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Discovery, Characterization, and Application of Chromosomal Integration Sites for Stable Heterologous Gene Expression in *Rhodotorula toruloides*

Background/Objective

Rhodotorula toruloides is an oleaginous yeast uniquely suited to produce acetyl-CoA-derived chemicals such as triacetic acid lactone (TAL), terpenes, and fatty alcohols. However, the lack of well-characterized genomic integration sites has impeded the metabolic engineering of this organism. Here, we report a set of computationally predicted and experimentally validated chromosomal integration sites in *R. toruloides*.

Approach

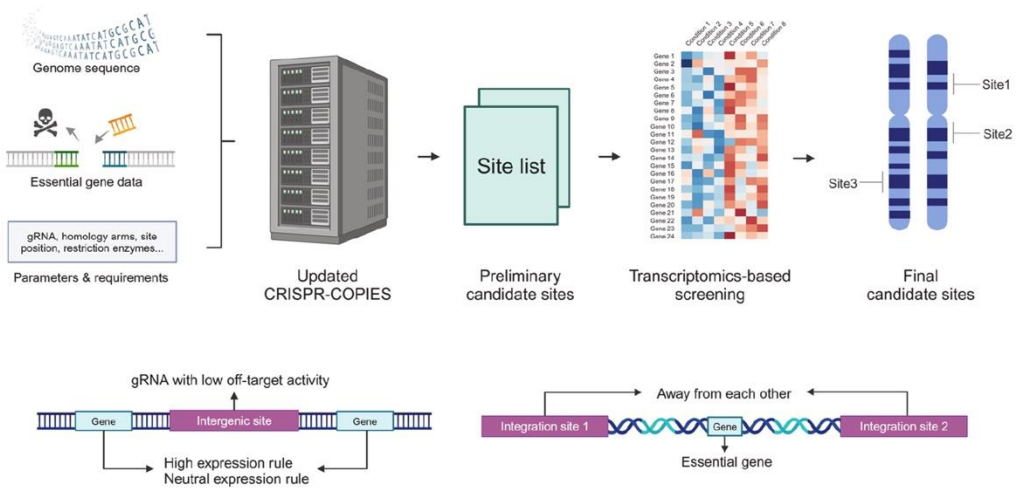
We incorporated essential gene information and transcriptomic data in an in-silico platform to identify candidate integration sites for CRISPR/Cas9-based gene integration in *R. toruloides*. The sites were experimentally characterized for integration efficiency, gene expression levels, impact on cell growth, and long-term stability. Applications with the integration sites were then tested.

Results

With selected sites, simultaneous double and triple integrations were achieved, and long functional pathways were efficiently integrated. Additionally, a new inducible marker recycling system was developed, allowing multiple rounds of integration at the characterized sites for fine-tuning the gene copy number.

Significance/Impacts

This study provides the first set of integration sites in *R. toruloides*, which can accelerate metabolic engineering efforts and genetic tool development.



***In silico* platform for selecting candidate integration sites.**

Xu et al. 2025. "Discovery, Characterization, and Application of Chromosomal Integration Sites for Stable Heterologous Gene Expression in *Rhodotorula toruloides*." *Metabolic Engineering*. DOI: 10.1016/j.ymben.2025.02.004.