Endogenous 2µ Plasmid Editing for Pathway Engineering in Saccharomyces cerevisiae

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In Saccharomyces cerevisiae, conventional 2µ-based plasmid (pC2µ), such as pRS425, have been widely adopted in pathway engineering for multi-copy overexpression of key genes. However, the loss of partition and copy number control elements of yeast endogenous 2µ plasmid (pE2µ) brings the issues concerning plasmid stability and copy number of pC2µ, especially in long-term fermentation. In this study, we developed a method based on CRISPR/Cas9 to edit pE2µ and built the pE2µ multi-copy system by insertion of the target DNA element and elimination of the original pE2µ plasmid. The resulting plasmid pE2µRAF1 and pE2µREP2 demonstrated higher copy number and slower loss rate than a pC2µ control plasmid pRS425RK, when carrying the same target gene. Then, moving the essential gene TPI1 (encoding triose phosphate isomerase) from chromosome to pE2µRAF1 could increase the plasmid viability to nearly 100% and further increase the plasmid copy number by 73.95%. The expression using pE2µ multi-copy system demonstrated much smaller cell-to-cell variation comparing with pC2µ multi-copy system. With auxotrophic complementation of TPI1, the resulting plasmid pE2µRT could undergo cultivation of 90 generations under non-selective conditions without loss. Applying pE2µ multi-copy system for dihydroartemisinic acid (DHAA) biosynthesis, the production of DHAA was increased to 620.9 mg/L at shake-flask level in non-selective rich medium. This titer was 4.73-fold of the strain constructed based on pC2µ due to the more stable pE2µ plasmid system and with higher plasmid copy number. This study provides an improved expression system in yeast, and set a promising platform to construct biosynthesis pathway for valuable products.

Keywords: 2µ plasmid, CRISPR, plasmid copy number, dihydroartemisinic acid, Saccharomyces cerevisiae

INTRODUCTION

For the high transformation efficiency and easy manipulation, plasmids have been developed as important tools and were widely applied in many kinds of organisms (Mignon et al., 2015; Lian et al., 2016). The multi-copy plasmids are always used to overexpress genes of interest (Kang et al., 2018). In Saccharomyces cerevisiae, conventional 2µ-based plasmid (pC2µ) such as pYES2,
pRS426, and pESC were widely used multi-copy plasmids for production of recombinant proteins as well as construction of metabolic pathway. For example, overexpression of Syn_ALD (aldehyde dehydrogenase of *Synechocystis* sp. PCC6803) and CCD2 (carotenoid cleavage dioxygenase of *Crocus*) by pRS426 increased crocetin production by about 1-fold (Chai et al., 2017). However, like all kinds of multi-copy plasmids, the plasmid viability in cells always depends on the selectivity pressure generated by the medium (with antibiotics or auxotroph medium) (Karim et al., 2013; Lian et al., 2016). In condition without selective pressure, pC2µ hardly keeps its high copy number and are difficult to be maintained in the host cell (Figure 1A). The loss frequency could reach to about 5% per generation (Gnügge and Rudolf, 2017), and nearly 50–60% of the cells lose their plasmid after about 24 h cultivation in non-selective medium (Christianson et al., 1992). For strain harboring pC2µ with auxotroph marker, the rich medium such as YPD is not suitable for long-term fermentation because of the loss of the plasmid, although the strain grows faster in these kinds of media; And for that with antibiotic marker, the addition of expensive drugs is inevitable and it is not economic for fermentation of industrial scale.

Plasmid pC2µ was derived from the yeast endogenous 2µ plasmid (pE2µ) which presents in most wild-type and laboratory *S. cerevisiae* strain (Gnügge and Rudolf, 2017). The pE2µ is a selfish episomal circular DNA element in *S. cerevisiae* (Rizvi et al., 2017a). It contains a special replication origin (2µ ori), two FRT sites (*FLP1* recognized target site), and sequences encoding four known genes: *REP1*, *REP2*, *RAF1*, and *FLP1* (Gnügge and Rudolf, 2017; McQuaid et al., 2017; Rizvi et al., 2017a; see Supplementary Figure 1). *FLP1* and both FRT sites are essential for the amplification of pE2µ. This amplification system is based on the *FLP1*-mediated recombination and follows the special Futcher’s model (Futcher, 1986; Rizvi et al., 2017a), thus each plasmid could reproduce more than one copy in one cell cycle to restore the steady-state for plasmid copy number (PCN) under missegregation and consequent perturbations (Rizvi et al., 2017a). Whereas, pC2µ plasmid contains only one FRT site. So that pC2µ cannot be amplified as Futcher’s model itself, and the amplification of pC2µ is not efficient as pE2µ. Therefore, the PCN of pC2µ cannot compete with native pE2µ, even though

**FIGURE 1** | Design and construction of pE2µ multi-copy system. (A) The comparison of the copy number of the inserted target gene using pC2µ (conventional 2µ-based plasmid) derivate plasmid and pE2µ (endogenous 2µ plasmid) derivate plasmid. (B) The process of pC2µ multi-copy system and pE2µ multi-copy system. The 2µ ori (original of replication of wild type endogenous plasmid) is shown in blue, other parts of endogenous plasmid are shown in purple, the Target gene of interest is shown in red. The scissors represent the CRISPR/Cas9 tool to edit the plasmid. (C) The construction and optimization of the pE2µ derivate plasmid.
weaken the expression of the selective marker by truncated or weaker promoter could increase the average plasmid copy number of pC2µ by eliminating the cells with less plasmid (Karim et al., 2013).

**REP1** and **REP2** are essential for the partition system of pE2µ. **REP1** and **REP2** form the REP1-REP2 complex which binds to the cis-acting locus (STB locus) (see Supplementary Figure 1) located within the 2µ ori to ensure equal segregation of plasmids from mother to daughter cells (Gnügge and Rudolf, 2017; Rizvi et al., 2017a); **RAF1** is responsible for the regulation of partition and amplification system to keep the plasmid copy number of pE2µ and minimize the cell-to-cell variations (McQuaid et al., 2017; Rizvi et al., 2017b). Based on the tightly controlled partition system and amplification system described above, pE2µ could be segregated equally and the copy number could be restored in some missegregation events, leading to very low loss frequency (<0.01%) (Gnügge and Rudolf, 2017). The pC2µ only contains 2µ ori and does not contain **REP1** and **REP2**, so the maintenance of pC2µ has to depend on pE2µ (Figure 1B). Rising from segregation instability, the loss frequency of pC2µ is about 50% per generation in strains without pE2µ (Gnügge and Rudolf, 2017). Therefore, to achieve stable and high-level expression of the targeted genes, we intended to employ pE2µ rather than pC2µ as an expression vector. Yukie Misumi once constructed the plasmid YHp (Misumi et al., 2019) based on the structure of pE2µ and applied in the S. cerevisiae strain without endogenous 2µ plasmid. However, because of the similar structure, YHp is not compatible to employ pE2µ as an expression vector.

In our work, we developed a novel multi-copy plasmid system which based on editing pE2µ by CRISPR/Cas9 genome engineering tools (Figures 1B,C). The laboratory strain CEN.PK2-1C was chosen as the host. It contains the natural wild type pE2µ plasmid. By using this pE2µ multi-copy plasmid system, the target gene could be overexpressed more stable and at higher level than using the commonly used pC2µ multi-copy plasmid system (Figure 1A). Although the edited pE2µ was still lost during long-term cultivation in non-selective medium, the plasmid viability was much higher than that of pC2µ.

To further reduce the loss frequency of pE2µ, auxotroph complementation strategy was applied by introducing the essential gene **TP11** (encoding triose phosphate isomerase) into pE2µ by CRISPR/Cas9 system as well as knocking out the **TP11** in the chromosome (Figure 1C). As a result, pE2µ plasmid (pE2µRT) could be maintained in cells for long-term cultivation. And its PCN was also increased. Although p425RT (pC2µ derived plasmid carrying **TP11**) in strain without chromosome **TP11** could also be maintained in cell after long-term cultivation in YPD, the average level and stability of p425RT could not compared with that of pE2µRT. At last, we applied the method for optimization of the metabolic pathway of dihydroartemisinic acid (DHALA) production in non-selective medium. The production was successfully increased to 620.9 mg/L which was 4.73-fold higher than the strain using pC2µ to overexpress the gene of the same biosynthetic pathway.

**MATERIALS AND METHODS**

**Strains and Medium**

*Escherichia coli* DH5α was used for construction of all the plasmids and was cultured at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 100 μg/ml ampicillin or 34 μg/ml chloramphenicol if necessary. All engineered yeast strains were derived from *S. cerevisiae* CEN.PK2-1C (Entian and Kiter, 2007) obtained from EUROSCARF (Frankfurt, Germany). The yeast strains were cultured at 30°C in YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or in synthetic complete (SC) drop-out medium. G418 was added to medium in final concentration of 200 μg/ml if necessary. The strains for DHAA production was derivative from Sc027 (Zeng et al., 2020). All *S. cerevisiae* strains used in this study were listed in Table 1.

**Construction of pE2µ Derivate Plasmid**

All the plasmids constructed in this study were listed in Table 2. The primer used in this study were listed in Supplementary Table 1. Three CRISPR/Cas9 plasmid pCasRAF1, pCasREP2, and pCasE2µ were constructed based on pRS416 (Supplementary Figure 2). Took the construction of pCasRAF1 for example: P_SNR52 (RAF1) was amplified from pNA034 by PCR using primer 18Q3-pSNR52-F and 18Qb-pSNR52-R; Cassette gRNA.2µ.DCA::pE2µ::T_SUP4_T_CYC1 was amplified from pNA034.

**TABLE 1 | Yeast strains used in this study.**

| Yeast Strains | Description | Source |
|---------------|-------------|--------|
| CEN.PK2-1C    | MAT a; ura3-52, trp1-289, leu2-3,112, his3Δ1, MAL2-8C, SUC2 | Invitrogen |
| Sc382         | CEN.PK2-1C derivative; Δ2µ::pE2µ::RAF1 | This study |
| Sc534         | CEN.PK2-1C derivative; pRS425SK | This study |
| Sc438         | CEN.PK2-1C derivative; Δ2µ::pE2µ::REP2 | This study |
| Sc519         | Sc382 derivative; ΔE2µ::RAFT::pE2µ::RT | This study |
| Sc594         | Sc591 derivative; ΔTPI1(chromosome)::leu2 | This study |
| Sc530         | CEN.PK2-1C derivative; p425RT; ΔTPI1(chromosome)::his3 | This study |
| Sc207         | CEN.PK2-1C derivative; leu2-3,112::KanMX6::P_GAL7::CYB5_TERG91(RC)-ERG19(RC)-P_GAL1-ERG8-TERG91(ADH1) (RC)-P_GAL1-TERG91-ADH1(ADH1)_P_ TPI1(chromosome)::hisd3 | Zeng et al., 2020 |
| Sc341         | Δgala80::P_GAL4-T_SUP4-T_CYC1; ΔKanMX6::P_GAL7::CYB5_TERG91(RC)-ERG19(RC)-P_GAL1-ERG8-TERG91(ADH1); Δgala10::P_GAL1-TERG91-ADH1(ADH1)_P_ TPI1(chromosome)::hisd3 | This study |
| Sc366         | Sc341 derivative; p425DCA | This study |
| Sc341         | Δ2µ::pE2µ::DCA | This study |
| Sc582         | Sc343 derivative; ΔE2µ::pE2µ::DCAT | This study |
| Sc584         | Sc582 derivative; ΔTPI1::leu2 | This study |
by PCR using primer 18Q0b-gRNA-F and 18Q3-cycl1t-R. Two DNA fragments were assembled together to construct cassette $P_{SNR52}$-gRNA-2p_raf1-Tsup4-TCYC1 by OE-PCR (overlap extension PCR) using primer 18Q3-cycl1t-R and 18Q3-pSNR52-F. Cassette $P_{TEF1}$-cas9-TCYC1 was amplified by PCR from pNA0306 using primer 18Q3-cas9-F and 18Q3-cas9-R. Cassette $P_{SNR52}$-gRNA-2p_raf1-Tsup4-TCYC1 was digested by Sphi I and NotI; Cassette $P_{TEF1}$-Cas9-TCYC1 was digested by KpnI and Sphi I; pRS416 was digested by KpnI and NotI. Three digested DNA fragments were ligated together by T4 ligase to construct pCasRAF1. pCasREP2 and pCasE2µ were constructed in similar way.

Plasmids pDonorRAF1, pDonorREP2, pDonorDCA were amplified by PCR using primer 18Q4a-kanMX6-F and 18Q4a-kanMX6-R. Cassette $P_{PGI1}$-homoraf1_raf1 was digested by PstI and XbaI; $P_{TDH3}$-RFP-TADH1 was digested by EcoRI and XhoI; KanMX6 was digested by XhoI and XhoI; plasmid pSB1C3 was digested by PstI and EcoRI. All 4 digested fragment were ligated together by T4 ligase to construct pDonorRAF1. Donor plasmid pDonorREP2 and pDonorDCA was constructed in similar way.

For construction of the S. cerevisiae strain Sc382, donor plasmid pDonorRAF1 was linearized by PmeI and was co-transformed to CEN.PK2-1C with CRISPR/Cas9 plasmid pCasRAF1 to insert the whole donor DNA into endogenous 2µ plasmid. The SC-Ura plate with 200 µg/L G418 was used for selection of the correct transformants. After keeping the plate at 30°C for about 72 h, all the transformants were visible. The single colonies were picked up and transferred to SC-Ura plate with 200 µg/L G418 and incubated at 30°C for 24 h. Then, the colonies were identified by colony PCR. For strain Sc382, the primer pairs 2µori-test-F/BioBrick-R and 18Q-test-5/18Q-test-15 were used for verification of the insertion of the donor DNA.

The DNA fragment followed by "(RC)" represented the orientation of the DNA fragment was reversed. CYP71AV1*: all CYP71AV1 used in this study was optimized by replacing the N-terminal membrane anchoring sequence (15-residue peptide) with the more hydrophilic 8-residue peptide from bovine (RBP) (Chen et al., 2017).
The primer pair 2µori-test-F/18Q-test-5 was used for verification of the elimination of all wild type endogenous 2µ plasmid. The result was used for characterization of the efficiency of the method of editing endogenous 2µ plasmid by CRISPR/Cas9 system. After verification, the correct colonies were streaked on 5-FOA (5-Fluoroorotic acid) plate with 200 mg/L G418 to lose the pCasRAF1 plasmid.

The process of construction of strain Sc438 and Sc343 were similar to that of strain Sc382. Donor plasmid pDonorREP2 and pCasREP2 were used for co-transformation into CEN.PK2-1C to construct strain Sc343 (harboring plasmid pE2µ). Donor plasmid pDonorDCA and pCasRAF1 were used for co-transformation into CEN.PK2-1C to construct strain Sc438 (harboring plasmid pE2µ DCA).

To introduce TPII to pE2µ, DNA fragment tpi-1 was amplified from genome of CEN.PK2-1C by PCR using primer 20QRcT-R6/20QRcT-F6, tpi-2 was amplified from tpi-1 by PCR using primer 20QRcT-R5/20QRcT-F5, tpi-1-3 was amplified from genome of CEN.PK2-1C by PCR using primer dTPI1-leu2-F1/dTPI1-leu2-R1; Then, leu2-2 was amplified from leu2-1 by PCR using primer dTPI1-leu2-F2/dTPI1-leu2-R2; and leu2-3 was amplified from leu2-2 by PCR using primer dTPI1-leu2-F3/dTPI1-leu2-R3. DNA fragment leu2-3 was introduced to Sc591 and Sc582 for deletion of TPII to construct strain Sc594 and Sc584. For construction of strain Sc530, p425RT was introduced into CEN.PK2-1C and then chromosome TPII was deleted by his3.

**Plasmid Stability Assay**

To characterize the property of plasmid pE2µ and pC2µ, strain Sc382, Sc438, Sc534, Sc530, and Sc594 were tested in non-selective YPD medium. The single colony from selective YPD+G418 medium plate was inoculated to 3 ml YPD+G418 medium (for Sc530, the medium was SC-Leu). The saturated culture was re-inoculated to 3 ml YPD+G418 medium (for Sc530, the medium was SC-Leu) at an optical density (OD) of 0.05 and grown for 10 h at 30°C. The culture was at 0 generation and was prepared for characterization. Then the culture was re-inoculated to non-selective YPD medium at OD = 0.05 and grown for 10 h at 30°C again to obtain the culture at 5 generation. Then serial sub-cultures were conducted every 5 generation until the 30th generation. The cultures at 5, 10, 20, and 30 generation were prepared for characterization.

For measurement of the fluorescence of RFP (Red fluorescence protein), plate reader (SpectraMax M2, Molecular Devices) was used with a 587 nm excitation filter and a 610 nm emission filter. The fluorescence of RFP at single cell level was measured by flow cytometer (NovoCyte D2040R). The culture of each generation was diluted and plated on YPD medium and YPD+G418 medium (for Sc530, the selective medium was SC-Leu) to calculate the viability of the plasmid by counting the colony number of each plate.

**Determination of Plasmid Copy Number (PCN)**

The plasmid copy number (PCN) was measured by quantitative PCR using the total DNA extracted from the strain. The process of total DNA extraction: the cells was harvest at mid-log phase, and were treated with lysis buffer (20 mM phosphate buffer at pH = 7.2, 1.2 M sorbitol, 15 U zymolyase) at 37°C for 20 min. The total DNA was extracted by boiling the sample for 15 min, –80°C for 15 min and then boiling again for 15 min (Lian et al., 2016). The suspension was diluted 10-fold before qPCR analyze. The absolute quantitative method referred to Lee et al. (2006). The plasmid stALG2 (contain ALG9, RFP, KanMX6) and p425DCA (containing ADS) was used for construction of the standard curve. TransStart™ Top Green qPCR SuperMix (purchased from TransGen Biotech Co., Ltd) were used for qPCR analyze on Real time fluorescent quantitative PCR (Molecular Machine MA-6000). The primers used for qPCR were listed in Supplementary Table 1.

**Fermentation and Measurement of the DHAA**

The medium used for fermentation was YPD medium and FM medium (as our previous work (Zeng et al., 2020)). The FM medium was composed of 8 g/L KH₂PO₄, 15 g/L (NH₄)₂SO₄, 6.2 g/L MgSO₄·7H₂O, 40 g/L glucose, 12 ml/L vitamin solution, and 10 ml/L trace metal solution and 10 ml/L Amino acid solution. Vitamin solution included 0.05 g/L biotin, 1 g/L calcium pantothenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl, 0.2 g/L p-aminobenzoic acid, and 2 g/L adenine sulfate. Trace metal solution was composed of 5.75 g/L ZnSO₄·7H₂O, 0.32 g/L MnCl₂·4H₂O, 0.32 g/L Anhydrous CuSO₄·5H₂O, 0.47 g/L CoCl₂·6H₂O, 0.48 g/L Na₂MoO₄·2H₂O, 2.9 g/L CaCl₂·2H₂O, 2.8 g/L FeSO₄·7H₂O, and 80 ml/L EDTA solution (containing 0.5 mol/L Na₂EDTA pH = 8.0). Amino acid solution is composed of 2 g/L methionine, 6 g/L tryptophan, 8 g/L isoleucine, 5 g/L phenylalanine, 10 g/L sodium glutamate, 20 g/L threonine, 10 g/L aspartate, 15 g/L valine, 40 g/L serine, and 2 g/L arginine. The selective medium for Sc566 was FM medium adding 50 mg/L uracil, the selective medium for Sc343 and Sc584 was FM medium adding 200 mg/L leucine, and 50 mg/L uracil and 200 mg/L G418. The non-selective medium for all strains were YPD medium. To test the productivity of the strains, the single colony from plate was inoculated to selective medium and cultured for 18–24 h at 30°C. The cells were re-inoculated to 3 ml selective medium at OD = 0.05 and cultured for another 18–24 h at 30°C. The seed culture of each strain was transferred 250 ml flask containing 25 ml selective medium and flask containing 25 ml non-selective medium at initial OD = 0.2. The cell was grown at 30°C with shaking at 200 rpm. After 24 h, 5 ml dodecane and 20 g/L ethanol was added to each flask. The whole fermentation process continued for 120 h until harvest.

After harvest, the fermentation broth was centrifuged at 12,000 × g for 2 min and the dodecane phase was collected. And then 50 μL organic phase was mixed with 950 μL methanol. After filtrated with 0.22 μm Nylon 66 filter, the sample was ready for HPLC analysis. The method for HPLC analysis and
the measurement of DHAA and other intermediates had been reported in previous work (Zeng et al., 2020).

**Measurement of Relative mRNA Level**

During the fermentation of Sc366 and Sc343, 1 ml samples was collected at 40 h. Total RNA was extracted from the cell using Yeast RNA kit (Omega bio-tek). The Reverse transcription procedure was used TransScript® First-Strand cDNA Synthesis SuperMix (purchased from TransGen Biotech Co., Ltd). The relative cDNA level of ADS, CYP71AV1, DBR2 were measured by qPCR. *ALG9* was the reference gene, and the result was relative to that of Sc366. The primers used for qPCR were listed in Supplementary Table 1.

**RESULTS AND DISCUSSION**

**The Construction of pE2µ Multi-Copy System**

In order to construct the multi-copy system, we intended to insert the target DNA into the wild type pE2µ plasmid of CEN.PK2-1C. The pDonor plasmid which harboring homologous arms of wild type pE2µ was designed to carry target DNA. After linearization, pDonor could be transformed into CEN.PK2-1C and inserted into wild type pE2µ by homologous recombination to form the recombinant plasmid. Since the incompatibility between the recombinant plasmid and the original wild type pE2µ plasmid, the CRISPR/Cas9 plasmid was designed to and enhance the recombination of pDonor with pE2µ and eliminate all the original wild type pE2µ without insertion of pDonor. The resulting strain only contained the recombinant plasmid with multi-copies (Figure 1C).

To insert the target DNA into pE2µ, the site for recombination and the editing target of CRISPR/Cas9 plasmid had to be determined. Beside the four known genes, there are several uncharacterized transcripts transcribed from wild type pE2µ plasmid (Rizvi et al., 2017a). All the elements described above covered almost the whole plasmid. To avoid the disruption of the plasmid function, only two sites can be chosen as the targets for insertion of foreign DNA fragment: One is at the downstream of the RAF1 (Supplementary Figure 1A), another is at the end of ORF of REP2 (Supplementary Figure 1B). The CRISPR/Cas9 plasmid pCasRAF1 and pCasREP2 were constructed for each target described above. Each of two plasmids encoded both the RNA-guided endonuclease Cas9 and the guide RNA (gRNA) of corresponding (see Supplementary Figure 2). The centromeric plasmid pRS416 was used as the backbone to construct the CRISPR/Cas9 plasmid. Although using multi-copy plasmid such as pRS426 might increase the efficiency of genome or plasmid editing and had been successfully applied for multi-genes disruption (Jakounas et al., 2015; Lian et al., 2018), the recombination between pRS426 and endogenous 2µ plasmid through FRT sites by FLP1 was not desired during the plasmid editing process.

In order to facilitate the introduction of foreign DNA and characterize the modified 2µ plasmid, we designed two donor plasmids pDonorRAF1 and pDonorREP2. The vector composed of backbone of pSB1C3, KanMX6 for selection, RFP (Red fluorescent protein) cassette as reporter, homologous arms and a terminator (Figure 1). Different from common genome editing by CRISPR/Cas9 system, KanMX6 is necessary selective marker for editing the wild type 2µ plasmid, because there is no essential gene on it. The terminator would help to finish the transcription of RAF1 or REP2 which might be influenced by inserted DNA. RFP cassette was used for characterization of the modified 2µ plasmid and two *BsaI* restriction endonuclease sites flanked by RFP were designed for substitute RFP cassette by other gene of interest. After linearized by *Pmel*, the donor plasmid pDonorRAF1 or pDonorREP2 was co-transformed with their corresponding CRISPR/Cas9 system plasmid (pCasRAF1 or pCasREP2) into host CEN.PK2-1C. After plasmid editing and recombination, the strain Sc382 (harboring recombinant plasmid pE2µ.RAF1) and Sc438 (harboring recombinant plasmid pE2µ.REP2) were constructed (see Figure 1C).

Take the construction of Sc382 for example, we randomly picked 36 single colonies for verification by colony PCR. Among them, 22 colonies (about 61.1%) were successfully transformed by linearized DNA. And no wild type endogenous 2µ plasmid remained in these colonies. The yeast plasmids extracted from strain Sc438 and Sc382 could be successfully transformed to *E. coli* and obtain the plasmid pE2µ.RAF1 and pE2µ.REP2. The sequencing result of the 2µ related part of pE2µ.RAF1 and pE2µ.REP2 were consistent with reported endogenous 2µ plasmid of CEN.PK113-7D (GenBank: CP025735.1) (Piroon et al., 2018), and the editing process was further proved to be successful.

Although CRISPR/Cas9 system has been successfully applied in genome editing, it was the first attempt to edit the multi-copy plasmid. Comparing with the efficiency of genome editing which was almost 100% (Jakounas et al., 2015; Generoso et al., 2016), the efficiency of multi-plasmid editing was not as high as that. Multi-copy target sequences and the special amplification system of endogenous 2µ plasmid (Gnügge and Rudolf, 2017) made the work of CRISPR/Cas9 system not easy. Some strategies for optimization CRISPR/Cas9 system increasing might be helpful for increasing the efficiency of 2µ plasmid editing, such as increasing activity of cleavage by using Cas9 variants (Bao et al., 2014), changing the promoter for optimization of gRNA expression (Gao and Zhao, 2013), and facilitating gRNA-transient expression system (Easmin et al., 2019). Anyway, the method for plasmid editing applied in this work had been proved to be viable and successful to insert the target DNA element into the pE2µ to form a new recombinant multi-copy plasmid and eliminate the original pE2µ plasmid.

**Characterization of the Plasmids From pE2µ Multi-Copy System**

To characterize the property of pE2µ, we chose pC2µ plasmid pRS425RK as a control. pRS425RK was constructed based on conventional 2µ plasmid pRS425. RFP cassette and KanMX6 of pRS425RK were used for characterization. The strain Sc534 which harboring pRS425RK was constructed. We evaluated the stability of Sc382 and Sc438 compared with control strain
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Sc534 by plasmid stability assay. The strains were culture in YPD media without selective pressure and were transferred to fresh media every 5 generation. The fluorescence at single cell level was measured at 5, 10, 20, and 30 generation (Figure 2). Under condition with selective pressure (at 0 generation), the cells of the strains with pE2µ (Sc382 and Sc438) showed less cell-to-cell variation (smaller CV %) than that with pRS425RK (Sc534) (Figure 2A). The average fluorescence of strains Sc438 and Sc382 was also 3.42 to 3.67-fold higher than that with pRS425RK (Supplementary Figure 3). The cell growth of Sc382 and Sc438 higher than that of Sc534 after 48 h fermentation in condition with selective pressure (Figure 2C). The PCN of Sc382 (pE2µ-RAF1) achieved 10.4 and was about 1.67-fold higher than that of Sc534, while the average PCN of Sc438 (pE2µ-REP2) was 10.8 copies and also higher than that of Sc534 (Figure 2D).

After culturing in the condition without selective pressure, all the strains began to lose the plasmid. Nearly 94% cells of the Sc534 (pRS425RK) lost their plasmid after culture of 10 generation and hardly to find cells with plasmid after 20 generation. The frequency of plasmid lost is lower at strain Sc382 and Sc438. Only about 20–23% of the cells lost their plasmid before 10 generation, but 88–89% of the cells had lost their plasmid until 30 generation (Figure 2B). There was no significant difference in PCN and plasmid stability between pE2µ-RAF1 and pE2µ-REP2. Although the stability of the strain with pE2µ is higher than the strain with conventional 2µ plasmid, it is much lower than the wild type endogenous 2µ plasmid.

The PCN control system of 2µ plasmid based on RAF1 and REP1-REP2 complex could tightly control its copy number (McQuaid et al., 2017; Rizvi et al., 2017a). REP1-REP2 complex is responsible for stability of plasmid and repression of FLP to control the PCN; RAF1 could repress the formation of REP1-REP2 to derepress the expression of FLP1 to increase the PCN (Rizvi et al., 2017b). The pC2µ is out of this copy number control

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**FIGURE 2** | Comparing the strains harboring pC2µ derivated plasmid (pRS425RK) and strains harboring pE2µ derivated plasmid (pE2µ-RAF1 or pE2µ-REP2) when carrying RFP expression cassettes. (A) Fluorescence at the single-cell analyzed by flow cytometry during cultivation of 30 generations in non-selective YPD medium. Strain CEN.PK2-1C which didn’t express RFP was set as the blank. Different plasmids harbored by strain were shown in the bracket. CV %, coefficient of variance. (B) Comparison of PCN of different strains in selective YPD+G418 medium (at 0 generation). (C) The cell growth of Sc534, Sc438, and Sc382 in selective YPD+G418 medium (D) Comparison of plasmid stability assay for strains Sc534, Sc382, and Sc438 in non-selective YPD medium.
system, and difficult to keep the stability and equality even in condition with selective pressure. While, the pE2µ could facilitate the PCN control system to keep its equality of PCN, and is more stable. The insertion of foreign DNA didn’t disrupt any known genes on the 2µ plasmid, but it might influence the expression level of these genes or other unknown function transcripts to reduce the stability of 2µ plasmid. In spite of that, pE2µ is might be a better choice than pC2µ plasmid to overexpress the genes of interest by increasing its copy number. Strain Sc382 was chosen as the host for further optimization.

**Optimization of pE2µ Multi-Copy System by Auxotrophy Complementation of Essential Gene TPI1**

The maintenance of the plasmid in the cell is very important for long-term fermentation. To eliminate the plasmid loss of pE2µ in non-selective condition, an auxotroph marker based on essential gene TPI1 was constructed. Gene TPI1 encodes triose phosphate isomerase which is required for growth on glucose and makes up about 2% of the soluble cellular protein (Scott and Baker, 1993). The strategy of using essential gene to substitute the one on the chromosome to construct the selective pressure-free system and optimize the plasmid system had been used in *E. coli* (Chen, 2012; Kang et al., 2018). However, fewer studies focused on the optimization of plasmid in *S. cerevisiae*. CRISPR/Cas9 plasmid pCasE2µ was constructed for insertion of TPI1 into plasmid pE2µRAF1 of strain Sc382. The editing target of pCasE2µ was on the pSB1C3 backbone of pE2µRAF1. After insertion of TPI1 into pE2µRAF1 (the resulting plasmid was pE2µRT) and deletion of native TPI1 in chromosome by his3, the strain Sc594 was constructed (see Figure 1C). For comparison, strain Sc530 which harboring plasmid p425RT (based on pRS425 and containing TPI1) and the deletion of the native TPI1 was constructed. Compared with wild type CEN. PK2-1C, both Sc594 and Sc530 showed similar cell growth for 48 h fermentation in non-selective YPD medium (see Figure 3B). The plasmid viability of both strains was evaluated by a very long-term cultivation (90 generation) without any selective pressure in YPD medium. For both strains, the viability was nearly 100% (see Figure 3D), and no plasmid-free cell was found at 90 generation, all cells plated on YPD plate were expressing RFP (see Supplementary Figure 4A). TPI1 was the key enzyme for both glycolysis and gluconeogenesis process, cells without TPI1 are inviable (Giaever et al., 2002). For strain Sc594, after moving the TPI1 from chromosome to plasmid, TPI1 became an auxotrophy selective marker of the plasmid. Since TPI1 is an essential gene, this auxotrophy selective marker have no requirement of the condition, and the plasmid-free cells was inviable. On the contrary, for strain Sc382 or Sc438, the selective marker of their plasmid was KanMX6, the plasmid only could be kept in condition with G418, the cells would lose the plasmid in non-selective YPD medium. Therefore, the plasmid viability of Sc594 or Sc530 was shown higher than that of Sc382 or Sc438. Optimization of the plasmid by auxotrophy complementation of essential gene TPI1 could also increase the PCN of both pC2µ and pE2µ derivate plasmid. The PCN of pE2µRT in Sc594 achieved to about 18.3 and was 1.76-fold higher than that of pE2µRAF1 in Sc382 at 0 generation; while the PCN of p425RT in Sc530 was also increased to 10.8 and was 1.80-fold higher than that of pRS425RK in Sc534 (Figure 3A). During the long-term cultivation in non-selective medium, the pE2µ derivate plasmid showed higher stability in PCN and less cell-to-cell variation comparing with pC2µ derivate plasmid. The CV% of Sc530 was increased by 37% from 132.32 (0 generation) to 182.59 (90 generation) (Figure 3C) and the average PCN of p425RT decreased by 38.2% to 6.67 (Figure 3A); While CV % of Sc594 was only increased by 15.90% from 94.95 to 110.05 (90 generation) and lower than that of Sc530 in any generations (see Figure 3B). The average PCN of pE2µRT only decreased by 8.74% to 16.7 after cultivation of 90 generation in non-selective medium (see Figure 3A), the average expression level of the RFP in Sc594 was obviously higher than that of Sc530 (Supplementary Figure 4B). The stability of the expression for the target gene using optimized pE2µ multi-copy system was proved to undergo very long-term cultivation. The strategy of introduction of TPI1 into plasmid to substitute the native TPI1 was proved to optimize both pC2µ derivate plasmid and pE2µ derivate plasmid. And the resulting plasmid pE2µRT showed higher PCN and less cell-to-cell variations. Therefore, pE2µRT was thought to be better choice for overexpression of target genes in high copy.

**Application pE2µ Multi-Copy System for DHAA Production**

To demonstrate pE2µ could be applied for optimization of metabolic pathway, dihydroartemisinic acid (DHAA) biosynthesis was chosen as an example. DHAA is the precursor of the anti-malaria drug artemisinin (Paddon and Keasling, 2014). The biosynthesis pathways of DHAA (see Figure 4A) starts from farnesyl pyrophosphate. In this pathway, ADS, CYP71AV1, and DBR2 are the key genes for biosynthesis of DHAA and are thought to be overexpressed in high level to increase the production of DHAA. Several studies about the biosynthesis of DHAA in *S. cerevisiae* had been reported (Yansheng et al., 2008; Chen et al., 2017; Zeng et al., 2020). All of these works facilitated pC2µ based plasmids such as pESC-LEU, pRS425, pYES260 to overexpress the key genes of the biosynthesis pathway. Therefore, pE2µ multi-copy system was thought to be helpful for further enhancing the biosynthesis of DHAA by optimization of the plasmid.

In this work, Sc366 was used as the control strain. Sc366 harbored plasmid p425DCA which was constructed based on pRS425 and contained three key genes ADS, CYP71AV1, and DBR2. Strain Sc343 harbored pE2µ plasmid pE2µDCA which contained ADS, CYP71AV1, and DBR2. And Sc584 was modified from Sc343 by introducing the TPI1 to pE2µDCA (resulting plasmid was plasmid pE2µDCAAT) to substitute the native chromosome TPI1. In non-selective rich medium (YPD medium), nearly 82% cells of Sc366 lost their plasmid during the fermentation (see Figure 4C), the production of DHAA was only 131.0 mg/L (Figure 4B); For strain Sc343 which harbored plasmid pE2µDCA, only 29.8% of cells lost their plasmid, and
FIGURE 3 | Effect of TPI1 auxotrophic complementation on strains Sc594 and Sc530 harboring pC2µ and pE2µ derivate plasmids, respectively. (A) Comparison of PCN for strain Sc530 and Sc594 before (0 generation) or after (90 generation) a very long-term cultivation in non-selective YPD medium. (B) Cell growth of strain Sc594 and Sc530 and the wild type CEN.PK2-1C in non-selective YPD medium. (C) Fluorescence at the single-cell analyzed by flow cytometry during cultivation of 90 generations. (D) The plasmid stability assay for strain Sc594 and Sc530.

FIGURE 4 | Comparison of the effects of pC2µ and pE2µ multi-copy system on DHAA production. (A) The biosynthesis pathway of DHAA. FPP, Farnesyl pyrophosphate; AD, amorpha-4,11-diene; AOH, artemisinic alcohol; AC, artemisic aldehyde; DHAO, dihydroartemisinic aldehyde; DHAA, dihydroartemisinic acid; AA, artemisic acid; ADS, amorphadiene synthase; CYP71AV1, amorpha diene P450 oxidase; CPR1, P450 cytochrome reductase from A. annua; CYBS, cytochrome b5 from A. annua; ADH1, artemisinic alcohol dehydrogenase of A. annua; DBR2, artemisic aldehyde 11(13) reductase of A. annua; ALDH1, artemisinic aldehyde dehydrogenase of A. annua. ADS, CYP71AV1, DBR2 (highlighted in the frame) were genes overexpressed by plasmid systems. (B) The DHAA production of strain Sc366, Sc343 and Sc584 in YPD medium. (C) The plasmid viability of strains Sc366, Sc343 and Sc584 which were measured at the end of the fermentation in YPD medium.
the production achieved to 519.2 mg/L and was 3.96-fold higher than that of strain harboring pC2µ plasmid (Figures 4B,C); After optimization with substitution the location of essential TPI1, the resulting strain Sc584 didn’t lose the plasmid, and got higher production to 620.9 mg/L. The average copy number of Sc366, Sc343, and Sc584 was 3.33, 6.57, and 9.32, respectively (Supplementary Figure 5). The relationship of mRNA levels of the key genes for different strain was consist with the result of average copy number (Supplementary Figure 6). Comparing with Sc594, the PCN of Sc584 was decreased by about 44.5%. The plasmid stability might be influenced by the overexpression of CYP71AV1 and CPR1, or the rapid accumulation of AA (artemisinic acid) and DHAA. It was consistent with the result of the work of Amyris (Paddon et al., 2013) which found the effect on the plasmid by overexpression of CYP71AV1 or accumulation of product.

The medium with selective pressure for yeast was auxotrophic synthetic medium or the rich medium. However, the non-selective pressure rich medium such as YPD or YPG would help strain to produce more production even the plasmid was not stable (Ro et al., 2008) during the large scale and long-time fermentation (Wang et al., 2017). In rich medium, approximately half of the proteome mass saved by amino acid could be redirected to protein engaged in translation (Bjørkeroth et al., 2020). It could promote cell growth and might increase the expression of the target gene. Therefore, the optimized pE2µ multi-copy system platform was suitable for S. cerevisiae to overexpress target gene in non-selective rich medium with higher PCN and has the patent for optimization of biosynthetic pathway and maximize the biochemical production.

In this work, we developed the toolbox for editing the 2µ plasmid to maintain and increase the copy number of the target gene for all S. cerevisiae with or without wild type pE2µ. (see Supplementary Figure 7). For strain without pE2µ, the plasmid pE2µRAFI or pE2µREP2 could be used as the backbone directly. The target DNA elements could be inserted into these plasmids in vitro or in vivo. For strains with wild type pE2µ, the CRISPR/Cas9 plasmid could be used for editing the wild type pE2µ plasmid. And the donor plasmid pDonorRAFI or pDonorREP2 could be used as the backbone to harbor the target DNA. The example of DHAA production in our work proved that this method could successfully increase the copy number of the target gene. The strain Sc594 was constructed as a convenient chassis for overexpression of the target gene efficiently (Supplementary Figure 7). The pE2µRT could also undergo a serial of editing steps to insert the DNA element of interest. During these steps, extra selective marker is not necessary to insert into the plasmid, and the whole editing process of pE2µ is same to the process for editing the genome of chromosome which had been widely used for optimization of S. cerevisiae. And higher copy number of the inserted genes could be easily obtained.

Considering the very low loss frequency of wild type pE2µ, the performance of the loss frequency for pE2µRAFI and pE2µREP2 was unexpected. Although introduction of essential gene TPI1 to substitute the original TPI1 on chromosome could eliminate the plasmid-free cells during the long-term cultivation, the reason for the increasing loss of the plasmid leaded by insertion of foreign DNA into wild type pE2µ plasmid was still needed to be studied. The future study might focus on the regulation of the gene or unknown transcript units of pE2µ plasmid and the stability and the copy number of the pE2µ might be further increased.

CONCLUSION

In this work, the endogenous 2µ plasmid had been successfully and perfectly edited by CRISPR/Cas9 system and the donor plasmid. The foreign gene could be inserted to 2µ plasmid in higher copy number for overexpression. The resulting plasmid pE2µ had higher PCN and higher stability than the conventional 2µ based plasmid. In single cell level, the distribution of pE2µ in each cell was more balanced than pC2µ plasmid. Taking these advantages, pE2µ was applied for the heterologous biosynthesis of DHAA, and increased the production titer by 4.73-fold higher than that of control. This study showed the potential of pE2µ for optimization of metabolic pathway by stable and efficiently overexpressing a serial of genes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

B-XZ and YW conceived the study as well as participated in strain construction and carried out the molecular genetic studies. B-XZ and W-HX participated in fermentation. Y-JY, M-DY, Y-ZL, and W-HX participated in design and coordination of the study as well as helped to draft the manuscript. YW supervised the whole research and revised the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.631462/full#supplementary-material
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Conflict of Interest: 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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