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Multi-gene precision editing tool using CRISPR-Cas12a/Cpf1 system in *Ogataea polymorpha*

Senqin Hou[1,2,3†], Shibin Yang[1,2†] and Wenqin Bai[1,2*]

Abstract

Background *Ogataea polymorpha*, a non-conventional methylotrophic yeast, has demonstrated significant potential for heterologous protein expression and the production of high-value chemicals and biopharmaceuticals. However, the lack of precise and efficient genome editing tools severely hinders the construction of cell factories. Although the CARISP-Cas9 system has been established in *Ogataea polymorpha*, the gene editing efficiency, especially for multiple genes edition, needs to be further improved.

Results In this study, we developed an efficient CRISPR-Cpf1-mediated genome editing system in *O. polymorpha* that exhibited high editing efficiency for single gene (98.1 ± 1.7%), duplex genes (93.9 ± 2.4%), and triplex genes (94.0 ± 6.0%). Additionally, by knocking out non-homologous end joining (NHEJ) related genes, homologous recombination (HR) efficiency was increased from less than 30% to 90 ~ 100%, significantly enhancing precise genome editing capabilities. The increased HR rates enabled over 90% integration efficiency of triplex genes, as well as over 90% deletion rates of large DNA fragments up to 20 kb. Furthermore, using this developed CRISPR-Cpf1 system, triple genes were precisely integrated into the genome by one-step, enabling lycopene production in *O. polymorpha*.

Conclusions This novel multiplexed genome-editing tool mediated by CRISPR-Cpf1 can realize the deletion and integration of multiple genes, which holds great promise for accelerating engineering efforts on this non-conventional methylotrophic yeast for metabolic engineering and genomic evolution towards its application as an industrial cell factory.

Keywords Ogataea polymorpha, CRISPR-Cpf1, Homologous recombination rates, Large DNA fragment deletion, Multiple genes integration

Introdution

Ogataea polymorpha is a non-conventional methylotrophic yeast with significant potential for industrial applications. It is generally regarded as safe (GRAS) and has been extensively explored as an excellent host for heterologous protein expression, including the production of recombinant enzymes and vaccines [1, 2]. *O. polymorpha* exhibits a broad substrate spectrum, encompassing glucose, sucrose, glycerol, xylose, and methanol, making it an attractive candidate for biosynthesis [3, 4]. Moreover, this yeast demonstrates remarkable heat resistance and can maintain sufficient vitality under harsh industrial

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conditions such as high temperature, low pH, and high osmotic pressure [5]. These exceptional characteristics highlight its immense potential in the efficient synthesis of high-value products using engineered microbial chassis cells. Therefore, *O. polymorpha* holds promise as an ideal host for industrial production.

The recent advancements in genetic manipulation tools have enabled efficient production of various chemical substances by *O. polymorpha* [6]. The development of effective genetic tools is crucial for constructing robust microbial cell factories [7, 8]. However, the lack of efficient gene editing tools has hindered genetic engineering in *O. polymorpha* thus far. To overcome this obstacle, the CRISPR (clustered regularly interspaced short palindromic repeats) -Cas (CRISPR-associated) nucleases system has emerged as a widely used gene editing tool in diverse microorganism [9–11]. This system primarily consists of Cas proteins that bind to target and cleave DNA sequences guided by gRNA to create double-strand breaks (DSBs) [12]. Subsequently, activated repair mechanisms such as non-homologous end joining (NHEJ) or homologous recombination (HR) facilitate gene deletion or integration. The CRISPR-Cas9 system has been successfully developed for *O. polymorpha*, enabling efficient genome editing through the evaluation of various codon-optimized Cas9 genes, sgRNAs, and promoters optimized for Cas9 and sgRNA expression [13]. However, there is a noticeable limitation in the ability of the CRISPR-Cas9 system for multiple genes editing, integration, and deletion of large DNA fragments, which highlights the urgent need to optimize and expand genetic engineering approaches to enhance metabolic engineering in *O. polymorpha*.

The CRISPR-Cas12a/Cpf1 system is a gene editing tool similar to CRISPR-Cas9, but Cpf1 possesses several advantages complementary to Cas9 [14]. Unlike Cas9, which primarily recognizes the PAM sequence rich in purines (NGG) for cleaving double-stranded DNA and creating blunt ends or cohesive ends with 5' overhangs of 1–3 nucleotides, Cpf1 predominantly recognizes the PAM sequence rich in thymine (TTTN), resulting in cleavage of double-stranded DNA and generation of sticky end with 5 nucleotides [15]. In comparison to the CRISPR-Cas9 system, CRISPR-Cpf1 does not require trans-activating CRISPR RNA (tracrRNA), and can target DNA and complete gene editing by binding with CRISPR RNA (crRNA), significantly shortening the gRNA expression cassette. Additionally, the Cpf1 protein possesses RNase activity and can specifically recognize the direct repeat (DR) sequence in crRNA and cleave at the 5' terminal of DR of pre-crRNA to form mature crRNA without additional nuclease [16]. This natural advantage suggests that the CRISPR-Cpf1 is well-suited for multiple genes editing. Although extensively used for genome editing in organisms such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytic* [10, 17, 18], the application of the CRISPR-Cpf1 system as a genome editing tool has not yet been established in *O. polymorpha*. With the simplified crRNA structure, production of sticky ends, and expanded PAM sequences, the Cpf1 system presents an attractive complementary approach that provides *O. polymorpha* with enhanced targeting efficiency and improved editing efficiency for multiple genes modifications.

The CRISPR-Cas system efficiently induces double-strand breaks (DSBs) through targeted DNA cleavage, followed by two major competing mechanisms for DSB repair: HR and NHEJ [19]. In *O. polymorpha*, NHEJ exhibits a clear advantage over HR, resulting in reduced accuracy for targeted genomic integration, even using larger homologous flanking regions exceeding several hundred base pairs [20]. The limited efficiency of HR leads to imprecise gene integration, significantly impeding the application of gene editing tools. Strategies such as overexpression of HR-related genes or deletion of NHEJ-related genes have proven effective in enhancing HR efficiency [21, 22]. For instance, overexpressing HR-related genes from *S. cerevisiae* including *RAD52*, *RAD59*, *MRE11* and *SAE2* effectively enhanced the HR efficiency in *P. pastoris* [23]. Similarly, deleting the key NHEJ gene *KU70* significantly improved gene integration efficiency in *Y. lipolytica* [24] while dynamically repressing the key gene *KU80* in the NHEJ pathway and enhancing the expression of HR-related proteins (*RAD51*, *RAD52* and *SAE2*) increased HR rates to 60–70% in *O. polymorpha* [13].

In this study, the engineered CRISPR-Cpf1 system demonstrated efficient and precise gene editing in *O. polymorpha* by screening both crRNA and Cpf1 promoters, enabling single, double, and triple genes disruption. The HR rates of *O. polymorpha* were significantly enhanced by eliminating the NHEJ key gene *KU70*, leading to a streamlined one-step integration of triple genes with high efficiency and accuracy (Fig. 1). Moreover, this system was successfully employed in *O. polymorpha* for large DNA fragment deletion for the first time, allowing the knockout of 10, 15, and 20 kb DNA fragments within the genome. Additionally, a novel CRISPR-dCpf1 mediated gene regulation system was developed to enable activation or repression of targeted gene expression. Overall, these findings highlighted that the CRISPR-Cpf1 system served as an efficient tool for genome editing in *O. polymorpha* with particular utility in one-step precise integration of multiple genes, and expanded its potential applications in metabolic engineering and cell factory construction.

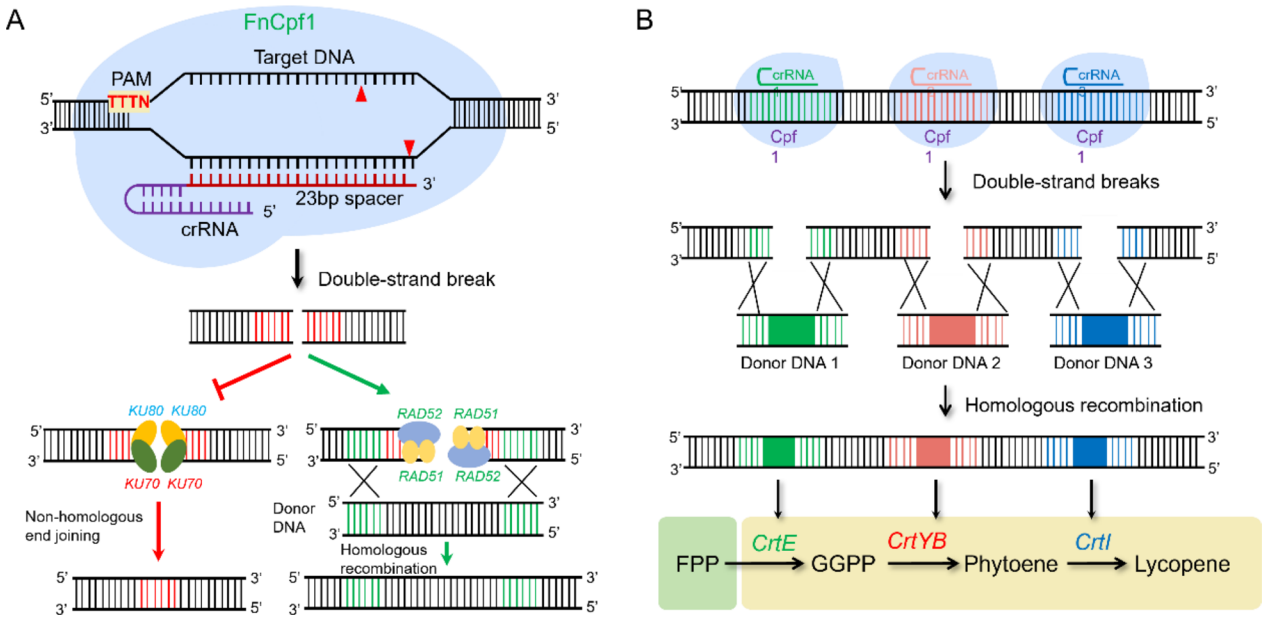

Fig. 1 Schematic diagram of CRISPR-*Fn*Cpf1-mediated gene editing mechanism in *O. polymorpha*. **A** DNA repair mechanisms induced by CRISPR-Cpf1. *Fn*Cpf1 forms a complex with crRNA that specifically targets DNA containing the appropriate protospacer adjacent motif (PAM). This assembly induces a double-strand break (DSB), resulting in a double-strand cut in the DNA. Within the cell, there are two competing DNA repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). Once the DSBs occurred, the NHEJ-related proteins Ku70 and Ku80 could form a complex that ligates the ends of the DNA break, often leading to deletions of base pairs at the junctions. Conversely, when homologous sequences are present, various HR-associated proteins, including Rad51 and Rad52, could collaboratively facilitate the integration of the homologous sequence into the genome via the homologous recombination pathway. The efficiency of HR was significantly improved by deleting the *KU70* gene in *O. polymorpha*. **B** Precise integration of multiple genes mediated by CRISPR-Cpf1. The crRNA array containing three distinct crRNAs is co-transformed with the corresponding donor DNAs, and FnCpf1 is able to cleave the crRNA array into multiple mature crRNAs. *Fn*Cpf1 forms complexes with different crRNAs that can create DSBs at multiple sites and simultaneously integrate multiple donor DNAs into the genome through homologous recombination. In this study, lycopene production was achieved by integrating three genes into the genome using one-step integration in *O. polymorpha*

Methods and materials

Strains and growth conditions

Escherichia coli DH5α was used as a cloning host for plasmid construction and cultured at 37 °C in Luria–Bertani (LB) broth containing 1% peptone, 0.5% yeast extract, 1% NaCl. *O. polymorpha* NCYC495 was used as host for genome-editing and routinely cultivated Yeast Extract Peptone Dextrose (YPD) medium containing 1% yeast extract, 2% peptone, and 2% Dextrose at 37 °C. The medium was supplemented separately with ampicillin (Amp, 100 μg/mL) in LB and nourseothricin (NAT, 30 μg/mL) in YPD for the selection of transformants. Leucine was added to Minimal Dextrose (MD) medium plates containing 1.34% (w/v) yeast nitrogen base (YNB), 2% (w/v) glucose, 0.00004% biotin, and 1.5% (w/v) agar for the selection of auxotrophic strains, and MG medium is the use of glycerol in place of glucose in MD medium while the other components are exactly the same. Uracil and leucine were added to Buffered Methanol-complex (BMMY) medium containing 1% yeast extract, 2% peptone, 1.34% YNB, 0.00004% biotin, 100 mM potassium phosphate (pH 6.0), and 2% methanol for fermentation. The lycopene standard was purchased from Sigma-Aldrich, St. Louis, MO. All strains used in this study were listed in Table S1.

Plasmid construction

All primers used in this study were listed in Table S2, and all primers were ordered from Tsingke Biotech (Beijing, China). The codon optimization of Cpf1 gene from *Francisella novicida* was performed for expressing in *O. polymorpha*, and the Cpf1 fused to the SV40 nuclear localization sequence (NLS) [25] was synthesized by Genscript Biotech (Nanjing, China), allowing Cpf1 to localize to the nucleus (Table S3). The GFP was linked to *Fn*Cpf1 via a P2A peptide at the 3'-terminal, and the expression level of Cpf1 was shown by detecting the green fluorescence intensity. *Fn*Cpf1 expression plasmid (pSH039) mainly contained recombinant *Fn*Cpf1 expression cassette controlled by the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) and the terminator of alcohol oxidase 1 (T_{AOX1}), NAT resistance

gene expression cassettes, and ampicillin resistance gene for screening in *E. coli* (Fig. S1A). Different promoter sequences were amplified from the genome and ligated to the vector backbone using the Gibson assembly method, allowing Cpf1 to be expressed under different promoters. The upstream and downstream sequences of the orotidine 5'-phosphate decarboxylase gene (*URA3*) were assembled as homologous arms on a Cpf1-expressing plasmid, enabling the integration of Cpf1 into the genome for expression and the acquisition of *URA3* auxotrophic strains (Fig. 2A).

The crRNA expression plasmids were episomal plasmids containing the autonomous replication start from *Kluyveromyces lactis* (panARS) [26], the crRNA expression cassette, the selection marker *URA3*, and a vector skeleton for plasmid construction containing the ampicillin gene and the prokaryotic replicon. To compare the transcription levels of crRNA, a combination of type II promoter/terminator and type III promoter/PolyT (Table S4) was used for crRNA transcription (Fig. S1B, C, D). To maximize the editing of multiple genes, the crRNA array is concatenated directly to construct the expression cassette $tRNA^{AGA}$-crRNA1-crRNA2-crRNA3-TSUP4 (Fig. 3A). And the homologous recombination DNA (donor DNA) contained roughly 500 bp 5′ upstream and 3′ downstream of the target gene, which were amplified from the genome of NCYC495. All crRNA sequences used in this study are listed in Table S5. The PCR amplifications of DNA fragments for cloning were obtained using PrimeSTAR Max Premix (TaKaRa), and the 2 × MultiF Seamless Assembly Mix (ABclonal) was performed to assemble plasmids in accordance with the manufacturer's instructions. All plasmids used in this study are listed in Table S6. The plasmid DNA and yeast genomic DNA were extracted using a TIANprep Mini Plasmid kit (TIANGEN) and TIANamp Yeast DNA kit (TIANGEN), respectively.

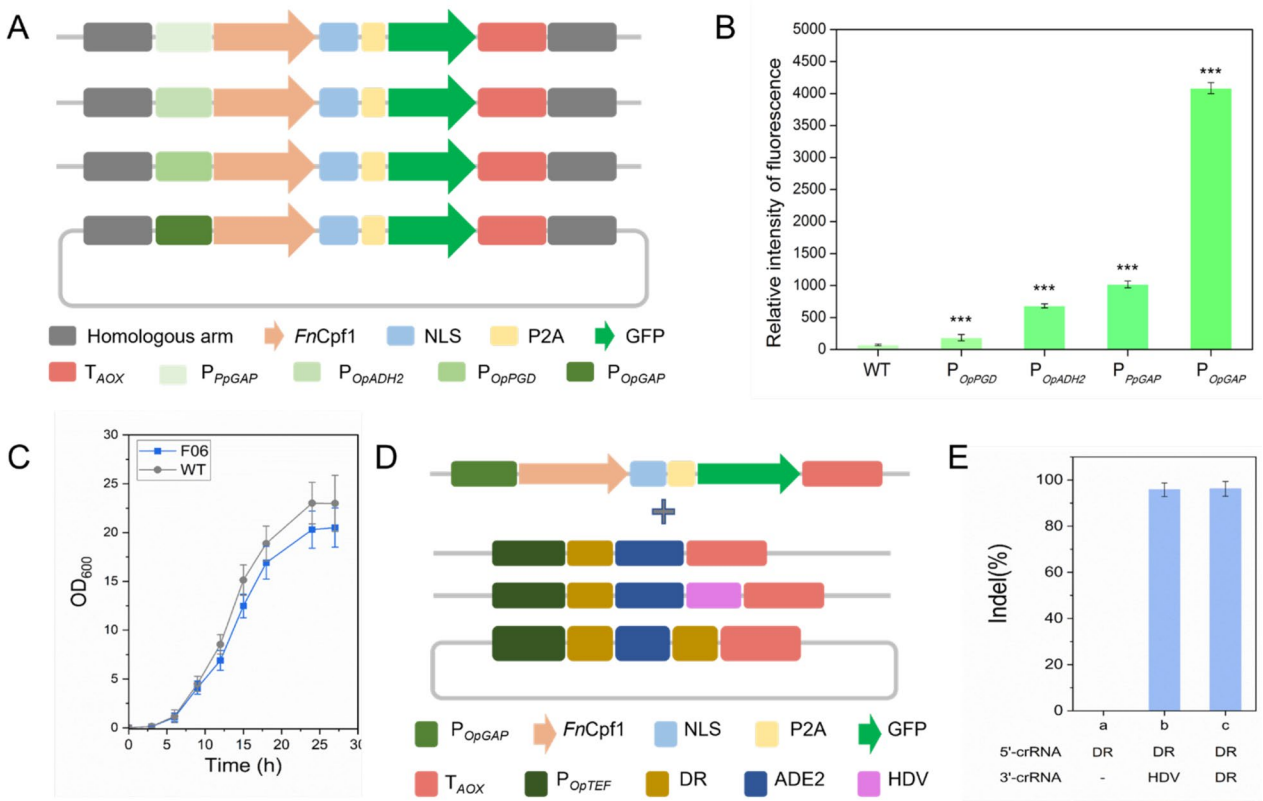

Fig. 2 Construction and editing efficiency of the CRISPR-Cpf1 system. **A** Construction of the expression plasmid of *Fn*Cpf1 under different promoters. And 1 Kb upstream and downstream of the open reading frame (ORF) of the *URA3* gene were used as Homologous arm. **B** The expression levels of *Fn*Cpf1 under different endogenous promoters by detecting relative fluorescence intensity of GFP. **C** Differences in growth curve between F06 strains and wild-type strains. F06 strains and WT strains were inoculated into YPD medium at 2% inoculum, and OD600 was recorded at different times, with three parallel sets for each group. **D** Construction of crRNA expression cassettes under Type II promoter P_{OpGAP} and terminator T_{AOX}, containing HDV and/or DR at 3′ terminal. **E** Editing efficiency of the CRISPR-Cpf1 system with crRNAs containing different elements at 3′ terminal on the *Ade2* gene without donor DNA. The data are represented as mean ± SD (n = 3 biologically independent samples). Black asterisks indicate statistical significance as determined using paired t test (***$p < 0.001$)

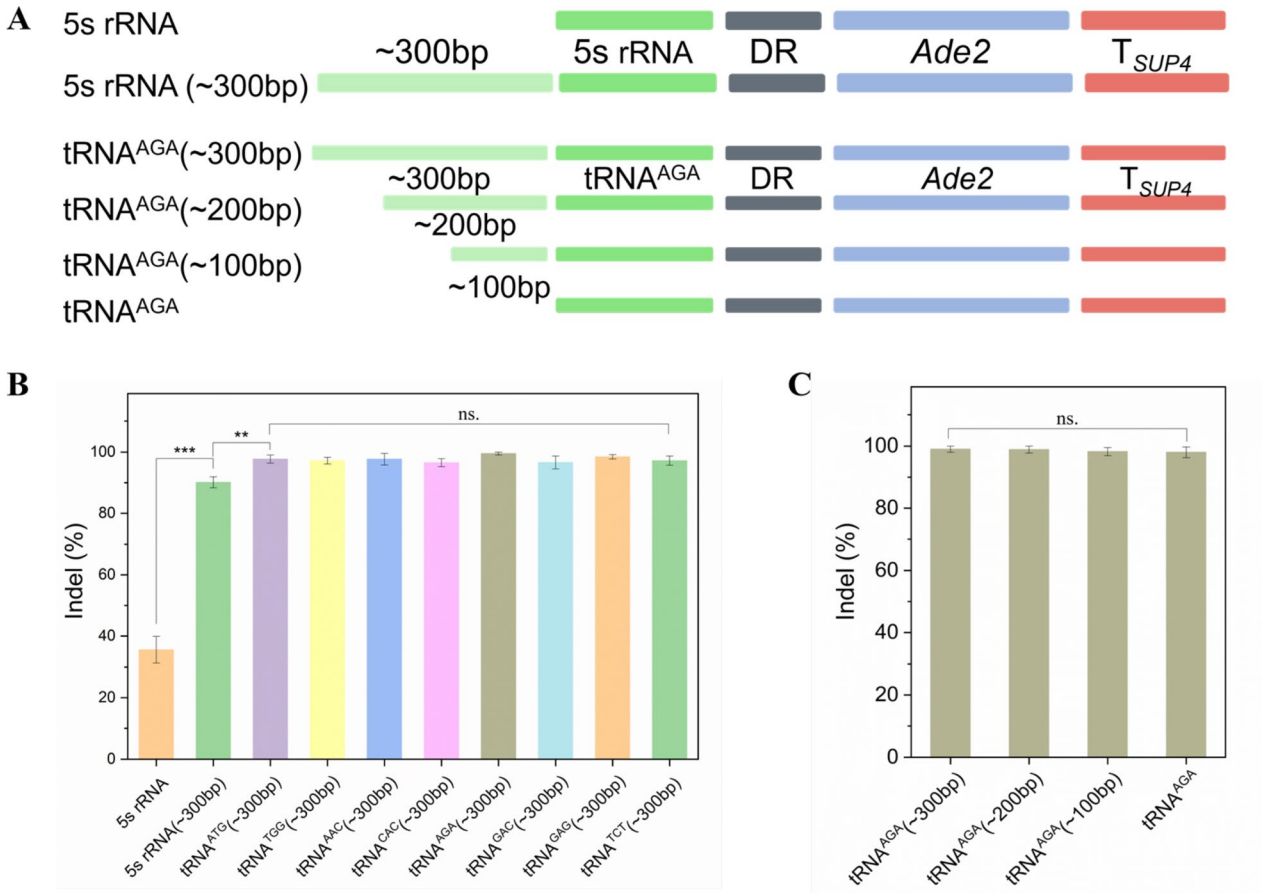

Fig. 3 Optimization of promoter sequences for crRNA array. **A** Schematic illustration of the construction of a crRNA expression cassette with Type III promoters and PolyT terminator containing different lengths of 5′UTR regions of unknown function. **B** Editing efficiency when transcribing crRNA using different Type III promoters (5 s rRNA and tRNA). **C** Effect of using $tRNA^{AGA}$ with different lengths of upstream regions as a promoter on editing efficiency during crRNA transcription. The data are represented as mean ± SD. Data are presented as means of three biologically independent samples. Black asterisks indicate statistical significance as determined using paired t test (**p < 0.01; ***p < 0.001; ns.: no significant difference)

Transformation and screening

The transformation process of the yeast was performed according to the optimized electroporation protocol as described previously [27]. To integrate the Cpf1 gene expression cassette into the genome, plasmids carrying Cpf1 controlled by different promoters were linearized using SacII and used for *O.* polymorpha transformation (by electroporation). After resuscitation, the recombinant yeast strains were selected on YPD plates containing 30 μg/mL of nourseothricin (NAT). Transformants were inoculated into YPD medium and incubated at 37 °C for 2 days. After incubation, 1 mL of the culture was centrifuged at 12,000 rpm for 5 min to harvest the cells. The pellets were then resuspended in 1 mL of 50 mM PBS buffer (pH7.2). The optical density at 600 nm (OD600) was detected and the GFP fluorescence intensity were measured using a multifunctional plate reader (excitation: 488 nm, emission: 509 nm). The expression level of Cpf1 was represented by the relative fluorescence intensity, calculated as the ratio of GFP fluorescence to OD600.

Circular plasmids containing various crRNAs and linear DNA donors obtained by PCR were co-transformed into F06 strain following the same electroporation method, and the transformants were screened on MD plates containing leucine, which was performed by auxotrophic. The transformants were validated by colony PCR and DNA sequencing. The efficiency of genome editing is calculated by comparing physiological traits resulting from the impairment of gene function. The destruction of the *ADE2* gene produced red colonies on the MD plate in the presence of low adenine concentrations, while in the absence of adenine, the colonies could not grow. The strain with disrupted *HIS4* was unable to grow on the MD plate without histidine, and when *GUT1*gene was broken, the growth of the strain was severely suppressed on the MG plate with glycerol as the sole carbon source [17]. Editing efficiency was identified as the ratio of the

number of correctly edited colonies to the total number of colonies.

Construction of lycopene producing strains and the production of lycopene

The F13 (NCYC495; Δ*ura3*; *FnCpf1*; Δ*Ku70*) strain was used to build the lycopene pathway. The carotenoid genes (*CrtE*, *CrtYB*, and *CrtI*) [28] were synthesized and produced with several endogenous constitutive promoters. The three genes were integrated into distinct locations in the genome, with neutral sites (NS18, NS12, and 3NS2) chosen as the integration sites. The corresponding homology arm sequences (500 bp) were added to both ends of the various genes as donor DNAs, and the crRNA array ($tRNA^{AGA}$-$crRNA_{NS18}$-$crRNA_{NS12}$-$crRNA_{3NS2}$-T_{SUP4}) was built to allow for simultaneous editing at three locations. The crRNA plasmid and donor DNAs were introduced into F13 competent cells together, then recovered and grown on MD plates. Five transformants were randomly selected and colony PCR was performed to verify whether the three genes had been integrated into the genome. Subsequently, three positive clones were randomly selected and inoculated into BMMY medium and fermented in flasks for three days with 2% inoculum, and the F13 strain was served as a control.

Extraction and detection of lycopene

To extract lycopene, 2 mL of the fermentation broth was transferred to an Eppendorf tube and centrifuged at 6000 g for 10 min. The lycopene was extracted from the pellets using hexane–acetone (2:3 v/v) according to previous report [29]. The extracted lycopene was quantified using high-performance liquid chromatography (HPLC), and the lycopene standard was used as the control. The quantification of lycopene was performed using a Waters 2695 series HPLC instrument (Waters Technologies, Milford, MA), equipped with a Waters C18 column (4.6×250 mm) and a PDA detector set to measure signals at 450 nm. For separation, samples were eluted with a mobile phase consisting of acetonitrile: isopropanol: methanol (5:2:3 v/v) at a flow rate of 1 mL/min and a temperature of 40 °C. The injection volume was set to 10 μL.

Results

Integrant expression of *Fn*Cpf1 with different promoters

Cpf1, originally derived from *Francisella novicida*, is recognized as a highly efficient genome editing alternative or complement to Cas9 [30]. Therefore, *Fn*Cpf1 was selected to establish the genome editing system. In this system, *Fn*Cpf1 fused with a nuclear localization signal (NLS) is connected to GFP via P2A peptide, and the recombinant *Fn*Cpf1 is expressed under the control of various promoters including P_{PpGAP} (P_{GAP} from *P. pastoris*), as well as P_{OpGAP} (P_{GAP} from *O. polymorpha*), P_{OpTEF1} (promoter of translation elongation factor EF-1α from *O. polymorpha*), and P_{OpPGD} (promoter of phosphogluconate dehydrogenase from *O. polymorpha*) (Fig. 2A and Fig. S1). During the translation of the recombinant Cpf1 protein, the ribosome undergoes a "skip" at the P2A peptide, facilitating the production of a cleaved protein [31]. The translation of GFP occurred only after the synthesis of the Cpf1 protein. Upon integration of the *Fn*Cpf1 expression cassette into the genome for expression, the presence of *Fn*Cpf1 was confirmed by the detection of relative fluorescence intensity of GFP. The results indicated that all four promoters were capable of expressing *Fn*Cpf1, and exhibited the highest expression levels with endogenous P_{OpGAP} promoter (Fig. 2B). To assess the potential toxicity of *Fn*Cpf1, the cell growth of F06 strain, in which the *Fn*Cpf1 was expressed using P_{OpGAP}, was compared to that of wild type. F06 strain revealed similar growth curves to the wild type in YPD medium, suggesting that the expression of *Fn*Cpf1 didn't exert a significant impact on the physiological activity of *O. polymorpha* (Fig. 2C). In order to ensure the editing efficiency of *Fn*Cpf1, the F06 strain was selected as the target strain for further research.

Genome editing using CRISPR-Cpf1 with different promoters for crRNA expression

In order to expediently evaluate the genome editing efficiency of the CRISPR-Cpf1 system in *O. polymorpha*, the *ADE2* gene, encoding phosphoribosylaminoimidazole carboxylase, was targeted to induce the formation of pink colonies upon its deletion [32]. The RNA polymerase II promoter, P_{OpTEF1}, was successfully used to express the crRNA for the CRISPR-Cas9 in *O. polymorpha* [13]. Consequently, the P_{OpTEF1} promoter was first used to express the crRNA of *ADE2* with the AOX terminator (Fig. 2D). The results showed no editing activity, suggesting that the function of crRNA was affected due to the presence of a longer tail at the 3' end produced by type II terminator, illustrating that ribozymes are essential when type II terminators are used. When adding HDV ribozyme or DR sequence at the 3' end of crRNA, the formation of red/pink colonies indicated that the *ADE2* gene was disrupted, suggesting the CRISPR-Cpf1 system was successfully implemented in *O. polymorpha* (Fig. 2E). When using type II terminator, DR sequence could be recognized and cleaved by Cpf1 to remove redundant tails and promote crRNA maturation instead of ribozymes. To further simplify crRNA expression and facilitate the operation, some endogenous RNA polymerase III promoters containing 5S rRNA and tRNAs with T_{SUP4} terminator (transcription terminator for the *S. cerevisiae* SUP4

tRNA gene) were tested the feasibility of express crRNA (Fig. 3A). 5S rRNA-crRNA displayed low editing efficiency (35.6%) (Fig. 3B). However, when adding 300 bp of 5′ upstream region of 5S rRNA, the editing efficiency increased to 90.1%, indicating that there were unknown elements upstream of 5S rRNA that could significantly affect 5S rRNA transcription in *O. polymorpha*, which is different from *Aspergillus niger* [33]. Based on this result, tRNAs with 5′ upstream region (300 bp) were tested as promoters. The result showed that tRNAs (~300 bp) displayed the higher editing efficiency (from 96.1% to 100%) than 5S rRNA and type II promoters, while the $tRNA^{AGA}$ (~300 bp) demonstrated the highest efficiency (from 98.1% to 100%) (Fig. 3B and Fig. S2). Contrary to 5S rRNA, the truncation of the 5′ upstream region of $tRNA^{AGA}$ gene has no effect on gene disruption efficiency, which ranged from 98 to 100% for different length of truncation, suggesting that the $tRNA^{AGA}$ alone was sufficient to efficiently and functionally initiate the crRNA transcription (Fig. 3C). The concise crRNA expression cassette was used to examine the editing efficiency of other genes, and the disruption efficiency of *HIS4* (histidinol dehydrogenase gene) and *GUT1* (glycerol kinase gene) was 96.2 ± 1.9% and 98.7 ± 1.2%, respectively (Fig. S3). These results suggested that this developed CRISPR-Cpf1 system was equally applicable to other genes.

Multiplex genome editing by the CRISPR-Cpf1 system using a single crRNA array

Compared to traditional genome engineering and CRISPR-Cas9, CRISPR-Cpf1 offers a more efficient multi-gene editing capability without biological markers for selection, which was contributed to the ability of Cpf1's mature cleavage of crRNA [34]. The multiplexed genome-editing induced by CRISPR system is commonly achieved by an array of sgRNAs or crRNAs [35]. To verify the capabilities of CRISPR-Cpf1 in multi-gene editing in *O. polymorpha*, three genes, *ADE2*, *HIS4* and *GUT1*, were target genes to investigate the simultaneous disruption of multi-gene. The crRNA array expression cassettes with different crRNAs were assembled in an episomal plasmid (Fig. 4A), and the multi-gene mutants were screened based on auxotroph marker (Fig. S4 and S5). It was found that the efficiency of double deletion of *ADE2-HIS4* reached 94.2 ± 1.9% (Fig. 4B). Similarly, the double deletion efficiency of *ADE2-GUT1* and *HIS4-GUT1* were found to be 96.0 ± 4.0% (Fig. 4C) and 91.3 ± 1.2%, respectively (Fig. 4D). Surprisingly, the triplex disruption efficiency with the crRNA targets for *ADE2*, *HIS4* and *GUT1* reached 94.7 ± 5.6% (Fig. 4E). This result represented the current highest efficiency of multi-gene editing in *O. polymorpha*, exhibiting comparable editing efficacy to the triplex genes efficiency achieved by CRISPR-Csa9 using sgRNA-tRNA strategy in *S. cerevisiae* [36], while employing a simplified crRNA array. With the increase of the number of target genes, the efficiency of multi-gene editing did not significantly decrease. It was hypothesized that Cpf1 and crRNA may have increased expression levels in the CRISPR-Cpf1 system developed in *O. polymorpha*, leading to efficient editing of multiple genes with equivalent efficacy.

Improving precision editing efficiency of CRISPR-Cpf1 by increasing the rates of the homologous recombination (HR)

The Cpf1 protein guided by crRNA induces double-strand breaks (DSBs), leading to two potential repair pathways: non-homologous end joining (NHEJ) in the absence of donor DNA and homologous recombination (HR) with donor DNA [37]. In *O. polymorpha*, DSB repair predominantly relies on NHEJ, resulting in low HR rates that impede accurate gene integration [38]. When CRISPR-Cpf1 and donor DNA were utilized to assess gene integration efficiency, which was verified by PCR and sequencing of the red clones. The validation primers were located in the upstream and downstream of the homology arm, resulting in a 1000 bp PCR product in the event of homologous recombination. However, three kinds of PCR products with different lengths were obtained (Fig. 5A). The 2700 bp bands represented the results of NHEJ, while the 1000 bp bands represented successful HR events, and the presence of 2300 or 2400 bp bands might imply an additional model: potentially semi-HR (Fig. 5B). The subsequent sequencing results also demonstrated three kinds of different repair patterns in the positive clones (Fig. S6). Modulating NHEJ-related genes or enhancing the expression of HR-related genes proved effective in boosting HR rates, thereby enhancing precision integration efficiency. Overexpression of HR-related genes *RAD51* and *RAD52* from *P. pastoris* and *O. polymorpha*, or deletion of NHEJ-related gene *KU70*, were employed to elevate HR rates [13, 23]. While overexpression of *RAD51* and *RAD52* had minimal impact on HR rates, deletion of *KU70* increased HR efficiency from less than 30% to more than 95% (F13 strain) (Fig. 5C), indicating deletion of the NHEJ key gene *KU70* or *KU80* is a simple and effective way to improve the efficiency of HR. The efficiency of HR was verified by integrating functional *LEU2* (β-isopropylmalate dehydrogenase gene from *O. polymorpha* CBS4732) expression cassette into *ADE2*, and the HR rates were detected by measuring the growth status of red/pink colonies on the MD plate without leucine (Fig. S7A). The results showed that the integration efficiency of *LEU2* gene was almost 100%, indicating that the deletion of *KU70* greatly improved the efficiency of HR in the strain (Fig. S7B). To further verify

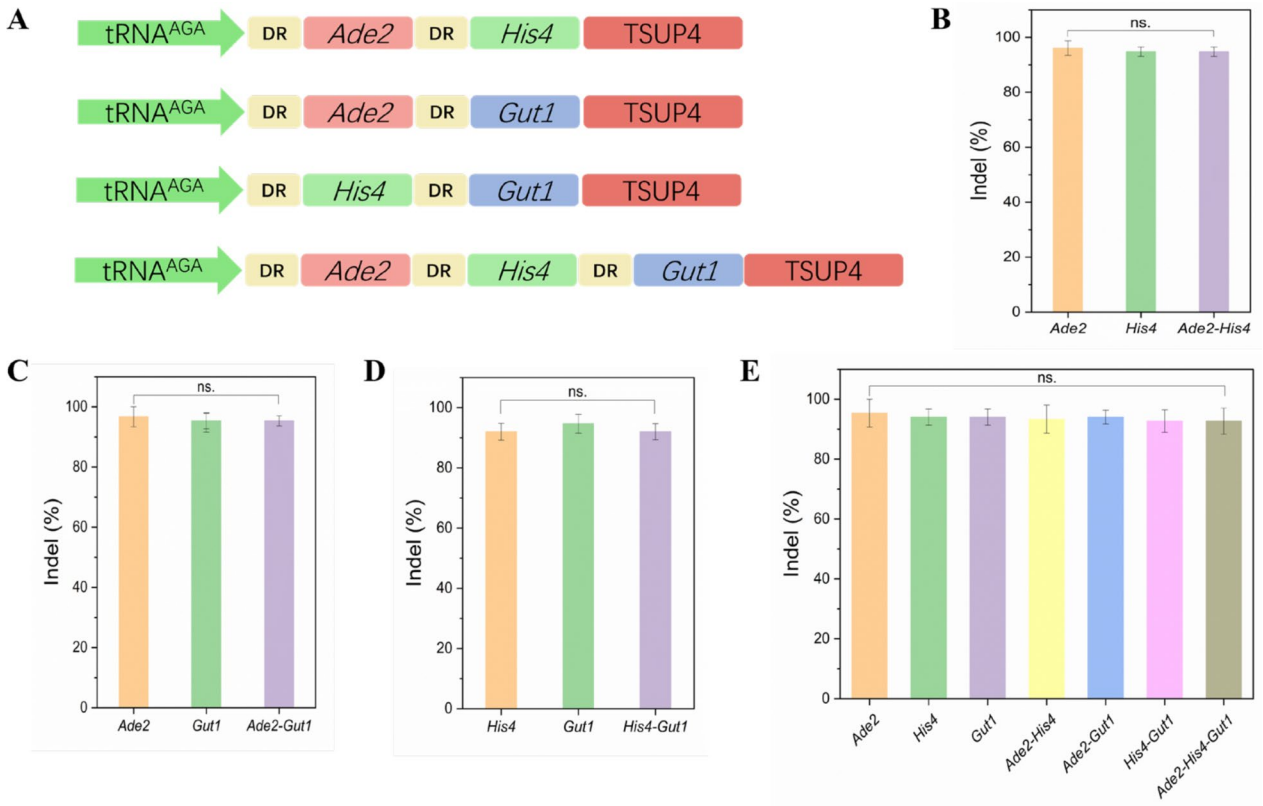

Fig. 4 CRISPR-Cpf1 system efficiency in editing multiple genes with a crRNA array. **A** Schematic representation of the construction of the crRNA array expression cassette used for multi-gene editing. **B** Editing efficiency of CRISPR-Cpf1 on single and dual gene *ADE2-HIS4*. **C** Editing efficiency of CRISPR-Cpf1 on single and double gene *ADE2-GUT1*. **D** Editing efficiency of CRISPR-Cpf1 on the dual gene *HIS4-GUT1*. **E** Editing efficiency of CRISPR-Cpf1 on single, double, and the triple gene *ADE2-HIS4-GUT1*. The data are represented as mean ± SD. Data are presented as means of three biologically independent samples. Black asterisks indicate statistical significance as determined using paired t test (ns.: no significant difference)

the precision editing efficiency of multiple genes, expression cassettes of *ADE2*, *HIS4* and *LEU2* with homologous arms were integrated into genome in one-step (Fig. 6A). The integration efficiency of the triplex genes exceeded 98% using nutritional markers for screening (Fig. 6C). These results suggested that NHEJ holds a distinct advantage in DNA repair competition, and HR can prevail by eliminating NHEJ-related proteins in *O. polymorpha*. The deletion of *KU70* did not significantly affect the strain's growth in YPD medium (Fig. 5D), underscoring that suppressing NHEJ-related proteins was an effective strategy for enhancing HR rates.

Large DNA fragments deletion using the CRISPR-Cpf1 system

The deletion of large chromosomal fragments has great potential application in basic research and engineering modification [39]. However, there was no related report on the deletion of large fragments in *O. polymorpha*. Here, CRISPR-Cpf1 was used to try to evaluate the potential for large fragment deletion. The large DNA fragment of 10, 15, and 20 kb in the chromosome VII was selected as the target (Fig. 6B), and the deletion efficiency was further verified by PCR. Plasmids containing double crRNA arrays were employed to guide Cpf1 for generating double-strand breaks (DSBs), which were subsequently repaired through HR using homologous arms. Primers located in the upstream and downstream of the homologous arm were utilized to detect deletions, resulting in a PCR product of 2 kb indicative of successful deletion, while no PCR product was obtained when the larger fragment remained intact. To ensure the reliability of the PCR results, a genomic fragment (500 bp) served as an internal reference. The presence of predominantly 2 kb PCR bands among the clones indicated successful deletion of large DNA fragments (10, 15, and 20 kb) (Fig. S8A). The sequencing results of three randomly selected positive clones also confirmed that large fragments had been successfully deleted (Fig. S8B). The deletion efficiencies of large DNA fragments reached 95.8 ± 3.6% (10 kb),

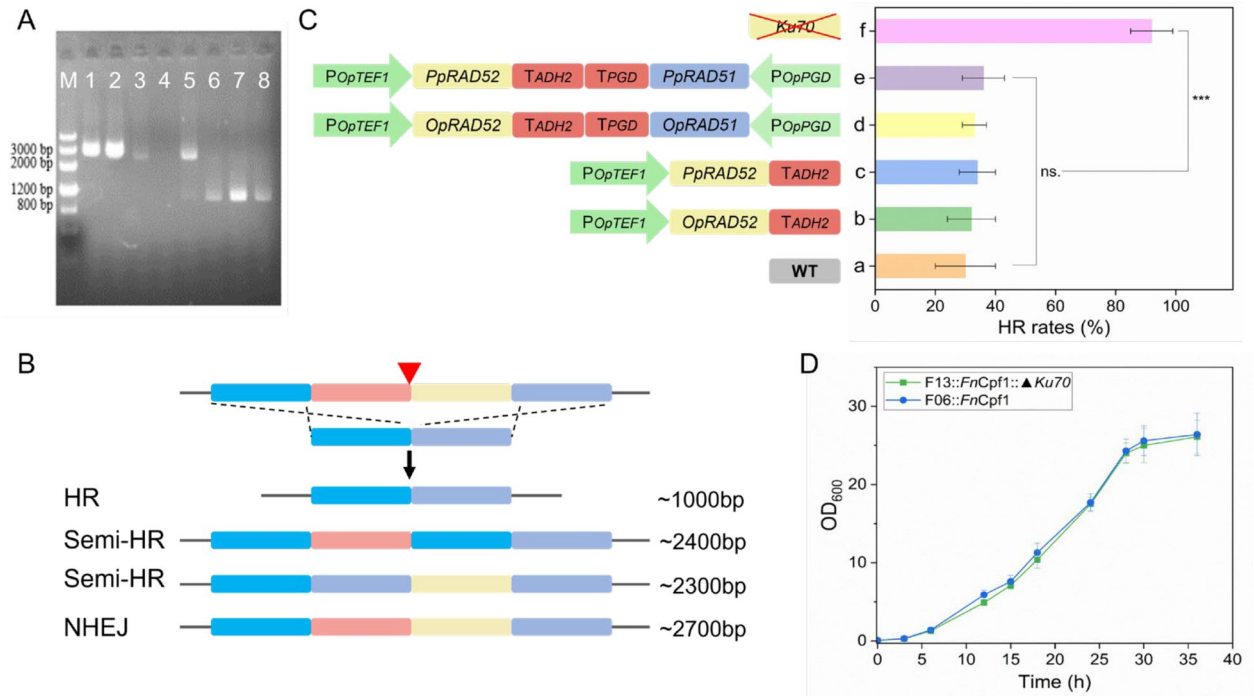

Fig. 5 Enhancing homologous recombination efficiency in *O. polymorpha*. **A** Colony PCR of red/pink clones. PCR products of three different lengths were obtained from the genome of red/pink colonies, indicating the presence of three different genotypes in positive clones upon addition of donor DNA (M: Marker; lanes 1–2: 2700 bp of NHEJ products; lanes 3–5: 2300/2400 bp of semi-HR products; lanes 6–8: 1000 bp of HR products.). **B** Schematic representation of the three genotypes (HR, semi-HR, and NHEJ) presented in pink clones. **C** Effect of overexpressing HR-related protein genes *RAD51* and *RAD52* from *O. polymorpha* and *P. pastoris*, as well as deletion of the NHEJ key gene *KU70*, on HR efficiency (n = 52). **D** Effects of deletion of the *KU70* gene on strain growth vitality. The data are represented as mean ± SD. (n = 3 biologically independent samples). Black asterisks indicate statistical significance as determined using paired t test (***$p < 0.001$; ns.: no significant difference)

89.9 ± 9.5% (15 kb) and 83.3 ± 13.0% (20 kb), respectively (Fig. 6D). In contrast to the significantly low efficiency of large fragment deletion in *P. pastoris* [17], CRISPR-Cpf1 exhibited a remarkably high deletion efficiency, which could be attributed to its superior editing capability and substantial enhancement in HR rates.

Application of multigene integration using the CRISPR-Cpf1 system

One-step integration of multiple genes could certainly facilitate the assembly of a complex metabolic pathway containing various genes. And CRISPR-Cas9-mediated multi-gene integration method has been successfully developed in *P. pastoris* [29]. In order to verify the feasibility of one-step multi-locus gene integration, the genes involved in lycopene synthesis, *CrtE*, *CrtYB*, and *CrtI* from *Phaffia rhodozyma* [28], were integrated into different genomic neutral sites using different endogenous strong promoters and homologous arms simultaneously (Fig. 7A). These neutral sites containing NS18, NS12 and 3NS2 had been shown to enable the genes expression and have no effect on cellular fitness (Fig. 7B) [40]. When all three genes were integrated into the genome synchronously, the engineered strain exhibited the ability to produce lycopene, and the integration efficiency of the three genes was calculated by counting the ratio of the red colonies in all transformants. Five red clones were randomly selected, and colony PCR was conducted to verify whether the three genes were correctly integrated at specific locations in the genome. The sizes of the PCR products were 3465 bp for *CrtE*, 4356 bp for *CrtYB*, and 4083 bp for *CrtI*, respectively, indicating successful precise integration of all three genes at pre-designated genomic locations in five clones (Fig. 7C). Three positive clones were randomly selected and inoculated into BMMY medium in conical flasks for a three-day fermentation process. After centrifugation, the cell pellets were collected, and all the clones exhibited a distinct red color, indicating that the strain (F41 strain) was capable of producing lycopene in BMMY medium with methanol as the sole carbon source. Further quantification of lycopene production was conducted using HPLC analysis, which demonstrated that the F41 strain was able to accumulate lycopene concentrations up to 48.2 ± 4.9 mg/L in BMMY medium (Fig. 7D). These results suggest that *O. polymorpha* has promising potential for lycopene production

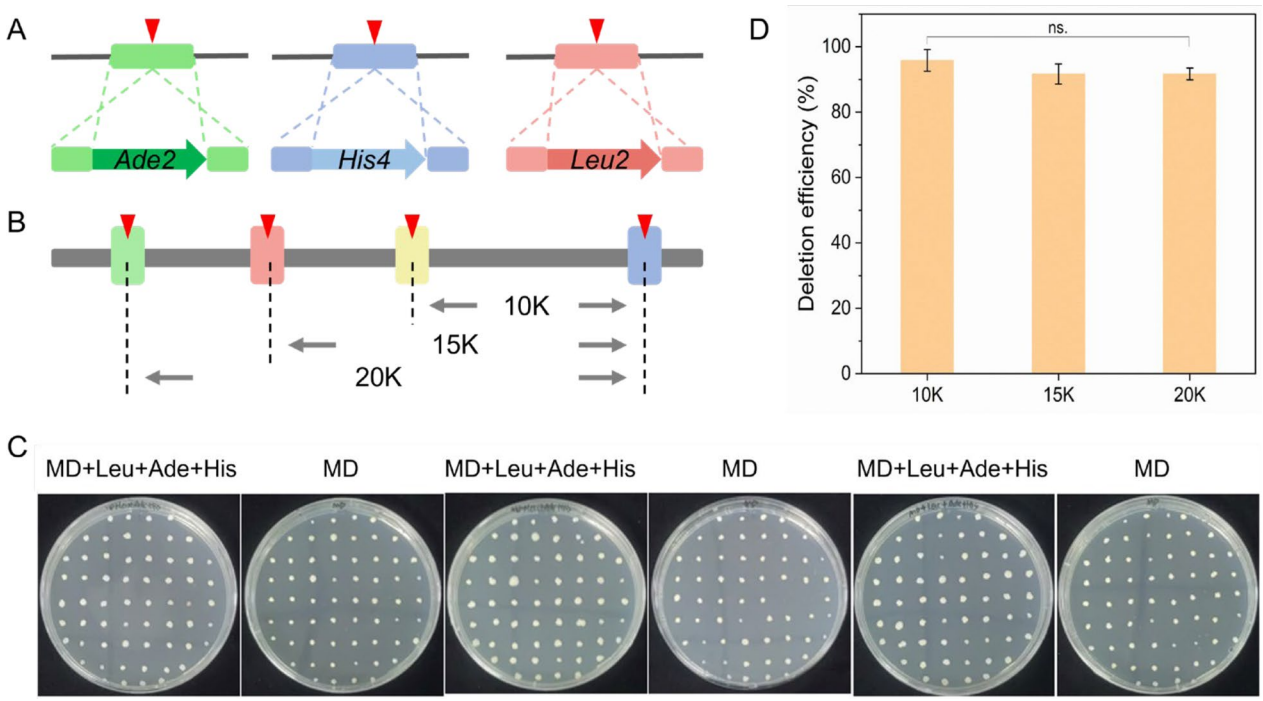

Fig. 6 Improving the efficiency of multi-gene integration and large DNA fragment deletion based on homologous recombination. **A** Schematic representation of the one-step integration of three genes *ADE2*, *HIS4*, and *LEU2*. **B** Diagram illustrating deletions of different large DNA fragment lengths (10 k, 15 k, and 20 k). **C** The efficiency of multi-gene integration. The efficiency of one-step integration of three genes was evaluated by comparing the growth status of transformants on MD plates and the MD plates containing leucine, adenine, and histidine after co-transformation of a plasmid containing a crRNAs array and donor DNA harboring the *ADE2*, *HIS4*, and *LEU2* expression cassettes (n = 180). **D** Efficiency of CRISPR-Cpf1-mediated deletion of large genomic fragments of varying lengths. The data are represented as mean ± SD. Statistical analysis using paired t test showed no significant difference (ns.) in deletion efficiency between 10 K, 15 K, and 20 K

utilizing methanol as the sole carbon source. Additionally, engineered strains capable of producing lycopene were successfully generated through a one-step integration process, demonstrating the high efficiency of the CRISPR-Cpf1-mediated multi-gene integration method.

Discussion

O. polymorpha is a promising industrial strain due to its exceptional thermostability and versatile carbon source utilization. Efficient genome editing tools are essential for metabolic engineering and constructing cell factories, but the low efficiency of homologous recombination (HR) complicates precise genome modifications. Previous studies have established CRISPR-Cas9 technology in *O. polymorpha*, employing RNA polymerase II-type promoters and ribozymes flanking the sgRNA for expression[13, 20, 38, 41]. Although single-gene editing efficiencies have reached 93.4%, multiple-gene editing remained challenging due to the limited availability of efficient RNA Pol III promoters [13]. In this study, we demonstrate that the direct repeat (DR) sequence of crRNA could be recognized and cleaved by Cpf1, thereby removing redundant sequences generated by type II terminators (Fig. 2D). This finding suggested that CRISPR-Cpf1 has the potential to facilitate multi-gene editing without the need for flanking ribozymes when using type II promoters and terminators. To further enhance editing efficiency and simplify the crRNA expression cassette, it is essential to screen efficient Pol III promoters. tRNA promoters exhibited higher editing efficiencies than Pol II promoters in *O. polymorpha*. The simplified crRNA expression cassette comprised a type III tRNA promoter and the type III terminator SUP4, which facilitated easier manipulation and required only a single-step PCR with long primers.

Typically, the editing efficiency of multiple genes decreases as the number of target genes increases [10, 17]. To enhance multi-gene editing efficiency, an optimized CRISPR-Cas9 system in *S. cerevisiae* incorporated tRNA sequences in crRNAs, achieving over 90% editing efficiency for triple gene targets [36]. However, this strategy was not equally successful in *O. polymorpha* [41]. In this study, a crRNA array was employed to simultaneously edit three genes using CRISPR/Cpf1 with an efficiency of over 90% in *O. polymorpha* (Fig. 6C). In contrast, the editing efficiencies of only 30.5% and 41.7%

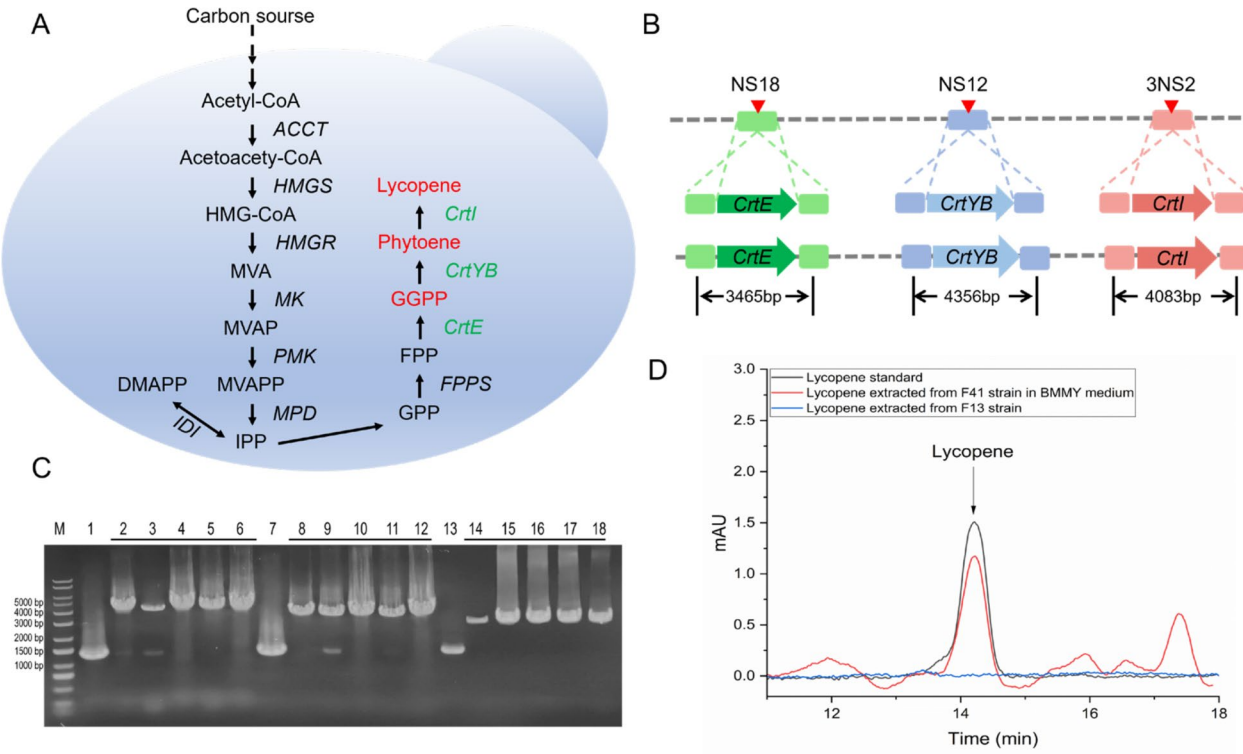

Fig. 7 The production of lycopene by engineered *O. polymorpha* and detection of lycopene. **A** The lycopene biosynthesis pathway in *O. polymorpha*. **B** A schematic representation of the integration of lycopene biosynthesis-related genes *CrtE*, *CrtYB*, and into three different neutral sites NS18, NS12, and 3NS2 in the genome using the CRISPR-Cpf1 system in one-step. **C** Verification of transformants using colony PCR. Five clones were selected and colony PCR was employed to confirm the integration of the three genes at their designated locations. The sizes of PCR products were 3465 bp for *CrtE*, 4356 bp for *CrtYB*, and 4083 bp for *CrtI*, indicating precise integration into the specified genomic sites (M: marker; lanes 1, 7, 13 were negative control; lanes 2–6 were products pf *CrtYB*; lanes 8–12 were products of *CrtI*; lanes 14–18 were products of *CrtE*.). **D** Analysis of lycopene extracted from F41 strain after 3 days of fermentation in BMMY media was performed using HPLC

were observed in *P. pastoris* and *Y. lipolytica* using three independent crRNA expression cassettes were respectively [17, 42]. The high editing efficiency for multiple genes mediated by CRISPR-Cpf1 could be attributed to the robust expression of *Fn*Cpf1 and the effective maturation of the crRNAs, leading to synchronized and highly efficient targeting of multiple genes. Compared to the CRISPR-Cas9/Cpf1 systems in *P. pastoris* and CRISPR-Cas9 in *O. polymorpha*, this system demonstrated superior efficiency in multi-gene editing and a simplified construction process. The improved multi-gene editing efficiency of CRISPR-Cpf1 facilitates the genetic engineering of *O. polymorpha* and demonstrates its potential application in the construction of cell factories and the optimization of expression systems in *O. polymorpha*.

HR efficiency in the host is crucial for precise gene editing, particularly for the accurate integration of multiple genes. Compared to *S. cerevisiae* and *P. pastoris*, *O. polymorpha* exhibited a lower HR rate of less than 30%. Enhancing HR rates in *O. polymorpha* is essential for improving gene editing precision. Increasing the expression of HR-related genes or downregulating NHEJ-related genes was effective in improving HR efficiency, which could potentially be increased to 60–70% through the overexpression of HR-related genes in *O. polymorpha* [13]. In this study, deletion of the NHEJ key gene *KU70* resulted in a remarkable 100% HR efficiency. This increase is attributed primarily to the disruption of the NHEJ pathway, compelling the host to rely exclusively on the HR pathway for gene repair, consistent with previous findings. The deletion of NHEJ key genes, such as *KU70* or *KU80*, represented a viable strategy for enhancing CRISPR-mediated precise gene editing. However, it could significantly inhibit the host's growth under stressful conditions [13, 24]. Therefore, after completing gene editing, it is important to restore the NHEJ pathway to ensure the growth viability of cells in different environments. Implementing an inducible expression system for the *KU70* or *KU80* gene, coupled with the use of inducers to repress *KU70* or *KU80* expression during gene editing, may be a more effective strategy.

Table 1 Genome editing by CRISPR-Cas9/Cpf1 in *Ogataea* strains

Strains	Cas9/Cpf1		sgRNA/crRNA		Editing rates	Integration		References
	Promoter	Type	Promoter	Type		Single-gene	Triple-gene	
O. polymorpha	P_{OpTDH3}	Episomal	$P_{OpSNR6\text{-}tRNA}$	Episomal	17–71%	–	–	[41]
O. polymorpha	P_{AaTEF1}	Episomal	P_{ScTDH3}	Episomal	< 9%	–	–	[20]
O. thermomethanolica	P_{AOX1}	Episomal	P_{AOX1}	Episomal	63–97%	–	–	[43]
O. parapolymorpha	P_{AaTEF1}	Episomal	P_{ScTDH3}	Episomal	< 63%	–	–	[20]
O. polymorpha	P_{ScTEF1}	Integrated	$P_{ScSNR52}$	Integrated	62–66%	–	28–34%	[38]
O. polymorpha	P_{KpGAP}	Integrated	P_{TEF1}	Episomal	90–95%	40–70%	–	[13]
O. polymorpha	P_{OpGAP}	Integrated	$P_{tRNAAGA}$	Episomal	94–100%	100%	100%	This study

The increased HR rates significantly improved the efficacy of CRISPR-Cpf1 in large fragment deletion and one-step integration of multiple genes. The deletion efficiency for 20 kb DNA fragments exceeded 90% (Fig. 6D), compared to only 11% in *P. pastoris* [17]. Furthermore, a lycopene-producing strain was successfully generated through one-step integration, highlighting the convenience of using CRISPR-Cpf1 in metabolic pathway construction. In summary, the CRISPR-Cpf1 system has a powerful multi-gene editing capability that complements the CRISPR-Cas9 system and shows excellent genome editing efficiency (Table 1). However, some limitations remained. It was generally necessary to remove the Cpf1 or Cas9 genes from the genome after editing to prevent any adverse effects on cellular function. Specifically, Cpf1 must be excised while simultaneously complementing essential genes, such as *URA3* and *KU70*, back into the genome upon completion of gene editing. Additionally, achieving efficient expression of Cpf1 and crRNA from the same episomal plasmid with high editing efficiency may be a more effective strategy. Nonetheless, further exploration is required to ensure the strength and stability of Cpf1 expression on episomal plasmids. Overall, the constructed CRISPR-Cpf1 system demonstrated distinct advantages in the precise editing of multiple genes, revealing its potential for constructing cell factories for protein expression and the production of high-value chemicals.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02654-8.

Additional file 1.

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Author contributions
W.B. conceived the project, W.B., S.Y., and S.H. designed the experiments. S.H. and S.Y. performed the experiments. W.B., S.Y., and S.H. analyzed the data. S.Y. wrote the manuscript. W.B. revised the manuscript. W.B. supervised the research. All authors have read and approved the final manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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