Highly efficient rDNA-mediated multicopy integration based on the dynamic balance of rDNA in Saccharomyces cerevisiae

Huihui Zheng, Kai Wang, Xiaoxiao Xu, Jing Pan, Xinhua Sun, Jin Hou, Weifeng Liu and Yu Shen* 
State Key Laboratory of Microbial Technology, Institute of Microbial Technology, Shandong University, Qingdao, China.

Introduction

Saccharomyces cerevisiae is a eukaryotic model organism that is also a good cell factory. It is 'generally recognized as safe' by the US Food and Drug Administration (FDA) and is easy to cultivate on a large scale partly because of its fast growth rate. S. cerevisiae is, thus, essential in industrial production of bioethanol and is highly competitive for producing other chemicals and pharmaceutical or industrial heterologous proteins (Ostergaard et al., 2000; Hou et al., 2012; Lian et al., 2018; Nielsen, 2019). Generally, genetic manipulation is necessary to construct cell factories that endow S. cerevisiae with the capability to effectively produce desired products or to use nutrients that are not optimal. Research efforts have, thus, been devoted to developing convenient and efficient genetic tools to express target genes in S. cerevisiae (Blazeck et al., 2012; Lian et al., 2016, 2018; Moon et al., 2016).

The most convenient way to express a gene is first ligating the target gene into a suitable expression vector plasmid and then transforming the recombinant plasmid into S. cerevisiae. Two types of plasmids, CEN-ARS and 2μ, which, respectively, maintain two to five copies and dozens of copies in S. cerevisiae cells, control the copy number of target genes in recombinant strains (Karim et al., 2013). Using the 2μ plasmid as an expression vector along with a strong promoter to control the target gene results in very high expression of the target gene. Maintaining selection pressure in a culture environment is a prerequisite to maintaining plasmids in S. cerevisiae cells, to avoid losing the target gene with the plasmids. However, maintaining the selection pressure obviously increases the cost of culturing, limiting the application of strains with plasmids in long-term and large-scale cultivation in poorly defined media. This limitation negatively affects industrial production (Lian et al., 2018).

Integrating a target gene into the genome allows for stable existence of the gene in S. cerevisiae. Moreover, repetitive sequences can be chosen as homologous recombination sites for genetic engineering to obtain a strain whose genome can incorporate multiple copies of a target gene (Mathiasen and Lisby, 2014). Two repetitive sequences, δ sequence and the rDNA region, are commonly used as homologous recombination sites for

Summary

Engineered Saccharomyces cerevisiae strains are good cell factories, and developing additional genetic manipulation tools will accelerate construction of metabolically engineered strains. Highly repetitive rDNA sequence is one of two main sites typically used for multicopy integration of genes. Here, we developed a simple and high-efficiency strategy for rDNA-mediated multicopy gene integration based on the dynamic balance of rDNA in S. cerevisiae. rDNA copy number was decreased by pre-treatment with hydroxyurea (HU). Then, heterologous genes were integrated into the rDNA sequence. The copy number of the integrated heterologous genes increased along with restoration of the copy number of rDNA. Our results demonstrated that HU pre-treatment doubled the number of integrated gene copies; moreover, compared with removing HU stress during transformation, removing HU stress after selection of transformants had a higher probability of resulting in transformants with high-copy integrated genes. Finally, we integrated 18.0 copies of the xylose isomerase gene into the S. cerevisiae genome in a single step. This novel rDNA-mediated multicopy genome integration strategy provides a convenient and efficient tool for further metabolic engineering of S. cerevisiae.

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*For correspondence. E-mail shenyu@sdu.edu.cn; Tel./Fax +86 532 5863 2401.
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multicopy integration (Liu et al., 2013; Semkviv et al., 2016; Fang et al., 2017; Choi and Kim, 2018). The δ sequence is the long terminal repeat sequence in the yeast retrotransposon Ty1. About 400 copies of δ sequences are thought to be scattered throughout the chromosomes of S. cerevisiae, with slightly different DNA sequences (Dujon, 1996; Bleykasten-Grosshans et al., 2013). The rDNA, which is the ribosomal RNA gene, is highly repetitive in all eukaryotic genomes with copies from 100 to 1000 (Petes, 1979; Long and Dawid, 1980). rDNA-mediated multicopy integration has been applied in various yeasts, such as S. cerevisiae (Fang et al., 2017), Yarrowia lipolytica (Lv et al., 2019) and Lipomyces starkeyi (Oguro et al., 2015).

Approximately 150 copies of rDNA are tandemly repeated on Chromosome XII of S. cerevisiae (Petes, 1979). A rDNA unit of S. cerevisiae is 9.1 kb, including 35S pre-rRNA and 55 rRNA genes and two intergenic spacers, IGS1 and IGS2, separated by 5S rDNA (Fig. 1A). Ageing and some compounds, such as hydroxyurea (HU), a ribonucleotide reductase inhibitor, reduce chromosomal rDNA copy number by causing intra-chromatid recombination between repeats (Fig. 1B) (Kobayashi, 2011; Nelson et al., 2019). Briefly, Fob1 protein binds the replication fork barrier (RFB), such that the replication fork can only pass through in one direction, generating double-strand breaks (DSBs) during the S phase of the cell cycle (Salim and Gerton, 2019). When the rDNA copy number is ~150, Sir2 represses E-pro transcription, and DSB repair follows the ‘maintenance’ status. However, fewer rDNA copies lead to derepression of E-pro transcription. Activated E-pro transcription then causes DSB repair under the ‘amplification’ status, in which the rDNA copy is recovered by unequal sister chromatid recombination (Ida and Kobayashi, 2019).

In the present work, we evaluated the integration efficiency of a heterologous gene to the rDNA sequence under regular and high selection pressure. Then, to increase integration efficiency, we developed a novel rDNA-mediated multicopy integration strategy based on dynamic balance of the rDNA gene copy number. In this novel strategy, yeast is pre-treated by HU to decrease the rDNA copy number (Salim et al., 2017). Then, DNA fragments with the heterologous gene are transformed into these yeast cells. The heterologous genes integrated into the rDNA region of transformants can increase their copy number with rDNA amplification when the transformants are released from HU pressure. Our results reveal that either the ratio of transformants with high copy numbers or the highest copy number reached by transformants are increased when this novel strategy was used instead of a strategy without HU pre-treatment. Moreover, we successfully integrated 18 copies of Ru-xylA, which encodes xylose isomerase (XI), into the rDNA sequences of an S. cerevisiae strain with a single transformation. The xylose consumption rate of the recombinant strain is 0.36 g l⁻¹ h⁻¹, providing a good example of how this novel rDNA-mediated multicopy integration strategy can be applied in metabolic engineering.

Results

Transformants screened under high selection pressure contain more copies of heterologous genes

Increasing selection pressure by adding more antibiotic in the medium is a way to select transformants that integrate more copies of a heterologous gene (S cooker et al., 1994). To clearly demonstrate the effect of increasing the screening pressure, we first evaluated the efficiency of yEGFP integrated into rDNA site under regular and high selection pressure. Several parameters were used for this evaluation, including transformation frequency (obtained transformants per 10⁷ cells heterologous relative fluorescence (RFU) of randomly selected transformants, and copy number of yEGFP in the chromosomes of transformants with the top five highest RFU values).

The DNA fragment rDNA_{xyr-GFP-KanMX4-rDNA_{down} is cut from plasmid pJK and transformed into S. cerevisiae strain MH001. The transformants were selected on the plate with YPD medium containing an additional 500 µg ml⁻¹ G418 (representing regular selection pressure, Fig. 2A) or 20 000 µg ml⁻¹ G418 (representing high selection pressure, Fig. 2B). Fewer transformants, a decrease of 30.4%, were obtained under high selection pressure of 20 000 µg ml⁻¹ G418 than those obtained under regular selection pressure of 500 µg ml⁻¹ G418 (Fig. 3A). The fluorescence intensity of all transformants that were randomly selected in 500 µg ml⁻¹ G418 was lower than 10 000, with the highest value of only 7852 RFU (Fig. 3B). On average, the fluorescence intensity of transformants selected in 20 000 µg ml⁻¹ G418 was higher than those selected in 500 µg ml⁻¹ G418; the highest fluorescence intensity of transformants selected in 20 000 µg ml⁻¹ G418 reached 10 688 (Fig. 3C), which was 1.4-fold higher than the highest transformant selected in 500 µg ml⁻¹ G418.

Transformants with the top five highest fluorescence intensities at both levels of selection pressure were selected out, and the copy number of yEGFP in chromosomes was determined. The transformants selected in 500 µg ml⁻¹ G418 contained 1.2–2.4 copies/cell of yEGFP, while the transformants selected in 20 000 µg ml⁻¹ G418 contained 1.3–4.9 copies/cell of yEGFP (Fig. 3D). Our results suggested that the high selection pressure decreased the number of transformants that we can obtain in a single transformation.
operation; however, it is beneficial to select transformants with a high copy number of a heterologous gene.

Decrease of the rDNA copy number following hydroxyurea treatment

We designed a novel process of rDNA-mediated multi-copy integration based on the theory that rDNA copies maintain homeostasis. In this process, the S. cerevisiae strain was treated with HU to decrease the number of rDNA repeats (Salim et al., 2017). Then, the heterologous genes were integrated into the remaining rDNA sequence. In theory, this provides the copy number of heterologous genes a chance to increase along with the restoration of the copy number of rDNA.

To achieve this design, strain MH001 was cultured in YPD medium with an additional 150 mM HU, and the culture was transferred to fresh medium every 2 days for

Fig. 1. Schematic diagram of rDNA structure in budding yeast (A), and recombination types of rDNA (B). The rDNA is located on yeast chromosome XII in a tandem repeat, occupying 60% of this chromosome, whose length varies with different rDNA copy numbers. Each rDNA unit includes 35S pre-rRNA and 5S rRNA genes, and two intergenic spacers IGS1 and IGS2 separated by 5S rDNA. The direction of 35S rDNA transcription is indicated by arrows. The diagram is not drawn to scale. A non-coding RNA (E-pro) and a replication fork barrier (RFB) are present in IGS1. Fob1 protein binds RFB and makes the replication fork can only pass in one direction, thereby generating double-strand breaks (DSBs). Normally, Sir2 inhibits E-Pro transcription, and cohesin binds to the broken ends, resulting in equal sister chromatid recombination and maintaining the rDNA copy number. When the copy number of rDNA is low, transcription of Sir2 is repressed, thereby activating E-Pro and preventing cohesin from binding to the broken end, promoting unequal sister chromatid recombination. As a result, one of the chromatids gains rDNA copies, and the copy number of rDNA gene maintains a dynamic balance. When cells are ageing or in a state of replication stress, the broken end is looped by intra-chromatid recombination, leading to the loss of rDNA copies.
about 25 generations. The rDNA copy number in the cell’s genome of each batch cultivation was determined. MH001 was shown to have 144.7 ± 28.6 copies/cell of rDNA, while after 2, 4, 6 and 8 days of culture in the medium with HU, the rDNA copy numbers, respectively, decreased to 132.9 ± 18.5, 101.4 ± 16.7, 83.5 ± 2.4 and 81.0 ± 8.3 copies/cell (Fig. 4A). This finding confirmed that the rDNA copy number decreased with HU treatment, and after 75–100 generations, half of the rDNA copies remained and no further loss occurred. This is consistent with the previous report (Salim et al., 2017). A single clone was separated from the cultivation of MH001 in the medium with HU for 8 days and named MH001-8d. To monitor the recovery of rDNA copies, MH001-8d was cultured in YPD medium (HU free) and transferred to fresh YPD every 12 h. The rDNA copy numbers of MH001-8d cells after 24, 48 and 72 h of culture were 187.5 ± 12.4, 138.1 ± 4.8 and 150.2 ± 5.6 copies/cell respectively (Fig. 4B). This suggested that the rDNA copy number of the strain first increased to a high level, and then fell back to normal level, when it moved to HU free condition. The high rDNA copy number at 24 h indicated the excessive recovery, like excessive reaction of cells to some kinds of stresses.

Then, the effect of HU treatment on strain MH001-8d and its progenitor MH001 was investigated. The growth curves of strains were determined in YPD medium with or without HU in a microplate reader. The maximum specific growth rate ($\mu_{\text{max}}$) of MH001-8d in YPD medium was $0.244 \pm 0.002$ h$^{-1}$, which is similar to the $\mu_{\text{max}}$ of MH001 (0.231 ± 0.001 h$^{-1}$). The $\mu_{\text{max}}$ of MH001-8d in YPD with additional 150 mM HU was 0.134 ± 0.002 h$^{-1}$, which is 54.9% of that in YPD; while the $\mu_{\text{max}}$ of MH001 was 0.038 ± 0.011 h$^{-1}$ in the present of HU, which is only 16.5% of that in YPD (Fig. 4C). This result indicated that although HU inhibits growth, the HU treatment does

Fig. 2. Four protocols for integrating a heterologous gene in the rDNA region.
A. general protocol, in which the DNA fragments are transformed into yeast cells and the transformants are selected on the plate with medium containing 400–800 μg ml$^{-1}$ G418; (B) high concentration antibiotics for selection, with DNA fragments transformed into yeast cells and the transformants are selected on the plate with medium containing 20 000 μg ml$^{-1}$ G418; (C) HU pre-treatment and high concentration antibiotics for selection, yeast cells are pre-treated by 150 mM HU for 6–8 days, then the DNA fragments are transformed into the pre-treated cells and the transformants are selected on the plate with medium containing 20 000 μg ml$^{-1}$ G418; (D) HU pre-treatment, high concentration antibiotics for selection and delayed HU pressure release timing, in which yeast cells are pre-treated with 150 mM HU for 6–8 days, then the DNA fragments are transformed into the pre-treated cells and the transformants are selected on a plate with medium containing both 20 000 μg ml$^{-1}$ G418 and 60 mM HU.
not affect strain growth in the HU free condition. The reason may be that having fewer rDNA copies has no effect on the strain growth as suggested by Ide et al. (2010), or the rDNA copy number was restored in a very short time (Fig. 4B), or a combination of the two. Furthermore, MH001-8d is more tolerant to HU compared with MH001, which may be because it adapted to the challenges of HU stress during the 8 days pre-treatment process.

**HU pre-treatment process increased the integration copy number of a heterologous gene in the rDNA region**

We then investigated the effect of HU pre-treatment on the integration copy number. The concentrated DNA fragment rDNA<sup>up</sup>-GFP-KanMX4-rDNA<sup>down</sup> used in MH001 was transformed into strain MH001-8d using the same transformation method. The only difference was that MH001-8d was cultured in YPD with an additional 150 mM HU before transformation. Then, the transformants were selected in YPD medium containing 20 000 µg ml<sup>-1</sup> G418, thus immediately releasing cells from HU pressure after transformation (Fig. 2C). About 1250 transformants per 10<sup>7</sup> cells were obtained in the YPD medium containing 20 000 µg ml<sup>-1</sup> G418 (~1277 transformants per 10<sup>7</sup> cells). This indicated that the HU pre-treatment did not decrease the transformation efficiency.

The fluorescence intensity (Fig. 5B) revealed that among the 96 randomly selected transformants, 9.8% showed a fluorescence intensity greater than 10 000, and the highest one was about twofold higher than the highest transformant derived from MH001 selected in the medium containing 20 000 µg ml<sup>-1</sup> G418. The copy

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**Fig. 3.** The effect of selection pressure on transformation. The DNA fragment containing yEGFP and the KanMX4 expression cassette was transformed into MH001. The transformants were selected on YPD containing 500 or 20 000 µg ml<sup>-1</sup> G418. A. the transformation efficiencies of different processes; (B), the fluorescence intensity of strains selected in 500 µg ml<sup>-1</sup> G418; (C), the fluorescence intensity of strains selected in 20 000 µg ml<sup>-1</sup> G418; (D), the yEGFP copy numbers in transformants with the top five highest fluorescence intensities under both selection pressures. The average copy number is depicted by the black dotted lines and error bars represent triplicates for copy number estimation.
numbers of yEGFP in the MH001-8d transformants with the top five highest fluorescence intensities were 2.5 to 7.3 copies/cell, with an average copy number of 5.0 copies/cell, which is higher than the previous two conditions. These results suggested that the HU pre-treatment increased the average and maximum copy number of the gene integrated into the rDNA region, directly increasing the probability of obtaining strains with high gene expression.

Furthermore, we investigated the effects of the release timing of HU pressure on the transformants. The MH001-8d transformants were first selected in medium containing both G418 and HU, and then cultured in the HU free condition (Fig. 2D). A preliminary experiment (Fig. S2) showed that the stress of 150 mM HU or 90 mM HU with 20 000 μg ml⁻¹ G418 killed almost all the cells. While 60 mM HU is a suitable selective condition, about 1076 transformants were obtained in YPD containing both 20 000 μg ml⁻¹ G418 and 60 mM HU, which was slightly less (14%) than the transformants obtained in YPD medium containing only 20 000 μg ml⁻¹ G418 (~1250 transformants per 10⁷ cells) (Fig. 5A). Among the 96 randomly selected transformants in YPD containing both 20 000 μg ml⁻¹ G418 and 60 mM HU, 17.4% transformants showed a fluorescence intensity greater than 10 000 (Fig. 5C), although the highest one was similar to the highest transformant selected in medium containing 20 000 μg ml⁻¹ G418.

The copy number of yEGFP in the transformants with the top five highest fluorescence intensities was 3.9–10.1 copies/cell, with an average copy number of 8.2 copies/cell, which is higher than the previous three conditions (Fig. 5D). These results suggested that keeping HU stress in the selected medium further increased the average and maximum copy number of the gene integrated into the rDNA region.

Construction of initial xylose metabolic pathway in S. cerevisiae by rDNA-mediated multicopy integration

Expression of heterologous xylose isomerase (XI) is an effective strategy to endow S. cerevisiae, which lacks an initial xylose metabolic pathway, with the capacity to utilize xylose, the second predominant sugar in lignocellulosic hydrolysate after glucose (Yinbo et al., 2006; Liu et al., 2010). Moreover, the expression levels of XI genes seriously affect the efficiency of xylose utilization (van Maris et al., 2007; Hou et al., 2017). In the present work, an XI gene Ru-xylA, cloned from the bovine rumen metagenome, was placed between the TEF1 promoter and ADH1 terminator (Fig. S1B) and separately introduced into MH001 and MH001-8d, using different methods of rDNA-mediated multicopy integration as examples. More specifically, the fragment rDNA_{up}-GFP-KanMX4-Ru-xylA-rDNA_{down} was cut from plasmid pJGKX and transformed into MH001. The transformants were selected on plates containing YPD medium with
500 and 20000 μg ml⁻¹ G418. The fragment rDNA_up-GFP-KanMX4-Ru-xylA-rDNA_down was also transformed into MH001-8d, and the transformants were selected on plates containing YPD medium with 20000 μg ml⁻¹ G418 and 60 mM HU. Ninety-six transformants obtained through each process were randomly selected and cultured in YPX medium and their fluorescence intensity was determined (Fig. S3). The highest RFU of the transformants that integrated fragment rDNA_up-GFP-KanMX4-Ru-xylA-rDNA_down obtained through processes (Fig. 2A–D) was, respectively, 5.8-, 5.0-, 2.5- and 3.2-folds of the highest RFU of the transformants that integrated fragment rDNA_up-GFP-KanMX4-rDNA_down obtained through the corresponding processes (Figs 3 and 5, Fig. S3). The average copy number of Ru-xylA in the transformants with the top five highest fluorescence intensities under both selection pressures. The average copy number is depicted by black dotted lines and error bars represent triplicates for copy number estimation.

Xylose fermentation profiles of recombinant strains

To evaluate the expression of Ru-xylA integrated into the rDNA region, the xylose fermentation profiles of two strains, dC2 and dD12, the MH001-8d transformants that were selected in YPD medium containing 20000 μg ml⁻¹ G418 plus 60 mM HU, were investigated. The Ru-xylA copy numbers of dC2 and dD12 were 18 and 13.5 copies/cell respectively. Meanwhile, aiming to obtain a reference strain with one copy per cell of Ru-xylA, the fragment HXT12_up-GFP-KanMX-Ru-xylA-HXT12_down was integrated into the HXT12 locus (a possible pseudogene encodes a non-functional member of the hexose transporter family, with no repeated
sequences on the genome) of strain MH001. A transformant E9, which showed 0.9 copy of Ru-xylA in the qPCR determination test, was chosen as the reference strain (more details are in Fig. S4). The fermentation was performed in YNBUX in shake flasks with an initial OD600 of 1.0 (Fig. 7). The xylose utilization rate of dC2 was 0.36 g l⁻¹ h⁻¹, which was, respectively, 12.5% and 260% higher than those of dD12 (0.32 g l⁻¹ h⁻¹) and E9 (0.1 g l⁻¹ h⁻¹). Meanwhile, the ethanol produced by dC2 was 6.9 g l⁻¹, which was, respectively, 15.0% and 245% higher than dD12 (6.0 g l⁻¹) and E9 (2.0 g l⁻¹), after 48 h of fermentation (Fig. 7). This result indicated that Ru-xylA was expressed in the rDNA region and supports the viewpoint of previous researches that higher copies of the xylose isomerase gene contribute to higher xylose utilization of the recombinant strain (Verhoeven et al., 2017; Papapetridis et al., 2018).

Genetic stability of multicopy gene in the chromosome

To test the genetic stability of integrated genes, the dD12 and dC2 were cultured in YPX/YPD medium and continuously transferred to the fresh medium every 12 h. The cells totally cultured 0, 24, 48, 72, 96 and 120 h in YPX were collected and their genomes were extracted to determine the copy number of Ru-xylA. The results showed that after 0, 24, 48, 72, 96 and 120 h of cultivation, the Ru-xylA copy number of dD12 cultures was, respectively, 11.8, 11.7, 10.3, 10.2 and 10.8 copies/cell; the Ru-xylA copy number of dC2 cultures was, respectively, 16.2, 17.8, 18.0, 16.3 and 16.4 copies/cell (Fig. 8A). This result indicated that the copy number of Ru-xylA in the YPX medium was stably maintained. However, the situation was different in YPD medium. The number of Ru-xylA in both dD12 and dC2 notably decreased after 48–72 h of cultivation in YPD (Fig. 8B). This is different to popular opinion that the gene integrated in rDNA loci is stable (Fang et al., 2017; Wang et al., 2018).

To investigate the factors affecting stability of integrated gene in rDNA loci, we deleted the FOB1 gene of dD12 and dC2. Then, the genetic stability of Ru-xylA in the result strain dD12 (fob1Δ) and dC2 (fob1Δ) cultured in YPD was determined. The result (Fig. 8C) showed that deletion of FOB1 delayed but did not eliminate the decrease of Ru-xylA copies. As we know, Fob1 binds to the replication fork barrier RFB site and triggers the induction of DSBs, which result in equal sister chromatid recombination or intra-chromatid recombination (Fig. 1) (Kobayashi and Sasaki, 2017). Our result indicated that the Fob1-dependent process may relate to the decrease of gene copy number. However, there are still reasons, which worth further study.

Discussion

Multicopy integration of genes into a genome contributes to stable and high-level expression. However, it is challenging to easily obtain the transformants integrated into high-copy number target genes. Repetitive rDNA sequence is commonly used as the homologous recombination site for multicopy integration (Liu et al., 2013;
The copy number of rDNA maintains a dynamic equilibrium under strict control. Copies lost due to ageing or drug stresses will be recovered by unequal sister chromatid recombination when the cells are released from the stress (Iida and Kobayashi, 2019). Here, based on these properties of rDNA, we provide a simple and high efficiency rDNA-mediated tool for genome editing and metabolic engineering in S. cerevisiae. The most efficient process we used was designed as follows. The number of rDNA repeats was first decreased through HU stress; then the heterologous genes were integrated into the remaining rDNA sequence, transformants were selected under stresses from a high concentration of antibiotic G418 plus HU; finally, following release from HU stress, the number of heterologous genes was increased by unequal sister chromatid recombination (Fig. 2D). Using this method, the maximum copy number of yEGFP increased twofold (from 4.9 copies/cell to 10.1 copies/cell) compared with the process without HU pretreatment (Fig. 2B). Furthermore, the maximum copy number of Ru-xylA increased ~1.7-fold (from 10.3 copies/cell to 18.0 copies/cell). This leads to a xylose utilization rate of one strain (0.36 g l⁻¹ h⁻¹ in YNB medium with additional uracil) that is comparable to the strain with the same background but expressing Ru-xylA through the 2 μ plasmid (0.28 g l⁻¹ h⁻¹ in YNB medium) (Zheng et al., 2020).
The δ-site integration is the other popular repetitive sequence for multicopy integration. A Di-CRISPR platform, which uses CRISPR-Cas to generate double-strand breaks (DSBs) in the δ sites of *S. cerevisiae* chromosome, was used to integrate up to 18 copies of 24 kb combined genes in the δ sites in a single step (Shi et al., 2016). The same strategy was also used for rDNA-integration, used CRISPR-Cas to generate DSBs, ~ 10 copies of GFP gene were integrated in rDNA region (Wang et al., 2018). Practically, the multiple modification is often unavoidable to obtain an excellent engineered strain. Using the method we provided, we can also integrate 18 copies of combined genes in rDNA regions in a single step, and no DNA fragment other than the integrated fragment is required to be introduced into yeast. Thus, we developed another simple and high efficiency tool for genome editing and metabolic engineering in *S. cerevisiae*. The use of this strategy may reduce tedious labour required for iterative gene integration and marker rescue (Choi and Kim, 2018; Lv et al., 2019).

In theory, many factors determined by the genetic background of a strain and the method of transformation may affect the copy number that is integrated. In this work, the rDNA integration efficiency of different processes using the same strain and transformation method under uniform criteria were evaluated. Our results support the viewpoint that copy number is closely related to drug resistance, and increasing selection pressure can increase the copy number of target genes (Scorer et al., 1994). The transformant with the highest copy number of *yEGFP* selected in 20 000 mg l⁻¹ G418 is 4.9 copies/cell (Fig. 2D), which is ~twofold higher than that selected in 500 mg l⁻¹ G418. However, it is obvious that a small number of resistance genes can cause very high resistance. The concentration of G418 increased 40-fold (from 500 to 20 000 mg l⁻¹), while the maximum copy number of the integrated gene increased only twofold (from 2.4 copies/cell to 4.9 copies/cell). Even 1.3 copies/cell (the copy number of *yEGFP*, which is expressed in tandem with the KanMX4 gene) supports cells survival under the stress of 20 000 mg l⁻¹ G418.

To fix the problem of small copy number genes leading to high antibiotic resistance, the attenuated KanMX4 gene was used. The δ-integration transformants with 3–10 copies of attenuated KanMX4 could be selected with moderate antibiotic concentrations (Semkiv et al., 2016). Using a truncated promoter to control the expression of the selection marker gene at a low level achieved a similar result (Lian et al., 2016; Moon et al., 2016; Cui et al., 2021). Since the mechanism of this method to enhance integration by weakening expression of a selection marker gene is completely different from the novel method we designed, these two methods could be

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**Fig. 8.** Genetic stability of multicopy *Ru-xylA* in the chromosome. *Ru-xylA* stability of dD12, dC2 recombinant strains in YPX medium (A) and in YPD medium (B); *Ru-xylA* stability of dD12 (*fob1Δ*), dC2 (*fob1Δ*) recombinant strains in YPD medium.
combined to further increase the copy number of the integrated gene.

We also noticed that the overall copy number of the transformants that integrated the fragment rDNAup-GFP-KanMX4-Ru-xylA-rDNA down was obviously higher than the transformants that integrated the fragment rDNAup-GFP-KanMX4-rDNA down. This may be because the length of the rDNAup-GFP-KanMX4-Ru-xylA-rDNA down fragment (7.3 kb between the two rDNA arms) is more conducive to efficient integration compared with the fragment rDNAup-GFP-KanMX4-rDNA down (5.2 kb between the two rDNA arms). The growth pressure from YPX medium, which uses xylose as the sole carbon source, may also increase the probability of obtaining strains with a high copy number of integrated genes. Furthermore, although fluorescence intensity is correlated with gene copy number, the relationship between them is not a linear proportion. This may be due to the conversion of rDNA loci between heterochromatin and euchromatin.

Conclusion

Using metabolically engineered strategies to build cell factories requires convenient and efficient tools for manipulating DNA. Here, we developed rDNA-mediated multicopy integration in S. cerevisiae, with an HU pretreatment process, to decrease the copies of rDNA. Genes were then integrated into the rest of the rDNA and their copies increased with the recovery of rDNA copies. Using this method, 18 copies of the xylose isomerase gene were integrated into the yeast genome in a single step and no other DNA except our integration fragment needed to be introduced into the cells. The resulting strain has a comparable xylose utilization rate to the strain expressing xylose isomerase gene by 2 μ plasmid. Our work, thus, provides another simple and efficient tool for genome editing and metabolic engineering in S. cerevisiae. Further work is worthwhile to improve the integration efficiency and the stability of integrated genes, as well as try larger DNA fragments.

Experimental procedures

Strains and media

E. coli DH5α was used for plasmid construction and subcloning. DH5α was cultured in LB (Luria–Bertani) medium (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract and 10 g l⁻¹ NaCl) at 37°C. Transformants with plasmids were screened in LB with 100 mg l⁻¹ ampicillin added.

Plasmid pJX7, which contains a xylose isomerase gene, in S. cerevisiae recombinant strain BSGX001 (CEN.PK 113-5d derivative; XK, gre3::PPP, cox4Δ, AEr, pJX7) (Wei et al., 2018) was removed. The resulting strain MH001 lost its xylose-utilizing capacity; however, the overexpressed state of xyulokinase genes and the non-oxidative part of the pentose phosphate pathway (PPP) were retained. Normally, MH001 is cultured in YPD medium (10 g l⁻¹ yeast extract, 20 g l⁻¹ tryptone and 20 g l⁻¹ glucose); MH001-8d, derived from MH001, was cultured in YPD medium with 150 mM HU. YPX medium (10 g l⁻¹ yeast extract, 20 g l⁻¹ tryptone and 20 g l⁻¹ xylose) and YNBUX medium (1.7 g l⁻¹ yeast nitrogen base, 5 g l⁻¹ ammonium sulfate, 20 mg l⁻¹ Uracil and 20 g l⁻¹ xylose) were, respectively, used for the culture and fermentation of strains derived from MH001 or MH001-8d and expressing the xylose isomerase gene. For knocking out FOB1 gene, YPD plus 200 mg l⁻¹ nourseothricin solid medium was used.

DNA manipulation and plasmid construction

Plasmids used in this study are listed in Table 1. yEGFP expression cassette (TEF1p-yEGFP-PGKt), used to identify the expression level of a heterologous gene in the present work, was amplified from plasmid pJFE1-TEF1-GFP. KanMX4 expression cassette (TEF1p-KanMX4-TEF1t), which is the antibiotic selection marker and confers G418 resistance to S. cerevisiae (Jimenez and Davies, 1980), was amplified from plasmid pUG6. The homologous arms of the rDNA fragment were

| Table 1. Plasmids used in this study. |
|--------------------------------------|
| **Plasmids** | **Description** | **Source** |
| pJFE1-TEF1-GFP | CEN4, AmpR, TEF1p-PGK1 t, URA3 | Yang et al. (2021) |
| pUG6 | AmpR, TEF1p-KanMX4-TEFI | (Guldener et al. 1996) |
| pJFE3 | 2μm, AmpR, TEF1p-PGK1 t, URA3 | Hou et al. (2016) |
| pJYCO4 | 2μm, PGK1p-CYC1 t, TEF1p-ADHl, HIS3 | Chen et al. (2013) |
| pJX7 | 2μm, AmpR, TEF1p-RuXl-PGK1 t, URA3 | Hou et al. (2016) |
| pJGK | pJFE3 derivative; 2μm, AmpR, rDNAup-GFP-KanMX4-rDNAdown, URA3 | This study |
| pJGXK | pJGK derivative; 2μm, AmpR, rDNAup-GFP-KanMX4-Ru-xylA-rDNAdown, URA3 | This study |
| Cas9-NAT | CENARS, AmpR, TEF1p-natMX6 TEF1 t | Zhang et al. (2014) |
| pEAZY-DNA-ALG9 | AmpR, NeoR/KanR, rDNA and ALG9 fragments | This study |
| pEAZY-GFP-ALG9 | AmpR, NeoR/KanR, yEGFP and ALG9 fragments | This study |
| pEAZY-RuXl-ALG9 | AmpR, NeoR/KanR, Ru-xylA and ALG9 fragments | This study |

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amplified from MH001 genomic DNA, with primers designed by referring to GenBank BK006945.2. The homologous arms are targeted to the 35S rDNA to avoid damaging elements in the intergenic spacer regions IGS1 and IGS2, which is important for rDNA replication, transcription and copy number maintenance (Fig. 1). yEGFP (Table S2), KanMX4 (Table S2) expression cassette and homologous arms of the rDNA were fused by overlap extension PCR resulting in fragment rDNA-up-gfp-KanMX4-rDNA-down (Fig. S1A). This fragment and plasmid pJFE3 were, respectively, digested with restriction enzymes EcoRI and HindIII and ligated together resulting in plasmid pJGK. Furthermore, the Ru-xylA (GenBank: JF496707.1), gene of Ru-XI, with promoter TEF1p was amplified from plasmid pJX7, the terminator ADH1t was amplified from the plasmid pJYC04, and we fused TEF1p-Ru-xylA with ADH1t to obtain the Ru-xylA expression cassette (TEF1p-Ru-xylA-ADH1t). Then, the Ru-xylA expression cassette was ligated into the restriction enzyme BclI of plasmid pJGK with the opposite direction of yEGFP, resulting in plasmid pJGKX (Fig. S1B).

In order to integrate Ru-XylA gene at HXT12 (Gene ID: 854636) site, the HXT12-up-gfp-KanMX4-Ru-XylA-HXT12-down fragment was constructed. It was amplified from pJGKX plasmid using primers Hxt12-U-F and Hxt12-D-R. Fragment fob1-up-nat-fob1-down was constructed by knocking out FOB1 gene (Gene ID: 851688). The fragment fob1-up and fob1-down were amplified from MH001 genomic DNA; the expression cassette of natMX6 that endow the nourseothricin resistance to transformant was amplified from plasmid Cas9-NAT (Zhang et al., 2014). Then, the three fragments were fused by overlap extension PCR resulting in fragment fob1-up-nat-fob1-down.

In addition to the above plasmids for gene integration, we also constructed three plasmids to build the standard curve of quantitative PCR (qPCR). Briefly, the 25S rDNA fragment, yEGFP fragment and Ru-xylA fragment were separately fused with the ALG9 fragment (Gene ID: 855502), and these fusion fragments were separately ligated into the pEASY-Blunt Cloning Kit (TransGen Biotech, Beijing, China), resulting in the plasmids pEASY-rDNA-ALG9, pEASY-GFP-ALG9 and pEASY-RuXI-ALG9 respectively.

Yeast transformation

The conventional lithium acetate method was used for yeast transformation (Gietz and Schiestl, 2007). For all transformation experiments in this study, the amount of DNA was controlled at 2 μg, which was determined by a spectrophotometer (Ailisheng; Nano-300, Hangzhou, China), and the density of cells used for transformation was about 1 OD.

Fluorescence intensity detection

The fluorescence intensity and cell density (OD_{600}) were simultaneously detected using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments Inc., Vermont, USA). The recombinants obtained through transformation were inoculated in a 96-well plate with an initial OD_{600} of about 0.1. The wavelengths of excitation and emission for detecting green fluorescence were 485 and 528 nm respectively (Cui et al., 2021). Relative fluorescence (RFU) was calculated by normalizing against the cell density detected at 600 nm.

Copy number estimation by quantitative real-time polymerase chain reaction

For the extraction of genomic DNA, cells were cultured in the corresponding medium until mid-log phase, and extraction was carried out according to the manufacturer’s protocol of the genome extraction kit (TIANGEN, Beijing, China). Quantitative real-time polymerase chain reaction (qPCR) was performed on an Applied Biosystems QuantStudio 3™ Real-Time PCR System (Thermo Fisher Scientific, California, USA) by using SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) reagents, and each sample had three techniques in parallel. The oligonucleotide primers used for qPCR are listed in Table S2.

The copy numbers of rDNA, yEGFP or Ru-xylA in strains were determined by q-PCR. ALG9, which is a single-copy gene (Shi et al., 2016), was used as the internal reference gene. Taking the determination of rDNA copy number as an example, the plasmid pEASY-rDNA-ALG9 was linearized by restriction enzyme HindIII and used to prepare the standard curve of linear correlation between CT value and copy number. Then, the copy number of rDNA in the genomic DNA extraction of strains was calculated using a previously described method (Shi et al., 2014).

Genetic stability detection

The determination of genetic stability was performed by following the previous report (Wang et al., 2018). Briefly, strains were cultured in a 100 ml shake flask containing 40 ml YPX orYPD medium and transferred to fresh medium every 12 h. The cells totally cultured 0, 24, 48, 72, 96 and 120 h were collected and their genomes were extracted to determine the copy number of Ru-xylA.
Fermentation and analysis of metabolites

Strains with Ru-xyIa were pre-cultured in YPX medium for 12 h, and then inoculated into 40 ml YNBUX medium with an initial OD600 of 1.0. The fermentation was performed in 100 ml shake flasks at 30°C and 200 rpm. Xylose, ethanol, glycerol, and acetic acid in the fermentation broth were detected by high-performance liquid chromatography equipped with a refractive index detector (RID) (Shimazu, Japan). The column Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) was used. The temperature of the column oven was maintained at 45°C and 5 mM sulfuric acid was used as the mobile phase at a flow rate of 0.6 ml min⁻¹ (Wei et al., 2018).

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Conflict of interest

The authors declare no conflict of interests.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Schematic representation of rDNA-based multiplex integration cassettes combined with KanMX4 marker.

**Fig. S2.** Transformants grown on plates with different concentrations of hydroxyurea in the presence of 20 000 μg ml⁻¹ G418.

**Fig. S3.** GFP fluorescence intensity of strains that integrated the rDNAup-GFP-KanMX4-Ru-xylA-rDNAdown fragment. (A) transformants of MH001 selected in 500 μg ml⁻¹ G418; (B) transformants of MH001 selected in 20 000 μg ml⁻¹ G418; (C) transformants of MH001-8d selected in 20 000 μg ml⁻¹ G418; (D) transformants of MH001-8d selected in 20 000 μg ml⁻¹ G418 plus 60 μM HU.

**Fig. S4.** The fragment HXT12up-GFP-KanMX4-Ru-xylA-HXT12down was integrated into the HXT12 locus. Transformants were randomly selected on the YPD plate with 400 μg ml⁻¹ G418. Basing on the relative fluorescence intensity (A), two strains B2 and G6 with low RU, and three strains E1, E7 and E9 with high RU were select out. The regular PCR was performed to amplify the Ru-xylA fragments from the genome DNA of trains B2, G6, E1, E7 and E9 (B) to make sure that the Ru-xylA was integrated into the genome of the transformants. Lane M is DNA marker; and lane B is the blank control which PCR reaction did not add any genome samples as template. Then the Ru-xylA copy number of them were determined (C), error bars represent standard deviations of triplicates.

**Table S1.** Oligonucleotides used in this study

**Table S2.** ORF sequences of yEGFP and KanMX4 used in this study.