



# Application of a Scalable Plant Transient Gene Expression Platform for Malaria Vaccine Development

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Despite decades of intensive research efforts there is currently no vaccine that provides sustained sterile immunity against malaria. In this context, a large number of targets from the different stages of the *Plasmodium falciparum* life cycle have been evaluated as vaccine candidates. None of these candidates has fulfilled expectations, and as long as we lack a single target that induces strain-transcending protective immune responses, combining key antigens from different life cycle stages seems to be the most promising route toward the development of efficacious malaria vaccines. After the identification of potential targets using approaches such as omics-based technology and reverse immunology, the rapid expression, purification, and characterization of these proteins, as well as the generation and analysis of fusion constructs combining different promising antigens or antigen domains before committing to expensive and time consuming clinical development, represents one of the bottlenecks in the vaccine development pipeline. The production of recombinant proteins by transient gene expression in plants is a robust and versatile alternative to cell-based microbial and eukaryotic production platforms. The transfection of plant tissues and/or whole plants using *Agrobacterium tumefaciens* offers a low technical entry barrier, low costs, and a high degree of flexibility embedded within a rapid and scalable workflow. Recombinant proteins can easily be targeted to different subcellular compartments according to their physicochemical requirements, including post-translational modifications, to ensure optimal yields of high quality product, and to support simple and economical downstream processing. Here, we demonstrate the use of a plant transient expression platform based on transfection with *A. tumefaciens* as essential component of a malaria vaccine development workflow involving screens for expression, solubility, and stability using fluorescent fusion proteins. Our results have been implemented for the evidence-based iterative design and expression of vaccine candidates combining suitable *P. falciparum* antigen domains. The antigens were also produced, purified, and characterized in further studies by taking advantage of the scalability of this platform.

**Keywords:** expression screening, heat stability, multi domain-fusion antigens, *Nicotiana benthamiana* plants, *Plasmodium falciparum*, red fluorescent protein

## INTRODUCTION

Even though the first malaria vaccine, GSK's *Plasmodium falciparum* circumsporozoite protein (CSP)-based Mosquirix (RTS,S), (Wilby et al., 2012) is expected to enter the market within the next 6–12 months, there is still an urgent demand for a malaria vaccine that reliably delivers long lasting protection against infection, clinical manifestation and transmission of the disease. RTS,S (Chattopadhyay et al., 2003; Richards et al., 2013; Penny et al., 2015; Reddy et al., 2015; RTS,S Clinical Trials Partnership, 2015) presents pre-erythrocytic stage epitopes and targets the parasite in an early phase of its life cycle within the human host. However, Mosquirix does not induce complete protection, and efficacy decreases rapidly over the first 24 months after immunization (RTS,S Clinical Trials Partnership et al., 2012; Moorthy et al., 2013). Various strategies must be considered to develop a better malaria vaccine, including the optimization of immune responses and immune memory against well-known and well-characterized targets like PfCSP, by testing different presentation strategies (e.g., viral vectored vaccines, virus-like particles), formulations (viriosomes), delivery, and immunization schemes, as well as the identification of alternative and/or additional antigens to target the blood and sexual stages of the parasite life cycle to overcome allelic diversity and also to prevent immune evasion.

Significant effort has been invested into the identification of a large number of potential malaria vaccine candidate antigens, domains, and epitopes, and several been taken through pre-clinical and even clinical development, but none of the candidates tested thus far provides long-lasting, strain-transcending sterile immunity. The available data indicate that the development of better malaria vaccines face two major challenges, i.e., the induction of a strong and long-lasting (durable) response, and the identification and combination of appropriate epitopes as targets to induce a neutralizing response.

Natural acquired immunity (NAI) against malaria (Doolan et al., 2009) is observed in individuals from holoendemic areas and requires several years and many (50–70) infections to develop. Its most important function seems to be the control of blood-stage parasitemia by IgG directed against merozoite surface proteins. NAI-mediated protection from episodes of clinical malaria rapidly ceases following reduced or diminished exposure to repeated infections. This highlights the importance of both the induction of strong and durable IgG responses as well as the identification of optimal target antigens.

The different stages of the parasite life cycle are characterized by sets of specific surface proteins (Druilhe et al., 1984), many of them involved in important functions such as host cell attachment and host cell invasion (Malpede and Tolia, 2014). Another feature of *P. falciparum* that complicates the development of sustainable protective immune responses is its ability to switch between different pathways of erythrocyte invasion, involving different sets of proteins from two invasion ligand families, i.e., erythrocyte-binding antigens (EBA) and reticulocyte-binding antigens (Rh) (Persson et al., 2008).

Many epidemiological studies involving donors from malaria endemic areas associate protective immunity with antibody

responses against various, predominantly merozoite antigens (Osier et al., 2008) including PfMSP1, PfAMA1, PfMSP4, PfMSP8, PfEBA175, and PfRIPR. Many of these proteins elicit *in vitro* parasite growth-inhibitory antibody responses after animal immunization experiments (Sim et al., 2001; Stowers et al., 2002; Moss et al., 2012), but despite substantial efforts (Richards et al., 2013; Penny et al., 2015; Reddy et al., 2015; RTS,S Clinical Trials Partnership, 2015) the roles of these proteins in protective immunity remains poorly understood, making it difficult to select rational targets for the design of an ultimate malaria vaccine candidate.

We therefore believe that one promising direction in malaria vaccine development is the generation and testing of single or multi-stage-specific, multi-domain constructs that combine promising antigens or antigen domains in the context of one or several fusion proteins, which should induce more diverse and ideally strain-transcendent immune responses against all three stages of the *P. falciparum* life cycle, and thus achieve a greater degree of protection than existing vaccine candidates. The successful design of such completely artificial fusion proteins that combine isolated protein domains will inevitably result in a completely new context for these elements and may affect both the expression level and stability. This is an unpredictable challenge that must be addressed empirically through iterative design cycles featuring cloning and expression experiments.

In addition to the classic molecular farming approaches (Fischer et al., 1999; Daniell et al., 2001; Fischer and Schillberg, 2006) using stable transgenic plants, different transient plant expression systems (Kapila et al., 1997; Gleba et al., 2005, 2007; Starkevicius et al., 2015) are gaining more and more attention as platforms for the manufacture of pharmaceutical proteins such as antibodies (Hull et al., 2005; Huang et al., 2010) and enzymes for emergency use (Rosenberg et al., 2015) and as conventional regular therapeutics (Aviezer et al., 2009). These transient expression systems are robust and scalable, and have been used for the inexpensive and the time and cost-efficient generation and testing of expression constructs including for many antibodies and vaccine antigens from different pathogens including targeting *Human immunodeficiency virus* (Rosenberg et al., 2013), *Ebola virus* (Huang et al., 2010), *Influenza virus* (D'Aoust et al., 2010; Landry et al., 2010), *Dengue virus* (Kim et al., 2015), *Yersinia pestis* (Santi et al., 2006), and *P. falciparum* (Davoodi-Semiromi et al., 2010; Feller et al., 2012; Jones et al., 2013; Boes et al., 2014, 2015; Voepel et al., 2014; Beiss et al., 2015).

Here, we demonstrate the application of the classical *Nicotiana benthamiana*/*A. tumefaciens* transient expression system to accelerate the development of malaria vaccine candidates, using as case studies a pre-erythrocytic stage multi-domain candidate, a dual-stage multi-domain candidate, and a multi-stage multi-domain candidate.

## MATERIALS AND METHODS

### Ethics Statement

The animal experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen

(LANUV), reference number 8.87.–51.05.30.10.077. All animals received humane care according to the requirements of the German Tierschutzgesetz, §8 Abs. 1 and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

## Bacteria, Plant Characterization, and Parasites

*A. tumefaciens* strain GV3101::pMP90RK [Gm<sup>R</sup>, Km<sup>R</sup>, Rif<sup>R</sup>] (Koncz and Schell, 1986) and *N. benthamiana* plants were used for transient expression by agroinfiltration. According to Bally et al. *N. benthamiana* isolates vary in the RNA-dependent RNA polymerase 1 gene (Rdr1) and a 72 bp insertion leading to a truncated version of the enzyme has been reported. To verify the genotype in respect to the Rdr1 locus, we used PCR, to amplify the respective region from genomic DNA prepared from *N. benthamiana* (Accession number: AY574374) and *Nicotiana tabacum* L. Petit Havana cultivar SR1 (Accession number: AJ011576, control). Primers (Rdr1 forward primer: 5'-GTAAACGTATCCAATCGGTTCTGCG-3' and Rdr1 reverse Primer: 5'-CTGATTTGCCGAAAATCACCCATCC-3') were designed to cover the region potentially containing the 72 bp insertion (found in the *N. benthamiana* isolates LAB, 16C and SA Bally et al., 2015) compatible to *N. benthamiana* as well as *N. tabacum* which does not carry the Rdr1 72 bp insertion. Fragment size analysis (Presentation 1 in Supplementary Material) revealed the insertion phenotype for the *N. benthamiana* used in our study. *P. falciparum* strain NF54 (MRA-1000, MR4, Manassas, USA) was used for the parasite assays.

## Construction of the Plant Expression Vector

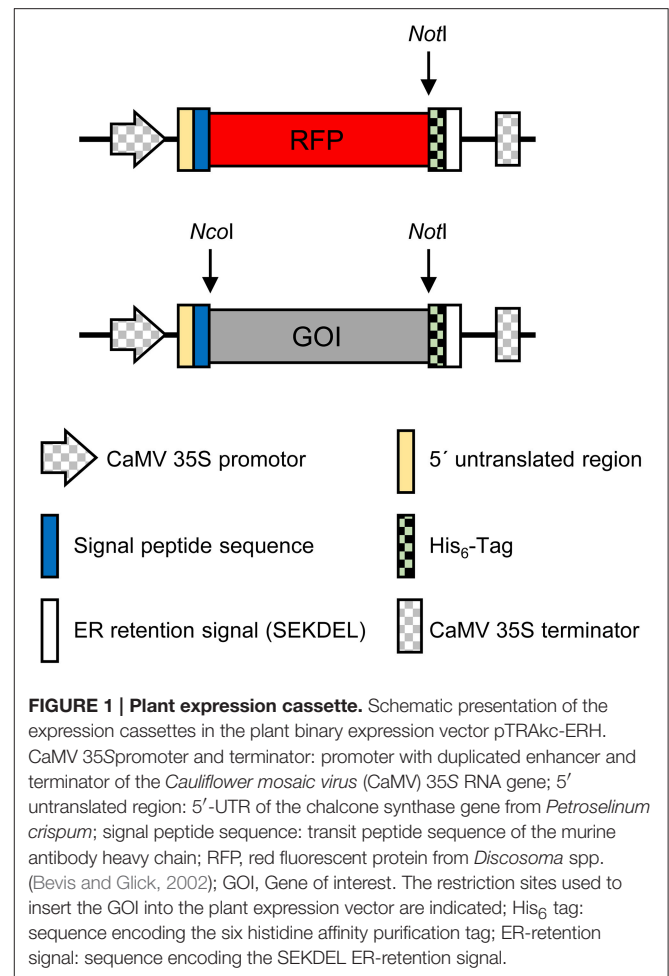
Synthetic genes or PCR products encoding selected antigens and antigen domains were inserted into pTRAc-ERH at the NcoI/NotI sites. Subsequent stacking of additional domains was achieved by inserting EagI/NotI fragments following plasmid linearization with NotI. Red fluorescent protein (RFP) fusion genes were inserted into a NotI-linearized plasmid carrying the RFP cDNA using the same approach (Figure 1). Restriction enzymes were used according to the manufacturers' instructions. The sequences of the *P. falciparum* antigens are summarized in Table 1.

## Transient Expression in *N. benthamiana*

The expression vectors were introduced separately into *A. tumefaciens* and were used for the infiltration of *N. benthamiana* plants as previously described by Feller et al. (2012).

## Extraction of Total Soluble Protein from Leaves and Heat Precipitation

For initial screening, two leaf discs (diameter 1 cm) were punched from infiltrated leaves, weighed, and extracted in 3 mL phosphate buffered saline (PBS) per gram of leaf material using an electropistol. Insoluble material was removed by centrifugation (16,000 g for 5 min) and the supernatants were used directly



for SDS-PAGE, western blot analysis, and the quantification of RFP fluorescence. An aliquot of the supernatant was heated (70°C for 5 min) and analyzed in the same manner. For the purification of E5, total soluble protein was extracted from 230 g of vacuum-infiltrated leaves in a Waring blender using 3 mL of extraction buffer (PBS containing 500 mM NaCl, pH 7.4) per gram of leaf material. The pH of the crude extract was adjusted to pH 8.0 using NaOH and the extract was heated to 65°C. Insoluble material was removed by a combination of centrifugation (14,000 g for 15 min) and filtration.

## Protein Purification

The E5 recombinant protein was purified from heat-treated extracts first by immobilized metal affinity chromatography (IMAC) using chelating Sepharose (GE Healthcare) charged with nickel (NiSO<sub>4</sub>, 200 mM). Unbound proteins were washed away with PBS and bound proteins were eluted in a two-step gradient using PBS containing 10 and 250 mM imidazole, respectively. IMAC elution fractions containing the target protein E5 were desalted against 20 mM Tris (pH 7.5) and E5 was further purified by ion exchange chromatography

**TABLE 1 | Overview of *P. falciparum* antigens and antigen domains.**

Antigen	Abbreviation	PlasmoDB ID	Amino acid	Size [kDa]	Size RFP-Fusion [kDa]	RFP fluorescence in leaves	RFP fluorescence in extract
<i>PfMsp1-19_EGF1</i>	19_1	PF3D7_0930300	I <sup>1608</sup> -V <sup>1648</sup>	4.8	32.4	+++	M
<i>PfMsp8_EGF1</i>	8_1	PF3D7_0502400	N <sup>489</sup> -D <sup>533</sup>	5.2	32.8	+++	H
<i>PfMsp8_EGF2</i>	8_2	PF3D7_0502400	D <sup>534</sup> -S <sup>576</sup>	4.8	32.4	+++	M
<i>PfMsp4_EGF</i>	4	PF3D7_0207000	L <sup>201</sup> -L <sup>247</sup>	5.3	32.9	+++	H
<i>PfMsp10_EGF1</i>	10_1	PF3D7_0620400	V <sup>414</sup> -P <sup>458</sup>	5.4	33.0	+++	L
<i>PfMsp10_EGF2</i>	10_2	PF3D7_0620400	K <sup>460</sup> -K <sup>501</sup>	4.5	32.1	+++	M
<i>PfRipr_EGF6</i>	R6	PF3D7_0323400	K <sup>771</sup> -I <sup>814</sup>	5.3	32.9	++	M
<i>PfMTRAP_TSR</i>	MT	PF3D7_1028700	T <sup>25</sup> -E <sup>98</sup>	8.6	36.2	++	M
<i>PfCelTOS</i>	Ce	PF3D7_1216600	F <sup>25</sup> -D <sup>182</sup>	17.4	45.0	++	M
<i>PfCSP_TSR</i>	CT	PF3D7_0304600	P <sup>311</sup> -S <sup>383</sup>	8.2	35.8	+++	M
<i>PfTRAP_TSR</i>	TT	PF3D7_1335900	E <sup>239</sup> -K <sup>289</sup>	5.8	33.4	+++	L
<i>PfSPATR_TSR</i>	ST	PF3D7_0212600	E <sup>168</sup> -C <sup>250</sup>	10.2	37.8	+	L
<i>Pfs25</i>	25	PF3D7_1031000	V <sup>24</sup> -T <sup>193</sup>	18.6	46.2	++	M
<i>Pfs230_CO</i>	CO	PF3D7_0209000	E <sup>443</sup> -N <sup>586</sup>	16.0	43.6	++	H

For each antigen, the table provides the name, a short abbreviation, the plasma DB identifier, the amino acid sequence, the calculated single domain size, the calculated size of the corresponding RFP-fusion protein as well as the categorized fluorescence observed in intact leaves (+++, high fluorescence in leaves; ++, moderate fluorescence in leaves; +, low fluorescence in leaves) and categorized concentration (based on RFP fluorescence) in crude extracts (H, Accumulation > 150 µg/g FLW; M, Accumulation between 25 and 150 µg/g FLW, and L, < 25 µg/g FLW).

(IEX) using a prepacked HiTrap Q FF column. The E5 protein was eluted using a three-step gradient (20 mM Tris pH 7.5 with 240, 500 and 1000 mM NaCl, respectively). The buffer was exchanged against PBS overnight at 4°C using a Spectra/Por membrane (MWCO 6000–8000 g/mol; SpectrumLabs). The samples were then concentrated using Vivaspin centrifugal concentrators (Sartorius-Stedim Biotech GmbH) and passed through a 0.22-µm sterile filter. The protein concentration was determined by UV spectroscopy at 280 nm and the recombinant protein was stored at 4°C.

### SDS-Page and Western Blot Analysis

SDS-PAGE and western blot analysis were carried out as previously described by Boes et al. (2011). The western blots were probed with rabbit anti His<sub>6</sub> (Jackson ImmunoResearch) and alkaline phosphatase (AP)-conjugated goat anti-rabbit serum (Jackson ImmunoResearch) were used for detection. The monoclonal antibody (mAb) 5.2 (MRA-94, MR4, Manassas, USA), which recognizes the first epithelial growth factor (EGF)-like domain of PfMsp1-19, was used for dot-blot analysis and was detected using AP-conjugated goat anti-mouse serum (Jackson ImmunoResearch).

### Quantification of RFP

The RFP concentration in tobacco supernatants was measured as previously described by Buyel and Fischer (2012) with minor modifications. The standard curve was generated with purified RFP ranging from 0 to 100 µg/mL and the concentration was determined by linear regression analysis.

### Binding of Soluble and Insoluble RFP-Fusion Proteins to Ni-NTA Magnetic Agarose Beads

To assess the solubility of RFP and selected RFP-fusion proteins, Ni<sup>2+</sup>-NTA magnetic agarose beads (Qiagen) were used in combination with fluorescence microscopy. Infiltrated leaves were homogenized as described above and 400 µL PBS and 50 µL of Ni-NTA magnetic agarose beads were added directly to the pulp. After incubation for 30 min with continuous shaking, the beads were collected with a magnet, washed intensively with PBS and analyzed under a fluorescence microscope (Leica DMI6000/AF6000).

### Mouse Immunization and Titer Determination

Three BALB/c mice 6–8 weeks of age (Taconic) were immunized intraperitoneally with 50 µg E5 emulsified in Gerbu MM adjuvant (Gerbu Biotechnik GmbH) on days 1, 14, 28, and 42. On each day, 5-µL blood samples were collected from the tail vein, diluted 1:10 in PBS and stored at –20°C. Fourteen days after the last immunization, the mice were narcotized, and blood was collected by cardiocentesis. After 1 h incubation at room temperature, the blood was centrifuged (200 g for 2 min at room temperature) and the serum was separated from the blood cells and stored at –20°C. The titers were determined in relation to the respective pre-immune samples taken directly before immunization, as previously described by Voepel et al. (2014) using the full fusion antigen, as well as the single domain RFP fusion proteins (100 ng/well), to coat microtiter plates for analysis by direct ELISA.

## Immunofluorescence Assays

Indirect immunofluorescence assays with E5-specific immune sera were carried out using the schizonts and macrogametes of *P. falciparum* strain NF54 as previously described by Boes et al. (2014), Voepel et al. (2014), Beiss et al. (2015).

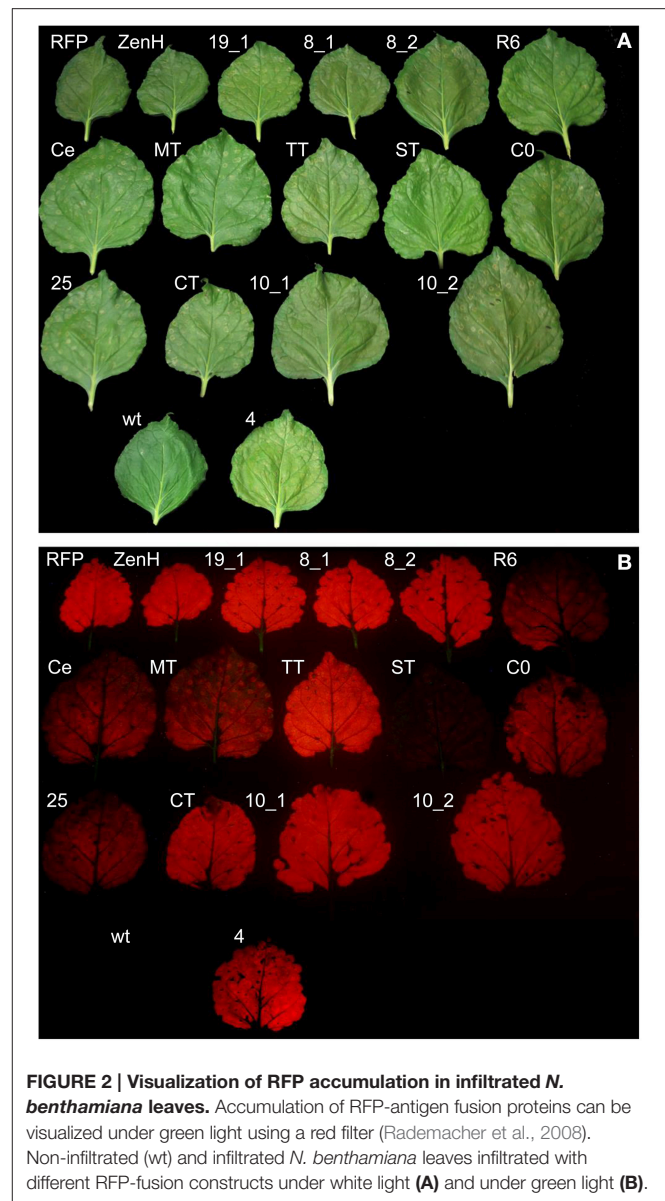
## RESULTS

### Expression Screening of Single-Domain RFP-Fusion Proteins

To select suitable antigens and/or antigen domains for a multi-domain fusion vaccine from a panel of candidates, we generated N-terminal RFP-fusion constructs to easily investigate the accumulation, solubility, integrity, and heat stability of each protein by transient expression in *N. benthamiana*.

The initial selection of antigens and antigen domains was primarily based on the availability of epidemiological and/or experimental data indicating the immunological relevance or potential protective efficacy of the antigens or antigen domains. The heat treatment of clarified or crude plant extracts is a valuable tool to simplify the downstream purification of recombinant protein from plant material because this step removes the majority of host cell proteins (HCPs) including RuBisCo (Buyel et al., 2014) but this approach is only suitable for recombinant proteins that resist temperature-induced denaturation. In this approach we wanted to avoid the heat sensitivity we observed in previous experiments with larger, more complex, or unstructured *P. falciparum* proteins (data not shown). Therefore, we focused on compact and stable domains featuring many disulfide bridges, including EGF-like (Beeby et al., 2005) and thrombospondin type 1 repeat (TSR)-domains that are present in many *P. falciparum* surface proteins.

We duly selected 14 *P. falciparum* antigens or antigen domains (Table 1) from different developmental stages of the parasite (pre-erythrocytic stage *Pf*CeTOS, *Pf*CSP\_TSR, *Pf*TRAP\_TSR, and *Pf*SPATR; blood stage *Pf*Msp1-19\_EGF1, *Pf*Msp4\_EGF, *Pf*Msp8\_EGF1, *Pf*Msp8\_EGF2, *Pf*Msp10\_EGF1, *Pf*Msp10\_EGF2, *Pf*MTRAP\_TSR, and *Pf*Ripr\_EGF6; and sexual stage *Pfs*25 and *Pfs*230-C0). Synthetic genes encoding each antigen were codon optimized for *N. benthamiana*, cloned into the expression cassette of the vector pTRAcK-RFP-ERH (Figure 1), introduced into *A. tumefaciens* which were then used for transient expression in *N. benthamiana* by the syringe infiltration of single leaves from intact plants. The vectors pTRAcK-RFP-ERH and pTRA-RFP-ZenH, the latter encoding a RFP fusion with the maize  $\gamma$ -zein domain that is targeted to protein bodies (Hofbauer et al., 2014) were used as controls for insoluble aggregates. After incubation for 4–5 days, the leaves were harvested and RFP fluorescence was visualized using a simple red filter with a cold light source and a green excitation filter. As shown in Figures 2A,B, representative leaves under normal and fluorescent light display different degrees of RFP fluorescence for each of the constructs. A strong signal was seen in the leaves infiltrated with the controls ER-retarded RFP, and protein body targeted RFP (ZenH), as well as RFP-*Pf*Msp1-19\_EGF1 (19\_1), RFP-*Pf*Msp8\_EGF1



**FIGURE 2 | Visualization of RFP accumulation in infiltrated *N. benthamiana* leaves.** Accumulation of RFP-antigen fusion proteins can be visualized under green light using a red filter (Rademacher et al., 2008). Non-infiltrated (wt) and infiltrated *N. benthamiana* leaves infiltrated with different RFP-fusion constructs under white light (A) and under green light (B).

(8\_1), RFP-*Pf*Msp8\_EGF2 (8\_2), RFP-*Pf*TRAP\_TSR (TT), RFP-*Pf*CSP\_TSR (CT), RFP-*Pf*Msp10\_EGF1 (10\_1), RFP-*Pf*Msp10\_EGF2 (10\_2), and RFP-*Pf*Msp4\_EGF1 (4). Moderate signals were observed for RFP-*Pf*Ripr\_EGF6 (R6), RFP-*Pf*CeTOS (Ce), RFP-*Pf*MTRAP\_TSR (MT), RFP-*Pf*230-C0 (C0), and RFP-*Pfs*25 (25). RFP-*Pf*SPATR\_TSR (ST) exhibited minimal RFP fluorescence (see also Table 1, column 7).

The soluble RFP fusion proteins were quantified by extracting total soluble protein from leaf discs taken from infiltrated leaves (three biological replicates) and taking spectroscopic fluorescence measurements before and after heat treatment (5 min at 70°C). The concentrations were determined relative to the RFP calibration curve as summarized in Figure 3 and also Table 1 (column 8). These results indicated that all the RFP-fusion proteins were heat stable because no significant reduction in the fluorescence signal was observed after heat treatment (Figure 3,

red columns). A strong difference between the visual appearance (**Figure 2B**) and measured fluorescence values was observed for RFP-*PfMsp10\_EGF1*, RFP-*PfMsp10\_EGF2*, RFP-*PfTRAP\_TSR*, and RFP-ZenH (**Figure 3**).

To investigate the possibility that insoluble aggregates may form during expression we analyzed six representative samples (non-infiltrated, RFP, RFP-ZenH, RFP-*PfMsp1-19\_EGF1*, RFP-*PfMsp10\_EGF1*, and RFP-*PfSPATR\_TSR*) by microscopy after capturing the recombinant protein and/or aggregates from the crude extract on Ni-NTA magnetic agarose beads. The images (**Figure 4** left and right panel) clearly illustrate the differences between predominantly soluble proteins like the ER-targeted RFP and the RFP-*PfMsp1-19\_EGF1* fusion (even distribution of fluorescence signal), and insoluble or aggregated proteins (fluorescent berry-like structures) like the protein body targeted RFP-ZenH and apparently the RFP-*PfMsp10\_EGF1* fusion. For the low-yielding RFP-*PfSPATR\_TSR* protein we observed the soluble phenotype without indication for insoluble aggregates.

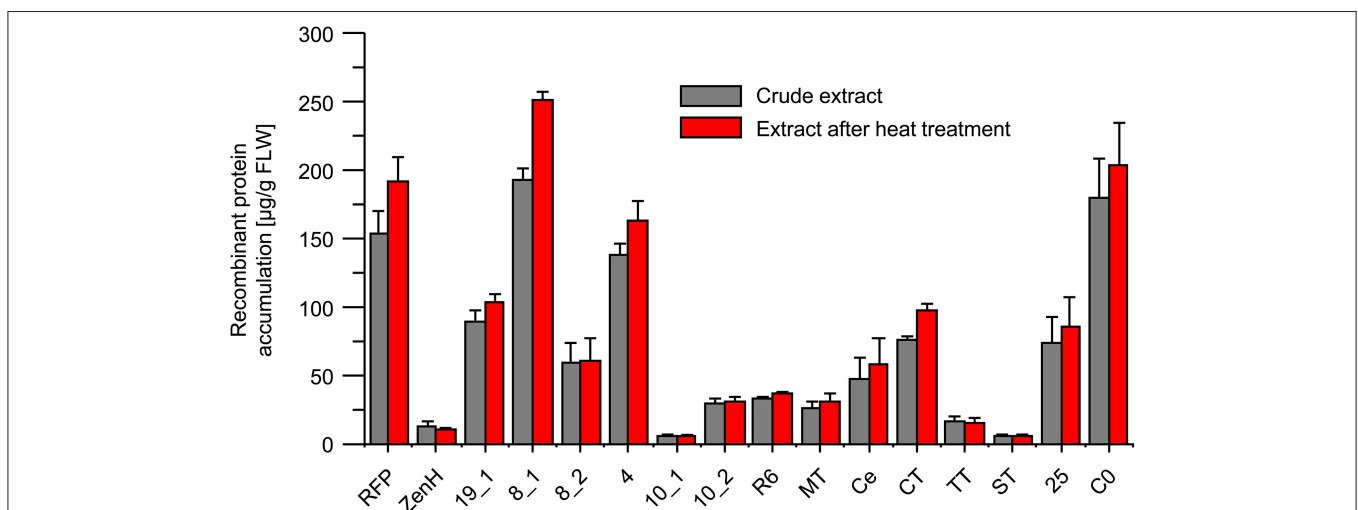
Finally, the expression, solubility, integrity, and heat stability of all RFP-fusion proteins was analyzed by reducing SDS-PAGE and western blot (**Figures 5A,B**) using the supernatant of heat-treated soluble extracts. Most of the recombinant proteins migrated in the region corresponding to their expected molecular weight (**Table 1**). Both methods also revealed the presence of covalently linked dimers of the RFP-fusion proteins, an observation we have made with almost all RFP-fusion proteins we have generated and tested under comparable conditions. In accordance with these observations, the RFP-*PfSPATR\_TSR* fusion protein (**Figures 5A,B**, lane 14) and also the insoluble RFP-*PfMsp10\_EGF1* fusion protein (**Figures 5A,B**, lane 7) could not be detected. For the RFP-*PfCelTOS* fusion protein (**Figure 5B**, lane 12) a western blot to detect the His<sub>6</sub> tag revealed the presence of small amounts of degradation products in the

regions around 40 kDa (D3, **Figure 5B**) and 18 kDa (D1, D2, **Figure 5B**) potentially representing proteolytic cleavage. The band intensities in Coomassie stained gels and on western blots did not always correlate well (e.g., RFP-*PfCSP\_TSR*, **Figure 5B**, lane 12, and *Pf230-C0*, **Figure 5B**, lane 16), possibly indicating differences in the accessibility of the His<sub>6</sub> tag for the different *P. falciparum* proteins. The pronounced difference between the in-gel and western blot signals representing *Pf230-C0* (**Figures 5A,B**, lane 16) may reflect the proteolytic cleavage of a C-terminal fragment comprising the His<sub>6</sub> tag (visible at >15 kDa on the western blot, D4, **Figure 5B**).

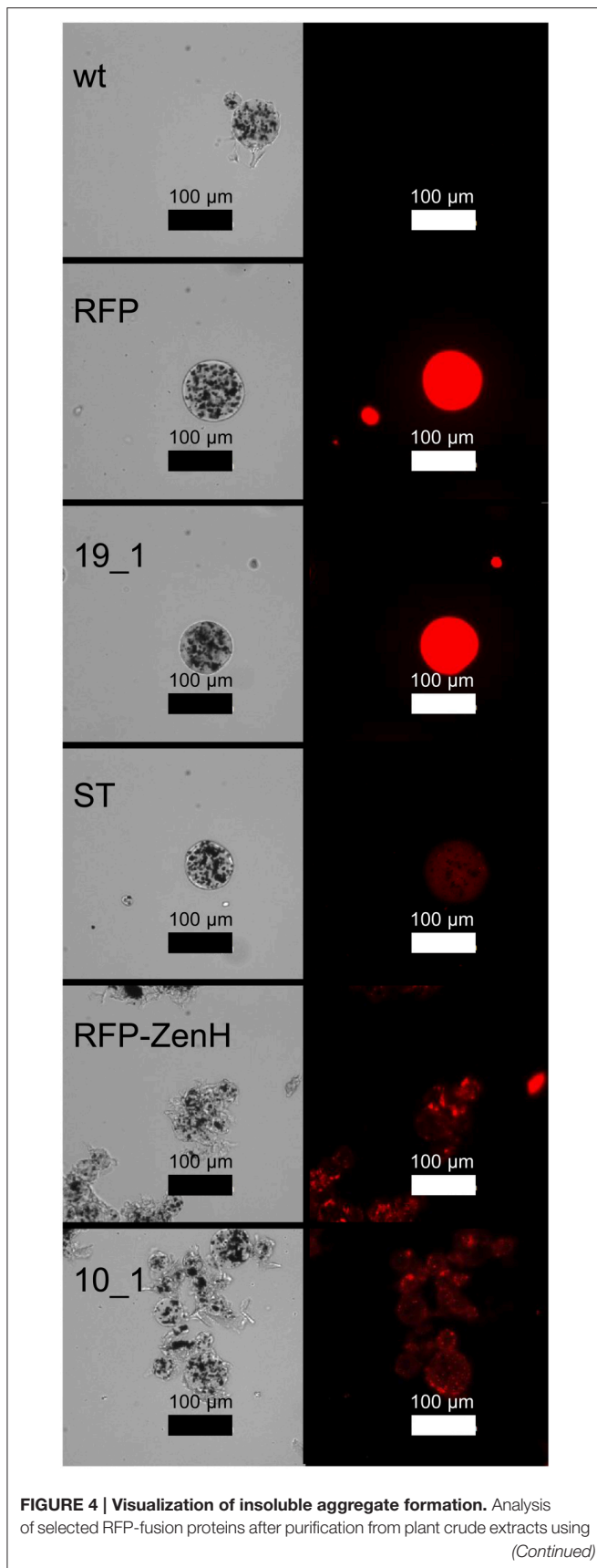
## Construction of the Pre-erythrocytic Vaccine Candidate P3

Based on the screening results for protein domains fused to RFP, we selected *PfCelTOS* (Ce), *PfCSP\_TSR* (CT), and *PfTRAP\_TSR* (TT) for the construction of a pre-erythrocytic vaccine candidate. The pre-erythrocytic antigen *PfSPATR\_TSR* was rejected due to its poor expression as a RFP-fusion protein, while the apparently poorly soluble *PfTRAP\_TSR* (TT) was included because of its clinical relevance and hoping for improved solubility in the different context of a new fusion protein.

To investigate the integrity, expression, and heat stability of the domains subsequently combined in the context of a fusion protein, we generated three expression constructs (P1, P2, and P3, **Figure 6A**) and tested them by transient expression followed by heat precipitation, SDS-PAGE and western blot. As shown in **Figure 6B**, all three variants, including the artificial fusion proteins P2 and P3, could be produced at satisfactory levels (>100 µg/g fresh leaf weight (FLW) based on our judgment of in-gel band intensities after Coomassie staining, according to our previous experiences) and remained in the soluble fraction



**FIGURE 3 | Quantification of RFP-fusion proteins in plant extracts before and after heat treatment by fluorescence detection.** The accumulation of RFP-fusion proteins was determined by fluorescence quantification compared to affinity-purified RFP-derived calibration curve. Values are expressed as mean of three biological replicates including standard deviations. Gray columns, crude extract; red columns, extract after heat treatment; lanes 3–16 contain the samples identified in **Table 1**. FLW, fresh leaf weight.

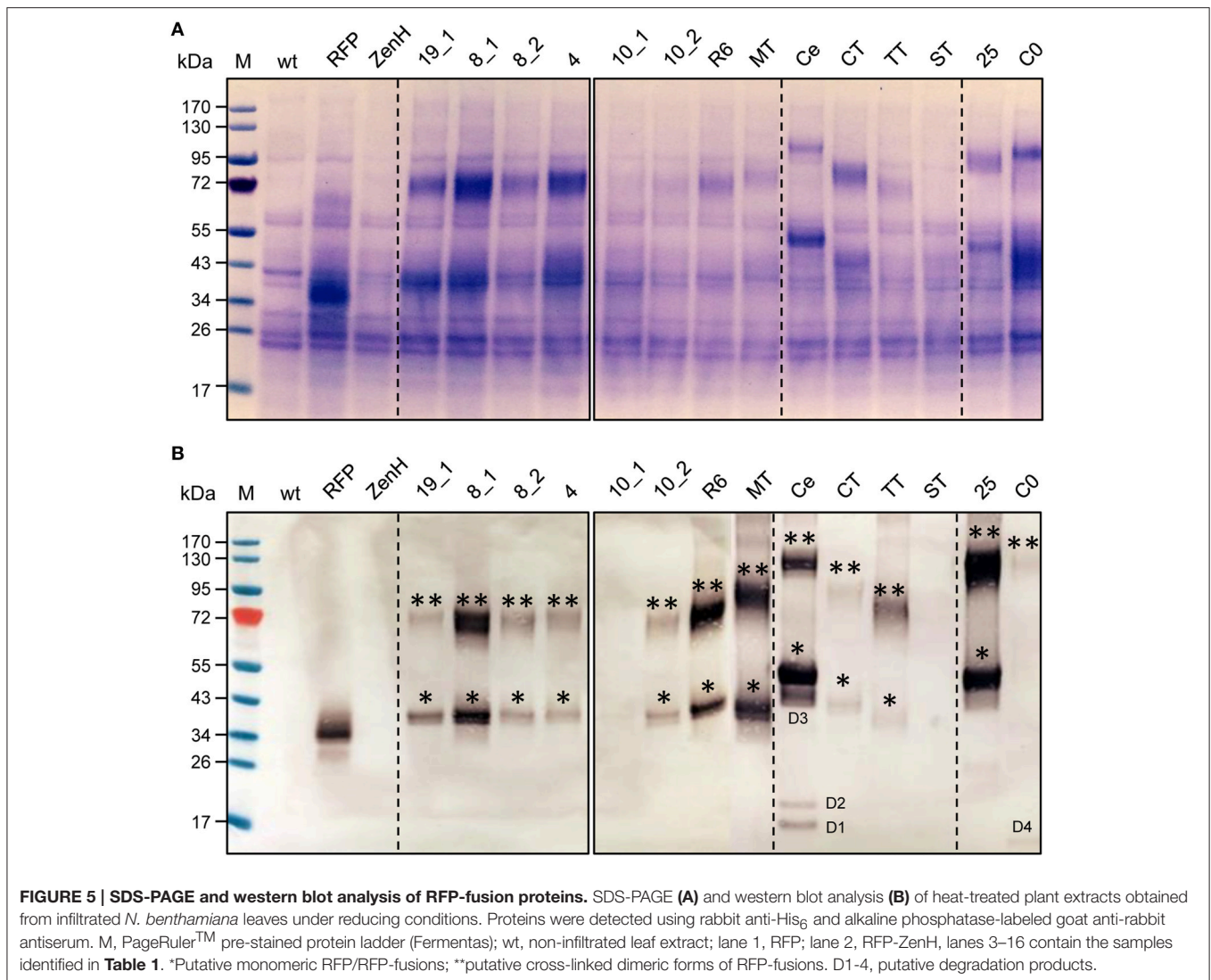
**FIGURE 4 | Continued**

Ni-NTA magnetic agarose beads, showing the difference between soluble and insoluble proteins. Wt, non-infiltrated leaf extract; RFP, RFP construct; 19\_1, RFP-*PfMsp1-19\_EGF1* construct; ST, RFP-*PfSPATR\_TSR*; RFP-ZenH; 10\_1, RFP-*PfMsp10\_EGF1*. Left panel, Transmission image; right panel, fluorescence image.

after heat treatment. Bands corresponding to P1 (calculated size 19.3 kDa, observed size 19–20 kDa), P2 (calculated size 27.7 kDa, observed size: 32–34 kDa), and P3 (calculated size 33.9 kDa, observed size 38–40 kDa) were clearly represented on the stained gel and corresponding His<sub>6</sub> tag-specific western blot representing extracts after heat treatment, indicating that the combination of *PfCelTOS* with the TSR domains of *PfCSP-TSR* and *PfTRAP\_TSR* within a fusion construct yields an intact and heat-stable protein that combines important complementary *P. falciparum* pre-erythrocytic antigens. For the fusion proteins P2 and P3, the western blot revealed the formation of reduction-insensitive higher-molecular-weight aggregates with an apparent molecular weight of the corresponding dimers (64–68 kDa for the P2 dimer and 76–80 kDa for the P3 dimer), whereas only a small amount of the C-terminal (His<sub>6</sub>-tagged) degradation product was observed at 11 kDa, suggesting that the partial proteolytic degradation of *PfCelTOS* as observed in the expression screening of single domain RFP-fusion proteins (Figure 5) does not occur to the same extent in the context of the P3 fusion protein.

### Construction, Expression, Purification, and Characterization of the Multi-EGF Dual-Stage Vaccine Candidate E5

EGF-like domains are present in several *P. falciparum* surface proteins, predominantly on the merozoites and schizonts (blood stage) but also on the zygotes, macrogametes, and ookinetes (sexual stage). Whereas GPI-anchored merozoite surface proteins such as *PfMsp4* and *PfMsp5* feature single EGF-like domains, *PfMsp1*, *PfMsp8*, and *PfMsp10* feature a tandem array of two EGF-like domains, and the sexual stage antigen *Pfs25* is composed of four EGF-like domains. The merozoite surface antigen *PfRipr* contains a total of 10 EGF-like domains arranged in a tandem followed by a separate cluster of eight EGF-like domains. It has been shown that EGF-like domains are the target of parasite growth inhibitory antibodies in humans (Egan et al., 1999; O'Donnell et al., 2001; Maskus et al., 2015) and/or immunized animals (Chappel and Holder, 1993; Chen et al., 2011) and improve the antigenicity of the antigens (Wang et al., 1999). The high expression levels and thermal stability of most of the EGF-like domain constructs in our panel prompted us to combine a number of such domains from *P. falciparum* merozoites with the sexual stage antigen *Pfs25* in the context of a multi-EGF dual-stage vaccine candidate antigen. Taking into account the expression levels and solubility data obtained in the RFP-fusion expression screening experiments, we omitted *PfMsp10\_EGF1/2*. Using a domain stacking approach, a series of five sequentially elongated constructs (E1–E5) was generated, the largest of which was E5

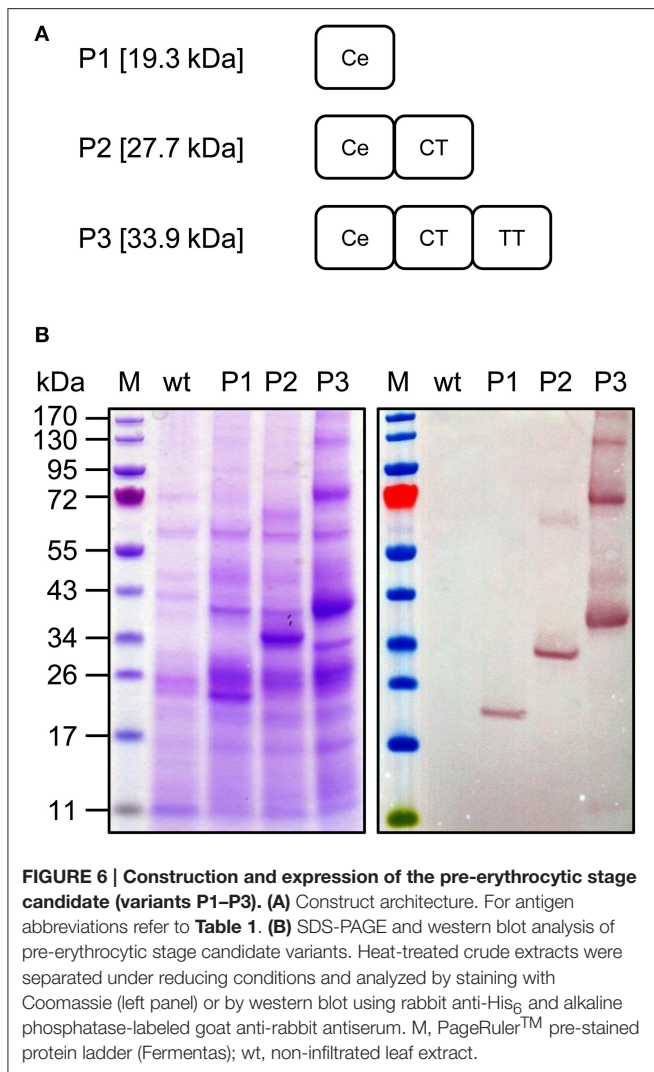


comprising *PfMsp1-19\_EGF1*, *PfMsp8\_EGF1/2*, *PfMsp4\_EGF*, and *Pfs25* (Figure 7A). Heat-treated extracts from syringe-infiltrated leaf material at 5 dpi were analyzed by SDS-PAGE and western blot. As shown in Figure 7B, the smallest expression construct (construct E1, featuring *PfMsp1-19\_EGF1* alone) was not detected in the heat-treated extract by a His<sub>6</sub> tag-specific western blot, but the presence of the protein was confirmed, alone and in the context of the different successor fusion proteins, using a conformational, reduction-sensitive *PfMsp1-19\_EGF1*-specific monoclonal antibody in a dot-blot under native conditions (Figure 7C). Although construct E2 combining *PfMsp1-19\_EGF1* and *PfMsp4\_EGF* showed weak expression, the larger variants E3, E4, and E5 accumulated to satisfactory levels that were easily detected by Coomassie staining. A 15 kDa C-terminal degradation fragment comprising the His<sub>6</sub> tag observed in the case of construct E3 and a weaker 25 kDa His<sub>6</sub>-tagged degradation fragment observed for construct E5, indicate that the fusion proteins are not completely resistant to proteolytic cleavage.

To provide material for immunization studies, E5 was produced and purified from 200 g of vacuum infiltrated *N. benthamiana* leaves. Purification by IMAC and IEX yielded a final 4 mg preparation of highly pure (>90%) protein (Figure 8A) corresponding to 15 μg of purified E5 per gram of fresh leaf material.

The purified E5 fusion antigen was used to immunize mice and the resulting immune sera were analyzed for E5 titers as well as single domain-specific titers by ELISA. As shown in Figure 8B, the immune response was directed against all domains and the average titer against the single domains ranged from around 70,000 for *PfMsp1-19\_EGF1* to around 450,000 for *Pfs25*. The titer measured against the full fusion antigen was >500,000. For crude ELISA data refer to data sheet 1. Additionally, the ability of the E5 fusion antigen to induce antibodies that recognize *P. falciparum* antigens in their native context was determined by immunofluorescence analysis involving different parasite preparations from the blood and sexual stages. Figures 8C,D show that the E5-specific mouse immune sera bind to parasite

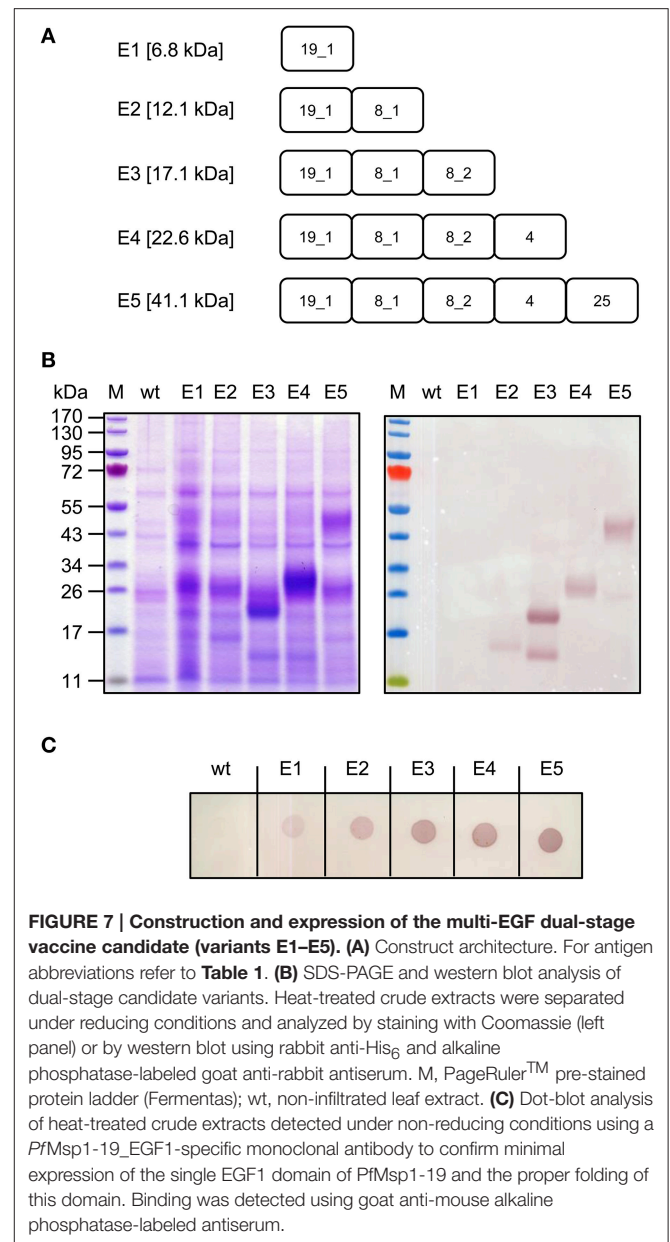




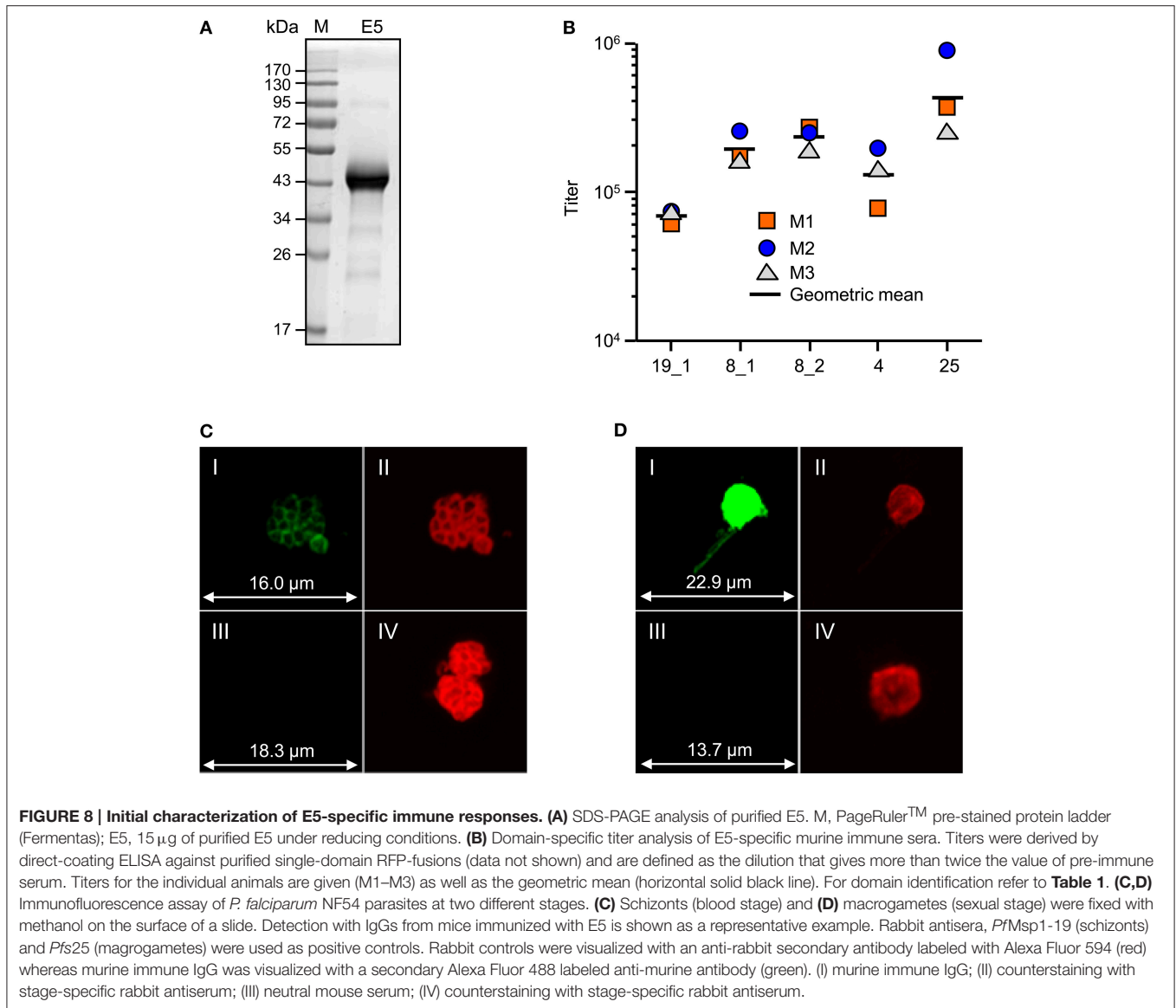
surface proteins in their native context (schizonts, blood stage parasites, **Figure 8C**, and macrogametes, sexual stage parasites, **Figure 8D**) and provide an indication that *Pf*EGFs are correctly folded when combined within the E5 fusion protein.

### Construction and Expression of the Multi-Domain, Multi-Stage Vaccine Candidate M8

After gaining promising results with the P3 and E5 fusion proteins, we next used the transient expression platform for the stepwise generation of the multi-domain, multi-stage vaccine candidate fusion protein M8. Starting from a four EGF-domain variant featuring *Pf*Msp1-19\_EGF1, *Pf*Msp8\_EGF1/2, and *Pf*Msp4\_EGF, we subsequently added seven additional previously screened domains (**Figure 9A**) representing all three main parasite stages, and analyzed the accumulation and heat stability of the fusion proteins by SDS-PAGE and western blot. **Figure 9B** shows that all eight multi-domain fusion proteins were detected in heat-treated crude extracts when the proteins



were separated by SDS-PAGE under reducing conditions and stained with Coomassie (**Figure 9B**, left side). The proteins were also detected by His<sub>6</sub> tag-specific western blot (**Figure 9B**, right side). In both cases, the proteins migrated as expected for their molecular weights. The western blot revealed a number of higher-molecular-weight aggregates especially for the larger fusion proteins (**Figure 9B**, right side, lanes 3–8) as well as small amounts of (His<sub>6</sub>-tagged) degradation products. Two of the degradation products previously observed for RFP-*Pf*CeTOS (D1, D2, **Figure 5B**) were also observed for the *Pf*CeTOS-containing fusion proteins (**Figure 9B**, right side, lanes 3, 7, and 8) with increasing size correlating with the size of the fusion partners distal to the *Pf*CeTOS component.

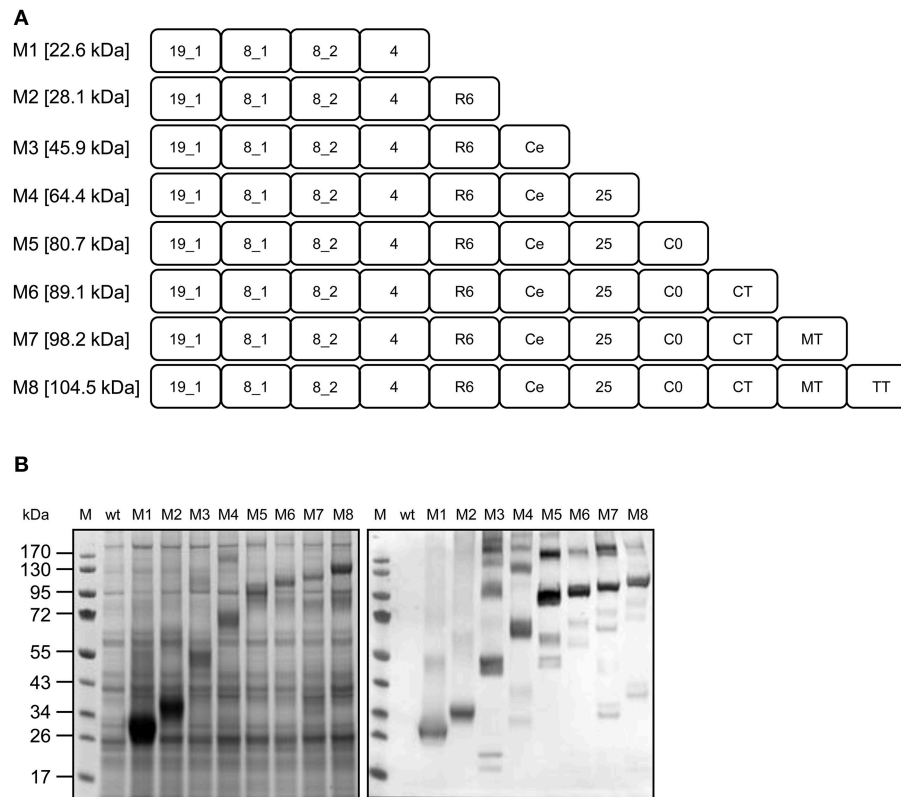


## DISCUSSION

### RFP-Fusion Protein Based Expression Screening as Vaccine Development Tool

Over the last few decades, a large number of *P. falciparum* antigens from different lifecycle stages have been proposed as potential malaria vaccine candidates (Moorthy and Hill, 2001; Todryk and Hill, 2007). Many of them have been tested in preclinical animal studies, some yielding promising results, others yielding ambiguous, or negative outcomes. Taken together, the available data suggest that one route toward a more efficacious malaria vaccine could be the combination of different antigens or antigen domains to broaden the immune response against multiple targets, even targets from different stages to achieve multi-stage protective efficacy (Hill, 2011).

Based on these considerations, we implemented a malaria vaccine development program using our well-established plant transient expression platform based on *A. tumefaciens*. The screening of RFP-fusion protein expression provided an efficient tool to rapidly assess the expression and solubility of large numbers of vaccine antigens. RFP supports both N-terminal and C-terminal fusions, accumulates to high levels in different subcellular compartments and has been used to analyze protein targeting and/or localization (Pasare et al., 2013), as a visual marker for transgene expression (Rademacher et al., 2008; Sack et al., 2015), to address and compare the efficiency of transfection methodologies (Leuzinger et al., 2013), and to develop predictive models for transient gene expression in tobacco (Buyel and Fischer, 2012). The strong fluorescence of the mature homotetrameric structure is simple to detect and quantify, making it an ideal reporter for expression screening.



**FIGURE 9 | Construction and expression of the multi-domain, multi-stage vaccine candidate (variants M1–M8). (A)** Construct architecture. For antigen abbreviations refer to **Table 1**. **(B)** SDS-PAGE and western blot analysis of multi-domain, multi-stage candidate variants. Heat-treated crude extracts were separated under reducing conditions and analyzed by staining with Coomassie (left panel) or by western blot using rabbit anti-His<sub>6</sub> and alkaline phosphatase-labeled goat anti-rabbit antiserum. M, PageRuler™ pre-stained protein ladder (Fermentas); wt, non-infiltrated leaf extract.

The syringe infiltration of single leaves allows the straightforward comparative testing of up to six constructs in a single plant, using an *A. tumefaciens* suspension prepared from a 5-mL culture grown in standard test tubes. Visual inspection of RFP fluorescence *in planta* provides the first readout of construct functionality, whereas solubility and heat stability can be assessed visually at the macroscopic or microscopic levels in planta, and after extract preparation, processing, and centrifugation. Heat stability is favorable because it facilitates the removal of HCPs during downstream processing (Buyel et al., 2014; Voepel et al., 2014; Beiss et al., 2015) and potentially translates into favorable storage and shelf-life properties. In the context of our panel of 14 candidates, this screen quickly identified poorly expressed proteins such as *PfSPATR\_TSR* and *PfMsp10\_EGF2*, as well as insoluble proteins such as *PfMsp10\_EGF1* and *PfTRAP\_TSR*.

In addition to immunological and clinical considerations, information about domain folding and the number of disulfide bridges was an important criterion during the selection of our panel of *P. falciparum* antigens. In contrast to the large, often redundant and unstructured characteristics of *P. falciparum* surface antigens (Feng et al., 2006), both EGF-like and TSR domains are highly structured and rich in disulfide bridges. This pre-selection process explains why we did not find any

heat-sensitive proteins during the screen. The use of RFP as a fusion partner provided an additional advantage because it enabled the analysis of small proteins (like single EGF-like domains) which sometimes do not accumulate to detectable levels when expressed alone. One example is *PfMsp1-19\_EGF1*, which was subsequently introduced successfully into multi-domain fusion proteins (Boes et al., 2015). Furthermore, RFP-domain fusion proteins are also needed for the deconvolution of antibody responses against vaccine candidates comprising multiple domains and components.

### Assembly of the Pre-erythrocytic Stage Candidate P3

After selecting soluble and heat stable pre-erythrocytic stage antigens using the transient expression platform to screen RFP-fusion proteins, we generated sequentially elongated fusion protein variants and developed the novel pre-erythrocytic multi-domain vaccine candidate P3. We confirmed that P3, the largest variant composed of three different antigens or antigen domains, was expressed as an intact fusion protein at satisfactory levels and remained soluble after heat treatment to remove HCPs.

P3 was thus regarded as a promising pre-erythrocytic vaccine candidate and was selected for further investigations. The production, purification, and characterization of P3 (renamed CCT in later studies) has been described in detail elsewhere, including results confirming the *in vitro* inhibition of pre-erythrocytic parasite stages by P3-specific mouse immune sera (Voepel et al., 2014). P3 was also selected as part of a plant-derived multi-stage vaccine cocktail that underwent detailed characterization in rabbit immunization studies and *in vitro* parasite inhibition assays at different life cycle stages (Boes et al., 2015). These studies confirmed its pre-erythrocytic stage efficacy (up to 80% inhibition of pre-erythrocytic parasites *in vitro*) following the immunization of rabbits. Upstream production, and downstream process development for the large-scale transient expression of P3 in *N. benthamiana* is presented in a separate publication in this issue, including the optimization of buffer conditions and the heat treatment process (Menzel et al., in preparation).

### Construction, Expression Purification, and Characterization of the Multi-EGF Dual-Stage Vaccine Candidate E5

By analogy to the construction of the single stage (pre-erythrocytic stage) multi-domain vaccine candidate P3 we performed the generation of the dual-stage (blood stage and sexual stage) multi-domain vaccine candidate E5 as an assembly of EGF-like domains from three different blood stage antigens and one sexual stage antigen. The EGF-like domain is a small (30–40 amino acids) and well-conserved fold found in many proteins from diverse species and typically features three intra-domain disulfide bounds (Wouters et al., 2005). Even though the EGF-like domain is stable, our initial experiments with the EGF-like domains from different *P. falciparum* antigens revealed large differences in expression and accumulation (data not shown). The RFP-fusion expression screening approach made it possible to reject problematic EGF-like domains such as PfMsp10\_EGF1 and 2 and only combine strongly-expressed soluble domains to construct the E5 vaccine candidate antigen.

The purified protein was used in an initial mouse immunization study to determine the titers against the full-size fusion protein and its individual domains. The overall titers of >500,000 indicated robust immunogenicity, and although the small number (three) of animals used in this immunization study prevented thorough statistical evaluation, the strong immune response against the largest component (Pfs25, four EGF-like domains) compared to the other components (PfMsp1-19\_EGF1, PfMsp8-EGF1, PfMsp8\_EGF2, and PfMsp4\_EGF, each featuring one EGF-like domain) matches our observations concerning multi-domain, multi component vaccines in previous studies (Boes et al., 2015; Spiegel et al., 2015) confirming the positive correlation between component size and immune response and thereby provides useful guidelines for the development of a multi-domain, multi-stage, multi-allele vaccine cocktail.

### Construction and Expression of the Multi-Domain, Multi-Stage Vaccine Candidate M8

We also used the workflow described above for the stepwise construction and expression of a large multi-domain, multi-stage vaccine candidate named M8. Based on the selection of *P. falciparum* antigen domains with confirmed strong expression, integrity, heat stability, and suitability as components of fusion antigens, we were able to produce a complex fusion protein consisting of 11 different promising vaccine candidate antigens and antigen domains in the context of a heat stable, moderately expressed, and proteolytically stable protein. Following the optimization of downstream processing, the M8 vaccine candidate will be produced, purified, and tested in animal immunization studies and parasite growth inhibition assays using the three main stages of the *P. falciparum* life cycle. These results will be particularly interesting with respect to conclusions that have been drawn in the context of results the authors have generated by immunization experiments with the pre-erythrocytic vaccine candidate P3 (Voepel et al., 2014), a sexual stage vaccine candidate (F0) containing Pfs25 and Pfs230\_Co (Beiss et al., 2015) as well as with a malaria multi-component vaccine cocktail (among others including P3 and F0; Boes et al., 2015) and multi-stage fusion proteins (Spiegel et al., 2015). In these studies it could be shown that the combination of candidates that provide good *in vitro* efficacy may lead to unwanted reduction of titers against single components by antigenic competition. On the other hand low immunogenic components like PfMsp1-19 seem to profit from presentation in the context of fusion proteins that provide additional t-cell epitopes. Taking the available data together it becomes clear that these effects are poorly predictable. The authors observed an unproportionally high immune response against PfCSP\_TSR in the context of P3, while in the studies working with larger fusion proteins and cocktails the distribution of component-specific titers did roughly correlate with the molar quantity of the component within the mixture and/or fusion. It will be interesting to see what advantages and disadvantages can be observed in the case of M8 that combines interesting pre-erythrocytic, sexual, and blood stage antigens in the context of a large fusion protein.

### CONCLUSIONS AND FUTURE PERSPECTIVES

In this study we have demonstrated the successful application of a plant-based transient expression platform as an essential tool for the development of multi-domain, multi-stage malaria vaccine candidates. The simple and robust workflow based on syringe infiltration requires only a small number of plants and small culture volumes. This allows the convenient analysis several samples in parallel in a time frame from gene to protein of <10 days. The method can easily be scaled up from 100 µg scale (leaf discs) realized by syringe infiltration of leaves up to 100 g (whole leaves) scale realized by vacuum infiltration of whole

plants, enabling the convenient generation of mg-amounts of target protein for advanced analytics or animal immunization studies. Providing a generic toolbox, the established workflow and methodologies can be easily translated to successfully address any vaccine candidate identification and development task.

## AUTHOR CONTRIBUTIONS

HS participated in the design of the experiments, performed the experiments, performed data interpretation, and drafted the manuscript. AB participated in the design of the experiments, performed the experiments, performed data interpretation, and drafted the manuscript. NV participated in the work related to the pre-erythrocytic stage vaccine candidate P3 and helped in drafting the manuscript. VB was involved in antigen selection and performed the cloning of sexual stage antigen constructs and helped in drafting the manuscript. GE was involved in antigen selection and performed the cloning of TSR-domain fusion constructs and helped in drafting the manuscript. TR participated in the design of the experiments and performed the cloning and testing of RFP-control constructs. MS participated in the study design and the data interpretation and revised the manuscript. SS participated in study design and coordination, and helped to draft the manuscript. AR participated in the study design and the data interpretation, and revised the manuscript. RF conceived of the study, participated in its design and revised the manuscript.

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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