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Production, characterisation and immunogenicity of a plant-made *Plasmodium* antigen—the 19 kDa C-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1

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Abstract Development of a safe, effective and affordable malaria vaccine is central to global disease control efforts. One of the most highly regarded proteins for inclusion in an asexual blood stage subunit vaccine is the 19-kDa C-terminal fragment of merozoite surface protein 1 ($MSP1_{19}$). As production of vaccine antigens in plants can potentially overcome cost and delivery hurdles, we set out to produce $MSP1_{19}$ in plants, characterise the protein and test its immunogenicity using a mouse model. *Plasmodium yoelii* $MSP1_{19}$ ($PyMSP1_{19}$) was produced in *Nicotiana benthamiana* using the MagnICON® deconstructed TMV-based viral vector. $PyMSP1_{19}$ yield of at least 23% total soluble protein (TSP;3–4 mg/g Fwt) were achieved using a codon-optimised construct that was targeted to the apoplast. Freeze-dried leaf powder contained at least 20 mg $PyMSP1_{19}$ per gram dry weight and the protein retained immunogenicity in this form for more than 2 years. Characterisation studies, including SDS-PAGE, mass spectrometry and circular dichroism, indicated that the plant-expressed $PyMSP1_{19}$ was similar to its *Escherichia coli*- and *Saccharomyces cerevisiae*-expressed counterparts. Purified plant-made $PyMSP1_{19}$ induced strong immune responses following intraperitoneal immunisation, although titres were lower than those induced by an equivalent dose of purified *E. coli*-expressed $PyMSP1_{19}$. The reason for this is uncertain but may be due to differences in the oligomerisation profile of the vaccines. The plant-made $PyMSP1_{19}$ vaccine was also found to be orally immunogenic when delivered alone or following immunisation with a $PyMSP1_{19}$ DNA vaccine. This study adds to an increasing body of research supporting the feasibility of plants as both a factory for the production of malaria antigens, and as a safe and affordable platform for oral delivery of a temperature-stable malaria vaccine.

Keywords Malaria · *Plasmodium yoelii* · Plant-made vaccines · $PyMSP1_{19}$

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Introduction

Malaria is prevalent in over 100 countries and territories around the world and remains one of the leading causes of morbidity and mortality, especially in children under the age of 5. Although drugs are available for the treatment of malaria, resistance to these drugs is widespread. A vaccine that can reduce the human toll would be a valuable resource in the fight against malaria. A number of subunit vaccine candidates have been proposed and tested, some with encouraging results (Ellis et al. 2009; Pichyangkul et al. 2009). For the asexual blood stage, one of the highly regarded candidate antigens is the 19-kDa C-terminal fragment of merozoite surface protein 1 ($MSP1_{19}$) (Daly and Long 1993; Hirunpetcharat et al. 1997; Mazumdar et al. 2010). This fragment is carried into red blood cells during merozoites invasion (Hodder et al. 1996) and the presence of antibody against the fragment is protective (Tian et al.

1997). The effectiveness of $MSP1_{19}$ has been demonstrated in a mouse model in which *Plasmodium yoelii* $MSP1_{19}$ ($PyMSP1_{19}$) was used as the major vaccine component (Holder and Freeman 1981; Daly and Long 1993; Hirunpetcharat et al. 1997). *P. falciparum* $MSP1_{19}$ ($PfMSP1_{19}$) is currently being tested in human clinical trials, either alone or in combination with other candidate antigens (Hu et al. 2008; Malkin et al. 2008; Peng et al. 2010).

Although vaccine antigens are traditionally produced by bacteria, yeast or mammalian cells, plants are emerging as a robust and reliable method of recombinant protein production (Ling et al. 2010; Yusiboy et al. 2011). The advantages of this method include low cost, safety, scalability, fewer constraints in distribution and the potential for oral delivery (Spok et al. 2008). Methods to express antigens in plants by stable transformation have been available for some time and a large number of vaccine antigens have been produced. However, the development cycle time is quite long and antigen yield has been low for malaria proteins (Ghosh et al. 2002; Wang et al. 2008). More recently, viral-based transient expression approaches have been used to substantially improve yields (Marillonnet et al. 2004; Webster et al. 2009). In this project, a deconstructed tobacco mosaic virus-based transient expression system, magnICON® from ICON Genetics (Germany), was employed to produce $PyMSP1_{19}$ (Marillonnet et al. 2004). High yields of recombinant protein were achieved and the plant-made $PyMSP1_{19}$ was shown to be immunogenic in laboratory mice by either intraperitoneal or oral immunisation.

Materials and methods

Generation of a plant codon-optimised $PyMSP1_{19}$ sequence

The gene encoding $PyMSP1_{19}$ 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, University of Sydney, Australia). To construct the codon-optimised $PyMSP1_{19}$ gene, 12 oligonucleotides (30–75 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 (Wang et al. 2008).

Plasmid construction

The coding sequence of $PyMSP1_{19}$, excluding the GPI anchor attachment signal, was amplified by PCR using primers PyMSP1-F (5′-GCCATGCCATGGATGGTATGGATTTATTA-3′) and PyMSP1-R (5′-ATCCGCGGATCCTACTTATTTA**ATGATGGTGATGGTGATG**GCTGGAAGAACTACAGAA-3′), which incorporated an *Nco*I site at the 5′ end and a *Bam*HI site at the 3′ end (underlined). The PyMSP1-R primer also contained a sequence encoding a hexahistidine tag (shown in bold). The PCR product was digested with *Nco*I/*Bam*HI and ligated into the 3′ viral module, pICH11599 (ICON Genetics, Halle, Germany), resulting in plasmid pICH-$PyMSP1_{19}$. The plant-codon optimised $PyMSP1_{19}$ gene was also cloned into pICH11599 using the same method following amplification with primers CO-PyMSP1-F (5′-GCCATGCCATGGATGGTATGGATCTCTTG-3′) and PyMSP1-R, yielding plasmid pICH-co$PyMSP1_{19}$. After the correct sequences of the $PyMSP1_{19}$ genes were confirmed by sequencing analysis, the plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 and used for agroinfiltration of *Nicotiana benthamiana* leaves. Plasmids pICH14011, pICH15879, pICH12190 and pICH8420 have been described previously (Marillonnet et al. 2004; Huang et al. 2006; Webster et al. 2009). pICH14011 contains the integrase module while pICH15879, pICH12190 and pICH8420 are the 5′ modules containing the viral replicase gene and targeting presequences for cytosol, chloroplast and apoplast, respectively.

Agroinfiltration

A. tumefaciens bacteria containing the relevant plasmids were cultured in LB broth supplemented with rifampicin (50 μg/ml) and carbenicillin (100 μg/ml) at 28°C. The cells from overnight cultures were sedimented by centrifugation at 2,500 × *g* for 5 min and resuspended in infiltration buffer (10 mM 2-(*N*-morpholino)ethane-sulphonic acid, 10 mM Mg_2SO_4, pH 5.5) to a final OD_{600} of 0.2. Equal volumes of *A. tumefaciens* suspension containing the three viral modules (5′ module, 3′ module and integrase) were mixed and injected/infiltrated into the abaxial leaf surface of 6- to 12-week-old *N. benthamiana* plants as described previously (Huang et al. 2006; Webster et al. 2009). To minimise the possible plant and environmental variability, a pair-wise comparison method was adopted, which involved inoculation of different halves of the same leaf for the comparison of different constructs (e.g., original vs. codon-optimised $PyMSP1_{19}$). For small-quantity analysis, leaf halves were kept separately. For large-quantity purification for immunisation, leaf halves of the same type were combined.

Total soluble protein and total protein extraction and analysis

N. benthamiana leaves were harvested at 12 days post-infiltration and snap frozen in liquid nitrogen, then ground

to a fine powder. To extract the total soluble protein (TSP), the leaf powder was mixed with ice-cold extraction buffer (PBS containing 100 mM ascorbic acid, 20 mM EDTA, 0.1% (v/v) Triton X-100, supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland) at a ratio of 2 ml buffer per gram of fresh leaf weight, followed by centrifugation at 4°C for 15 min. Total protein (TP) was extracted by adding sample buffer (0.38 M Tris–HCl, 2% (v/v) SDS, 20% (v/v) glycerol, 10% (w/v) bromophenol blue) to the leaf powder instead of TSP extraction buffer. For samples intended for purification by Ni-NTA affinity chromatography, EDTA was omitted from the extraction buffer and the pH was adjusted to 8.0. TSP and TP samples were fractionated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with polyclonal antibodies raised to $PyMSP1_{19}$ expressed in *Escherichia coli* ($EcPyMSP1_{19}$) or HRP-conjugated anti-His tag antibodies. Protein expression in percentage TSP was quantified by densitometry measurements with known quantities (range, 0.1–1.5 μg) of $PyMSP1_{19}$ expressed in *Saccharomycescerevisiae* ($ScPyMSP1_{19}$) as a standard on Western blots using Image J (http://rsb.info.nih.gov/ij/). Quantitation of expression in mg/g dry weight was determined by similar densitometry measurement of TP in 10 mg freeze-dried leaf powder.

$PyMSP1_{19}$ purification and characterisation

$PyMSP1_{19}$ produced in *N. benthamiana* leaves ($NbPyMSP1_{19}$) was purified from TSP by Ni-NTA affinity chromatography (Qiagen, Valencia, CA), according to the manufacturer's instruction. Two rounds of Ni-NTA affinity chromatography were performed, the resultant protein was analysed by SDS-PAGE and the concentration was determined by absorbance at 280 nm. Expression of the hexahistidine-tagged $EcPyMSP1_{19}$ protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and purified with TALON metal affinity resin under native conditions according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA). Expression and purification of $ScPyMSP1_{19}$ was described previously (Kaslow et al. 1994). $NbPyMSP1_{19}$, $EcPyMSP1_{19}$ and $ScPyMSP1_{19}$ were all subjected to MALDI-MS on a 4700 Proteomics Discovery System instrument (Applied Biosystems, Calsbad, CA). Circular dichroism (CD) spectra were recorded on a Jasco J-815 CD Spectrometer (Essex, UK) over a wavelength range of 190–260 nm, at a temperature of 20°C. Samples were analysed in 50 mM sodium phosphate pH 7.2. Data were collected using a step size of 0.1 nm at a scan rate of 50 nm/min in a 1-mm quartz cuvette. Data were analysed using Spectra Manager II software (Jasco, Essex, UK).

Immunisation of mice and antibody analyses

All mice were purchased from the Central Animal Services of Monash University and the procedures were performed in accordance with the Monash University Animal Ethics Committee requirements and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Groups of female BALB/c mice (n=8, power 85%, $p<0.05$), aged from 6 to 8 weeks, were immunised either intraperitoneally (with purified protein) or orally (with freeze-dried leaf powder). For intraperitoneal injection, mice were immunised with 25 μg of protein emulsified in Freund's adjuvant (complete adjuvant for the primary immunisation and incomplete for the subsequent immunisations) at weeks 0, 3 and 6. Oral immunisation with freeze-dried leaf powder was conducted as a prime-boost regime. Mice were immunised with plasmid DNA (VR1020 vector from Vical with $NbPyMSP1_{19}$ gene inserted) using a gene gun (1 μg/dose) at week 0 and gavaged with 10 mg leaf powder (containing 200 μg of $PyMSP1_{19}$) suspended in 250 μl PBS containing 100 mM ascorbic acid and 1 mg saponin (from *Quillaja* bark; Sigma 84510) at weeks 6, 7, 8, 9, 13, and 14. The $PyMSP1_{19}$-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Wang et al. 2008) with $EcPyMSP1_{19}$ as the coating antigen for determination of antibody titre and IgG profiles, or with TSP for comparison of stability of $NbPyMSP1_{19}$. For antibody titre determination, sera were tested at a 2-fold serial dilution and titre was defined as the reciprocal of the highest dilution that gave a specific absorbance greater than 0.1. For determination of IgG profiles, a panel of alkaline phosphotase-conjugated anti-mouse immunoglobulin subclass, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associate Inc., Birmingham, AL) were used as the secondary antibodies. For comparison of stability of $NbPyMSP1_{19}$, $EcPyMSP1_{19}$-specific mouse sera were used as primary antibody and alkaline phosphotase-conjugated anti-mouse IgG was used as secondary antibody.

Statistical analysis

The difference between two groups of plant materials (freeze-dried leaf powder either frozen at −70°C or stored at room temperature) or animal data (mice immunised with $EcPyMSP1_{19}$ or $NbPyMSP1_{19}$, as well as mice gavaged with either wild type leaf powder or with $NbPyMSP1_{19}$-containing leaf powder) was evaluated by a t-test with unequal variance using GraphPad Prism 5 software (GradhPad Software, Inc., La Jolla, CA). For comparisons between the effect of native and codon-optimised sequences under pairwise infiltration conditions, paired t-tests were applied.

Results

Optimisation of the codon usage for PyMSP1$_{19}$ significantly increased expression

A synthetic PyMSP1$_{19}$ gene was constructed to more closely reflect the codon bias of the target species used for expression but with minimal alteration to the amino acid sequence (Fig. 1). Codons with a usage frequency of <5/1,000 in plants were removed unless they were also rare in *P. yoelii*. The AT content was reduced from 66.4% to 53.5%, one mRNA destabilising motif ATTTA was removed and two potential *N*-linked glycosylation sites were removed by replacing the threonine residues with alanine.

PyMSP1$_{19}$ was successfully expressed in *N. benthamiana* leaves (NbPyMSP1$_{19}$) using the magnICON® viral vector expression system. Codon optimisation significantly increased the yield of NbPyMSP1$_{19}$ compared to the native sequence (Fig. 2). Increases of between 2- and 4-fold were achieved for NbPyMSP1$_{19}$ harvested at 12 days post infiltration (dpi) using the cytosol- and apoplast-targeting 5′ modules (Fig. 2). Interestingly, NbPyMSP1$_{19}$ expression was undetectable when the chloroplast-targeting 5′ module was used with either the native or the codon-optimised gene sequences. Maximum yields were obtained at 12 dpi using the codon-optimised sequence with the apoplast-targeting 5′ module. NbPyMSP1$_{19}$ from the apoplast targeted construct is clearly visible on a Coomassie stained SDS-PAGE gel, which also serves as loading control (Fig. 2b) and quantification indicated yields of 25±3% (mean ± SD) TSP. Freeze-dried leaf powder contained 20±6 mg (mean ± SD) NbPyMSP1$_{19}$ per gram of dry weight.

Plant-derived PyMSP1$_{19}$ was structurally similar to the same protein produced in *E. coli* or *S. cerevisiae*

NbPyMSP1$_{19}$ was compared with EcPyMSP1$_{19}$ or ScPyMSP1$_{19}$. Recombinant PyMSP1$_{19}$ from all sources reacted with PyMSP1$_{19}$-specific antibodies as well as anti-hexahistidine antibodies (Fig. 3). The observed molecular mass of ~19 kDa on SDS-PAGE was consistent with previous studies. Precise determination by mass spectrometry recorded sizes of 12,233, 12,644 and 12,617 Da for EcPyMSP1$_{19}$, ScPyMSP1$_{19}$ and NbPyMSP1$_{19}$, respectively (Fig. 4). The slight differences were likely due to four extra amino acids (LDKR) in ScPyMSP1$_{19}$ and three extra residues (EVG) in NbPyMSP1$_{19}$ at the Ntermini of the proteins as a result of sequences added to assist in cloning of the gene into the expression vectors. Mass spectrometry plots also showed that recombinant PyMSP1$_{19}$ was predominantly present as monomer for all three species. Small amounts of dimer were also present in all three species but the amount in EcPyMSP1$_{19}$ was higher than those in ScPyMSP1$_{19}$ and NbPyMSP1$_{19}$ (Fig. 4). A putative higher order oligomer for EcPyMSP1$_{19}$ is also visible by Western blot (Fig. 3a). No significant difference was observed in the molar ellipticity among the three proteins as determined by CD spectra, which indicated a secondary structure of random coil (Fig. 3c).

Plant-derived PyMSP1$_{19}$ was highly stable in freeze-dried leaf powder

Leaf material containing NbPyMSP1$_{19}$ was snap frozen, ground and freeze-dried. A vaccine batch was created by mixing the freeze-dried powder to ensure delivery of a consistent dose. Aliquots of the leaf powder were stored at

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ATGGATGGTATGGATTTATTAGGTGTAGACCCTAAACATGTATGTGTTGATACAAGAGAT   60
---------------C-C--G--A--G--------G--C--C------------------

ATTCCTAAAAATGCTGGATGTTTTAGAGATGATAATGGTACTGAAGAATGGAGATGTTTA  120
-----C--G--C-----T--C--C--------C-----AG-------G-----G-----G

TTAGGTTACAAAAAAGGTGAAGGTAATACATGTGTAGAAAATAATAATCCTACTTGTGAT  180
C-G--C-----G----------G--------C--G--G-----C------G-------C

ATCAACAATGGTGGATGTGATCCAACTGCTAGTTGTCAAAATGCGGAAAGTACGGAAAAT  240
-----------------C----------CTC------G--C------TCC--C--G---

TCCAAAAAAATTATATGTACATGTAAAGAACCAACCCCTAATGCATATTATGAAGGTGTA  300
AGT-----G--A--C--C--------G--G----------C-----C--------A-G

TTCTGTAGTTCTTCCAGC                                            318
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Fig. 1 Alignment of the endogenous PyMSP1$_{19}$ gene sequence and the codon-optimised PyMSP1$_{19}$ sequence. The nucleotides are shown for the endogenous PyMSP1$_{19}$, whereas for codon-optimised PyMSP1$_{19}$ only mutated nucleotides are shown, with identical nucleotides marked by *dashed lines*. Both sequences encode the identical PyMSP1$_{19}$, except that two threonine residues (*underlined*) are replaced with alanines in codon-optimised PyMSP1$_{19}$ to remove the two *N*-linked glycosylation sites

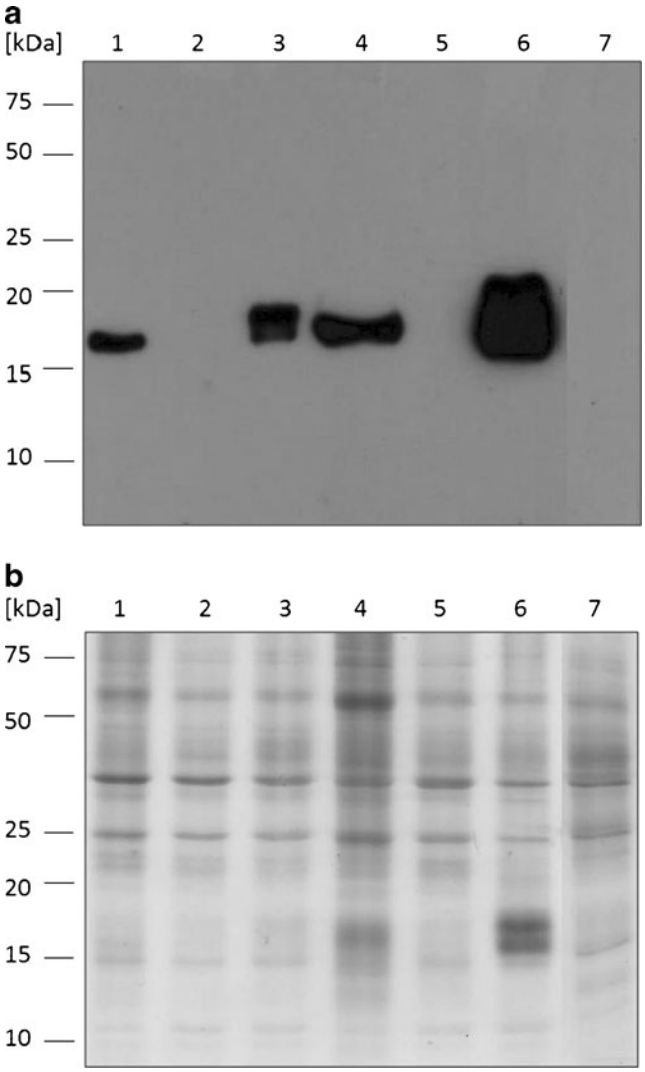

Fig. 2 Comparison of NbPyMSP1$_{19}$ expression in agroinfiltrated leaves for different targeting signals and codon optimisation. **a** Detection of NbPyMSP1$_{19}$ protein by Western blot (under reduced condition) using EcPyMSP1$_{19}$-specific antibodies. *Lanes 1–3* total proteins from leaves infiltrated with native PyMSP1$_{19}$ sequence; *lanes 4–6* leaves infiltrated with codon-optimised PyMSP1$_{19}$ sequence. *Lanes 1 and 4* cytosol targeting; *lanes 2 and 5* chloroplast targeting; *lanes 3 and 6* apoplast targeting. *Lane 7* (negative control) was from wild-type leaves. Protein loading ranged from 15 to 20 μg. **b** Confirmation of protein loading of the samples in **a** (*same lanes*) by Coomassie stain

room temperature or −70°C. After 2 years, an equal amount of leaf powder from both aliquots was analysed for the presence of NbPyMSP1$_{19}$ by Western blot. As shown in Fig. 5a, both preparations had the same amount of NbPyMSP1$_{19}$, indicating that NbPyMSP1$_{19}$ in freeze-dried leaf powder was well preserved at room temperature. ELISA analysis also confirmed the equal reactivity of both preparations (as coating antigens) with PyMSP1$_{19}$-specific antibodies (Fig. 5b).

Plant-derived PyMSP1$_{19}$ was immunogenic

Immunogenicity of NbPyMSP1$_{19}$ was investigated via two immunisation experiments. In the first experiment, mice were immunised with purified NbPyMSP1$_{19}$ emulsified with Freund's adjuvant via intraperitoneal injection. As shown in Fig. 6a, mice immunised with NbPyMSP1$_{19}$ developed PyMSP1$_{19}$-specific antibodies with a mean titre of 262,144 compared to 50 for the control mice ($p<0.0001$), demonstrating the parenteral immunogenicity of NbPyMSP1$_{19}$. The antibody responses induced by NbPyMSP1$_{19}$ were lower than those induced by EcPyMSP1$_{19}$ (average titre of 262,144 compared to 2,753,000, $p<0.0001$). Further analyses of the antibody by isotype profiles (Fig. 6b) revealed that IgG1 was the predominant subclass for all PyMSP1$_{19}$ immunised mice. However, EcPyMSP1$_{19}$ induced a more balanced profile with significant amounts of IgG2b and IgG3 compared to NbPyMSP1$_{19}$.

A second experiment was designed to examine the oral immunogenicity of the plant-made PyMSP1$_{19}$ in the context of a prime-boost vaccination schedule. Mice were immunised on day 0 with DNA plasmid to prime the immune system. This was followed by six boosting doses of leaf powder by gavage with a mucosal adjuvant. As shown in Fig. 7, immunisation with NbPyMSP1$_{19}$ resulted in a significant increase in end-point PyMSP1$_{19}$ specific antibody titres compared to gavage with wild-type leaves, irrespective of the DNA vaccine used ($p<0.0001$). Immunisation with the PyMSP1$_{19}$ DNA vaccine followed by gavage with wild-type leaves resulted in an average titre of only 38. Whereas mice immunised with the control or PyMSP1$_{19}$ DNA vaccine followed by NbPyMSP1$_{19}$ recorded average titres of 64,520 and 14,592, respectively. The impact of prior immunisation with the DNA vaccine on PyMSP1$_{19}$ titres was not statistically significant ($p=0.1537$). However, the smaller standard deviation observed for the PyMSP1$_{19}$ DNA primed group indicates a more consistent response to the mucosally delivered NbPyMSP1$_{19}$ vaccine. Collectively, this data demonstrates that NbPyMSP1$_{19}$ was orally immunogenic alone and in combination with the PyMSP1$_{19}$ DNA vaccine.

Discussion

Production of malaria antigens in plants is a promising approach as the technology has developed to a state where the yields of recombinant proteins are much enhanced. In this study, we described the rapid expression of PyMSP1$_{19}$ in *N. benthamiana* leaves using the magnICON® viral vector system. We achieved an optimal yield of more than23% TSP or 3–4 mg/g fresh weight (using the result of 20 mg/g dry weight and the ratio of dry weight to wet weight from 0.15 to 0.2) by use of the apoplast-targeting 5′ module and

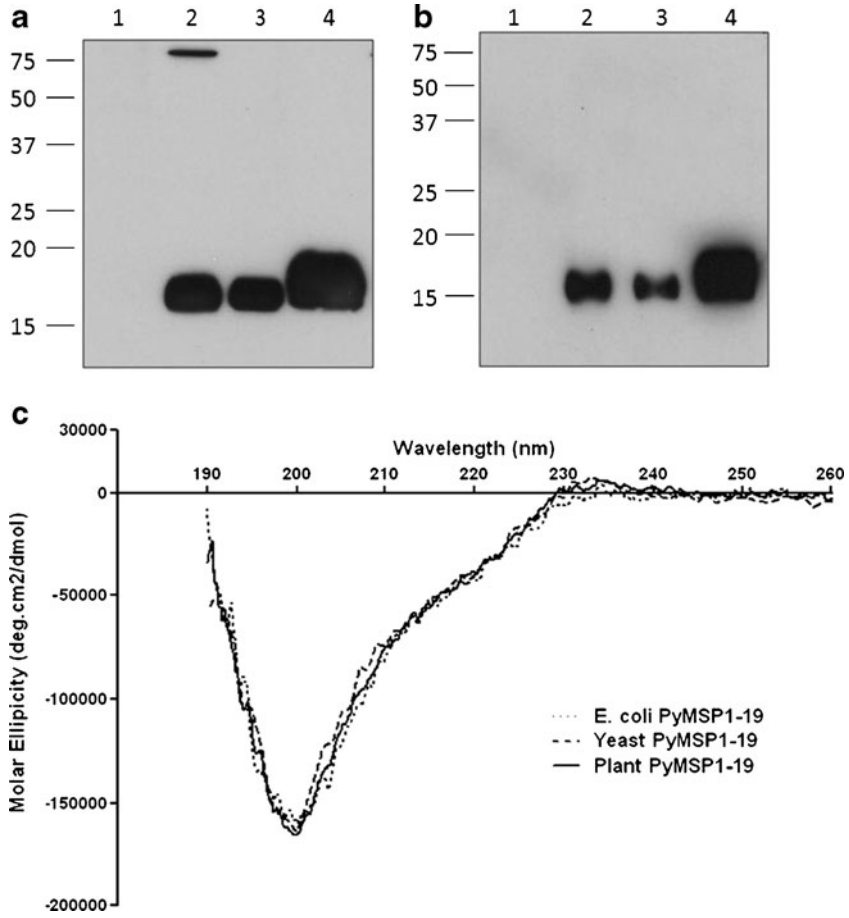

Fig. 3 Characterisation of $NbPyMSP1_{19}$. **a**, **b** Western blots (under reduced conditions) of negative control (*lane 1*), purified $EcPyMSP1_{19}$ (*lane 2*), purified $ScPyMSP1_{19}$ (*lanes 3*) and purified $NbPyMSP1_{19}$ (*lanes 4*) detected by anti-$PyMSP1_{19}$ antibodies (**a**) and anti-hexahistidine antibody (**b**). Protein loading ranged from 1 to 3 μg of $PyMSP1_{19}$. **c** Overlay of circular dichroism spectra for the three purified recombinant proteins, corrected for protein concentration (80–100 mM). *Solid line* indicated $NbPyMSP1_{19}$, *dotted lines* and *dashed lines* indicate $EcPyMSP1_{19}$ and $ScPyMSP1_{19}$, respectively

the plant-codon optimised $PyMSP1_{19}$ gene sequence. This is a significant improvement in expression compared to stable transformation. We have previously tried various combinations of promoters/terminators (promoter: cauliflower mosaic virus 35S; terminators: cauliflower mosaic virus 35S, potato *pin*II gene and soybean vegetative storage protein) for the stable transformation of $PyMSP1_{19}$ and the maximum yield achieved was 0.1% TSP (unpublished data). A yield of 23% TSP for $NbPyMSP1_{19}$ is also much higher than published data for *Plasmodium* antigen expression in plants. For example, a maximum yield of 0.0035% TSP for $PfMSP1_{19}$ and 0.25% TSP for PyMSP4/5 were achieved in stably transformed tobacco plants (Ghosh et al. 2002; Wang et al. 2008). The maximum reported yield of *Plasmodium* antigens expressed by stable nuclear transformation was for $PfMSP1_{42}$ produced in *Arabidopsis thaliana* seeds, with a yield of up to 5% total protein (Lau et al. 2010). Recent studies involving stable integration of transgenes into the chloroplast genome have also resulted in the accumulation of large quantities of foreign protein in various plant species, for example, fusion proteins of CTB-MSP1 and CTB-AMA1 were expressed at up to 13% and 10% TSP in tobacco and up to 7.3% and 6.1% TSP in lettuce, respectively (Davoodi-Semiromi et al. 2009). Our study has achieved the highest reported expression of *Plasmodium* antigens in plants and further highlights the importance of codon optimisation for expression of genes from organisms with unusual codon usage (e.g., the high AT content in *Plasmodium* species). It will be of interest to determine whether these high levels of expression are seen with other *Plasmodium* antigens from blood stage and other stages.

Using the same magnICON® viral vector system, we have previously described the rapid expression of *P. yoelii* MSP4/5 in *N. benthamiana* leaves (Webster et al. 2009). A maximum yield of 10% TSP or 2 mg/g fresh weight was achieved by using a plant codon optimized PyMSP4/5 gene sequence and the cytosolic transport 5′-viral module. $PyMSP1_{19}$ and PyMSP4/5 have similar structural features: both are surface proteins with one or two epidermal growth factor-like domains. The higher yield of $PyMSP1_{19}$ compared to that of PyMSP4/5 is probably due to its smaller size, 106 aa for $PyMSP1_{19}$ compared to 170 aa for PyMSP4/5. However, their maximum yields were achieved in different cellular compartments: the optimal expression for $PyMSP1_{19}$

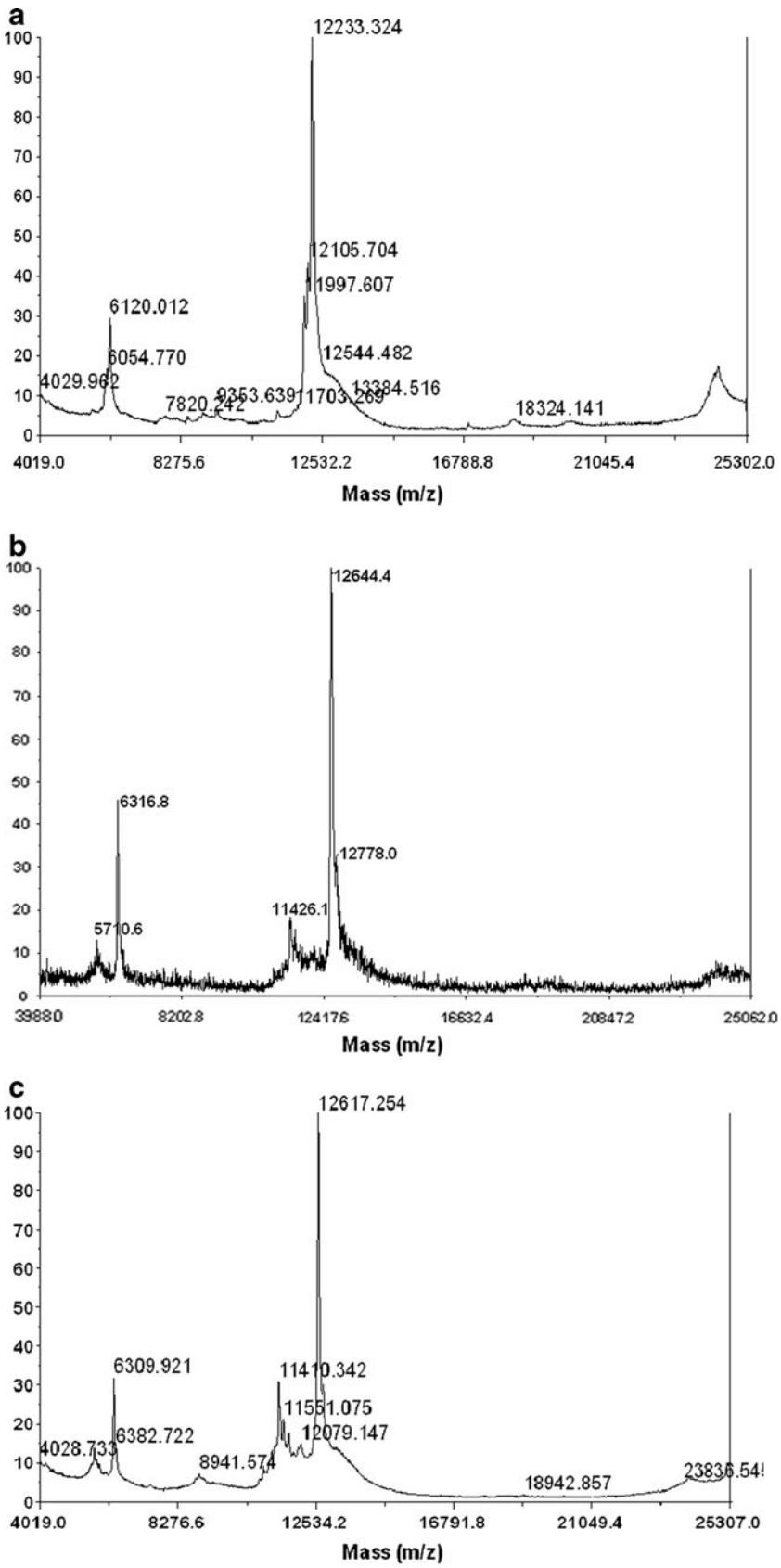

Fig. 4 Results of mass spectrometry for $EcPyMSP1_{19}$ (**a**), $ScPyMSP1_{19}$, (**b**) and $NbPyMSP1_{19}$ (**c**)

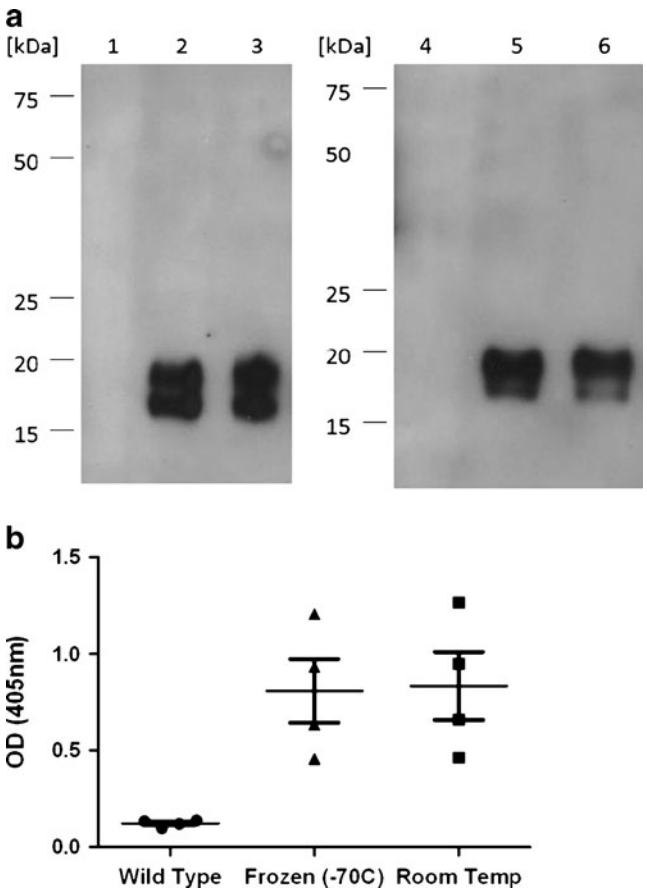

Fig. 5 Stability of NbPyMSP1$_{19}$ in freeze-dried leaf powder. Aliquots of freeze-dried leaf powder were either frozen at −70°C or stored at room temperature for 2 years. **a** Total protein extracted from aliquots stored in the two conditions were fractionated by SDS-PAGE and detected by anti-PyMSP1$_{19}$ (*left panel*) and anti-hexahistidine (*right panel*) antibodies. *Lanes 1 and 4* total protein from wild-type leaves (10 μg each). *Lanes 2 and 5* total protein from leaf powder stored at −70°C; *lanes 3 and 6* were from those stored at room temperature (5 μg each). **b** Total soluble protein from aliquots stored in the two conditions was used to coat 96-well plates for ELISA. Testing sera from mice immunised with EcPyMSP1$_{19}$ were diluted 1 in 3,000

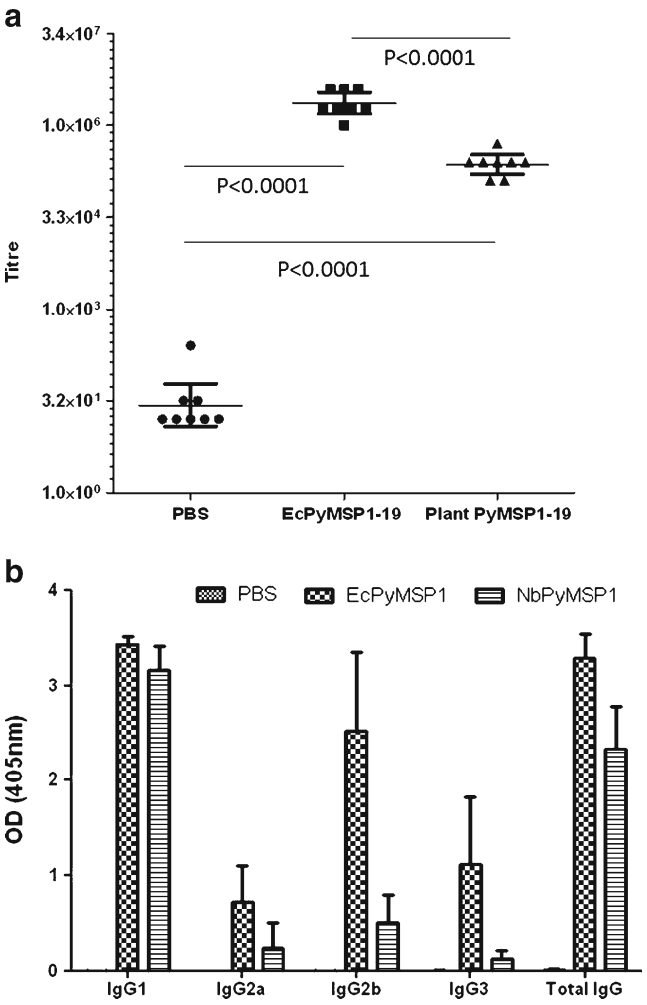

Fig. 6 Antibody responses in mice parenterally immunised with PBS, purified EcPyMSP1$_{19}$ or purified NbPyMSP1$_{19}$, all were emulsified in Freund's adjuvant. **a** Mean antibody titres. *Dots* represent individual mice. **b** Antibody isotype profiles. Sera from mice were diluted 1 in 3,000. The error bars represent 1 standard deviation

was achieved when it was targeted to apoplast, whereas the optimal PyMSP4/5 expression was produced by targeting to cytosol. This result indicates the inherent difficulty of predicting expression, even for proteins with a similar structure, and suggests that empirical verification is required for any particular protein. Expression of PfMSP1$_{42}$ in *A. thaliana* has also demonstrated the importance of sequence optimisation and targeting strategy on protein accumulation (Lau et al. 2010). By synthesizing a plant-optimised PfMSP1$_{42}$ cDNA and either targeting the recombinant protein to protein storage vacuoles or fusing it with a stable plant storage protein, Lau et al. have achieved an impressive improvement in PfMSP1$_{42}$ expression, from an undetectable level to 5% of total extractable protein.

No significant difference in protein structure was revealed between NbPyMSP1$_{19}$ and its counterparts produced by *E. coli* or *S. cerevisiae*. All these proteins have a similar secondary structure as determined by CD and are essentially random coils which are also referred as 'intrinsically unstructured proteins' (Tompa 2002). However, EcPyMSP1$_{19}$ contained a higher proportion of dimers and multimers than NbPyMSP1$_{19}$. This may explain the higher antibody titres induced with purified EcPyMSP1$_{19}$, as multimers are generally more stable and immunogenic than monomers (Ferro et al. 2002; Peralta et al. 2009). Although the presence of multimers increases the immunogenicity of a vaccine, they are difficult to include in GMP antigen production procedures. This is because the ratio of multimers to monomer needs to be consistent from batch to batch of produced vaccine for the formulation to be acceptable for human use. It should also be noted that NbPyMSP1$_{19}$ exists as a doublet, which may be due to internal initiation of translation, and this may contribute to the differences in antibody titres as well. Since glycoprotein

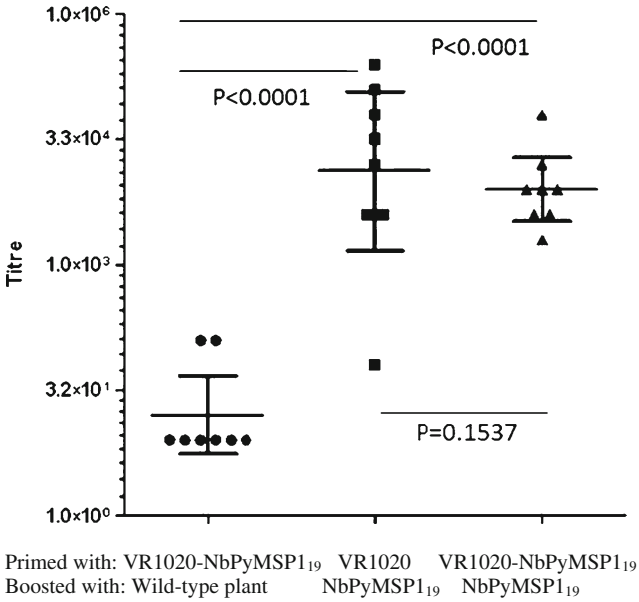

Fig. 7 Antibody titres in mice orally immunised with $NbPyMSP1_{19}$. Mice were immunised with either VR1020 or VR1020-$NbPyMSP1_{19}$ plasmids by gene gun and gavaged with either wild type leaf powder or with $NbPyMSP1_{19}$-containing leaf powder

staining did not suggest glycosylation of the doublet (data not shown), further characterisation may be needed. It may represent internal initiation within the coding sequence and this issue could be resolved by N-terminal sequencing of the products.

From mass spectrometry results, it was apparent that a protein fragment of approximately 6 kDa appeared in all three species of $PyMSP1_{19}$ (Fig. 4). The size of the fragment in each of the samples was half of the size of each protein. We speculate that $PyMSP1_{19}$ protein may have a region that is susceptible to breakage/degradation in the plant cell or following lysis. Further studies are required to determine if a genetic approach can be employed to remove this sequence and reduce the loss of recombinant $PyMSP1_{19}$. The 6-kDa fragment detected during MS analysis clearly contained the hexahistidine tag as they were retained in the purified preparations.

$NbPyMSP1_{19}$ stored as freeze-dried leaf powder was stable for at least 2 years at room temperature, demonstrating the stability of recombinant proteins made in plants, one of the major advantages of plant-made proteins. Similar results have been observed for PyMSP4/5 expressed using the same technology (Webster et al. 2009). The effectiveness of freeze-drying as a preservation method has also been demonstrated in other plants; the measles virus hemagglutinin protein was found to be stable in freeze-dried lettuce powder for at least 13 months at room temperature (Webster et al. 2006). Furthermore, the integrity and antigenicity of freeze-dried powder of the fruit of tomato expressing a fusion of *Yersinia pestis* F1 and V antigens was also maintained (Alvarez et al. 2006). Stability of antigen in vaccines is one of the major factors affecting its deployment in the field. If antigens are stable at room temperature then refrigeration is not required for storage and distribution (known as the cold-chain). This is particularly important for a malaria vaccine as most of the endemic countries are resource poor and maintaining a cold-chain can be uniquely challenging.

The immunogenicity of $NbPyMSP1_{19}$ was studied in two different settings. Purified $NbPyMSP1_{19}$ was immunogenic when delivered intraperitoneally with Freund's adjuvant, although the antibody titres were lower than those induced with the same dose of $EcPyMSP1_{19}$, presumably due to differences in the oligomerisation profile of the vaccines, discussed above. The dosage and antibody responses obtained in the current study were consistent with other studies (Hirunpetcharat et al. 1997, 1998). Although the predominant IgG1 responses were consistent with the isotype profiles of recombinant proteins emulsified in Freund's adjuvant for Balb/c mice (Kenney et al. 1989), the IgG2a responses to $NbPyMSP1_{19}$ were not as strong as those elicited by $EcPyMSP1_{19}$ suggesting that the response to the plant-made vaccine is skewed towards a Th2-type response, rather than the more balanced Th1/Th2 profile induced by $EcPyMSP1_{19}$. As IgG2a is more effective in conferring protection against parasites, other methods, such as vaccine formulation and the choice of adjuvant, may be needed to enhance IgG2a responses against the plant-made protein (Kenney et al. 1989).

To examine the potential for mucosal vaccine delivery using a non-purified minimally processed vaccine formulation, mice were immunised by gavage using a DNA-protein prime-boost vaccination schedule. When leaf powders containing the $NbPyMSP1_{19}$ were delivered orally, modest titres of $PyMSP1_{19}$-specific antibodies were induced alone, and in combination with the DNA vaccine. As this was the first study to test for antibody responses following oral delivery, the immunisation schedule, one dose per week for 4 weeks and two booster doses 4 weeks later, was not optimised, and further refinement leading to increased titres may be possible (Davoodi-Semiromi et al. 2009). Another potential method to enhance responses is the addition of other *Plasmodium* antigens and this is in line with the approach of developing a multivalent vaccine targeting different life cycle stages of the malaria parasite. A trivalent malaria vaccine containing AMA1, EBA-175 and $MSP1_{42}$ has been used to immunise *Aotus* monkeys and it was found that antibody titres to whole parasites were 3- to 12-fold higher than those induced by any of the single antigen vaccines (Jones et al. 2002).

Oral delivery represents one of the greatest challenges in vaccine development (Pelosi et al. 2011). Successful

vaccination via the oral route is hampered by the harsh environment of the gastrointestinal tract as well as the balancing of complex processes that govern the induction of immunogenicity or immune tolerance following presentation of a protein at a mucosal surface. However, the potential advantages of oral delivery to vaccination campaigns are immeasurable in terms of compliance and vaccine coverage (Clements et al. 2008).

In summary, we have shown that high yields of $PyMSP1_{19}$ can be achieved in *N. benthamiana* leaves using a deconstructed viral vector (3–4 mg/g Fwt). The $NbPyMSP1_{19}$ protein is structurally identical to *E. coli* and *S. cereviseae* made recombinant $PyMSP1_{19}$. However, differences were observed in immunogenicity following intraperitonal immunisation, presumably due to differences in the multimer to monomer ratios in the *E. coli* and plant-made protein preparations. Plant-made $PyMSP1_{19}$ is well preserved in freeze-dried power form and induced malaria-specific antibodies via oral immunisation. This study adds to an increasing body of research supporting the feasibility of plants as both a biofactory for the production of malaria antigens, and as a safe and affordable platform for oral delivery of a temperature-stable malaria vaccine.

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Authors' contributions CM performed all the laboratory/greenhouse/animal house works and analyses; LW was involved in training of techniques in laboratory/animal facility and advisory of the results; DW provided training to infiltration/greenhouse techniques and protein characterisation techniques; AC produced the codon-optimised $PyMSP1_{19}$ sequence; RC planned the experiments, gave guidance and support to the project. All authors have read and approved the final manuscript.

References

Alvarez ML, Pinyerd HL, Crisantes JD, Rigano MM, Pinkhasov J, Walmsley AM, Mason HS, Cardineau GA (2006) Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice. Vaccine 24:2477–2490

Clements CJ, Nshimirimanda D, Gasasira A (2008) Using immunization delivery strategies to accelerate progress in Africa towards achieving the Millennium Development Goals. Vaccine 26:1926–1933

Daly TM, Long CA (1993) A Recombinant 15-kilodalton Carboxyl-Terminal Fragment of *Plasmodium yoelii yoelii* 17XL merozoite surface protein 1 induces a protective immune response in mice. Infect Immun 61:2462–2467

Davoodi-Semiromi A, Schreiber M, Nalapalli S, Verma D, Singh ND, Banks RK, Chakrabarti D, Daniell H (2009) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. Plant Biotechnol J 8:223–242

Ellis RD, Mullen GE, Pierce M, Martin LB, Miura K, Fay MP, Long CA, Shaffer D, Saul A, Miller LH, Durbin AP (2009) A Phase 1 study of the blood-stage malaria vaccine candidate AMA1-C1/Alhydrogel with CPG 7909, using two different formulations and dosing intervals. Vaccine 27:4104–4109

Ferro VA, Harvey MJ, Colston A, Stimson WH (2002) Part II: influence of dimerization of a modified GnRH-I peptide sequence on a male antifertility vaccine. Am J Reprod Immunol 48:372–380

Ghosh S, Malhotra P, Lalitha PV, Guha-Mukherjee S, Chauhan VS (2002) Expression of *Plasmodium falciparum* C-terminal region of merozoite surface protein (PfMSP119), a potential malaria vaccine candidate, in tobacco. Plant Science 162:335–343

Hirunpetcharat C, Tian JH, Kaslow DC, van Rooijen N, Kumar S, Berzofsky JA, Miller LH, Good MF (1997) Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of *Plasmodium yoelii* expressed in *Saccharomyces cerevisiae*: correlation of protection with antigen-specific antibody titer, but not with effector CD4+ T cells. J Immunol 159:3400–3411

Hirunpetcharat C, Stanisic D, Liu XQ, Vadolas J, Strugnell RA, Lee R, Miller LH, Kaslow DC, Good MF (1998) Intranasal immunization with yeast-expressed 19 kD carboxyl-terminal fragment of *Plasmodium yoelii* merozoite surface protein-1 (yMSP119) induces protective immunity to blood stage malaria infection in mice. Parasite Immunol 20:413–420

Hodder AN, Crewther PE, Matthew MLSM, Reid GE, Moritz RL, Simpson RJ, Anders RF (1996) The disulfide bond structure of *Plasmodium*apical membrane antigen-1. J Biol Chem 271:29446–29452

Holder AA, Freeman RR (1981) Immunization against blood-stage rodent malaria using purified parasite antigens. Nature 294:361–364

Hu J, Chen Z, Gu J, Wan M, Shen Q, Kieny MP, He J, Li Z, Zhang Q, Reed ZH, Zhu Y, Li W, Cao Y, Qu L, Cao Z, Wang Q, Liu H, Pan X, Huang X, Zhang D, Xue X, Pan W (2008) Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. PLoS One 3:e1952

Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS (2006) Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. Vaccine 24:2506–2513

Jones TR, Gramzinski RA, Aguiar JC, Sim BK, Narum DL, Fuhrmann SR, Kumar S, Obaldia N, Hoffman SL (2002) Absence of antigenic competition in Aotus monkeys immunized with *Plasmodium falciparum* DNA vaccines delivered as a mixture. Vaccine 20:1675–1680

Kaslow D, Bathurst I, Lensen T, Ponnudurai T, Barr P, Keister D (1994) *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of *Plasmodium falciparum*. Infect Immun 62:5576–5580

Kenney JS, Hughes BW, Masada MP, Allison AC (1989) Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. J Immunol Methods 121:157–166

Lau OS, Ng DW, Chan WW, Chang SP, Sun SS (2010) Production of the 42-kDa fragment of *Plasmodium falciparum* merozoite surface protein 1, a leading malaria vaccine antigen, in *Arabidopsis thaliana* seeds. Plant Biotechnol J 8:994–1004

Ling HY, Pelosi A, Walmsley AM (2010) Current status of plant-made vaccines for veterinary purposes. Expert Rev Vaccines 9:971–982

Malkin E, Hu J, Li Z, Chen Z, Bi X, Reed Z, Dubovsky F, Liu J, Wang Q, Pan X, Chen T, Giersing B, Xu Y, Kang X, Gu J, Shen Q, Tucker K, Tierney E, Pan W, Long C, Cao Z (2008) A phase 1

trial of PfCP2.9: an AMA1/MSP1 chimeric recombinant protein vaccine for *Plasmodium falciparum* malaria. Vaccine 26:6864–6873

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. PNAS 101:6852–6857

Mazumdar S, Mukherjee P, Yazdani SS, Jain SK, Mohmmed A, Chauhan VS (2010) *Plasmodium falciparum* merozoite surface protein 1 (MSP-1)-MSP-3 chimeric protein: immunogenicity determined with human-compatible adjuvants and induction of protective immune response. Infect Immun 78:872–883

Pelosi A, Shepherd R and Walmsley A (2011) Delivery of plant-made vaccines and therapeutics. Biotechnol Adv, in press

Peng H, Hu Y, Zhou A, Jin C, Pan W (2010) Solution structure of a *Plasmodium falciparum* AMA-1/MSP 1 chimeric protein vaccine candidate (PfCP-2.9) for malaria. Malar J 9:76

Peralta A, Molinari P, Taboga O (2009) Chimeric recombinant rotavirus-like particles as a vehicle for the display of heterologous epitopes. Virol J 6:192

Pichyangkul S, Tongtawe P, Kum-Arb U, Yongvanitchit K, Gettayacamin M, Hollingdale MR, Limsalakpetch A, Stewart VA, Lanar DE, Dutta S, Angov E, Ware LA, Bergmann-Leitner ES, House B, Voss G, Dubois MC, Cohen JD, Fukuda MM, Heppner DG, Miller RS (2009) Evaluation of the safety and immunogenicity of Plasmodium falciparum apical membrane antigen 1, merozoite surface protein 1 or RTS, S vaccines with adjuvant system AS02A administered alone or concurrently in rhesus monkeys. Vaccine 28:452–462

Spok A, Twyman RM, Fischer R, Ma JK, Sparrow PA (2008) Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. Trends Biotechnol 26:506–517

Tian JH, Kumar S, Kaslow DC, Miller LH (1997) Comparison of protection induced by immunization with recombinant proteins from different regions of merozoite surface protein 1 of *Plasmodium yoelii*. Infect Immun 65:3032–3036

Tompa P (2002) Intrinsically unstructured proteins. Trends Biochem Sci 27:527–533

Wang L, Webster DE, Campbell AE, Dry IB, Wesselingh SL, Coppel RL (2008) Immunogenicity of *Plasmodium yoelii* merozoite surface protein 4/5 produced in transgenic plants. Int J Parasitol 38:103–110

Webster DE, Smith SD, Pickering RJ, Strugnell RA, Dry IB, Wesselingh SL (2006) Measles virus hemagglutinin protein expressed in transgenic lettuce induces neutralising antibodies in mice following mucosal vaccination. Vaccine 24:3538–3544

Webster DE, Wang L, Mulcair M, Ma C, Santi L, Mason HS, Wesselingh SL, Coppel RL (2009) Production and characterization of an orally immunogenic *Plasmodium* antigen in plants using a virus-based expression system. Plant Biotechnol J 7:846–855

Yusiboy V, Streatfield S, Kushnir N (2011) Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies, and beyond. Human Vaccines 7:313–321