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Article

CRISPR-Mediated rDNA Integration and Fluorescence Screening for Pathway Optimization in *Pichia pastoris*

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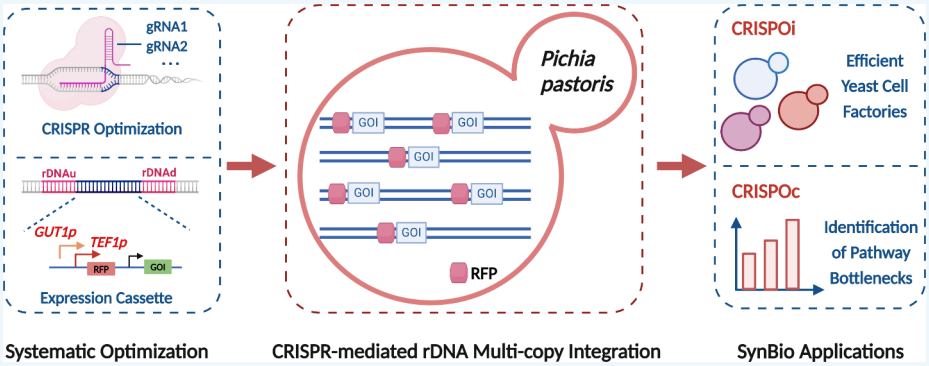

ABSTRACT: Gene dosage amplification is an effective strategy to improve the performance of heterologous genes and pathways. *Pichia pastoris* is an excellent recombinant protein expression host with high efficiency in protein folding and glycosylation. However, the traditional iterative multicopy integration method typically faces challenges such as being time-consuming and having high cost and potential gene mutations. Accordingly, we established CRISPR-mediated rDNA integration and fluorescence screening for pathway optimization (CRISPO) for multicopy pathway integration in a single-step and antibiotic-free manner. With geraniol biosynthesis as a case study, we designed CRISPO based on the use of glycerol-induced and glucose-repressed promoters (CRISPOi) or strong constitutive promoters (CRISPOc) to drive the expression of the red fluorescent protein mCherry as the screening marker. We employed CRISPOi for stable strain construction by multicopy integration of the geraniol synthase encoding gene, achieving a 19.5-fold increase in geraniol production. We demonstrated CRISPOc for visualizing and determining the rate-limiting steps of the mevalonate pathway, with HMG1 and ERG12 identified as the major rate-limiting enzymes through two rounds of exploration. Ultimately, CRISPO enabled us to construct an engineered *P. pastoris* strain producing 1.66 g/L geraniol (with a total of 2.12 g/L monoterpenoids) and 6.27 g/L geraniol (with a total of 6.48 g/L monoterpenoids) in 24-well plates and 5 L fermenters, respectively, representing the highest titer and productivity of geraniol ever reported. CRISPO is an important addition to the synthetic biology toolbox for the construction and optimization of *P. pastoris* cell factories.

KEYWORDS: *Pichia pastoris, CRISPR, Multicopy integration, Geraniol, Synthetic biology*

1. INTRODUCTION

Pichia pastoris has attracted increasing attention in biotechnology for its remarkable ability to express heterologous proteins. Its superiority lies in the high production of recombinant proteins with precise folding and glycosylation.[1,2] Owing to recent advances in synthetic biology, there is a growing interest in establishing *P. pastoris* for the synthesis of certain small molecules, particularly natural products.[3,4] This includes but is not limited to lycopene,[5] β-carotene,[6] astaxanthin,[3] menaquinone-4,[7] lovastatin,[8] and catharanthine.[9] Notably, the highest production of α-santalene[10] and *cis*,*trans*-nepetalactol[11] has been achieved in *P. pastoris*. Some natural product biosynthesis enzymes (such as polyketide synthases and cytochrome P450s) often suffer from low catalytic activities,[12] which should be expressed at high levels to maximize metabolic flux towards the

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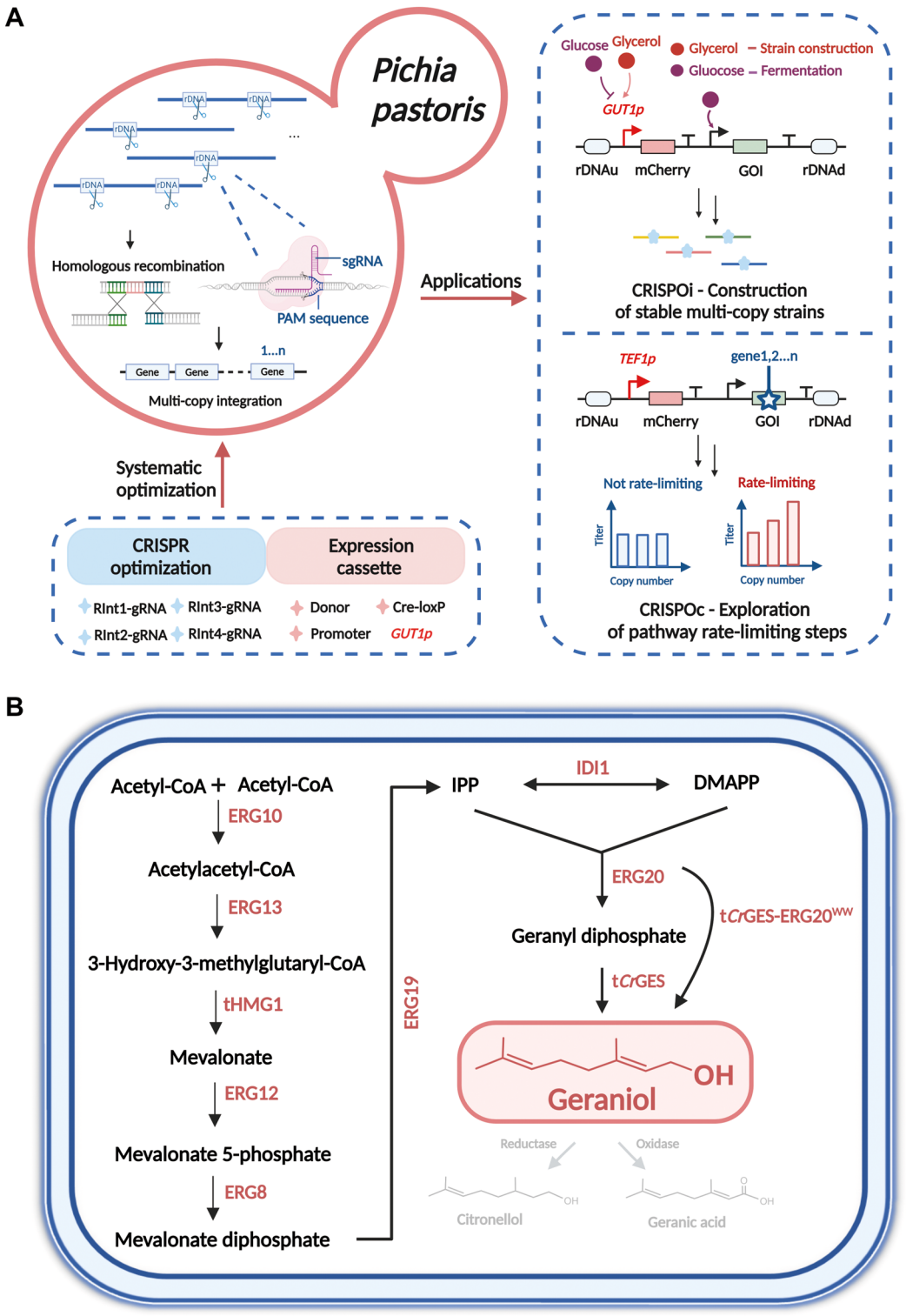

Figure 1. Schematic diagrams for the design and application of CRISPO. (A) Construction and application of CRISPO. Multicopy integration is mainly based on the CRISPR/Cas9 system and repeated NTS sequences at the rDNA loci. CRISPO optimization is mainly divided into two modules: the CRISPR/Cas9 module and the *mCherry* expression cassette module. Two sets of promoters were employed for different applications. Glycerol-induced and glucose-repressed promoter *GUT1p* was used for strain construction (CRISPOi), where the screening marker was expressed during the screening stage in glycerol while silenced during the fermentation state in glucose. Constitutive promoter *TEF1p* was used to quantify heterologous gene copy numbers (CRISPOc), facilitating the exploration of pathway rate-limiting steps. GOI: gene of interest. (B) Geraniol biosynthetic pathway. ERG10: acetoacetyl-CoA thiolase; ERG13: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; tHMG1: truncated HMG-CoA reductase without the regulatory domain; ERG12: mevalonate kinase; ERG8: phosphomevalonate kinase; ERG19: mevalonate pyrophosphate decarboxylase; IDI1: isopentenyl diphosphate isomerase; $ERG20^{WW}$: ERG20 (F96W, N127W) mutant acting as a geranyl pyrophosphate (GPP) synthase; tCrGES: truncated geraniol synthase from *Catharanthus roseus*; tCrGES-$ERG20^{WW}$: fusion of tCrGES and $ERG20^{WW}$. Citronellol, a reduction product of geraniol. Geranic acid, an oxidative product of geraniol.

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desired products. Traditionally, gene dosage amplification is a commonly employed strategy to boost foreign gene expression levels.[13−15] Considering the instability of episomal plasmids in *P. pastoris*, multicopy genome integration should be performed for gene dosage amplification. As the traditional stepwise integration strategy is time-consuming and labor-intensive, there is an urgent need to implement multicopy integration technology to construct stable and high-expression strains efficiently.

In *P. pastoris*, multicopy integration is typically achieved through a process known as post-transformational vector amplification (PTVA),[16] which involves the transformation of a vector carrying an antibiotic marker, such as zeocin or G418, and selection for multicopy clones by gradually increasing the antibiotic concentration. PTVA, combined with the rDNA non-transcribed sequences (NTS) as the integration target, has been successful in generating multicopy *P. pastoris* strains for high-level expression of recombinant proteins.[17] However, this method has been criticized for being time-consuming and costly and often leading to an abnormal increase in antibiotic resistance, probably through adaptive evolution. The PTVA process has also been found to result in the amplification of a fragment with the resistant gene flanked by two homologous arms instead of the entire vector containing the target gene.[18] In addition, antibiotic treatment (e.g., Zeocine) has been associated with elevated mutation rates and genomic alterations in yeast,[19] which are undesired features for biotechnological applications. In other words, PTVA does not necessarily result in the construction of stable multicopy strains and high-level expression of recombinant proteins, highlighting the need for alternative strategies to achieve multicopy integration in an antibiotic-free manner in *P. pastoris*.

On the other hand, many novel strategies for achieving multicopy integration have been reported in other yeast species, such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica*.[20−22] For example, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has enabled marker-less multicopy integration in *S. cerevisiae*, allowing for the insertion of up to 18 copies of a 24 kb pathway in a single transformation event.[23] The combination of δ sequences for site-specific integration with the *TPI1* gene from *Schizosaccharomyces pombe* (POT1) as a selection marker has resulted in high-copy integration and stable expression of heterologous proteins in *S. cerevisiae*.[21] In *Y. lipolytica*, the repetitive rDNA sequences and the high recombination efficiency of the Cre-loxP system for recycling selection markers were combined to establish a versatile method to integrate multicopy metabolic pathways iteratively.[20,24] However, relatively less research has been conducted in *P. pastoris*.

In the present study, we established CRISPR-mediated rDNA integration and fluorescence screening for pathway optimization (CRISPO) for efficient single-step, antibiotic-free, and multicopy integration. Instead of conventional iterative antibiotic selection, CRISPO resulted in the construction of multicopy strain in a quantitative manner, with the use of the red fluorescent protein mCherry for screening. Then, using geraniol biosynthesis as a case study, we demonstrated the applications of CRISPO for the construction of stable-producing strains and the identification of pathway rate-limiting steps (Figure 1). For strain construction, we designed CRISPO with the expression of *mCherry* driven by glycerol-induced yet glucose-repressed promoters (CRISPOi), enabling efficient screening under glycerol conditions and high production under glucose conditions. Through single-step multicopy integration of geraniol synthase, we achieved a maximal 19.5-fold higher geraniol titer than that of the single-copy strain. For identifying the mevalonate (MVA) pathway bottlenecks, we designed CRISPO with the expression of *mCherry* driven by constitutive promoters (CRISPOc) and quantified the copy number effect of each pathway gene on geraniol biosynthesis. Through two rounds of CRISPOc investigation, we identified HMG1 and ERG12 as the major rate-limiting enzymes of the MVA pathway for geraniol biosynthesis. The resultant debottlenecked strain produced 1.66 g/L geraniol (with a total of 2.12 g/L monoterpenoids) in 24-well plates and 6.27 g/L geraniol (with a total of 6.48 g/L monoterpenoids) in a 5 L fed-batch fermenter, representing the highest titer and productivity of geraniol ever reported.

2. METHODS

2.1. Strains, Media, and Reagents. *Escherichia coli* DH5α (Tsingke Biotech, China) was used for gene cloning and plasmid amplification. Recombinant *E. coli* strains were cultured in LB medium containing 100 mg/L ampicillin at 37 °C. Yeast strains were routinely cultivated in YPD (1% yeast extract, 2% peptone, and 2% glucose), YPM (1% yeast extract, 2% peptone, and 2% methanol), YPG (1% yeast extract, 2% peptone, and 2% glycerol), and YPO (1% yeast extract, 2% peptone, 2% mannitol, and 0.2% oleic acid) at 30 °C and 250 rpm. When necessary, G418 sulfate, Hygromycin B (Sangon Bio-tech, Shanghai, China), and Zeocin (Thermo Fisher Scientific) were supplemented with a final concentration of 200 mg/L, 200 mg/L, and 100 mg/L, respectively. All restriction enzymes, DNA polymerase, and T4 DNA ligase were purchased from NEB (New England Biolabs). Analytical standards of geraniol, citronellol, citral, and geranic acid were purchased from Yuanye Bio-tech (Shanghai, China). All chemicals were from BBI Life Sciences unless specifically mentioned.

2.2. Plasmid Construction. The gRNA sequences were designed using the Benchling CRISPR tool (https://benchling.com) and inserted into the corresponding helper plasmids (HZP-sgRNA, HHP-sgRNA, and HGP-sgRNA) constructed in our previous studies.[3] The integration donor helper plasmids were constructed by cloning the upstream (approximately 500 bp) homology arms, expression cassettes, and downstream (approximately 500 bp) homology arms into pUC19 using Golden-Gate Assembly. The geraniol pathway genes, *ERG8*, *ERG10*, *ERG12*, *ERG13*, *ERG19*, *tHMG1*, and *IDI1*, were amplified from the genome of *S. cerevisiae* BY4741. $ERG20^{F96W-N127W}$ (abbreviated as $ERG20^{WW}$) and *t43CrGES-*$ERG20^{F96W-N127W}$ (abbreviated as *tCrGES-*$ERG20^{WW}$) were obtained from previous studies.[25] They were all cloned into the donor helper plasmids of rDNA-HAup-loxP-*TEF1p-mCherry-0547t*-loxP-*GAPp-AOX1t*-HAdown. All the plasmids used in this study are listed in Supplementary Table S1, and the Benchling links for some important plasmid maps and sequences are listed in Supplementary Table S2. The corresponding oligonucleotides are listed in Supplementary Table S3.

2.3. Yeast Transformation and Strain Construction. All genetic modifications in *P. pastoris* were performed using the CRISPR/Cas9 system. *P. pastoris* were transformed using Lin-Cereghino's method.[26] Heterologous gene expression cassettes were amplified by PCR with 40 bp or 500 bp homology arms to the target chromosomal loci and co-transformed with the corresponding gRNA plasmids to the Cas9-expressing *P. pastoris* strains. All the yeast strains constructed in this study are listed in Supplementary Table S4.

2.4. Measurement of mCherry Fluorescence Intensity. *P. pastoris* colonies were randomly selected from the selection plates and inoculated into 1 mL of YPD medium. They were cultured at 30 °C and 800 rpm in 96 deep-well plates in a high-speed shaker. After 48 h of culture, 200 μL of culture broth (diluted 10 times) was transferred to a 96-well plate for fluorescence detection. By using a

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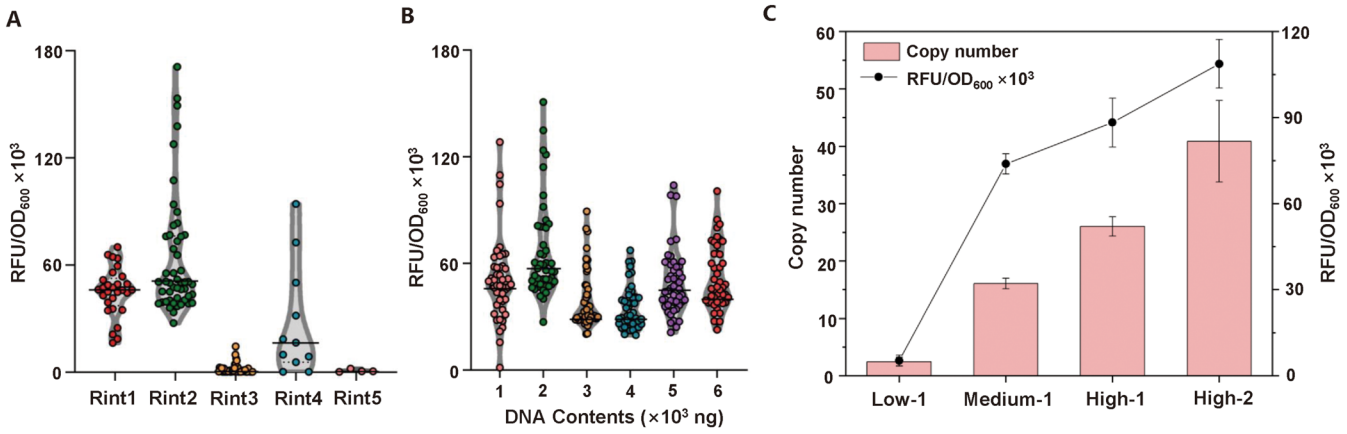

Figure 2. CRISPO design and execution using *mCherry* as a reporter. (A) Fluorescence intensities of multicopy integrated strains constructed with different gRNAs. The black line shows the average fluorescence intensity. (B) Optimization of the amount of donor DNA. The black line shows the average fluorescence intensity. (C) Heterologous gene copy numbers of the five randomly selected colonies. One colony with low fluorescence (Low-1), one colony with medium fluorescence (Medium-1), and two colonies with high fluorescence (High-1, High-2) were selected for qPCR analysis.

Tecan Infinite 200 PRO plate reader (Trading AG, Switzerland), the fluorescence intensity of mCherry was measured with an excitation of 561 nm and an emission of 620 nm. Cells devoid of mCherry were used as a nonfluorescent control.

2.5. Quantitative PCR (qPCR). The genome of *P. pastoris* was extracted using a Yeast DNA Extraction Kit (Thermo Fisher Scientific). To determine gene copy numbers, qPCR was conducted using a Thermo Fisher Scientific StepOnePlus Real-Time PCR Instrument with the PowerUp SYBR Green Master Mix. Gene copy numbers were determined using the $2^{-\Delta\Delta Ct}$ method, with *TDH3* (glyceraldehyde-3-phosphate dehydrogenase) included as the reference gene.

2.6. Geraniol Fermentation and Quantification. Different colonies of the transformants were picked up from YPD or YPG screening plates and inoculated into 24-well plates containing 2 mL of fresh medium, followed by cultivation at 30 °C, 250 rpm for 20−24 h to the exponential phase. Preculture was transferred into a new 24-well plate containing 2 mL of fresh medium at 30 °C, 250 rpm to minimize the effects of varying growth stages among different strains. Then, the seed culture was transferred into another 24-well plate containing 2 mL of fresh medium with 1% inoculum and cultured at 30 °C, 250 rpm for 72 h. 20% (v/v) isopropyl myristate (Sigma-Aldrich) was added to the culture at the beginning of the fermentation to extract the produced geraniol to the organic phase.

The organic layer was harvested by centrifugation of the fermentation broth at 12 000 rpm for 5 min and diluted 25−100-fold in hexane for Gas Chromatography and Mass Spectrometry (GC-MS) analysis. Geraniol was quantified using SHIMADZU QP2010SE GC-MS (SHIMADZU, Tokyo, Japan). Samples were injected by an AOC-20i autosampler into GC, which is equipped with a fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 mm RTX-5MS stationary phase). The injector and ion source temperature were 250 °C and 200 °C, respectively. The GC temperature program was started at 80 °C, increased to 330 °C at a rate of 9 °C/min, and held for an additional 2 min. Mass spectrometry was conducted using electrospray ionization in selected ion monitoring (SIM) scan mode with retention times and diagnostic ions monitored as follows: geraniol (7.111 min, the mass fragments observable at *m/z* 69, *m/z* 41, and *m/z* 93), citronellol (6.749 min, *m/z* 69, *m/z* 41, and *m/z* 95), citral (7.406 min, *m/z* 69, *m/z* 41, and *m/z* 94), and geranic acid (8.573 min, *m/z* 69, *m/z* 41, and *m/z* 100).

2.7. Fed-Batch Fermentation. Seed culture was prepared by plating frozen yeast stocks preserved in 25% glycerol onto YPD agar. A single colony was picked up and inoculated into 5 mL of YPD medium and incubated at 30 °C and 250 rpm for 12−16 h. The preculture was then transferred to four flasks containing 50 mL of fresh YPD medium with 1% inoculum, culturing at the same conditions. Then, 150 mL seed cultures were transferred into a 5 L fermenter containing 1.5 L of YPD or YPG medium supplemented with 3.2 g/L $MgSO_4$, which could increase the production of terpenoids.[27,28] A 30% volume of isopropyl myristate (IPM) was added to the fermenter initially. Fermentation conditions were controlled as follows: temperature was maintained at 30 °C; pH was automatically regulated at 5.8 via 25% ammonia or 36% acetic acid addition; dissolved oxygen was kept near 40% saturation by variable agitation (300−800 rpm). When the glycerol or glucose concentration in the fermenter was reduced to 5 g/L, 800 g/L glucose or glycerol was fed into the fermenter to supplement the fermentation substrate. An aliquot of 10 g/L yeast extract solution was intermittently supplied to the fermenter at 12 and 24 h. Samples were taken from the fermenter to determine OD_{600}, glucose or glycerol contents, ethanol contents, and geraniol and other monoterpenoid titers. Due to the interference of isopropyl myristate with OD_{600} measurements, the organic phase was removed by centrifugation prior. In addition, with the aqueous phase volume increasing and the organic phase volume decreasing in fed-batch fermentation, the geraniol titer was calibrated against fluctuating two-phase volumes to adjust for changing phase ratios over time.

3. RESULTS AND DISCUSSION

3.1. Design and Execution of CRISPO. We designed CRISPO based on the CRISPR/Cas9 system, using the repeated NTS sequences at the rDNA loci of *P. pastoris* as genome integration targets. We could achieve simultaneous cleavage and homologous recombination (HR) repair at multiple loci on the chromosome, leading to the construction of stable multicopy integrated strains. Using red fluorescent protein mCherry as a screening reporter, we first optimized the CRISPO system, mainly including the CRISPR/Cas9 module and the *mCherry* expression cassette module, followed by the demonstration of CRISPO for applications in the construction of stable strains and the determination of pathway rate-limiting steps. We designed CRISPOi with the expression of *mCherry* driven by a glycerol-induced and glucose-repressed promoter (e.g., *GUT1p*) for the generation of multicopy producing strains: strain construction in glycerol medium with *mCherry* expression for the screening of multicopy strains and fermentation in glucose medium with the expression of *mCherry* was strictly inhibited for minimal metabolic burden. We also designed CRISPOc with the expression of *mCherry* driven by a strong constitutive promoter (e.g., *TEF1p*) for the

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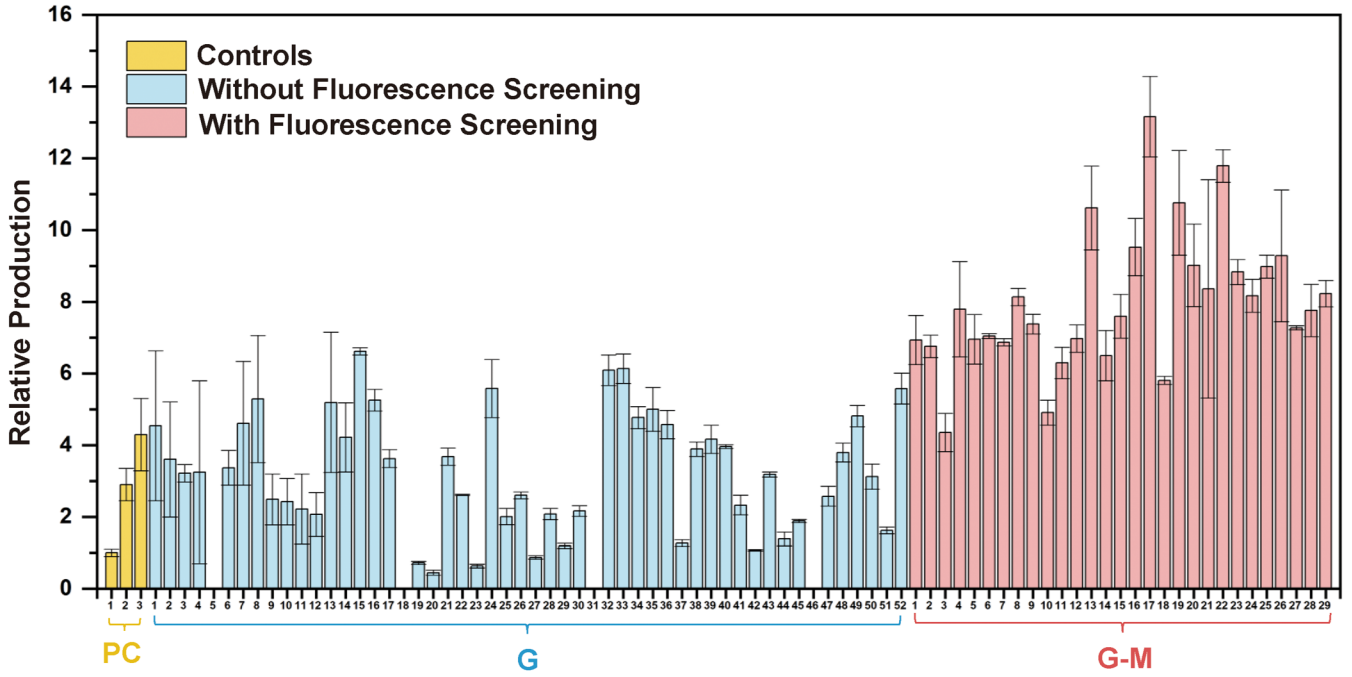

Figure 3. Validation of CRISPO for geraniol production. Yellow columns indicated the results of the control group, with PC-1, -2, and -3 representing yeast strains containing single-, two-, and three-copies of *tCrGES-ERG20*^{WW} expression cassette, respectively. Blue columns indicated the results of randomly selected clones, with G-x representing different strains with multiple copies of integrated *tCrGES-ERG20*^{WW} expression cassette. Pink columns indicated the results with fluorescence screening, with G-M-x indicating different strains with multiple copies of integrated *tCrGES-ERG20*^{WW} and *mCherry* expression cassettes. All results were expressed as titers relative to the single-copy control strain.

identification of pathway rate-limiting steps, which was featured with a positive relationship between gene dosage and product titer (Figure 1A).

We chose PpHR3 with enhanced HR efficiency[29] as our parent strain. With *mCherry* as the reporter gene, we designed five gRNAs with varying targeting efficiency: RInt1-gRNA, RInt2-gRNA, RInt3-gRNA, RInt4-gRNA, and RInt5-gRNA. As shown in Figure 2A, we obtained fewer transformants with RInt3-gRNA and RInt5-gRNA, none of which exhibited notable fluorescence intensity. While we observed detectable fluorescence with RInt1-gRNA and RInt4-gRNA, yeast strains with RInt2-gRNA resulted in the highest fluorescence intensities. Therefore, we chose RInt2-gRNA for subsequent studies. Optimization of the amount of donor DNA (Figure 2B) resulted in 2000 ng as the most effective for CRISPO. Interestingly, a further increase of the donor DNA contents failed to enhance the fluorescence intensity of the transformants proportionately. To validate the superior capability of CRISPO for multicopy integration, we randomly selected five transformants with varied fluorescence intensities for copy number determination (Figure 2C). The results indicated that the transformant with the strongest fluorescence intensity had as many as 40 copies, while the weakest had only 2 copies. The possible explanation for this phenomenon could be attributed to the activity of the CRISPR system to generate double strand breaks and HR efficiency of the chassis strain. To further investigate, different gRNA plasmids were introduced into the chassis strain PpHR3 without the addition of repair template DNA (Supplementary Figure S1). Notably, for RInt1-gRNA, RInt2-gRNA, and RInt4-gRNA, there were approximately 68, 54, and 81 transformants, respectively, which were much lower than the control experiment with repair template. These results indicate that, although HR is the major mechanism, non-homologous end joining (NHEJ) is also effective in repairing double strand breaks. As a result, during the strain construction process, both HR and NHEJ were functional in repairing CRISPR-generated double strand breaks, and yeast strains with varying copy numbers were obtained, necessitating the development of an effective screening method for multicopy strains. As the cutting efficiency of RInt3-gRNA and RInt5-gRNA was low, it resulted in the formation of a higher number of transformants.

3.2. Validation of CRISPO for Geraniol Production.

Having established an effective CRISPO system, we proceeded to validate its utility in the biosynthesis of geraniol in *P. pastoris*, which is synthesized through the MVA pathway and a geraniol synthase t*Cr*GES-ERG20^{ww}, a fusion of *N*-terminal truncated *Cr*GES and ERG20 mutant.[25] While CRISPO enabled multicopy integration of the *tCrGES-ERG20*^{WW} expression cassette, the production of geraniol varied from clone to clone. We could only achieve gene dosage amplification in a portion of yeast clones. In addition, we failed to identify clones with the highest copy numbers via random selection, as none of the strains significantly outperformed the rationally constructed control strain with three copies of *tCrGES-ERG20*^{ww} (Figure 3). In other words, we should combine CRISPO with selection or screening for the construction of multicopy integrated strains.

In order to establish an antibiotic-free screening system, we chose the red fluorescent protein mCherry as the screening marker, which is suitable for precise and reliable cell tracking.[30] The Cre-loxP system is a powerful genetic engineering tool widely used for selection marker recycling.[31] It has been reported that the combination of the 26S rDNA and Cre-loxP system resulted in the construction of multicopy strains via iterative integration and removal of the screening marker in *Y. lipolytica*.[24] Therefore, we initially designed CRISPO with *mCherry* driven by the constitutive *TEF1* promoter and flanked

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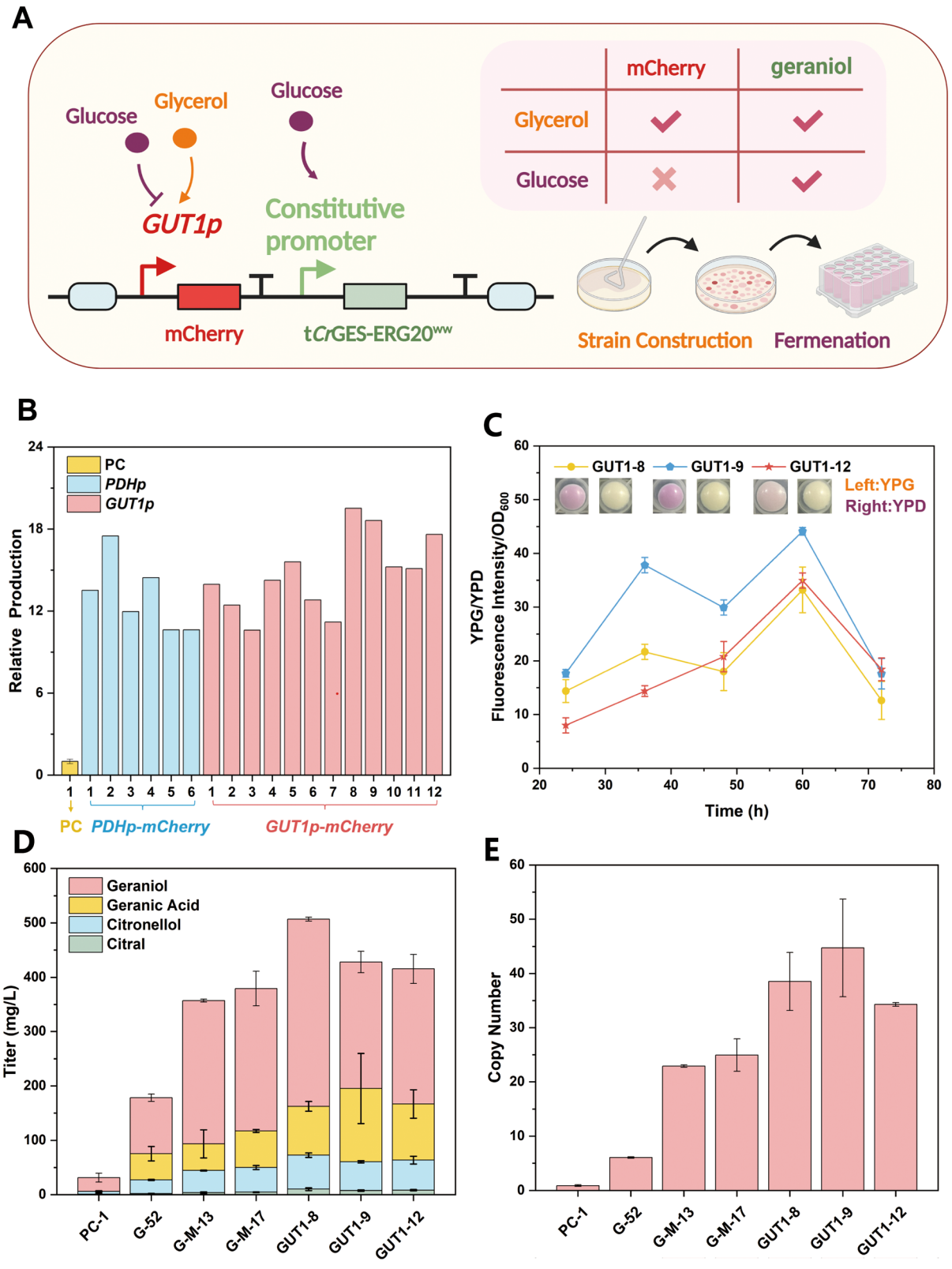

Figure 4. CRISPO for the construction of stable multicopy strains. (A) A schematic diagram describing the performance of *GUT1p* during the strain construction stage and fermentation stage. The orange shows the process of strain construction, using glycerol medium. The purple indicates the fermentation process, using glucose medium. (B) Screening and characterization of glycerol-induced and glucose-repressed promoters. The yellow column was the single-copy control strain. Pink columns were the results with *GUT1p* and blue columns were the results with *PDHp*. All results were expressed as titers relative to the single-copy control strain. (C) The expression level of *mCherry* driven by *GUT1p* in YPG and YPD. (D) Comparison of multicopy strains obtained by different screening methods, including non-fluorescence screening (G-52), screening with constitutive fluorescence (G-M-13, G-M-17), and screening with glycerol-induced fluorescence (GUT1-8, GUT1-9, GUT1-12). All strains were tested after 30 generations. (E) Copy numbers of *tCrGES-ERG20^{WW}* in different multicopy strains.

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by loxP sites, which enabled effective screening of multicopy strains via visible red coloration on agar plates or fluorescence-activated cell sorting in liquid culture, followed by eliminating the screening marker via Cre-mediated recombination.

In this process, we found that mCherry screening significantly improved screening efficiency (Figure 3). Compared with the control strain, the multicopy integrated strains resulted in as high as a 13.2-fold increase in the titer of total monoterpenoids, including geraniol, citronellol, citral, and geranic acid, which reached a total of 356.12 mg/L. In addition, we observed a positive correlation between fluorescence intensity and geraniol production (Supplementary Figure S2), indicating that we could evaluate the copy number effect in a quantitative manner. We also employed CRISPO for the biosynthesis of patchoulol, a tricyclic sesquiterpene widely used in perfumes and cosmetics. The corresponding biosynthetic pathway and results are shown in Supplementary Figure S3. The titer of patchoulol reached up to 12.5 times higher than that of the single-copy control strain, further showcasing the high efficiency and practicality of CRISPO.

We then employed the Cre-loxP system to excise the screening marker (Supplementary Figure S4A). Effectively controlling the expression level of Cre recombinase has always been a key aspect of this system.[32] Firstly, overexpressing Cre recombinase causes toxicity and inhibits host growth.[33] Secondly, continuous expression of Cre recombinase may lead to repetitive recombination between loxP sites, resulting in genomic instability. An inducible system is commonly used to regulate the expression of Cre recombinase.[34] Hence, we first evaluated four methanol-induced promoters including *AOX1p* (alcohol oxidase I promoter), *AOX2p* (alcohol oxidase II promoter), PAS_chr1-1_0319*p* (fructose-1,6-bisphosphate aldolase promoter), and PAS_chr1-4_0547*p* (peroxidase promoter) to drive *Cre* expression,[3] with the promoter PAS_chr1-1_0319*p* being chosen for its minimal leakage expression.[35] Unfortunately, direct induction of *Cre* expression in YPM led to a significant decrease in geraniol production despite successful mCherry excision (Supplementary Figure S4B). For better control of the induction condition, we cultured the yeast strains in YPD until the exponential phase, followed by Cre induction in YPM (rapid and high induction) or YPO (slow and mild induction). In addition, we also attempted to integrate the *Cre* expression cassette into the chromosome locus (IntE2 or IntE10) with a low expression level.[9] However, all the strategies failed to prevent the decline in geraniol production, probably due to *Cre* leakage expression and chromosomal rearrangement (Supplementary Figure S4C−F). The same phenomenon had also been discovered in other systems: the more loxP sites on the chromosome, the more prominent was this phenomenon.[32,36] These findings suggested that the Cre-loxP system was unsuitable for removing screening markers in *P. pastoris* and an alternative strategy should be established for CRISPO.

3.3. CRISPO for the Construction of Stable Multicopy Strains. As the Cre-loxP system failed to function as expected in *P. pastoris*, we employed a new CRISPO design via conditional *mCherry* expression, which was activated during screening and silenced under fermentation conditions. Accordingly, we designed CRISPO with glycerol-induced and glucose-repressed promoters to modulate *mCherry* expression (CRISPOi). In this case, CRISPOi was bifurcated into two distinct stages: strain construction stage and fermentation stage. We performed strain construction in a glycerol medium, where *mCherry* was expressed for the screening of multicopy strains. Meanwhile, we carried out fermentation in a glucose medium, where the expression of *mCherry* was strictly inhibited by glucose to minimize the heterologous protein expression burden (Figure 4A).

Here, we evaluated three glycerol-induced and glucose-repressed promoters: *GUT1p*, *GUT2p*, and *PDHp*. After transformation, we found no transformants with obvious fluorescence in *GUT2p* strains, probably due to the low transcriptional activity of *GUT2p* even under glycerol conditions. On the other hand, we observed obvious fluorescence in *GUT1p* and *PDHp* strains on YPG plates. Unfortunately, *PDHp* demonstrated leaky expression in YPD, which was not ideal for CRISPOi. In previous studies, it has also been shown that *PDHp* in *P. pastoris* was active under glucose conditions.[37] Therefore, *GUT1p* was chosen for subsequent studies. The monoterpenoid titer of the selected strain GUT1-8 (GUT1 indicated the strains obtained by fluorescence screening with promoter *GUT1p*) reached up to 535.47 mg/L, which was 19.5-fold higher than that of the single-copy control strain (Figure 4B). In addition, *GUT1p* demonstrated significantly higher activity in YPG than YPD, as evidenced by the distinctive red pigmentation of colonies under glycerol-induced conditions (Figure 4C).

To evaluate the effectiveness and stability of these methods, strains with the highest titer were selected from each screening method and fermented under the same conditions after 30 generations of serial transfers in the non-selective medium. These included strains G-52 (screened without fluorescence), G-M-13, and G-M-17 (screened with constitutive fluorescence) and GUT1-8, GUT1-9, and GUT1-12 (screened with glycerol-induced fluorescence). Notably, the GUT1 strains outperformed those screened with constitutive fluorescence, with GUT1-8 achieving the highest monoterpenoids titer of 506.87 mg/L (Figure 4D), comprising 344.40 mg/L geraniol, 89.80 mg/L geranic acid, 62.48 mg/L citronellol, and 10.19 mg/L citral in a 24-well plate. Supplementary Figure S5 shows the GC-MS profiles of these products, which were derived from the conversion of geraniol. The production of citronellol has previously been confirmed in *P. pastoris*.[38] Citral is likely to be an intermediate metabolite for the conversion of geraniol to citronellol (a reduction product) and geranic acid (an oxidation product), which has been observed in many microorganisms.[39,40] Additionally, we found that the production of geranic acid and geraniol in this process was strongly associated with fermentation conditions, especially the concentration of the carbon source, consistent with that reported in *Y. lipolytica*.[41] Then, after quantifying gene copy numbers with qPCR, we found that the strains screened with glycerol-induced fluorescence had higher copy numbers than those screened with constitutive fluorescence (Figure 4E), reaching up to 44 copies. This might be because the strength of *GUT1p* even under glycerol-induced conditions was weaker than that of the strong constitutive promoter *TEF1p*, which is more conducive to screening strains with higher copy numbers. Overall, CRISPOi represents a substantial advancement in the construction of multicopy yet stable-producing *P. pastoris* strains.

3.4. CRISPO for Identifying and Eliminating Pathway Bottlenecks. In addition to the construction of multicopy strains, the quantitative feature of CRISPO with *mCherry* driven by a constitutive promoter (CRISPOc) rendered the exploration of pathway rate-limiting steps. If higher copy

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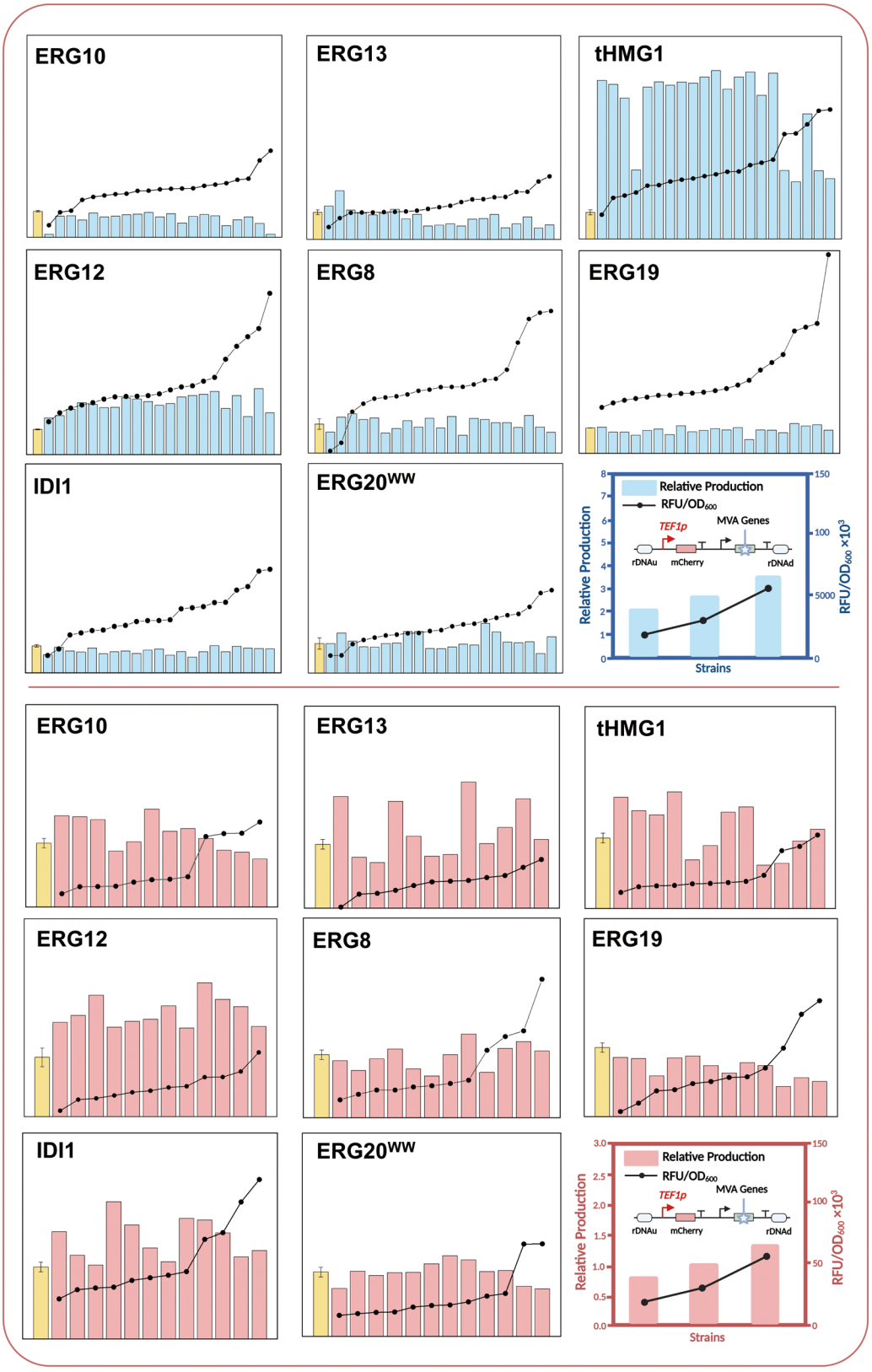

Figure 5. Two rounds of CRISPO for identifying MVA pathway bottlenecks in *P. pastoris*. The blue bar chart above indicated the 1st round of CRISPO. The chassis strain S1 was constructed by integrating three copies of the *tCrGES-ERG20^{WW}* expression cassette into the chromosome of *P. pastoris*. The pink bar chart below indicated the 2nd round of CRISPO. The chassis strain S2 was constructed by integrating four copies of the *tHMG1* expression cassette into the chromosome of S1. CRISPO-mediated multicopy integration of each MVA pathway gene, including *ERG8*, *ERG10*, *ERG12*, *ERG13*, *ERG19*, *ERG20^{ww}*, *tHMG1*, and *IDI1*, and 21 colonies for each construct were randomly selected for the characterization of mCherry fluorescence and monoterpenoid production. The results were presented as the total titer of monoterpenoids relative to the corresponding chassis strain.

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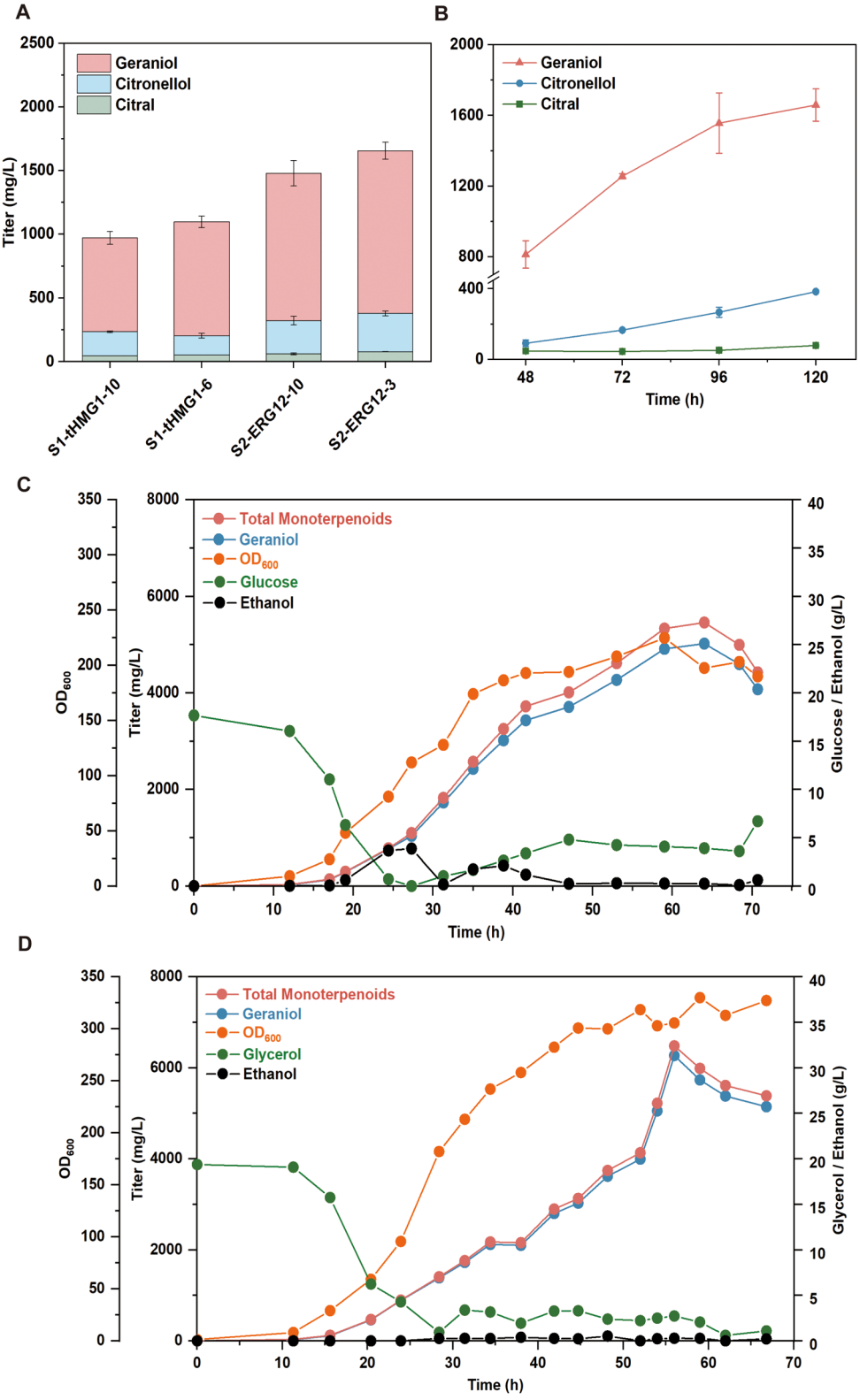

Figure 6. Characterization of the fermentation performance of multicopy strains. (A) Two rounds of screening strains were verified by fermentation under the same conditions. S1 indicated the parent strain for the first round of CRISPO screening. S2 indicated the parent strain for the second round of CRISPO screening. (B) The fermentation profile of S2-ERG12-3 in shake flasks. Fed-batch fermentations were performed by the engineered *P. pastoris* strain S2-ERG12-3 in a 5 L bioreactor in YPD (C) or YPG (D). All fermentation titers are corrected for the volume changes of aqueous and organic phases during fed-batch fermentation.

numbers (higher fluorescence) resulted in higher production, the corresponding gene product represented the rate-limiting enzyme for the biosynthetic pathway. Although there have been many studies on the optimization of the MVA pathway with HMG-CoA reductase HMG1 generally regarded as the rate-limiting enzyme,[42,43] less research has been done in

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P. pastoris. Therefore, with geraniol biosynthesis as a case study, we explored the rate-limiting steps through CRISPOc-mediated multicopy integration of eight genes in the MVA pathway (*ERG8*, *ERG10*, *ERG12*, *ERG13*, *ERG19*, *ERG20^{ww}*, *tHMG1*, and *IDI1*). The chassis strain S1 was constructed by integrating 3 copies of *tCrGES-ERG20^{ww}*. As shown in Figure 5, multicopy integration of *tHMG1* markedly increased geraniol production, while multicopy integration of other pathway genes only resulted in a marginal increase in geraniol titer. These results indicated that tHMG1 was the rate-limited enzyme for the MVA pathway in *P. pastoris*, which was consistent with that in other yeast species, indicating the efficacy of CRISPOc in identifying pathway bottlenecks. The highest strain S1-tHMG1-11 increased the production of total monoterpenoids by 6.6-fold to 1.25 g/L (comprising 1.23 g/L geraniol and 0.02 g/L citronellol), compared with the parent chassis strain. Our result also found that the gene dosage effect could be saturated, as a further increase of *tHMG1* copy numbers failed to yield significant gains, probably due to the presence of other pathway bottlenecks.

After identification of the tHMG1 bottleneck, we would like to explore the copy number effect of other pathway genes. We first eliminated the known bottleneck by rationally integrating additional copies of *tHMG1*, with 4 copies determined to be sufficient to drive the maximal production of geraniol (Supplementary Figure S6). Based on the S2 chassis strain, we proceeded to overexpress the eight genes of the MVA pathway following the same CRISPOc operation. As depicted in Figure 5, multicopy integration of *tHMG1* failed to increase geraniol production further, indicating the bottleneck was successfully addressed. On the contrary, the second round of CRISPO identified ERG12 as the rate-limiting step, whose multicopy integration further increased geraniol titer by an additional 2-fold. Interestingly, some clones of the *ERG13* and *IDI1* multicopy integrated strains also increased geraniol production. In other words, although *ERG13* and *IDI1* overexpression was beneficial for geraniol production, the expression levels or copy numbers should be carefully fine-tuned to achieve optimal production. Through two rounds of rate-limiting step exploration, we discovered the two major rate-limiting steps in the MVA pathway. CRISPOc could help us rapidly obtain strains containing different gene dosages, which significantly shortened the process of strain construction compared to the traditional stepwise integration method. This approach held promise for applications in determining and subsequently eliminating rate-limiting steps in other natural product biosynthetic pathways.

3.5. Characterization of the Fermentation Performance of Multicopy Strains. Through our exploration of the rate-limiting steps of the MVA pathway, we have constructed *P. pastoris* strains with substantially improved geraniol titer. We fermented the 1st round CRISPO strains (S1, with *tHMG1* multicopy integrated) and the 2nd round CRISPO strains (S2, with *ERG12* multicopy integrated) under the same fermentation conditions (Figure 6A). In a 24-well plate containing 2 mL of YPD with 40 g/L glucose, the top-performing strain S2-ERG12-3 produced a total monoterpenoid titer of 1.66 g/L (comprising 1.30 g/L geraniol, 0.30 g/L citronellol, and 0.06 g/L of citral). Extension of the fermentation time to 120 h further increased the total monoterpenoid titer up to 2.12 g/L, containing 1.66 g/L geraniol, 0.38 g/L citronellol, and 0.08 g/L citral (Figure 6B). This result has currently reached the highest production level in yeast, which was also comparable to that reported in *E. coli* through mutating and screening of the *N*-terminal tag.[44] Agrawal et al. achieved 1 g/L geraniol production at the shake flask level by screening geraniol synthases from different sources and overexpression of the MVA pathway in *Y. lipolytica*.[41] Zhao et al. effectively increased geraniol production in *S. cerevisiae* by dynamically regulating *ERG20* expression.[42] Jiang et al. truncated the geraniol synthase GES and fused the mutant protein ERG20^{WW}, achieving a geraniol production of 523.96 mg/L at the shake flask level.[25]

The optimal strain S2-ERG12-3 was employed for fed-batch fermentation in a 5 L bioreactor (Figure 6C,D). We individually evaluated the fermentation performance with glycerol and glucose as carbon sources in Figure 6C,D, respectively. The initial glycerol or glucose concentration was 20 g/L, followed by constant rate feeding to maintain the concentration at ∼5 g/L. In glucose-fed culture, we obtained an OD_{600} of 197.6 after 64 h of fermentation, with the production of geraniol and monoterpenoids reaching 5.01 g/L and 5.45 g/L, respectively. In glycerol-fed culture, we obtained an OD_{600} of 305 after 56 h of fermentation, with the production of geraniol and monoterpenoids reaching up to 6.27 g/L and 6.48 g/L, respectively, representing the highest geraniol titer ever reported. We corrected the product titer for volume changes of the aqueous and organic phases during fed-batch fermentation, and the product concentration in the organic phase was provided in Supplementary Figure S7. As for the biosynthesis of geraniol, Jiang et al. reported a titer of only 1.68 g/L in a 5 L fermenter.[25] In another study, a titer of 5.52 g/L was achieved via fed-batch fermentation for more than 800 h,[45] whose productivity (0.0069 g/L/h) was 16-fold lower than our constructed strain (0.11 g/L/h). Furthermore, it could be seen from the results that the titer of geraniol and monoterpenoids in the glycerol medium is higher than that in the glucose medium. This could be because, under glycerol conditions, the cell growth rate or initial substrate utilization rate was slower, and relative to the metabolic overflow caused by glucose, the metabolism was smoother and gradual. Consequently, under glycerol conditions, the metabolic burden on cells was smaller, and the final OD_{600} and titer ended up being higher. We could also observe that, relative to the titer per cell, the titer under glucose conditions was higher.

4. CONCLUSIONS

In this work, we established CRISPO for the construction of multicopy integrated strains in an antibiotic-free and single-step manner in *P. pastoris*. We designed CRISPOi for the construction of stable multicopy integrated strains using a glycerol-induced and glucose-repressed promoter to drive screening marker *mCherry* expression, leading to a 19.5-fold increase in geraniol production relative to the single-copy strain. We also designed CRISPOc using a constitutive promoter to explore pathway rate-limiting steps, with two rounds of investigation leading to the identification of tHMG1 and ERG12 as the major bottlenecks of the MVA pathway. The final engineered strain synthesized 6.27 g/L geraniol (with a total monoterpene titer of 6.48 g/L) in a 5 L bioreactor, representing the highest titer and productivity reported to date. As an efficient genome editing and pathway optimization tool, CRISPO provides a valuable reference for the construction of microbial cell factories for the production of terpenoids and other valuable chemicals.

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■ ASSOCIATED CONTENT

Supporting Information

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Figure S1, CRISPR-mediated rDNA integration with no repair template; Figure S2, correlation between fluorescence intensity and geraniol production; Figure S3, CRISPO for the biosynthesis of patchoulol; Figure S4, Cre-loxP system; Figure S5, gas chromatogram and mass spectra; Figure S6, construction of the chassis strain S2; Figure S7, concentration of monoterpenoids in the organic phase of 5 L bioreactors; Tables S1−S4, the plasmids, benchling links, oligonucleotides, and yeast strains, respectively, used in this study (PDF)

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Author Contributions

X.J. and J.L. designed the study, analyzed the data, and wrote the manuscript. X.J., M.L., Z.W., C.Y., J.G., X.A., J.B., and J.C. conducted the experiments. All authors revised and approved the manuscript.

Notes

The authors declare no competing financial interest.

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