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BIOTECHNOLOGY METHODS

Multiplex gene editing of the *Yarrowia lipolytica* genome using the CRISPR-Cas9 system

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Abstract *Yarrowia lipolytica* is categorized as a generally recognized as safe (GRAS) organism and is a heavily documented, unconventional yeast that has been widely incorporated into multiple industrial fields to produce valuable biochemicals. This study describes the construction of a CRISPR-Cas9 system for genome editing in *Y. lipolytica* using a single plasmid (pCAS1yl or pCAS2yl) to transport Cas9 and relevant guide RNA expression cassettes, with or without donor DNA, to target genes. Two Cas9 target genes, *TRP1* and *PEX10,* were repaired by non-homologous end-joining (NHEJ) or homologous recombination, with maximal efficiencies in *Y. lipolytica* of 85.6 % for the wild-type strain and 94.1 % for the *ku70*/*ku80* double-deficient strain, within 4 days. Simultaneous double and triple multigene editing was achieved with pCAS1yl by NHEJ, with efficiencies of 36.7 or 19.3 %, respectively, and the pCASyl system was successfully expanded to different *Y. lipolytica* breeding strains. This timesaving method will enable and improve synthetic biology, metabolic engineering and functional genomic studies of *Y. lipolytica*.

Keywords *Yarrowia lipolytica* · CRISPR · Genome-editing

Introduction

Yarrowia lipolytica is a generally recognized as safe (GRAS) organism and a well-studied unconventional yeast species [24, 29]. It is widely used in multiple industrial fields to produce valuable biochemicals, including organic acids [24] and omega-3 eicosapentaenoic acids (EPA) [44]. The metabolic engineering of *Y. lipolytica* is heavily documented [13, 22, 26, 38, 44, 45, 47], but traditional

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genome-editing tools, such as the ura3-blaster [26] and Cre-lox [12] systems, make the process laborious and time-consuming.

Clustered regularly interspaced short palindromic repeats (CRISPR) were originally discovered as an immunological mechanism in bacteria and archaea [2, 42]. The type II CRISPR-Cas9 system has been extensively developed as a powerful genome-editing tool because of its high on-target efficiency in numerous prokaryotes and eukaryotes, including (but not limited to) *E. coli* [16], *Streptomyces* spp. [7], *Clostridium cellulolyticum* [43], *Lactobacillus reuteri* [30], *Saccharomyces cerevisiae* [11], *Bombyx mori* [41], *Filamentous fungi* [23, 28], *Drosophila* [46], *Candida albicans* [40], higher plants [32], and multiple human cell lines [8, 19, 25]. The CRISPR-associated protein (Cas9) endonuclease is guided by a mature CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) towards a target DNA sequence (known as a protospacer). The dual-tracrRNA:crRNA also can be engineered as a single guide RNA (sgRNA) to direct sequence-specific Cas9 dsDNA cleavage [18]. A protospacer-adjacent motif (PAM) immediately follows the 3′ end of the protospacer (NGG in *Streptococcus pyogenes*, where N represents any nucleotide) [10]. In eukaryotes, DNA double-stranded breaks (DBSs) are induced by the Cas9–RNA complex and are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ). The NHEJ pathway recruits multiple proteins, including *KU70* and *KU80,* and does not require a homologous template [9]. When a homologous template is present, the HR pathway recruits multiple proteins to repair the DSBs through a homology-based repair process [36]. It has been previously reported that frequency of NHEJ-mediated DSBs repair is high in *Y. lipolytica* [31].

CRISPR-Cas9 systems have been implemented in three species of yeast, *S. cerevisiae* [11], *Schizosaccharomyces pombe* [15] and *Kluyveromyces lactis* [14]. These tools have enabled single-gene and simultaneous multigene editing [1, 14, 34]. The CRISPR-Cas9 system used in *S. cerevisiae* was successfully extended to *K. lactis* [14]. However, this system could not function in *Y. lipolytica* (data not shown). It is possible that biological impediments exist that would render the *S. cerevisiae* constructs inapplicable to *Y. lipolytica*. Schwartz et al. [33] developed a two plasmid-based CRISPR system, in which the sgRNA was expressed by an architectural synthetic RNA polymerase III promoter, and the system successfully achieved single-gene knockouts and knockins in *Y. lipolytica*.

In this study, we demonstrated a single plasmid-based CRISPR-Cas9 system using pCASyl that successfully achieved single or multiple gene disruption via NHEJ or HR in wild-type, *ku70*-deficient and *ku70*/*ku80* double-deficient strains of *Y. lipolytica* (Fig. 1). The pCASyl can be easily cured to enable multiple rounds of gene editing. The pCASyl system was successfully expanded to different *Y. lipolytica* strains for gene disruption.

Materials and methods

Strains, media and culture conditions

The *Y. lipolytica* strains used in this study are listed in Table 1. The cells used for transformations were cultivated overnight in culture tubes and transferred to culture flasks for incubation and continuous shaking in a fresh YPD medium, as previously described [13]. After

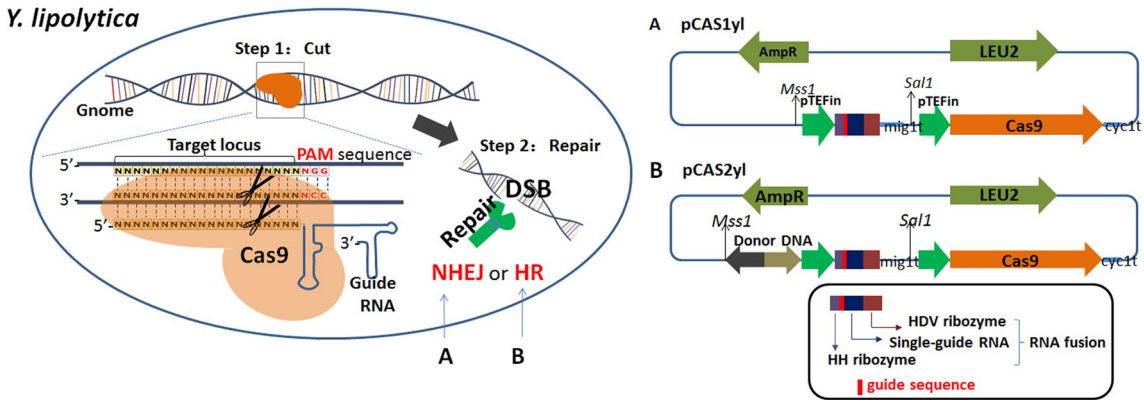

Fig. 1 Overview of genome editing by the CRISPR system in *Y. lipolytica*. **a** Cells transformed with pCAS1yl without donor DNA, inducing DSBs that were repaired by NHEJ. **b** Cells transformed with pCAS2yl with donor DNA, inducing DSBs that were repaired by HR. *NHEJ* non-homogeneous end-joining, *HR* homologous recombination, *DSB* double-stranded break. pCASyl plasmids derived from pMCSCen1 backbone. The *LEU2* was used for selection in *Y. lipolytica* and the *AmpR* was used in *E.coli*

Table 1 Strains and plamids used in this work

Strains or plasmids	Characteristics	Source or references
Strains		
ATCC201249	*MATA ura3-302 leu2-270 lys8-11 PEX17-HA*	ATCC
ATCC MYA-2613	*MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2*	ATCC
CIBTS1604	ATCC MYA-2613 △*ku70::hisG-URA3-hisG*	This work
CIBTS1605*	ATCC MYA-2613 △*ku70::hisG*	This work
CIBTS1961	ATCC MYA-2613 △*ku70::hisG*△*ku80::URA3*	This work
Plasmids		
pTAs	pMD18T simple vector	TAKARA
pTA	pMD18T vector	TAKARA
pTA-HUH	4.2 kb HisG-URA3-hisG recycling type marker in pTAs	This work
pTAs-ku70-HUH	7.2 kb Ku70 deletion cassette in pTAs	This work
p415-GalL-Cas9-CYC1t	Carrying Cas9 gene	[10]
pMCSCen1	Replicative plasmid for *Y. lipolytica*	[1]
pTAs-TinCas9	Cas9 expression cassette in pTAs	This work
pCen1-Cas9	Only Cas9 expression cassette in pMCSCen1	This work
pCen1-inCas9	Cas9 gene ORF deleted 641 bp by insertion of 1.1 kb hisG	This work
pCAS1yl-trp	*TRP1* Guide RNA module and Cas9 expression cassette in pMCSCen1	This work
pCAS2yl-trp	1.0 kb *TRP1* donor DNA in pCAS1yl-trp	This work
pCen1-Cas9-donorTRP1	1.0 kb *TRP1* donor DNA and Cas9, no guide RNA module	This work
pCAS1yl-pex	*PEX10* guide RNA module and Cas9 expression cassette in pMCSCen1	This work
pCAS2yl-pex	0.88 kb *PEX10* donor DNA in pCAS1yl-pex	This work
pCAS1yl-TP	*TRP1* and PEX10 guide RNA modules and Cas9 expression cassette in pMCSCen1	This work
pCAS1yl-GTP	*GUT2, TRP1* and *PEX10* guide RNA modules and Cas9 expression cassette in pMCSCen1	This work

* Used as △*ku70* strain

transformation, the cells were grown in an appropriate synthetic complete (SC) medium without the auxotrophic compound supplemented by the plasmids [13]. SC-ura (SC-uracil) plates were used to select the *ku70*- and *ku80*-disrupted cells. SC-leu (SC-leucine) plates were used to select the pMCSCen1 plasmid and to derive the transformants. SCG-leu indicates that glucose was the unique carbon source in the SC-leu medium. SCO-leu indicates that oleic acid was the unique carbon source in the SC-leu medium. Glycerol was the unique carbon source in the SCGly-leu medium. All *Y. lipolytica* cells were cultured at 30 °C. Continuous shaking of the liquid cultures was performed at 250 rpm. To cure the pCASyl plasmid, a single colony was cultured for approximately 15 h up to early log-phase, with cell densities reaching an optical absorbance of approximately 0.5 at 600 nm. The culture was diluted to approximately 1000 cells per milliliter and was plated onto YPD agar.

Construction of plasmids

Plasmids are listed in Table 1; primers are listed in Table S1.

HisG-URA3-hisG cassette construction

(1) The HisG-*Bam*HI-F and HisG-*Hin*dIII-R primer set was used to amplify a 1.1-kb hisG fragment from *Salmonella* genomic DNA. The resulting hisG fragment was digested with *Bam*HI and *Hin*dIII and inserted into the pTA vector. (2) The URA3 cassette fragment was amplified from NRRL Y-1095 genomic DNA with the Ura3-*Kpn*I-F and Ura3-*Bam*HI-R primer set and was digested with *Bam*HI and *Kpn*I for insertion into the plasmid generated in step 1. (3) Another hisG fragment was amplified with the HisG-*Eco*RI-F/HisG-*Kpn*I-R primer set and was digested with *Kpn*I and *Eco*RI for insertion into the plasmid generated in step 2, yielding the pTA-HUH plasmid.

ku70 and ku80 deletion cassette construction

For *ku70,* the upstream and downstream homologous arms were amplified from MYA2613 genomic DNA using the KU70-UP-F/R and KU70-DN-F/R primer pairs, respectively. This was followed by overlap extension polymerase chain reaction (OE-PCR) mediated assembly to generate a fusion fragment. The fusion fragment was cloned into pTAs

to generate pTAs-ku70. The larger fragment, hisG-ura3-hisG, was excised from pTAs-hisGura3hisG with *Eco*RI and *Hin*dIII and ligated via *Eco*RI/*Hin*dIII into the pTAs-ku70 backbone to generate the pTAs-ku70-HUH plasmid. pTAs-ku70-HUH was digested by *Swa*I to generate a larger fragment (the cassette for the *ku70* knockout), which was used for MYA-2613 transformation.

For *ku80*, the upstream and downstream homologous fragments were amplified from MYA-2613 genomic DNA with the Ku80-up-F/Ku80-up-R and Ku80-dn-F/Ku80-dn-R primer pairs, respectively. URA3 was amplified using the URA3-△ku80-F/URA3-△ku80-R primer set and was assembled with the upstream and downstream homologous fragments by OE-PCR to generate the Ku80 deletion cassette for the transformation used for the *ku80* deletion.

Cas9 expression cassette

(1) Three fragments were amplified from MYA-2613 genomic DNA and a p415-GalL-Cas9-CYC1t template using the pTEFin-Sal1-F/pTEFin-hCas9-OE-R, hCas9-TEFin-OE-F/hCas9-Sal1 mutation-R and hCas9-Sal1 mutation-F/hCas9-region BamH1-Mlu1-R primer pairs. (2) The three fragments were assembled by OE-PCR and cloned into pTAs. A 2336-bp fragment, generated by digesting p415-GalL-Cas9-CYC1t with *BamH*I and *Mlu*I, was inserted into pTAs-TinhC to generate pTAs-TinCas9, which contained the Cas9 expression cassette. The 4875-bp Cas9 expression cassette was generated by digesting pTAs-TinCas9 with *Sal*I and *Mlu*I.

CRISPR plasmid construction

The backbone was amplified using the pCen1-Sal1-F/pCen1-Mlu1-R primer set to include the *Mlu*I and *Sal*I sites to linearize the backbone, resulting in a 59-bp deletion from 652 to 710 bp. The 4875-bp Cas9 expression cassette (Fig. S1) was ligated into the backbone that was previously digested by *Sal*I and *Mlu*I, yielding the plasmid pCen1-Cas9.

sgRNA expression cassette construction

The sgRNA-HDV sequence was ordered from Genscript (Nanjing, Jiangsu, CN). TEFin-HH-sgRNA-HDV-mig1t was generated by assembling the TEFin fragment (amplified with TEF1p-Pme1-F/TEF-TRP1.1-HH-R) with the HH-g RNA-HDV fragment (amplified with TRP1.1-HH-F/HDV-mig1t-R) and the mig1t terminator (amplified with Mig1t-HDV-F/Mig1t-Sal1-R). The assembly fragments, sgRNA expression cassette (Fig. S2), and pCen1-Cas9 backbone were ligated together after being digested with *Sal*I and *Mss*I to generate the pCAS1yl-trp plasmid (Addgene No. 73226). The upstream and downstream regions of the donor DNA were fused with the sgRNA expression cassette fragment by OE-PCR, and the fusion products were ligated with pCen1-Cas9 after digestions with *Sal*I and *Mss*I. The resulting plasmid was pCAS2yl-trp (Fig. S3). The pCASyl-pex plasmids were constructed as described for pCASy-trp.

The *TRP1* sgRNA expression cassette fragment, which was amplified using the TEFin-Pme1Sph1-F/Mig1t-Avr-2Sal1-R primer set to establish *Mss*I/*Sph*I and *Avr*II/*Sal*I sites at its 5′ and 3′ ends, respectively, was ligated into pCen1-Cas9 via *Sal*I/*Mss*I to generate the pCAS1y-trp(Sph1Avr2) plasmid (Fig. S4A). The *PEX10* sgRNA expression cassette fragment was amplified using the TEFin-Avr2-F/Mig1t-Sal1-R primer set and digested with *Avr*II and *Sal*I. The *Avr*II/*Sal*I fragment was ligated into the pCAS1y-trp(Sph1Avr2) vector backbone via *Avr*II/*Sal*I to generate pCAS1y-TP (Fig. S4B). The *GUT2* sgRNA expression cassette was amplified with TEF1p-Pme1-F/Mig1t-Sph1-R, digested with *Mss*I and *Sph*I and inserted into pCAS1y-TP via *Mss*I/*Sph*I to generate pCAS1y-GTP (Fig. S4C). The *PEX10* and *GUT2* guide sequences used in this study are shown in Fig. S5.

Yeast transformation

The transformation procedure was performed as previously described [13]. Briefly, the 50-μL transformation mixture was plated onto SC-leu solid media for colony counting and for the calculation of transformation efficiency. The remaining transformation mixture was transferred to a tube containing SC-leu liquid media for 2–4 days incubation, and the culture was diluted as needed for plating onto SC-leu solid media.

Phenotype verification

Colonies from the SC-leu plate were randomly picked and streaked onto SC-leu and SC-leu-trp plates, and incubated at 30 °C for 2 days. SCG-leu and SCO-leu media were used in the PEX10 experiments; the SCGly-leu medium was used in the GUT2 experiments.

Colony PCR confirmation and sequencing

The high efficient and success rate DNA polymerase KOD FX Neo (TOYOBO, CO.,LTD) was used for confirmation by colony PCR. The products were sequenced as needed by Sangon Biotech (Shanghai) Co., Ltd. The TRP1-ORF-YZ-F/TRP1-YZ-R primer set was used for confirmation by PCR for the *TRP1* experiments. The PEX10-donor-440up-F/PEX10-dn-YZ-R primer set was used for confirmation by PCR for the PEX10 experiments. The

Gut2-donor-470up-F/△GUT2-YZ-dn-R primer set was used for gut2 confirmation by colony PCR. All PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Results and discussion

Construction of the *Y. lipolytica* CRISPR system

Because NHEJ-mediated DSB repair dominant *Y. lipolytica* [31] and can be weakened by disrupting one or both of the *ku70* and *ku80* genes [20, 39], two CRISPR-Cas9 plasmids were developed with or without donor DNA. pCAS1yl was designed by utilizing a codon-optimized nuclease Cas9 gene from *S. pyogenes* MGAS5005 [11] that was controlled by a strong, endogenous TEFin promoter [37], a sgRNA containing a 20-bp guide sequence flanked upstream by the hammerhead (HH) ribozyme and downstream by the hepatitis delta virus (HDV) ribozyme [28] (transcribed by the TEFin promoter), and a replicative vector backbone (pMCSCen1) [4] (Fig. 1). pCAS2yl was designed by inserting the donor DNA upstream of the sgRNA expression cassette (Fig. 1). Three different host strains were examined, and they included (1) wild-type MYA-2613 (WT), (2) MYA-2613△*ku70* (△*ku70*) and (3) MYA-2613△*ku70*△*ku80* (△*ku70*△*ku80*).

The CRISPR-Cas9 system was initially tested for Cas9-mediated toxicity. The total colony-forming units (cfu) were calculated based on the number of colonies that grew on the SC-leu agar. There were no apparent differences between the transformation efficiencies of the plasmid containing Cas9 and the plasmid containing an inactivated Cas9 gene, indicating that Cas9 had no effect on *Y. lipolytica* growth (Fig. 2).

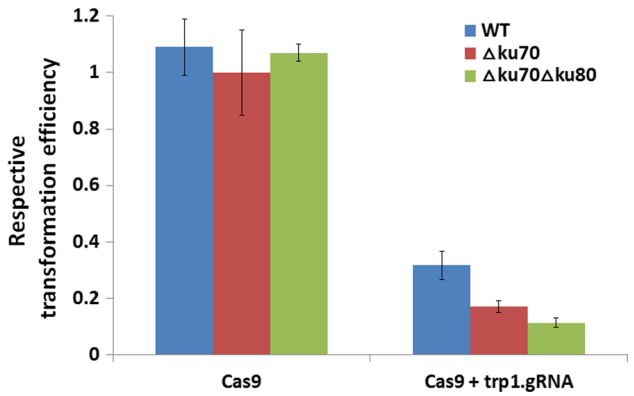

Fig. 2 CRISPR-Cas9-mediated death of *Y. lipolytica* cells. Relative transformation efficiency with inactivated Cas9, Cas9, and Cas9 + TRP1.gRNA. Inactive Cas9, the pCen1-inCas9 (11.2 kb) plasmid containing an inactivated *cas9* mutant was used as the null control. *Cas9* the pCen1-Cas9 (10.7 kb) plasmid carrying a Cas9 expression cassette. Cas9 + trp1.g RNA, the pCAS1yl-trp (11.9 kb) plasmid carrying Cas9 and the sgRNA gene expression cassettes, which targeted to the *TRP1*. One microgram per plasmid was used for the transformation. The transformation mixture without the outgrowth step was plated on SC-leu agar. The *values* and *error bars* represent the average readings and standard deviations for three experiments

The *TRP1* gene, which encodes *N*-(5-phosphoribosyl)-anthranilate isomerase, was selected as a target for editing. When Cas9 was expressed with the guide RNA targeted the *TRP1* locus in the genome, the cfu values dramatically decreased in the WT, △*ku70* and △*ku70*△*ku80* strains (31.7 ± 5, 17.2 ± 2.3 and 11.4 ± 1.7 %, respectively, Fig. 2). The differences in the survival rates may be due to the NHEJ-mediated repairs of the DSBs generated by Cas9 in the WT and △*ku70* strains [9]. The low survival rate observed in the △*ku70*△*ku80* strain was a likely result of insufficient Cas9 or sgRNA activity [1, 11].

Disruption of *TRP1* by the pCAS1yl system via NHEJ in the WT and *ku70* deficient strains

When donor DNA is unavailable in *Y. lipolytica,* Cas9-induced DSBs can be repaired only by NHEJ, which involves nonspecific insertions or deletions. This results in target gene mutations and precludes continual cleavage by Cas9 [11, 23, 28, 40].

Inactivation of *TRP1* (*trp*$^-$) resulted in *Y. lipolytica* tryptophan auxotrophy, and the *trp*$^-$ mutant was unable to grow on a synthetic complete (SC) medium lacking tryptophan [6]. The disruption efficiency achieved with pCAS1yl-trp was calculated based on the number of cells on the agar plate with or without tryptophan. Without outgrowth, a disruption efficiency of 12.5 ± 7.4 % (15/84, 5/69) was observed for the WT strain (Fig. 3a, b), and no mutations were detected in the △*ku70* (0/54, 0/40) and △*ku70*△*ku80* (0/26, 0/52) strains (Fig. 3a).

A previous study showed that disruption efficiency clearly improved after an extended outgrowth step following transformation [1]. We tested cell outgrowth times of 2 and 4 days before plating for WT, which resulted in efficiencies of 62.5 ± 38.5 % (30/85, 35/39) and 85.6 ± 7.1 % (66/88, 29/32), respectively. In the *ku70*-deficient strain, the disruption efficiencies were 1.7 ± 0.9 % (1/97, 2/82) for the 2-day outgrowth and 26.1 ± 19.6 % (16/40, 5/41) for the 4-day outgrowth (Fig. 3a). However, no *trp*$^-$ mutants (0/88, 0/34) were detected in the *ku70* and *ku80* double-deficient strains (Fig. 3a), indicating that *KU* factor deletions also drastically decreased NHEJ in *Y. lipolytica* [9]. More than 100 mutants of auxotrophic for tryptophan were sequenced in our work. The sequencing results showed that 100 % of colonies had indels in expected *TRP1* gene locus as some were listed in Fig. 3c. When sgRNA was absent, no *trp*$^-$ mutants were detected (Fig. S6).

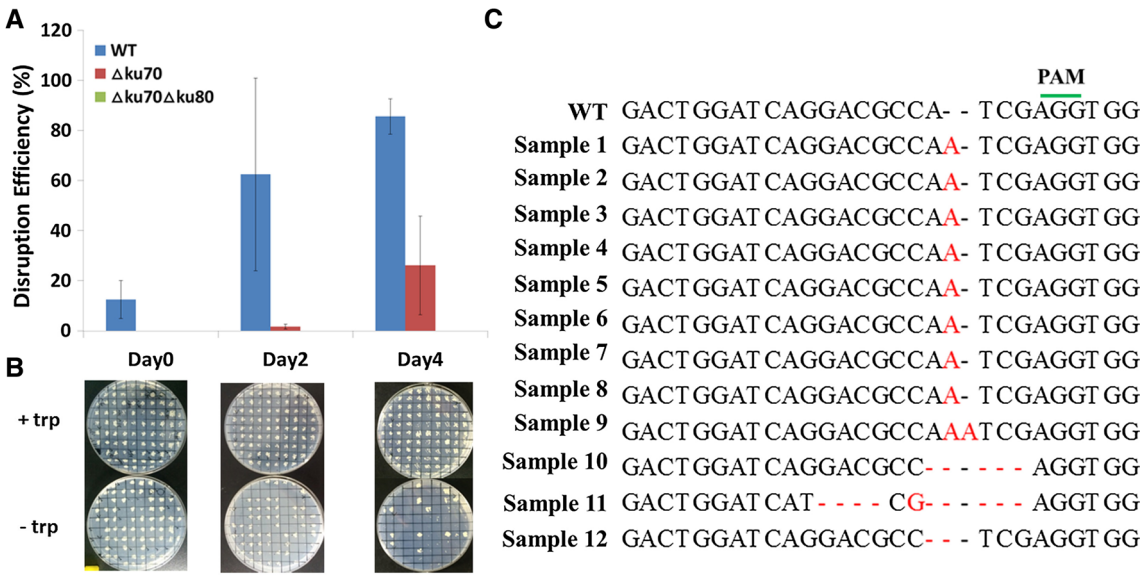

Fig. 3 CRISPR-Cas9-mediated *TRP1* gene disruption by NHEJ. **a** *TRP1* gene disruption efficiency in WT, △*ku70* and △*ku70*△*ku80* via pCAS1yl-trp. **b** A representative WT transformant phenotype-confirmation is shown. All confirmations were conducted following a similar procedure. Transformants were seeded onto agar with (+trp) or without (−trp) tryptophan. **c** A representative sample is shown for the alignments of the *TRP1* gene sequence from selected *trp*$^-$ mutants in WT *Y. lipolytica*. Indels were induced, resulting in *TRP1* disruptions, and were indicated in red color. The *values* and *error bars* represent the average readings and standard deviations for two experiments

Deletion of the *TRP1* gene via HR in *ku70*-deficient or *ku70/ku80* double-deficient *Y. lipolytica*

To determine the HR frequency, we initially co-transformed pCAS1yl-trp and linear donor DNA. Without the outgrowth, the HR efficiency was approximately 10 % in the WT *trp*$^-$ mutants, and there was no improvement with 2 days of outgrowth. For Δku70 and Δku70Δku80, the frequencies of the *trp*$^-$ mutation were under 10 % using the approach described above (Fig. 4). We speculated that the linear DNA was not sufficiently sustained inside the cell, thus hampering HR after outgrowth. Therefore, we delivered the donor DNA using a replicative plasmid, as previously described [1, 17]. The donor DNA containing 500 bp upstream and 500 bp downstream sequences of *TRP1* was fused with the sgRNA expression cassette and cloned into pCen1-Cas9 to form pCAS2yl-trp (Fig. S3).

The phenotypes of the transformants with pCAS2yl-trp were assessed as described above, and their genotypes were confirmed by colony PCR. After 4 days of outgrowth, total *trp*$^-$ mutation efficiencies of 72.3 ± 15 % (54/88, 68/82), 58.9 ± 3.5 % (49/87, 54/88) and 94.1 ± 4.3 % (79/81, 80/88) via NHEJ or HR were observed for WT, △*ku70* and △*ku70*△*ku80*, respectively (Fig. 4a). The colony PCR results indicated that only 11.1 ± 3.6 % (7/51, 4/47) were generated by HR in the WT *trp*$^-$ mutants, whereas HR-mediated efficiencies were increased to 100 % in the two NHEJ-defective strains (△*ku70*, 3/3,16/16, △*ku70*△*ku80,* 2/2, 13/13) (Figs. 4b, S3B). When sgRNA was absent in these two above conditions, there were no *trp*$^-$ mutants being detected (Figs. S7, S8).

PEX10 deletion with pCASyl and expansion of the pCASyl system to more breeding strains

We investigated whether our CRISPR-Cas9 system could be expanded to other genes and *Y. lipolytica* strains by focusing on *PEX10*, another key metabolic gene [5, 33, 44]. *PEX10* disruption causes a cell to lose its ability to grow on medium containing oleic acid as the unique carbon source. In this study, WT and △*ku70*△*ku80* were transformed with pCAS1yl-pex and pCAS2yl-pex, respectively, to target the *PEX10* gene. pCAS2yl-pex contained the donor DNA, which consisted of the following *PEX10* components: a 444-bp upstream sequence, a 450-bp downstream sequences (Fig. S4A) and the intended 200-bp deletion (Fig. S4B). The resulting mutation efficiencies for MYA-2613 and △*ku70*△*ku80* were 62.5 ± 21.4 % (17/22, 16/34) and 28.3 ± 2.4 % (3/10, 4/15), respectively (Table 2; Figs. S9, S10). Additionally, *TRP1* was successfully disrupted in the ATCC201249 strain derived from the E122 parent strain [35], unlike the MYA-2613 parent strain (W29) [21], efficiency was 98.1 ± 2.5 % (2/2, 26/27) (Table 2).

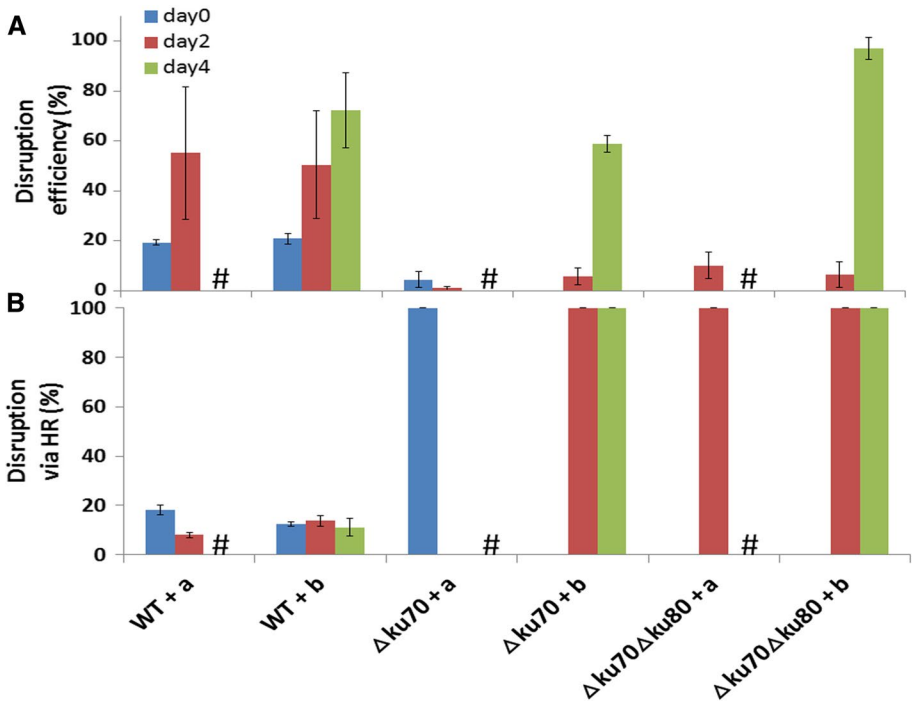

Fig. 4 CRISPR-Cas9 mediated HR. **A** Disruption efficiency of the *TRP1* gene mediated by CRISPR-Cas9 with different outgrowth times. **B** Percentage of *TRP1* gene disruption via HR in *trp*$^{-}$ mutants. *a* pCAS1yl plus the co-transformed linear donor DNA; *b* pCAS2yl alone transformed. Mean ± standard deviation, $n = 2$. # These detections were not performed

Table 2 CRISPR mediated disruption efficiency of different genes in different strains

Strain	Gene	Mutation efficiency
MYA-2613	*PEX10*	62.5 ± 21.4 % (17/22, 16/34)
CIBTS1961	*PEX10*	28.3 ± 2.4 % (3/10, 4/15)
ATCC 201249	*TRP1*	98.1 ± 2.5 % (2/2, 26/27)

One-step multiplex gene disruption with pCAS1yl system

Simultaneous double and triple multigene disruption efficiencies were also investigated for the pCAS1yl system. At first, we tried two plasmids (pCAS1yl-trp and pCAS1yl-pex) co-transformation into the WT strain for *TRP1* and *PEX10* disruption. However, only 1.6 % (1/60) double gene disruption efficiency was achieved (Fig. S11), while single gene disruption efficiency reached 40 % (*TRP1,* 24/60) or 56.6 % (*PEX10,* 34/60). The pCAS1yl-TP plasmid (Fig. S4B), which contained two tandem sgRNA expression cassettes, was thus constructed. A double-disruption efficiency of 36.7 ± 8.5 % (18/42, 12/39) was achieved in 4 days, which was drastically higher than that of the two-plasmid co-transformation procedure (Fig. S12A). The sequencing results indicated that indels were introduced into both target loci (Fig. S12B). To determine whether the system enables simultaneous three targets, an additional *GUT2*(YALI0B13970g) gene, encoding the mitochondrial *sn*-glycerol-3-phosphate dehydrogenase, was chosen as the third target gene. The mutant that contained an inactivated *gut2* exhibited growth on glucose but not on glycerol [3, 27]. The pCAS1yl-GTP plasmid (Fig. S4C), carrying an sgRNA in tandem targeting *GUT2*, *TRP1* and *PEX10* gene cassettes, was constructed and transformed into the WT strain. A triple-gene disruption efficiency of 19.3 ± 9.2 % (5/39, 8/31) was achieved after 4 days, and the mutation was confirmed by sequencing (Fig. 5).

Multi-round genome editing by plasmid curing

To enable multiple rounds of genome editing, plasmid curing was necessary [17]. The pCAS1yl transformant was cultured in non-selective liquid medium (YPD) and plated on YPD agar. Colonies were picked from a plate and confirmed by replica dripping on SC medium agar (+leu) and SC-leu medium agar (−leu). The resulting frequency of colonies that do not grow on -leu from the total was calculated as 38 % (24/63) (Fig. S13A). We observed that smaller colonies on YPD medium did not grow on medium lacking leu (−leu) (Fig. S13B), which agreed with the previous report [12]. The isolations of colonies from +leu and −leu plates were further transformed into *E. coli* DH5α. We did not get any *E. coli* transformants through those isolates from colonies which only grew on +leu (Fig S13C), indicating the loss of plasmid pCAS1yl. Thus, single, double or triple target modification could be accomplished via pCASyl within 6 days, and 2 additional days were needed for pCASyl curing to realize next round of genome modification (Fig. S14).

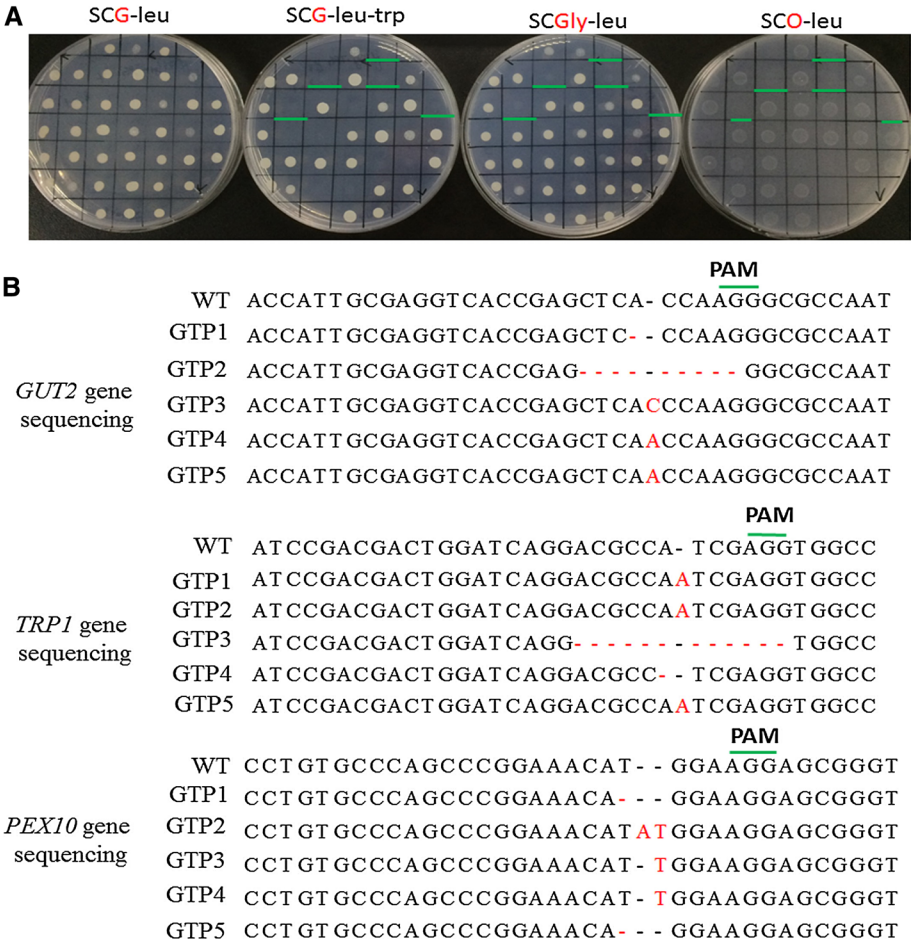

Fig. 5 Triple simultaneous disruption. Colonies were picked from transformant plates and diluted in 10 μL of sterilized water were inoculated onto agar plates containing one of four different media to confirm phenotypes. Triple-disruptants only grew on SCG-leu. **a** Representative image of the phenotype confirmations for the GTP triple disruptants. *Green lines* indicate positive disruptants. **b** Alignments of gene sequences from five disruptants shown in **a** against wild-type sequences. *Red* indicates variations from the wild-type sequence (WT). One of two replicates is shown

Conclusions

In this report, we have described the development of a pCASyl-based CRISPR-Cas9 system for *Y. lipolytica*. This genetic tool enabled efficient, scarless, single or multigene editing in several *Y. lipolytica* strains through NHEJ and HR. The pCASyl CRISPR-Cas9 system established here is more efficient than traditional genome-editing methods and will strongly facilitate synthetic biology, metabolic engineering and functional genomic studies of *Y. lipolytica*.

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