloroplasts and the high copy number of chloroplast genomes (up to 10,000 copies per cell), which resulting in very high levels of PyMSP1$_{19}$ expression in mature leaves.

4a



4b



Note: No of times the experiment was repeated (n=3)

In each experiment: - no of replicates are 2(replicates=2)

Figure 4: Quantification of expression of level of PyMSP1$_{19}$ in T_0 generation

(a) Standard curve for ELISA quantification

(b) Expression levels in %TSP of PyMSP1$_{19}$ expressing leaves (Young, Mature and Old).

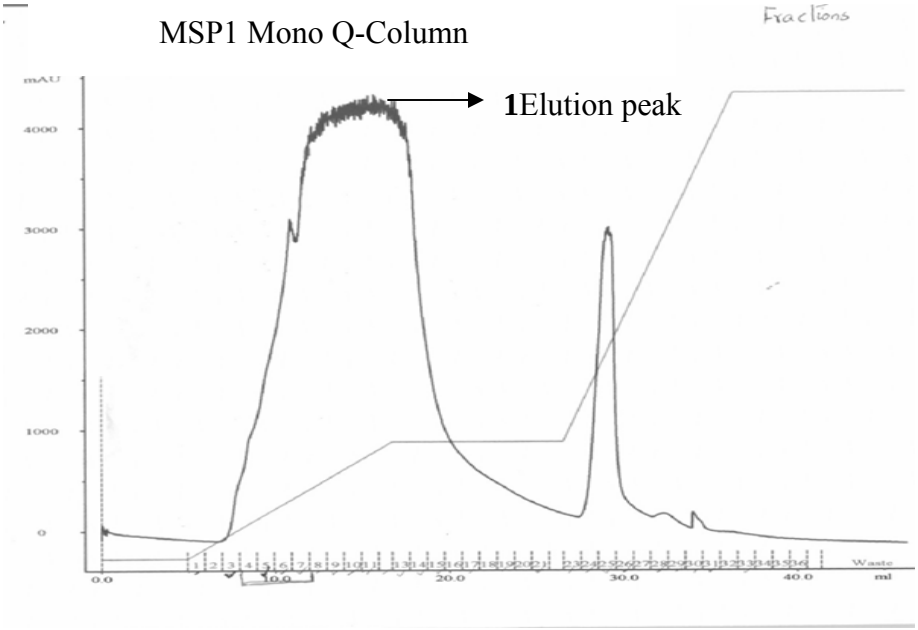
Note: %TSP is calculated = $\frac{\text{TSP of PyMSP1}_{19}\text{ protein}}{\text{TSP of total crude extract}}$ X 100

Enrichment of chloroplast-derived PyMSP119

A. Using Mono-Q and Superdex columns by FPLC

The $PyMSP1_{19}$ protein was eluted using Buffer B in a 25% gradient manner (**fig. 5a**). $PyMSP1_{19}$ protein was eluted at 25% Buffer B in 8 fractions (4-10). Some amount of protein was observed in flow through as well. All 8 fractions with flow through were pooled and loaded onto 10K MW protein concentrator to obtain concentrated protein. The concentrated protein was then run through superdex column to separate the protein based on its molecular weight (**fig. 5b**). All the fractions were collected and the alternate samples were tested with immunoblot. Since the expression levels of $PyMSP1_{19}$ protein was low and the amount of protein obtained at the end of the purification was not sufficient to carry out further experiments, therefore, the protein was enriched using lyophilization.

5a


MSP1 Mono Q-Column
Fractions
mAU
4000
3000
2000
1000
0
1Elution peak
0.0
10.0
20.0
30.0
40.0
ml
Waste

5b

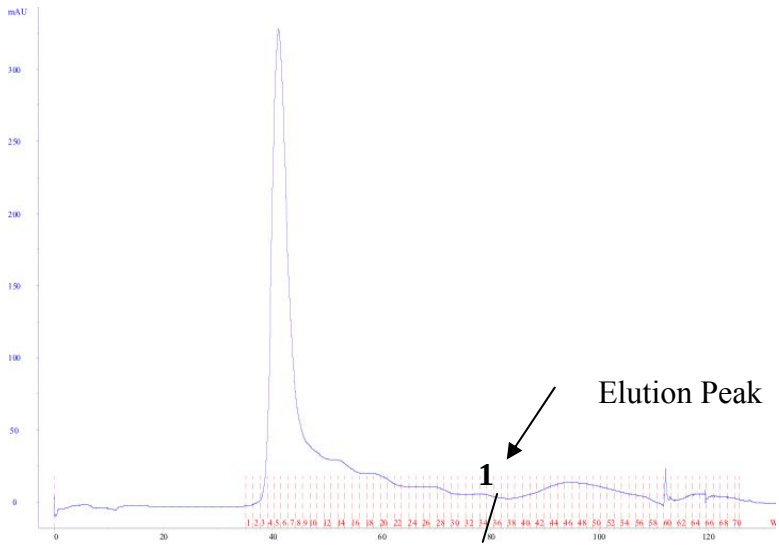

mAU
300
250
200
150
100
50
0
Elution Peak
1
0
20
40
60
80
100
120

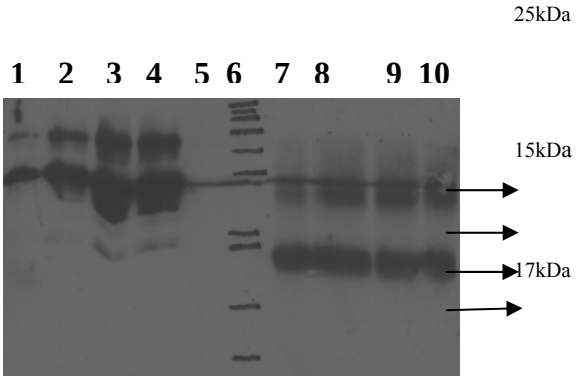
Figure 5: Enrichment of chloroplast-derived PyMSP1$_{19}$ by Mono-Q and Superdex columns by FPLC method

5a: 1. Elution peak where PyMSP1$_{19}$ protein was eluted using Mono-Q column **Fig 5b:** 1. Elution peak where PyMSP1$_{19}$ protein was eluted using superdex column**.**

B. Using Lyophilizer and Electro-elutor

The immunoblot assay was performed on the concentrated protein. The blot showed a 17kDa PyMSP1$_{19}$ protein which was used as guidance to excise the corresponding band from the coomassie stained SDS-PAGE. The PyMSP1$_{19}$ protein was eluted from the gel using an electro-eluter. The eluted protein was quantified by western blot using a densitometry (**fig. 6**).

6a



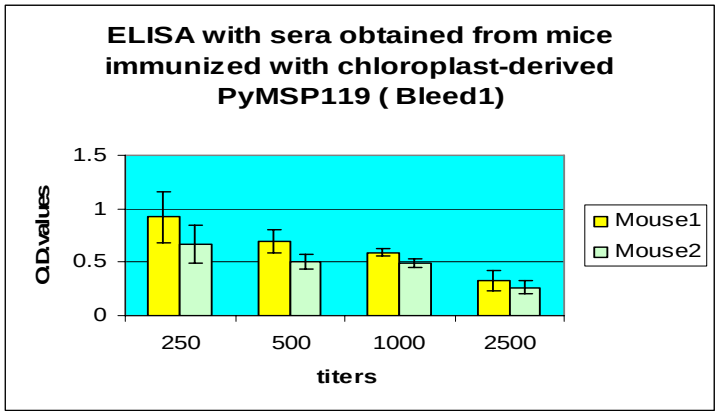
After Protein Elution

Figure 6: Westerns of eluted PyMSP1-19 protein: Lanes: 1-4 different concentrations of positive control GST-PyMSP1-19 (50ng/ul, 100ng/ul, 200ng/ul and 250ng/ul respectively). Lane5- wildtype, Lane6-ladder, Lane7-10-eluted PyMSP1$_{19}$ protein.

Immunization in mice

The first test bleeds for two immunized mice and one-control mice were sent at 0.1ml volume each at 1:10 dilution. The serum was tested for anti- PyMSP1$_{19}$ antibodies by conducting ELISA. Mouse#1 was showing higher immune response with IgG titers of 1:2500 than mouse#2 with IgG titers of 1:1000 (**Fig. 7a**). After the final boost, the immunized mouse-1 showed 1:7000 IgG titers whereas immunized mouse-2 showed a titer of 1:4000 (**Fig. 7b**).The control mouse injected with wild type crude extract did not show any significant anti- PyMSP1$_{19}$ IgG titers.

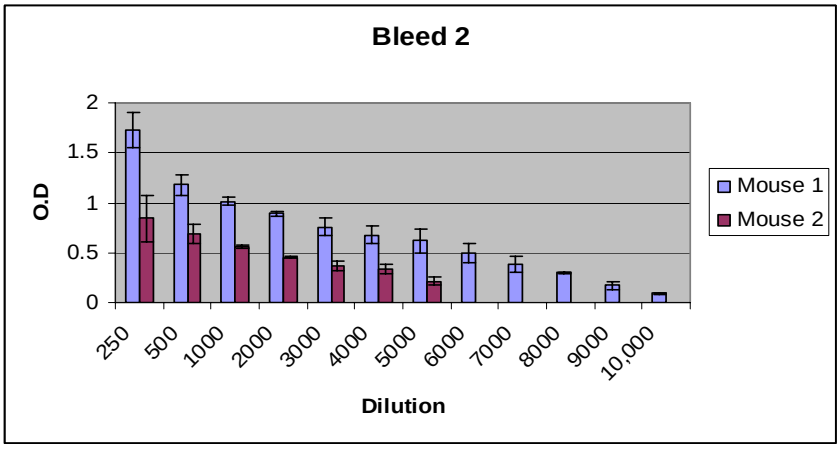
Figure: 7a



No of times the experiment was performed (n=3)

No of replicates per experiment: (r=2)

Figure: 7b



Number of times the experiment was repeated (n=3)

No. of replicates per experiment (r=2)

Figure 7: ELISA for the detection of anti- PyMSP1$_{19}$ antibody titers from serum samples collected 7 days after the first boost. Immune response between two mice is shown. Also the table is given to show the statistics.

In vitro Plasmodium falciparum inhibition assay with anti- PyMSP119 sera

To test the ability of anti-PyMSP1$_{19}$ antibodies in inhibiting the parasite entry into the red blood cells, an *in vitro assay* was conducted. After the incubation with synchronized *Plasmodium falciparum* culture (2% parasitemia and 2% hematocrit) and trophozoite-schizont stage infected RBC's (**fig. 8a**) for 48 hours, the parasitemia was estimated. The percentage of parasitemia = number of infected cells/ number of infected cells+ number of uninfected cells. The experimental investigations showed that the percentage of average parasitemia with anti-PyMSP1$_{19}$ antibodies was very similar with

the negative control (sera from mice immunized with wild type crude extract) and higher than the blank (untreated but incubated culture with media) (**fig. 8b**), indicating the failure of parasite inhibition into red blood cells by anti-PyMSP1$_{19}$ antibodies (**Table 1**). Also, the red blood cells treated with anti-PyMSP1$_{19}$ antibodies and the sera of wild type crude extract predominantly showed stressed late-trophozoites to early schizonts(**fig 8c and 8d**) as opposed to the positive control (treated with anti- PfMSP1$_{19}$), which showed 100% protection from parasite attack (**fig 8e**). The failure of parasitic inhibition could be attributed to the lack of cross reactivity across plasmodium Sp. (Between *yoelli* and *falciparum*).

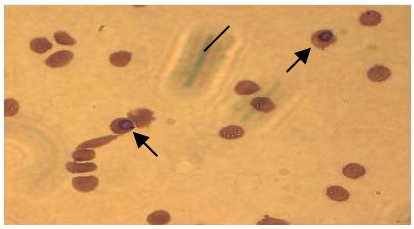
Table 1: Calculation of average parasitemia in RBC after *in vitro* inhibition assay:

Sample	Wells (infected/uninfected+infected RBC)	Wells (infected/uninfected+infected RBC)	Average parasitemia
Blank(RPMI)	12/254.Mostly consisted of rings and early trophozoites in infected RBC	14/286.Predominently consisted of rings and early trophozoites in infected cells	4.8%
Negative control (anti-sera from unimmunized mice)	35/300. Mostly stressed trophozoites were seen in infected RBC	19/223. Infected RBC were with stressed trophozoites	10.3%
Anti-PyMSp1$_{19}$	26/237.Moslty stressed	16/220.Stressed	9.2%

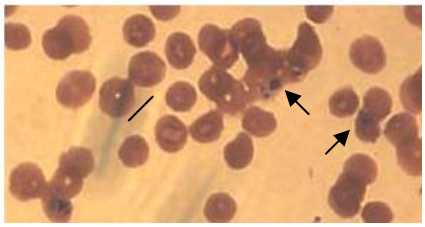
	early schizonts were seen	trophozoites and schizontswere observed.	
Positive control(anti-PfMSP119)	1/206	0/205	0%

Figure 8

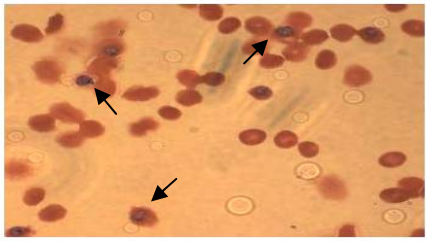
a) pre-treatment



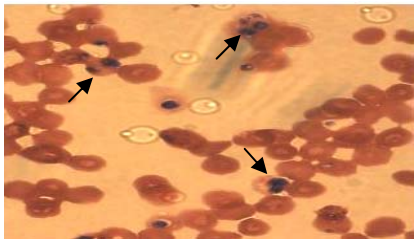
b) Blank –RPMI treated



c) Anti-PyMSP1$_{19}$ treated



d) Treated with un-immunized sera



e) Anti-PfMSP1$_{19}$ treated

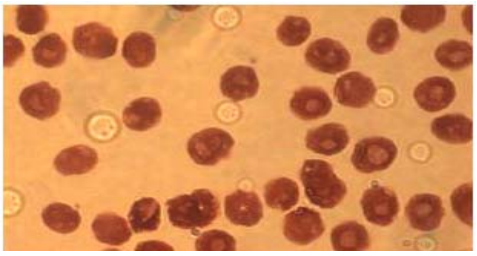


Figure 8: In vitro *Plasmodium falciparum* parasite inhibition assay using sera obtained from mice immunized with chloroplast-derived PyMSP1$_{19}$

a) Synchronized pretreated healthy and infected RBC consisting of trophozoites and schizonts.
b) Untreated culture consisting of uninfected and infected RBC with rings and early trophozoites- treated as blank
c) Treated with sera from the mice immunized with chloroplast-derived PyMSP1$_{19}$.Infected RBC mostly consisted of late trophozoites and early schizonts.
d) Treated with sera obtained from mice immunized with wild type crude extract. Infected cells showed late trophozoites
e) Treated with anti-PfMSP119 antibodies as a positive control. No infected cell was seen.

Arrows- represents the infected red blood cells. **Lines**- indicate the oil smear.

ELISA of PfMSP119 coated plates with anti- PyMSP119 antibodies

Since the *in vitro* assay with anti-PyMSP1$_{19}$ antibodies was not successful, we have decided to test whether the sera obtained from the mice immunized with chloroplast-derived PyMSP1$_{19}$ cross reacts with MSP1$_{19}$ antigen of *Plasmodium falciparum*. The

ELISA results showed that the average O.D values (0.123) obtained with sera from mice immunized with chloroplast-derived $PyMSP1_{19}$ were not significantly different from the O.D. values (0.117) obtained from negative control. Thereby, showing that anti-$PyMSP1_{19}$ antibodies were not cross-reacting with the $MSP1_{19}$ antigen of *Plasmodium falciparum*.

In vivo *P.yoelii* challenge into immunized mice

After challenging the immunized mice and control mouse with infected red blood cells, the percentage parasitemia for all three mice was monitored for every alternate day by collecting blood as described in Material and methods. The percentage parasitemia for immunized mouse-1 was 0.53%, 4.6%, 39.1%, 57.6% on day 3, 5, 7, 9 respectively after post-infection whereas IM-2 showed 0.39%, 20.1% on day3 and 5 respectively. The unimmunized control mouse showed 0.97%, 20.4%, 78.3% parasitemia on day 3, 5, 7 and 9 respectively after post-infection (**Table 2**). The days of survival of immunized mouse-1 were 10 days as opposed to immunized mouse-2 (8 days), while the unimmunized control mouse survived for 9 days. There is inverse correlation between between the parasitemia and the antibody titers.

Days after post-infection	Immunized Mouse-1	Immunized mouse-2	Control mouse
3	0.53%	0.39%	0.97%
5	4.6%	20.1%	20.4%
7	39.1%	Died	78.3%
9	57.6%		Died
10	Died		

Table2: Percentage parasitemia of immunized mice after challenge with *P.yoelii* infected RBC.

DISCUSSION

The main objective of expressing $PyMSP1_{19}$ antigen in chloroplast system using biolistic method was to develop a safer vaccine in plants and also the rodent model was used to establish a human malaria vaccine eventually. The final chloroplast pLD vector with pLD-g10- $PyMSP1_{19}$ cassette was integrated into the chloroplast genome via homologous recombination of trnA and trnI flanking sequences. The transcription and translation of $PyMSP1_{19}$ gene was regulated by 16srRNA promoter and bacteriophage 5'UTR-g10 with a ribosomal binding region respectively. The 3'UTR region downstream the gene stabilizes the transcripts. The integration of the cassette into the chloroplast genome was selected under 500mg/l of spectinomycin antibiotic pressure. Due to the presence of the *aadA* gene that encodes for the enzyme aminoglycoside 3' adenyltransferase in the pLD vector (Daniell *et al*., 2001a, Svab and Maliga, 1993), the plant cells with pLD-g10- $PyMSP1_{19}$ cassette would overcome the spectinomycin selection pressure. The viable cells undergo cell division along with the chloroplast genome including the pLD-g10 -$PyMSP1_{19}$ sequence. The integration of the gene within chloroplast genome was confirmed by PCR using 3P/3M and gene-specific primers. However, under the influence of the resistance developed by aadA gene product there will be simultaneous regeneration of wild type cells. As a result, the first round of selection would still contain both wild type and transgenic cells in the viable tissue. Therefore, the transgenic leaf tissues were subjected to second round of selection by placing them on a regeneration media containing 500mg/l spectinomycin.

The percentage of homoplasmy within the transgenic lines of pLD-g10- $PyMSP1_{19}$ was tested by performing southern analysis using flanking and gene-specific sequences as

probes. Although, a slight degree of heteroplasmy was seen in the transgenic lines, the presence of transformed genome was higher than the wild type genome and the complete transformation of wild type genome is expected by next generation (T1).

The translation of $PyMSP1_{19}$ gene was ensured by bacteriophage 5'UTR-g10 with ribosomal binding region and the expression of $PyMSP1_{19}$ protein was confirmed by western blot. The protein was seen at 17kDa size under denaturing conditions. Upon quantification, the $PyMSP1_{19}$ protein levels were 1.5-2% of TSP (**Fig 5**) The expression levels obtained were 1000 fold higher than the nuclear expressed $PyMSP1_{19}$ (Ghosh *et al.*, 2002). Although the expression levels of the protein in chloroplast were significantly higher than the nuclear expressed protein, compared with other chloroplast-derived vaccine antigens such as anthrax protective antigen-14%(Koya *et al*., 2005), CTB-33% (De cosa *et al*.,2001), Tetanus antigen-25%(Tregoning *et al*., 2003), the expression levels of $PyMSP1_{19}$ were very low. These low levels of $PyMSP1_{19}$ protein expression could be attributed to the absence of light-regulated 5'UTR- *psbA* promoter. The cloning of $PyMSP1_{19}$ gene with 5'UTR-psbA promoter was difficult and therefore, it had to be replaced with 5' UTR-bacteriophage g10. Since, the bacteriophage-g10 sequence consists of only ribosomal binding region and do not contain the promoter sequence, it will not hyper-express the gene but only ensures its proper translation.

Due to low levels of protein expression, the enrichment procedure was done for several rounds and also the functional studies had to be limited to two mice.100 μg of enriched protein was injected in two mice and anti-sera obtained from these mice were tested for anti- $PyMSP1_{19}$ antibodies using yeast expressed $PyMSP1_{19}$ protein. The titers obtained for immunized mouse-1 were 1:7000 and for immunized mouse-2 were 1:4000.

To prove the functionality of PyMSP1$_{19}$ gene, the *in-vitro P.falciparum* inhibition assays were performed based on the rationale of sequence homology (30%) between *P.yoelii* and *P.falciparum* (**Fig 10**). The expected outcome of the assay was the inhibition of *P.falciparum* invasion into the RBC, by the anti-sera. However, the parasite inhibition was not observed in *in vitro* assays. This could be attributed to the lack of cross reactivity between the anti-sera and *P.falciparum* MSP1$_{19}$. This was further confirmed by performing ELISA with anti-sera from the first bleed, where no cross reactivity was observed between anti-sera and P.falciparum antigen. Therefore, the in vivo experiments were carried out to test the functionality of anti- PyMSP1$_{19}$ antibodies developed against chloroplast-derived PyMSP1$_{19}$.

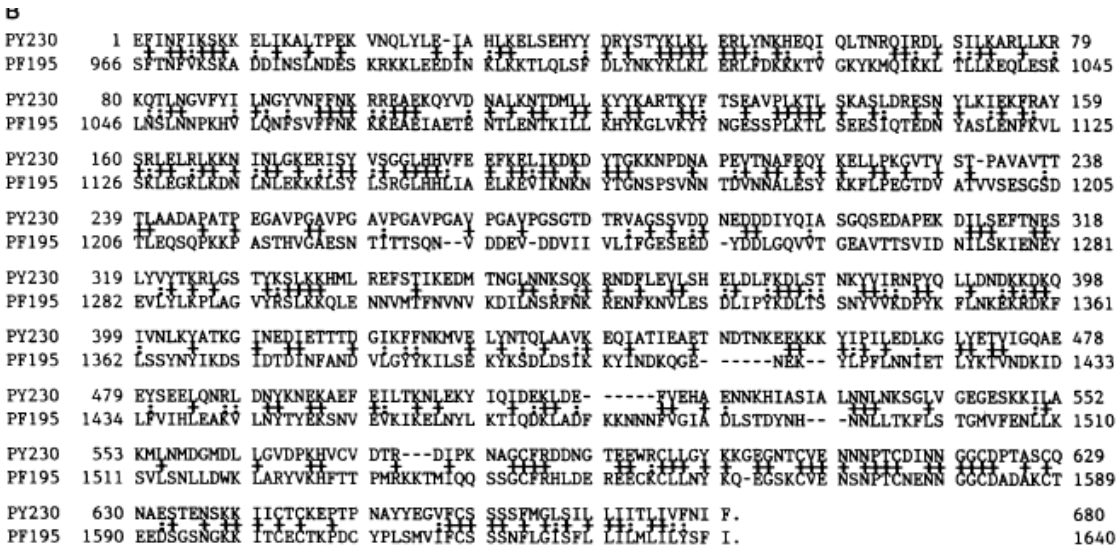


Figure 9: Homology between the C-terminal portion of Plasmodium yoelii(Py230) and Plasmodium falciparum (PF195) MSP1. Courtesy from Burns *et al*., 1988

The immunized mice were subjected to parasite challenge. When compared with unimmunized control mouse, the percentage parasitemia for immunized mouse-1 on day 5 after post-infection was five fold lower (4.6% parasitemia in IM-1 and 20.4% in

unimmunized control mouse) and two fold lower (39.1% in IM-1 and 78.3% in Mc) on day 7 after post-infection. On the other hand, the percentage parasitemia in immunized mouse-2 were parallel with unimmunized control mouse. This relative difference in parasitemia between immunized mouse-1 and immunized mouse-2 could be inversely correlated with the relatively higher immune titers of immunized mouse-1(1:7000) and relatively lower immune titers of immunized mouse-2(1:4000). The days of survival of immunized mouse-1 were 10 days as opposed to immunized mouse-2 (8 days), while the unimmunized control mouse survived for 9 days. However, for effective immunoprotective titers the dose protocol needs to be optimized with larger animal group to make a concrete conclusion.

CONCLUSION

The C-terminal 19kDa merozoite surface antigen of *Plasmodium yoelii* has been expressed in chloroplast system for the first time successfully. Due to 65% homology between the amino acid sequence of *Plasmodium falciparum* and *Plasmodium yoelii* (Burns *et al*., 1988), the production of chloroplast-derived $PyMSP1_{19}$ transgenic plants could create hope for developing a low cost human malaria vaccine in plants, which will eliminate some of the major constraints such as laborious purification techniques, extensive storage facilities and high technical skills.

The site-specific integration of $PyMSP1_{19}$ gene within chloroplast genome was confirmed by PCR using specific primers and the degrees of homoplasmy was measured by southern blot. To ensure proper expression of the transgene, immunoblot blot using anti- $PyMSP1_{19}$ antibodies was performed, where a 17kDa protein was detected under denatured, reduced conditions. The expression levels of $PyMSP1_{19}$ protein was detected by ELISA using anti-$PyMSP1_{19}$ antibodies and they showed a maximum expression of about 2% of TSP in mature leaves. The immunization studies in mice confirm that the chloroplast-derived $PyMSP1_{19}$ is immunogenic in nature as it elicited 1:2500 anti-$PyMSP1_{19}$ IgG titers with the sera collected from 7 days after first boost in mice. Although, the *in vitro Plasmodium falciparum* parasite inhibition assay was not successful with anti- $PyMSP1_{19}$ antibodies, the lack of cross reactivity between the two species explained the experimental outcome. The in vivo studies suggested that there was an inverse correlation between the parasitemia and the immune titers. However, concrete conclusions can be made with the studies using larger animal groups.

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