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Review Article

Overview of Plant-Made Vaccine Antigens against Malaria

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This paper is an overview of vaccine antigens against malaria produced in plants. Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability. At present, different *Plasmodium* antigens and expression strategies have been optimized in plants. Furthermore, malaria antigens are one of the few examples of eukaryotic proteins with vaccine value expressed in plants, making plant-derived malaria antigens an interesting model to analyze. Up to now, malaria antigen expression in plants has allowed the complete synthesis of these vaccine antigens, which have been able to induce an active immune response in mice. Therefore, plant production platforms offer wonderful prospects for improving the access to malaria vaccines.

1. Introduction

Malaria is a major global health problem caused by parasites of the genus *Plasmodium*. This disease is responsible for the death of over one million people annually, and approximately three billon people are at risk of infection by both *Plasmodium falciparum* and *Plasmodium vivax* parasites [1, 2]. About 90% of the disease burden occurs in the most poverty-stricken sub-Saharan African countries. However, countries of Asia, Latin America, Middle East, and Europe are also affected [3]. Among the species of *Plasmodium* known to cause disease in humans: *P. falciparum* and *P. vivax* have received special attention for vaccine development. *P. falciparum* is responsible for most malaria-induced deaths and has therefore been the focus of the greater part of research [4]. On the other hand, *P. vivax* is often the most prevalent malaria infection in tropical and subtropical areas [5, 6].

Vaccination against malaria is considered the most efficient method to prevent this infectious disease [7]. However, although several vaccines are under development, a completely effective vaccine against malaria is not yet available. In particular, RTS,S, a potential vaccine targeting the preerythrocytic stage of the parasite, has shown the most promising results so far [8]. However, this vaccine is not fully effective, and thus more efficacious, second-generation vaccines are needed. In this sense, good progress is being made in the development of potential vaccines directed to the asexual blood and sexual stages of the parasite [9, 10]. In the first case, vaccines are designed either to stop invasion of erythrocytes or to kill intraerythrocytic parasites [8]. In the second case, vaccines aim to prevent mosquitoes from becoming infected by preventing parasite fertilization and/or by inhibiting early development in the insect vector. This is referred to as transmission-blocking (TB) immunity and is mediated by specific antibodies and other factors ingested during the blood meal, which inhibit parasite development in the mosquito [11, 12]. These antibodies recognize proteins expressed on either gametocytes or parasite stages that develop in the mosquito midgut and are considered to be potential malaria vaccine candidates [13]. This interruption of parasite transmission from patients to mosquitoes provides the basis for the feasibility of TB vaccines (TBV) of stopping transmission from infected to noninfected people [10]. These findings have stimulated research into the human immune mechanisms involved in

protection against infection and disease [14, 15] and into the blockage of parasite transmission from the host to the mosquito [13, 16].

Most of the vaccines available today belong to one of three categories: attenuated microbes, a killed version of the original pathogen, or protein subunits [17]. Each of them has its own associated advantages and disadvantages in terms of safety, immunogenicity, and general feasibility in terms of the ability to develop in to a commercial product. The disadvantages of live, attenuated vaccines in primates and humans include their side effects and risks for breakthrough infection. In contrast, recombinant subunit vaccine approaches offer one of the safest alternatives [18, 19].

Since a malaria vaccine based on attenuated parasites is not feasible in humans, several antigens from malaria parasites are being expressed in different heterologous expression systems like bacteria, yeast, insects, and mammalian cells [2, 8, 20–23]. However, the high costs estimated for a successful immunization strategy and scalable methods for vaccine production, distribution and delivery, together with the lack of infrastructure in malaria-endemic areas, are the main challenges in the development of a subunit malaria vaccine [20]. In this context, the capacity of transgenic plants to produce properly folded proteins at low costs makes them attractive expression systems [24, 25].

Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability. In addition, plants have the ability to generate complex recombinant proteins with desired structures, maintaining biological functions and offering greater safety because plants do not harbor mammalian pathogens or microbial toxins [22, 26–29]. However, plant-expression systems have been poorly explored for development of vaccines against human parasite pathogens. In fact, only few antigens from *Plasmodium* ssp. and *Toxoplasma gondii* have been expressed in plants [25, 30–39]. At present, whereas *T. gondii* antigen expression in plants is just beginning, different *Plasmodium* antigens and expression strategies have been optimized in plants. Furthermore, malaria antigens are one of the few examples of eukaryotic proteins with vaccine value expressed in plants, making plant-derived malaria antigens an interesting model to analyze. For this reason, this paper is an overview of vaccine antigens against malaria produced in plants.

2. Plant-Made Vaccines

In the last twenty years, plants have been used as bioreactors for the biosynthesis of products with biotechnological interest, such as subunit vaccines [40, 41]. An increasing number of antigens derived from either viruses or bacteria have been expressed using transgenic and transient expression in whole plants or plant cell cultures [42]. Among them, mucosal vaccines for diarrheal diseases, hepatitis B and rabies, as well as injectable vaccines for non-Hodgkin's lymphoma, H1N1 and H5N1 strains of influenza A virus, and Newcastle disease have been assayed [43]. Many of these antigens have demonstrated efficacy and safety in animal models and preclinical studies [42]. In fact, Dow AgroSciences [44] obtained the first commercial plant-made vaccine approved for Newcastle illness in chicken. This vaccine was developed and purified from tobacco cell cultures [44].

In addition to their use as bioreactors, plants can be used as potential delivery systems for oral vaccines [43]. In particular, plant tissues provide protection and prevent degradation of the antigen when it passes through the gut [45, 46]. It has been demonstrated that plant-made vaccines applied to mucosal surfaces in the absence of adjuvant are able to induce a protective immune response, suggesting that some phytochemicals could synergistically affect the immunogenicity of plant-expressed antigens acting as endogenous adjuvants [47–53]. In addition, plants are known for their natural immune-stimulating or antimicrobial activity due to secondary metabolites as lectins, saponins, alkaloids, phenolic compounds, and flavonoids [54]. Moreover, some commonly occurring plant components, such as unmethylated CpGs motifs of DNA, carotenoids and immunogenic proteins, have adjuvant properties [55, 56]. Hence, it might be assumed that plants can be used in the short term as oral or injectable vaccine producers and as a source of endogenous adjuvants as well.

3. Plant-Made Antigens against the Asexual Blood Stage of *Plasmodium*

A small number of merozoite surface proteins have been analyzed as vaccines against the asexual bloodstage of *Plasmodium* by inducing an immune response that either blocks invasion of erythrocytes or inhibits its subsequent multiplication [57–59]. Apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1), and erythrocyte surface antigens are under clinical trials [11, 60–64]. Some of them have been expressed in plants, and their immunogenicity assayed (Table 1) [25, 33, 35, 36, 39, 65].

The 19-kDa C-terminal fragment of *P. falciparum* merozoite surface protein 1 ($PfMSP1_{19}$) was the first malaria antigen expressed in plants by stable transformation, with a very low expression level (~0.0035% of the total soluble protein (TSP)) [30]. Afterwards, a plant-codon-optimized version of the 42 kDa C-terminal fragment of PfMSP1 ($PfMSP1_{42}$) was designed and synthesized, and expressed in transgenic *Arabidopsis thaliana* seeds [35]. Also, for protein-stable accumulation, the plant-optimized $PfMSP1_{42}$ gene was fused to the phaseolin peptide signal [66] or to the lysine-rich protein [35]. These strategies resulted in a substantial improvement of $PfMSP1_{42}$ expression (approximately 5% of TSP) [35].

Plant codon optimization has also been implemented to improve the expression level of *Plasmodium yoelii* merozoite surface protein 4/5 (PyMSP4/5) [25], another important vaccine candidate with high effectiveness in protecting mice against a lethal challenge [67, 68]. In this case, this optimized PyMSP4/5 version was expressed in transgenic tobacco plants and its expression level was six-fold higher than that obtained with the native protein [25]. In general,

Table 1: Malaria vaccine antigens expressed in plants.

Malaria antigens	Plant/expression system	Expression level	Functional evaluation	Refs.
Asexual blood stage antigens				
P. falciparum $MSP1_{19}$ ($PfMSP1_{19}$)	Tobacco/nuclear transformation	0.0035% TSP	Not reported	[30]
P. falciparum $MSP1_{42}$ ($PfMSP1_{42}$)	*A. thaliana* seeds/nuclear transformation	5% TSP	Immunoreactivity to sera from malaria-infected patients	[35]
P. yoelii MSP4/5 (PyMSP4/5)	Tobacco/nuclear transformation Tobacco/magnICON	0.02–0.04% TSP 10% TSP (1-2 mg/g FW)	Immunogenic by i.p. and oral delivery to mice Induction of specific antibodies by oral delivery or primed by a DNA vaccine to mice	[25] [33]
P. yoelii $MSP1_{19}$ ($PyMSP1_{19}$)	Tobacco/magnICON	23% TSP (3-4 mg/g FW)	Immunogenic by i.p. delivery with Freund's adjuvant or oral administration without any adjuvant	[65]
P. falciparum AMA1 (PfAMA1)	Tobacco/chloroplast transformation Lettuce/chloroplast transformation	13.1% TPS 7.3% TSP	Induction of specific antibodies in s.c. and orally immunized mice Immunoreactivity to native parasite proteins	[36]
P. falciparum MSP1 (PfMSP1)	Tobacco/chloroplast transformation Lettuce/chloroplast transformation	10.1% TSP 6.1% TSP	Induction of specific antibodies in s.c. and orally immunized mice Immunoreactivity to native parasite proteins	[36]
P. vivax MSP1 (PvMSP1)	*B. napus*/nuclear transformation	Not reported	Induction of antigen-specific IgG1 and increase in Th1-related cytokines IL-12 (p40), TNF, and IFN-γ in orally immunized mice	[39]
P. vivax CSP (PvCSP)	*B. napus*/nuclear transformation	Not reported	Induction of antigen-specific IgG1 and increase in Th1-related cytokines IL-12 (p40), TNF, and IFN-γ in orally immunized mice	[39]
Sexual stage antigens				
P. falciparum P230 (Pfs230)	Tobacco/agroinfiltration	800 mg/kg FW	Induction of transmission-blocking antibodies in s.c. and i.m. immunized mice Immunoreactivity to native parasite proteins	[38]
P. falciparum P25 (Pfs25)	Tobacco/agroinfiltration	0.25 and 0.4 mg/g FW	Induction of transmission-blocking antibodies in i.m. immunized mice Immunoreactivity to native parasite proteins	[37]

TSP: total soluble protein. FW: fresh weight. i.p.: intraperitoneal. i.s.: intrasubcutaneous. i.m.: intramuscular.

rare codons, AU-rich destabilizing sequences and putative polyadenylation and splicing signals may contribute to rapid mRNA decay, thus limiting the expression of foreign genes in plants. In the plant codon-optimized PyMSP4/5 antigen, the A+T content is reduced from 67% to 53%, and the AT-rich regions are disrupted, allowing the reduced A+T gene version to be more efficient in antigen production than the native version [25]. Codon optimization has also been implemented in protozoan parasite *T. gondii* antigens expressed in plants [31]. In this last case, the A+T content (54%) in the optimized *T. gondii* SAG1 version is increased, negatively affecting SAG1 expression [31]. These results, together with the results obtained in *Plasmodium*, support the idea that a high A+T content is correlated with a low protein expression.

More recently, Webster et al. [33] have expressed the plant codon-optimized PyMSP4/5 in *Nicotiana benthamiana* leaves, using the deconstructed tobacco mosaic virus-based transient expression system, magnICON, from ICON Genetics (Germany) [69] and found expression levels of up to 10% TSP (~1-2 mg/g of fresh weight) [33]. The use of magnICON has also been found to increase the expression level of the plant codon-optimized *P. yoelii* $MSP1_{19}$ ($PyMSP1_{19}$) to at least 23% TSP (~3-4 mg/g of fresh weight) [65]. All together, these results show that not only codon optimization but also the viral provector system improves the expression level of plant-made malaria antigens [33, 65]. Indeed, plant codon optimization combined with the magnICON expression system has been shown to allow an increase of up to 100 fold with respect to the transgenic tobacco system [25, 33].

Studies on the immunogenicity of these plant-made malaria antigens have shown that PyMSP4 and $PyMSP1_{19}$ expressed in plants are able to elicit an immune response by intraperitoneal (i.p.) or oral vaccination in a murine model [25, 33, 36, 39, 65]. These findings provide a rationale for the development of a plant-made oral vaccine against malaria. The report by Wang et al. [25] was the first to demonstrate the immunogenic properties of PyMSP4/5. First they found that oral immunization with *E. coli*-produced PyMSP4/5 in the presence of the cholera toxin B subunit of *Vibrio cholera* (CTB) induced a significant increase in the systemic-specific antibodies and a partial protection against a lethal challenge with *P. yoelii* [24]. Then, they found that oral immunization of mice using plant-made malaria antigens elicited an effective immunity against this parasite [25].

They demonstrated that oral immunization using plant-made malaria antigens elicited an effective immunity against this parasite [25]. However, immunization with plant-made antigens elicited a lower immune response than *E. coli*-made antigen immunization. Thus, Webster et al. [33] proposed increasing the antigen dose using the magnICON system [33]. This strategy allowed them to increase the antigen dose orally administered. However, a higher antigen dose (leaf powder containing 100 μg of PyMSP4/5) was not enough to induce a protective response against *P. yoelli* [33].

Thus, they hypothesized that the presence of dimeric antigen forms produced in *E. coli* enhances its immunogenicity, while the presence of monomeric forms in the plant-made antigen would explain its inability to induce protection. Similar results were obtained by Ma et al. [65]: although the plant-made $PyMSP1_{19}$ was immunogenic when delivered i.p. with Freund's adjuvant or orally without any adjuvant, the specific antibody titers were lower than those induced with the same dose as that of *E. coli*-made antigens [65]. Like that observed in PyMSP4/5, the higher proportion of dimers and multimers contained in *E. coli*-made $PyMSP1_{19}$ suggests that the multimers are more stable and immunogenic [65]. In case that this hypothesis is demonstrated, further innovations will be required to induce a protective immunity.

Finally, *P. falciparum* apical membrane antigen-1 (PfAMA1) and PfMSP1, both fused to CTB, have been expressed in transplastomic plants [36]. This strategy allowed accumulating CTB-PfAMA1 and CTB-PfMSP1 fusion proteins up to 13.1% and 10.1% of TSP in tobacco plants. In addition, since the expression of antigenic proteins in tobacco plants is not suitable for oral delivery in humans, Davoodi-Semiromi et al. [36] expressed these same chimeric proteins in lettuce chloroplasts and found that the expression levels of these malaria antigens fused to CTB in lettuce were up to 7.3% and 6.1% of TSP, sufficient for oral delivery evaluation [36, 39]. These authors combined two attractive strategies to improve the level of expression: plastid expression and chimeric protein production. While many chloroplasts in each cell and multiple copies of the plastid genome per cell make the high level of protein expression possible [20, 23], the *carrier* proteins contribute to a higher stability of the target protein in plants [43]. The levels of dual cholera-malaria vaccine antigens were ~50 fold higher in tobacco and ~30 fold higher in lettuce than other malaria antigens expressed in tobacco transgenic lines [36]. On the other hand, the lettuce chloroplast transformation opens the possibility of producing a green vaccine that could be orally administered without cold chain requirements. Indeed, Davoodi-Semiromi et al. [36] also found that subcutaneous and oral vaccination with candidate PfAMA1 and PfMSP1 genes fused to CTB are highly immunogenic in mice. Moreover, they also showed a significant parasitic inhibition, providing evidence that the antibodies generated from immunized mice are effective in preventing parasite invasion of red blood cells [36].

Several reports have assessed plant production of chimeric proteins as potential vaccines [36, 43]. The expression of chimeric proteins carrying epitopes from different pathogens, linkers, or adjuvant sequences would allow increasing the immunogenicity of the recombinant antigen [43]. In addition, a great number of models have demonstrated the feasibility to elicit broad cellular and humoral immune responses using such a type of molecules [43]. In particular, Davoodi-Semiromi et al. [36] demonstrated that CTB-PfAMA1 and CTB-PfMSP1 are able to elicit a humoral response by mucosal routes. However, they did not evaluate the cellular immune response. In addition, it is necessary to identify new, safe, and nontoxic adjuvants, particularly for those capable of strongly boosting cellular immune responses. In this context, leaves have a large number of heat shock proteins (HSPs), which have been demonstrated to have adjuvant properties in other organisms [70, 71]. Indeed, plants expressing HSP90 are able to induce *in vitro* B-cell proliferation from naïve mice, suggesting that these proteins could be excellent *carriers* of interesting vaccine antigens and peptides expressed in plants [72].

Other edible plant species has been explored to produce malaria antigens [39]. *Brassica napus* has been used to express *P. vivax* MSP1 (PvMSP1) and the *P. vivax* circumsporozoite protein (PvCSP), and to synthesize a chimeric recombinant gene containing PvMSP-1, a Pro-Gly linker motif, and PvCSP [39]. Oral immunization of mice with plant-made MLC chimeric recombinant protein successfully induced antigen-specific IgG1 production [39]. Additionally, the Th1-related cytokines IL-12, TNF, and IFN-γ were significantly increased in the spleens of BALB/c mice, supporting the idea that oral vaccines could be developed based on the use of food plant platforms for the production and delivery of vaccine antigens, contributing to a substantial cost reduction [22].

4. Plant-Made Antigens against Sexual Blood Stage of *Plasmodium*

As mentioned above, an alternative strategy to develop an efficient malaria vaccine is to obtain vaccines directed against proteins found in the sexual stages of *P. falciparum* parasites present in the *Anopheles* mosquito midgut, known as transmission-blocking vaccines (TBVs). A positive aspect of this type of TBV is that they attack the parasite at

a vulnerable stage of its life cycle, when it has to transfer from one host to another and when its numbers are very small [73]. Clinical trials have been conducted with vaccines based on the ookinete antigens of *P. falciparum* and *P. vivax* with induction of TB antibodies [10, 74].

Farrance et al. [38] used the transient *N. benthamiana* expression system to express the *P. falciparum* gamete surface antigen P230 (Pfs230) (Table 1). They demonstrated that antibodies against this protein block the progression of the parasite's life cycle in the mosquito and thus block the transmission to the human host [8, 75]. Pfs230 is present on the surface of *P. falciparum* gametocytes and gametes and is expressed during human infection [8, 75]. The N-terminal region of Pfs230 spanning aminoacids 444 to 730 was fused to the ER retention sequence (KDEL) and produced using plant-based transient-expression system by agroinfiltration [38]. The recombinant protein was expressed in approximately 800 mg/kg in whole fresh leaf tissue, being 100% soluble [38]. This plant-made Pfs230 elicited high titers of anti-Pfs230 antibodies when administered to rabbits in the presence of complete/incomplete Freund's adjuvant. Furthermore, the antibodies generated combined with this adjuvant were shown to bind specifically to the native protein in *P. falciparum* on the surface of gametes/zygotes, resulting in significant TB activity (TBA) [38].

Farrance et al. [37] also expressed an ookinete antigen present in the sexual form of the parasite—Pfs25—using the transient *N. benthamiana* expression system (Table 1). Pf25 is able to induce TB antibodies and is present in the zygote and ookinete stages but is not expressed during human infection [8]. In order to evaluate the role of the Lichenase molecule (LicKM) as a *carrier* [54] and the effects of N-glycosylation on the generation of a functional immune response, Farrance et al. [37] obtained four constructs carrying the recombinant Pfs25 protein for transient expression in plants. Independently of the protein versions evaluated, Pfs25 expression levels were between 0.25 and 0.4 mg/g of fresh leaf tissue [37]. Also, these authors observed that this recombinant protein with N-glycans had the lowest titers, while the Pfs25 lacking glycans (Pfs25-LG) showed the highest IgG titers in mice immunized intramuscularly (i.m.). However, the fusion to LicKM elicited a higher titer than that of the Pfs25 lacking glycans alone. The authors concluded that nonglycosylated antigens generated high antibody titers and enhanced TBA. In addition, they were able to eliminate the negative effect of the presence of glycans on the Pfs25 protein's ability to induce a functional immune response when Pfs25 was fused to LicKM [38]. Glycosylation of some antigens can affect their immunogenicity [37]. According to this, the plant-produced nonglycosylated and glycosylated versions determined that the nonglycosylated antigens generated higher antibodies titers and enhanced TBA [37]. Since glycosylation does not occur in chloroplasts and the natural pathogen organism (*Plasmodium*) does not glycosylate the target proteins, these results support the idea that the chloroplast would be the best compartment to express this group of proteins, where the glycosylation may affect their antigenicity.

Conclusion

Development of an effective malaria vaccine has been a great challenge for medical science. Up to now, malaria antigen expression in plants has allowed the complete synthesis of these vaccine antigens, which have been able to induce an active immune response in mice. Therefore, plant production platforms offer wonderful prospects for improving the access to malaria vaccines. Indeed, plant-derived multiantigen malaria vaccines could be formulated through mixing transgenic plant materials or polycistronic operons in chloroplasts expressing several malaria antigens. In addition, efficient malaria antigen expression in the lettuce chloroplast for oral immunization is a significant advancement that allows the oral delivery of subunit vaccines combined with a potent mucosal adjuvant. However, the local immune response induced by plant-made malaria antigens has not been analyzed yet. Indeed, most of these published reports are centered in the systemic humoral response. In this sense, further research is needed to understand the mucosal and systemic cellular immune response elicited by plant-made malaria antigens orally delivered.

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