Generation of Novel *Plasmodium falciparum* NF135 and NF54 Lines Expressing Fluorescent Reporter Proteins Under the Control of Strong and Constitutive Promoters

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Transgenic reporter lines of malaria parasites that express fluorescent or luminescent proteins are valuable tools for drug and vaccine screening assays as well as to interrogate parasite gene function. Different *Plasmodium falciparum* (*Pf*) reporter lines exist, however nearly all have been created in the African NF54/3D7 laboratory strain. Here we describe the generation of novel reporter lines, using CRISPR/Cas9 gene modification, both in the standard *Pf* NF54 background and in a recently described Cambodian *P. falciparum* NF135.C10 line. Sporozoites of this line show more effective hepatocyte invasion and enhanced liver merozoite development compared to *Pf* NF54. We first generated *Pf* NF54 reporter parasites to analyze two novel promoters for constitutive and high expression of mCherry-luciferase and GFP in blood and mosquito stages. The promoter sequences were selected based on available transcriptome data and are derived from two housekeeping genes, i.e., translation initiation factor SUI1, putative (*sui1*, PF3D7_1243600) and 40S ribosomal protein S30 (*40s*, PF3D7_0219200). We then generated and characterized reporter lines in the *Pf* NF135 line which express GFP driven by the *sui1* and *40s* promoters as well as by the previously used *ef1α* promoter (*GFP@efs*, *GFP@sui1*, *GFP@40s*). The *GFP@40s* reporter line showed strongest GFP expression in liver stages as compared to the other two lines. The strength of reporter expression by the 40s promoter throughout the complete life cycle, including liver stages, makes transgenic lines expressing reporters by the 40s promoter valuable novel tools for analyses of *P. falciparum*.

**Keywords:** *Plasmodium falciparum*, malaria, reporter lines, liver stage, NF135, CRISPR/Cas9
INTRODUCTION

Transgenic rodent and human malaria parasites expressing fluorescent and bioluminescent proteins are used extensively to interrogate parasite biology and malaria pathology as well as tools to evaluate anti-parasite inhibitors and vaccines (Othman et al., 2017). For Plasmodium falciparum, transgenic reporter lines have been used to quantify the effect of inhibitors and antibodies on in vitro asexual blood stage parasite development (Cui et al., 2008), to evaluate the effect of inhibitors on gametocyte and mosquito stage development (Adjalley et al., 2011; Lucantoni et al., 2013, 2016; Wang et al., 2014; Vos et al., 2015) and to analyze sporozoite infection of hepatocytes in immune-deficient humanized mice engrafted with human liver tissue (Sack et al., 2014; Flannery et al., 2018; Foquet et al., 2018) and sporozoite movement in skin model (Hopp et al., 2019; Winkel et al., 2019).

Most transgenic P. falciparum reporter lines have been generated in the widely used laboratory strain NF54 (PfNF54), or its clone 3D7, which originates from an African isolate (Ponnudurai et al., 1981). Reporter expression in the transgenic lines has been driven by various promoters from different genes which are either constitutively expressed in multiple life cycle stages (e.g., the housekeeping genes ef1α or hsp70) (Talman et al., 2010; Vaughan et al., 2012; Vos et al., 2015) or from stage-specific genes (e.g., pfs16, gep02, or etramp10.3/peg4) (Adjalley et al., 2011; Lucantoni et al., 2016; Marin-Mogollon et al., 2019; McLean et al., 2019; Portugaliza et al., 2019). To analyze liver-stage development a parasite line with strong and constitutive expression of the reporter is essential. The ef1α promoter drives expression in liver stages and luciferase reporter expression using this promoter has been used to quantify liver infection in humanized mice (Vaughan et al., 2012). However, low activity of the ef1α promoter in sporozoites and early liver stages hampers its use for early liver stage analyses (Vaughan et al., 2012; Lucantoni et al., 2016; Marin-Mogollon et al., 2019). Similarly a transgenic PfNF54 reporter line expressing GFP-luciferase under control of the hsp70 promoter (Vos et al., 2015) has limited reporter expression in liver stages (M.W. Vos. personal communication). Recently, we generated a PfNF54 reporter line (mCherry-Luc@etramp) using the promoter from the Pfetramp10.3 gene (Marin-Mogollon et al., 2019), which is related to rodent Plasmodium uis4 gene. In rodent malaria reporter lines the uis4 promoter has been used to drive high transgene expression in both sporozoites and in liver-stages (Marin-Mogollon et al., 2019). Although expression of the reporter mCherry-luciferase was high in the PfNF54 mCherry-Luc@etramp sporozoites, luciferase expression in liver stages was lower than observed in a P. falciparum transgenic line expressing GFP-luciferase under control of the ef1α promoter (Marin-Mogollon et al., 2019).

In this study we have examined new P. falciparum promoters to drive high reporter expression throughout the parasite life cycle, in particular in mosquito and liver stages. In addition to using the PfNF54 strain we also generated reporter lines in a recently characterized clone from a Cambodian isolate, NF135:C10 (Teirlinck et al., 2013), hereafter referred to as PfNF135. Sporozoites of PfNF135 show a higher invasion rate of hepatocyte in vitro and produce more liver merozoites per infected hepatocyte than PfNF54 parasites (McCall et al., 2017). High reporter expression in liver stages of transgenic PfNF135 parasites in combination with increased liver-infectivity should improve liver stage quantification both in inhibitor studies in vitro and in humanized mice models, as well as permitting efficient sorting of infected hepatocytes by flow cytometry, as has been achieved using rodent Plasmodium reporter lines. To generate the different transgenic PfNF54 and PfNF135 parasites we used a recently developed CRISPR/Cas9 methodology for introducing transgenes into the genome without retaining a drug selectable marker (Mogollon et al., 2016). In this study we describe the characterization of reporter expression in multiple life cycle stages using novel strong promoters of two housekeeping genes (sui1 and 40s) and describe for the first time transgenic parasites in the PfNF135 background which demonstrate high GFP expression throughout the parasite life-cycle, including liver-stage development as shown in in vitro studies performed in primary human hepatocytes.

MATERIALS AND METHODS

P. falciparum: Parasites and in vitro Cultivation of Blood Stages

P. falciparum NF54 (PfNF54) (Mogollon et al., 2016) and P. falciparum NF135 (PfNF135) (Teirlinck et al., 2013) parasites were cultured using standard culture conditions in a semi-automated shaker incubator system as previously described (Mogollon et al., 2016). Fresh human serum and red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology; tested for safety). RBC and human serum from different donors were pooled. Cloning of parasites was performed by the method of limiting dilution as described previously (Mogollon et al., 2016). Gametocyte cultures were prepared using standard culture conditions as described (Mogollon et al., 2016). Briefly, parasites from asexual stage cultures were diluted to a final parasitemia of 0.5% and cultures were followed during 14–17 days with daily medium change twice, but without replenishing fresh RBCs. At day 14–17 the cultures were analyzed for stage V gametocytes and fed to Anopheles stephensi mosquitoes (see below). The growth rate of asexual blood-stages (parasitemia) of PfNF135 parasite lines was monitored by determination of parasitemia in standard in vitro cultures (in a semi-automated shaker incubator system) for a period of 3 days with a starting parasitemia of 0.1%. Parasitemia was determined by counting infected red blood cells in Giemsa-stained thin blood films in three independent experiments.

Generation of Transgenic Lines Expressing Reporters

We used the recently developed two plasmid CRISPR/Cas9 method for generation of the transgenic PfNF54 parasites without retaining a selectable marker in the genome (Mogollon
et al., 2016). The Cas9 construct pLf0019 (Mogollon et al., 2016), that contains the Cas9 expression cassette and a blasticidin (BSD) drug-selectable marker cassette, was used in combination with the donor DNA plasmids, that are based on the published plasmid pLf0047 (Marin-Mogollon et al., 2019) and contain the p47 sgRNA/U6 cassette, the hdhfr-yfcu drug selectable marker (SM) cassette, the two p47 homology regions and the transgene expression cassettes. Three different donor DNA constructs were generated, pLf0117, pLf0123, and pLf0128 that contain the mCherry-luciferase (mCherry-Luc) fusion gene under control of the ef1α (PF3D7_1357000), 40s (PF3D7_0219200), and sui1 (PF3D7_1243600) promoters respectively (see below). In addition, two DNA donor constructs were generated, pLf0116 and pLf0127 that contain the GFP gene under control of the ef1α and 40s promoter.

To generate the transgene-expression cassettes, we first PCR amplified (KOD Hot Start DNA Polymerase, Merck Millipore) 800 bp of the ef1α 5′UTR (primers P1/P2, Table S1), 1,493 bp of the sui 5′UTR (primers P3/P4, Table S1), and 1,097 bp of the 40s 5′UTR (primers P5/P6, Table S1) using Pf NF54 genomic DNA as template. These 5′UTR regions (promoters) were introduced by SacII/Xhol sites into either the intermediate plasmid, pLf0188 that contains an mCherry-luciferase fusion gene (under control of the gapdh promoter; Marin-Mogollon et al., 2019) or in the intermediate plasmid pLf0187 that contains a GFP gene (under control of the gapdh promoter; Marin-Mogollon et al., 2019). The transgene expression cassettes from these plasmids were subsequently removed by Apai/SacII digestion and ligated into plasmid pLf0047, resulting in the final mCherry-Luc donor DNA plasmids pLf0117, pLf0123, and pLf0128 (Figure 1 and Figure S1) and the final GFP donor DNA plasmids pLf0116, pLf0122, and pLf0127 (Figures S1, S2).

Plasmids for transfection were isolated from 250 ml cultures of Escherichia coli, XL10-Gold Ultracompetent Cells (Stratagene) by maxi-prep [using HiSpeed Plasmid Maxi Kit (Qiagen 1)] to generate 50 µg of DNA used per transfection. Transfection with CRISPR/Cas9 constructs was performed using the “spontaneous uptake method” as previously described (Deitsch et al., 2001). Briefly uninfected red blood cells (300 µl of packed RBCs) were transfected with CRISPR constructs (a mixture of ~50 µg of each circular plasmid (Cas9 and donor DNA constructs in 200 µl cytomix) using the Gene Pulser Xcell electroporator (BioRad) with a single pulse (310 V, 950 µF and ∞ Capacity). Subsequently, the transfected RBCs were washed with complete medium and mixed with P/NF54-infected RBCs to a parasitemia of 0.1–0.5% and a hematocrit of 5%. These cells were transferred into a 10 ml culture flask of the semi-automated shaker system.

Selection of transfected P/NF54 parasites was performed by applying double positive selection for a period of 6–19 days by adding the drugs WR99210 (2.6 nM) and BSD (5.µg/ml) starting 3 days after transfection. After the days of treatment with double drugs, parasites were maintained in drug-free medium until parasites were detectable in Giemsa-stained thin blood films (~12–24 days). In independent experiments, we obtained parasite populations mCh-Luc@ef1α (Pf-Exp. 156), mCh-Luc@sui1 (Pf-Exp. 159), mCh-Luc@40s (Pf-Exp. 187), GFP@ef1α (Pf-Exp. 155), and GFP@40s (Pf-Exp. 175) expressing mCherry or GFP as determined by fluorescence microscopy analysis of mixed blood stages. For the mCh-Luc@sui1 parasites, different clonal lines were obtained by limiting dilution (see below). For mCh-Luc@ef1α, mCh-Luc@40s line, mCherry-positive cells were collected by flow cytometry (FACS) sorting (see below) using mixed blood stages, resulting in parasite populations in which more than 90% of blood stages were mCherry-positive (determined by fluorescence microscopy).

Selection of transfected P/NF135 parasites was performed by applying double positive selection by adding the drugs pyrimethamine (100 ng/ml) and BSD (5 µg/ml). After transgenic parasites stably grew in the semi-automated shaker system (usually after 5 to 8 days of positive selection), both drugs were removed from the cultures for 2–4 days, and negative selection was applied by addition of 5-Fluorocytosine as described except for GFP@40s (Mogollon et al., 2016). In independent experiments we obtained the following parasite populations GFP@ef1αNF135 (Pf-Exp. 160), GFP@40sNF135 (Pf_Exp. 165), and GFP@sui1 NF135 (PL_Exp. 164) expressing GFP as determined by fluorescence microscopy analysis of mixed blood stages. For the GFP@40sNF135 and GFP@sui1NF135 populations we performed two rounds of FACS-sorting (see below) to enrich for transgenic parasites expressing GFP, resulting in parasite populations where more than 99% of the blood stages were GFP positive (determined by fluorescence microscopy). From the GFP@ef1αNF135 population we obtained a GFP-expressing clone (clone 2) by the method of limiting dilution (see below).

Genotyping of Transgenic P. falciparum Reporter Lines

For genotyping and Southern blotting genomic DNA was isolated as previously described using phenol/chloroform DNA isolation (Mogollon et al., 2016). Correct integration of reporter constructs was analyzed by PCR amplification of the fragments: 5′-integration, 3′-integration and the p47 gene (see Table S1 for primer sequences). The PCR fragments were amplified using KOD Hot Start Polymerase (Merck Millipore) following standard conditions with annealing temperatures of 50, 55, 60°C for 10 s and an elongation step of 68°C. Southern blot analyses of digested DNA were performed with HpaI-digested genomic DNA (overnight at 37°C). Digested DNA was hybridized with probes targeting the p47 homology region 1 (HR1), amplified from NF54 genomic DNA by PCR (primers P19/P20) and a second probe targeting ampicillin (Amp) gene, amplified from a plasmid by PCR (primers P21/P22; see Table S1 for primer sequences).

Flow (FACS) Sorting of GFP- and mCherry-Expressing Parasites

To enrich for transfected parasites obtained after drug selection (see above) we selected GFP- and mCherry-expressing blood stages by FACS sorting. Briefly, 200 ±1 samples of mixed blood stage cultures (parasitemia 1 to 8%) were diluted in 1 ml of complete medium supplemented with 3% of human serum and 1% penicillin/streptomycin (Gibco). Parasites were sorted using a BD FACS AriaTM III (Becton Dickinson, Mountain View, CA, USA). RBCs were selected by gating on Forward and Side
FIGURE 1 | Generation of *P. falciparum* NF54 reporter lines expressing mCherry-luciferase under control of *ef1α*, *sui1*, or *40s* promoter. (A) Schematic representation of the Cas9 (pLf0019) and donor DNA plasmids (pL0117, pLf0123, pLf0128) constructs used to introduce the mCherry-luciferase expression cassette into the
Scatter parameters (FSC and SSC, respectively). Doubledts were excluded by using FSC-Area and FSC-height parameters. For GFP, a blue laser (488 nm excitation) was used in combination with band pass filter 530/30 nm. For mCherry, a yellow/green laser (561 nm excitation) was used in combination with band pass filter 615/20 nm. A total of 1 × 10^5 fluorescent cells were selected and collected in a well of a 24 wells plate in complete medium supplemented with 20% of human serum and 1% penicillin/streptomycin. Then the sorted cells were diluted to 10 cells or 100 cells per culture and transferred to 10 ml flask. FACs-sorted parasites were cultured under standardized conditions for a period of 7 to 14 days in the semi-automated shaker system. At a parasitemia of 5 to 10%, parasites were collected for genotyping, fluorescence analyses (see below) and storage in liquid nitrogen.

Flow Cytometric Analysis of GFP-Expression of Asexual Blood Stages

The relative GFP-fluorescence intensities of different asexual blood stages were determined as previously described (Janse and van Vianen, 1994; Mogollon et al, 2016) with a few modifications. Briefly, triplicate samples of 100 µl of infected RBC were collected from cultures that had been synchronized with sorbitol as described previously (Mogollon et al, 2016). Samples were collected at 12 and 42 h after synchronization and resuspended in 3 ml of culture medium containing 3% serum. Cells were stained with the DNA-specific dye Hoechst-33258 at 37°C for 30 min (Janse and van Vianen, 1994; Mogollon et al, 2016). GFP- and Hoechst-fluorescence intensity was determined using an LSRII flow cytometer (Becton Dickinson, Mountain View, CA, USA), data were generated using the FlowJo DIVA software (Becton Dickinson) and analyzed with FlowJo software (Treestar, Ashland, OR, USA). Per sample 100,000 cells were evaluated and RBCs were selected by gating on FSC and SSC and excluding doublets. Excitation of cells for Hoechst-33258 was performed with a UV laser (355 nm) and band pass filter 450/50 nm and for GFP with a blue laser (488 nm) and a band pass filter of 530/30 nm. The GFP fluorescence intensity was determined of the haploid blood stages [rings and trophozoites; Gate 1 (G1)] and polyploid blood stages [schizonts; Gate 2 (G2)]. Haploid and polyploid blood stages were distinguished based on Hoechst-fluorescence intensity and the values were used to calculate the number of nuclei per schizont. G2 is set at 8-45x the mean Hoechst-fluorescence value of ring forms, calculated from G1.

Analysis of Oocyst and Sporozoite Production

For analysis of mosquito stages (oocysts and sporozoites), Anopheles stephensi mosquitoes were infected with day 14 gametocyte cultures using the standard membrane feeding assay (SMFA) (Ponnudurai et al, 1989; Marin-Mogollon et al, 2018). Oocysts and salivary gland sporozoites were counted at days 8–14 and 14–21 post feeding, respectively. Oocyst numbers and GFP/mCherry expression (see next section) in oocysts were determined at day 8 to 14 after feeding. Isolation of salivary gland sporozoites for counting numbers and expression of GFP/mCherry (see next section) was performed at day 14 to 21 after feeding. For counting sporozoites, salivary glands from 10 to 30 mosquitoes were dissected, collected in 100 µl of PBS and homogenized using a grinder. Sporozoites were counted using a Bürker cell counter using phase-contrast microscopy.

Analysis of GFP and mCherry Expression by Fluorescence Microscopy

GFP- or mCherry-expression in different blood stages, oocysts and sporozoites was analyzed by standard fluorescence microscopy as previously described (Mogollon et al, 2016; Marin-Mogollon et al, 2019). In brief, for blood stages 200 µl samples of iRBC were collected from 10 ml cultures with a parasitemia between 4 and 10% and stained with the DNA-specific dye Hoechst-33342 by adding 4 µl of a 500 µM stock-solution (final concentration 10 µM) for 20 min at 37°C. Subsequently, 10 µl were placed on a microscope slide (mounted under a cover slip) and GFP/mCherry fluorescence of live infected RBC was analyzed using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera using ColourPro software and with the following exposure times: GFP 0.7 s; mCherry 1s; Hoechst 0.2 s; bright field 0.1 s (1x gain).

GFP/mCherry-expression in oocysts was determined at day 8 and 14 after infection of A. stephensi mosquitoes as described in the previous section. GFP/mCherry expression in sporozoites was determined at day 14 and 21 after infection of mosquitoes. Isolated sporozoites (see previous section) were pelleted by centrifugation (800 × g; 5 min). The pellet was suspended in [40 µl of PBS] and sporozoites stained with Hoechst-33342 (10 µM). Of this solution, 10 µl were placed on a microscopic slide (mounted under a cover slip) and live fluorescence of sporozoites was analyzed as described above. GFP/mCherry...
fluorescence of live oocysts and sporozoites was analyzed using a Leica fluorescence DMR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using ColourPro software and with the following exposure times: GFP 0.7 s; mCherry 1s; Hoechst 0.2 s; bright field 0.1 s (1x gain).

**Luciferase Assay**

Luciferase expression in mCh-Luc@sui1 and mCh-Luc@40s asexual blood-stages was determined as follows. Samples of asexual blood-stages (in triplicate) were prepared from blood-stage cultures with $1.0 \times 10^8$ parasites per sample. Complete lysates (100 µl) were collected in a black 96-well plate (flat bottom) and luciferase activity was measured after adding 50 µl of Luciferase substrate ([Promega](https://www.promega.com)). Luciferase activity (in relative light units; RLU) was measured using the Glomax multi detection system Luminometer (Promega) and the Instinct software (Promega).

**Primary Human Hepatocyte Infection and Analyses**

Liver segments from surgical patients were obtained 2 days prior to infection with sporozoites. Hepatocytes were isolated by perfusing the tissue with increasing concentrations of collagenase as described (Lecluyse and Alexandre, 2010). Viable cells were resuspended in complete William’s B medium (William’s E medium with Glutamax (Gibco, 32551-087), supplemented with 1% (v/v) insulin/transferrin/selenium (Gibco, 41400-045), 1% (v/v) sodium pyruvate (Gibco, 11360-036), 1% (v/v) MEM-NEAA (Gibco, 1140-035), 2.5 µg/ml Fungizone Antimycotic (Gibco, 15290-018), 200 Units/ml penicillin/streptomycin (Gibco, 15410-122), and 1.6 µM dexamethasone (Sigma Aldrich D4902-100MG), stained with trypsin blue and counted. Approximately 62,000 cells were seeded (per 125 µl total volume) into each well of black 96 well plates (Falcon, 353219) at 37°C in an atmosphere of 5% CO$_2$. The parasites were spun down onto the monolayer at 100 g for 10 min with low brakes. The culture was incubated at 37°C in an atmosphere of 5% CO$_2$ for a further 3 h, after which there was a medium refreshment with complete William’s B media supplemented with 10% HIHS. The culture was incubated at 37°C in an atmosphere of 5% CO$_2$ with daily medium refreshment. A total of 62,000 sporozoites (MOI of 1:1) were added onto the hepatocytes 48 h post plating. The sporozoites were dissected in complete William’s B media and then supplemented with heat inactivated human serum (HIHS) upon addition to the monolayer at 100 µl of Luciferase substrate (Luciferase Assay System Promega). Luciferase activity (in relative light units; RLU) was measured using the Glomax multi detection system Luminometer (Promega) and the Instinct software (Promega).

**RESULTS AND DISCUSSION**

**In silico Identification of Constitutive and Strong Promoters for Reporter Expression**

To select promoters for driving constitutive expression of reporters, we first searched for genes that are highly transcribed in multiple life cycle stages, specifically housekeeping genes that show strong expression, not only in blood stages but also in sporozoites and liver stages and whose transcript levels are similar or higher in comparison with the housekeeping genes $ef1$ (elongation factor 1-alpha; PF3D7_1357000) and $hsp70$ (heat shock protein 70; PF3D7_0818900). Promoters of these latter two genes have been used previously to drive reporter expression in P. falciparum (Pf); however, reporter expression with the $ef1$ promoter in sporozoites and early liver stages is weak and reporter expression in liver stages with the $hsp70$ promoter was not successful (see Introduction section). For analyses of transcript levels we used published genome-wide transcriptome data available from the PlasmoDB database ([www.PlasmoDB.org](http://www.PlasmoDB.org)) for Pf (see Table S2) (Otto et al., 2010; Lasonder et al., 2016; Zanghi et al., 2018) and from published transcriptome data of blood, mosquito and liver stages of *Plasmodium berghei* (Otto et al., 2014; Caldelari et al., 2019). In the absence of Pf transcriptome data of liver stages, we also analyzed transcriptome datasets of liver stages of other human/primate Plasmodium species *Plasmodium vivax* and *Plasmodium cynomolgi* (Cubi et al., 2017; Voorberg-van der Wel et al., 2017; Gural et al., 2018). Based on analyses of these different datasets we selected two housekeeping genes involved in protein synthesis, i.e., translation initiation factor SU11, putative (sui1, PF3D7_1243600) and 40S ribosomal protein S30 (40s, PF3D7_0219200). Both genes show high transcript levels in most life cycle stages (Table S2) with particularly high transcript abundance in sporozoites and liver stages of human/primate *Plasmodium* species.
Analysis of Promoter Regions of the Housekeeping Genes *sui1* or 40s to Express the Reporters GFP and mCherry-Luciferase in *P. falciparum* NF54

First we tested reporter expression using the 40s and *sui1* promoters in blood stages and mosquito stages of the NF54 laboratory strain (*Pf*NF54) since this strain is well-characterized with respect to genome sequence and has been used for generation of transgenic parasites using CRISPR/Cas9 genetic modification (Mogollon et al., 2016; Marin-Mogollon et al., 2019). In these experiments we used GFP and the fusion protein mCherry-luciferase as reporter proteins. As a control promoter we included the *ef1α* promoter that has been used previously for reporter expression in *Pf*. For introduction of the different transgene expression cassettes into the genome we selected the *p47* gene (PF3D7_1346800) as target locus since this locus has been shown to be suitable for introduction of transgenes in *Pf*NF54 parasites without compromising blood stage development and development in *A. stephensi* mosquitoes (Vaughan et al., 2012; Vos et al., 2015; Lucantoni et al., 2016; Marin-Mogollon et al., 2019).

We used the recently developed two plasmid CRISPR/Cas9 method for generation of the transgenic *Pf* parasites without retaining a selectable marker in the genome (Mogollon et al., 2016). The Cas9 construct pLf0019, that contains the Cas9 expression cassette and a blasticidin (BSD) drug-selectable marker cassette (Mogollon et al., 2016), was used in combination with the donor DNA plasmids, that are based on the published plasmid pLf0047 (Marin-Mogollon et al., 2019) and contains the
p47 sgRNA, the hdhfr-yfcu drug selectable marker (SM) cassette, the two p47 homology (targeting) regions and the transgene expression cassettes. Three different donor DNA constructs were generated, pLf0117, pLf0123, and pLf0128 that contain the mCherry-luciferase (mCherry-Luc) fusion gene under control of the efla, sui1, and 40s promoters respectively. In addition, two DNA donor constructs were generated, pLf0116 and pLf0127 that contain the GFP gene under control of the efla and 40s promoters, respectively (see the Materials and Methods section and Figure S1 for more details of the generation of the plasmids). These five plasmids are designed to introduce the transgene expression cassettes into the p47 gene locus by double crossover homologous recombination (Figure 1A and Figure S2).

Transfection of PfNF54 parasites was performed by the method of pre-loading of erythrocytes with the plasmids, which are subsequently mixed with parasite-infected erythrocytes (Deitsch et al., 2001). After mixing, transfected parasites were selected by double positive selection using the drugs WR99210 and BSD. In independent experiments we obtained the following parasite populations, mCh-Luc@ef1α, mCh-Luc@sui1, mCh-Luc@40s, GFP@ef1α, and GFP@40s, expressing mCherry or GFP as determined by fluorescence microscopy analysis of mixed blood stages. For the mCh-Luc@sui1 parasites, different clonal lines were obtained by limiting dilution. For mCh-Luc@ef1α, mCh-Luc@40s line, mCherry-positive cells were collected by flow cytometry (FACS) sorting using mixed

![FIGURE 3](image-url)
FIGURE 4 | Generation of *P. falciparum* NF135 reporter lines expressing GFP under control of *ef1α*, *sui1* or 40s promoter. (A) Schematic representation of the Cas9 (pLf0019) and sgRNA/donor (pL0116, pLf0122, pLf0127) constructs used to introduce the GFP expression cassette into the *P. falciparum* NF135 p47 gene locus. (Continued)
FIGURE 4 | The GFP gene is under the control of the promoter of the ef1α, sui1, or 40s genes. The p47 homology regions (HR1, HR2) used to introduce the donor DNA (i.e., the GFP expression cassette), location of primers (p), sizes of restriction fragments (H: HpaI; s, in red) and PCR amplicons (in black) are indicated. Primer sequences (shown in black and bold) are shown in Table S1. WT, wild type; bsd, blasticidin selectable marker (SM); hdhfr: yfcu—SM in donor plasmid. (B) Diagnostic PCR confirms the correct 5′ integration into the genome of GFP@ef1α135, GFP@sui1135, or GFP@40s135 (5-Int; primers p7/p8 for ef1α 1,009 bp, p7/p9 for sui1 1,106 bp, p7/p10 for 40s 1,087 bp) and correct 3′ integration (3-Int; primers p11/p12; 2,188 bp). In addition, it shows absence of the p47 gene in GFP@ef1α135 clone 2 and in the FACS sorted line of GFP@40s135 (p47 primers p13/p14; 216 bp). Primer locations and product sizes are shown in (A) and primer sequences in Table S1. The arrow indicates PCR product of WT p47 gene amplified by p13/p14 primers. The weak 1.5 kb band with the 5-Int@40s-primer is a non-specific fragment which is only present in WT with and not in the transgenic lines with the GFP cassette. (C) Southern analysis of HpaI restricted DNA to confirm correct integration of the plasmids in the three transgenic lines. Digested DNA was hybridized with a probe targeting the homology region 1 of p47 [HR1; shown in red; see (A)] and with a probe recognizing ampicillin (Amp; fragment of ∼10 kb). Left panel: WT NF135 shows the expected 5.8 kb fragment. In both uncropped and clone 2 of GFP@ef1α135 parasites the expected 3.6 kb fragment is present after double crossover (DoxO) integration. In uncloned and FACS-sorted populations of GFP@40s135 parasites two fragments with the expected sizes of 3.8 kb and ∼10 kb of single crossover integration are present whereas in the uncloned GFP@sui1 the expected fragment of 4.3 kb of double crossover integration and the fragment of 5.8 kb of WT is present (see arrows). Hybridization with the Amp probe shows the single crossover events in the GFP@40s populations. Right panel: After additional FACS sorting of GFP-positive parasites Southern analysis shows the expected 5.8 kb fragment in GFP@sui1 of double crossover integration whereas in GFP@40s the two fragments are present with the expected sizes of 3.8 kb and ∼10 kb of single crossover integration. The presence of SXO parasites in GFP@40s135 is confirmed by hybridization with a probe recognizing ampicillin (Amp; fragment of ∼10 kb).

blood stages, resulting in parasite populations in which more than 90% of blood stages were mCherry-positive (determined by fluorescence microscopy; Figure 2). Diagnostic PCR analyses on genomic DNA of the three mCherry-Luc lines indicate integration of the transgene-expression cassettes into the p47 locus (Figure 1B). Southern analysis confirmed double crossover integration in parasites of these three lines and absence of episomal donor plasmids (Figure 1C). The GFP@ef1α and GFP@40s parasite populations were not cloned or enriched for transgenic parasites by FACS-sorting. In these populations more than 90% of mixed blood stages were GFP-positive (determined by fluorescence microscopy; Figure 2). Diagnostic PCR on genomic DNA of these two GFP lines indicates integration of the transgene expression cassettes into the p47 locus (Figure S2). In addition, PCR amplification of the WT p47 locus indicates the presence of WT parasites as was expected based on the presence of GFP-negative parasites in mixed blood stage populations of these two lines.

We examined mCherry and GFP expression in blood stages and mosquito stages of the various transgenic lines. The different asexual blood stages (trophozoites, schizonts) and gametocytes (stage III) of all five lines expressed mCherry or GFP (Figure 2). In addition, we confirmed expression of luciferase in blood stages of two lines with the mCherry-luciferase fusion protein (Figure S3). We next passaged two of the lines, mCh-Luc@sui1 and GFP@40s through A. stephensi mosquitoes. Oocysts and salivary gland sporozoites of both lines were mCherry- and GFP-positive, respectively (Figure 3), showing that both the sui1 and 40s promoter drive expression in mosquito stages. Combined these analyses show that the two selected promoter regions of the housekeeping genes, sui1 and 40s, are able to drive expression of reporter genes and both promoters drive expression in all blood stages and mosquito stages examined. Based on fluorescence intensity of these life cycle stages, the level of reporter expression with these two novel promoters appears to be similar or higher compared to reporter expression with the ef1α promoter (see also the next section describing transgenic Pf NF135 parasites expressing GFP using the three promoters).

TABLE 1 | Gametocyte, oocyst and sporozoite production of three transgenic PYNF135 reporter lines.

| Lines         | Stage V Gametocytemiaa | No. of oocystsb | No. of sporozoites (×10b) c |
|---------------|------------------------|-----------------|------------------------------|
|               | Average (SD)           | Average (SD)    | Average (SD)                |
| NF135 WT      | 0.9                    | 5.4             | 8.0                          |
| (1 exp.)      | (1 exp.)               | (1 exp.)        |                             |
| GFP@ef1α135  | 0.7 (0.3)              | 15 (7.4)        | 16 (4.0)                     |
| (3 exp.)      | (2 exp.)               | (3 exp.)        |                             |
| GFP@sui1135  | 0.5 (0.3)              | 13 (0.2)        | 11 (2.4)                     |
| (3 exp.)      | (2 exp.)               | (2 exp.)        |                             |
| GFP@40s135   | 0.6 (0.4)              | 9.2 (1.3)       | 11 (3.3)                     |
| (4 exp.)      | (3 exp.)               | (2 exp.)        |                             |
| NF54 WT      | 0.8–1.2 (0.2)          | 21–67 (17)      | 8–45 (19)                    |
| (4 exp.)      | (8 exp.)               | (4 exp.)        |                             |

aPercentage of stage V gametocytes (per 100 red blood cells) in day 14 cultures.
bMean number of oocyst per mosquito at day 7–12 after feeding (10–30 mosquitoes per exp.).
cMean number of salivary gland sporozoites per mosquito at day 21 after feeding (20–30 mosquitoes per exp.).

Generation and Genotyping of P. falciparum Transgenic NF135 Lines Expressing GFP Under Control of the sui1 and 40s Promoters

PYNF135 parasites (PYNF135) have a higher invasion rate of hepatocyte in vitro and produce more liver merozoites per infected hepatocyte as compared to PYNF54 parasites (McCall et al., 2017). Transgenic PYNF135 parasites with high reporter expression in liver stages may therefore be valuable tools for liver stage analyses. Since both the sui1 and 40s genes are highly transcribed in liver stages of the rodent parasite P. berghei and the human/primate Plasmodium species P. vivax and P. cynomolgi (Table S2), we generated two transgenic PYNF135 lines expressing GFP under control of either the sui1 or the 40s promoter. As a control line, we also
generated a PfNF135 line expressing GFP under control of the eflα promoter.

We first compared the published genome sequences of the promoter regions of eflα, sui1, and 40s in the PfNF54 genome (www.GeneDB.org) and the PfNF135 genome (Moser et al., 2019). The promoter regions selected from the PfNF54 (see above) were almost identical (99–100% similarity) to those of PfNF135. Also, the sequence of the p47 target regions were more than 99% similar and the p47 guide RNA sequence was identical in both lines (data not shown). Based on the sequence similarities, we decided to use the same sequences/basic plasmids for generation of the donor DNA constructs to create the PfNF135 reporter lines as were used for the PfNF54 reporter lines (see above). We used the final DNA donor plasmids plfl0116 and plfl0127 for introduction into the genome of the eflα-GFP and 40s-GFP expression cassettes (as described in the previous section). For introduction of the sui1-GFP expression cassette, we generated the final donor DNA plasmid plfl0122 (Figure 4A; see the Materials and Methods section and Figure S1 for more details of the generation of the plasmid). Transfection with the final donor DNA constructs and the Cas9 construct plLfo019, followed by positive drug selection (pyrimethamine and BSD) and negative selection (5-FC) was performed as described in the Materials and Methods section and resulted in selection of the three parasite populations GFP@eflαNF135, GFP@40sNF135, and GFP@sui1NF135 expressing GFP as determined by fluorescence microscopy analysis of mixed blood stages. For the GFP@40sNF135 and GFP@sui1NF135 populations we performed two rounds of FACS-sorting using mixed blood stages to enrich for transgenic GFP-positive parasites, resulting in parasite populations where more than 99% of the blood stages were GFP-positive. From the GFP@eflαNF135 population we obtained a GFP-expressing clone (clone 2) by the method of limiting dilution. For all three lines we performed a PCR analysis that distinguishes between PfNF54 and PfNF135 parasites. This analysis confirmed the PfNF135 background of the three GFP-expressing lines (Figure S4).

Diagnostic PCR on genomic DNA of the three GFP lines indicates correct integration of the transgene-expression cassettes into the p47 locus (Figure 4B). We could not detect a PCR fragment of the p47 open reading frame in the FACS-sorted GFP@40sNF135 and GFP@eflαNF135 clone2,
FIGURE 6 | GFP and Hoechst33258 fluorescence intensities of ring forms and schizonts in transgenic PfNF135 reporter lines based on FACS analysis. Fluorescence intensity of rings (left panel) and mixed blood stages (right panel) as determined by flow cytometry. Infected red blood cells (RBC) were stained with the DNA-specific (Continued)
indicating homogenous and pure populations of transgenic parasites with the GFP-expression cassettes integrated into the p47 locus. Southern analysis confirmed that the GFP@ef1α NF135 clone2 consists of a pure population of transgenic parasites containing the GFP-expression cassette integrated by double crossover recombination into the p47 locus (Figure 4C). Southern analysis indicates that both FACS-sorted GFP@40sNF135 and GFP@sui1NF135 populations consist of transgenic parasites with the GFP-expression cassette integrated either by double crossover (GFP@sui1NF135) or single crossover recombination (GFP@40sNF135) (Figure 4C). This Southern analysis cannot distinguish between episomal plasmid and single crossover integration. However, in these parasites the 12 kb fragment is most likely integrated plasmid since episomal plasmid is usually lost during in cultures without drug-selection for prolonged periods.

Growth Characteristics of Blood and Mosquito Stages of Transgenic PfNF135 Lines Expressing GFP by the sui1 and 40s Promoters

We first examined growth characteristics of blood stages and mosquito stages of the three transgenic GFP-expressing PfNF135 lines. Though it has been shown that disruption of the p47 gene locus in PfNF54 parasites does not compromise parasite development in blood stages and liver stages as well as in A. stephensi mosquitoes (Talman et al., 2010; Vaughan et al., 2012; Marin-Mogollon et al., 2019), the effect of p47 disruption in PfNF135 parasite is unknown. For use of these reporter lines in future studies it is important that they retain growth and development kinetics of the parental PfNF135 line.

*In vitro* growth of asexual blood stages of the three lines was comparable to the growth of asexual blood-stages of the parent WT PfNF135 line (Figure S5) and they produced WT-comparable numbers of mature stage V gametocytes in standardized gametocyte cultures (Table 1). We next passaged the three lines through A. stephensi mosquitoes and analyzed production of oocysts and salivary gland sporozoites. All three lines produced oocysts and sporozoites and oocyst and sporozoite numbers were in the same range as WT PfNF135 parasites (Table 1). Since we have passaged WT PfNF135 only once through mosquitoes, we compared oocysts and sporozoite production also with those of PfNF54 parasites, which have been frequently transmitted through mosquitoes in our laboratory. Oocyst and sporozoite production of the three transgenic lines (mean of 5–15 oocyst per mosquito) is lower than those of PfNF54 parasites (mean of 21–67) which is in agreement to published observations on mosquito transmission of PfNF135 (Teirlinck et al., 2013). Combined, these results indicate that the three transgenic lines have growth characteristics of blood stages and mosquito stages that are comparable to the parent PfNF135 line and all three lines produce salivary gland sporozoites, which will permit further analyses of sporozoite infectivity and liver stage development (see below). These results also demonstrate that the p47 gene can be used as a target locus for introduction of transgenes in PfNF135 parasites.

Analysis of GFP-Expression Throughout the Complete Life Cycle of Transgenic PfNF135 Lines Expressing GFP by the sui1 and 40s Promoters

We examined GFP expression in the different reporter lines during blood stage development by fluorescence microscopy. Expression of GFP was detectable throughout blood stage development, from ring forms to mature schizonts of GFP@40sNF135, GFP@sui1NF135 and the control line GFP@ef1αNF135 (Figures 5A–C). Gametocytes were also GFP-positive with gametocytes of the GFP@40sNF135 line showing brightest fluorescence (Figures 5D–F). The GFP-expression in all blood stages, including gametocytes, is in agreement with data on transcription of the three genes from which the promoters were used (Table S2). Next, we more precisely compared the GFP-fluorescence intensity of the different lines by examining mixed blood stages by flow cytometry as described previously for GFP-expressing PfNF54 reporter lines (Mogollon et al., 2016). In this analysis the different blood stages are distinguished based on their fluorescence intensity (DNA content) after staining with the DNA-specific dye Hoechst-33258. In all three lines GFP-fluorescence intensity increased during the period of development of rings into mature schizonts (Figure 6). Haploid ring forms (1N) of both GFP@40sNF135 and GFP@sui1NF135 showed 3–5 times higher fluorescence intensity than GFP@ef1α NF135 ring forms with highest intensity of GFP@sui1NF135 rings. GFP@40sNF135 schizonts (mean of 11 nuclei) showed highest fluorescence intensity with 1.6–2.8 times the intensity of GFP@ef1α NF135 and GFP@sui1NF135 schizonts, respectively.

We next examined GFP expression in oocysts and sporozoites, collected from A. stephensi mosquitoes, which were fed with gametocytes of the three transgenic lines using the standard membrane feeding assay. Like oocysts and sporozoites of PfNF54 lines that expressed reporters by the sui1 and 40s promoter, oocyst and sporozoites of both GFP@sui1NF135 and GFP@40sNF135 expressed GFP and these life cycle stages were strongly GFP-positive (Figure 7).

Finally, we examined reporter expression during liver stage development of the three PfNF135 reporter lines in primary human hepatocytes. Primary human hepatocytes (PHH) were infected with $5 \times 10^4$ sporozoites of the GFP@40sNF135,
GFP@sui\textsubscript{1NF135}, and GFP@ef\textsubscript{1\alpha NF135} per well of a 96-wells plate. Live imaging for GFP expression was performed using confocal fluorescence microscopy on day 3, 5, 7, and 10 (Figure 8A).

Both GFP@40s\textsubscript{NF135} and GFP@sui\textsubscript{1NF135} had increased GFP expression on day 3 compared to GFP@ef\textsubscript{1\alpha NF135}. While the GFP expression levels for GFP@sui\textsubscript{1NF135} decreased to similar
FIGURE 8 | GFP expression and development in three NF135 reporter lines in the liver stage. (A) Representative GFP-fluorescence microscopy images of liver stage parasites of GFP@ef1αNF135, GFP@sui1NF135, and GFP@40sNF135 at different days after adding sporozoites to primary human hepatocytes. Scale bar, 25 µm. (B–E) (Continued)
levels compared to GFP@ef1αNF135 as liver stage development progressed, it remained strong for GFP@40sNF135.

To check for proper development of the reporter lines in PHH, protein expression of standard liver stage markers such as Exp1 (PF3D7_1121600), Exp2 (PF3D7_1477100), GAPDH (PF3D7_1462800), and the late stage marker MSP1 (PF3D7_0930300) were examined on day 7 post invasion and compared with expression in livers stages of wild type P. falciparum (Figures 8B–E). Additionally, to examine the infection rate of and maintenance of developing liver stages of the reporter lines in PHH compared to the PNF54 WT, the number of infected host cells were calculated using high content fluorescence microscopy after staining with anti-PfHSP70 (PF3D7_0818900) on Day 3, 5, 7, and 10 (Figure 8F). Unexpectedly, all three PNF135 reporter lines infected significantly fewer PHH on all 4 time points examined (Figure 8F). Furthermore, intracellular GFP@40sNF135 and GFP@sul1αNF135 parasites were significantly smaller than the PNF135 parental line and were more comparable in size to PNF54 when examined on day 5 and day7 post invasion (Figure 8G and Figure 5E). The reason for the lower infectivity of sporozoites of the PNF135 reporter lines is unclear. It is possible that high expression of the reporter protein GFP slightly affects both sporozoite infectivity and development of liver stages. However, in previous experiments with PNF135 parasites a relatively large variation has been observed in size of liver stages, measured at day 6 where the size of PNF54 livers stage schizonts (6.3–16.1 μm) overlap with that of PNF135 schizonts (12.5–28.6) (McCall et al., 2017). A similar variation and overlap was found in sporozoite infectivity between the two strains as determined by measuring infected hepatocytes at day 2 and 5 after adding sporozoites to freshly isolated human hepatocytes in 3 independent experiments (McCay et al., 2017). Therefore, it would be important to confirm in future experiments with these reporter lines whether the lower infectivity is a characteristic of the reporter lines or is due to inter-experimental variation.

CONCLUDING REMARKS

Combined our analyses identified novel promoter regions of two housekeeping genes, sul1 and 40s, that can drive strong expression of reporter proteins throughout the complete Pf life cycle. These promoter sequences are conserved in sequence between PNF54 and PNF135.G10 parasites and, as expected, we found that the promoters selected and derived from the PNF54 parasite genome drive expression of reporters in PNF135 parasites with a similar pattern as in PNF54 parasites. We report for the first time the generation of transgenic parasites in Cambodian PNF135 parasites and show that CRISPR/Cas9 constructs can be used that are based on constructs containing only PNF54 genome sequences. In addition, our analyses demonstrate that, like in PfNF54 parasites, the p47 gene locus can be used as a silent target locus for introducing transgenes in PNF135 parasites without reducing oocyst and sporozoite production in A. stephensi mosquitoes. We found that specifically the 40s promoter drives high expression in all life cycle stages with higher expression in gametocytes and liver stages compared to the sul1 promoter. In view of the high-level reporter expression in liver stages, the GFP@40s reporter lines may be beneficial for further analysis of liver stage development, both for analyses in vitro in primary hepatocytes and in vivo in humanized mice, for example in screening assays for novel drugs/small molecule inhibitors. In addition, it may also enhance collection of infected hepatocytes by flow cytometry/FACS sorting, as has been performed in rodent malaria parasites, which will permit genome wide analyses of P. falciparum liver stage such as proteomics, transcriptomic and lipidomics. Such larger scale analyses are still lacking for P. falciparum liver stages but are essential for a better understanding in this part of the Plasmodium life cycle (Vaughan and Kappe, 2017).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

Primary human liver cells were freshly isolated from remnant surgical material. The samples are anonymized and general approval for use of remnant surgical material was granted in accordance to the Dutch ethical legislation as described in the Medical Research (Human Subjects) Act, and confirmed by the Committee on Research involving Human Subjects, in the region of Arnhem-Nijmegen, the Netherlands.

AUTHOR’S NOTE

This paper is dedicated to the memory of our friend and colleague, SMK, who recently passed away.

AUTHOR CONTRIBUTIONS

SM, CJ, and SMK came up with the study concept and design. SM, AM, FG, CM-M, YM, TI, SKK, JR, SC-M, AS, and YW acquired the data. SM, AM, BF-F, CJ, and SMK conducted analysis and interpretation of the data. SM, AM, and CJ wrote the draft.
of the manuscript. SM, AY, BF-F, and CJ critically revised the manuscript for important intellectual content. G-JG, BF-F, AH, and RS provided technical and/or material support. CJ and SMK supervised the study. All authors reviewed the manuscript.

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**SUPPLEMENTARY MATERIAL**

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