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Development of an efficient genetic manipulation strategy for sequential gene disruption and expression of different heterologous *GFP* genes in *Candida tropicalis*

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Abstract The diploid yeast *Candida tropicalis*, which can utilize *n*-alkane as a carbon and energy source, is an attractive strain for both physiological studies and practical applications. However, it presents some characteristics, such as rare codon usage, difficulty in sequential gene disruption, and inefficiency in foreign gene expression, that hamper strain improvement through genetic engineering. In this work, we present a simple and effective method for sequential gene disruption in *C. tropicalis* based on the use of an auxotrophic mutant host defective in orotidine monophosphate decarboxylase (URA3). The disruption cassette, which consists of a functional yeast *URA3* gene flanked by a 0.3 kb gene disruption auxiliary sequence (*gda*) direct repeat derived from downstream or upstream of the *URA3* gene and of homologous arms of the target gene, was constructed and introduced into the yeast genome by integrative transformation. Stable integrants were isolated by selection for Ura^+ and identified by PCR and sequencing. The important feature of this construct, which makes it very attractive, is that recombination between the flanking direct *gda* repeats occurs at a high frequency (10^{-8}) during mitosis. After excision of the *URA3* marker, only one copy of the *gda* sequence remains at the recombinant locus. Thus, the resulting *ura3* strain can be used again to disrupt a second allelic gene in a similar manner. In addition to this effective sequential gene disruption method, a codon-optimized green fluorescent protein-encoding gene (*GFP*) was functionally expressed in *C. tropicalis*. Thus, we propose a simple and reliable method to improve *C. tropicalis* by genetic manipulation.

Keywords *Candida tropicalis* · Gene disruption · Gene disruption auxiliary sequence · *URA3* gene · Carnitine acetyltransferase · Dodecanedioic acid · GFP

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Introduction

Candida tropicalis is a diploid yeast which does not undergo a sexual reproductive stage (Blandin et al. 2000). In recent years, growing attention has been devoted to *C. tropicalis* especially for its use in chemical and fuel production. Moreover, unique physiological features such as the possibility to use *C. tropicalis* for the production of long-chain α,ω-

dicarboxylic acids (DCA) (Liu et al. 2004; Picataggio et al. 1992) and xylitol (Ahmad et al. 2013; Guo et al. 2013; Misra et al. 2013) have enhanced the academic and commercial value of this diploid yeast.

In yeast molecular biology, gene disruption plays an important role in physiological studies and gene function research. Having an appropriate selection marker is a key element, being necessary for the isolation of a gene-disrupted mutant. An integrative transformation system for *C. tropicalis* that depends on a uracil-auxotrophic mutant was reported for the first time by Haas et al. (1990). In this system, both alleles of the *POX4* and *POX5* genes, which encode distinct isozymes of acyl coenzyme A involved in DCA metabolism, were disrupted in a *C. tropicalis* strain, enhancing the yield and efficiency of DCA production (Picataggio et al. 1992). It is noteworthy that it was difficult and time consuming to isolate *ura3* mutant and re-use the *URA3* gene as a selection marker. In *Saccharomyces cerevisiae*, a cassette was developed through the introduction of direct repeats of *Salmonella hisG* DNA segments at both ends of the *URA3* marker for the re-utilization of the selection marker (Alani et al. 1987). After cassette integration into the yeast genome, the *URA3* gene was deleted by spontaneous intra-chromosomal recombination between the *hisG* fragment repeats. This resulted in a uracil-auxotrophic mutant that can be selected on plates containing 5-fluoroorotic acid (5-FOA), and the marker gene can be reused repeatedly. A similar approach was applied to gene disruption in *C. tropicalis* strains (Ahmad et al. 2013), where direct repeats of *hisG* sequences were replaced by other DNA sequences (*glu*, *leu*, *trp*, or *arg*) isolated from the *Bacillus subtilis* genome.

A mutated hygromycin resistance gene has also been used as a selection marker for *C. tropicalis* transformation (Hara et al. 2001). However, we had difficulty in repeating these results with our yeast host (unpublished data). Recently, a *SAT1* flipper method for gene disruption developed in *C. albicans* (Reuß et al. 2004) was directly applied to *C. tropicalis* MYA3404 (Chen et al. 2014). The *SAT1* flipper cassette contains a dominant nourseothricin resistance marker (Ca*SAT1*) for the selection of integrative transformants, and the Ca*SAT1* marker was subsequently deleted based on the FLP-*FRT* recombination system with a *C. albicans*-adapted *FLP* gene. However, there are still several issues associated with the use of antibiotic-resistant genes as selection markers. One of the most common problems is that resistance genes often used as selection markers in other yeast transformation are ill suited to *C. tropicalis* as, for example, *C. tropicalis* is highly resistant to G418 (Hara et al. 2000). Moreover, codon usage bias is common in *C. tropicalis* strains. For example, the CTG codon, which would be mistranslated to serine instead of leucine in *C. tropicalis* (Santos et al. 2011; Ueda et al. 1994), adds difficulty in the genetic manipulation of *C. tropicalis*. A conventional Ura-blaster that contains direct repeats of *hisG* enables re-use of the marker gene, as already reported. However, the *hisG* repeats resulted in *URA3* pop-out via homologous annealing during PCR (Wilson et al. 1999, 2000). In addition, the *hisG* repeats led to difficulties in the construction of the large disruption cassette because of low amplification efficiency of large DNA fragments.

In this study, we developed an efficient gene disruption strategy for *C. tropicalis*, in which the selection marker can be easily re-utilized. Using this method, two *C. tropicalis* carnitine acetyltransferase encoding genes (*CAT*) were sequentially deleted. Furthermore, we demonstrated that this technology can integrate and functionally express an exogenous gene into the chromosome at the location of the deleted gene.

Materials and methods

Strains, media, and culture conditions

C. tropicalis strains used in this study are listed in Table 1. The diploid strain of *C. tropicalis* ATCC 20336 was maintained on yeast peptone dextrose (YPD) medium (10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 20 g L^{-1} peptone). *C. tropicalis* XZX, a derivative of *C. tropicalis* ATCC 20336 generated by random mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), was used as a host strain for transformation. The mutagenesis process is described below. For genetic manipulation of *C. tropicalis* XZX, minimal medium (MM, 0.67 g L^{-1} yeast nitrogen base (YNB), 20 g L^{-1} glucose, 10 g L^{-1} [$(NH_4)_2SO_4$]), supplemented medium (SM, MM supplemented with uracil at a concentration of 0.06 g L^{-1}) and FOA-SM (SM supplemented with 5-fluoroorotic acid at a concentration of 2 g L^{-1}) were used. All *C. tropicalis* strains were routinely cultured at 30 °C. *Escherichia coli* strain JM109 (TaKaRa, Dalian, China) was used as a host for all cloning experiments and was cultured in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl) at 37 °C. Ampicillin was supplemented at 100 μg mL^{-1}. When necessary, agar was added to a final concentration of 20 g L^{-1} for solid media.

Auxotrophic mutant isolation

The uracil-auxotrophic mutant was derived from *C. tropicalis* ATCC 20336 by chemical mutagenesis with slight modifications to a double selection method (Haas et al. 1990). A colony of *C. tropicalis* ATCC 20336 was inoculated in YPD medium and incubated at 30 °C and 200 rpm to prepare log growth phase cells. The resulting cells were washed once with sterile H_2O and re-suspended for 40 min in 10-mL YPD medium containing 1–4 mg NTG. After NTG treatment, the culture was washed twice with 50-mL PBS buffer (NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.42 g L^{-1}, KH_2PO_4 0.27 g L^{-1}, pH 7.4) and re-suspended in 50-mL YPD medium for 3 h at 30 °C.

Table 1 *C. tropicalis* strains used in this study

Strain	Genotype	Reference
C. tropicalis ATCC 20336	*URA3/URA3 CAT/CAT*	ATCC
C. tropicalis XZX	*ura3/ura3 CAT/CAT*	This study
C. tropicalis 01-1	*ura3/ura3 cat::gda143-URA3/CAT*	This study
C. tropicalis 01-2	*ura3/ura3 cat::gda245-URA3/CAT*	This study
C. tropicalis 01-3	*ura3/ura3 cat::gda324-URA3/CAT*	This study
C. tropicalis 01-4	*ura3/ura3 cat::gda488-URA3/CAT*	This study
C. tropicalis 01-5	*ura3/ura3 cat::gda302-URA3/CAT*	This study
C. tropicalis 01-6	*ura3/ura3 cat::gda305-URA3/CAT*	This study
C. tropicalis 01-7	*ura3/ura3 cat::gda325-URA3/CAT*	This study
C. tropicalis 01-8	*ura3/ura3 cat::hisG-URA3-hisG/CAT*	This study
C. tropicalis 02-1	*ura3/ura3 cat::gda143/CAT*	This study
C. tropicalis 02-2	*ura3/ura3 cat::gda245/CAT*	This study
C. tropicalis 02-3	*ura3/ura3 cat::gda324/CAT*	This study
C. tropicalis 02-4	*ura3/ura3 cat::gda488/CAT*	This study
C. tropicalis 02-5	*ura3/ura3 cat::gda302/CAT*	This study
C. tropicalis 02-6	*ura3/ura3 cat::gda305/CAT*	This study
C. tropicalis 02-7	*ura3/ura3 cat::gda325/CAT*	This study
C. tropicalis 02-8	*ura3/ura3 cat::hisG/CAT*	This study
C. tropicalis 03	*ura3/ura3 cat::gda324/cat::gda324-URA3*	This study
C. tropicalis 04	*ura3/ura3 cat::gda324/cat::gda324*	This study
C. tropicalis 05-1	*ura3/ura3 cat::gda324/cat::gda324-URA3-P-wtGFP-T*	This study
C. tropicalis 05-2	*ura3/ura3 cat::gda324/cat::gda324-URA3-P-GFP$_{CTC}$-T*	This study
C. tropicalis 05-3	*ura3/ura3 cat::gda324/cat::gda324-URA3-P-yeGFP3-T*	This study
C. tropicalis 06-1	*ura3/ura3 cat::gda324/cat::gda324-P-wtGFP-T*	This study
C. tropicalis 06-2	*ura3/ura3 cat::gda324/cat::gda324-P-GFP$_{CTC}$-T*	This study
C. tropicalis 06-3	*ura3/ura3 cat::gda324/cat::gda324-P-yeGFP3-T*	This study

Then, cells were washed twice with MM and incubated in 50 mL MM for 6–8 h. For enrichment of auxotrophs, nystatin was added to a concentration of 50 μg mL^{-1} and cells were incubated with shaking for 2 h. Cells were then washed twice with 50 mL sterile PBS buffer and incubated in YPD medium for 10 h. The culture was then spread onto FOA-SM plates, and colonies that appeared 2 to 3 days later were transferred to MM and SM plates by replica plating. Colonies that reappeared on SM agar plates, but not on MM plates, exhibited uracil auxotrophy. The *URA3* genes of uracil auxotrophs were isolated and sequenced.

Construction of Tm-*gda-URA3* and Tm-*URA3-gda*

A series of plasmids Tm-*gda-URA3* containing different sized *gda* fragments, were constructed as follows. The 1.6 kb *URA3* gene to be used as marker was amplified by PCR from the genomic DNA of *C. tropicalis* ATCC 20336 using primers URAU and URAR based on the *URA3* sequence (GenBank accession number AB006207). The PCR product was ligated into the pMD 18-T (TaKaRa, Dalian, China) to generate plasmid Tm-*URA3*. *gda* fragments from the 3′ terminus of the *URA3* gene were amplified by PCR with primers Ugda143 and Dgda (yielding a 143 bp product), Ugda245 and Dgda (yielding a 245 bp product), Ugda324 and Dgda (yielding a 324 bp product), and Ugda488 and Dgda (yielding a 488 bp product). A 325 bp segment (*gda325*) located before the terminator codon (TAA) in *URA3* was also amplified with primers Ugda325 and Dgda325. These fragments were then ligated to the 5′ end of the *URA3* gene as follows: all the *gda* fragments were digested with *Pst*I and *Sal*I and inserted into Tm-*URA3* at the corresponding sites. The resulting plasmids were named Tm-*gda143-URA3*, Tm-*gda245-URA3*, Tm-*gda324-URA3*, Tm-*gda488-URA3*, and Tm-*gda325-URA3*. The primers used in this study are listed in Table 2.

In addition, a 305 bp *gda* segment (*gda305*) located in the promoter region of *URA3* and a 302 bp *gda* segment (*gda302*) located after the initiation codon (ATG) of *URA3* were amplified from the *URA3* gene by PCR with primers Ugda305 and Dgda305, Ugda302 and Dgda302, respectively. The *gda305* and *gda302* fragments were ligated to the 3′ end of the *URA3* gene by digestion with *Xba*I and *Eco*RI and inserted into Tm-*URA3*, yielding plasmids Tm-*URA3-gda305* and Tm-*URA3-gda302*.

Table 2 Primers used in this study

Primer	Sequence[a](5′ to 3′)	Restriction site
URAU	TACTCTAACGACGGGTACAAC	
URAR	ACCCGATTTCAAAAGTGCAG	
CATU	GTTTAACTTTAAGTTGTCGC	
CATR	TACAACTTAGGCTTAGCATCA	
rCATU	AACTGCAGCCAAAATTCAGCCAACCAGT	*Pst*I
rCATR	GCTCTAGAAGATGATTCAACCAGGCGAAC	*Xba*I
CAT2ndU	CTGAAGGCTCCGACATCACC	
CAT2ndR	CAACCTTGTCGGCGCTGCTA	
rCAT2ndU	AACTGCAGATCTGTTTTGACCGTCCCCGTG	*Pst*I
rCAT2ndR	GCTCTAGATCTTGCCTTTCGACATCAACCC	*Xba*I
Ugda143	AACTGCAGTGCTTGAAGGTATCCACGTA	*Pst*I
Ugda245	AACTGCAGAATGGATGTAGCAGGGATGGT	*Pst*I
Ugda324	AACTGCAG GGATCCACTAAGCTTCTAGGACGTCAT	*Pst*I and *Bam*HI
Ugda488	AACTGCAGTTCTGACTGGTACCGAT	*Pst*I
Dgda	GCGTCGACACCCGATTTCAAAAGTGCAGA	*Sal*I
Ugda325	AACTGCAGTCGTGATTGGGTTCATCGC	*Pst*I
Dgda325	GCGTCGACCAATGACGTCCTAGAAGC	*Sal*I
Ugda305	GCTCTAGATCTAACGACGGGTACAACGA	*Xba*I
Dgda305	CGGAATTCACGTGACTAGTATGGCAAT	*Eco*RI
Ugda302	GCTCTAGACATACACAGAAAGGGCATC	*Xba*I
Dgda302	CGGAATTCGTACTGCAACATCACGG	*Eco*RI
HisG-F1	CCGGAATTCTTCCAGTGGTGCATGAACGC	*Eco*RI
HisG-R1	CGCGGATCCGCTGTTCCAGTCAATCAGGGT	*Bam*HI
HisG-F2	ACGCGTCGACTTCCAGTGGTGCATGAACGC	*Sal*I
HisG-R2	AACTGCAGGCTGTTCCAGTCAATCAGGGT	*Pst*I
CATLD	AATAGAAACTAGCAATCGGAA	
GFP-U	CCCAAGCTTATGGTAGATCTGACTAGT	*Hin*dIII
GFP-D	GCGTCGACCCCGATCTAGTAACAT	*Sal*I
PgapF	GCTCTAGAAACGTGGTATGGTTGTAAGAAAC	*Xba*I
PgapR	CCCAAGCTTTGTTTAAATTCTTTAATTG	*Hin*dIII
TgapF	GCGTCGACGCTATCCAACAAACTCTAG	*Sal*I
TgapR	CGGAATTCTCTGGTTTAGAAGTAGGGACTGTATG	*Eco*RI
ACT1-F	GACCGAAGCTCCAATGAATC	for RT-qPCR
ACT1-R	AATTGGGACAACGTGGGTAA	for RT-qPCR
CAT-F	TTTGTCATTGCCTTGGACT	for RT-qPCR
CAT-R	ACCAGAGTTACCGTTAGCA	for RT-qPCR
GFP-F	TGAATTAGATGGTGATGTTA	for RT-qPCR
GFP-R	TAAGAGAAAGTAGTGACAAG	for RT-qPCR
yeGFP3-F	TTCGGTTATGGTGTTCAAT	for RT-qPCR
yeGFP3-R	GCTCTGGTCTTGTAGTTAC	for RT-qPCR

[a] Restriction sites introduced into the primers are underlined

Construction of Ts-*CAT1-gda-URA3* and Ts-*CAT1-URA3-gda*

The plasmid Ts-*CAT1-gda-URA3* containing the cassette for *CAT* allele 1 disruption was constructed as follows. Based on the *C. tropicalis* ATCC 20336 *CAT* gene sequence (GenBank accession number D84549.1), a 1881 bp *CAT* fragment was amplified from *C. tropicalis* XZX genomic DNA by PCR using primers CATU and CATR (Table 2), which was termed *CAT1*. The *CAT1* fragment was ligated into the pMD 18-T Simple vector (TaKaRa, Dalian, China) to yield plasmid Ts-*CAT1*. The primers rCATU and rCATR (Table 2) were then used to eliminate the middle region (approximately 1.2 kb) by inverse PCR. The PCR product (*CAT1*-Ts-*CAT1*) was then

digested with *Pst*I and *Xba*I and ligated to various *gda-URA3* cassettes, yielding plasmids Ts-*CAT1-gda143-URA3*, Ts-*CAT1-gda245-URA3*, Ts-*CAT1-gda324-URA3*, Ts-*CAT1-gda488-URA3*, and Ts-*CAT1-gda325-URA3*.

The *URA3-gda305* fragment was separated from plasmid Tm-*URA3-gda305* by digestion with *Pst*I and *Eco*RI, and the PCR product *CAT1*-Ts-*CAT1* was digested with *Pst*I and *Xba*I. The cloning process included two steps: firstly, the *Eco*RI and *Xba*I sticky ends were made blunt by *pfu* DNA polymerase (TaKaRa, Dalian, China); secondly, *URA3-gda305* and *CAT1*-Ts-*CAT1* fragments having the same cohesive end and a blunt end were ligated together by T4 DNA ligase. The resulting plasmid was termed Ts-*CAT1-URA3-gda305*. A similar strategy was used for construction of the plasmid Ts-*CAT1-URA3-gda302*.

Disruption cassettes for the first *CAT* allele were isolated from plasmids Ts-*CAT1-gda-URA3* or Ts-*CAT1-URA3-gda* by PCR using primers CATU and CATR. The resulting PCR products of *CAT1-gda-URA3-CAT1* and *CAT1-URA3-gda-CAT1* were purified using the Axyprep PCR cleanup kit (Axygen, Suzhou, China).

Construction of Ts-*CAT1-hisG-URA3-hisG*

The plasmid Ts-*CAT1-hisG-URA3-hisG* was constructed as described previously (Ko et al. 2006) with slight modifications. Two 1.1 kb *hisG* fragments were amplified by PCR from the plasmid pCUB6 (Lu et al. 2008) using HisG-F1 and HisG-R1, HisG-F2 and HisG-R2 as primers. The *hisG1* fragment was digested with *Eco*RI and *Bam*HI, and inserted into Tm-*URA3* digested with *Eco*RI and *Bam*HI, yielding the plasmid Tm-*URA3-hisG1*. Then, the *hisG2* fragment was inserted into the plasmid between the *Pst*I and *Sal*I sites. The resulting plasmid was named Tm-*hisG2-URA3-hisG1*. Finally, the *hisG2-URA3-hisG1* fragment was flanked by homologous regions as in the construction of Ts-*CAT1-URA3-gda305*, yielding plasmid Ts-*CAT1-hisG-URA3-hisG*.

Construction of Ts-*CAT2-gda324-URA3*

A similar method was adopted for the construction of Ts-*CAT2-gda324-URA3*, a plasmid containing the cassette for the disruption of the second *CAT* allele. In short, a 906 bp *CAT2* fragment was amplified by PCR from the genomic DNA of *C. tropicalis* XZX using primers CAT2ndU and CAT2ndR, which were designed from sequences inside the disrupted region of the *CAT1* gene (Fig. 5a). The *CAT2* fragment was inserted into the pMD 18-T Simple vector, which was linearized and ligated to the *gda324-URA3* cassette to generate plasmid Ts-*CAT2-gda324-URA3*. A 2.6 kb disruption cassette for the second *CAT* allele *CAT2-gda324-URA3-CAT2* was obtained by PCR using primers CAT2ndU and CAT2ndR.

Construction of GFP expression cassette

The open reading frame (ORF) of the wild-type green fluorescent protein-encoding gene (*GFP*) (*wtGFP*) gene was amplified from plasmid pCAMBIA1302 (Cambia, Canberra, Australia) by PCR with primers GFP-U and GFP-D. The *wtGFP* gene was then expressed under the control of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) promoter and terminator derived from *C. tropicalis* ATCC 20336 genomic DNA by PCR with the primers PgapF, PgapR, TgapF, and TgapR (Jeon et al. 2012). The promoter-*wtGFP*-terminator fragment was inserted into pMD 18-T Simple to obtain plasmid Ts-P-*wtGFP*-T.

The plasmid Ts-P-*wtGFP*-T was digested with *Xba*I and *Eco*RI to obtain the P-*wtGFP*-T cassette, which was then inserted into plasmid Tm-*gda324-URA3*, following digestion with the same restriction enzymes. The resulting plasmid was designated as Tm-*gda324-URA3*-P-*wtGFP*-T. Finally, the *gda324-URA3*-P-*wtGFP*-T cassette was flanked by homologous regions as in the construction of Ts-*CAT2-gda324-URA3*. For this construct, only the site for the restriction enzyme *Xba*I was changed to that of *Eco*RI in the rCAT2ndR primer. The resulting plasmid Ts-*CAT2-gda324-URA3*-P-*wtGFP*-T was linearized by restriction enzyme *Dra*II and transformed into *C. tropicalis* 01-3 cells to generate transformant *C. tropicalis* 05-1. After removing the *gda324-URA3* fragment in the *C. tropicalis* 05-1 genome, the resulting strain *C. tropicalis* 06-1 was obtained.

The codon-optimized *GFP* (GFP_{CTC}) gene (GenBank accession number KU763376) was chemically synthesized based on the sequence in plasmid pCAMBIA1302 in which two CTG codons were changed to optimal leucine codons (CTC). The GFP_{CTC} gene was then digested with *Hin*dIII and *Sal*I and inserted into plasmid Ts-P-*wtGFP*-T that had previously been digested with the same restriction endonucleases to replace the *wtGFP* gene. The resulting plasmid was named Ts-P-GFP_{CTC}-T. Finally, the plasmid Ts-*CAT2-gda324-URA3*-P-GFP_{CTC}-T was constructed as described above.

In an earlier report, *C. albicans* codon-adapted *SAT1* and *FLP* genes were functionally expressed in *C. tropicalis* (Chen et al. 2014). Based on this observation, a 717 bp *yeGFP3* gene was chemically synthesized in the light of the NCBI sequence (GenBank accession number U73901.1) where all 239 codons for amino acids were optimized according to the *C. albicans* codon usage bias (Cormack et al. 1997). The plasmid Ts-*CAT2-gda324-URA3*-P-*yeGFP3*-T, which contains *yeGFP3* expression cassette, was constructed as described above.

Transformation of *C. tropicalis*

Yeast transformation was performed using lithium chloride according to a previously described method (Haas

et al. 1990), with slight modifications. We used the PCR products from plasmids Ts-*CAT1-gda-URA3*, Ts-*CAT1-URA3-gda*, Ts-*CAT1-hisG-URA3-hisG*, Ts-*CAT2-gda324-URA3*, or Ts-*CAT2-gda324-URA3*-P-*yeGFP3*-T. Cells from a SM pre-culture were diluted to a density of 0.08 at 600 nm (OD_{600}) in 50-mL fresh SM. After 7–9 h of growth to an OD_{600} of 1.0, the cells were washed once with 2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Cells were re-suspended in 1 mL 100 mM LiCl solution, and the suspension was incubated at 30 °C and 200 rpm for 1 h. A mixture containing 88 μL of cell suspension, 2 μL salmon testis DNA (10 g L^{-1}), and 1.5 μg transforming DNA was incubated at 30 °C for 30 min. Then, 900 μL of PEG3350 solution (40 % polyethylene glycol, 100 mM LiCl) was added to each sample, followed by mixing and incubation at 30 °C for 1 h with shaking. Samples were incubated at 42 °C for 5 min and then subjected to a cold shock step in an ice bath for 3–5 min. The cells were washed once with sterile water and spread onto MM plates. The plates were incubated at 30 °C for 2–3 days until colonies appeared.

Marker gene pop-out

Clones of individual *C. tropicalis* strains transformed by a gene cassette were cultured in SM at 30 °C and 200 rpm until the cell concentration reached 15 (OD_{600}), spread onto a FOA-SM plate, and cultured at 30 °C for 3–4 days. Colonies resistant to 5-FOA were inoculated onto an SM plate and into a liquid culture to extract chromosomal DNA. PCR analysis was performed to identify excision of the *URA3* marker from the genome of recombinant cells.

Preparation of crude enzyme and enzyme assay

Strains were cultured for 48 h in SM and then inoculated into SM supplemented with 2 % dodecane and cultured for 48 h for induction. Crude enzyme was extracted from *C. tropicalis* strains as described previously (Ueda et al. 1982). CAT activity was assayed at 30 °C by the 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) method (Kawamoto et al. 1978). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of acetyl-CoA per minute at 30 °C.

Real-time quantitative PCR

The recombinant strains were cultured in 20 mL SM. Total RNA isolation and synthesis of cDNA were performed with the Yeast RNAiso Kit and PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) as recommended by the manufacturer. Real-time quantitative PCR (RT-qPCR) was performed to measure the relative levels of messenger RNA (mRNA) from target genes. The *ACT1* gene (accession no. FM864204.1) was used as the reference housekeeping gene. For RT-qPCR, a CFX96 real-time PCR instrument with a SYBR® *Premix Ex Taq*™ Kit (TaKaRa) and designed primers (ACT1-F and ACT1-R, CAT-F and CAT-R, GFP-F and GFP-R, yeGFP3-F and yeGFP3-R, corresponding to *ACT1*, *CAT*, *wtGFP*, GFP_{CTC}, and *yeGFP3*, respectively) were used. The relative quantification of target gene transcription was performed using the comparative Ct method (Silva et al. 2011). All experiments were performed in triplicate, and mean values were analyzed for target gene expression.

Fermentation and analytical methods

The fermentation experiments for long-chain α,ω-dodecanedioic acid (DCA12) production were performed in a 750-mL shaking flask with 120-mL fermentation medium at 200 rpm and 30 °C. The fermentation medium for DCA12 production consisted of 6.7 g YNB, 3 g yeast extract, 75 g glucose, 3 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, and 0.06 g uracil L^{-1} (Picataggio et al. 1992). Yeast cells were pre-cultured in 250-mL Erlenmeyer flasks in SM until the OD_{600} value reached 20 (approximately 36 h). This stationary phase culture was inoculated into the fermentation medium at a final concentration of 5 % (*v*/*v*), and the pH was maintained at 5.5–6.0 by addition of 5 M NaOH or 2 M H_2SO_4 during the growth phase (36 h). During the DCA12 production phase, the pH was maintained at 7.5–8.0. In each run, a total of 4 % dodecane was added to each flask, in which 2 % was added twice, first at 0 and then at 72 h. During the conversion phase, glucose was added at a final concentration of 5 g L^{-1} every 24 h to the yeast culture. The concentration of DCA12 was determined by an HP 4890D gas chromatograph with an FID-detector (Agilent, Shanghai, China) (Picataggio et al. 1992).

Fluorescence observation

C. tropicalis 06-1, 06-2, 06-3, and XZX strains were incubated in SM overnight on a rotary shaker at 200 rpm at 30 °C and then stored at 4 °C for several hours. The culture sample was washed twice with sterile PBS buffer, re-suspended in PBS buffer, and the fluorescence intensity was observed using a Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan).

Results

Isolation of *ura3* mutant strains

Using *C. tropicalis* ATCC 20336 as the parent strain, 13 auxotrophic mutant strains defective in the *URA3* gene were isolated by chemical mutagenesis and a double selection procedure that combined nystatin enrichment

selection and 5-FOA resistance selection. Three *ura3* mutant strains were chosen and designated *C. tropicalis* XZW, XZX, and XZB. Mutant strains and the wild-type strain were streaked on SM plates and MM plates. After 4 days of incubation, the wild-type strain was able to grow well on both SM plates and MM plates (Fig. 1). On the other hand, the three mutant strains only exhibited growth on SM plates (Fig. 1). The *URA3* alleles were amplified from the three mutant strains and sequenced. The results showed single-base mutations with amino acid substitutions in all the *URA3* alleles. The residue change at Gly203 to Asp was found in all three mutant *URA3* alleles, thus indicating that Gly203 might reside in a particular domain or in the active site of the URA3 protein.

Sequential disruption of the two *CAT* alleles in *C. tropicalis* XZX

Physical maps of disruption cassettes and the disruption strategy used for the two *CAT* alleles are represented schematically in Fig. 2a, c. The miniature Ura-blaster cassette was constructed as described in the "Materials and methods" section. For the deletion of the allele 1 of the *CAT* genes, the deletion cassette *CAT1-gda-URA3-CAT1* was generated by PCR using Ts-*CAT1-gda-URA3* as a template and CATU and CATR as primers, and the linear DNA fragment was used to transform the *URA3* mutant strain *C. tropicalis* XZX. Uracil prototrophic colonies were isolated and gene replacement was confirmed by PCR using the primers CATU and CATLD (Fig. 2b) and sequencing. A correct transformant in which one of two *CAT* genes was disrupted was designated *C. tropicalis* 01-3 (*ura3*/*ura3*, *cat::gda324-URA3/CAT*) (lane 1, Fig. 2b). After the elimination of the *URA3* marker from *C. tropicalis* 01-3, the resulting strain was designated as *C. tropicalis* 02-3 (*ura3*/*ura3*, *cat::gda324/CAT*) (lane 2, Fig. 2b).

Using a similar strategy, the second *CAT* disruption cassette (*CAT2-gda324-URA3-CAT2*) was transformed into *C. tropicalis* 02-3. The Ura^+ transformants were verified by PCR using primers CAT2ndU and CATR, and a positive transformant was isolated and designated *C. tropicalis* 03 (*ura3*/*ura3*, *cat::gda324*/*cat::gda324-URA3*) (lane 4, Fig. 2b). After elimination of the *URA3* marker from *C. tropicalis* 03, the resulting strain was designated *C. tropicalis* 04 (*ura3*/*ura3*, *cat::gda324*/*cat::gda324*) (lane 5, Fig. 2b).

Characterization of *CAT*-disrupted engineered strains

When incubated in SM, *C. tropicalis* XZX and *C. tropicalis* 02-3 demonstrated a similar growth rate while the growth rate and the final biomass of *C. tropicalis* 04 was much lower when compared to *C. tropicalis* XZX and *C. tropicalis* 02-3 (Fig. 3a). Furthermore, *C. tropicalis* 04 was unable to grow using dodecane as a sole carbon source (Fig. 3b). These results indicated that the *CAT* gene is essential for dodecane metabolism in *C. tropicalis* and the pathway for glucose metabolism might also be affected by the disruption of the *CAT* alleles.

The CAT enzymatic activity of *C. tropicalis* 02-3, *C. tropicalis* 04, and *C. tropicalis* XZX was measured as reported in the "Materials and methods" section (Fig. 3c). Our results showed that the CAT activity of *C. tropicalis* 02-3 decreased by 80 %. In addition, there was a trace amount of CAT activity in *C. tropicalis* 04 (Fig. 3c). A previous study indicated that the DTNB method for the CAT assay may be affected by acetyl coenzyme A hydrolase in *C. tropicalis* (Zhang et al. 2005), which might lead to residual CAT activity. Therefore, RT-qPCR was conducted to further confirm the deletion of the two *CAT* alleles (Fig. 3d). In *C. tropicalis* 04, *CAT* mRNA could not be detected by RT-qPCR. The results indicated that the two *CAT* alleles were deleted successfully in *C. tropicalis* 04.

In addition, effects of deleting the *CAT* alleles on DCA12 accumulation were investigated. Fig. 3e shows that *C. tropicalis* XZX and 02-3 could accumulate a small amount

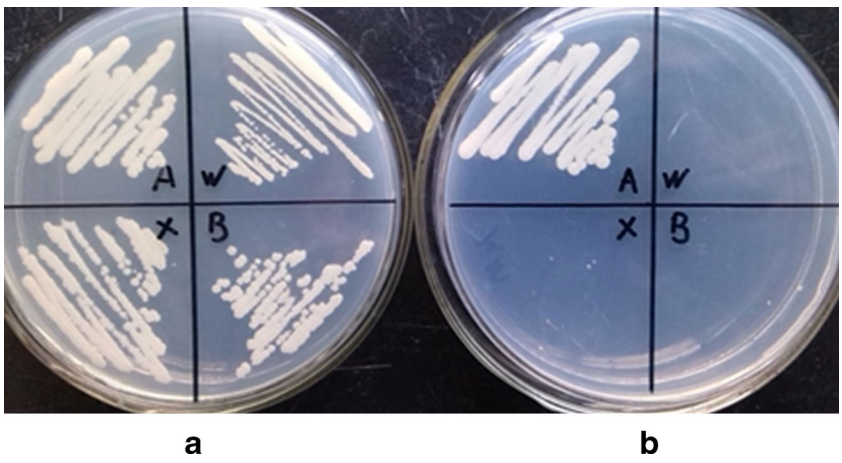

Fig. 1 Phenotypes observed on an SM plate (**a**) and an MM plate (**b**) of the parent and mutant strains. *A C. tropicalis* ATCC20336, *W C. tropicalis* XZW, *X C. tropicalis* XZX, *B C. tropicalis* XZB

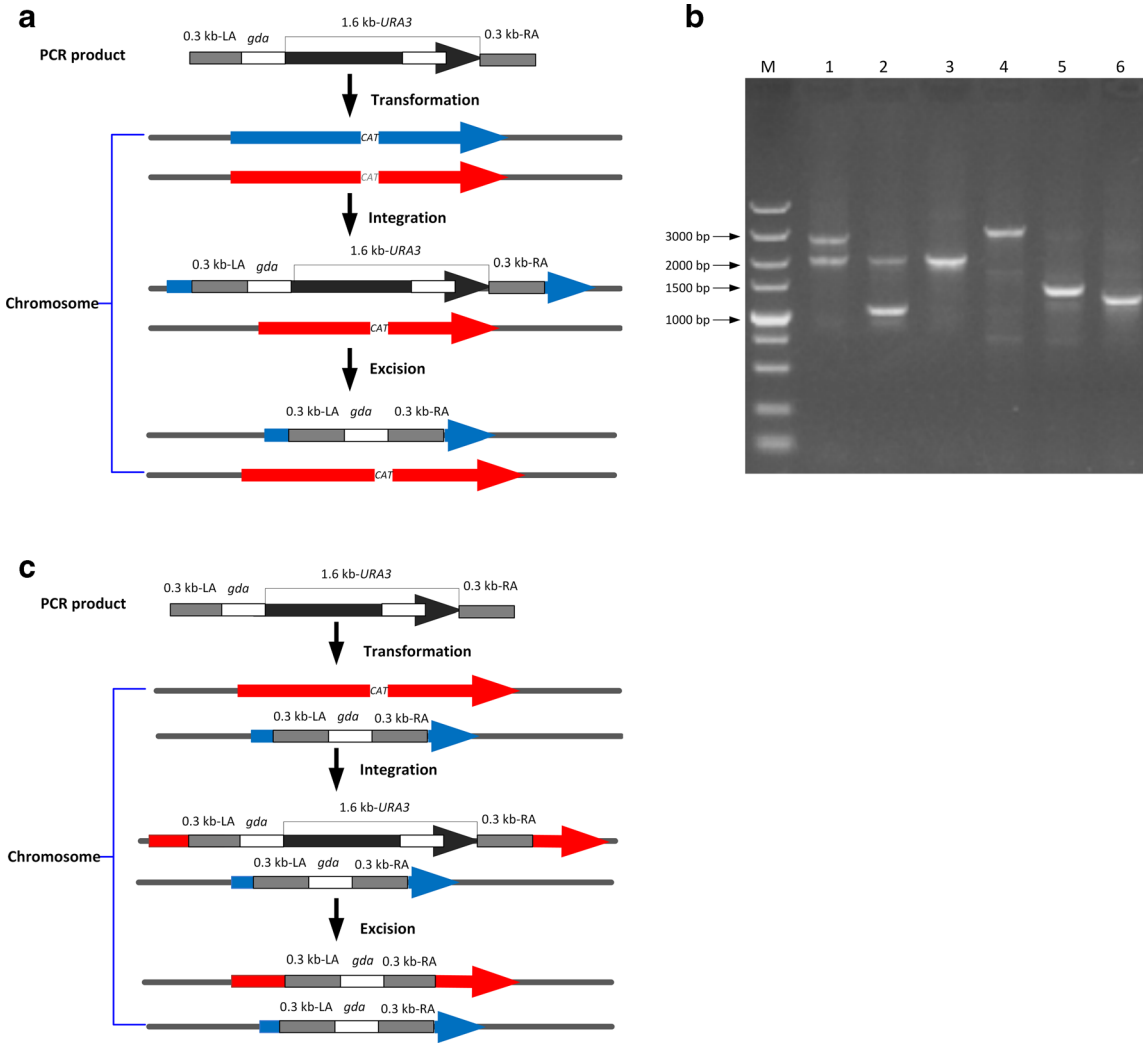

Fig. 2 Sequential gene disruption of *CAT* in *C. tropicalis*. **a** Deletion of *CAT* allele 1 using the miniature disruption cassettes. The *gray boxes* represent the homologous arms LA (*left arm*) and RA (*right arm*). The *white boxes* represent the *gda* fragments. The *black arrow* indicates the *URA3* gene. *CAT* allele 1 and *CAT* allele 2 are represented by the *blue arrow* and the *red arrow*, respectively. **b** Agarose gel electrophoresis of PCR products generated with identification primers. Lanes *1*, *2*, and *3* indicate PCR with primers CATU and CATLD; lane *1* represents *C. tropicalis* 01-3, lane *2* represents *C. tropicalis* 02-3, and lane *3* represents *C. tropicalis* XZX. Lanes *4*, *5*, and *6* indicate PCR with primers CAT2ndU and CATR. Lane *4* represents *C. tropicalis* 03, lane *5* represents *C. tropicalis* 04, and lane 6 represents *C. tropicalis* XZX. Lane *M* represents DNA marker. **c** Deletion of *CAT* allele 2 using the miniature disruption cassettes. (Color figure online)

of DCA12, whereas *C. tropicalis* 04 could produce higher amounts of DCA12 and a final concentration of DCA12 of 12.9 g L^{-1} was reached with this clone. These results indicated that the deletion of both *CAT* alleles of *C. tropicalis* can lead to significantly higher DCA12 production rates.

Effect of different *gda* elements on the *URA3* removal efficiency

To evaluate the effect of different *gda* elements on the *URA3* marker removal efficiency, a series of disruption cassettes for the *CAT* allele 1 were constructed (Fig. 4a) and transformed into *C. tropicalis* XZX. Transformants designated *C. tropicalis* 01-1, *C. tropicalis* 01-2, *C. tropicalis* 01-3, *C. tropicalis* 01-4, *C. tropicalis* 01-5, *C. tropicalis* 01-6, *C. tropicalis* 01-7, and *C. tropicalis* 01-8 were cultured in SM at 200 rpm and 30 °C for 36 h. The viable cell numbers were counted by spreading cultures onto YPD plates. Similarly, the same culture volumes were directly spread onto FOA-SM plates. The elimination of *URA3* in clones grown on FOA-SM plates was confirmed by PCR using primers CATU and CATLD (Fig. 4b–f) and sequencing. The frequencies of *URA3* gene excision of various disruption cassettes were compared and are reported in Table 3. In particular, Table 3 shows that *gda143* is sufficiently long to promote *URA3* gene excision. The *gda324* fragment in the disruption cassette *CAT1-gda-URA3-CAT1* was the most efficient in the excision of the *URA3* marker when compared to the other *gda* fragments in

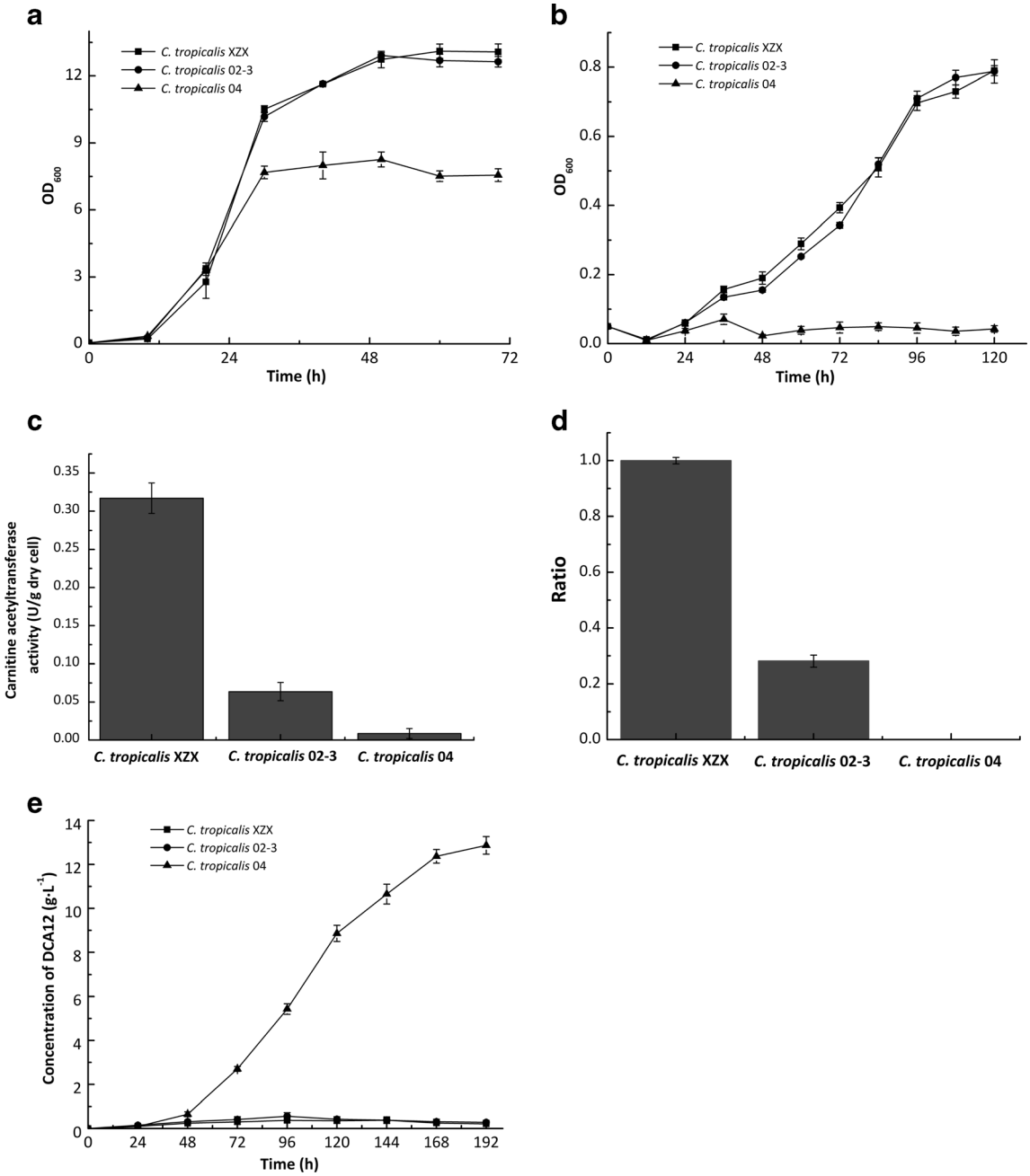

Fig. 3 Physiological and biochemical confirmation of *CAT* disruption. **a** Growth curve of *C. tropicalis* strains in SM. **b** Growth curve of different *C. tropicalis* strains using dodecane as a sole carbon source. **c** Assay for CAT activities in *CAT*-disrupted engineered strains and the parental strain. **d** Transcription levels of the *CAT* gene in various *C. tropicalis* strains. **e** Comparison of DCA12 production for three *C. tropicalis* strains. The data points represent the average of three independent experiments (mean ± standard deviations)

other cassettes. An excision frequency of approximately 10^{-8} is in the same order as the long *hisG* fragments.

Integrative expression of a foreign gene combined with target gene disruption

C. tropicalis 02-3 was transformed with the *yeGFP3* expression cassette (Fig. 5a), which was amplified from Ts-*CAT2-gda324-URA3*-P-*yeGFP3*-T by PCR using primers CAT2ndU and CAT2ndR and was selected on the basis of uracil prototrophy. Ura^+ transformants were verified by PCR using the identification primers CAT2ndU and CATR (Fig. 5b). Thus, we confirmed that both *CAT* alleles were destroyed and the *yeGFP3* expression cassette was specifically integrated into the *CAT* locus. A transformant that had the expected structure was designated as *C. tropicalis* 05-3 (lane 1, Fig. 5b). The strain that lost *URA3* was designated as *C. tropicalis* 06-3 (lane 2, Fig. 5b). Similarly, the recombinant strains

Table 3 Frequency of *URA3* gene pop-out based on different *gda* fragments

Strains	Size of *gda* fragment (bp)	Colony number of *Ura*$^-$ clones on FOA-SM plates[a]	Number of unicellular yeast cells plated on FOA-SM plates	Percentage of expected strains	Frequency of *URA3* pop-out
01-1	143	0, 1, 3	2.715×10^9	3/4	3.7×10^{-10}
01-2	245	0, 0, 2	2.86×10^9	2/2	2.33×10^{-10}
01-3	324	92, 99, 132	2.055×10^9	8/8	5.58×10^{-8}
01-4	488	73, 82, 112	5.395×10^9	6/6	1.65×10^{-8}
01-5	302	61, 72, 87	5.16×10^9	12/12	1.42×10^{-8}
01-6	305	62, 99, 145	5.84×10^9	12/12	1.73×10^{-8}
01-7	325	145, 146, 188	2.33×10^9	9/11	5.61×10^{-8}
01-8	*hisG*	744, 711, 657	9.43×10^9	12/12	7.5×10^{-8}

[a] Indicates that three samples (each 200 μL) cultured in SM were spread onto three FOA-SM plates for every strain. Colony numbers were counted for every plate

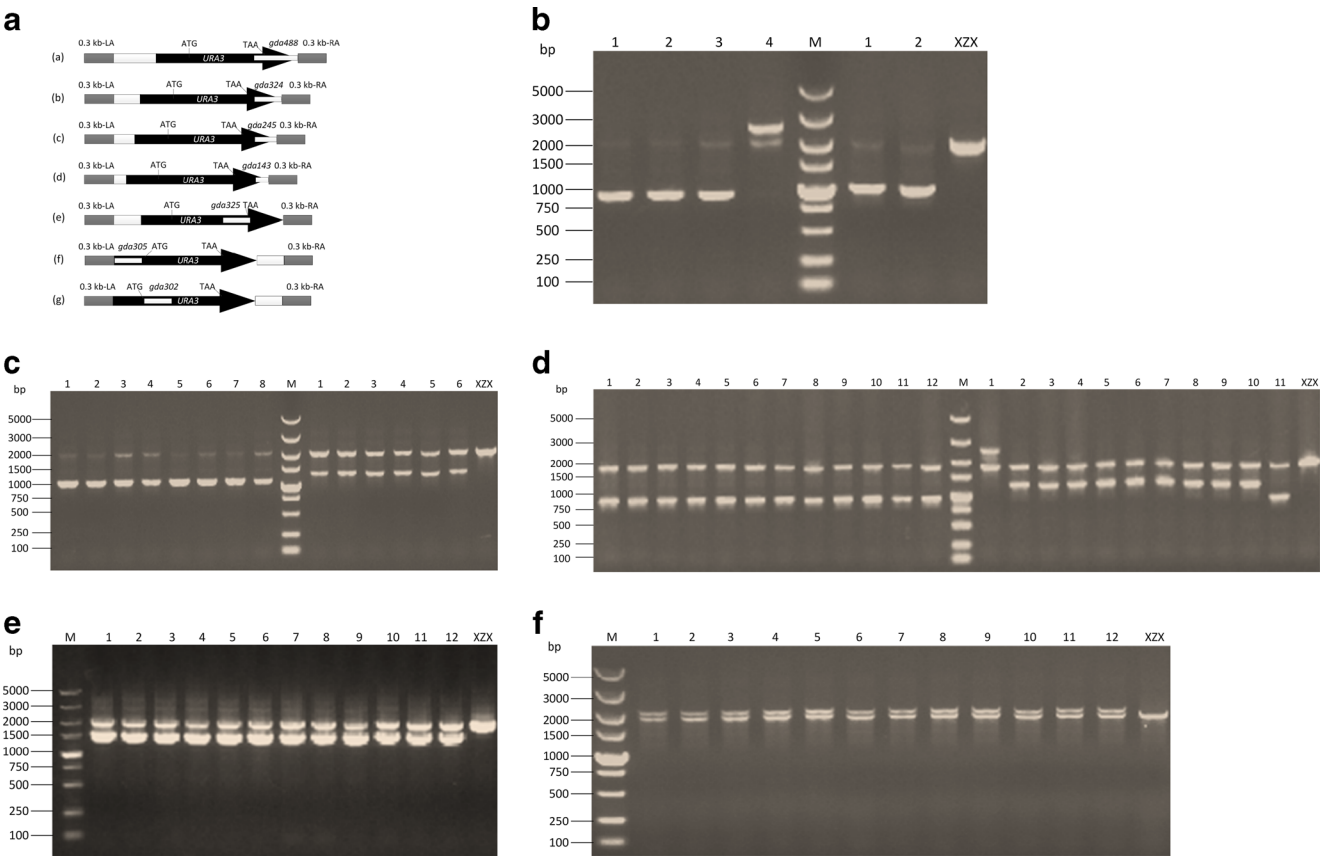

Fig. 4 Effect of different *gda* fragments on *URA3* marker excision. **a** Physical map of different disruption cassettes. The *gray boxes* represent the homologous arms LA (*left arm*) and RA (*right arm*). The *white boxes* represent the *gda* fragments. The *black arrow* indicates the *URA3* gene. **b** PCR confirmation of *URA3* marker excision in *C. tropicalis* 01-1 and *C. tropicalis* 01-2 strains. Lanes on the *left* of marker represent four different *C. tropicalis* 01-1-type samples cultured in FOA-SM; lanes on the *right* of the marker represent two different *C. tropicalis* 01-2-type samples cultured in FOA-SM. **c** PCR confirmation of *URA3* marker excision in *C. tropicalis* 01-3 and *C. tropicalis* 01-4 strains. Lanes on the *left* of marker represent eight different *C. tropicalis* 01-3-type samples cultured in FOA-SM; lanes on the *right* of the marker represent six different *C. tropicalis* 01-4-type samples cultured in FOA-SM. **d** PCR confirmation of *URA3* marker excision in *C. tropicalis* 01-6 and *C. tropicalis* 01-7 strains. Lanes on the *left* of marker represent 12 different *C. tropicalis* 01-6-type samples cultured in FOA-SM; lanes on the *right* of the marker represent 11 different *C. tropicalis* 01-7-type samples cultured in FOA-SM. **e** PCR confirmation of *URA3* marker excision in *C. tropicalis* 01-5. Lanes *1–12* represent 12 different *C. tropicalis* 01-5-type samples cultured in FOA-SM. f PCR confirmation of *URA3* marker excision in *C. tropicalis* 01-8. Lanes *1–12* represent 12 different *C. tropicalis* 01-8-type samples cultured in FOA-SM. Lane *M* indicates DNA markers. *XZX* represents *C. tropicalis* XZX. All PCR reactions were carried out using primers CATU and CATLD (locations are shown in Fig. 5a)

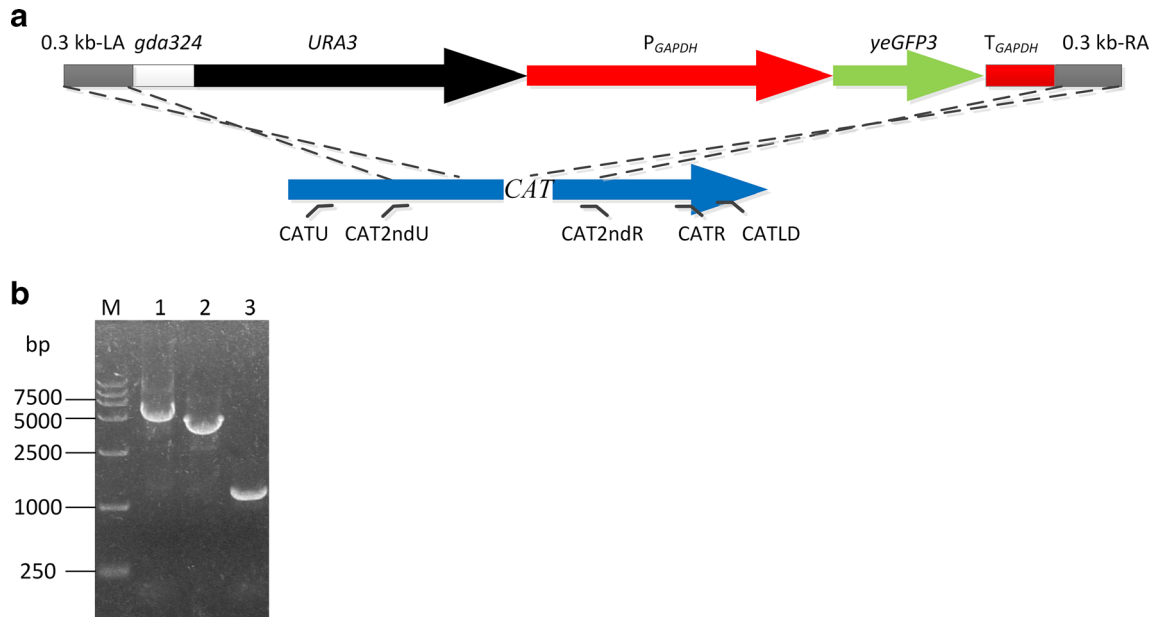

Fig. 5 Construction of a *GFP* expressing strain. **a** Physical map of the *GFP* expression cassette and integration of the cassette into the *CAT* locus of *C. tropicalis* 02-3. The *gray boxes* represent the homologous arms. The *white boxes* represent the *gda* fragments. The *black arrow* indicates the *URA3* gene. The *green arrow* represents the ORF of *yeGFP3*. The *red arrow* represents the *GAPDH* promoter, while the *red box* represents the *GAPDH* terminator. **b** Agarose gel electrophoresis of PCR products generated with identification primers. Lanes *1*, *2*, and *3* indicate PCR with primers CAT2ndU and CATR; lane 1 represents *C. tropicalis* 05-3, lane 2 represents *C. tropicalis* 06-3, lane 3 represents *C. tropicalis* XZX

C. tropicalis 06-1 expressing the *wtGFP* gene and *C. tropicalis* 06-2 expressing the GFP_{CTC} gene were also constructed.

To examine the expression of different *GFP* genes in transformant cells, *C. tropicalis* 06-1, 06-2, 06-3, and the control strain XZX were cultured and the fluorescence intensity of cells was observed by using a fluorescence microscope (Fig. 6a). High levels of green fluorescence were observed in *C. tropicalis* 06-3, and only a slight amount of fluorescence was observed in *C. tropicalis* 06-2 after a long exposure time. However, no fluorescence was observed in *C. tropicalis* 06-1 and the control strain XZX. Thus, *yeGFP3* is functionally expressed in *C. tropicalis*. Furthermore, RT-qPCR analysis showed that *yeGFP3* mRNA levels increased significantly when compared to a transformant containing GFP_{CTC} (Fig. 6b), while no *GFP* mRNA was detected in *C. tropicalis* 06-1.

Discussion

C. tropicalis represents an attractive yeast strain involved in an increasing number of applications. However, it has several characteristics that hamper strain improvement through genetic engineering. A common approach for sequential gene disruption in *C. tropicalis* is based on the use of *ura3* auxotrophic mutant hosts. In previous reports, gene disruption in *C. tropicalis* and *C. albicans* has been performed with the *hisG-URA3-hisG* ("Ura-blaster") cassette, which can be reused in successive transformations of a single strain after homologous excision of *URA3* (Fonzi and Irwin 1993; Ko et al. 2006). However, the large *hisG* repeats can lead to difficulties in the amplification of the disruption cassette and yeast integrative transformation. Here, we developed an efficient and simple sequential gene disruption method for stable unlabeled gene insertion into *C. tropicalis* chromosomes. Moreover, foreign genes were successfully expressed in *C. tropicalis* using this method.

The disruption cassette, which consists of a functional *C. tropicalis URA3* gene flanked by 0.3 kb *gda* direct repeats derived from regions upstream or downstream of the *URA3* gene and of homologous arms to the target gene, was constructed and introduced into the yeast genome by integrative transformation. Using this strategy, a pair of *CAT* alleles was successfully disrupted. In addition, in further experiments, two copies of *POX4* and *POX5* alleles, encoding distinct isozymes of the acyl-CoA oxidase that catalyzes the first reaction in the ß-oxidation pathway, were also knocked out (data not shown). This new strategy is highly efficient and time saving as compared to the previously described process (Picataggio et al. 1991). Another advantage of the miniature Ura-blaster is that the recombination efficiency can be increased by almost one order of magnitude when compared to the traditional Ura-blaster. Overall, the results presented in this paper demonstrated that the miniature Ura-blaster can be used in a highly efficient way to destroy genes of *C. tropicalis* with a single marker gene.

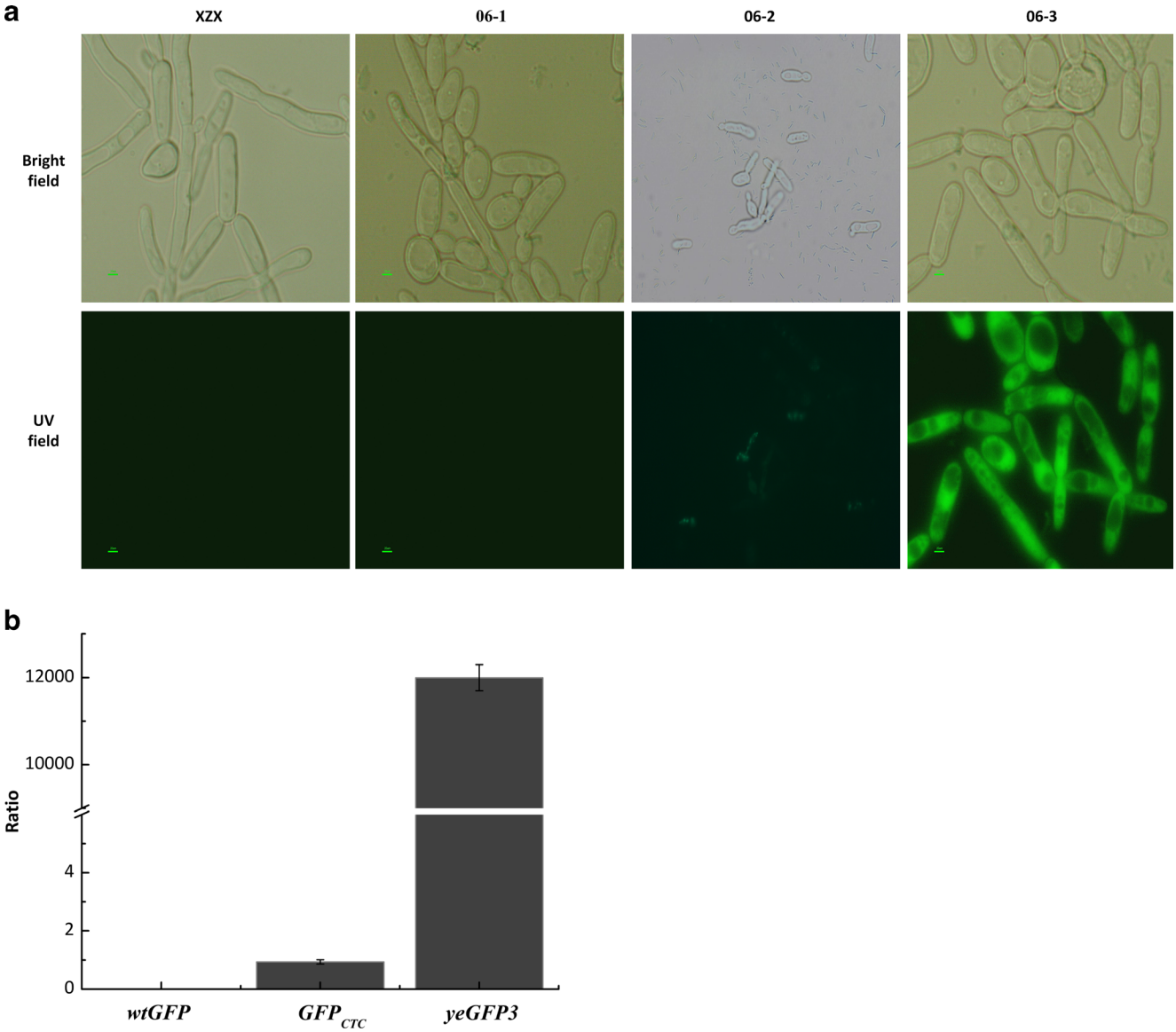

Fig. 6 Confirmation of different *GFP* gene expression in the strain *C. tropicalis* 02-3. **a** Phase contrast and UV fluorescence images of *C. tropicalis* XZX, 06-1, 06-2, and 06-3 strains. (**b**) Transcription levels of the different GFP genes in *C. tropicalis*; *wtGFP* represents a transformant with the wild-type *GFP* gene; GFP_{CTC} represents a transformant with the CTG codons optimized *GFP* gene; and *yeGFP3* represents a transformant with the *yeGFP3* gene

To evaluate the effect of the *gda* fragment on excision of the *URA3* gene from the deletion cassette, different sized *gda* fragments were employed and evaluated in this gene disruption system. Our results showed that 143 base pairs are enough for marker recycling, whereas *gda324* with 324 base pairs has the highest excision frequency of the *URA3* gene (Table 3). Moreover, *gda* fragments from different locations of *URA3* (*gda305*, *gda302*, *gda324*) were cloned and investigated for their effects on the excision of *URA3* marker (Table 3; Fig. 3). The frequencies of the *URA3* gene excision were similar for different *gda* fragments isolated from the 5′ or the 3′ terminus of the *URA3* gene. This indicated that the *gda* sequence position does not significantly affect *URA3* marker removal. A traditional Ura-blaster (Alani et al. 1987) disruption cassette (*CAT1-hisG-URA3-hisG-CAT1*) for *CAT* allele 1 disruption was also constructed to be compared to the frequency of *URA3* gene excision. When *hisG* was used as repeat fragment, the frequency of *URA3* excision was similar with *gda324*; however, it was much lower than the rate reported previously for *S. cerevisiae* (10^{-4}) (Alani et al. 1987) and *C. albicans* (10^{-5}–10^{-6}) (Fonzi and Irwin 1993). This difference might be due to the complicated genetic background of *C. tropicalis.*

Expression of heterologous proteins is of great academic value and of commercial significance. However, it has proven difficult to functionally express a heterologous protein in *Candida* species and other fungi (Gustafsson et al. 2004;

Tanaka et al. 2012). In the present study, *wtGFP*, GFP_{CTC}, and *yeGFP3* were used as reporter genes under the control of the *GAPDH* promoter and integrated into the chromosome of *C. tropicalis* 02-3. The recombinant strains were confirmed and cultured in SM for fluorescence detection. No fluorescence was observed in transformants expressing *wtGFP*. When wild-type red fluorescent gene (*RFP*) was integrated in *C. tropicalis* 02-3 chromosome using a similar strategy, also no fluorescence was observed (data not shown). Moreover, mRNA levels of *wtGFP* could not be detected by RT-qPCR, which might be explained by a nonstop mRNA degradation mechanism (Frischmeyer et al. 2002; van Hoof et al. 2002).

For transformants expressing GFP_{CTC}, only scant fluorescence was observed. Also, transcriptional analysis showed lower mRNA levels of the GFP_{CTC} gene. As mentioned by Cormack et al. (1997), the CTG codons in *GFP* are not the only reason for the lack of expression of this gene in *C. albicans*. A second problematic codon may also be present in *wtGFP*. Therefore, we synthesized *yeGFP3* in which all the codons were optimal for expression in *C. albicans* and integrated it into a *C. tropicalis* chromosome. As for other *C. albicans* codon-optimized genes that have been shown to function in *C. tropicalis* (Chen et al. 2014), the *C. albicans* codon-optimized *yeGFP3* gene also functioned in *C. tropicalis* and strong fluorescence was observed in all transformed cells (Fig. 6a). Furthermore, RT-qPCR showed that the mRNA levels of *yeGFP3* significantly increased when compared to GFP_{CTC} (Fig. 6b).

Although *yeGFP3* is functionally expressed in *C. tropicalis*, the mechanism behind codon optimization is unclear and the effect on transcription levels has not yet been elucidated. To the best of our knowledge, the mechanism behind codon optimization on mRNA levels has been reported in many microorganisms such as in *E. coli* (Kudla et al. 2009), *Aspergillus oryzae* (Tanaka et al. 2012), and *S. cerevisiae* (Doma and Parker 2006; Tsuboi et al. 2012). However, little is known regarding *C. tropicalis*. It is meaningful in the future to give insight into the mechanism of transcription and translation in *C. tropicalis*.

In summary, we developed an efficient sequential gene disruption strategy using a *gda* segment as gene disruption auxiliary sequence for *C. tropicalis*. The *gda* sequence employed in this system shortened the length of the disruption cassette and increased the efficiency of integration into the target locus. The two *CAT* alleles were deleted successfully using this approach. Moreover, the *yeGFP3* gene was functionally expressed in *C. tropicalis* by integration through homologous recombination at the target locus, using this effective strategy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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