Supplementary materials

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Transcriptome analysis of *Kluyveromyces marxianus* under succinic acid stress and development of a robust strain

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Construction of plasmids and strains

For the construction of plasmids, pUCC001 (Rajkumar and Morrissey 2022) was utilized as the initial plasmid (Table S1). The backbone of the plasmid, which included the hygromycin resistance cassette, was obtained by removing the Cas9 expression cassette via *Bst*EII and *Afl*II digestion. The expression cassettes of the fluorescent protein reporter genes (*yeGFP* and *mcherry*) were ligated to the plasmid backbone in the following steps. Firstly, the fragments *IMTCP2*p, *yeGFP*, and *IMTT1*t were amplified from the *K. marxianus* NBRC1777 genome and plasmid pKT0209 (Sheff and Thorn 2004) using the primer pairs from Table S2. Then, the expression cassette for green fluorescent protein (*IMTCP2*p-*yeGFP*-*IMTT1*t) was created using the overlap extension polymerase chain reaction (OE-PCR) technique. This cassette was then ligated to the plasmid skeleton between the *Bst*EII and *Afl*II sites by Gibson assembly to obtain plasmid pUCZ01 (see Table S1, line 2). Similarly, the red fluorescent protein mcherry (donated from Prof. Xie at Shanghai jiaotong University) expression cassette comprises promoter sc*TDH3* (glyceraldehyde 3-phosphate dehydrogenase), *mcherry*, and sc*CYC1* terminator to obtain plasmid pUCZ02 (see Table S1, line 3). This cassette was ligated to the skeleton of plasmid pUCZ01 after digested with *Bsm*BI for removing the originally existed guide RNA expression cassette and the plasmid cyclized by Gibson assembly to obtained plasmid pUCZ03 (Table S1, line 4). Subsequently, the hygromycin marker of pUCZ03 was removed after digestion by *Psp*XI and *Bam*HI, then replaced by gene *URA3* coding orotidine-5'-phosphate decarboxylase amplified from NBRC1777 genome between the *Psp*XI and *Bam*HI sites to obtain the plasmid

pUCZ04 (Table S1, line 5). Finally, a series of plasmids (from Table S1 line 5 to line 17) derived from pUCZ04 by replacing the original promoter *IMTCP2*p of *yeGFP*. The plasmid pUKDN132 (Zhou et al. 2018) removed the original promoter and signal peptide sequences and replaced by fragment of the *IMTCP2* promoter through digestion of *Sac*II and *Not*I to obtain the episomal plasmid pUTCP2, which was used as the vector for the overexpression of transcription factors (Fig. S2).

The *URA3* gene encoding orotidine-5'-phosphate decarboxylase was disrupted in *K. marxianus* NBRC1777 to obtain strain ZW01 by the CRISPR/Cas9-mediated genome editing method (Rajkumar et al. 2019). Yeast transformation was performed using the lithium acetate transformation method (Lyu et al. 2021).

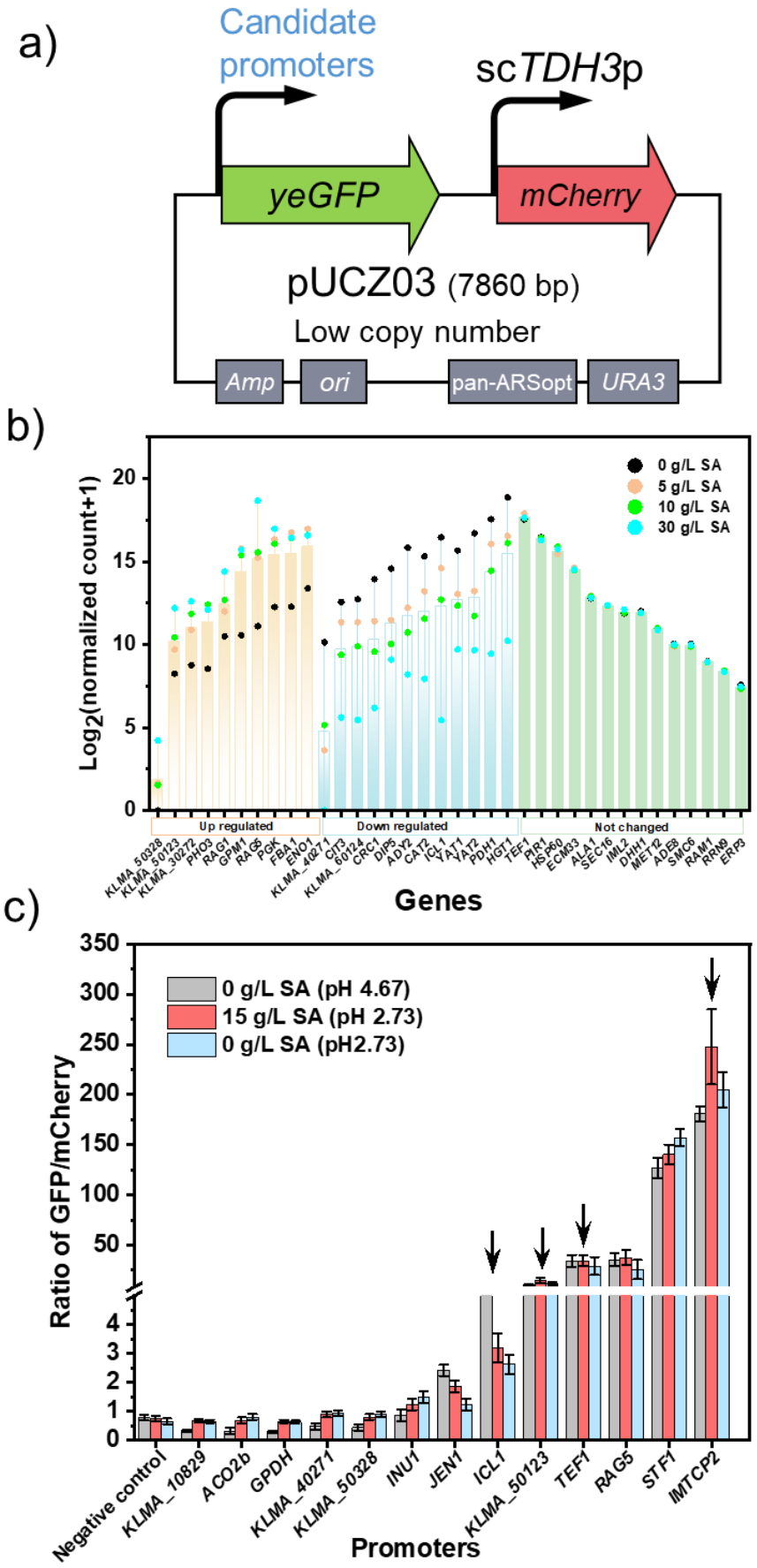
a)
Candidate promoters
scTDH3p
yeGFP
mCherry
pUCZ03 (7860 bp)
Low copy number
Amp
ori
pan-ARSopt
URA3
b)
Log2(normalized count+1)
0 g/L SA
5 g/L SA
10 g/L SA
30 g/L SA
Up regulated
Down regulated
Not changed
Genes
c)
Ratio of GFP/mCherry
0 g/L SA (pH 4.67)
15 g/L SA (pH 2.73)
0 g/L SA (pH2.73)
Negative control
KLMA_10829
ACO2b
GPDH
KLMA_40271
KLMA_50328
INU1
JEN1
ICL1
KLMA_50123
TEF1
RAG5
STF1
IMTCP2
Promoters

Figure S1 The selection and verification of promoters responding to SA. (a) The candidate SA-responsive promoter verification by the double fluorescence expression system on low copy plasmid pUCZ03, in which the candidate promoters and sc*TDH3* control the green and red fluorescent protein expression respectively. (b) The relative transcription level of a gene under different concentrations of SA selected from transcriptome data. The x-axis divided into three types including upregulated (orange bars), downregulated (blue bars) and not changed (green bars), in which the ordinate values of the small balls (black, yellow, green and blue) represent the transcription levels of a candidate gene under the 0, 5, 10, and 30 g/L SA, respectively. (c) Ratio of green and red fluorescent intensity at 15 g/L SA (pH 2.73) or without SA but with pH adjustment to 2.73 by hydrochloric acid.

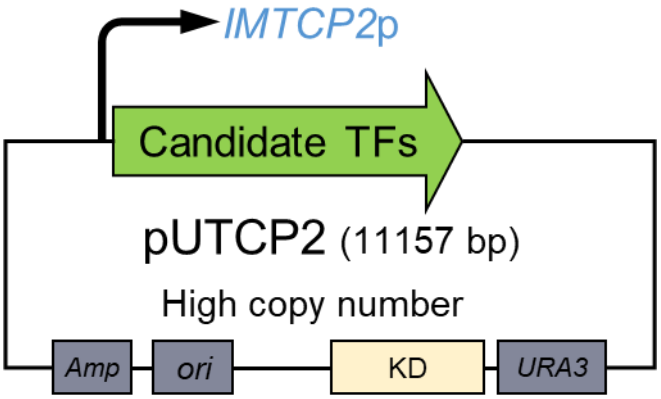

Figure S2 Transcription factors overexpression system, which is under the control of promoter *IMTCP2* on the high copy number plasmid pUTCP2 with *URA3* as selected marker.

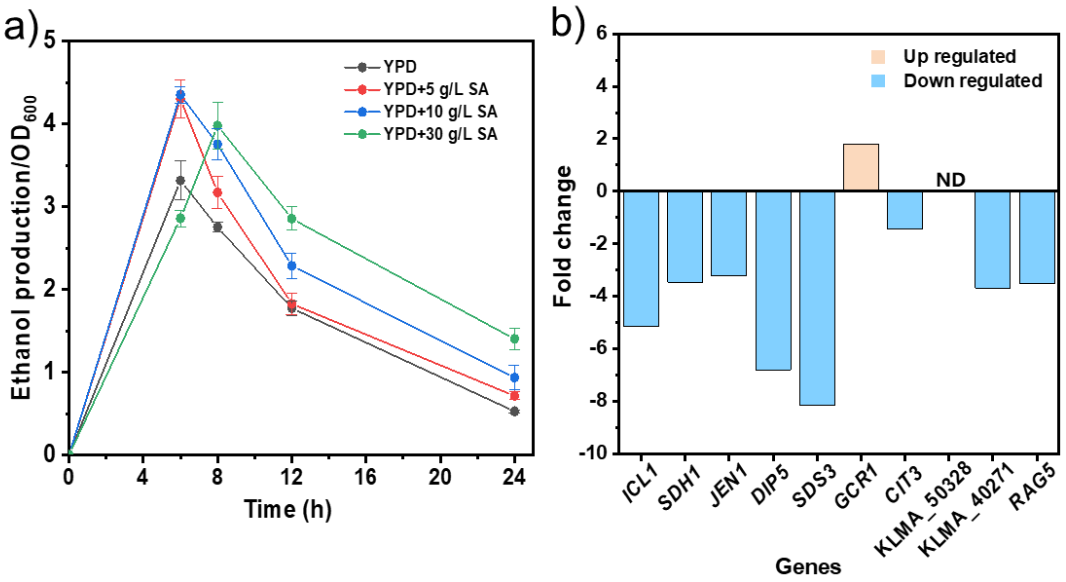

Figure S3 The glycolytic pathway was enhanced and the tricarboxylic acid cycle was repressed under SA stress. (a) The ethanol production capacity of per OD600 cells of NBRC1777 was improved under the different concentrations of SA including 0, 5, 10, and 30 g/L in YPD medium cultured in 37 ºC and 200 rpm for 24 h. (b) Verification of transcription level change of key genes selected from transcriptome data by qPCR under 30 g/L SA in YPD medium cultured in 37 ºC and 200 rpm for 6 h.

Table S1 Plasmids and strains

Plasmids	Genotype	Resource
(1) pUCC001	Centromeric plasmid; *HygR*, pan-ARS, (Beta-galactosidase)-gRNA scaffold, *CAS9*	(Rajkumar and Morrissey 2022)
(2) pUCZ01	pUCC001 derivative; *KmURA3*, pan-ARSopt, *IMTCP2*p-*IMTT1*t	This study
(3) PUCZ02	pUCZ01 derivative; *KmURA3*, pan-ARSopt, *IMTCP2*p-*yeGFP-IMTT1*t	This study
(4) pUCZ03	pUCZ02 derivative; *KmURA3*, pan-ARSopt, *scTDH3*p- *mCherry -CYC1*t	This study
(5) pUCZ04	pUCZ03 derivative; *KmURA3*, pan-ARSopt, *IMTCP2*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(6) pUCZ04-INU1	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *INU1*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(7) pUCZ04-TEF1	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *TEF1*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(8) pUCZ04-JEN1	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *JEN1*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(9) pUCZ04-K50328	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *KLMA_50328*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC*t	This study
(10) pUCZ04-ICL1	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *ICL1*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(11) pUCZ04-K40271	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *KLMA_40271*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(12) pUCZ04-RAG5	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *RAG5*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(13) pUCZ04-STF2	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *STF2*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(14) pUCZ04-Aco2b	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *Acocb*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(15) pUCZ04-K10829	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *KLMA_10829*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(16) pUCZ04-GPDH	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *GPDH*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(17) pUCZ04-K50231	pUCZ04 derivative; *KmURA3*, pan-ARSopt, *KLMA_50123*p-*yeGFP-IMTT1*t, *scTDH3*p-*mCherry-CYC1*t	This study
(18) pUKDN132	Episomal plasmid; *KmURA3*, KD, *INU1*p-(INU1 signal peptide)-*INU1*t	(Zhou et al. 2018)
(19) pUTCP2	pUTCP2 derivative; *KmURA3*, KD, *IMTCP2*p-*INU1*t	This study
(20) pUTCP2-GCR1	pUTCP2 derivative; *KmURA3*, KD, *IMTCP2*p-*GCR1-INU1*t	This study
(21) pUTCP2-UPC2	pUTCP2 derivative; *KmURA3*, KD, *IMTCP2*p-*UPC2-INU1*t	This study
(22) pUTCP2-NRG1	pUTCP2 derivative; *KmURA3*, KD, *IMTCP2*p-*NRG1-INU1*t	This study
(23) pUTCP2-NDT1	pUTCP2 derivative; *KmURA3*, KD, *IMTCP2*p-*NDT1-INU1*t	This study
Strains		

K. marxianus		
(24) NBRC1777	Wild type, haploid	NBRC
(25) ZW01	Derived from NBRC1777, *ura3*Δ	This study
(26) NBKD	Derived from NBRC1777, *ku70*Δ :: *KmIMTCP1*p-*scRAD52-TIMTT2*t	This study
(27) ZW01-none	ZW01 derivative; empty plasmid {pUCZ01}/(*KmURA3*)	This study
(28) ZW01-gfp	ZW01 derivative; plasmid {pUCZ02}/(*KmURA3*, *IMTCP2*p-*yeGFP*)	This study
(29) ZW01-mch	ZW01 derivative; plasmid {pUCZ03}/(*KmURA3*, *mCherry*)	This study
(30) ZW01-TCP2p	ZW01 derivative; plasmid {pUCZ04}/(*KmURA3*, *mCherry*, *IMTCP2*p-*yeGFP*)	This study
(31) ZW01-INU1p	ZW01 derivative; plasmid {pUCZ04-INU1}/(*KmURA3*, *mCherry*, *INU1*p-*yeGFP*)	This study
(32) ZW01-TEF1p	ZW01 derivative; plasmid {pUCZ04-TEF1}/(*KmURA3*, *mCherry*, *TEF1*p-*yeGFP*)	This study
(33) ZW01-JENp	ZW01 derivative; plasmid {pUCZ04-JEN1}/(*KmURA3*, *mCherry*, *JEN1*p-*yeGFP*)	This study
(34) ZW01-K50328p	ZW01 derivative; plasmid {pUCZ04-K50328}/(*KmURA3*, *mCherry*, *KLMA_50328*p-*yeGFP*)	This study
(35) ZW01-ICL1p	ZW01 derivative; plasmid {pUCZ04-ICL1}/(*KmURA3*, *mCherry*, *ICL1*p-*yeGFP*)	This study
(36) ZW01-K40271p	ZW01 derivative; plasmid {pUCZ04-K40271}/(*KmURA3*, *mCherry*, *KLMA_40271*p-*yeGFP*)	This study
(37) ZW01-RAG5p	ZW01 derivative; plasmid {pUCZ04-RAG5}/(*KmURA3*, *mCherry*, *RAG5*p-*yeGFP*)	This study
(38) ZW01-STF2p	ZW01 derivative; plasmid {pUCZ04-STF2}/(*KmURA3*, *mCherry*, *STF2*p-*yeGFP*)	This study
(39) ZW01-Aco2bp	ZW01 derivative; plasmid {pUCZ04-Aco2b}/(*KmURA3*, *mCherry*, *Aco2b*p-*yeGFP*)	This study
(40) ZW01-K10829p	ZW01 derivative; plasmid {pUCZ04-K10829}/(*KmURA3*, *mCherry*, *KLMA_10829*p-*yeGFP*)	This study
(41) ZW01-GPDHp	ZW01 derivative; plasmid {pUCZ04-GPDH}/(*KmURA3*, *mCherry*, *GPDH*p-*yeGFP*)	This study
(42) ZW01-K50231p	ZW01 derivative; plasmid {pUCZ04-K10829}/(*KmURA3*, *mCherry*, *KLMA_50231*p-*yeGFP*)	This study
(43) ZW01-TCP2	ZW01 derivative; plasmid {pUTCP2}/(*KmURA3*, *KD*)	This study
(44) ZW01-GCR1	ZW01 derivative; plasmid {pUTCP2-GCR1}/(*KmURA3*, *KD*, *GCR1*)	This study
(45) ZW01-UPC2	ZW01 derivative; plasmid {pUTCP2-UPC2}/(*KmURA3*, *KD*, *UPC2*)	This study
(46) ZW01-NRG1	ZW01 derivative; plasmid {pUTCP2-NRG1}/(*KmURA3*, *KD*, *NRG1*)	This study
(47) ZW01-NDT1	ZW01 derivative; plasmid {pUTCP2-NDT1}/(*KmURA3*, *KD*, *NDT1*)	This study

Table S2 Primers used in this study

Primer name	Sequence (5'→3')	Purpose
BstE2-sbf1-TCP2-F	CCGGGTAACCCCTGCAGGCAAAACAGGACAAAA CAAAACAATACAGTAC	Amplification of *IMTCP2* promoter
TCP2-eGFP-R	AATTCTTCACCTTTAGACATGTCGACTTTTGATTT GTGTTTAAGCGAGTGACTG	
eGFP-TCP2-F	CTTAAACACAAATCAAAAGTCGACATGTCTAAAG GTGAAGAATTATTCACTGG	Amplification of *yeGFP*
eGFP-MTT1-R	GATTTTATCAACAAAGCTTCTCTAGATTATTTGTA CAATTCATCCATACCATGGG	
MTT1-F	TCTAGAGAAGCTTTGTTGATAAAATCTAACTACTG	Amplification of *IMTT1* terminator
Alf2-Asc1-MTT1-R	GGGCTTAAGGGCGCGCCGTTCAAATACGGAAGC AAAGGAAC	
scTDH3p-F	CAAATCATAATCAGCACTAACGTCTCATCATTATC AATACTGCCATTTCAAAGAATACG	Amplification of sc*TDH3* promoter
scTDH3-R	TTTGTTTGTTTATGTGTGTTTATTCGAAACTAAG	
mCherry-F	CGAATAAACACACATAAACAAACAAAATGGTTTC TAAGGGTGAAGAAGAC	Amplification of mCherry
mCherry-R	ACAAAGGAAAAGGGGCCTGTTTACTTATACAATT CATCCATACCACCAG	
tCYC1-F	ACAGGCCCCTTTTCCTTTGTC	Amplification of *CYC1* terminator
tCYC1-R	CTTAGATTGTCGCTACGGCATATACGAGAGACGG TCCCAAAACCTTCTCAAGCAAGG	
km50123-F	AGGACGACCGGGTAACCCCTGCAGGTATTCTTCC TCCCTTGCTATTCTTTC	Amplification of KLMA_50123 promoter
km50123-R	GAATAATTCTTCACCTTTAGACATGTCGACGATTA CTGTGTTATTTTTCGATTCG	
Sbf1-50328p-F	AGGACGACCGGGTAACCCCTGCAGGCTTACTCTG CCTGCTGAGCTTG	Amplification of KLMA_50328 promoter
SalI-50328p-R	GAATAATTCTTCACCTTTAGACATGTCGACGATTA CTGTGTTATTTTTCGATTCG	
Sbf1-ICL1p-F	AGGACGACCGGGTAACCCCTGCAGGCTACGTCAT GAGTCTGCCAT	Amplification of *ICL1* promoter
SalI-ICL1p-R	AATAATTCTTCACCTTTAGACATGTCGACTCTTCT TCTTGAATATTGTTCGTTTGTATG	
Sbf1-INUIp-F	AGGACGACCGGGTAACCCCTGCAGGGTTGCAAG TTGCACGCTGGA	Amplification of *INU1* promoter
salI-INU1p-R	AATAATTCTTCACCTTTAGACATGTCGACATCTAA CAAAAAAAAAATTAAATGTGTCAC	
SbfI-JEN1p-F	GGACGACCGGGTAACCCCTGCAGGGGCTTGGAT GGAAACTCGCC	Amplification of *JEN1* promoter

SalI-JEN1p-R	GAATAATTCTTCACCTTTAGACATGTCGACTATGC CTATAGTAGCTTGTGTATTAGCTG	
Sbf1-TEFp-F	AGGACGACCGGGTAACCCCTGCAGGAGATTAAA AAAAAAGTACAGTTAGTTAGAGCAGG	Amplification of *TEF1* promoter
SalI-TEFp-R	GTGAATAATTCTTCACCTTTAGACATGTCGACCTT TAATGTTACTTCTCTTGGAGTTAG	
Sbf1-40271p-F	AGGACGACCGGGTAACCCCTGCAGGGGTAACAC AGAACTCTGACTCTCC	Amplification of KLMA_40271 promoter
Sal1-40271p-R	GAATAATTCTTCACCTTTAGACATGTCGACGGCA ATCCTGTTCTCATACCTGATG	
STF2p-F	AGGACGACCGGGTAACCCCTGCAGGGACAAAGT CCAATGCCTCTC	Amplification of *STF2* promoter
STF2p-R	AATTCTTCACCTTTAGACATGTCGACGATGTAATG TAGTATTTGTTGTGTAAATG	
Sbf1-ACO2bp-F	CAAAGGACGACCGGGTAACCCCTGCAGGAACTT GCTCCGATCCTCGGA	Amplification of *ACO2b* promoter
Sbf1-ACO2bp-R	GTGAATAATTCTTCACCTTTAGACATGTCGACTAT CAACAGATGATATGGTTTG	
Sbf1-GPDHp-F	AGGACGACCGGGTAACCCCTGCAGGAAGCGTCT CGTCAAGGACCG	Amplification of *GPDH* promoter
Sbf1-GPDHp-R	ATAATTCTTCACCTTTAGACATGTCGACCTTTTCT AACTACTACGCAATTGCTGCTG	
Sbf1-RAG5p-F	AGGACGACCGGGTAACCCCTGCAGGCCGAGTCC TACGCCAGCTAC	Amplification of *RAG5* promoter
Sbf1-RAG5p-R	AATAATTCTTCACCTTTAGACATGTCGACTTTTGT AAGTGTGTGTGTTTGTAATAATTG	
Sbf1-km10829-F	AGGACGACCGGGTAACCCCTGCAGGAATTGAAA ACACTATTAAAATTTGTTGTAAGACC	Amplification of KLMA_10829 promoter
Sbf1-km10829-R	GTGAATAATTCTTCACCTTTAGACATGTCGACGTC TGCTAAAAGTTCAAAATTAATTCG	
KD-pTCP2-F	ATTACCGTGCCGATTCGCACGCTGCAACCGCGGC AAAACAGGACAAAACAAAACAATACAG	Amplification of *IMTCP2* promoter
INUt-IMTCP2-R	GATCAGATCAAAGCTTGCGGCCTTAAGCGGCCGC ACTAGTCCTGCAGGTTTTGATTTGTGTTTAAGCGA GTGACTG	
TCP2-UPC2-F	CTTCAGTCACTCGCTTAAACACAAATCAAAACCT GCAATGAGTACAGAAAGAATGCAATCTTC	Amplification of *UPC2* promoter
UPC2-R	GCTTGCGGCCTTAAGCGGCCGCACTAGTCCTGCA CTAGAAAATTCCAAAATCCGACAAGTTTG	
TCP2-NRG1-F	TCAGTCACTCGCTTAAACACAAATCAAAACCTGC AATGAGTATTGTTCGCCCAAATATG	Amplification of *NRG1* promoter
NRG1-R	GCTTGCGGCCTTAAGCGGCCGCACTAGTCCTGCA TCATGTGTCGATTGGAGGTG	

TCP2-NDT80-F	CTTCAGTCACTCGCTTAAACACAAATCAAAACCTGCAATGATATCTACTCTGCAGCAGTTTATTG	Amplification of *NDT80* promoter
TCP2-NDT80-R	GCGGCCTTAAGCGGCCGCACTAGTCCTGCATTAGTGCCGGAATATGCTCG	
CP2-GCR1-F	ACTCGCTTAAACACAAATCAAAACCTGCAATGGACAAAGACAATTTGAATATCAATTTG	Amplification of *GCR1* promoter
CP2-GCR1-R	CGGCCTTAAGCGGCCGCACTAGTCCTGCATTATTCATTATCATCTTGCGAGTAATTATC	
pIMTCP1-F	TGCGGAGGTCTCCTACGCTTGTTTCTCCATTAGGATC	*IMTCP1* promoter
pIMTCP1-R	TCCGCAGGTCTCGTCATTTTTAACTTCTTTGTGTTGGTTTTTTAGGC	
G-URA3△-F	GAGGACGAAACGAGTAAGCTCGTCTGGTTTGAAGCAAGGTGCCGGTTTTAGAGCTAGAAATAGCAAG	gRNA of *URA3* deletion
G-URA3△-R	CTTGCTATTTCTAGCTCTAAAACCGGCACCTTGCTTCAAACCAGACGAGCTTACTCGTTTCGTCCTC	
Dor_ura3_up1000-F	TTAAGTAGTCAAACAAATTGTGTTGAAAAG	Donor DNA of *URA3*
Dor_ura3_dn1000-R	GTCTGCAACACCGATTTATCGG	
ACT-F	ACGGTATCGTTACCAACTGGG	For qPCR
ACT-R	GGGGCTTCGGTCAACAAAAC	
ICL1-F	TTCTTCGACTGGGACTTGCC	For qPCR
ICL1-R	TCGACTCCATCCAGCAGAGA	
SDH1-F	CCCGTGAAGCTCCAAAGTCT	For qPCR
SDH1-R	ATGCTTGACCACCCTTACCG	
JEN1-F	GCCATGGGTGGTATCTACGG	For qPCR
JEN1-R	TTGACGTGCAAGAAGGCTCT	
DIP5-F	GTATCCCAGTGCTCCCATCG	For qPCR
DIP5-R	CGATAGCCAAGCCGTAGAGG	
SDS3-F	CAAAGCTGAACACGAACGCA	For qPCR
SDS3-R	AAGATTGAGCGTTTGCGACG	
GCR1-F	GCCGTAACTGCAGAAGGTCT	For qPCR
GCR1-R	AGTTAATGGCTGCTTGGGGG	
CIT3-F	ACGTTTCTGCCCACACTACC	For qPCR
CIT3-R	CTTCTTGTGCCGCTAAACCG	
KLMA_50328-F	GAAACCGATCCTACCTGCGT	For qPCR
KLMA_50328-R	TGCAGGCCTCTTAGAATCGC	
KLMA_40271-F	GTACGGTGTCTCCGGAATG	For qPCR
KLMA_40271-R	GAACCCTCGGGGTATTGCTC	
RAG5-F	CACCTACCCAGCCAAGATCG	For qPCR
RAG5-R	TGTTCCGACCAATTCAGCCA	

Reference for the supplementary materials

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