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Research Article

RESEARCH ARTICLE

Free fatty acids promote transformation efficiency of yeast

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One sentence summary: The external supplementation of FFAs could improve yeast transformation efficiency, which is the prerequisite in genetic engineering for optimizing metabolic pathways and enhancing the robustness.

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ABSTRACT

High transformation efficiency is essential in genetic engineering for functional metabolic analysis and cell factory construction, in particular in construction of long biosynthetic pathways with multiple genes. Here, we found that free fatty acid (FFA)-overproducing strain showed higher transformation efficiency in *Saccharomyces cerevisiae*. We then verified that external supplementation of FFAs, to the culture media for competent cell preparation, improved yeast transformation efficiency significantly. Among all tested FFAs, 0.5 g/L C16:0 FFA worked best on promoting transformation of *S. cerevisiae* and *Komagataella phaffii* (previously named as *Pichia pastoris*). Furthermore, C16:0 FFA improved the assembly efficiency of multiple DNA fragments into large plasmids and genome by 100%, which will facilitate the construction and optimization of multigene-containing long pathways.

Keywords: DNA transformation; free fatty acids; *Saccharomyces cerevisiae*; *Komagataella phaffii*

INTRODUCTION

Yeast cell factories play increasingly important roles in the synthesis of renewable biofuels (Nielsen *et al.* 2013; Zhou *et al.* 2016b), food additives (Kallscheuer 2018) and drugs (Guo *et al.* 2013) with the development of synthetic biology. Establishing robust cell factories requires extensive rewiring of the cellular metabolism and regulation (Gong, Nielsen and Zhou 2017), which depends on efficient genome editing and genetic engineering. Recently, several novel genetic manipulation tools such as CRISPR-Cas9 have been developed for the precise genome editing with high efficiency (Cai, Gao and Zhou 2019). In almost all genetic manipulations, high transformation efficiency is a prerequisite in efficient genetic engineering for optimizing metabolic pathways and enhancing the robustness.

For transformation, the exogenous DNA should pass through the cell wall and the cell membrane, and then be delivered across the cytoplasm to the nucleus (Kawai, Hashimoto and Murata 2010; Mitrikeski 2013). Previous studies revealed that

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foreign DNA could be taken up by yeast cells through endocytosis (Kawai *et al.* 2004) with several proposed by not well-characterized mechanisms (Zheng *et al.* 2005; Chen *et al.* 2008; Kawai *et al.* 2009). The yeast transformation efficiency has been significantly improved by optimizing the transformation conditions. For example, Ito *et al.* (1983) established a chemical transformation method by optimizing four key factors including PEG (polyethylene glycol), LiAc, heat shock and the cell state. Chen, Yang and Kuo (1992) revealed that the use of 100 mM DTT could promote transformation in the LiAc/single-stranded carrier DNA/PEG method. Furthermore, the LiAc/single-stranded carrier DNA/PEG method has been showed to be much more convenient for DNA transformation of multiple samples in comparison with other methods such as electroporation, agitation with glass beads and bombardment with DNA-coated microprojectiles (Gietz and Woods 2002). These attempts significantly improved the transformation efficiency of external (plasmid) DNA to *Saccharomyces cerevisiae*. However, it is still challenging in integrating larger DNA fragments, especially the multigene-containing pathways, into *S. cerevisiae* and other non-conventional yeasts. Therefore, further improvement of the transformation efficiency should be made to ensure the efficient integration of multigene fragments into yeast cells. We previously accidentally found that the fatty acid-overproducing *S. cerevisiae* cells always had high amount of transformation clones, during genetic engineering of yeast cell factories for overproduction of fatty acid-derived chemicals and biofuels (Zhou *et al.* 2016a). Therefore, it is worth studying whether fatty acids could promote the transformation of yeasts.

This study revealed that free fatty acid (FFA) significantly improved the transformation efficiency in *S. cerevisiae* and *Komagataella phaffii*. With screening of the effective fatty acids and optimizing of the fatty acid concentrations, 0.5 g/L palmitic acid (C16:0) showed the highest improvement of transformation for both *S. cerevisiae* and *K. phaffii*. In addition, C16:0 fatty acid could promote transformation of large and multiple DNA fragments into *S. cerevisiae*. This study is of great significance to further improve the transformation of *S. cerevisiae* and *K. phaffii*, and provides a method for the construction of multigene pathway yeast platform strains.

MATERIALS AND METHODS

Yeast strains, plasmids and reagents

S. cerevisiae CEN.PK113-11C and *K. phaffii* GS115 are wild-type strains, YJZ01 (*MATa MAL2-8c SUC2 his3*Δ*1 ura3-52 hfd1*Δ), YJZ03 (*MATa MAL2-8c SUC2 his3*Δ*1 ura3-52 hfd1*Δ *pox1*Δ), YJZ08 (*MATa MAL2-8c SUC2 his3*Δ*1 ura3-52 hfd1*Δ *pox1*Δ *faa1*Δ *faa4*Δ) and XC01 (*MATa MAL2-8c SUC2 his3*Δ*1 ura3-52 gal80*Δ; XI-5::Cas9) in this study were constructed based on the CEN.PK113-11C strain (Zhou *et al.* 2016b). The pAOH9 (Zhou *et al.* 2016b), pPICZA-panARS and gRNA plasmids for targeting *HIS3* gene were used for yeast transformation in this study. gRNA plasmids were constructed following a previously described method (Mans *et al.* 2015). The Yeastriction webtool (http://yeastriction.tnw.tudelft.nl) was used to select specific guide RNAs. The construction of the guide RNA expression cassettes was performed by fusion PCR. Plasmid backbone was cloned by a single primer 6005 (Mans *et al.* 2015). The gRNA plasmid was constructed by Gibson assembly. The pPICZA-panARS plasmid was constructed based on the pPICZA plasmid, in which the AOX1t sequence was replaced by autonomously replicating sequences panARS (Camattari *et al.* 2016). pPICZA–panARS can be expressed stably in *K. phaffii* GS115.

FFAs used in this study including octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), tetradecanoic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) were all purchased from Sangon Biotech company (Shanghai, China). Tween 80 was added to dissolve the FFAs. The transformation mix for *S. cerevisiae* included 36 μL lithium acetate (1 M), 240 μL PEG 3350 (50% w/v), 10 μL single-stranded carrier DNA (10 mg/mL), 34 μL plasmid DNA (100 ng) and water (distilled/deionized).

Yeast transformation

S. cerevisiae yeast cells were incubated overnight in YPD medium on a rotary shaker at 200 rpm and 30°C. The harvested cells were then grown in 5 mL YPD medium at 30°C and 200 rpm until the OD_{600} of the culture reached 0.4–0.8 with supplementation with various FFAs with different concentrations. Then, 1 mL carrier DNA was denatured in a boiling water bath for 5 min and was chilled immediately. Cells were harvested by centrifugation and the pellets were washed twice using sterile water. Cell pellets were resuspended in 1 mL sterile water and were transferred to a sample of 10^7 cells for each transformation. Transformation mix (T mix) of 360 μL was added to a sample of 10^7 cells and the cells were resuspended by vortex. Sample of cells in each transformation was placed in tubes and incubated in a water bath at 30°C for 30 min and then at 42°C for 15 min. T mix was removed by centrifugation with a micropipettor. In the transformation tube, 1 mL sterile water was added to resuspend the cells. Cell suspensions of 100 μL were inoculated onto selection medium plates and incubated at 30°C for 2–3 days. Transformation efficiency and yield of transformants were calculated.

K. phaffii cells were inoculated in nonselective 20 mL YPD medium at 30°C until the OD_{600} of the culture reached 0.4–0.6. Cells were harvested by centrifugation at 1600 *g* for 5 min and were washed twice with sterile water. Cell pellets were resuspended in 1 mL 0.1 M LiAc/TE (0.1 M LiAc, 10 mM Tris/HCl, 1 mM EDTA and pH 8.0), and then harvested by centrifugation. Pellets were resuspended by 0.5 mL 0.1 M LiAc/TE. Yeast suspensions were dispensed. Then, 240 μL freshly prepared PEG3350 solution (40% w/v), 36 μL 1 M LiAc (pH 8.0), 100 μg sheared salmon sperm DNA and 0.1 μg plasmid constructs were added. The suspensions were mixed by vortex and then incubated at 30°C for 30 min. DMSO (40 μL) was added into the cell suspensions. Cells were kept at 42°C for 20 min before recovering in 1 mL YPD at 30°C for 2 h. Finally, cells were plated on selection medium and incubated at 30°C for 2–4 days (Qian *et al.* 2009). The numbers of colony forming units (CFU) were counted, and transformation efficiencies (*E*) were calculated with the following formula.

$$E = \frac{\text{CFU}}{\mu\text{gDNA} \times \text{total numbers of cells used for transformation}}$$

Fatty acid extraction and analysis

FFA extraction and methylation from cell cultures of *S. cerevisiae* were carried out according to a previous study (Zhou *et al.* 2016b). After that, the extracted FFAs were analyzed by gas chromatography (Focus GC, ThermoFisher Scientific) equipped with a Zebron TG-5MS GUARDIAN capillary column (30 m × 0.25 mm × 0.25 μm). The GC program was as follows: initial temperature of 40°C, hold for 2 min; ramp to 180°C at a rate of 30°C

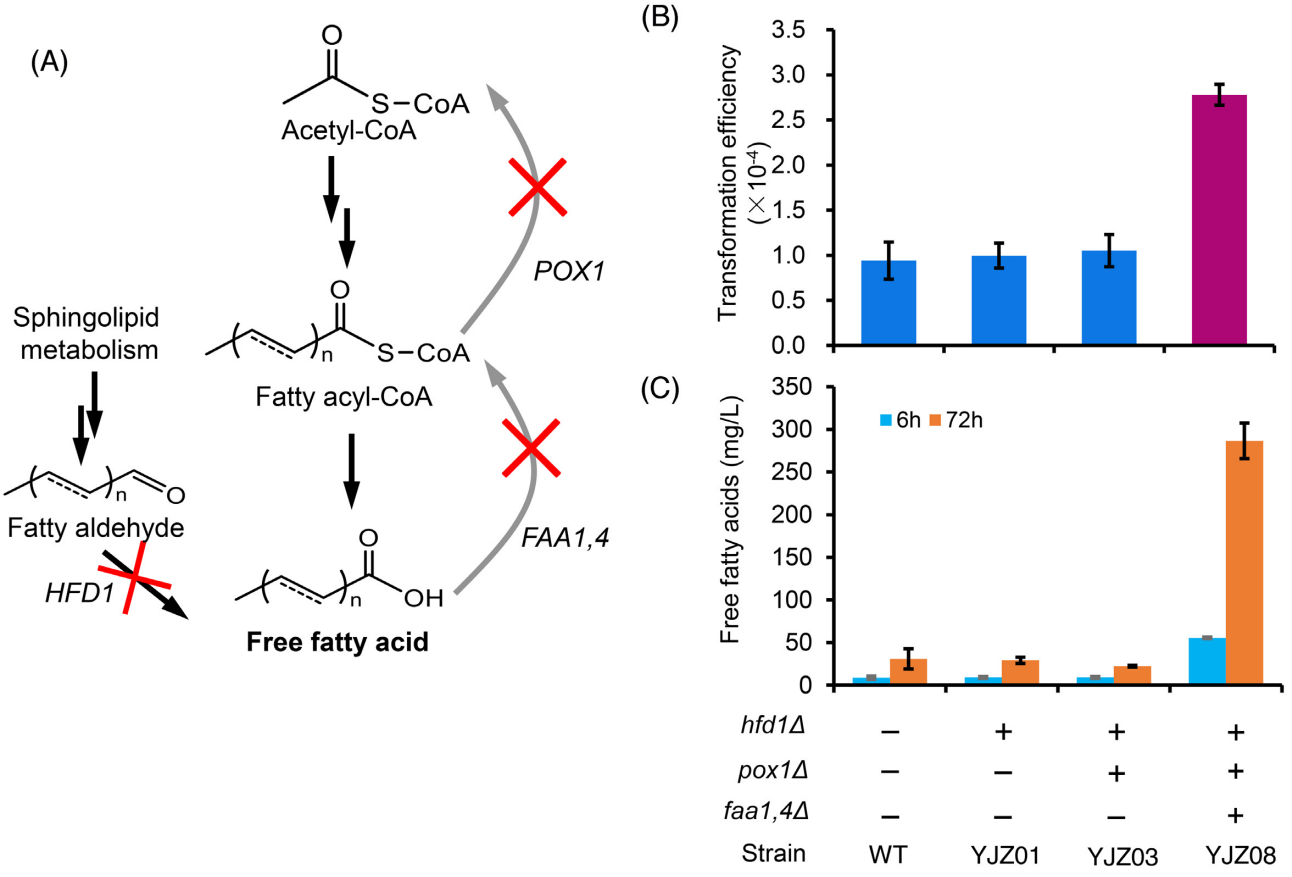

Figure 1. FFA overproduction improved the transformation efficiency. (A) The metabolic pathways for enhancing fatty acids accumulation; (B) the DNA transformation efficiency of various strains with engineered fatty acid metabolism; (C) fatty acid production of various strains at 6 h (yeast cells collected for competent cell preparation) and 72 h. All data represent the mean ± s.d. of biological triplicates.

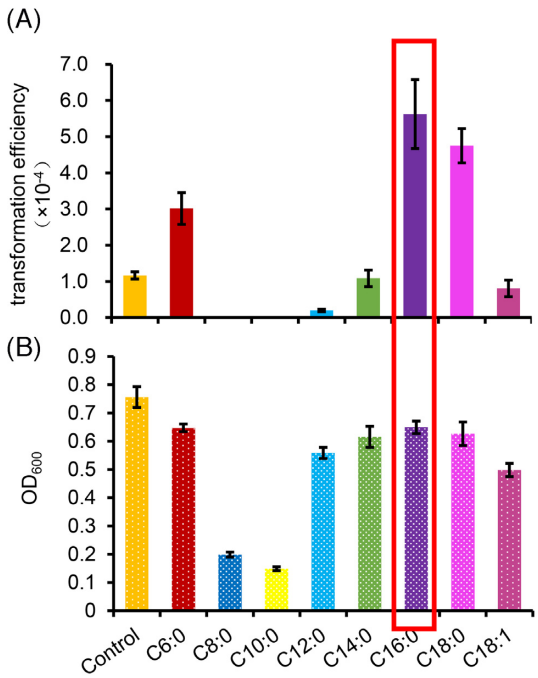

Figure 2. Evaluation of various FFAs on the transformation of *S. cerevisiae*. (A) The DNA transformation efficiency of CEN.PK113-11C when adding various types of fatty acids at 0.5 g/L. (B) The optical densities at 600 nm (OD_{600}) of cell cultures with treatment of different fatty acids after 6 h cultivation. All data represent the mean ± s.d. of biological triplicates.

per min, then raised to 200°C at a rate of 4°C per min and hold for 1 min, and finally raised to 240°C at a rate of 2°C per min and hold for 10 min. The temperature of inlet was kept at 250°C. The flow rate of the carrier gas (helium) was set to 1 mL/min. The data acquisition frequency was acquired at 50 Hz. Final quantification was performed using the Xcalibur software.

Data analysis

Data were analyzed with SPSS17.0. Data that obey normal distribution were displayed as X ± S and were analyzed using independent-samples *t*-test or one-way ANOVA. Equal variances and unequal variances were tested using Bonferroni and Games-Howell, respectively. Statistical significance was defined as $P < 0.05$.

RESULTS

Saccharomyces cerevisiae fatty acids overproducing strain displayed higher transformation efficiency

During our previous construction of *S. cerevisiae* cell factories for production of fatty acid derivatives, we accidentally found that there were big variations of clone number on the selection plates when transforming the same plasmid to several yeast backgrounds with different fatty acid production. In particular, the fatty acid overproducing yeast cells always had high amount of transformation clones. We thus speculated that high-level fatty acids were beneficial for transformation of external DNA into *S.*

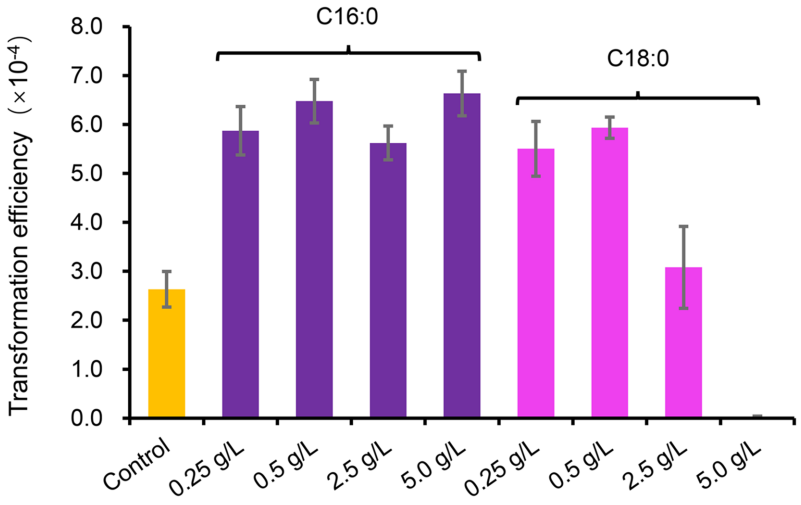

Figure 3. Optimization of C16:0 and C18:0 FFA concentration for improving the transformation of *S. cerevisiae*. All data represent the mean ± s.d. of biological triplicates.

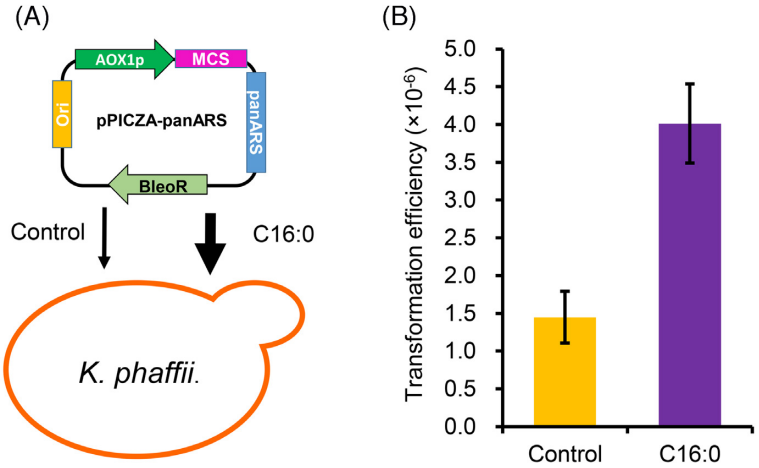

Figure 4. C16:0 FFA promotes transformation of *K. phaffii*. (A) Schematic illustration of transformation of *K. phaffii*; (B) C16:0 FFA promotes transformation of *K. phaffii*. 0.5 g/L C16:0 FFA was added to the YPD medium for cultivating *K. phaffii* cells, which were cultivated for 4–6 h (OD_{600} = 0.4–0.8) and then collected for competent cell preparation. All data represent the mean ± s.d. of biological triplicates.

cerevisiae. To verify this speculation, we investigated several *S. cerevisiae* strains with different rewired fatty acid metabolism. Strain YJZ08 (Zhou *et al.* 2016b), with the quaternary knockout of *pox1*Δ, *faa1*Δ, *faa4*Δ and *hfd1*Δ, had a 2.9 times higher transformation efficiency than the wild-type strain CEN.PK 113–11C when transforming the plasmid pAOH9 (Zhou *et al.* 2016a) harboring a fatty alcohol biosynthetic pathway (Fig. 1A). Consistently, YJZ08 had much higher fatty acid production of 56 mg/L at the time point of competent cell preparation and 286 mg/L at 72 h than the wild-type strain CEN.PK 113-11C (8.7 mg/L at the time point of competent cell preparation and 31 mg/L at 72 h) (Fig. 1B). Deletion of *Hfd1* gene encoding fatty aldehyde dehydrogenase (YJZ01) had no significant difference in plasmid transformation efficiency when compared to the wild-type strain, which was consistent with that there was similar fatty acids in YJZ01 and wild-type strain. Actually, we previously showed that *Hfd1* deletion had marginal effect on FFA accumulation (Zhou *et al.* 2016a), though its deletion was essential for production of fatty aldehyde-derived alkanes and fatty alcohols. *Pox1* gene, which is essential for β-oxidation of fatty acids, was knocked out based on the YJZ01 strain. *Pox1* gene knockout could lead to increased production of fatty acids through reducing degradation of fatty acids by β-oxidation. *FAA1* and *FAA4* genes, encoding the main fatty acyl-CoA synthetases, are responsible for the fatty acid activation and degradation in *S. cerevisiae*. Knockout of *Pox1* had little effect on the fatty acid yield of *S. cerevisiae* in the presence of *FAA1* and *FAA4* genes. As a result, the transformation efficiency did not improve significantly in strain YJZ03 when compared to YJZ01. These results clearly showed that the transformation efficiencies were positively correlated with the FFA accumulation in different genetic background strains (Fig. 1C).

External supplementation of C16 and C18 fatty acids promotes transformation of *S. cerevisiae*

Since YJZ08 produced high level of mixed fatty acids with various chain lengths, we thus investigated whether external supplementation of FFA could improve yeast transformation and which fatty acids played the major role in transformation promotion. Thus, fatty acids with different chain length of C8:0, C10:0, C12:0, C14:0, C16:0, C18:0 and C18:1 were added into YPD medium for cultivating wild-type strain CEN.PK113-11C. After 4–6 h cultivation, the competent cells were prepared and chemically transformed with the plasmid pAOH9. It was shown

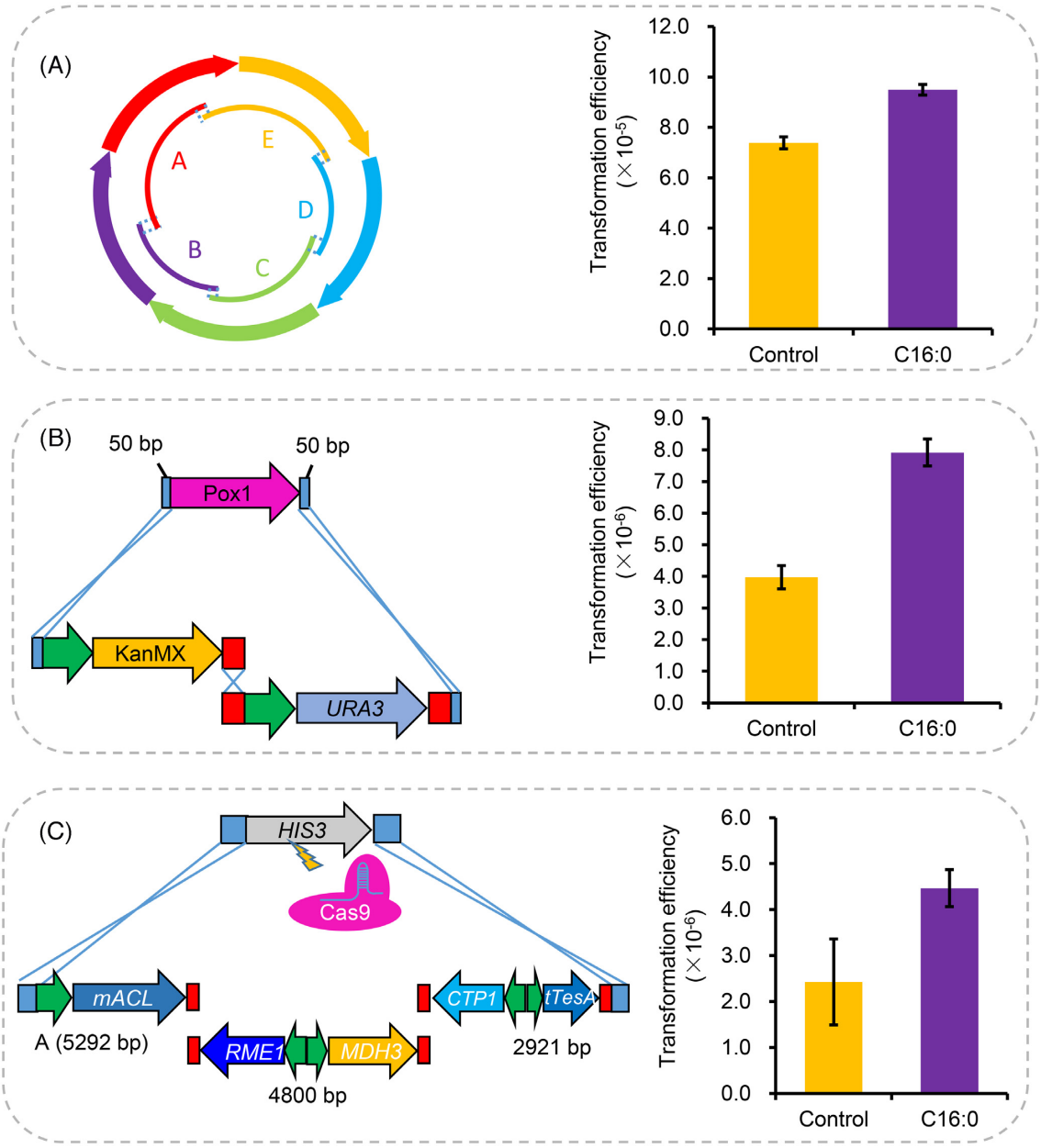

Figure 5. C16:0 promoted multiple DNA fragment assembly in *S. cerevisiae*. (A) C16:0 promoted assembly of multiple DNA fragments into pAOH9 in *S. cerevisiae*; (B) C16:0 promoted transformation of two fragments with short homologous arms (50 bp) for integration into genome of *S. cerevisiae*; (C) C16:0 could promote genome integration of a metabolic pathway with multiple fragments into *S. cerevisiae*. The acetyl-CoA pathway, consisting of *mACL*, *RtME1*, *MDH3*, *CTP1* and *tTesA*, was assembled into HIS3 site of *S. cerevisiae*. All data represent the mean ± s.d. of biological triplicates.

that C8:0 and C10:0 fatty acids were toxic to *S. cerevisiae* strain (Fig. 2B), while C16:0 and C18:0 fatty acids significantly improved the transformation efficiency by 4.8 and 4.1 times, respectively, when compared to the control cell without fatty acid treatment (Fig. 2B). These data showed that external supplementation of fatty acid could significantly improve the transformation efficiency of *S. cerevisiae*, and C16:0 and C18:0 fatty acids were the key components for this transformation promotion.

Optimization of the concentration of C16:0 and C18:0 fatty acids

To further improve the transformation efficiency, we optimized the concentration of C16:0 and C18:0 fatty acids in the YPD media for competent cell preparation. For C16:0 fatty acids, the higher concentration rendered higher transformation efficiency and 0.25–5 g/L had similar transformation efficiency. In contrast to C16:0 fatty acid, the higher concentration of C18:0 fatty acids had negative effect on the transformation efficiency, which suggested that *S. cerevisiae* was more sensitive to C18:0 fatty acid. We thus fixed 0.5 g/L of C16:0 fatty acid for further analysis when studying its effect on transformation efficiency of different yeast strains (Fig. 3).

We also investigated whether C16:0 improved transformation efficiency of *K. phaffii*, a very important methyltrophic yeast for protein expression and a potential versatile host for production of chemicals (Duan, Gao and Zhou 2018). The result showed that 0.5 g/L C16:0 was also able to promote transformation

efficiency of *K. phaffii* by 2.8 times compared with yeast cells without C16:0 treatment (Fig. 4), which should facilitate genetic engineering of *K. phaffii* for industrial application.

C16:0 could promote plasmid-based assembly and genomic integration of multiple DNA fragments

In vivo assembly of multiple DNA fragment is essential for construction and optimization of long biosynthetic pathways (Shao, Zhao and Zhao 2009; Zhou *et al.* 2012), which always require extremely high transformation efficiency. Here, we thus explore whether the external FFA could improve multiple DNA fragment assembly with enhanced transformation efficiency by *in vivo* assembly plasmid pAOH9 (19.5 kb) harboring a fatty alcohol biosynthetic pathway (Zhou *et al.* 2016a). Five fragments in pAOH9 with overlapping sequences (188–632 bp) were amplified and transformed into *S. cerevisiae*. External supplementation of 0.5 g/L C16:0 FFA improved the plasmid assembly by 29% (2279 clones vs 1832 clones without FFA), which indicated that C16:0 facilitated the assembly of large plasmids for long pathway construction (Fig. 5A).

Genomic integration of multiple fragments is more challenging due to the complex structure of chromosome and single copy of genome, and thus requires high amount of external DNA fragment to increase the recombination probability. We first co-transformed the *KanMX* (1357 bp) and *KIURA3* (1284 bp) cassettes into *POX1* site of *S. cerevisiae* by using 50 bp homologous arms. C16:0 fatty acid significantly improved the integration efficiency by 100% compared to the control competent cell without C16:0 treatment (Fig. 5B). We then tried to assemble an acetyl-CoA pathway into HIS3 site (Zhou *et al.* 2016b) by co-transformation of five cassettes expressing ATP:citrate-lyase from *Mus musculus* (*mACL*), malic enzymes from *Rhodosporidium toruloides* (*RtME*), endogenous mitochondrial citrate transporter *Ctp1*, malate dehydrogenase *Mdh3* and truncated *Escherichia coli* thiosterase (*tTESA*). C16:0 fatty acid also significantly improved the integration efficiency by 99.3% compared to the control competent cell without C16:0 treating (Fig. 5C).

DISCUSSION

High transformation efficiency is essential for *in vivo* DNA manipulation toward robust cell factory construction. We here found that external FFA addition improved transformation efficiency significantly inspired from the observation that the high FFA-overproducing strain had higher transformation efficiency (Fig. 1). We also revealed that C16:0 and C18:0 fatty acids were the major component in transformation improvement and 0.5 g/L C16:0 was the best condition. This FFA treatment facilitated the assembly of multiple DNA fragment into episomal vectors and yeast genome.

The transformation improvement suggested that fatty acids could promote the uptake of exogenous DNA into yeast cells through possible ubiquitin-mediated cell membrane endocytosis or cell wall structure alteration (Dell'angelica *et al.* 1996; Kawai, Hashimoto and Murata 2010; Yu *et al.* 2018). Several fatty acids were found to repress (1,3) β-glucan synthase, which is required for biosynthesis of cell wall component in *S. cerevisiae*. It was speculated that the supplementation of fatty acids could affect cell wall structure, which would then promote DNA uptake by *S. cerevisiae* (Dell'angelica *et al.* 1996). Besides, it has been shown that ubiquitin-mediated endocytosis of cell membrane enhanced yeast cell transformation (Yu *et al.* 2018). Since C16:0 and C18:0 are the main component of saturated fatty acids in the yeast cell membrane, external C16:0 and C18:0 FFA might loosen the cell wall structure through inhibiting the activity of (1,3) β-glucan synthase in the cell wall, which was beneficial for uptake of external DNAs (Ko *et al.* 1994).

In summary, we found FFA treatment, during the yeast cell cultivation for component cells preparation, significantly improved the transformation of external DNAs and facilitated the construction of multiple gene pathways in yeast. We expect that this strategy will be helpful in genetic engineering of yeast for basic research and cell factory construction.

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Conflicts of interest. None declared.

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