Disruption of Plasmodium Falciparum Histidine-Rich Protein II can affect Heme Metabolism in the Blood Stage

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Abstract

Background. Heme is a key metabolic factor in the life of malaria parasite. In the blood stage it acquires host hemoglobin to generate amino acids for its own protein synthesis and by-product heme for metabolic use. The malaria parasite also can *de novo* synthesize heme by itself. *Plasmodium falciparum*-specific histidine-rich protein 2 (*Pf*HRP2) is a histidine-rich protein and has a heme-binding site to mediate hemozoin formation, a bio-crystallized form of heme-aggregates. It is interesting to investigate the vibration of hemoglobin-derived heme metabolism and *de novo* heme-biosynthetic pathway in the *Pfhrp2* disrupted parasites during the intraerythrocytic stages.

Methods. A CRISPR-Cas9 system was used to disrupt the gene locus of *Pfhrp2*. DNA was extracted from the transgenic parasites and polymerase chain reaction (PCR), western blotting and southern blotting were used to manifest the successful establishment of transgenic parasites. RNA-seq and comparative transcriptome analysis were performed to identify the difference in gene expression between 3D7 and *Pfhrp2*$^-$ 3D7 parasites.

Results. *Pfhrp2*$^-$ transgenic parasites were successfully established by the CRISPR/Cas9 system. The disruption of the exon 2 of *Pfhrp2* can down-regulate the gene expression of *Pfhrp3* which involved in hemoglobin-derived heme metabolism. It also up-regulates the gene expression level of enzymes of heme biosynthesis.

Conclusion: These data support that although *Pfhrp2* is a dispensable gene for intraerythrocytic stages parasite but heme metabolism's stabilizing is very important. The disruption of *Pfhrp2* can both affect the pathway of heme metabolism and biosynthesis. A co-operation mechanism may exist between the heme biosynthesis and metabolism pathways for parasite growth in blood stage.

Introduction

Malaria remains one of the most significant health challenges for human beings. *Plasmodium falciparum*, as one of the most deadly human malaria diseases, is the most burdensome form and causes about 228 million cases of malaria and result in 405 000 deaths in 2018 worldwide [1].

Heme is a crucial metabolic factor and it derives primarily from the parasite's heme biosynthesis pathway [2] at the early ring stage and from hemoglobin digestion at the latter stages [3]. Malaria parasite ingests more than 75% of its host cell hemoglobin in a short period for its nutritional requirements in the blood stage [3, 4]. It was digested in the food vacuole to generate amino acids, releasing the toxic heme moiety[5]. Since heme is toxic, it stores the excess heme as hemozoin pigment, a bio-crystallized form of heme-aggregates[6].

Plasmodium falciparum-specific histidine-rich protein 2 (*Pf*HRP2, PlasmoDB: PF3D7_0831800, www.plasmodb.org) constitutes two exons and it is a water-soluble protein and was released from infected erythrocytes and circulated in the malaria-infected patient [7, 8]. HRPII and HRP III as a

homologous protein could bind heme in the digestive vacuole and play a role in hemozoin formation [9–11]. Antimalarial drugs chloroquine, it binds to toxic heme metabolites and thereby prevents their conversion and deposition to the inert hemozoin [12]. However, the malaria parasite also has a heme-biosynthetic pathway. Studies with *Plasmodium berghei*-infected mice and *Plasmodium falciparum* in cultures using knockout (KO) parasites generated for δ-aminolevulinate synthease (ALAS) and ferrochelatase (FC) have indicated that the heme biosynthetic pathway is nonessential for parasite survival in the blood stages[13, 14].

The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) has been successfully using in genome-editing for human malaria parasite *Pf* and *Pv*[15–17]. Briefly, a single guide RNA (sgRNA) guides the Cas9 endonuclease to cause double-strand breaks (DSBs), and DSBs can be repaired by homologous recombination using donor DNA. Transgenic parasites can be obtained after 3–6 weeks [15, 16]. It has exhibited as highly precise and efficient in genome editing.

The present study aims to specific disruption of the gene locus of *Pfhrp2* from wild type 3D7 parasites and investigates how explicit gene disruption affects the hemoglobin-derived heme metabolism and heme-biosynthetic pathway.

Methods

Parasite culture, Synchronization, and Pellets collection. *P. falciparum* asexual stages (3D7 strain) were cultured in vitro in human erythrocytes (blood group O+) obtained from the Beijing Red Cross Blood Center. It was grown under 5% O_2, and 5% CO_2 in RPMI-1640 media supplemented with 5 g/L Albumax II (Life Technologies), 2 g/L sodium bicarbonate, 25 mM HEPES pH7.4 (pH adjusted with potassium hydroxide), 1 mM hypoxanthine and 50 mg/L gentamicin as previously described[18].

For synchronization, parasites were cultured to at least 10% parasitemia in T-75 flasks containing 50 ml medium at 1% hematocrit. Then it was moved from a flask to a 50 mL tube, centrifuged for 5 min at 500x g and removed supernatant. 15 mL 5% D-Sorbitol solution was added to the pellet and incubated at 37℃ for 10 min, centrifuged, and removed supernatant. The culture was synchronized with three rounds of sorbitol treatments. Then after the invasion at 8 h, 16 h, 24 h, 32 h, 40 h, 46/0 h collected and centrifuged culture solution, pellets were stored at -80℃ until to use.

Plasmid constructs and plasmodium **transfection.** Based on pUF1-Cas9 and pL6cs plasmids, kindly provided by Jose-Juan Lopez-Rubio, pUF1-BSD-Cas9 and pL6CS-hDHFR-*hrp2* were constructed to disruption of the *Pfhrp2* locus. The two plasmids and plasmodium transfection described as previous[19].

Briefly, pUF1-BSD-Cas9 expresses Cas9 nuclease and blasticidin S deaminase (BSD). The pL6CS-hDHFR-*hrp2* plasmid, which offers donor DNAs and sgRNAs, targeting the *Pf*hrp2 gene (guide^{hrp2}). The left and right homologous arms were amplified separately by PCR from the genomic DNA of *P. falciparum* 3D7 (primers P1/P2 for the left arm and P3/P4 for right arm). The construct of transitional- pL6CS-hDHFR-

hrp2 was transformed into competent cells, and plasmids were extracted and checked with restriction enzyme and sequencing. After the correct transitional- pL6CS-hDHFR-*hrp2* was obtained, this transitional plasmid was linearized with *AvrII & XhoI*. The sgRNAs of *hrp2* were annealed and inserted into linearized transitional- pL6CS-hDHFR-*hrp2* plasmids using the in-fusion kit. The construct was transformed into competent cells again and extracted. The final plasmid was confirmed by restriction enzyme digestion and DNA sequencing. The confirmed plasmid was isolated and used for electroporation to generate *P. falciparum* transgenic strains.

At the electroporation step, the two plasmids were carried out by the spontaneous uptake method using ~ 50 µg of maxi-prepped plasmid DNA, and 8 square wave electroporation pulses of 356 V for 1 ms each, separated by 0.1 seconds. Drugs (final concentration is 2.5 mg/L blasticidin S and 250 mg/L G418) were added into complete medium post-transfection to kill those parasites without episomal pUF1-BSD-Cas9 and pL6CS-hDHFR-*hrp2*. All primers and sgRNA sequences used for constructing plasmids can be seen in Additional file 1.

Confirmation of transgene success via PCR. Twenty days after electroporation, live *P. falciparum* appeared, and genomic DNA was extracted from harvest parasite pellets using the Qiagen DNA extraction kit (QIAGEN, Valencia, California USA). A PCR was performed in 20 µl total volume consisting of 10 × buffer with 15 nM MgCl2, 200 µM dNTPs, 15 µM forward and reverse primers (P1/P2, as indicated in Fig. 2), 0.69 units of *Taq* DNA Polymerase, and 2 µl of DNA template. An in vitro cultured *P. falciparum* parasite 3D7 was used as a positive control for *Pfhrp2* gene amplification experiments. All PCR products were separated and visualized on agarose gels, and products with the expected size were sent for sequencing to confirm.

Western blotting analysis. Successfully transgenic parasites were cultured in flasks. For the analysis of Pfhrp2 expression, when parasitemia passed 5%, iRBCs were collected and incubated with 0.15% saponin lysis solution on ice for 7 min. After centrifugation at 500 × g for 5 min at room temperature (RT), the supernatants was collected and added an appropriate amount of SDS-PAGE sample buffer, and denatured at 95℃ for 8 min, and resolved by electrophoresis in a 12.5% polyacrylamide gel (Life Technologies) and transferred onto a 0.2-um polyvinylidene difluoride (PVDF) membrane (Hybond LFP; GE Healthcare). HRP2 was specifically detected by using the Anti-Plasmodium falciparum monoclonal antibody [MPFG-55P] (HRP) (Abcam, Massachusetts, USA).

Southern Blot Analysis. The genomic DNA from transgenic parasites was isolated, as described above. For parasites, 5 µg of genomic DNA was digested overnight using the *PstI* or *SacI* restriction endonucleases (TaKaRa Bio companies). The DNAs were separated on a 1.0% agarose gel and transferred to Hybond™ $-N^+$ membrane (GE Healthcare Amersham™) using the high salt capillary transfer method. Probes were PCR-amplified, cleaned, and labeled with DIG-dUTP using a PCR DIG Probe Synthesis Kit (Roche). The blots were hybridized with the labeled probes, washed, and exposed to film and detected by in a cassette.

Total RNA extraction. Total RNA was extracted using TRIzol® according to the manufacturer's protocol. Briefly, different time collected pellets from transgenic parasites were ground into powder by liquid nitrogen and transferred into a new tube with Trizol reagent. The mix was shaken and kept for 5 min at room temperature, then centrifuged at 10,000 × g for 5 min at 4℃. The supernatant was added chloroform/isoamyl alcohol (24:1) with lysis reagents. After centrifuged at 10,000 × g for 10 min at 4℃, the supernatant was transferred into a new tube with an equal volume of isopropanol and kept at -20℃ for 1 h. After centrifuged at 13600 × g for 20 min at 4℃, the supernatant was precipitated by ethanol and dry for 3 min. The RNA pellet was dissolved with RNase-free water.

mRNA Library Construction. Oligo(dT)-attached magnetic beads were used to purified mRNA from parasite pellets. Purified mRNA was fragmented into small pieces with buffer at the appropriate temperature. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis. Afterward, A-Tailing Mic and RNA Index Adapters were added by incubating to end repair. The cDNA fragments were amplified by PCR, and purified by Ampure XP Beads, then dissolved in EB solution. The double-stranded PCR products were heated to denature and circularized by the splint oligo sequence to get the final library. The single-strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoball (DNB), which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray, and pair-end 100 bases reads were generated on BGISEQ500 platform (BGI-Shenzhen, China).

Sequencing Data Analysis. The sequencing data was filtered with SOAPnuke (v1.5.2)[20] by (1) removing reads containing sequencing adapter; (2) removing reads whose low-quality base ratio (base quality ≤ 5) is more than 20%; (3) removing reads whose unknown base ('N' base) ratio is more than 5%, the clean reads were stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4)[21]. Bowtie2 (v2.2.5)[22] was applied to align the clean reads to the reference coding gene set, then expression level of the gene was calculated by RSEM (v1.2.12)[23] and drawn by pheatmap (v1.0.8). Primally, differential expression analysis was performed using the DESeq2(v1.4.5)[24] with Q value ≤ 0.05.

Results

Successful construct plasmids for gene knockdown

The pUF1-BSD-Cas9 plasmid was constructed from the pUF1-Cas9 plasmid. It offered Cas9 endonuclease and blasticidin S deaminase (BSD) by changing the drug selection marker from yDHODH into BSD. The plasmid which provide donor DNAs and sgRNA were constructed from the pL6cs plasmid by two steps. First, gene-specific DNA cassettes expressing sgRNAs were chosen from gene sequences and inserted into pL6cs vector. Secondly, two ~ 550 bp genomic DNA sequences from the upstream and downstream of *the Pfhrp2* gene were chosen as the left and the right homologous arms. The construct was screened with enzyme digestion and DNA sequencing to ensure its correct. After that, *P. falciparum* 3D7 strain was subsequently transfected with 50 μg pL6cs-hDHFR-hrp2 (donor DNA) and 50 μg pUF1-BSD-Cas9 plasmids via electroporation. To select the successfully transfected parasite, BSD and WR99210 were added to the culture medium one day after electroporation.

Figure 1 Schematic illustration of the *hrp2* gene deletion principle using CRISPR/Cas9. The *hrp2* gene was replaced by hDHFR sequences through homologous recombination that happened at the left and right arms. Primers for PCR to check the hDHFR are labeled as P1 and P2.

Successful checked by PCR and DNA sequencing

Around 20 days after electroporation, live parasites could be seen in the culture under selection with the above described two drugs. A portion of the live parasite population was collected for genomic DNA isolation, and a PCR was performed to validate the modification of the *Pfhrp2* gene. In these PCRs, two primers were designed at the genomic DNA sequences beyond the left and right homologous arms (P1/P2, Fig. 2), to prevent contamination from the episomal plasmid template. PCR products were analyzed by agarose electrophoresis and sequenced to confirm. Therefore, CRISPR/Cas9 system was successfully used to knock out the *Pfhrp2* gene.

Figure 2 Genomic DNA PCR to confirm the gene disruption. (a) Genomic DNA PCR to confirm the success modification of the *Pfhrp2* gene by sgRNA. (b) Genomic DNA PCR to confirm the *hrp2*$^{-}$ monoclonal parasites. 3D7 was the wild type of *Plasmodium falciparum*. L and L2 were the transgenic parasites which disruption of exon 1 plus exon 2 and only exon 2 of *Pfhrp2*, respectively. The PCR product sizes of whole-fragment (w), semi-fragment (s) were 2903 bp, 725 bp, respectively.

Successful checked by western blot

Transgenic parasites that had been checked by PCR and DNA sequencing were further confirmed by Western blot. Theoretical molecular weights of the proteins were 32.41KD (Fig. 3a). Western blot result showed that the molecular weight of HRP2 was not the same as its theoretical molecular weight. The band corresponding to HRP2 appeared to be slightly larger than its theoretical molecular weight, which may be caused by post-translational modification.

Successful checked by Southern blot

Transgenic parasites that had been checked by PCR, DNA sequencing, and western blot were further confirmed by Southern blot. Two experiments were performed to verify that the gene of *Pfhrp2* was replaced by drug resistance gene hDHFR. The one experiment is to prove that wild strain 3D7 parasite still owns the gene of *Pfhrp2*, while transgenic parasite does not (Fig. 3b). The other experiment is to prove that the gene of *Pfhrp2* was replaced by the gene of hDHFR (Fig. 3c). The theoretical molecular weights of the proteins were 39KD. Southern blot results showed that only the gene of hDHFR was detected in the transgenic parasite. Therefore, the gene of *Pfhrp2* was successfully knocked out from *P. falciparum* 3D7 strain using CRISPR/Cas9 system.

Figure 3 (a) Western blot to confirm the expression of PfHRP2 protein. The supernatants of parasite culture medium were separated on SDS-PAGE, and mouse monoclonal [MFPG-55P] to Plasmodium falciparum (HRP) was 1st antibody used for the Western Blot to confirm the HRP2 protein express. HRP-goat anti-mouse was the second antibodies. (b) Southern blot to confirm the gene disruption of *Pfhrp2*. The genomic DNA was digested overnight using the *SacI* restriction endonucleases. The DNAs were

separated on agarose gel and transferred to membrane. The blots were hybridized with the labeled Pfhrp2 probe and exposure 10 min. (c) Southern blot to confirm the drug resistance gene of hDHFR. The genomic DNA was digested overnight using the *PstI* restriction endonucleases. The DNAs were separated on agarose gel and transferred to membrane. The blots were hybridized with the labeled hDHFR probe and exposure 40 min. L2 was the transgenic parasites which only disruption of exon 2 of *Pfhrp2*.

An overview of the RNA-Seq data

To test the possible role for *Pfhrp2* in transcriptional regulation, we compared the global transcript levels in transgenic parasites to wild type 3D7 at six stages during the intraerythrocytic developmental cycle (IDC). Three biological replicates were used for each stage and *P. falciparum* species. After RNA sequencing, the quality of the data was assessed. An overview of the sequencing and assembly is shown in Supplementary Material Table 1.

After filtering the low-quality reads, clean reads were obtained. Total clean reads from the RNA-Seq data were mapped uniquely to the reference genome in all the samples. More than 97% of the total clean reads had Phred-like quality scores at the Q20 level. Via comparative transcriptome analysis, this RNA-Seq data provided a solid foundation for identifying the genes participating in heme metabolism (Table 1).

Analyses of differentially expressed genes

The gene of enzymes participated in hemoglobin-derived heme metabolism, and *the de novo* heme-biosynthesis pathway was selected and analyzed. Most of their gene expression was affected by the deficiency of the gene of *Pfhrp2*. Transcripts of these enzymes fluctuated at different time points. It is notable that the first enzyme,δ-aminolevulinate synthease (ALAS, PlasmoDB:PF3D7_1246100), and the last enzyme, ferrochelatase (FC, PlasmoDB:PF3D7_1364900), in the heme-biosynthetic pathway were up-regulated in the trophozoite and schizont stages. Most of the proteins that participated in hemoglobin-derived heme metabolism were up-regulated in the ring and trophozoite stages. The gene expression of *Pfhrp3* (PlasmoDB:PF3D7_1372200) was down-regulated in all the time points (Fig. 4).

Figure 4 The transcription pattern of the genes involved in heme metabolism. Heat map representation of the relative transcription activity of 21 genes at six points during the asexual erythrocytic growth. Horizontal axis is the expression level of each gene and calculated by Log2(FPKM + 1). Its value from − 4 (lowest, blue) to + 4 (highest, red).

Discussion

The traditional method to edit *P. falciparum* genes is very inefficient and it always required several months to knock in/out target genes. It greatly limits molecular studies in malaria parasite. Recently, CRISPR/Cas9 has been used for gene editing in various organisms including Plasmodium [15, 16, 25]. The Cas9 endonuclease is guided to target DNA site by a sgRNA and induces DSBs at this site. Then the induced DSBs is repaired by homologous recombination using donor DNAs.

Our CRISPR/Cas9 system contains homologous arms (donor DNA fragments), sgRNA, and a selectable marker in one plasmid while Cas9 nuclease with selectable marker in another plasmid. Twenty days after electroporation, specific gene disruption parasites appeared. In this study, we successfully applied this CRISPR/Cas9-based genome editing system to disrupt the gene of *Pfhrp2* from P. falciparum 3D7.

The growth of the parasite in red cells seems not be affected by the deletion of *Pfhrp2* (data not shown). Furthermore, multiple genetic origins of histidine-rich protein 2 gene deletion in *P. falciparum* parasites were previously found in South America, Asia, and Africa [26–29]. These all proved that *Pfhrp2* is not an essential gene for the survival of parasite during intraerythrocytic stages.

Malaria parasite owns hemoglobin-derived heme metabolism and *de novo* heme-biosynthesis pathway. During the intraerythrocytic stages, parasite ingests host cell hemoglobin within the food vacuole to supply amino acids for its growth and release the toxic heme. The released by-product heme could bind with HRPII and HRP III to become hemozoin [12]. *Plasmodium* Heme Detoxification Protein (HDP) has also been proved the extremely potent in converting heme into hemozoin[30]. According to the RNA-Seq data, with disruption of *Pfhrp2* the transcript level of *Pfhrp3* was also completely down-regulated, the process of heme converted into hemozoin was influenced, however the transcript level of HDP was up-regulated at the schizont stage could partially take place the role of *Pfhrp2* and *Pfhrp3*. The vast majority of enzymes relate to hemoglobin-derived heme metabolism in *P. falciparum* 3D7, such as falcipain (FP), plasmepsin (PM) and falcilysin (FLN) up-regulate their gene transcript level in at least one time point to offset the impact and provide heme for the growth of parasite. So heme is definitely needed by intraerythrocytic stage parasites.

Chloroquine was discovered and derived from quinine in 1934[31]. It is effective against the malarial parasite during its intraerythrocytic stages. It could inhibit the HRP-mediated synthesis of hemozoin and breakdown the heme pathway that occurs within the acidic digestive vacuole of the parasite [12].From our research data, because parasites can acquire heme from different pathway only disruption of *Pfhrp2* cannot exterminate the heme metabolism completely. The comprehensive regulatory mechanism may exist in the heme metabolism and synthesis. We believe this network will be beneficial for understanding of heme acquisition and the drug resistance during intraerythrocytic stages.

Abbreviations

BSD
blasticidin S deaminase; sgRNA:single guide RNA; hDHFR:human dihydrofolate reducatase; DSB:double-strand break;

Declarations

Author Contributions

YY and QC designed research. YY, TT, BF, SL, and NH performed research. XM, XX, and LJ analyzed data; YY wrote the paper. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

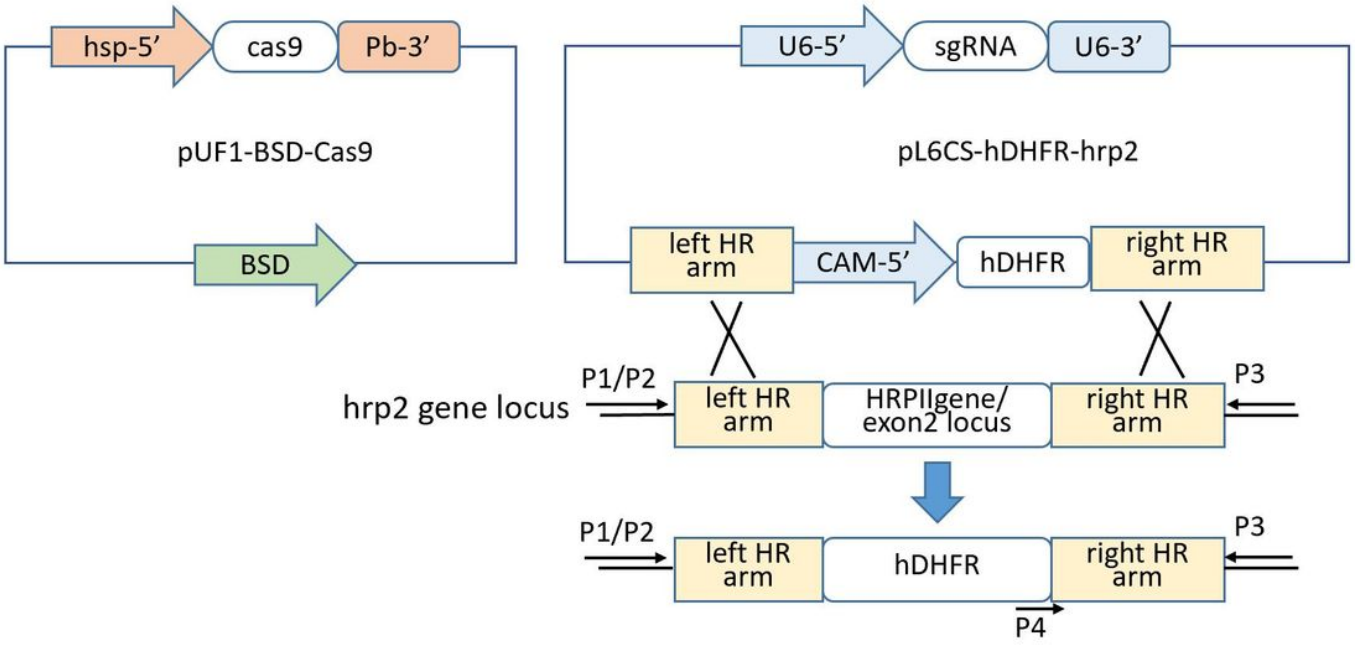

Figure 1

Schematic illustration of the hrp2 gene deletion principle using CRISPR/Cas9.

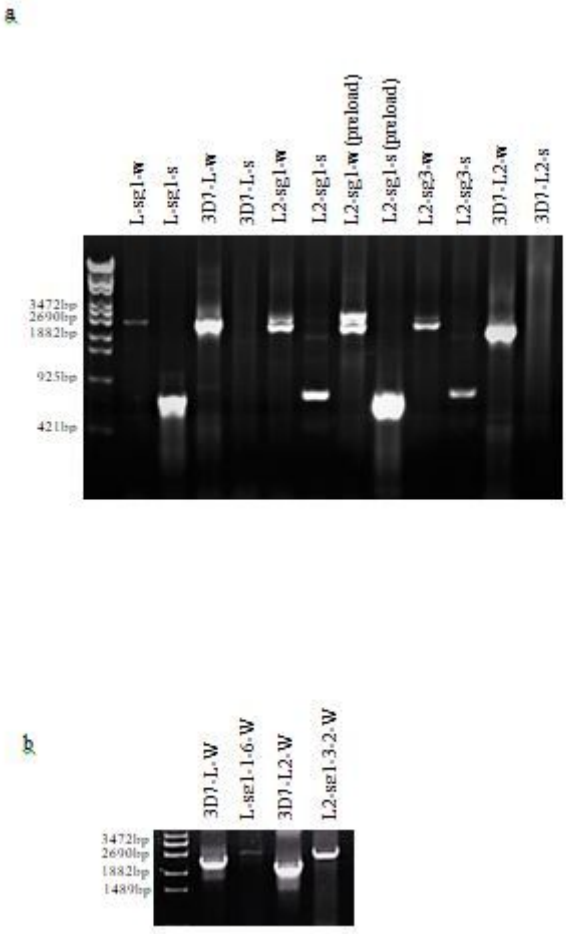

Figure 2

Genomic DNA PCR to confirm the gene disruption.

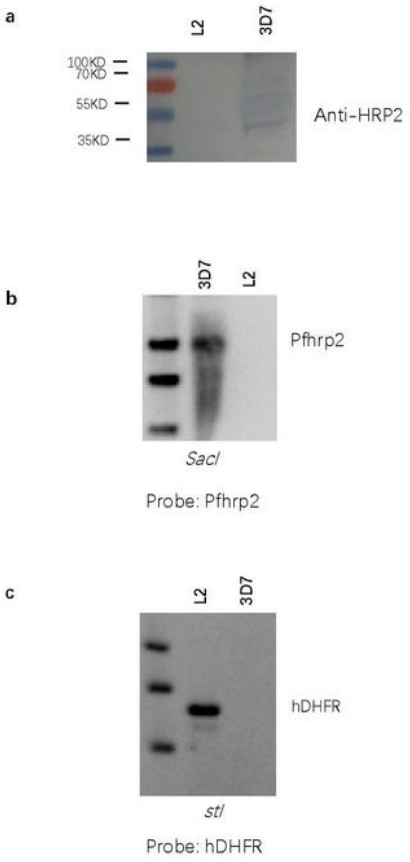

Figure 3

Western blot and Southern blot to confirm the gene disruption.

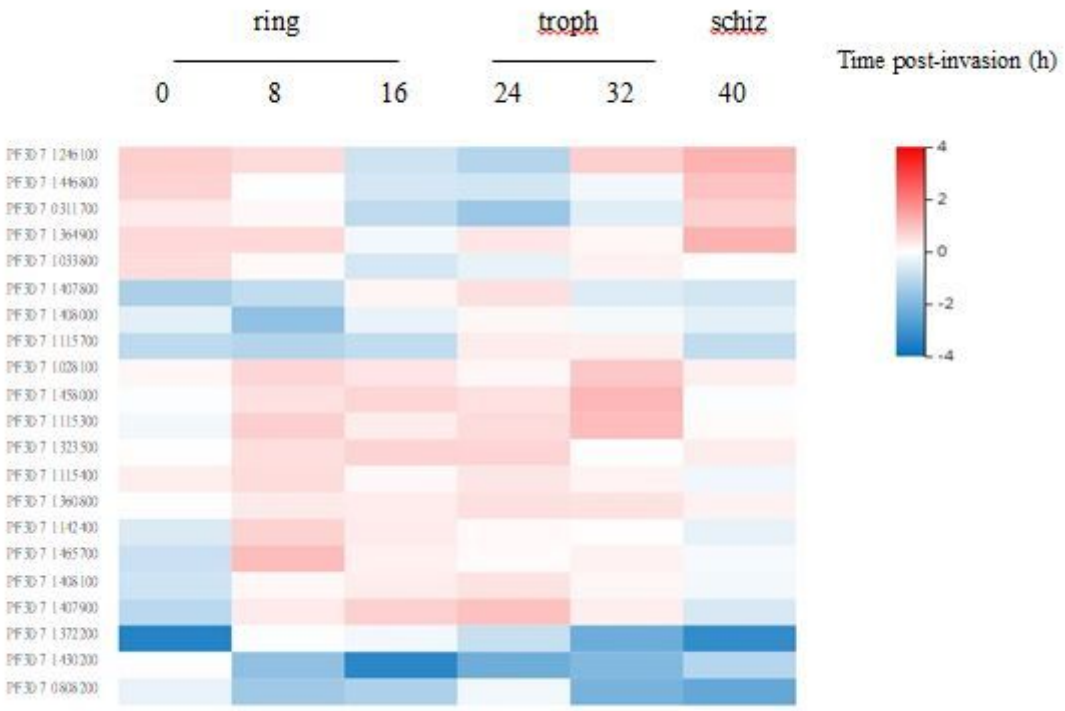

Figure 4

The transcription pattern of the genes involved in heme metabolism.

Supplementary Files

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- supplement9.docx