Background
Genetic modification of *Plasmodium falciparum*, the most virulent human malarial parasite, is an essential technology to analyze the gene function of the parasite, which can provide fundamental information for identifying vaccine candidates and drug targets. Recently, genome-editing techniques using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9 nuclease) was developed in *P. falciparum*. In this method, parasites are co-transfected with two plasmids; one contains the Cas9 nuclease gene, and the other contains the DNA fragment encoding the sgRNA and the donor template DNA. Thus, the success of genetic modification is dependent on whether these two plasmids co-exist in the parasite. However, because the transfection efficiency of *P. falciparum* is very low, only few parasites are co-transfected, resulting in a low modification efficiency. Therefore, the current method using the CRISPR/Cas9 nuclease in *P. falciparum* needs to be further improved.

Method
To improve the CRISPR/Cas9 nuclease system for *P. falciparum*, we developed a novel method using the transgenic parasite, PfCAS9, which stably expresses the Cas9 nuclease using the centromere plasmid. To prove the experimental concept, site-directed mutagenesis of the *kelch 13* gene was performed in PfCas9.
The sequence analysis showed that transgenic PfCas9 \textit{kelch} 13 gene mutation could be introduced almost 100% when transgenic PfCas9 was treated with two drugs. In contrast, 50-80% of total parasites still possessed wild-type sequence when transgenic PfCas9 was treated with one drug.

Discussion
The present study demonstrated that the genetic modification was performed in almost 100% when transgenic PfCas9 was treated with two drugs. This high efficiency is likely due to the fact that \textit{P. falciparum} lack of non-homologous end joining (NHEJ). When Cas9 nuclease cleaves the target genomic sequence, the parasite can only repair the double stand breaks with homology directed repair (HDR) using the donor template DNA. Therefore, only parasites in which mutation is introduced can survive when transgenic PfCas9 was treated with two drugs. When transgenic PfCas9 was treated with one drug, 50-80% of parasites still possessed wild-type sequence. Probably, a proportion of transfected parasites would enter the schizogony phase before undergoing genomic cleavage by Cas9 nuclease, and a small number of those parasites would lose the pfCas9 plasmid during the schizogony. These parasites would survive during single-drug selection, resulting in the observed low efficiency of genetic modification. The current genetic modification by the CRISPR/Cas9 system in \textit{P. falciparum} is carried out by co-transfection with two plasmids followed by single-drug treatment. The segregation efficiency of the conventional plasmid is estimated to be less than 90 %, which is much lower than that of the centromere plasmid. Thus, this may be why the efficiency of genetic modification in the current method is low.

Conclusions
Stable expression of the Cas9 nuclease improves the efficiency of genetic modification in \textit{P. falciparum}. This CRISPR/Cas9 nuclease system is a useful tool to overcome the present technical limitation.