Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 38 (2008) 103–110
www.elsevier.com/locate/ijpara

Immunogenicity of *Plasmodium yoelii* merozoite surface protein 4/5 produced in transgenic plants

Lina Wang [a,*,1], Diane E. Webster [b,c,1], Alison E. Campbell [a], Ian B. Dry [d], Steve L. Wesselingh [b], Ross L. Coppel [a,e]

[a] *Department of Microbiology, Monash University, Clayton, Vic. 3800, Australia*
[b] *Children's Vaccines Group, Macfarlane Burnet Institute for Medical Research and Public Health, Prahran, Vic. 3004, Australia*
[c] *School of Biological Sciences, Monash University, Clayton, Vic. 3800, Australia*
[d] *Commonwealth Scientific and Industrial Research Organisation, Plant Industry, Glen Osmond, SA 5064, Australia*
[e] *Victoria Bioinformatics Consortium, Monash University, Clayton, Vic. 3800, Australia*

Received 12 April 2007; received in revised form 15 June 2007; accepted 20 June 2007

Abstract

Malaria is a major global health problem for which effective control measures are urgently needed. Considerable effort has been focused on the development of effective vaccines against the causative parasite and protective vaccine trials are now being reported. Due to the relative poverty and lack of infrastructure in malaria-endemic areas, a successful immunisation strategy will depend critically on cheap and scaleable methods of vaccine production, distribution and delivery. One promising technology is transgenic plants, both as a bioreactor for the vaccine-manufacturing process as well as a matrix for oral immunisation. In this study, we investigated the feasibility of using transgenic plants to induce protective immunity against malaria infection using *Plasmodium yoelii* merozoite surface protein 4/5 (PyMSP4/5) in a mouse model of malaria infection. Our data show that the PyMSP4/5 protein can be produced in plants in a configuration that reacts with protective antibodies. Optimisation of codon usage for the PyMSP4/5 gene resulted in significantly increased antigen expression in plants. PyMSP4/5 protein from the codon-optimised construct accumulated to 0.25% of total soluble protein, a sixfold increase over the native gene sequence. Tobacco-made PyMSP4/5 was able to induce antigen-specific antibodies in mice following parenteral delivery, as well as boost the antibody responses induced by DNA vaccination when delivered parenterally or orally. We believe this is the first report to show that plant-made malaria antigens are immunogenic. However, the antibody levels were not high enough to protect the immunised mice against a lethal challenge with *P. yoelii*. Further strategies are needed to achieve a protective dose, including improvements to antigen expression levels in plants and strategies to enhance the immunogenicity of the expressed antigen.

Keywords: *Plasmodium yoelii*; PyMSP4/5; Transgenic plants; Oral vaccine; Malaria

1. Introduction

Malaria, an infectious disease caused by species of the parasite genus *Plasmodium*, is a major world health problem. Development of vaccines targeting various stages of the parasite life cycle holds great promise for assisting the control of the disease. A number of malaria proteins have been demonstrated to be potential vaccine candidates, and are in various stages of pre-clinical and clinical evaluation (Malkin et al., 2006; Moorthy et al., 2004). Due to the relative poverty and lack of infrastructure in malaria-endemic areas, a successful immunisation strategy will depend critically on cheap and scaleable methods of vaccine production, distribution and delivery. Affordable and effective vaccine formulations which are stable at ambient temperatures and able to be delivered without the use of needles would significantly enhance widespread vaccine deployment.

* Corresponding author. Tel.: +61 3 9905 1500; fax: +61 3 9905 4811.
E-mail address: lina.wang@med.monash.edu.au (L. Wang).
[1] These authors contributed equally to this work.

0020-7519/$30.00
doi:10.1016/j.ijpara.2007.06.005

One promising technology is the use of transgenic plants as bioreactors for the production and possible delivery of vaccine antigens. Studies have shown that plants can express, fold, assemble and process foreign proteins in a way that preserves their antigenicity and can provide both a simple vaccine-manufacturing process as well as a matrix suitable for oral immunisation (Schillberg et al., 2005). Plant-based vaccines are currently being developed for a number of human and animal diseases, several of which have been successfully tested in end-point species, including humans (Ma et al., 2005; Twyman et al., 2005). The feasibility of this platform technology has been validated recently with the licensing of an injectable plant-made poultry vaccine in the USA by Dow AgroSciences (http://www.dowagro.com/animalhealth/resources/firstlic.htm).

The capacity of transgenic plants to produce properly conformed proteins at low cost makes their application in malaria vaccine development attractive, particularly now that it has been demonstrated that oral immunisation can induce effective immunity against malaria (Wang et al., 2003). Furthermore, the successful induction of hepatitis B virus-specific serum antibodies in mice and humans fed with plants expressing hepatitis B surface antigen (Kapusta et al., 1999; Thanavala et al., 2005; Huang et al., 2006) provides a precedent for using plant-based oral vaccines against non-enteric pathogens. Thus, we set out to examine the feasibility of using transgenic plants to produce a subunit vaccine and induce protective immunity in a mouse system of *Plasmodium yoelii* infection of the laboratory mouse.

Plasmodium yoelii merozoite surface protein 4/5 (PyMSP4/5) is the homologue of *Plasmodium falciparum* merozoite surface proteins 4 (MSP4) and 5 (MSP5) (Kedzierski et al., 2000a). Both MSP4 and MSP5 are glycosylphosphatidylinositol (GPI)-anchored integral membrane proteins with similar structural features (Marshall et al., 1998). They are both immunogenic during natural infection (Wang et al., 2001) and antibodies raised to them are capable of inhibiting parasite growth in vitro (our unpublished data), suggesting their potential as components of a subunit malaria vaccine. Their single homologue in the rodent malaria species *P. yoelii*, PyMSP4/5, is highly effective at protecting mice against lethal challenge (Kedzierski et al., 2000b, 2001). MSP4 and MSP5 are now in the process of pre-clinical development and manufacture (http://www.malariavaccine.org/ab-current_projects.htm) for testing in humans. We have previously shown that oral immunisation with *Escherichia coli*-expressed recombinant PyMSP4/5 is able to induce serum antibodies at comparable levels with those achieved with parenteral immunisation, and provide significant protection against malaria infection (Wang et al., 2003). This finding provides a rationale for the development of a plant-made oral vaccine against malaria. In this report, we show that PyMSP4/5 can be produced by transgenic tobacco and that it is immunogenic in mice.

2. Materials and methods

2.1. Construction of plant expression vectors

The entire coding sequence of PyMSP4/5 (Kedzierski et al., 2000a) lacking only the C-terminal GPI attachment signal was amplified by PCR using primers P922 and P924, and cloned into pRTL2 (Huang et al., 2001) downstream of the cauliflower mosaic virus 35S promoter and tobacco etch virus leader (Fig. 1a). Primer P922 (5′-GCG CCATGGAAATCACAAATTACTTATC-3′) includes the native PyMSP4/5 secretion signal sequence and an NcoI site which changes the second amino acid from lysine to glutamic acid. Primer P924 (5′-CGCGGATCCTCATAGCTCATCTTTCTCAGATGAATGCGCACTGAGTAAT-3′) includes the sequence coding for the SEKDEL motif (underlined), which is an endoplasmic reticulum (ER) retention signal (Munro and Pelham, 1987). In a second construct, the putative PyMSP4/5 secretion signal was removed during PCR using primers P923 and P924. P923 (5′-CGCGAATTCTTAAGAATGTTCATGAAATTTCT-3′) includes an AflII site to facilitate the introduction of the secretion signal from the tobacco Prla protein (Hammond-Kosack et al., 1994). The secretion signal was amplified with a 5′ NcoI site and a 3′ AflII site using primers P925 (5′-GCGAAGCTTCCATGGGATTTGTTCTCTTTTCA-3′) and P926 (5′-GCGCTTAAGGGCACGGCAAGAGTGG-3′), and cloned in-frame upstream of the truncated PyMSP4/5 gene. This resulted in the addition of a lysine between the signal peptide and the truncated PyMSP4/5 sequence. Following sequence verification, the PyMSP4/5 expression cassettes were transferred into the binary vector pBIN19plus, which also contains a neomycin phosphotransferase II expression cassette that confers kanamycin resistance (van Engelen et al., 1995).

The PyMSP4/5 gene, lacking the putative secretion signal and the GPI anchor, was optimised for expression in plants using tobacco (*Nicotiana tabacum*) and lettuce (*Lactuca sativa*) codon usage tables from the Codon Usage Database (http://www.kazusa.or.jp/codon/). Design of the optimised sequence was undertaken manually with the assistance of software provided by the Australian National Genomic Information Service (ANGIS) (Fig. 1b). To construct the codon-optimised PyMSP4/5 gene, 16 oligonucleotides (59–70 bp in length) were synthesised, covering both strands with 7 bp overlap. The complementary oligonucleotides were annealed at 65 °C for 30 min then allowed to cool to room temperature. The resultant double-strand DNA fragments were ligated together and the assembled gene was cloned into pRTL2 to replace the original PyMSP4/5 gene (Fig. 1a). The expression cassette was then transferred to pBINplus.

2.2. Tobacco transformation

The recombinant pBINplus vectors were electroporated into *Agrobacterium tumefaciens* LBA 4404 and used