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Original Research Article

Establishment of a selectable marker recycling system for iterative gene editing in *Fusarium fujikuroi*

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ABSTRACT

Gibberellic acid (GA3) is a vital plant growth hormone widely used in agriculture. Currently, GA3 production relies on liquid fermentation by the filamentous fungus *Fusarium fujikuroi*. However, the lack of an effective selection marker recycling system hampers the application of metabolic engineering technology in *F. fujikuroi*, as multiple-gene editing and positive-strain screening still rely on a limited number of antibiotics. In this study, we developed a strategy using pyr4-blaster and CRISPR/Cas9 tools for recycling orotidine-5′-phosphate decarboxylase (Pyr4) selection markers. We demonstrated the effectiveness of this method for iterative gene integration and large gene-cluster deletion. We also successfully improved GA3 titers by overexpressing geranylgeranyl pyrophosphate synthase and truncated 3-hydroxy-3-methyl glutaryl coenzyme A reductase, which rewired the GA3 biosynthesis pathway. These results highlight the efficiency of our established system in recycling selection markers during iterative gene editing events. Moreover, the selection marker recycling system lays the foundation for further research on metabolic engineering for GA3 industrial production.

1. Introduction

Gibberellins (GAs) are a class of tetracyclic diterpene compounds that serve as vital natural plant hormones in regulating plant growth [1,2]. Over 136 structurally distinct gibberellins (GA1-GA136) have been identified to date [3]. Among these, particular attention has been given to GA3 due to its significant physiological bioactivity, leading to its extensive application in agriculture [4,5]. GA3 could be produced by three ways including phytoextraction, chemical synthesis and microbial fermentation [6]. The first two methods were limited to low content, high cost and environment pollution and were quickly abandoned [7]. Currently, industrial production of GA3 relies on the fermentation of the filamentous fungus *F. fujikuroi*, which is renowned for its high-level production of GA3 [8]. In the past few decades, medium optimization and strain mutagenesis have been commonly employed strategies to enhance the titer of gibberellic acids [9–13]. However, these methods often involve a lengthy process with limited improvement in yield, which has led researchers to seek alternative approaches.

In recent years, genome editing technology has emerged as a significant contributor to enhancing productivity and designing genetically manipulated strains with increased efficiency [14–16]. In 2019, the CRISPR/Cas9 genome editing system was firstly established in *F. fujikuroi* by my team, which solves the problem of lacking genetic manipulation tools to a large extent [17]. However, the lack of stable autonomous replication sequences for self-replicative plasmid construction has posed a challenge for iterative gene editing [14]. This limitation has resulted in dependence on a limited number of antibiotic markers. Among various organisms, the Cre/loxP system and *pyr4*-blaster have proven to be effective selection marker recycling systems. Cre/loxP system consists of Cre recombinase and 34-bp loxP sites. Cre recombinase is an enzyme capable of site-specific recombination between two 34-bp loxP sites. LoxP sites with the same orientation contribute to excision of the flanked sequence such as selection markers [18]. This system has been applied in virous filamentous fungi including *Aspergillus niger* and *Fusarium graminearum* [19,18]. The *pyr4*-blaster involves the incorporation of the 5-phosphoglycoside decarboxylase gene (*pyr4*) expression cassette flanked by identical direct repeats [20–22]. In summary, this system involves integrating it

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into a *pyr4* gene deletion mutant to create the chassis cell, which is then grown on solid medium containing 5-fluoroorotic acid (5-FOA) for counter-selection purposes. Through homologous recombination between the direct repeats, the *pyr4* gene expression cassette is excised, restoring the uracil auxotroph phenotype. This strategic approach allows for the *pyr4* marker to be reused in subsequent metabolic engineering. Compared with two rounds of protoplast transformations using Cre/loxP system, the advantage of *pyr4*-blaster is that only one-round transformation was required. What's more, the expression cassette of Cre recombinase needs to be cloned into a self-replicative plasmid, which could ensure the Cre recombinase can be loss after the selection marker was excised. However, the fact is that *F. fujikuroi* lacks a stable self-replicative plasmid. Taking these factors into consideration, *pyr4*-blaster is more suitable for the establishment of a selectable marker recycling system. Of course, in order to realize the purpose of iterative gene editing and improve editing efficiency, *pyr4*-blaster can be used by combining with CRISPR system, which could thoroughly solve previous challenges faced.

In this study, we investigated the potential of the *pyr4*-blaster system in *F. fujikuroi* and found that it functions effectively. Building upon this, we combined the pyr4-blaster system with the CRISPR/Cas9 system to rewire the complex metabolic pathways of *F. fujikuroi*. This included the deletion of the bikaverin gene cluster (17 kb) and the fusaric acid gene cluster (14 kb). Furthermore, we identified key enzymes from the mevalonate pathway, which led to an increase in GA3 production. Overall, this research expands the available genetic manipulation tools for selection marker recycling systems and significantly facilitates metabolic engineering applications in the non-model filamentous fungus, *F. fujikuroi*.

2. Materials and methods

2.1. Strains and culture media

The strains and plasmids used in this study were listed in Table S1. The *Escherichia coli* DH5α (ATCC 53868) was utilized for transformation. The *F. fujikuroi* NRF-C1 (CCTCC M20231195) was provided by Jiangxi New Reyphon Biochemical Co., Ltd. (Jiangxi, China). The seed medium consisted of (g/L): glucose, 40; soybean powder, 10; peanut powder, 8; dextrin, 20; $(NH_4)_2SO_4$, 0.2; KH_2PO_4, 1; $MgSO_4$, 0.5; The fermentation medium consisted of (g/L): starch, 50; soybean powder, 10; peanut powder, 15; soybean oil, 1.2; $(NH_4)_2SO_4$, 0.25; KH_2PO_4, 2; $MgSO_4$, 1.5; $ZnSO_4$, 0.05; $MnSO_4$, 0.075. The *F. fujikuroi* seed liquid was cultivated for 2 days at 200 rpm and 28 °C. The seed liquid (10 %, v/v) was inoculated the fresh fermentation medium for 7 days at 200 rpm and 28 °C. The potato dextrose broth (PDB) was used for genome extraction. Regeneration solid medium consisted of (g/L): glucose, 20; yeast extract, 3; agar, 15; sucrose, 205. Yeast nitrogen base medium (YNB, a synthetic medium) consisted of (g/L): glucose, 30; yeast nitrogen base without amino acid and $(NH_4)_2SO_4$, 1.7; $(NH_4)_2SO_4$, 5; agar, 30; sucrose, 170. The potato dextrose agar (PDA) supplemented with 4 g/L 5-FOA was utilized for the excision of *pyr4* gene selection marker. The G medium consisted of (g/L): glycine, 20; maltodextrin, 25; sucrose, 20; CH_3COONH_4, 4.5; KH_2PO_4, 1.5; $MgSO_4$, 1. Complete supplemental mix (CSM) purchased from MP Biomedicals Co., Ltd. (USA) consisted of various amino acids. CSM-URA consisted of various amino acids apart from the uracil.

2.2. Plasmids and recombinant strains construction

The primers used in this study were listed in Table S2. *F. fujikuroi* NRF-C1 genome was used as the template to amplify the gene from mevalonate (MVA) pathway and these NCBI Gene IDs were listed in Table S3. The FnCpf1 gene fused with the histone H2B nuclear localization signal (HTB_{NLS}) was synthesized by Synbio-tech Co., Ltd., China. The strong PgpdA promoter, TtrpC terminator and hygromycin expression cassette were amplified from the vector pUC-fFuCas9-HTB_{NLS}-hph [17]. All plasmids were constructed by TEDA cloning method [23]. All N20, N23 protospacers, gRNA and crRNA sequences for targeted gene editing were listed in Data S1. The upstream and downstream homologous arm sequences of *fub1* and *bik1* used for the long gene clusters deletion were listed in Data S2.

2.3. F. fujikuroi protoplast transformation method

The transformation protocol was optimized as follows: *F. fujikuroi* was cultured in G medium at 200 rpm and 28 °C for 2 days. The inoculum, prepared as a 10 % (v/v) seed liquid, was then added to fresh G medium and grown under the same conditions for 16 h. Subsequently, four layers of filter paper were used to filtrate wet mycelia which were added into the phosphate buffered saline (PBS) supplemented with 0.5 M KCl, 5 g/L lysing enzyme and 5 g/L driselase for 3 h incubation at 60 rpm and 28 °C to prepare the protoplast. In total 10 μg plasmid was introduced into 1×10^7 protoplasts, which was then plated on selection plates for 3–6 days at 28 °C. The positive transformations were identified using colony PCR. To eliminate the *pyr4* selection marker, the positive transformation was plated on YPD solid medium supplemented with 1 g/L uracil, 1 g/L uridine, and 4 g/L 5-FOA. The excision of the *pyr4* selection marker was confirmed through colony PCR and plate selection method.

2.4. Analytical methods of GA3

After cultivation, 2 mL of fermentation broth was filtered using a 0.45 μm filter membrane to collect the supernatant fraction. The GA3 content was quantified using HPLC LC-20AT coupled with an ODS-C18 column. The pretreated samples were separated using 100 % methanol as the organic phase and 0.05 % formic acid as the aqueous phase at a flow rate of 0.3 mL/min. Detection was performed at a wavelength of 210 nm, with an injection volume of 10 μL. The retention time for GA3 was approximately 24 min.

3. Results and discussion

3.1. Comparing the genome editing efficiency between CRISPR/Cas9 and CRISPR/Cpf1 system in the industrial strain F. fujikuroi NRF-C1

The utilization of an effective genome editing tool has the potential to significantly reduce the duration of genetic breeding. In our previous study, we successfully established the CRISPR/Cas9 technology in the wild strain *F. fujikuroi* NJtech 02. We systematically optimized the strategies for sgRNA and Cas9 expression, enabling the generation of a three-gene mutant strain through a single round of transformation.

To investigate the applicability of the CRISPR/Cas9 system in industrial *F. fujikuroi* strains, we selected three target loci, the Fusarium cyclin C1 gene (*fcc1*), bikaverin transcription factor (*bik5*) and fusaric acid polyketide synthase (*fub1*), for knockout experiments in order to generate the mutants *YCL002*, *YCL004* and *YCLf1*. The sequencing results revealed that the mutation efficiency of the *fcc1, bik5* and *fub1* loci in the industrial *F. fujikuroi NRF-C1* strain reached approximately 62.5 %, 45.8 % and 83.3 %, respectively. This indicated that the CRISPR/Cas9 system remains effective in the industrial *F. fujikuroi NRF-C1* strain, further highlighting its potential for application in industrial settings (Fig. 1A).

Recently, the CRISPR/Cpf1 system has gained increasing attention from researchers due to its many advantages. These include a smaller endonuclease size, staggered DNA overhang distal to the PAM site, and simplified multiplex genome editing enabled by single crRNA processing [24,25]. Therefore, we aimed to establish the CRISPR/Cpf1 system in *F. fujikuroi NRF-C1* to investigate its potential. Specifically, we utilized the 5S rRNA promoter and the strong gpdA promoter to drive the expression of crRNA and *FnCpf1*, respectively, following a similar

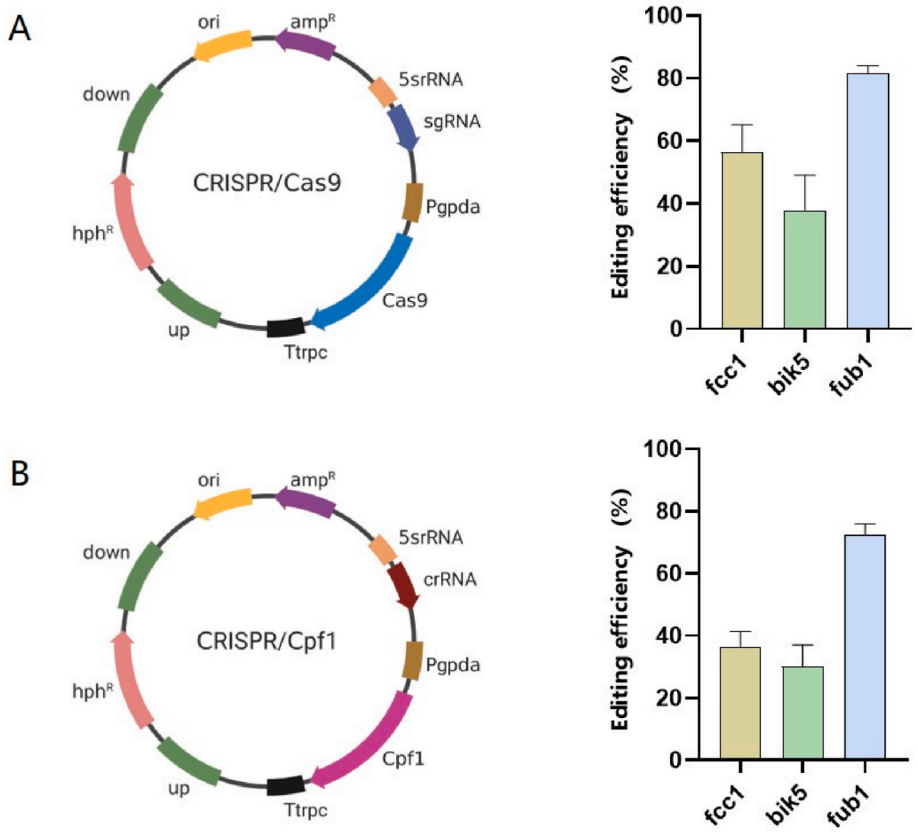

Fig. 1. The comparison of *fcc1*, *bik5* and *fub1* editing efficiency through CRISPR/Cas9 and CRISPR/Cpf1 systems. (A) The plasmid map and the editing efficiency medicated by CRISPR/Cas9. (B) The plasmid map and the editing efficiency medicated by CRISPR/Cpf1.

strategy employed in the CRISPR/Cas9 system (Fig. 1B). The *fcc1*, *bik5* and *fub1* genes were chosen for knock-out experiments to generate the mutant strains *YCL003*, *YCL005* and *YCLf2*. Our results demonstrated the effectiveness of CRISPR/Cpf1, as evident from the observed editing events confirmed through sequencing analysis. Notably, the disruption efficiencies of *fcc1*, *bik5* and *fub1* were approximately 33.3 %, 25.0 % and 75 %, respectively. However, due to higher efficiency achieved with CRISPR/Cas9, this system was selected for subsequent experiments.

3.2. Deleting the pyr4 gene in F. fujikuroi NRF-C1 via CRISPR/Cas9 resulted in a stable uracil auxotrophic mutant

Since *F. fujikuroi* lacks an efficient autonomously replicating plasmid, the availability of antibiotics remains limited for gene editing. However, it is worth noting that the *pyr4*-blaster system has been successfully utilized in various organisms, including *Hypocrea jecorina* [26], *Mucor circinelloides* [27] and *Aspergillus* sp. [13] for recycle of *pyr4* selection marker, we attempted to explore whether this system could be also established in *F. fujikuroi*. In order to reduce the size of plasmid and improve the transformation efficiency, *Cas9* and hygromycin resistance (*HPH*) expression cassettes were first introduced into the *pyr4* gene locus (FFUJ_13391) to construct an uracil auxotrophic mutant *YCL00*6 by CRISPR/Cas9 technology (Fig. 2A). To confirm the uracil auxotrophic phenotype, three synthetic solid media were utilized. These included YNB supplemented with 0.4 g/L CSM lacking uracil (CSM-URA), YNB supplemented with 0.4 g/L CSM-URA and 4 g/L 5-FOA, and YNB supplemented with 0.4 g/L CSM-URA, 4 g/L 5-FOA, 1 g/L uracil, and uridine. The growth of strain *NRF-C1* and *YCL006* was examined on these media. As shown in Fig. 2B, *YCL006* failed to grow on the synthetic medium without uracil and uridine supplementation. On the other hand, *NRF-C1* exhibited normal growth on the synthetic medium but was unable to survive in the presence of 5-FOA due to the lethal conversion

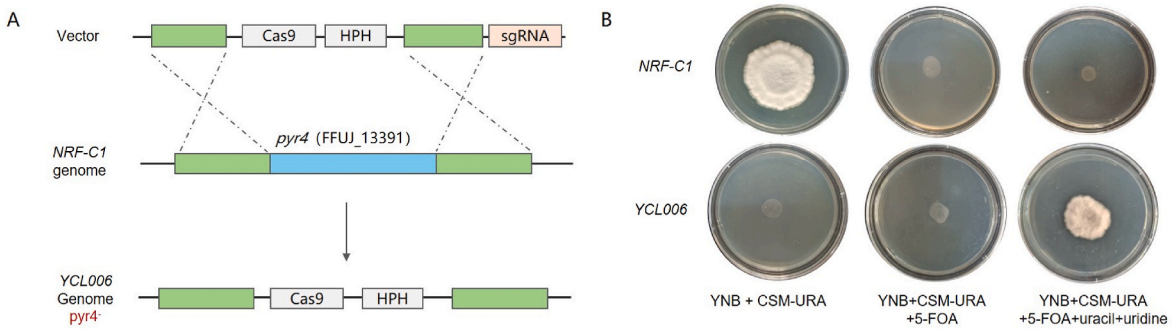

Fig. 2. Deleting the *pyr4* gene in *F. fujikuroi* via CRISPR/Cas9. (A) Flow chart of deleting *pyr4* gene. (B) The phenotype comparison of control strain NRF-C1 and YCL006 on three kinds of synthetic base medium.

of 5-FOA into highly toxic substances by orotidine-5′-phosphate decarboxylase Pyr4. This confirms the successful knockout of the *pyr4* gene in *YCL006*.

3.3. Establishment of a selection marker recycling system in F. fujikuroi NRF-C1 by combining CRISPR/Cas9 with pyr4-Blaster

To validate the effectiveness of *pyr4*-blaster, *YCL006* was employed for further experiments targeting the *fcc1* gene. A CRISPR plasmid, pUC-HUH-FCC1-sgRNA, was constructed. This plasmid contained 0.5 kb upstream and downstream homologous arms flanking the *hisG-pyr4-hisG* cassette, as well as an sgRNA specific to *fcc1*. Four primers were designed to verify the integration of the *hisG-pyr4-hisG* cassette into the *fcc1* locus (Fig. 3A). As shown in Figs. 3B and 2.5 kb and 3.7 kb DNA fragment could be amplified by two pairs of primers F1/R1 and F2/R2 with the efficiency of 58.3 % (14/24), which demonstrated that the integration has happened. The mutant was entitled as *YCL007*. Subsequently, in order to verify whether the *pyr4* marker could be excised by counter-selection, the mutant *YCL007* was coated on PDA solid plate supplemented with 1 g/L uracil, uridine and 4 g/L 5-FOA. After several days of cultivation, seven transformants were selected for the next verification. Among these, the PCR result displayed that the 3.4 kb fragment could be amplified from four transformants, which indicated that *pyr4* marker and a *hisG* fragment was excised through homologous interaction between the *hisG* repeats (Fig. 3C). Subsequently, *YCL008*, one of the selected transformants, was chosen for phenotype verification. *YCL008* exhibited an inability to grow on the uracil-deficient synthetic medium, while *YCL007* displayed normal growth on YNB (Fig. 3D). These dates demonstrated that *pyr4*-blaster system could been successfully applied in *F. fujikuroi*.

3.4. The long gene clusters in F. fujikuroi can be deleted by combining CRISPR/Cas9 with pyr4-Blaster system

Since *pyr4*-blaster system could been applied in *F. fujikuroi*, we next verified whether the selection marker recycling system could be used for iterative gene editing. On the basis of *YCL008* in which the *hisG and pyr4* marker was previously excised, we attempted to again introduce a *hisG-pyr4-hisG* cassette to iteratively editing target genes. Considering that it is very difficult to deleting the long gene clusters in *F. fujikuroi*, our purpose was on knocking out bikaverin and fusaric acid gene clusters by integrating *hisG-pyr4-hisG* cassette. In *F. fujikuroi*, the bikaverin pathway comprises six proximal genes, named *bik1* to *bik6*, while the fusaric acid gene cluster consists of five adjacent genes, named *fub1* to *fub5* [28,29]. The CRISPR plasmids pUC-FfHUH-BIK1-HR and pUC-FfHUH-FUB1-HR (Fig. 4A) were constructed in which *hisG-pyr4-hisG* cassette was flanked by 1.0 kb upstream and downstream homologous arms as well as the sgRNA targeting to the *bik1* or *fub1*. The two plasmids were individually introduced into the uracil auxotrophic *YCL008* protoplast to generate the *YCL009* and *YCL009F*. By PCR sequence, we found bikaverin (17 kb) and fusaric acid gene cluster (14 kb) were successfully knocked out with the efficiency of 16.7 ± 4.2 and 29.3 ± 4.3 (Fig. 4B). Moreover, as a control, *pyr4*-blaster plasmids lacking the sgRNA cassette were utilized, revealing that no mutants were obtained during the transformation process due to the low efficiency of homologous recombination. Consequently, the combined approach of CRISPR/Cas9 with the *pyr4*-blaster system proves to be an effective method for deleting long gene clusters.

3.5. Identifying key enzymes from the mevalonate pathway to increase the GA3 titer

The diterpene GA3 used geranylgeranyl pyrophosphate (GGPP) as the precursor [30]. Generally, the high GGPP content is favorable for the accumulation of diterpenes [31,32]. As shown in Fig. 5A, in eukaryotic microorganisms, GGPP can be biosynthesized using the acetyl-CoA as the substrate by mevalonate pathway [33], which consists of nine enzymes including acetoacetyl-CoA thiolase (*Erg10*), hydroxymethylglutaryl-CoA synthase (*Erg13*), hydroxymethylglutaryl-CoA reductase (*Hmgr*), mevalonate kinase (*Erg12*), phosphomevalonate kinase (*Erg8*), mevalonate diphosphate decarboxylase (*Erg19*), isopentenyl diphosphate isomerase (*Idi*), geranyl/farnesyl diphosphate synthase (*Erg20*), and geranylgeranyl pyrophosphate synthase (*Ggs2*). However, there are few reports on which enzyme plays an important role in enhancing the supply of precursor GGPP at present. We therefore constructed the CRISPR/Cas9-based integrated plasmids of single gene which was controlled under the strong promoter gpdA. These plasmids were individually introduced into *F. fujikuroi NRF-C1* and these genes would therefore be integrated into the *bik5* locus in the genome to generate the strains *YCL010-YCL0*18 by CRISPR/Cas9 gene editing tool. As shown in Fig. 5B, overexpression of *Erg10*, *Erg13*, *Erg8*, *Erg20*, and *Ggs2* improved the GA3 titer by 5.43 %、2.47 %、4.51 %、2.00 % and 9.22 %. while overexpression of *Hmgr*, *Erg12*, *Erg19*, *Idi* reduced the GA3 titer by 12.80 %, 22.36 %, 10.96 % and 7.52 %, which may result from the negative feedback suppression. In addition, truncating *Hmgr* (*tHmgr*) by deleting the N-terminal domain is generally considered to be

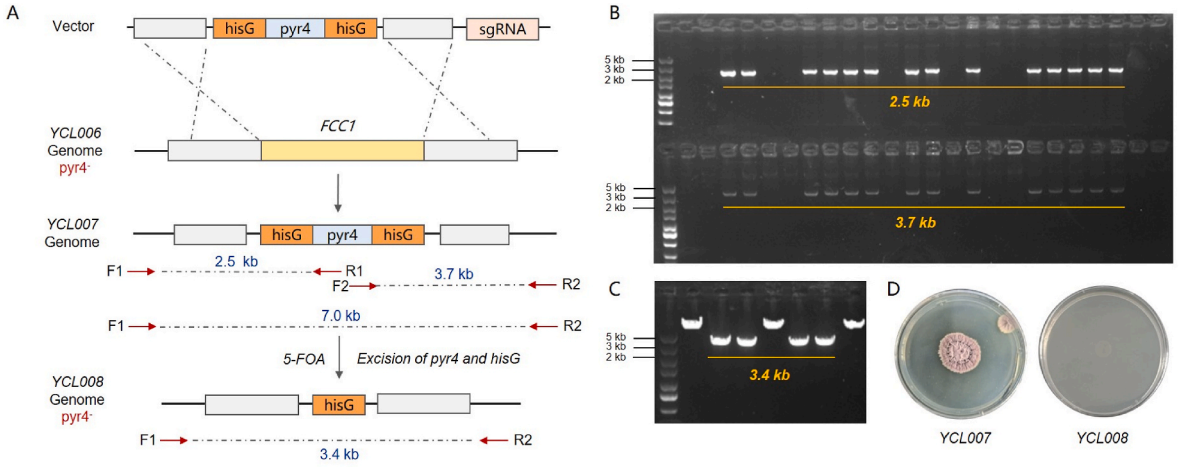

Fig. 3. Establishment of a selection marker recycling system by combining CRISPR/Cas9 with *pyr4*-blaster in *F. fujikuroi*. (A) Introducing a *hisG-pyr4-hisG* cassette in the *fcc1* locus to generate strain YCL007 and obtaining an uracil auxotrophic mutant YCL008 by 5-FOA counter-selection of YCL007. (B) Verifying the efficiency of obtaining the mutant YCL007 by PCR. (C) Verifying the efficiency of *pyr4* and *hisG* excision. (D) The phenotype comparison of control strain YCL007 and YCL008 on the synthetic base medium.

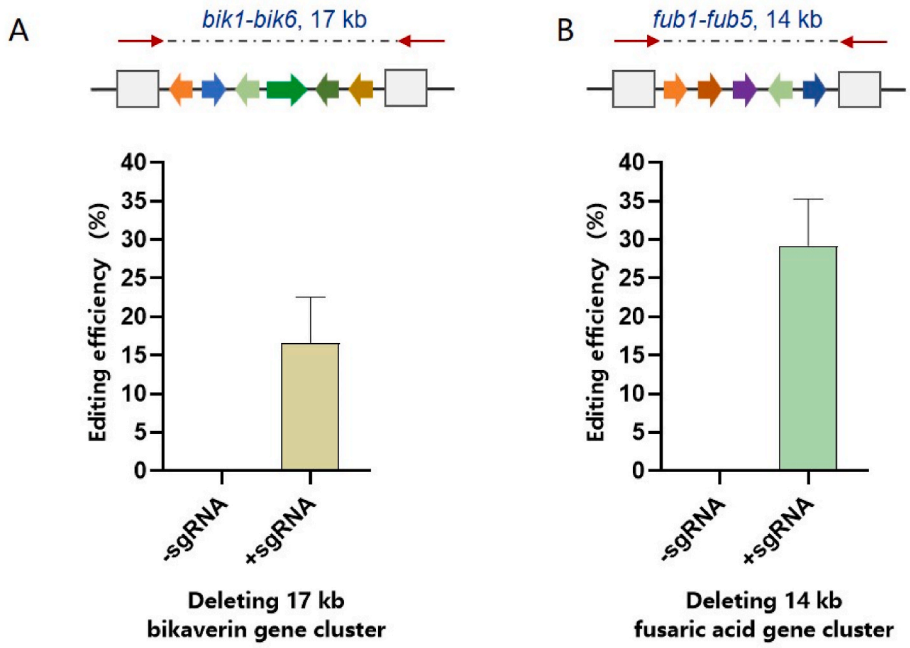

Fig. 4. Deleting large gene clusters by combining CRISPR/Cas9 with *pyr4*-blaster in *F. fujikuroi*. (A) Verifying the efficiency of deleting bikaverin gene cluster. (B) Verifying the efficiency of deleting fusaric acid gene cluster.

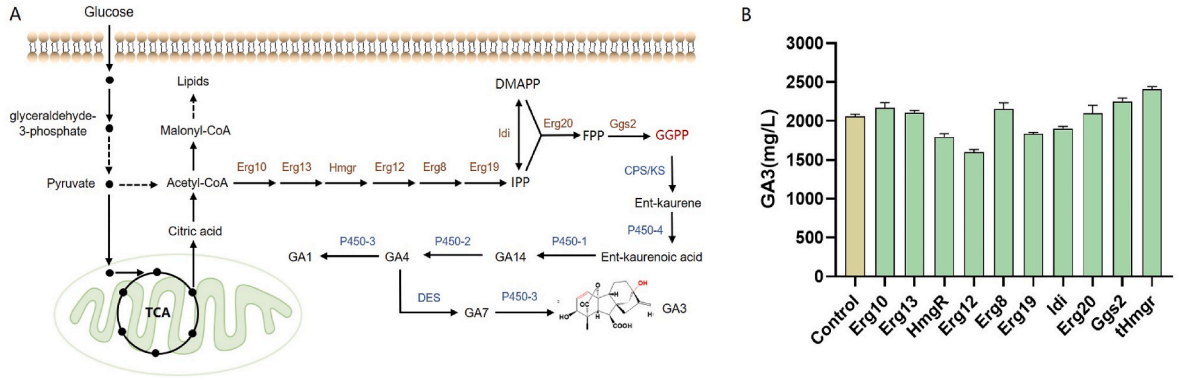

Fig. 5. Identifying key enzymes from the mevalonate pathway to increase the GA3 titer (A) GA3 biosynthesis pathway in *F. fujikuroi*. (B) the effect of single gene overexpression in the mevalonate pathway on the GA3 titer.

beneficial for protein soluble expression [34]. Consequently, we constructed the *YCL019* strain overexpressing *tHmgr*, resulting in a 10.98 % enhancement in GA3 titer, reaching 2.255 g/L compared to the control. These findings underscore the significant impact of *Ggs2* and *tHmgr*, acting as crucial rate-limiting genes, on bolstering the precursor GGPP supply. Moreover, these results lay the groundwork for achieving further improvements in GA3 titer through metabolic engineering.

4. Conclusion

Due to the lack of self-replicative plasmids in *F. fujikuroi*, the iterative gene editing events still depend on limited number of selection markers, which hindered the development of metabolic engineering. Therefore, it is very necessary to establish a selection marker recycling system to meet above requirements. In this study, the system was developed by combining the *pyr4*-blaster and CRISPR/Cas9 technology. This toolbox has been successfully used for multi-round gene editing including long gene clusters deletion, which was greatly favorable for facilitating the genetic manipulation of the non-model filamentous fungus *F. fujikuroi* and shortening the research and development cycle of high-producing strains. In addition, in order to identify the key rate-limiting enzymes in the MVA pathway, ten genes were individually overexpressed to generate the strain *YCL010-YCL018*. The result showed that *GGS2* and *tHMGR* play an important role in increasing the supply of precursor GGPP, the GA3 titer was improved by 9.22 % and 10.98 %, separately. These statistics provided the efficient targets for construction of GA3-overproducing strains using the metabolic engineering strategies. In the further research, *pyr4*-blaster and CRISPR systems were expected to be applied into all kind of *F. fujikuroi* for GA3 titer improvement by systems metabolic engineering.

CRediT authorship contribution statement

Tian-Qiong Shi: developed the research plan, performed the experiments, analyzed the statistics and did, Methodology, Validation, Supervision, the experiments, Writing – review & editing, the manuscript. **Cai-Ling Yang:** performed the experiments. **Dong-Xun Li:** performed the experiments. **Yue-Tong Wang:** analyzed the statistics and did, Methodology, Validation, Supervision, the experiments, Writing – review & editing, the manuscript. **Zhi-Kui Nie:** developed the research

plan, analyzed the statistics and did, Methodology, Validation, Supervision, the experiments, Writing – review & editing, the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest in the publication of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.01.010.

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