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Molecular genetic techniques for gene manipulation in *Candida albicans*

Qiu-Rong Xu[1,†], Lan Yan[2,†], Quan-Zhen Lv[2], Mi Zhou[2], Xue Sui[3], Yong-Bing Cao[2,*], and Yuan-Ying Jiang[2,*]

[1]Department of Traditional Chinese Medicine; College of Pharmacy; Fujian University of Traditional Chinese Medicine; Fuzhou, Fujian PR China; [2]Center for New Drug Research; School of Pharmacy; Second Military Medical University; Shanghai, PR China; [3]School of Life Science and Bio-pharmaceutics; Shenyang Pharmaceutical University; Shenyang, Liaoning PR China

[†]These authors contributed equally to this work.

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Candida albicans is one of the most common fungal pathogen in humans due to its high frequency as an opportunistic and pathogenic fungus causing superficial as well as invasive infections in immunocompromised patients. An understanding of gene function in *C. albicans* is necessary to study the molecular basis of its pathogenesis, virulence and drug resistance. Several manipulation techniques have been used for investigation of gene function in *C. albicans*, including gene disruption, controlled gene expression, protein tagging, gene reintegration, and overexpression. In this review, the main cassettes containing selectable markers used for gene manipulation in *C. albicans* are summarized; the advantages and limitations of these cassettes are discussed concerning the influences on the target gene expression and the virulence of the mutant strains.

Introduction

Candida albicans is one of the most common opportunistic pathogen in humans that causes a number of clinical diseases ranging from superficial infections to lifethreatening systemic disease in immunocompromised patients,[1] such as those infected with HIV, undergoing cancer chemotherapy and organ transplantation, as well as premature infants.[2,3] This pathogenic fungus is becoming the leading cause of nosocomial infections.[4] In Kunming, southwest China, the oral yeast colonization rate in AIDS patients (49.5%) was higher than that of healthy people (20.7%).[5] *C. albicans* constituted the most frequent species, accounting for 82.2% of yeast isolates and is the most common lethal species (48.8%) in patients with cancer,[5] endotracheal intubation or hypoproteinemia.[6] Azole antifungal agents, especially fluconazole (FLC), are used to treat candidiasis. However, the overuse and long-term treatment with FLC have resulted in the emergence of resistance in *C. albicans*.[7-10] It is remaining largely mysterious toward the drug resistance mechanism and the pathways of virulence and pathogenesis in *C. albicans*.[11-14]

In recent years, the *Candida* Genome Database is established to offer the genome sequences of *C. albicans* for researchers to study freely.[15] Several gene manipulation techniques are available to allow powerful genetic approaches to study function of the genes in *C. albicans*, including gene disruption,[16-19] controlled gene expression,[16,20,21] protein tagging, gene reintegration and overexpression.[16,18,22,23] The principles of these gene manipulation techniques are all based on homologous recombination between the complementary sequences and the genomic sequences in *C. albicans*. But their applications and the use of selectable markers in these gene manipulation approaches are different. In this review, the main cassettes used for gene manipulation in *C. albicans* are summarized (**Fig. 1**; **Table 1**); the advantages and limitations are discussed.

*Correspondence to: Yong-Bing Cao; Email: ybcao@vip.sina.com; Yuan-Ying Jiang; Email: 13761571578@163.com
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Cassettes Used in Gene Disruption

The method of targeted gene deletion has enabled the identification of several unique aspects of *C. albicans* genes that play roles in pathogenesis, virulence, and resistance. Genes that are not essential for survival in *C. albicans* can be disrupted or replaced by transformation with exogenous DNA. These DNA cassettes used for gene disruption contain the selectable marker, auxotrophic or drug-resistant marker, flanked by sequences homologous to your target gene (YTG).

Gene disruption with nutritional marker for selection

URA *blaster cassette*

In *C. albicans*, the *URA3* gene encodes the orotidine 5′-monophosphate (OMP) decarboxylase enzyme, catalyzing the conversion of OMP to uridine 5′-monophosphate in the de novo pyrimidine biosynthesis pathway.[24] The strain CAI4 (Ura^-) (**Table 2**) with two alleles' deletion of *URA3* is used as the parental strain. The *URA* blaster cassette carries two direct repeats of the *hisG* sequences from *Salmonella* Typhimurium flanking the *URA3* gene (**Fig. 1.1**). After the strain CAI4 being transformed with the *URA* blaster cassette, the Ura^+ transformants are selected on uracil-deficient medium. The two direct repeats of the *hisG* sequences can spontaneously recombine, and then

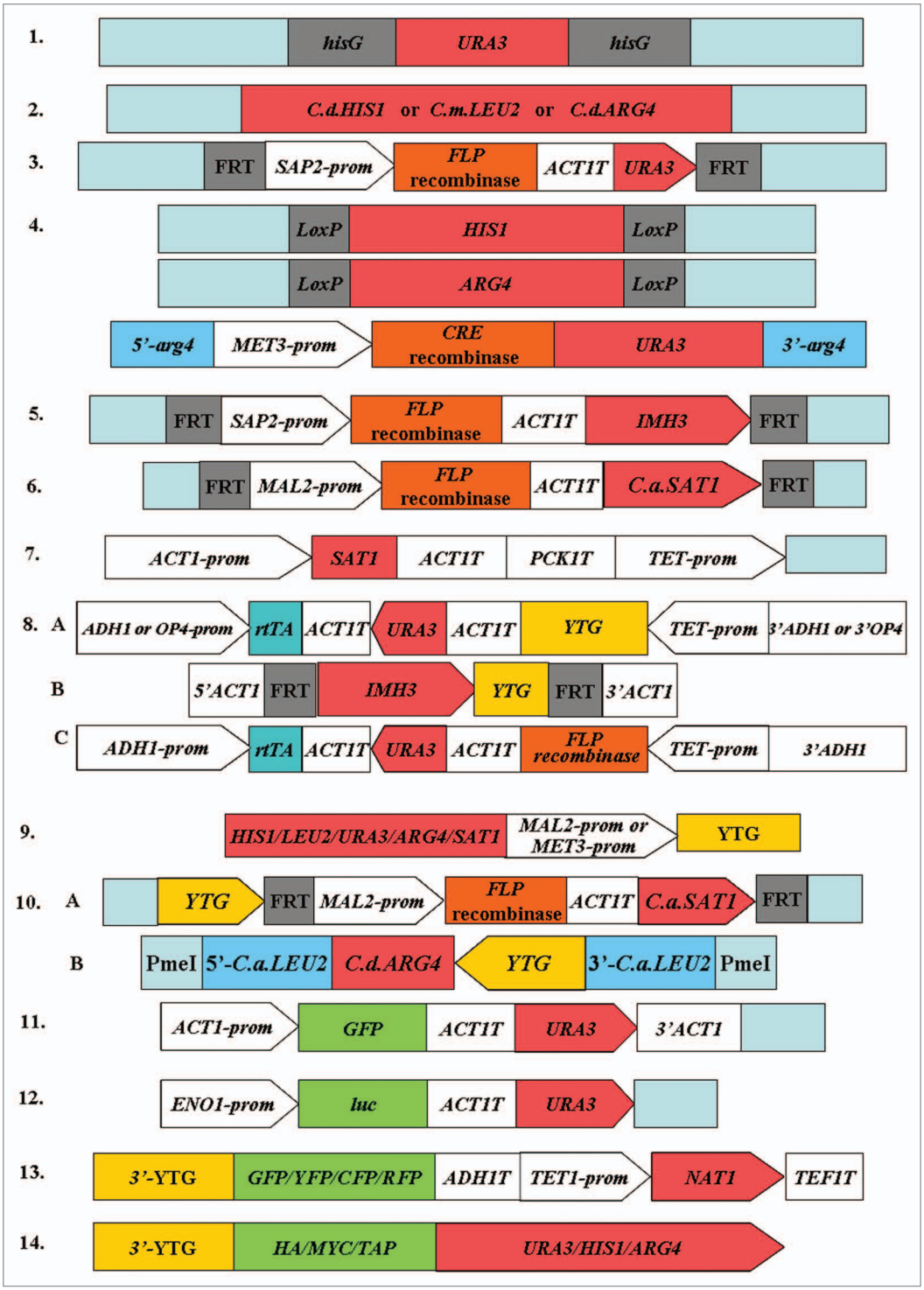

Figure 1. Cassettes used for gene manipulation in *C. albicans*. The figure shows the necessary elements in these cassettes, the light blue color of the elements represents the 5′ and 3′ DNA flanking your target gene (YTG) or the upstream and downstream sequences of YTG. (**1**) *URA* blaster cassette.[24] (**2**) PCR amplifiable marker cassettes from non-*C. albicans Candida* species.[45] *C.d.HIS1*, *Candida dubliniensis HIS1*; *C.m.LEU2*, *Candida maltosa LEU2*; *C.d.ARG4*, *Candida dubliniensis ARG4*. (**3**) *URA* flipper cassette.[38] prom, promoter region given for gene; T, termination sequence of given gene. (**4**) *Cre-loxP* system.[46] (**5**) *MPA*^{R} flipper cassette.[47] (**6**) *SAT1* flipper cassette.[51] *C.a.SAT1*, *C. albicans SAT1*. (**7**) "Tet-Off" system.[20] (**8**) "Tet-On"system.[54] (**9**) Promoter exchange cassette.[18,60] (**10**) Cassettes used for gene reintegration.[34] *C.a.SAT1*; *C. albicans SAT1*; *C.a.LEU2*, *C. albicans LEU2*; *C.d.ARG4*, *C. dubliniensis*. (**11**) ACT1P-GFP-ACT1T cassette.[76] (**12**) The firefly luciferase selectable marker cassette.[77] (**13**) PCR-mediated gene-tagging cassette.[16] (**14**) Epitope tagging cassette.[23]

Table 1. Cassettes used for gene manipulation in *C. albicans*

Cassettes	Purposes	Parent strains	Selective markers	Application examples	References
URA blaster cassette	Gene disruption	CAI4	*URA3*	Disrupting gene in auxotrophic strains	24
URA flipper cassette	Gene disruption	CAI4	*URA3*	Disrupting gene in auxotrophic strains	38
PCR amplifiable *URA3* cassette	Gene disruption	RM1000, BWP17	*URA3*	Disrupting gene in auxotrophic strains	19
UAU1 cassette	Gene disruption	BWP17	*URA3*, *ARG4*	Disrupting gene in auxotrophic strains	43
PCR amplifiable marker cassettes from non-*C. albicans Candida* species	Gene disruption	SN87, SN95, SN152	*HIS1*, *LEU2*, *ARG4*	Disrupting gene in auxotrophic strains	45
Cre-*loxP* system	Gene disruption	BWP17	*HIS1*, *URA3*, *ARG4*	Disrupting gene in auxotrophic strains	46
MPA^R flipper cassette	Gene disruption	Any strain	*IMH3*	Disrupting gene in any strains	47
SAT1 flipper cassette	Gene disruption	Any strain	*SAT1*	Disrupting gene in any strains	51
"Tet-Off" system	Controlled gene expression	CaSS1	*SAT1*, *URA3*	Repressing the expression of the target gene	20
"Tet-On"system	Controlled gene expression	CAI4	*SAT1*,*URA3*	Inducing the normally not expressed gene or inducing the deletion of essential gene	54
Promoter exchange cassette	Controlled gene expression	BWP17, SN148, SN152	*URA3*, *HIS1*, *ARG4*, *LEU2*, *SAT1*	Repressing the expression of target gene	18 and 60
PCR-directed recombination in *S. cerevisiae*	Gene reintegration and overexpression	SN152, SN147	*ARG4*, *SAT1*	Inducing overexpression of target gene	65
ACT1P-GFP-ACT1cassette	Protein tagging	CAI4	*URA3*	To locate the target protein	76,110, and 111
The firefly luciferase selectable marker cassette	Protein tagging	CAI4	*URA3*	To locate the target protein	77 and 78
PCR-mediated gene-tagging cassette	Protein tagging	CAI4, NGY152	*SAT1*	To locate the target protein	16
Epitope tagging for antibody detection	Protein tagging	BWP17, SN76	*HIS1*, *URA3*, *ARG4*	To analyze the protein complex	23

loop out the *URA3* gene and leave behind a short *hisG* sequence in the genome. Cells that have lost *URA3* by homologous recombination can be selected on medium containing 5-fluoroorotic acid (5-FOA), because 5-FOA is toxic to Ura$^+$ cells. After another round of transformation and homologous recombination, the cells that are null for the target gene can be generated (**Fig. 2**).[24,25]

The *URA* blaster method is a classical and highly effective approach to disrupt the target gene in *C. albicans*. Several parental strains lacking additional nutritional markers such as RM1000 (Ura3$^-$ His1$^-$) and BWP17 (Ura3$^-$ His1$^-$ Arg4$^-$) (**Table 2**) are constructed by this method.[25,26] However, the *URA* blaster method has several shortcomings. First, a portion of the 3′ end of the *IRO1* involving in iron utilization was removed during the construction of the parental strain CAI4 (Ura$^-$), which activated the alternative mechanisms of iron uptake in CAI4. The growth of CAI4 is better than that of the wild-type SC5314 strain in an iron-restricted environment.[27-29] Second, one copy of *hisG* sequences left in the chromosome decreases the integration frequency of the disruption cassette at the target locus because of the competition between the endogenous *hisG* sequences and the successive *hisG* cassettes in the second round of transformation.[30,31] Third, the flanking direct repeats of this cassette integrated at the *HWP1* locus can negatively influence the expression of the *URA3* in *C. albicans* and affect the virulence phenotype of the *hwp1* null mutants in *C. albicans*.[32] Finally, exposure to 5-FOA is potentially mutagenic and could introduce chromosomal rearrangements.[32,33] Particularly, the copy number and the changed chromosomal location of the *URA3* gene reduce capability of hyphal morphogenesis, adherence and virulence in *C. albicans*.[24,29,33] To ameliorate this problem, it is recommended to place the remaining *URA3* copy at a highly expressed locus, including *ENO1*, *RPS10*, *ARG4*, or at the *URA3* itself native locus.[29,34-37] Otherwise, researchers also improve the efficiency of the *URA3* cassette and have applied the PCR amplifiable *URA3* cassette (*URA3-dpl200*) and the *UAU1* cassette (*ura3*Δ3′-*ARG4-ura3*Δ5′) to disrupt the target gene in *C. albicans*.[19,26,41,42] The *UAU1* cassette on the Tn7 transposon has been used successfully to disrupt several genes in *C. albicans*.[44]

Table 2. The commonly used auxotrophic strains in gene manipulation in *C. albicans*

Auxotrophic strains	Phenotype	Features	References
CAI4	Ura$^-$	Decreased virulence; having chromosome 2 trisomy	24, 29, 32, 33, 63, 80, 81, and 86
RM1000	His$^-$, Ura$^-$	Decreased virulence; lacking the right arm of chromosome 5	25, 26, 29, 32, 33, 63, 80, 81, and 86
BWP17	Arg$^-$, His$^-$, Ura$^-$	Decreased virulence; lacking the right arm of chromosome 5	25, 26, 29, 32, 33, 63, 80, 81, and 86
CaSS1	His$^-$, Ura$^-$	Decreased virulence	20, 29, and 33
NGY152	Ura$^-$	Full virulence	16 and 29
SN76	Arg$^-$, His$^-$, Ura$^-$	Decreased virulence; having no chromosome change	23, 45, and 87
SN87	Leu$^-$, His$^-$	Full virulence	45
SN95	Arg$^-$, His$^-$	Full virulence; having no chromosome change	45 and 87
SN100	His$^-$	Full virulence	45
SN148	Arg$^-$, Leu$^-$, His$^-$, Ura$^-$	Decreased virulence; having no chromosome change	18, 45, 60, and 87
SN152	Arg$^-$, Leu$^-$, His$^-$	Mild virulence defect; having no chromosome change	18, 45, 60, and 87

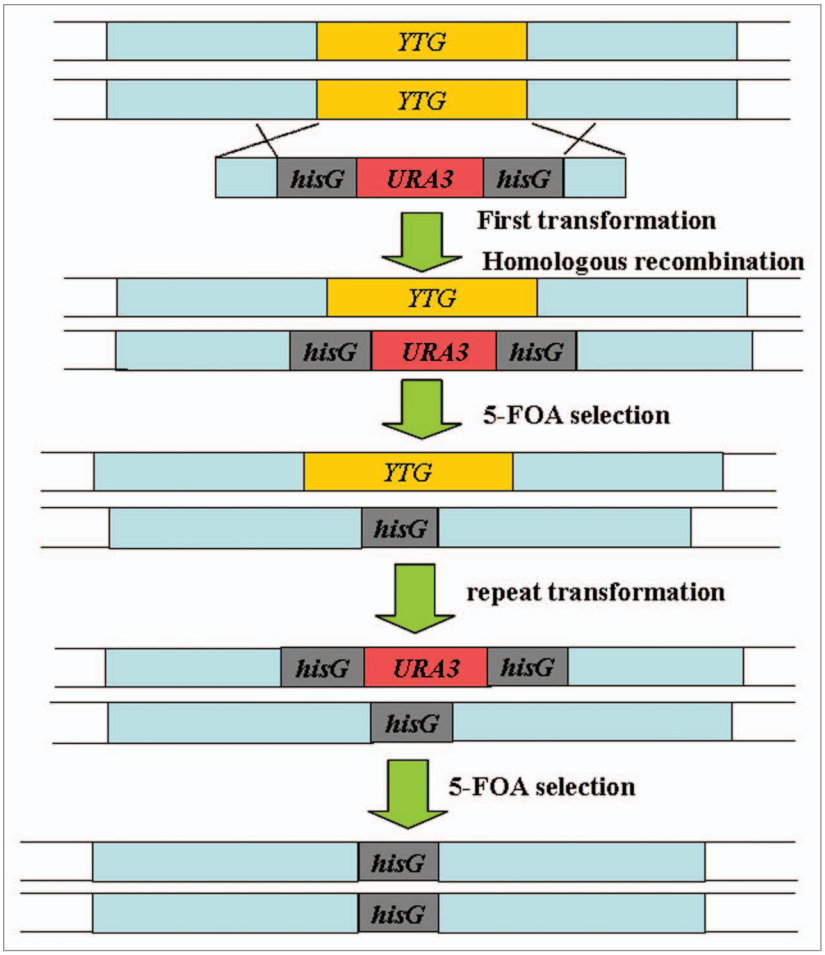

Figure 2. *URA* blaster strategy used for gene disruption in *C. albicans*.[39] YTG, your target gene.

*PCR amplifiable marker cassettes from non-*C. albicans Candida *species*

These cassettes contain *Candida dubliniensis HIS1*, *Candida maltosa LEU2*, or *C. dubliniensis ARG4* as selectable markers flanked by two short repeat sequences homologous to the target gene in *C. albicans* (**Fig. 1.2**). The auxotrophic parental strains, including SN87 (Leu2$^-$ His1$^-$), SN95 (His1$^-$ Arg4$^-$), or SN152 (Leu2$^-$ His1$^-$ Arg4$^-$) (**Table 2**), are transformed with these cassettes (**Fig. 3**). These cassettes have been successfully used for large-scale gene deletion studies in *C. albicans*. The parental strains used for these cassettes display no karyotypic changes and have one copy of *URA3* expressed at the native locus, which don't affect the virulence in *C. albicans*. So far, there is no evidence showing that the ectopic expression of any of the above nutritional markers would affect the virulence in *C. albicans*.[41,45] However, these cassettes cannot be used to disrupt the genes in the wild-type strains.

URA *flipper cassette*

The *URA* flipper cassette contains two direct repeats of the minimal *FLP* recombination target (FRT) sequence flanking the *URA3* marker. The site-specific recombinase *FLP* gene is regulated by secreted aspartyl proteinase (*SAP2*) promoter (**Fig. 1.3**). After the insertion of *URA3* flipper into the target locus in the parental strain CAI4 (Ura$^-$) (**Table 2**), the transformants are grown in the yeast carbon base and bovine serum albumin (YCB-BSA; pH 4.0) medium (**Table 3**) to induce the expression of *FLP* recombinase which promotes the homologous recombination between the *FRT* sites to loop out the *URA3* gene, making the *URA3* marker recyclable for another round of transformation.[38]

Table 3. The commonly used regulatable promoters in gene manipulation in *C. albicans*

Cassettes	Promoters	Inducer	Gene expression	References
"Tet-On"system	TET	Doxycycline	Induced	55
"Tet-Off"system	TET	Tetracycline	Repressed	20
Marker gene-MET3p	MET3	Methionine, cysteine	Repressed	18, 59, and 60
Marker gene-MAL2p	MAL2	Glucose	Repressed	18, 59, and 60
Marker gene-MAL2p	MAL2	Maltose	Induced	18, 59, and 60
G1G1p-GFP cassette	G1G1	GlcNAc	Induced	61
URA flipper cassette	SAP2	YCB-BSA pH 4.0	Induced	38 and 47–49
MPA^R flipper cassette				
SAT1 flipper cassette	MAL2	Maltose or glucose	Induced	51–53
Cre-*loxP* system	MET3	Methionine, cysteine	Repressed	46

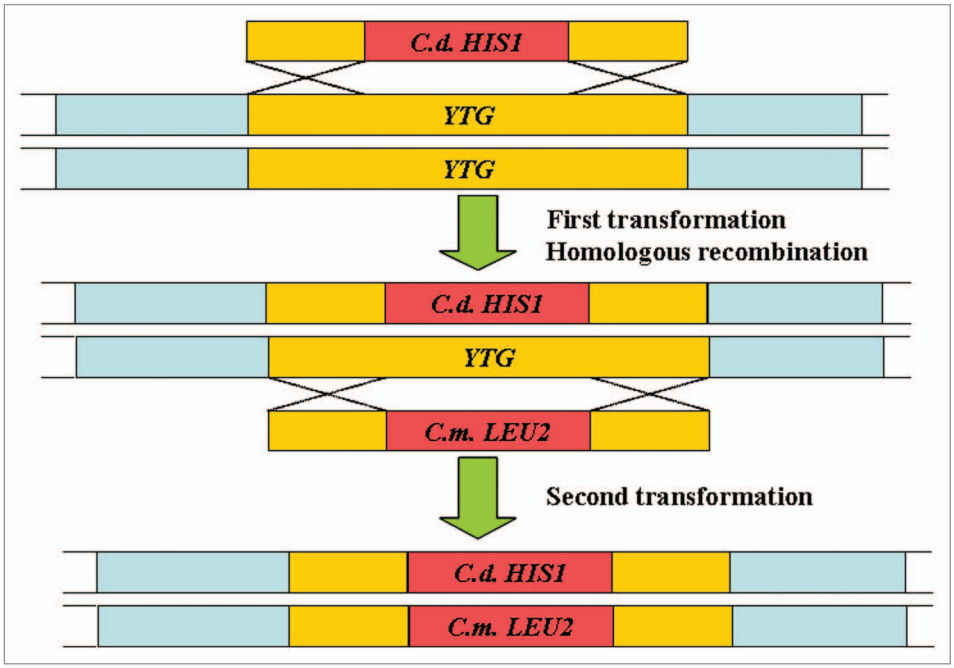

Figure 3. Strategy of the fusion PCR and heterologous markers used for gene disruption in *C. albicans*.[45] YTG, your target gene. *C. d*, *Candida dubliniensis*; *C. m.*, *Candida maltosa*.

Compared with the excision of the *URA3* gene by spontaneous recombination between the *hisG* repeats, the *FLP*-mediated recombination between the *FRT* sites to loop out the *URA3* marker is more efficient.[39] The *URA* flipper method does not need to use 5-FOA to select the *ura3*$^-$ clones, which would avoid mutagenic potentiality. However, this cassette leaves behind a single *FRT* sequence in the genome after the excision of *URA3* marker. The multiple *FRT* sequences left in the genome might recombine induced by the *FLP* recombinase, resulting in unexpected deletions or chromosome rearrangements.[30,40] The generation of *ura3*$^-$ clones would exclude the possibility of interchromosomal recombination.[38,41] Otherwise, the use of the BSA activates the *SAP2* promoter to induce the expression of *FLP* recombinase in the *URA* flipper cassette, whereas the BSA also represses the expression of other genes of *SAP* family except for *SAP2* gene.[38,41]

Cre-loxP *system*

This system is based on the site-specific recombination between two *loxP* elements which are catalyzed by the Cre recombinase. This system contains three components, including the disruption cassettes (using of *HIS1* and *ARG4* as auxotrophic markers flanked by 34 bp *loxP* elements), the resolving cassette (containing a synthetic, codon-optimized cre gene regulated by *MET3* promoter and the reintegration *URA3* marker gene) (**Fig. 1.4**). After two separate transformations, the two alleles of the target gene in parent strain BWP17 (Ura3$^-$ His1$^-$ Arg4$^-$) (**Table 2**) are disrupted by *HIS1* and *ARG4* markers; then these transformants are selected on medium lacking of histidine during the first transformation or histidine/arginine during the second transformation, but containing methionine and cysteine to repress the expression of the *MET3*-cre fusion (**Table 3**).[46] In the third transformation, the segment of the Cre recombinase and the *URA3* marker flanked by *ARG4* sequences is integrated into the *ARG4* gene that has disrupted one copy of the target gene in *C. albicans*. Then, the transformants that are auxotrophic for *HIS1*, *ARG4*, and *URA3* were incubated in medium lacking of methionine and cysteine, which can induce the synthetic *MET3-cre* fusion to produce Cre recombinase and subsequently catalyze the recombination between *loxP* sites to loop out the *HIS1* and the Cre recombinase-*URA3* fragment (**Fig. 4**).[46]

The Cre-*loxP* system doesn't need to use mutagen 5′-FOA to select the transformants. The synthetic *MET3-cre* fusion of this system to loop out the *LoxP*-marker-*LoxP* cassettes in *C. albicans* is efficient and avoids using positive screen method to select for the desired mutants. The knockout strains generated by the

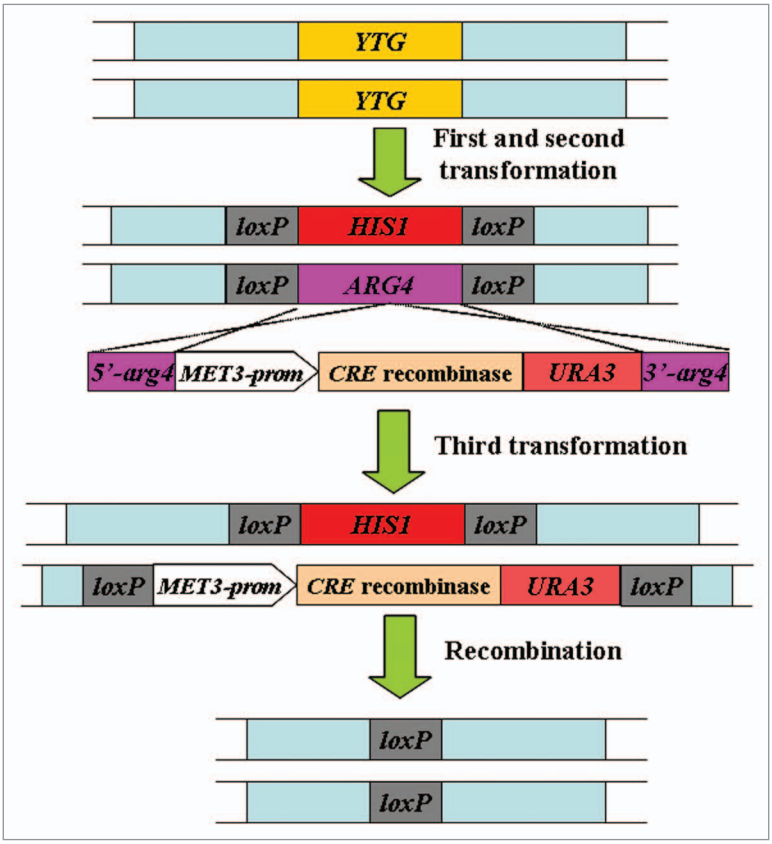

Figure 4. Strategy of the Cre-*loxP* system used for gene disruption in *C. albicans*.[46] YTG, your target gene; prom, promoter region given for gene.

Cre-loxP system are auxotrophic, unlike those by the positive selection markers.[41,46] Moreover, the future uses of Cre-*loxP* system in *C. albicans* are not limited to gene disruptions; it can be used in the tagging of multiple ORF in situ. However, the Cre-mediated recombination of this system might occur in the absence of explicit *MET3-cre* fusion induction, which will reduce the stability of *cre*-containing strains. This method leaves a short *loxP* sequence in the genome after excision of markers. The multiple *loxP* sequences left in the genome might also recombine induced by the *Cre* recombinase, which results in unexpected deletions or chromosome rearrangements.[30,40]

Gene disruption with drug-resistant marker for selection

*Mycophenolic acid-resistant (*MPA^R*) flipper cassette*

Using the drug-resistant markers, genes of *C. albicans* can be disrupted in any strains even in clinical isolates. It would be more benefit by using of the drug-resistant marker to study the virulence mechanism of the target genes in *C. albicans* than using the nutritional markers which might affect the pathways status for the genes of interest.

The inosine monophosphate dehydrogenase (Imh3) directs de novo synthesis of GMP in *C. albicans*. The mycophenolic acid (MPA) can inhibit the activity of Imh3. The transformants overexpressing *IMH3* due to the mutated *IMH3* gene are more resistant to MPA. The *MPA^R* flipper cassette contains the mutated *IMH3* gene and two *FRT* direct repeat sequences flanking the genetically engineered *FLP* gene regulated by *SAP2* promoter (**Fig. 1.5**). After the first transformation, the flanking sequences directly integrate the cassette into the target locus. The transformants are grown in the YCB-BSA medium to activate the *SAP2* promoter (**Table 3**) which promotes the expression of the *FLP* gene. Then the *FLP* recombinase mediates site-specific recombination between *FRT* sites to loop out the *MPA^R* flipper cassette and makes *IMH3* marker recyclable for another round of transformation.[47-49]

This *MPA^R* flipper method has been successfully used to study the causal relationships between specific genes and drug resistance in clinical isolates.[44,47-51] However, this cassette leaves behind a short *FRT* sequence in the genome after the excision of the *MPA^R* flipper cassette.[47,50] Furthermore, It is time-consuming to screen the *MPA^R* flipper cassette integrated at the target site of the MPA^R transformants.[41,47]

SAT1 *flipper cassette*

This cassette consists of a nourseothricin resistance marker *C. albicans SAT1* gene and the FLP-mediated recyclable marker system regulated by the *MAL2* promoter (**Fig. 1.6**). After the first transformation, the flanking sequences direct integration of the cassette into the target locus. The resistant transformants are picked on medium containing nourseothricin. Then, the correct transformants are grown in medium containing either maltose or glucose to active the *MAL2* promoter (**Table 3**) because the *MAL2* promoter contained in the *SAT1* flipper cassette is leaky, resulting in the expression of *FLP* recombinase to induce the homologous recombination between *FRT* sites and the excision of the *FLP* recombinase modulate system including the *SAT1* marker. The medium containing a low concentration of nourseothricin can be used to select the nourseothricin-sensitive (Nou^S) mutants. After another round of transformation and selection, the desired homozygous mutants can be generated (**Fig. 5**).[41,51-53]

Compared with the *MPA^R* flipper cassette, the integration of the *SAT1* cassette into the correct locus occurs with high specificity.[52,54] Otherwise, this *SAT1* flipper cassette can also be used to reintegrate the intact copy of the target gene into the genome of the null mutant to complement the phenotypes of the mutant.[51] However, this cassette leaves a short *FRT* sequence in the genome after the excision of the marker.

Cassettes Used in Controlled Gene Expression

Usually the inability to obtain homozygous mutants is considered that the target gene might be essential in *C. albicans*. To test this, one copy of the target gene can be constructed under a promoter and the other allele is disrupted or replaced. The essentiality of the gene is established by measuring survival of *C. albicans* after a shift to repressive conditions.

"Tet-Off" system

The "Tet-Off" system combines gene replacement and conditional expression (GRACE) to assess gene essentiality. This

system contains a chimeric transactivator protein and a tetracycline responsive promoter system. In the first step, the precise gene replacement of one allele of the target gene in the parental strain CaSS1[20] (Ura3$^-$ His3$^-$) (**Table 2**) is made by a cassette containing the *HIS3* selectable marker, these transformants were selected on YNB medium to obtain the *HIS3* prototrophs which can be used to additional transformation. In the second step, the controllable expression of the remaining allele is made by a Tet regulatable promoter system using a codon optimized *SAT1* selectable maker which is driven by the *ACT1* promoter (**Figs. 1.7 and 6**). The desired transformants can be obtained by selecting on medium containing nourseothricin. The transactivator protein binding to the Tet promoter results in constitutive expression of the target gene regulated by the Tet promoter system in the absence of tetracycline. In contrast, in the presence of tetracycline, the association between the transactivator protein and the Tet responsive promoter is disrupted, which leads to repressing of the expression of the target gene (**Table 3**).[20]

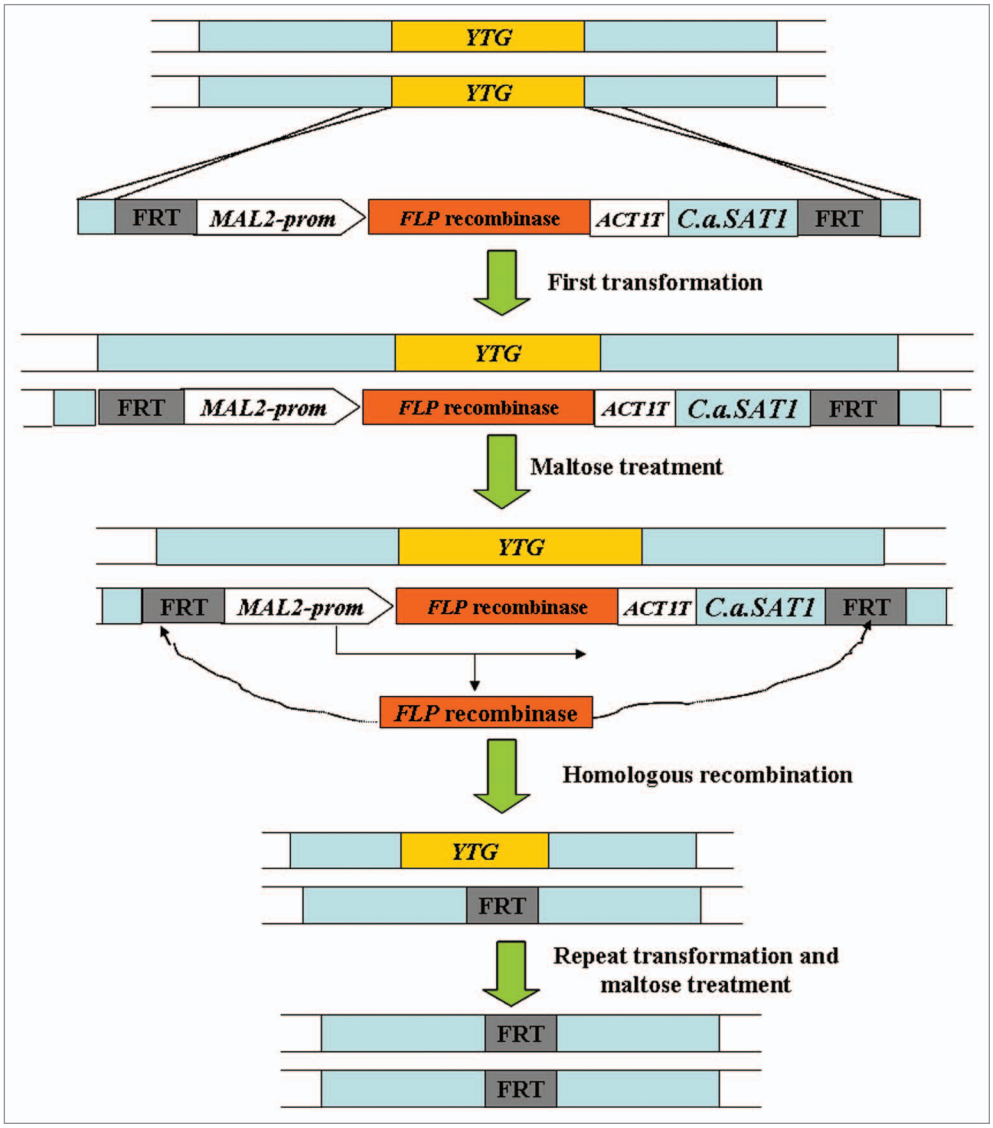

Figure 5. The *SAT1* flipper strategy used for gene disruption in *C. albicans*.[51] YTG, your target gene; prom, promoter region given for gene; T, termination sequence of given gene.

In the GRACE strains, the transactivator and the *URA3* marker gene are integrated into the *LEU2* locus. Therefore, GRACE strains can be forced to lose the transactivator and the *URA3* marker gene on 5-FOA-containing medium. Thus strains that are unable to survive in the medium containing 5-FOA may be identified as those carrying essential gene.[20] Furthermore, the strains obtained by this method have been used for high throughput drug screening. However, this method is less suitable for inducing the expression of specific target genes under conditions where they are normally not expressed, because this system is not always feasible to efficiently remove the tetracycline from cells grown in the presence of the drug environment.[20,54]

"Tet-On" system

The "Tet-On" system contains different elements and can be applied to induce or repress genes in *C. albicans*. It permits to induce expression of genes in conditions where they are normally not expressed in the wild-type genome. In such case, the Tet-On system contains reverse tetracycline-controlled transactivator (rtTA) regulated by *ADH1* or *OP4* promoter, the *URA3* or *SAT1* gene, the target gene regulated by the rtTA-dependent promoter, and the flanking *ADH1* sequences (**Fig. 1.8A**). The cassette would integrate into one copy of the *ADH1* in a single transformation step. In the presence of the doxycyline, the binding of the rtTA to the Tet responsive promoter leads to the expression of the target gene.

Tet-inducible gene expression system is also useful to induce the deletion of the essential genes and to create conditional lethal *C. albicans* mutants. In such case, the "Tet-On" system contains rtTA regulated by *ADH1* promoter, the *URA3* gene, and the *FLP* recombinase gene regulated by the rtTA-dependent promoter (**Fig. 1.8C**). Before using this inducible gene deletion system, the parental strain CAI4 (Ura3$^-$) (**Table 2**) is transformed with the *URA* flipper cassette (**Fig. 1.3**) to delete one allele of target gene. Then one copy of target gene is reintegrated into the *ACT1* gene locus by a cassette that contains the target gene and the *MPA^R* marker flanked by *FRT* sites (**Fig. 1.8B**).[55] This is followed by the

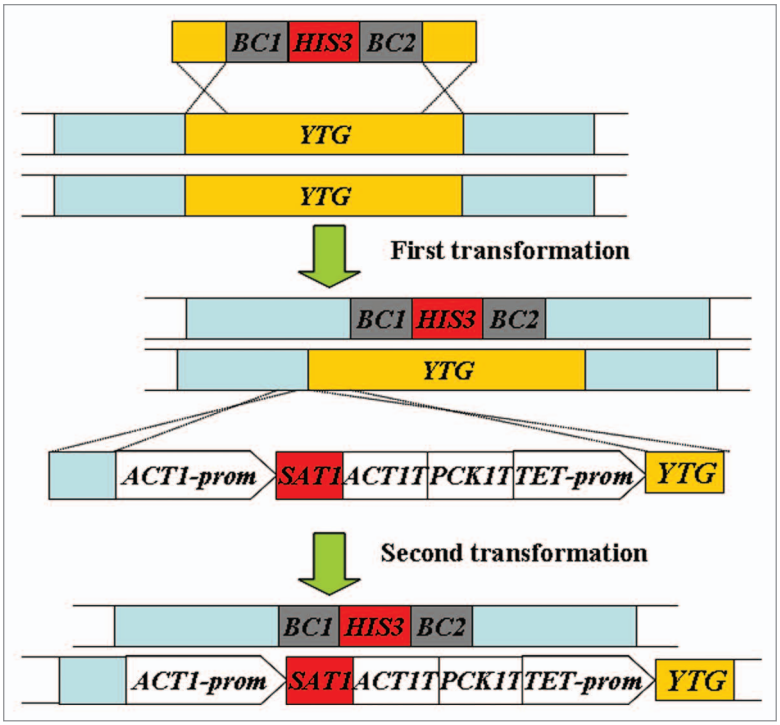

Figure 6. Strategy of the "Tet-Off" system used to repress the expression of the target gene in the presence of tetracycline.[20] YTG, your target gene; prom, promoter region given for gene; T, termination sequence of given gene; BC1, up tag; BC2, down tag.

disruption of the remaining allele of target gene by the *URA* flipper cassette. Subsequently, the "Tet-On" cassette is transformed to integrate into *ADH1* locus (**Fig. 7**).[54,55] Providing doxycycline for the cells, the binding of the rtTA to the Tet responsive promoter results in the expression of the recombinase *FLP* gene, which induces the homologous recombination between the *FRT* sites to excise the target gene along with the MPA^R marker (**Table 3**). The mutants losing the target gene can be identified by the gain of MPA susceptibility. On the contrary, cells remain MPA resistance in the absence of doxycycline, demonstrating that the *FLP* recombinase is not expressed and that no excision occurs.

This method does not depend on nutrient changes in the medium but simply supplies an inducible or repressible substance which does not affect metabolism. In contrast to the "Tet-Off" system, the "Tet-On" system also allows an inducer to express the genes of interest in conditions. However, the "Tet-On" system requires higher concentration of doxycycline to induce gene expression.[54] Due to the doxycycline is cytotoxic,[56] its concentration should be controlled. And dimethyl sulfoxide can be used to enhance doxycycline-dependent protein expression in Tet-On cells.[57] Furthermore, the chelation between the doxycycline and iron interferes with iron homeostasis and thus reduces resistance to FLC in *C. albicans*.[58] Otherwise, this system requires more transformation steps to disrupt two copies of the target gene than the "Tet-Off" system does.[54]

Promoter exchange cassette

The regulatable *MAL2* or *MET3* promoter following one marker gene (**Fig. 1.9**) has been successfully used to regulate the expression of target gene in *C. albicans* heterozygous strains. Marker genes include *ARG4*, *CaHIS1*, *CdHIS1*, *CmLEU2*, *SAT1*, and *URA3*. The strains BWP17 ($Ura3^-$ $His1^-$ $Arg4^-$), SN148 (Ura^- His^- Leu^- Arg^-), and SN152 (His^- Leu^- Arg^-) (**Table 2**) are usually used as parental strains. The promoter exchange cassette carries its 5′ end flanking sequences homologous to the noncoding upstream sequences of the target gene and its 3′ end flanking sequences homologous to the starting sequences of the target ORF. After the transformation, the cassette integrates into the genome of heterozygous strain, where the *MAL2* or *MET3* promoter is right before the target gene. The methionine and cysteine would shut off the *MET3* promoter and repress the target gene (**Table 3**). Since the *MAL2* promoter is shut down by glucose and activated by maltose, glucose-containing medium is used to repress the target gene (**Table 3**). These promoter exchange modules provide rapid way to study the gene function in *C. albicans*. Furthermore, this method does not recycle marker gene and is very convenient.[18,59,60] In addition, the P_{G1G1}-GFP cassette can be induced by N-acetylglucosamine (GlcNAc) (**Table 3**) to express the GFP.[61]

Cassettes Used for Gene Reintegration and Overexpression

In the process of constructing gene deletion mutants in *C. albicans*, the ectopic expression of selectable marker can mislead phenotypes of the null mutants.[35,62,63] It is necessary to reintegrate one copy of the intact target gene into the null mutant to restore the wild-type phenotypes. Several integrating vectors such as CIp10, CIp20, and CIp30 have been used efficiently for generating the complemented strains.[46] These plasmids carrying the *HIS1*, *URA3*, and *ARG4* markers are usually used for reintegrating the markers or target genes into the *RP10* locus of the auxotrophic strains.[34] Otherwise, the *SAT1* flipper cassette containing the complete ORF as well as upstream and downstream flanking sequences of the target gene can also be applied to reintegrate one allele of target gene into the native locus of the homozygous mutants (**Fig. 1.10A**).[51] Furthermore, the technique of the PCR-directed recombination in *S. cerevisiae* can be used to construct the integrating cassette which reintegrates one copy of the intact target gene into the *LEU2* locus of the null mutants in *C. albicans* (**Table 1**). The homologous recombination of the PCR fragments and the linearized pRS316 vector in *S. cerevisiae* can be constructed as the integrating cassette containing (5′ to 3′) a PmeI restriction site, the upstream sequences of *LEU2* ORF in *C. albicans*, the *ARG4* gene of *C. dubliniensis* (used as a selectable marker), the intact target gene ORF plus its promoter sequences, the downstream sequences of the *LEU2* ORF in *C. albicans*, and another PmeI site (**Fig. 1.10B**).[64-66] Meanwhile, the specific gene overexpression strains created by using of the highly active promoter to replace the endogenous promoter of the target gene is important as well for studying the gene function

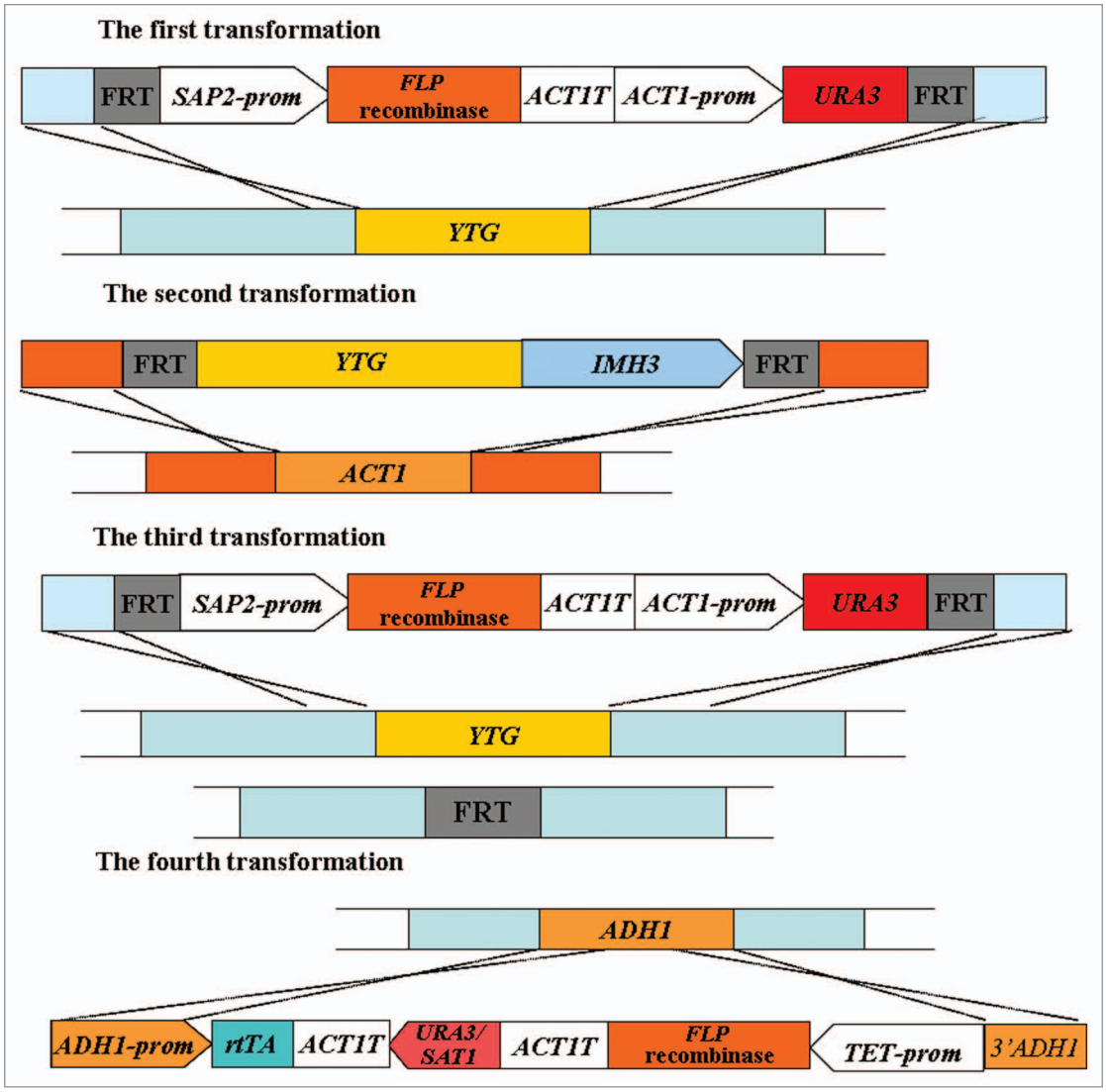

Figure 7. Strategy of the "Tet-On" system used to induce the deletion of essential genes.[54] YTG, your target gene; prom, promoter region given for gene; T, termination sequence of given gene.

Table 4. The commonly used tagging in *C. albicans*

Epitope tagging for antibody detection		Fluorescent markers	
Tagging	**Maker genes**	**Tagging**	**Maker genes**
HA	*URA3, HIS1, ARG4*	GFP	*URA3, CaHIS1, CmHIS1, ARG4, CmLEU2, SAT1*
MYC	*URA3, HIS1, ARG4*	YFP	*SAT1, URA3*
TAP	*URA3, HIS1, ARG4*	CFP	*SAT1, URA3*
GST	*URA3*	RFP	*SAT1, URA3*
His9	*URA3*	luc	*URA3*
V5–6 × His	*URA3, SAT1*		

in *C. albicans*. The highly active promoters such as NAD-linked glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) promoter, PEP carboxykinase (*PCK1*) promoter, and hexosaminidase (*HEX1*) promoter are often fused with the ORF of target gene to promote the expression of the target gene in *C. albicans*.[65,67,68]

Cassettes Used in Protein Tagging

The molecular techniques of epitope tags are usually applied to protein purification, localization and detection.[23,69-72] The cassettes used for tagging proteins with yellow, cyan, and green

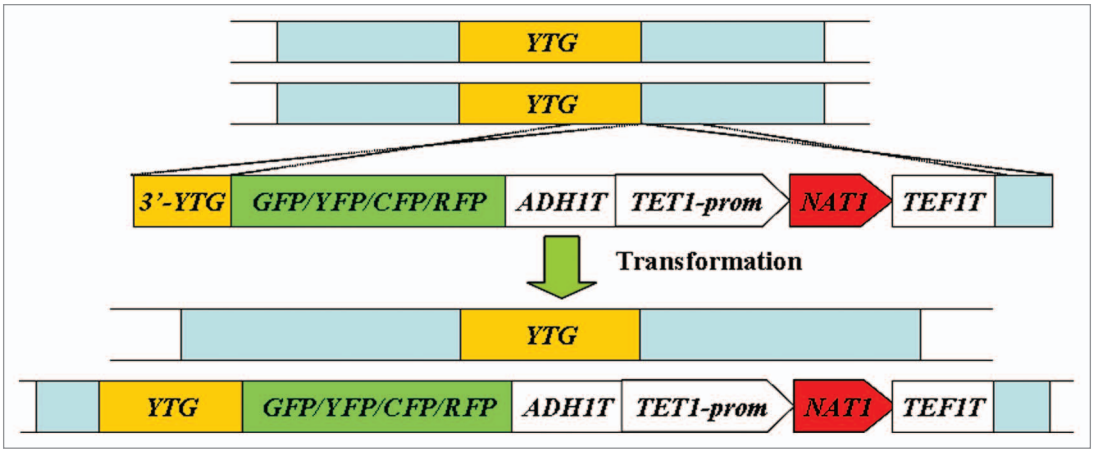

Figure 8. Strategy of protein tagging at the C-terminus of the target gene used in *C. albicans*.[16] YTG, your target gene; prom, promoter region given for gene; T, termination sequence of given gene.

fluorescent proteins (YFP, CFP, and GFP) at C-terminus has been applied to analyze the expression and localization of target protein within living cells in *C. albicans*.[18,73] Tagging at the N-terminus usually replace the native promoter with a regulatable or constitutive promoter cassette.[74] The small epitopes poly-His, V5, GST, HA, MYC, and FLAG are applied to research the assemble process of target protein complexes in *C. albicans* (**Table 4**).[18,23,75]

ACT1P-GFP-ACT1T cassette

This cassette contains *GFP* gene regulated by *ACT1* promoter and *URA3* used for selectable marker (**Fig. 1.11**). The flanking sequences of the cassette insert into the target locus through homologous recombination. Then the *GFP* gene of the integrative transformants exhibits a homogeneous, constitutive fluorescent phenotype, which can be applied to study the gene expression and evaluate the gene activation during pathogen–host cell interactions. The major advantage of this cassette is that gene induction can be monitored in a single cell of living organisms.[76]

The firefly luciferase selectable marker cassette

This cassette contains the firefly luciferase gene regulated by *ENO1* promoter and the *URA3* gene as a selectable marker gene (**Fig. 1.12**). The cassette inserts into the 5′ end of one allele of the target gene in *C. albicans* through homologous recombination. Then the expression of the firefly luciferase increases the light emission of the transformed colonies. The major feature of this cassette is that it can be used as a sensitive reporter to analyze gene function both in laboratory and clinical isolates of *C. albicans*. Furthermore, the expression of luciferase might be used as a selection strategy to introduce other genes into *C. albicans* cells to investigate the effect of these genes in the cells. Otherwise, the strains that constructed by this cassette can be used in animal infection models to monitor the location of the infection by the virtue of their bioluminescence.[77,78]

PCR-mediated gene-tagging cassette

The PCR-mediated gene-tagging cassette is based on combing the RFP, CFP, YFP, and GFP variants with the nourseothricin resistance marker *CaNAT1* regulated by the heterologous *TEF1* promoter and terminus (**Fig. 1.13**). The parental strain CAI4 (Ura^-) or NGY152 (Ura^-) (**Table 2**) is transformed with this cassette which inserts into the 3′ end of the target gene directly. Then the strains grow in selective medium to select for nourseothricin-resistant transformants (**Fig. 8**).[16] The major feature of this cassette is to be applied to study protein functional in *C. albicans*. Furthermore, this cassette requires the using of lower concentrations of nourseothricin. However, similar to the use of the *SAT1* marker, the expression of the *CaNAT1* marker takes time.[16]

Epitope tagging cassette

The construct for in vivo protein tagging in *C. albicans* combines the selectable marker genes such as *URA3*, *HIS1*, and *ARG4* with the protein tagging of TAP, HA, and MYC (**Fig. 1.14**) by PCR-mediated homologous recombination.[23] The insertion of epitope tags enables detection of target gene by immunodetection. The use of the tagged proteins is to perform chromatin immunoprecipitation coupled with microarray analysis (ChIP-ChIP) or tandem affinity purification (TAP). The MYC and HA epitope tags can be used for co-immunoprecipitation (co-IP) or immunoprecipitation (IP) experiments to validate formation of protein complex, while the TAP tag is often applied to obtain high purified protein samples for mass spectrometry analysis of protein complexes. In such case, the strain BWP17 ($Ura3^-$ $His1^-$ $Arg4^-$) or SN76 (Ura^- $His1^-$ Arg^-) (**Table 2**) can be used as parental strain. After the transformation, the tag is integrated into 3′ terminus of one allele of the target gene. This PCR tagging cassettes allow the rapid biochemical analysis of tagged proteins and have been successfully used to analyze the genome-wide location in *C. albicans*.[23,79]

Conclusion

Nowadays, several cassettes have been used to manipulate the *C. albicans* genome to study the function of the genes. The *URA* blaster cassette is the first used method. However, the expression of selectable auxotrophic marker *URA3* affects the virulence,

adhesion, and morphogenesis in *C. albicans*. The *ura3* null mutant strains such as CAI4, RM1000, and BWP17 are deficient in virulence.[24,29,32,33,63,80,81] The ectopic expression of *URA3* results in 30% of published virulence-deficient mutants having misattributed to the deletion of genes.[29] To overcome these problems, researchers proposed other solutions which are the reason that generated many other gene manipulation cassettes, such as the PCR amplifiable markers from non-*C. albicans Candida* species. This method has reintegrated the *URA3* gene into its native locus, so the constructed strains by this method have full virulence such as SN95 (His^- Arg^-), SN87 (His^- Leu^-), SN100 (His^-), and SN152 (His^- Leu^- Arg^-) (**Table 2**).[45] Among the use of *URA3* as a selectable marker for gene deletion, the *UAU1* cassette is the prominent method, because it is useful to create *C. albicans* null mutants with a single transformation step without leaving behind any chromosomal rearrangements. Nevertheless, the use of *ARG4*, *LEU2*, and *HIS1* as selectable markers for deleting the gene of interest in *C. albicans* is the best way among avoid using *URA3* as nutritional selectable marker, because this method needs two transformation steps to create *C. albicans* null mutants and doesn't need additional steps to excise the markers. So far, there is no evidence showing that the ectopic expression of the remaining nutritional markers *LEU2*, *HIS1*, or *ARG4* affects the virulence of *C. albicans*. However, the auxotrophic markers in laboratory strains can still be restrictive because of the target genes are in pathways affected by nutritional status. Therefore, the nutritional markers don't apply to analysis clinical *C. albicans* isolates which are not auxotrophic directly. In such case, drug-resistant markers, such as MPA^R flipper cassette and *SAT1* flipper cassette, can be applied to clinical isolates without creating any auxotrophic mutations. Moreover, if the target gene has three alleles in the parental strains such as CAI4 (Ura^-) and SGY-243 (Ura^-) which carry three copies of chromosome 1 (Chr 1),[82,83] researchers should apply recyclable markers, including *URA* blaster and drug-resistant markers, and reuse three cycles to create knockout strains, because disrupting triploid with two different auxotrophic markers will not succeed in acquiring homozygous mutants.[84] Furthermore, the "Tet-On" system and "Tet-Off" system can be both used to conditionally control the essential genes expression in *C. albicans*. Otherwise, the protein tagging cassettes are used to locate the target protein or analyze the target protein complex. Therefore, researchers should pay attention to select the cassette fitting for their purpose of research.

On the other hand, researchers should keep an eye on the chromosomal aneuploidy in *C. albicans* mutants. It is reported that approximately 35% of over 100 published microarray data sets of *C. albicans* mutants have chromosomal aneuploidy.[85] Aneuploidy can influence the virulence of *C. albicans*.[82] For example, the commonly used laboratory strains CAI4 has three alleles of Chr 1, as well as BWP17 and RM1000 which shows a heterozygous deletion in the right arm of Chr 5.[86] However, the strains SN76, SN95, SN152, and SN148 are initially free of aneuploidies.[87] There are many factors leading to aneuploidies of strains. The strains grown in L-sorbose can often lead to lose a copy of Chr 5 while grown in D-arabinose may result in Chr 6 trisomy.[88,89] In addition, the strains exposed to 5-FOA can lead to lose one copy of Chr 1.[82] The genome of *C. albicans* has remarkable tolerance for a wide variety of different chromosomal aneuploidies and changes in chromosomal copy numbers often arise as a response to stress.[85,87,90-93] Therefore, *C. albicans* mutants should be tested for aneuploidy before being used in further studies.

In general, the perfect cassette of gene manipulation should be that the expression of selectable markers do not affect the virulence of strains, the constructed parent strains have no karyotypic changes, as well as the use of reagents do not generate the potential mutant.

To modify and improve the available methods is the first way to conceive the perfect method. Gene expression at an ectopic site is lower relative to that at the native locus, which could lead to virulence defect and growth defects.[94-96] However, Gerami-Nejad et al.[97] have identified a neutral intergenic region *NEUT5L*, which facilitates the integration and expression of ectopic genes. They constructed a series of integrated shuttle vectors containing 550 bp sequences homologous to *NEUT5L* and three selectable markers (*NAT1*, the recyclable *URA3-dpl200* or *URA3*). Ectopic genes integrated at *NEUT5L* by these vectors do not influence growth rates and allow native-locus expression levels. Thus the *NEUT5L* is an ideal genomic locus for the integration of exogenous DNA in *C. albicans*. Otherwise, Lai et al.[98] have modified the vector pTET25 into the pTET25M, so that the *URA3* gene flanked by *dpl200* can be used repetitively. The pTET25M vectors not only allow ectopic expression of target proteins in a "Tet-On" system with either a C-terminal 6× His epitope or N-terminal or a C-terminal GFP tag, but also possess a Ura-blaster cassette to allow reintroducing a *URA3* marker.

The second way is to optimize and apply the gene manipulation methods used in other fungal species. For example, RNA interference techniques are used to disrupt the target genes in other fungal species such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Aspergillus nidulans*. Nowadays, this method is also proving to be successful to disrupt the gene of interest in *C. albicans*.[99] Moreover, Vieira et al.[100] have constructed three *C. albicans* integrative vectors such as CIp10, CIp20, and CIp30 which are ligated to 2 μ *S. cerevisiae* sequences, so that they are able to in vivo maintain and clone by gap repair within *S. cerevisiae*. These vectors are especially useful for the integration of genes into *C. albicans* genome that cannot be reproduced in *Escherichia coli* because of their toxic effects. Furthermore, Su-Kim et al.[101] have adopted double-joint PCR with *NAT*-split markers to construct the gene disruption cassettes in *C. neoformans*, which generates higher targeted-integration frequency. The *NAT*-split marker is referred as the two portions of DNAs of the *NAT* dominated selectable marker containing 200bp overlapping truncations in between, named the 5′-*NAT*-split marker and the 3′-*NAT*-split marker. During the double-joint PCR, the 5′-flanking region of target gene joints with the 5′-*NAT*-split marker and the 3′-flanking region of target gene joints with the 3′-*NAT*-split marker. Then, the two double-joint PCR fragments

are combined and are introduced into the strains. To obtain the desired deletion mutants in *C. neoformans*, researchers only have to screen a small number of transformants.

More recently, the transcription activator-like effector nucleases (TALENs) which contain a modular DNA-binding domain and a FokI endonuclease monomer, are fusion proteins and work in pairs. When two TALENs bind to their target DNA sequences, the FokI monomers will dimerize and introduce a double-strand DNA breaking within the specific binding site. Then the disrupted DNA can either be repaired by non-homologous end-joining (NHEJ) or homologous recombination, which leads to deletion/insertion mutations, specific site mutations or specific sequence additions. This technology have been successfully used for gene manipulation in zebrafish, *Drosophila*, *Arabidopsis*, mice, human cell lines, and so on.[102-105] Otherwise, in the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system, the targeting CRISPR RNA (crRNA) fused with the trans-activating crRNA can generate double stranded DNA breaks (DSDBs) in the target site. Then the DSDBs can be repaired either through NHEJ or through homologous recombination, which would result in random deletions or insertions. This system has also been used for genome editing in human cell lines, mouse, zebrafish, and *Caenorhabditis elegans*, and so on.[106-109]

In summary, there are many other novel gene manipulation strategies that should be considered for future investigations in *C. albicans*. We should make full use of our resources to improve the method used in gene manipulation in *C. albicans*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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