

Creation of a eukaryotic multiplexed site-specific inversion system and its application for metabolic engineering

Corresponding Author: Professor Yi Wu

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Combinatorial optimization is very essential to improve chemical production in microbial cell factories, which normally was realized by promoter replacement/engineering, terminator engineering and RBS regulation. This study constructed an efficient multiplexed site-specific inversion system for promoter shuffling by evolving the tyrosine recombinase Rci enzyme, which enabled the improvement production of β -carotene by more than 7-fold. Overall, this manuscript is very straightforward in establishing and engineering the site-specific inversion system and is well written. However, there are some concerns should be addressed for publishing in the journal Nature Communications.

1. Though this system is functional in optimizing the β -carotene with shuffling the promoters of three biosynthetic genes. however, this titer is still relatively low (< 2 mg/gDCW) compared to literature, which make no sense for demonstrating the feasibility of this approach in metabolic engineering. Suggesting to apply this strategy in a β -carotene overproducing chassis (if I am right, this group has this kind of strain), which can really show if this strategy is synergetic or compromised with traditional metabolic engineering strategies;
2. Fig.4 PCR based genotype verification might be not enough, since there might be some recombination events during the inversion procedure. Suggest conducting the complete genome sequencing (at least sequencing the whole integration cassettes) to verify the correlation between the production and gene inversion.
3. The manuscript can be polished further. For example, there are some duplicated descriptions. For example, line 218-219: However, most recombinases inevitably cause the deletion of DNA fragments among sites when multiple sites coexist, potentially leading to the loss of exogenous genes, is duplicated with line 198-200. Thus, this sentence can be deleted. Line 225-226, 'of metabolic pathway' can be deleted. I can't point out all these kinds of problem. Please kindly check the whole manuscript.
4. Some literatures can be compared and discussed: DOI:10.1002/biot.202300285; DOI:10.1016/j.synbio.2024.02.001; DOI:10.1002/biot.202300137
5. Figure 1 and 4, the gene and promoter names should be italicized

Reviewer #2

(Remarks to the Author)

The manuscript introduces a multiplexed site-specific inversion Rci51-5/multi-sfxa101 system in yeast *Saccharomyces cerevisiae*. The system resulted in significantly increased inversion efficiency and a noticeably lower deletion rate compared to existing Cre/multi-loxP systems, highlighting the effectiveness and potential impact of the established system. Its application in metabolic pathway optimization, showed by a 7-fold increase in beta-carotene yield, highlights the system's applicability. Overall, the text is clear and easy to follow.

Based on Rci8 recombinase and sfxa101 sites, the authors previously developed a DNA inversion system in yeast and mammalian cells (ACS Synth Biol, 2021). In the current study, the authors have developed a mutant Rci protein and several variants of inverted repeats that increase inversion efficiency (by 1000-fold).

The authors tested the efficiency of this enhanced system by inverting various promoters controlling the expression of marker genes and beta-carotene enzymes placed in different orientations between the repeats. It would be interesting to see its potential to improve the production of other chemicals with longer biosynthetic pathways (showing colorful products or can be screened using a biosensor) rather than model three-enzymatic beta-carotene pathway in yeast.

The long-term goal for such systems would be industrial-scale production when application focuses on engineering biosynthetic pathways. It would be insightful to ensure the established system's long-term stability and regulation of inversion frequency between reverse-oriented site regions.

The reported high efficiency could lead to frequent inversion events, disrupting the balance of gene expression and thereby resulting in non-optimal output. The authors showed in Fig. 3 that the Rci system resulted in a low white colony percentage compared to the Cre system in 24 h and didn't compare the numbers of dark orange colonies over time. Adding a reliable metric experiment for the number of inversions within a given timeframe would be beneficial. It is interesting to incorporate inducible backup pathways to prevent the loss of essential functions, as some inversion events might not be non-functional. To improve the system's robustness, showing the result of genomic integration (to stabilize system performance) would be the preferred strategy compared to expressing Rci variants on a centromeric plasmid. Production could also be controlled using orthogonal promoters, allowing more tight regulation of the recombinase enzyme expression than galactose inducible promoter of yeast GAL1.

While the system showed a lower deletion rate than others, deletion leakage cases were mentioned. The authors should discuss potential strategies to minimize these occurrences in more detail, possibly integrating findings from other studies on recombination specificity.

Others:

Fig. 1a: The schematic of the Cre recombinase is unclear; the blue, purple, and brown parts are shown twice without any change.

Fig. 4: A proper description for P promoters is missing, hindering the figure's understanding.

There are a few minor grammatical mistakes: Lines 86: enzymes, 151: a single, 174: changes in amino acid, 404: was used.

Reviewer #3

(Remarks to the Author)

This paper presents an innovative multiplexed site-specific inversion system for eukaryotes, showcasing its significant potential in the field of metabolic engineering. The system, based on the mutant Rci51-5 recombinase and sfxa101 sites, achieves impressive improvements in DNA inversion efficiency while reducing undesired deletion events. The study demonstrates a compelling application of this system in optimizing the metabolic pathway for β -carotene production in yeast, yielding substantial increases in product yield. The work is comprehensive, methodologically sound, and provides valuable insights for future advancements in genome engineering.

Although Han et al., 2021 (ref. 20) is briefly mentioned in the conclusion and discussion, it should be noted that this study shares significant similarities in process and methodology with the current research. Since this study builds upon that foundation, it somewhat diminishes the novelty of the current work. Therefore, the authors should emphasize the advancements made in this study within the discussion section to better highlight its innovative aspects. While the paper does not provide deep insights into the mechanism of Rci-mediated rearrangement, it offers valuable strategies for genome rearrangement applications in eukaryotes, which should be a key focus for revision.

Major Comments:

1. The paper describes two rounds of progressively stringent directed evolution that resulted in the development of the Rci51-5 mutant. However, there are two questions here. Firstly, why not start mutating from the Rci8 mutant, which is a highly specific DNA reversal mutant previously discovered by the author. Secondly, in the Methods section, the authors detail that in the second round of mutation, the induction time was reduced compared to the first round to increase the probability of selecting beneficial mutants. However, in the third round, the induction time was reverted to 12 hours. Please provide an explanation.
2. The authors report that after two rounds of directed evolution, the total inversion efficiency of Rci51-5 reached 36%. However, this is not the primary focus of the study. The emphasis should instead be on the two/three-markers co-inversion efficiency. Although the authors mention the three-markers co-inversion efficiency in the supplementary materials, this information should be integrated into the main text for discussion. Consider separating the analysis of single-marker inversion efficiency from multi-marker inversion efficiency. Evaluating the total inversion efficiency alone makes it difficult to assess the advantages of this mutant, as it is possible that the inversion efficiency at individual sites may be lower than that of the previous Rci8 mutant.
3. The authors identified that the single mutation F246V determines whether Rci has co-inversion capability. After identifying this key site, it is common practice to perform saturation mutagenesis at this site to further improve catalytic efficiency. However, the authors did not carry out this work. Please explain the reason or provide appropriate additional content.
4. The study primarily focuses on the application of this multi-site inversion system in *Saccharomyces cerevisiae*. Given the broad potential of this system, it is recommended that the manuscript briefly verify the feasibility of applying this system to other eukaryotic models (such as mammalian cells). This would broaden the scope and impact of the manuscript. Alternatively, it might be more appropriate to modify the title to specify *S.cerevisiae* rather than eukaryotes.
5. The study focuses on the short-term outcomes following the induction of the recombinase. However, for practical applications in metabolic engineering, it would be valuable to assess the long-term stability of these inversions. This is particularly important in hosts like *S.cerevisiae*, where the high efficiency of homologous recombination could lead to unintended recombination at repeat sequences even without the induction of recombinase. Experiments should be conducted to demonstrate the stability of this system, as this is crucial for its future applications.

Minor Comments:

1. Some figures, particularly Figure 1, could benefit from higher resolution and more detailed labeling to enhance readability. Additionally, please carefully check if the legends for all figures are correctly labeled. For example, there is an error in the legend for Figure 4C.
2. Please explain why chromosome X was chosen for integration. Is the integration location a neutral site? Will different sites

affect the efficiency of DNA inversion?

3. Please clarify the induction conditions for the two recombinases. For example, in the Methods section, it is mentioned that Rci mutants were tested using galactose induction, while in the comparison with the Cre/loxP system, β -estradiol induction is mentioned. Please provide detailed information on the types of inducible promoters to facilitate reproducibility for others.

4. Please clarify in the β -carotene validation section whether promoter inversion requires the pressure of resistance or auxotrophic markers to enhance transformation efficiency. Whether or not selection markers are necessary is crucial for determining the broader applicability of the system.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed my concerns well and the manuscript is ready for publishing.

Reviewer #2

(Remarks to the Author)

The authors made significant efforts to address the concerns and provided thorough data on the long-term stability and robustness of their multiplexed inversion system. Their comparison with the widely used Cre/loxP system demonstrated a significantly lower deletion rate, adding depth to the manuscript. Additionally, they implemented a genetic AND gate to tightly regulate recombinase expression by combining transcriptional control (pGAL1 promoter) with estradiol-regulated nuclear translocation of Rci51-5. This approach effectively minimized leakage and ensured recombinase activity only in the presence of both inducers.

To expand the system's applicability, the authors applied it to a more complex biosynthetic pathway, violacein, which involves five exogenous genes. Their results demonstrated diverse promoter inversion events and colony color variations, indicating different gene expression combinations. While this was a valuable demonstration, one major issue remains: the unpredictability of DNA shuffling outcomes, even over multiple generations. More rounds of shuffling do not necessarily increase the probability of generating colonies (variants) with higher production. Therefore, the solution is to screen the entire (or a significant portion of the) library using high-throughput methods. The authors relied on phenotype-based screening, but distinguishing subtle differences (such as between "dark" and "darker" colony colors) is inherently difficult. This limitation likely explains why they could not identify highly productive variants for violacein and β -carotene.

I believe the authors could potentially discover many higher-producing variants within their library if they systematically assessed the productivity of all variants. For this method, implementing a biosensor-based screening system would provide a more reliable solution. For example, the author could use a transcription factor-based biosensor for other natural product pathways involving nearly the same number of genes as violacein. Biosensors, combined with fluorescence-activated cell sorting (FACS), would enable the sorting of top producers from the entire library. Once the best producer is identified, the inversion system (loxP or Rci51-5) must be deactivated or removed from the strain. This will result in a long-term stable producer suitable for industrial-scale production. This part is still missing, so I suggest that the authors at least acknowledge this limitation and discuss the potential benefits of biosensor-driven screening for this specific methodology in the discussion section.

Reviewer #3

(Remarks to the Author)

I am highly satisfied with the way the authors have addressed these comments, effectively emphasizing the manuscript's novelty. The revisions are outstanding, and I am confident they will attract a wide readership in the relevant field.

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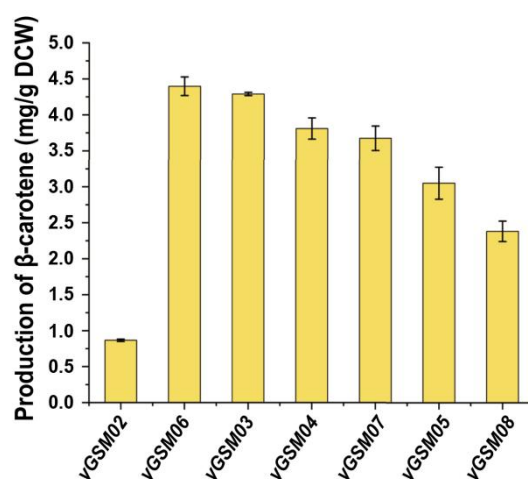
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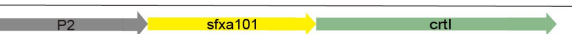
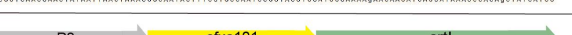
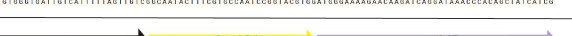
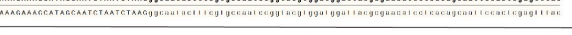
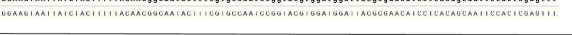
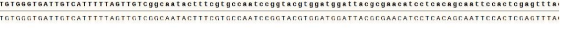
1. Though this system is functional in optimizing the β -carotene with shuffling the promoters of three biosynthetic genes. however, this titer is still relatively low (< 2 mg/gDCW) compared to literature, which make no sense for demonstrating the feasibility of this approach in metabolic engineering. Suggesting to apply this strategy in a β -carotene overproducing chassis (if I am right, this group has this kind of strain), which can really show if this strategy is synergetic or compromised with traditional metabolic engineering strategies;

Response: Thank you for the suggestions. We have applied our strategy into the yeast strain *SyBE_Sc118030*, which was donated by Xiao Wenhai (Doi: 10. 13523/ j. cb. 20160712). The strain has been systematically modified, involving the introduction of the biosynthetic pathway of β -carotene, the knockout of the endogenous gene *ypl062w*, and the overexpression of *thmgr* and the fusion gene *bts1-erg20*. To assess the the feasibility/universality of this approach, our multi-gene promoter regulatory system of β -carotene was introduced into the strain *SyBE_Sc118030* (*yGSM02*). For the uninduced *yGSM02*, the production of β -carotene is 0.87 mg/g DCW in SC-URA culture conditions (not YPD). Upon inducing enzyme expression, we selected colonies with various colors and tested the β -carotene production of these strains. The results of high-performance liquid chromatography (HPLC) showed that the strains show a 2.38-4.40 mg/g DCW (2.75 - 5.08 fold change compared to the control) for the production of β -carotene. Given that our system is plasmid-based, the strain was cultivated in SC-URA medium conditions, which results in a lower yield compared to growth in YPD medium. In the future, the yield might be enhanced by optimizing the fermentation conditions and extraction methods. These results demonstrate that our strategy is synergetic with traditional metabolic engineering strategies to optimize the production.



2. Fig.4 PCR based genotype verification might be not enough, since there might be some recombination events during the inversion procedure. Suggest conducting the complete genome sequencing (at least sequencing the whole integration cassettes) to verify the correlation between the production and gene inversion.

Response: Thank you for your suggestions. Given that our multi-gene promoter regulation system for the β -carotene metabolic pathway was located on the plasmids, we can assess the outcomes of inversion events through plasmid sequencing instead of whole-genome sequencing. By transforming the yeast plasmids back into *E. coli*, we could sequence to verify the substitution of the exogenous gene promoter on the plasmids through first-generation sequencing. We were able to clarify the inversion of the promoter sequence mediated by the *sfx101* site and the sequencing result of 11 plasmids indicated the consistent results with PCR verification (see below). For instance, the replacement of original promoter *pHXT7* of *crtE* gene by promoters P2 was verified in strain *yLJY045*. We also send these plasmids (~ 20 kb) to companies for whole-plasmid third-generation sequencing. Regrettably, none of the companies were able to produce a final sequencing assembly report. This may be attributed to the presence of repetitive multiple *sfxa101* sites in our system, as sequencing of large repetitive sequences has consistently been a challenge (doi: 10.1038/nrg3117, doi: 10.1146/annurev-genom-101722-103045). In the future, it will be necessary to explore more methods to obtain the complete plasmid sequencing. Regarding the possibility of some recombination events during the inversion process that you mentioned, we have indeed detected deletion events within our system and have discussed the details further in the revision.

yLJY081	Reference sequence	
	Sequencing result	AAACAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG AAACAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY045	Reference sequence	
	Sequencing result	CAAGAACTTAATTCGAATAAGACACAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG CAAGAACTTAATTCGAATAAGACACAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY028	Reference sequence	
	Sequencing result	CGGTCAACCAATTAATTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG CGGTCAACCAATTAATTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY037	Reference sequence	
	Sequencing result	TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY044	Reference sequence	
	Sequencing result	ATTCGTGTTGTAATTAACCTTCTGTTTCTAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG ATTCGTGTTGTAATTAACCTTCTGTTTCTAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY055	Reference sequence	
	Sequencing result	AAAGAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG AAAGAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY033	Reference sequence	
	Sequencing result	TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY074	Reference sequence	
	Sequencing result	AAAGAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG AAAGAAGCATAGCAATCTAATCTAAAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY024	Reference sequence	
	Sequencing result	GGAACTTAATTAATCTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG GGAACTTAATTAATCTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY031	Reference sequence	
	Sequencing result	CAAGAACTTAATTCGAATAAGACACAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG CAAGAACTTAATTCGAATAAGACACAGGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
	Reference sequence	
yLJY025	Reference sequence	
	Sequencing result	TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG TGTGGGTGATTGTGCAATTTTAAATTTCTGCGCAATCTTCTGCGCAATCCGGTACGTGGATGGGAAAGCAAGATCAGGATAAACCCACAGCTATCTCTG
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Line 225-226, 'of metabolic pathway' can be deleted. I can't point out all these kinds of problem. Please kindly check the whole manuscript.

Response: Thank you for your suggestions. We have carefully checked and revised the manuscript.

4. Some literatures can be compared and discussed: DOI:10.1002/biot.202300285; DOI:10.1016/j.synbio.2024.02.001; DOI10.1002/biot.202300137

Response: Thank you for your suggestions. We have compared and discussed these literatures within the manuscript in the revision.

5. Figure 1 and 4, the gene and promoter names should be italicized.

Response: Thank you for your suggestions. we have italicized to the names of gene and promoter in the Fig.1 and Fig.4 in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript introduces a multiplexed site-specific inversion Rci51-5/multi-sfxa101 system in yeast *Saccharomyces cerevisiae*. The system resulted in significantly increased inversion efficiency and a noticeably lower deletion rate compared to existing Cre/multi-loxP systems, highlighting the effectiveness and potential impact of the established system. Its application in metabolic pathway optimization, showed by a 7-fold increase in beta-carotene yield, highlights the system's applicability. Overall, the text is clear and easy to follow.

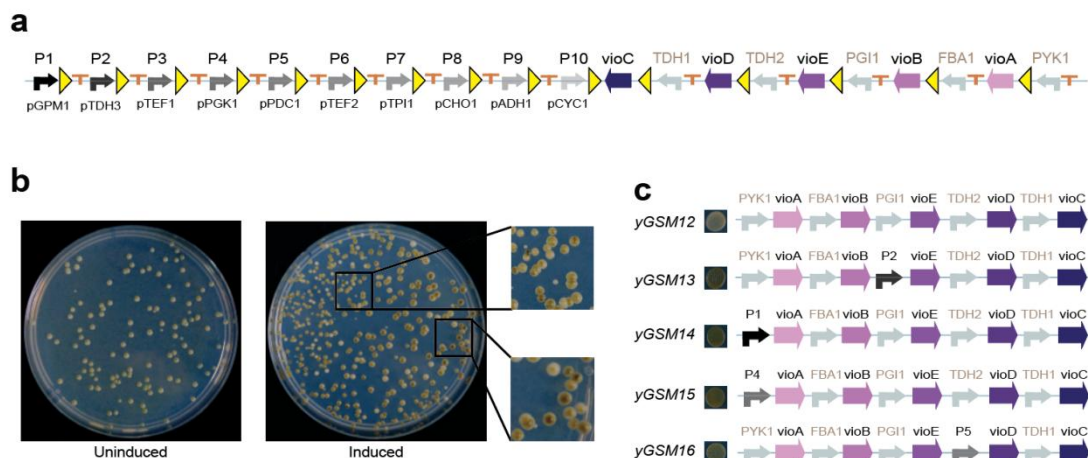
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Response: Thank you for your comments and affirmation of our work.

The authors tested the efficiency of this enhanced system by inverting various promoters controlling the expression of marker genes and beta-carotene enzymes placed in different orientations between the repeats. It would be interesting to see its potential to improve the production of other chemicals with longer biosynthetic pathways (showing colorful products or can be screened using a biosensor) rather than model three-enzymatic beta-carotene pathway in yeast.

Response: Thank you for the suggestions. We have applied the multiplexed site-specific inversion system to the biosynthetic pathway of violacein, which involves five exogenous genes (Figure below). After induced expression with galactose, we observed colonies with various colors. Previous studies have indicated that the darker the color of strains is, the higher the yield of violacein will be (doi: 10.1038/srep11301.). We randomly selected 4 clones with darker colors compared to the control and evaluated the substitution of promoters via PCR. The result showed

that inversions mediated by recombinase Rci51-5 occurred in these strains. For instance, the original promoter *pPYK1* of *vioA* gene was replaced by promoters P1 and P4 in strains *yGSM14* and *yGSM15*, respectively. These results validate that our multiplexed site-specific inversion system, when applied to longer biosynthetic pathways, is also capable of generating diverse combinations of gene expressions and potentially improving the production of other chemicals. We have updated this data in the revised manuscript.



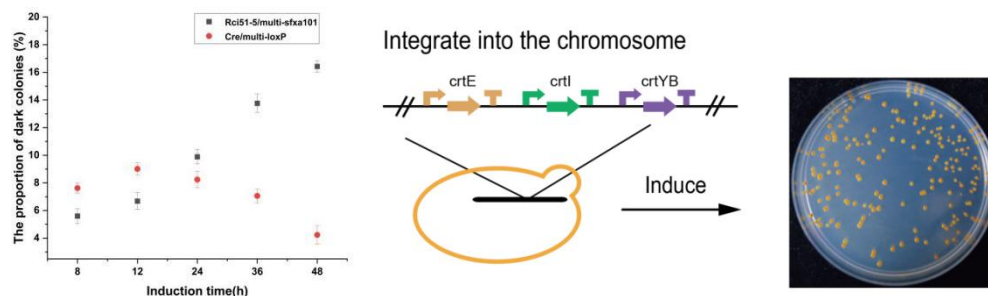
The long-term goal for such systems would be industrial-scale production when application focuses on engineering biosynthetic pathways. It would be insightful to ensure the established system's long-term stability and regulation of inversion frequency between reverse-oriented site regions.

Response: Thank you for your suggestion. Our multi-gene promoter regulation system was located on the plasmids. To test the long-term stability of the system, we subjected the yeast strain (*yLJY023*) to six serial passages for 14 days of growth in the presence of auxotroph screening (SC-Ura medium). Subsequently, we conducted PCR to verify the stability of the system. We randomly selected individual colonies from solid agar plates in each passage to validate the sequence integrity. The PCR results indicated positive bands for all PCR assays present in tested strains.-We also conducted the whole genome sequencing of the passaged strains, and the results demonstrated that no elements were lost after passage. In addition, we evaluated the capability of multiple inversions for the post-passage system. The induced yeast was tested by 33 of PCRTag assays for the pooled cells using the similar method described in the main text (Fig. 4a). The result showed that 33 primer pairs yielded positive bands, indicating that the system could generate inversions after 14 days of passages. We have incorporated these data into the supporting materials. We have updated this data in the revised manuscript.

Figure Redacted

The reported high efficiency could lead to frequent inversion events, disrupting the balance of gene expression and thereby resulting in non-optimal output. The authors showed in Fig. 3 that the Rci system resulted in a low white colony percentage compared to the Cre system in 24 h and didn't compare the numbers of dark orange colonies over time. Adding a reliable metric experiment for the number of inversions within a given timeframe would be beneficial. It is interesting to incorporate inducible backup pathways to prevent the loss of essential functions, as some inversion events might not be non-functional.

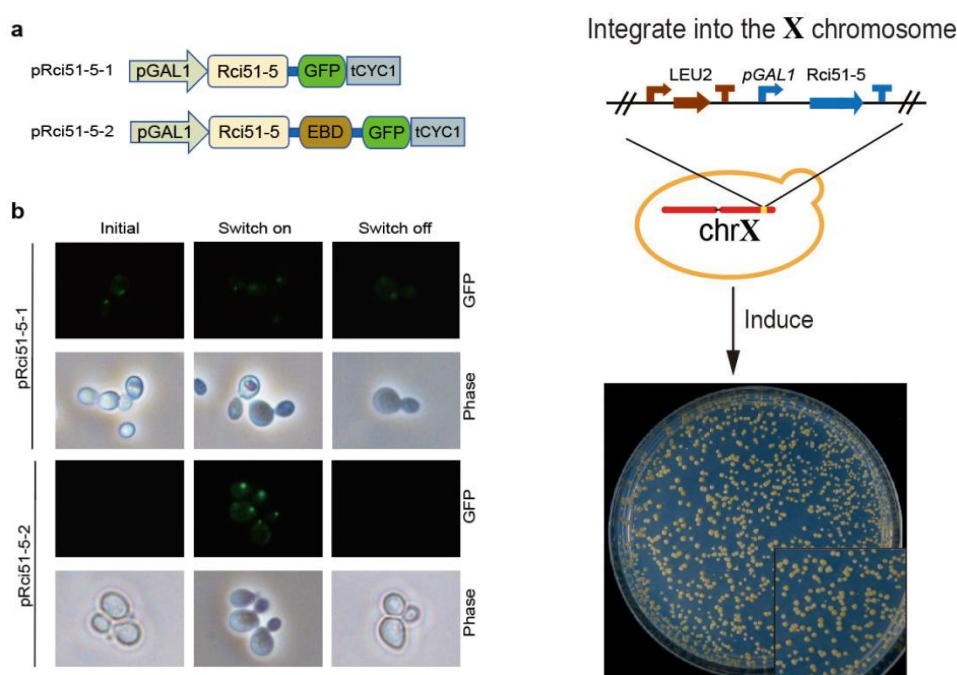
Response: Thank you for the suggestions. We have counted the proportions of dark orange colonies for the Rci51-5/multi-*sfx101* system and the Cre/multi-*loxP* system at various time points. Within 48 h, the numbers of dark orange colonies for Rci51-5/multi-*sfx101* system increased over time, whereas the Cre/multi-*loxP* system demonstrated a decline after 24 h, which was most likely attributed to the accumulation of deletions. We have incorporated these data into the supporting materials. We counted the number of inversions within a given timeframe and this data is displayed in Fig. 4c of the main text. In addition, according to your suggestion, we have integrated the biosynthetic pathway of β -carotene into the yeast genome. The result showed that no white colonies were observed in the induced yeast with incorporated backup pathway. This indicates that the strategy of incorporating a backup pathway is beneficial for optimizing metabolic pathways. Thank you for your valuable suggestions again.



To improve the system's robustness, showing the result of genomic integration (to stabilize system performance) would be the preferred strategy compared to expressing Rci variants on a centromeric plasmid. Production could also be controlled using orthogonal promoters, allowing

more tight regulation of the recombinase enzyme expression than galactose inducible promoter of yeast *GAL1*.

Response: Thank you for the suggestions. To control the expression of recombinase more tightly, we used a genetic AND gate (pGAL1-gene of interest-EBD), referring to our previous work (Nature Communication, 2019, 10(1): 839.). The switch combines the transcriptional control of *pGAL1* and the control of cellular localization of gene of interest-EBD. We constructed a pRci51-5-2 (pGAL1-Rci51-5-EBD-GFP-tCYC1) and another plasmid pRci51-5-1 (pGAL1-Rci51-5-GFP-tCYC1) as a control. The yeasts containing pRci51-5-1 and pRci51-5-2 were cultured under three conditions respectively: (1) The “initial state” media were SC-Leu glucose medium without estradiol; (2) The “switch on” media for pRci51-5-1 and pRci51-5-2 were SGal-Leu medium and SGal-Leu medium containing 1 μ M estradiol, respectively; (3) The “switch off” media were SC-Leu glucose medium without estradiol. For pRci51-5-1, GFP fluorescence were observed under the “initial state” and “switch off”, indicating the leakage of *GAL1* promoter. The cells of pRci51-5-2 exhibited the strong emission clearly from the “switch on”, whereas no fluorescence was detectable in the “initial” and “switch off”. These results indicate that the genetic AND gate can regulate the expression of inversion enzyme tightly. We have integrated the mutant Rci51-5 into the genome and conducted induction without screening enzymes. The result showed that colonies with various colors were observed on the solid agar medium, which was plated the induced yeast cultures. We have updated these data in the revised manuscript.



While the system showed a lower deletion rate than others, deletion leakage cases were mentioned. The authors should discuss potential strategies to minimize these occurrences in more detail, possibly integrating findings from other studies on recombination specificity.

Response: Thank you for the suggestions. We have discussed more potential strategies to

minimize such occurrences in the revision.

Others:

Fig. 1a: The schematic of the Cre recombinase is unclear; the blue, purple, and brown parts are shown twice without any change.

Response: Thank you for the suggestions. We have revised the Fig. 1a in the revision.

Fig. 4: A proper description for P promoters is missing, hindering the figure's understanding.

Response: Thank you for the suggestions. We have revised the Fig. 4a and complemented the description for P promoters in the legend.

There are a few minor grammatical mistakes: Lines 86: enzymes, 151: a single, 174: changes in amino acid, 404: was used.

Response: Thank you for the suggestions. we have revised the description in the revised manuscript.

Reviewer #3 (Remarks to the Author):

This paper presents an innovative multiplexed site-specific inversion system for eukaryotes, showcasing its significant potential in the field of metabolic engineering. The system, based on the mutant Rci51-5 recombinase and sfxa101 sites, achieves impressive improvements in DNA inversion efficiency while reducing undesired deletion events. The study demonstrates a compelling application of this system in optimizing the metabolic pathway for β -carotene production in yeast, yielding substantial increases in product yield. The work is comprehensive, methodologically sound, and provides valuable insights for future advancements in genome engineering.

Although Han et al., 2021 (ref. 20) is briefly mentioned in the conclusion and discussion, it should be noted that this study shares significant similarities in process and methodology with the current research. Since this study builds upon that foundation, it somewhat diminishes the novelty of the current work. Therefore, the authors should emphasize the advancements made in this study within the discussion section to better highlight its innovative aspects. While the paper does not provide deep insights into the mechanism of Rci-mediated rearrangement, it offers valuable strategies for genome rearrangement applications in eukaryotes, which should be a key focus for revision.

Response: Thank you for the suggestions. We have added our description of the advancements made in this study and highlight its innovative aspects of multiplexed genetic engineering, high throughput screening strategy and innovative application scenarios in eukaryotes.

Major Comments:

1. The paper describes two rounds of progressively stringent directed evolution that resulted in the development of the Rci51-5 mutant. However, there are two questions here. Firstly, why not start mutating from the Rci8 mutant, which is a highly specific DNA reversal mutant previously

discovered by the author. Secondly, in the Methods section, the authors detail that in the second round of mutation, the induction time was reduced compared to the first round to increase the probability of selecting beneficial mutants. However, in the third round, the induction time was reverted to 12 hours. Please provide an explanation.

Response: Thank you for the suggestions. In the initial experiment, we indeed constructed two mutant libraries using both Rci and Rci8 as templates, respectively. However, we failed to obtain the mutant with the ability of co-inversion from the mutant library of Rci8. Similar results were also observed in the study by Rix G et al., where the superior mutant BB-5k obtained in the fifth round of mutation was not derived from the optimal mutant in the previous round (doi: 10.1101/2023.11.13.566922). Therefore, in the subsequent studies presented in the paper, we used the superior mutant Rci51-5, which was selected from the mutant library of Rci. In the third round of directed evolution, we tried both the induction time of 8 h and 12 h, but no mutant superior to Rci51-5 was obtained. We have marked both 8 h and 12 h in the Supplemental Figure 2.

The authors report that after two rounds of directed evolution, the total inversion efficiency of Rci51-5 reached 36%. However, this is not the primary focus of the study. The emphasis should instead be on the two/three-markers co-inversion efficiency. Although the authors mention the three-markers co-inversion efficiency in the supplementary materials, this information should be integrated into the main text for discussion. Consider separating the analysis of single-marker inversion efficiency from multi-marker inversion efficiency. Evaluating the total inversion efficiency alone makes it difficult to assess the advantages of this mutant, as it is possible that the inversion efficiency at individual sites may be lower than that of the previous Rci8 mutant.

Response: Thank you for your suggestions. We have integrated the efficiency of co-inversion into the main text for discussion in the revision (Figure. 2b). Furthermore, we have calculated the inversion efficiency of Rci51-5 at a pair of sites, which was ~ 32%, higher than the 8.6% for the mutant Rci8 (doi: 10.1021/acssynbio.1c00132).

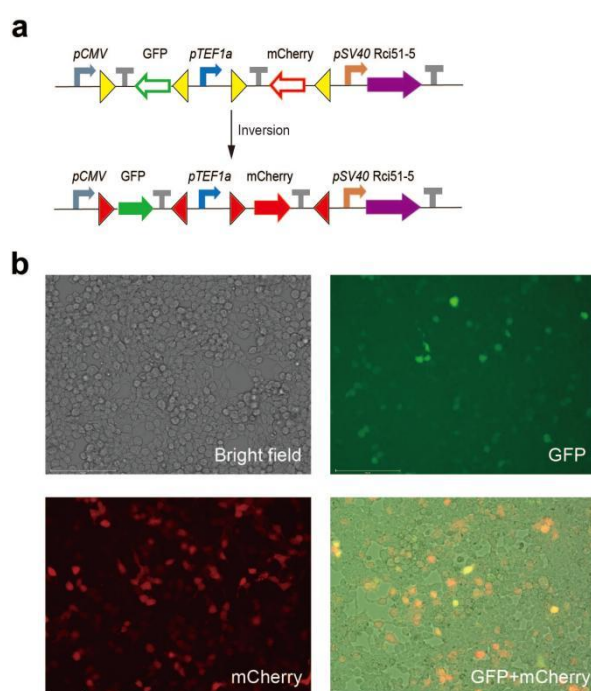
2. The authors identified that the single mutation F246V determines whether Rci has co-inversion capability. After identifying this key site, it is common practice to perform saturation mutagenesis at this site to further improve catalytic efficiency. However, the authors did not carry out this work. Please explain the reason or provide appropriate additional content.

Response: Thank you for your suggestion. Our results show that the multi-site inversion efficiency for synergy of five mutations is significantly higher than that of the single mutation F246V, with an ~ 48-fold increase. Previous reports have indicated that when the contribution of a single mutation to the efficiency is minor, the additive mutational effects should be considered further (doi: 10.1002/anie.201207842, doi: 10.1002/anie.202404880). Therefore, we have directed our subsequent research efforts towards the synergistic effects of multiple mutations supported by the data of combination mutation assay. In the future, the application of various enzyme engineering strategies holds the potential to further enhance the performance of multiple inversion enzymes.

3. The study primarily focuses on the application of this multi-site inversion system in *Saccharomyces cerevisiae*. Given the broad potential of this system, it is recommended that the manuscript briefly verify the feasibility of applying this system to other eukaryotic models (such as mammalian cells). This would broaden the scope and impact of the manuscript. Alternatively, it

might be more appropriate to modify the title to specify *S.cerevisiae* rather than eukaryotes.

Response: Thank you for the suggestions. We have applied the multiplexed site-specific inversion system to mammalian cells. We designed and constructed another inversion screening system in HEK-293T cell. The composition of the screening system is as follows: the promoters and the fluorescent proteins (GFP and mCherry) are oriented in opposing directions, with the GFP and mCherry arranged between a pair of reverse-oriented *sfxa101* sites, respectively. Upon enzyme expression, inversions among the *sfxa101* sites regulate on/off transcription of the fluorescent proteins, enabling cells switch to green and red. We transfected the system into HEK-293T cells and the transfected cells were cultured for 48 h and imaged by fluorescence microscopy. In randomly selected fields, cells were observed to be green, red or to exhibit both colors. The result indicated that the multiplexed site-specific inversion system can apply to other eukaryotic models (such as mammalian cells). We have updated this data in the revised manuscript.



4. The study focuses on the short-term outcomes following the induction of the recombinase. However, for practical applications in metabolic engineering, it would be valuable to assess the long-term stability of these inversions. This is particularly important in hosts like *S.cerevisiae*, where the high efficiency of homologous recombination could lead to unintended recombination at repeat sequences even without the induction of recombinase. Experiments should be conducted to demonstrate the stability of this system, as this is crucial for its future applications.

Response: Thank you for your suggestion. Our multi-gene promoter regulation system was located on the plasmids. To test the long-term stability of the system, we subjected the yeast strain (*yLJY023*) to six serial passages for 14 days of growth in the presence of auxotroph screening (SC-Ura medium). Subsequently, we conducted PCR to verify the stability of the system. We randomly selected individual colonies from solid agar plates in each passage to validate the sequence integrity. The PCR results indicated positive bands for all PCR assays present in tested strains. We also conducted the whole genome sequencing of the passaged strains, and the results

demonstrated that no elements were lost after passage. In addition, we evaluated the capability of multiple inversions for the post-passage system. The induced yeast was tested by 33 of PCRTagassays for the pooled cells using the similar method described in the main text (Fig. 4a). The resultshowed that 33 primer pairs yielded positive bands, indicating that the system could generateinversions after 14 days of passages. We have updated these data in the revised manuscript.

Figure Redacted

Minor Comments:

1. Some figures, particularly Figure 1, could benefit from higher resolution and more detailed labeling to enhance readability. Additionally, please carefully check if the legends for all figures are correctly labeled. For example, there is an error in the legend for Figure 4C.

Response: Thank you for your suggestions. We have carefully checked and revised the Figures in the revision.

2. Please explain why chromosome X was chosen for integration. Is the integration location a neutral site? Will different sites affect the efficiency of DNA inversion?

Response: This integration site selected in this study has been validated as a neutral site in our previous study (doi: 10.1038/s41467-020-18222-0). As for the effect of different sites on the efficiency of DNA inversion, our previous research has found that different chromosomal loci exhibit varying frequencies of rearrangements induced by the SCRaMbLE system (doi: 10.1093/nsr/nwad073). Therefore, we think it is reasonable to consider the position effect for future study.

3. Please clarify the induction conditions for the two recombinases. For example, in the Methods section, it is mentioned that Rci mutants were tested using galactose induction, while in the comparison with the Cre/loxP system, β -estradiol induction is mentioned. Please provide detailed

information on the types of inducible promoters to facilitate reproducibility for others.

Response: Thank you for your suggestions. We have updated the detailed descriptions in the revision.

4. Please clarify in the β -carotene validation section whether promoter inversion requires the pressure of resistance or auxotrophic markers to enhance transformation efficiency. Whether or not selection markers are necessary is crucial for determining the broader applicability of the system.

Response: In our research, the selection markers were not used to screen the inversion events itself, and only used to prevent the loss of plasmids. If an efficient screening system of inversion is developed, it might broader applicability of the system. Thank you for your suggestions. We have updated the detailed descriptions in the revision.

All revisions were highlighted in the revised manuscript.

Reviewer #1 (Remarks to the Author):

The authors addressed my concerns well and the manuscript is ready for publishing.

Response: Thank you for your valuable suggestions to improve the quality of our manuscript.

Reviewer #2 (Remarks to the Author):

The authors made significant efforts to address the concerns and provided thorough data on the long-term stability and robustness of their multiplexed inversion system. Their comparison with the widely used Cre/loxP system demonstrated a significantly lower deletion rate, adding depth to the manuscript. Additionally, they implemented a genetic AND gate to tightly regulate recombinase expression by combining transcriptional control (pGAL1 promoter) with estradiol-regulated nuclear translocation of Rci51-5. This approach effectively minimized leakage and ensured recombinase activity only in the presence of both inducers.

To expand the system's applicability, the authors applied it to a more complex biosynthetic pathway, violacein, which involves five exogenous genes. Their results demonstrated diverse promoter inversion events and colony color variations, indicating different gene expression combinations. While this was a valuable demonstration, one major issue remains: the unpredictability of DNA shuffling outcomes, even over multiple generations. More rounds of shuffling do not necessarily increase the probability of generating colonies (variants) with higher production. Therefore, the solution is to screen the entire (or a significant portion of the) library using high-throughput methods. The authors relied on phenotype-based screening, but distinguishing subtle differences (such as between "dark" and "darker" colony colors) is inherently difficult. This limitation likely explains why they could not identify highly productive variants for violacein and β -carotene.

I believe the authors could potentially discover many higher-producing variants within their library if they systematically assessed the productivity of all variants. For this method, implementing a biosensor-based screening system would provide a more reliable solution. For example, the author could use a transcription factor-based biosensor for other natural product pathways involving nearly the same number of genes as violacein. Biosensors, combined with fluorescence-activated cell sorting (FACS), would enable the sorting of top producers from the entire library. Once the best producer is identified, the inversion system (loxP or Rci51-5) must be deactivated or removed from the strain. This will result in a long-term stable producer suitable for industrial-scale production. This part is still missing, so I suggest that the authors at least acknowledge this limitation and discuss the potential benefits of biosensor-driven screening for this specific methodology in the discussion section.

Response: Thank you for your valuable suggestions to improve the quality of our manuscript. We have discussed the limitations of our system in aspects of high-throughput screening method and long-term stability.

Reviewer #3 (Remarks to the Author):

I am highly satisfied with the way the authors have addressed these comments, effectively emphasizing the manuscript's novelty. The revisions are outstanding, and I am confident they will attract a wide readership in the relevant field.

Response: Thank you for your valuable suggestions to improve the quality of our manuscript.

All revisions were highlighted in the revised manuscript.