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Selection systems based on dominant-negative transcription factors for precise genetic engineering

Raphaël Dutoit[1,*], Evelyne Dubois[1,2] and Eric Jacobs[1,*]

[1]Institut de Recherches Microbiologiques JM Wiame and [2]Laboratoire de Microbiologie de l'Université Libre de Bruxelles, 1 avenue Emile Gryson, BE1070 Belgium

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ABSTRACT

Diverse tools are available for performing genetic modifications of microorganisms. However, new methods still need to be developed for performing precise genomic engineering without introducing any undesirable side-alteration. Indeed for functional analyses of genomic elements, as well as for some industrial applications, only the desired mutation should be introduced at the locus considered. This article describes a new approach fulfilling these requirements, based on the use of selection systems consisting in truncated genes encoding dominant-negative transcription factors. We have demonstrated dominant-negative effects mediated by truncated Gal4p and Arg81p proteins in *Saccharomyces cerevisiae*, interfering with galactose and arginine metabolic pathways, respectively. These genes can be used as positive and negative markers, since they provoke both growth inhibition on substrates and resistance to specific drugs. These selection markers have been successfully used for precisely deleting *HO* and *URA3* in wild yeasts. This genetic engineering approach could be extended to other microorganisms.

INTRODUCTION

In the past decades, various systems have been established for genetic engineering of prototroph microorganisms, including wild yeasts. Powerful tools have been developed, in first instance using *Saccharomyces cerevisiae*, in the context of the collaborative genome sequencing and functional analysis projects (1–3). Classically, the first step consists in selecting linear vector integration by homologous recombination at the target locus. Then, internal vector elements are removed by homologous recombination between appropriately designed, directly repeated, flanking sequences (4). To perform genomic modifications in prototroph yeasts dominant selective markers leading to drug resistance are available, such as *neo*, which confers resistance to G418 (5), but convenient negative selection systems for subsequent elimination of vector sequences remained to be developed. Indeed, excision of the selectable marker spontaneously occurs by recombination between short directly repeated flanking sequences, but its frequency is very low (6). Therefore, genes encoding specific recombinases have been additionally used, together with specific pairs of target sequences included in the integrative vectors, which allowed increasing the homologous recombination frequency (7,8). Then, only a short piece of foreign DNA including the recombinase recognition sequence remains at the modified locus. However, for genetic engineering of various functional elements, but also for several applications involving industrial strains, vector excision leading to the elimination of all foreign DNA sequences is desired. Such requirements exclude the use of systems involving specific recombinases. Some years ago, Akada *et al.* (9) and Olesen *et al.* (10) developed a counter-selection method for vector recycling in *S. cerevisiae* based on conditioned over-expression of two particular genes (*GIN11* and *PKA3*) encoding proteins which cause lethality when overproduced (9,10). These systems, combined with a positive marker, were successfully used in some industrial strains (9–11). However, they need to be adapted according to the behavior of a conditional promoter but also to the potency of the particular growth-inhibitory protein in the yeast host strain of interest (10). Considering these constraints and the diverse metabolic features of target hosts, it is obvious that new tools still need to be developed.

The approach described here is based on the use of expression cassettes encoding dominant-negative truncated transcription factors designed for interfering with specific regulatory pathways. Such defective transcription factors have been extensively used for fundamental research in higher eukaryotic cells, due to the difficulties of reverse genetics, or in view of therapeutic applications (12).

*To whom correspondence should be addressed. Tel: + 322 526 7279; Fax: +322 526 7273; Email: rdutoit@ulb.ac.be
Correspondence may also be addressed to Eric Jacobs. Tel: +33 681 53 43 88; Email: eric.jacobs@ulb.ac.be

However, in yeast as well as in many other microorganisms, this tool has only been marginally used, for studying metabolic functions (13–16). For genetic engineering of prototroph microorganisms, dominant-negative transcription factors would provide ideal tools. Indeed, this approach should allow the design of both positive and negative selection systems that are very efficient and should be appropriate for performing any precise genomic modification. Using such strategy, we performed several precise genetic modifications in two wild strains isolated from banana leaves in Thailand (17).

Two systems were established, respectively interfering with the catabolism of galactose and with the metabolism of arginine. In the absence of galactose, the transcriptional activator Gal4p is bound to specific sites (UAS) upstream the structural *GAL* genes, but is unable to activate their transcription, since the activation domain is masked by Gal80p (18). In the presence of galactose, the Gal4p activation domain is liberated. Consequently, expression of *GAL1*, *GAL2*, *GAL7* and *GAL10* is induced (19). To be efficient, the induction requires the relief of carbon catabolite repression.

A truncated transcription factor, Gal4pΔ (aa 1–147) is known to be localized in the nucleus and to bind to the UAS upstream the *GAL* genes, while being unable to activate their transcription in the presence of galactose (20–23). Overproduction of Gal4pΔ was expected to lead to competition with the endogenous Gal4p (which is produced at very low level) for binding to the DNA target sites, which should give rise to inhibition of induction in the presence of galactose, and to inhibition of growth on galactose as carbon source. We also expected that, when grown on a medium containing appropriate amounts of glucose and galactose, a yeast producing Gal4pΔ should be less sensitive to 2-deoxy-galactose (2DG), a pro-drug whose toxicity was previously demonstrated to be increased in strains constitutively producing the enzymes of the pathway (24). Therefore, a gene encoding Gal4pΔ was placed under the control of a strong constitutive promoter, in view of designing both positive and negative selection systems.

The arginine biosynthetic pathway consists of five enzymes allowing conversion of glutamate to ornithine, which is then converted in arginine in three steps [ornithine carbamoyltransferase (*ARG3*), argininosuccinate synthetase (*ARG1*) and lyase (*ARG4*)] (25). Arginine can be degraded by arginase (*CAR1*), giving rise to urea and ornithine which is modified by a transaminase (*CAR2*) into glutamate semialdehyde, giving rise to glutamate and ammonium after several steps. Regulation of arginine anabolic and catabolic pathways is dependent on the availability of arginine or ornithine as nitrogen source. Several regulatory systems are involved, including a specific transcriptional control which regulates the production of both anabolic and catabolic enzymes and involves the 'ArgR–Mcm1p' complex (26). When arginine or ornithine is used as nitrogen source, this complex binds specific sequences and activates the catabolic pathway, the anabolic pathway being simultaneously downregulated. In the absence of arginine or ornithine, the complex is formed but does not bind to DNA. Deletion of *ARG81* leads to a growth defect on arginine or ornithine and to constitutive expression of genes encoding anabolic enzymes (27). In addition, such a mutant strain appeared to be resistant to canavanine (an arginine analog) toxicity (28), probably due to the accumulation of intracellular arginine. It has been shown that a truncated Arg81p (aa 1–180) can be included in a functionally defective complex (29). The dominant-negative factor evaluated in this study was then constitutively overproduced, and was expected to compete with the endogenous Arg81p for complex formation.

MATERIALS AND METHODS

Strains, culture media and chemicals

Escherichia coli strain XL1blue (Stratagen) was used for all cloning steps and was cultivated on LB medium. For selective medium, ampicillin was added at 100 μg/ml. *Saccharomyces cerevisiae* strains BY4709 (*MATα ura3Δ0*) (30), Σ1278b (*MATα*) (28), TR2 and TR3 (17) were used as model strains through the development of dominant negative marker. DNA of FY1679 (*MATa ura3-52 his3Δ200/MATα his3Δ200 ura3-52*) (31) has served as DNA template for cloning. These *S. cerevisiae* strains were cultivated on YPD and on minimum medium YNB with 80 mM ammonium and 20 mg/ml glucose (except for selection media and growth conditions for transcriptional analysis, see below). 2-Deoxy-D-galactose was purchased from MP Biomedicals and L-canavanine from Sigma-Aldrich.

Plasmid construction

All plasmids are described in Table 1. Cloning procedures are largely described in Supplementary Data.

Transformation, positive and negative selection media

Saccharomyces cerevisiae strains were transformed using the lithium acetate procedure (32). For 10^8–10^9 harvested cells concentrated in 100 μl, at least 5 μg of integrative vector were added with 5 μg of carrier DNA (10 mg/ml fish sperm DNA solution, MB grade from Roche Diagnostics GmbH). For basic *URA3* selection, transformants were selected on YNB, 20 mg/ml glucose. For *GAL4*Δ, the positive selection medium was YNB, 30 mg/ml glycerol, 20 mg/ml 2-deoxygalatose, 5 mg/ml casaminoacids, 1 mg/ml galactose, 200 μg/ml glucose, supplemented with 50 mg/l leucine or 25 mg/ml uracil if required. For *ARG81*Δ, the positive selection medium was YNB, 20 mg/ml glucose, 200 μg/ml ornithine and 25 μg/ml canavanine. Cells were not incubated in a non-selective growth medium before plating. Geneticin selection was performed as previously described (5).

The loss of integrative plasmid or deletion cassette was selected depending on the negative marker. First, transformants were cultivated overnight at 30°C during 24 h on YPD, diluted in fresh media to $OD_{660\,nm}$ 0.05 and then grown for 24 h. Second, 10^4, 10^6 and 3×10^8 cfu were plated on selective media: YNB, 4 mg/ml

Table 1. List of plasmids used in this work

Plasmid name	Plasmid description
p0int	*CYC1*-integrative plasmid derived from pFL44 L with an empty expression cassette (*TDH3* promoter and *CYC1* terminator). The restriction by MluI targets integration in genomic *CYC1*.
pGint	p0int carrying *GAL4*Δ encoding Gal4p (aa 1–147).
pGVint	p0int carrying *GAL4ΔV5* encoding Gal4p (aa 1–147) with C-terminal V5 epitope.
pGVGint	p0int carrying *GAL4ΔV5GFP* encoding Gal4p (aa 1–147) with C-terminal V5 epitope followed by eGFP.
pAint	p0int carrying *ARG81*Δ encoding Arg81p (aa 1–180).
pAVint	p0int carrying *ARG81*Δ encoding Arg81p (aa 1–180) with C-terminal V5 epitope.
pGintL	pGint with a 613-bp sequence of *LEU2* (from nt 24 to 636). The restriction by KpnI targets integration in genomic *LEU2*.
pGVintL	pGVint with a 613-bp sequence of *LEU2*. The restriction by KpnI targets integration in genomic *LEU2*.
pGVdelHO	pUC19 carrying the *GAL4ΔV5* expression cassette flanked by two sequences homologous to upstream and downstream of *HO* ORF domains, with a short *HO* direct repeat for vector excision (Figure 4).
pGVdelURA	pUC19 carrying the *GAL4ΔV5* expression cassette flanked by two sequences homologous to upstream and downstream of *URA3* ORF, with a short *URA3* direct repeat for vector excision (Figure 4).
pGVKdelHO	pGVdelHO with *KanMX* cassette depending on a bidirectional *TDH3* promoter (Figure 4).
pGVKdelURA	pGVdelURA with *KanMX* cassette depending on a bidirectional *TDH3* promoter (Figure 4).
pAVdelHO	pUC19 carrying the *ARG81ΔV5* expression cassette flanked by two sequences homologous to upstream and downstream of *HO* ORF domains, with a short *HO* direct repeat for vector excision (Figure 4).

galactose for *GAL4*Δ loss and YNB glucose, 1 mg/ml ornithine for *ARG81*Δ loss.

PCR characterization

All yeast transformants were characterized by PCR screening directly with colonies. First, cells were added to 10 µl of 0.25% SDS and heated at 95°C during 15 min. After this rapid DNA extraction, 90 µl of water were added and, after 1 min of centrifugation at 12 000*g*, 1 µl of supernatant was used as DNA template for PCR. The 25 µl-reaction mixture contained 0.4 µM of each primer, 0.8% Triton X100 and DreamTaq PCR MasterMix (Fermentas).

Sequencing of PCR fragments carrying genomic *HO* and *URA3* deletions

Fragments carrying genomic *HO* and *URA3* deletions were amplified by PCR with primer pairs ocej359/360 and ocej317/320, respectively (see Supplementary Table S1 for primer sequences). Fragments were purified by using High Pure PCR Product Purification Kit (Roche Diagnostics GmbH) and sequenced using the same primers (DNAvision S.A.).

Immunodetection by Western blot

After separation by SDS–PAGE, proteins were electroblotted onto a Hybond nitrocellulose membrane (GE Healthcare) with XcellII module (Invitrogen) and with NuPage Transfer Buffer (Invitrogen)/10% methanol. Tagged proteins were immunodetected with Anti-V5 antibodies (Invitrogen) and revealed by using Western-Breeze Chemiluminescent Kit Anti-Mouse (Invitrogen).

Quantitative qRT–PCR

Quantification of mRNA was performed as described previously (33). For inducing *GAL* genes expression, yeast strains were cultivated on YNB with 80 mM ammonium, 20 mg/ml raffinose until $OD_{660\,nm}$ 0.5 in which 2 mg/ml galatose were added for a further 1-h growth. The anabolic and catabolic gene transcripts were quantified after growth on YNB with 80 mM ammonium, 20 mg/ml glucose in the presence or not of 1 mg/ml arginine. Total RNA was extracted as described previously (34). Primers amplified a 167-bp fragment of *GAL1* (ocej235, 5′-CCGAAAAGTGCCCGAGCATA-3′; ocej236, 5′-GACGGCGCAAAGCATATCAA-3′), a 145-bp fragment of *GAL2* (ocej237, 5′-TGAATTGAAAGCCGGTGAGT-3′; ocej238, 5′-GGTATCCCAGCCAAACATGA-3′), a 169-bp fragment of *GAL10* (GAL10-O1, 5′-TGGGTTCCGGTAAAGGTTCT-3′; GAL10-O2, 5′-TGCAACTCGGTCTGCCATTT-3′), a 149-bp fragment of *CAR1* (CAR1-O1, 5′-AATACCCCGATGCTGGTCTT-3′; CAR1-O2, 5′-TTTGAGCGACTCGGGACAAT-3′), a 100-bp fragment of *ARG3* (ARG3-O1, 5′-GCCCGTGTGAACAAACATGA-3′; ARG3-O2, 5′-TTGCTTGCAAAGGGTGGAAT-3′) and a 125-bp fragment of *TBP1* (TBP1-O1, 5′-TATAACCCCAAGCGTTTTGC-3′; TBP1-O2, 5′-GCCAGCTTTGAGTCATCCTC-3′).

Confocal imaging

Cells were imaged on LSM510-Axiovert 100 M confocal microscope (Zeiss) by exciting eGFP fusion at 488 nm.

RESULTS

Dominant-negative effects resulting from overexpression of *GAL4*Δ or *ARG81*Δ

To ensure a constitutive overproduction of truncated protein, the *S. cerevisiae GAL4*Δ sequence coding for Gal4pΔ (aa 1–147) was placed under the control of the strong *TDH3* promoter. The ORF was fused or not to the V5 epitope coding sequence allowing immunodetection with V5-specific antibodies. The expression cassette was cloned in integrative vectors. The *S. cerevisiae* strain BY4709 was transformed with *CYC1* integrative plasmids (described in Table 1) by conventional *URA3* selection. Transformants harboring a single copy of the empty vector (p0int), plasmid with *GAL4*Δ (pGint) or

plasmid carrying *GAL4Δ:V5* (pGVint) were chosen after PCR characterization of genomic DNA (data not shown). The production of the truncated Gal4p was verified by immunodetection with V5-specific antibodies (Supplementary Figure S1). The phenotypes were determined by streaking the selected strains on minimal medium containing either glucose or galactose as sole carbon source. The growth of strains producing truncated Gal4p was significantly inhibited on galactose, indicating the occurrence of a dominant-negative effect (Figure 1). It should be noticed that this phenotype was slightly more pronounced with the *GAL4Δ:V5* construct. This possibly results from a higher production level of Gal4pΔ-V5 (expression or half-live) or from a functional benefit due to this particular C-terminal modification of the protein (conformation, interaction with other transcription factors, etc.). Another plasmid, pGVGint, was constructed in order to overproduce Gal4pΔ as a fusion with GFP. The phenotype of a strain carrying such plasmid was similar to the one observed for pGVint. Observation by fluorescence microscopy revealed very efficient accumulation of the protein in the nucleus when *GAL4Δ* is expressed under the control of the strong *TDH*3 promoter (Figure 2). According to experiments described hereafter, the dominant-negative effect is due to interference of

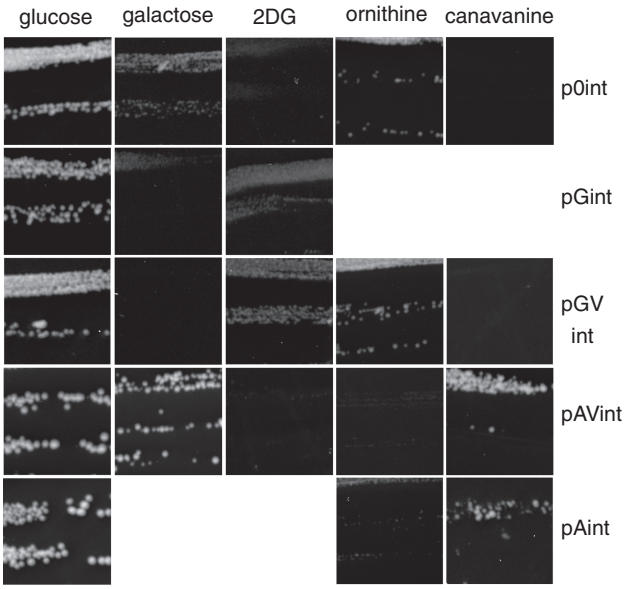

Figure 1. Phenotype determination of *GAL4Δ* and *ARG81Δ* expressing *S. cerevisiae* strains. BY4709-derived strains carrying the various integrated vectors were streaked on diverse media for identifying phenotypes. P0int: negative control vector; pGint: *GAL4Δ* expression vector; pGVint: *GAL4Δ:V5* expression vector; pAVint: *ARG81Δ:V5* expression vector; pAint: *ARG81Δ* expression vector. First column: minimal medium (YNB) with 20 mg/ml glucose as carbon source (no significant effect resulting from expression of *GAL4Δ* or *ARG81Δ*). *GAL4Δ*-associated phenotypes were observed on YNB with 20 mg/ml galactose (second column) and on 2DG selection medium (YNB with 30 mg/ml glycerol, 20 mg/ml 2DG, 5 mg/ml casaminoacids, 1 mg/ml galactose and 0.2 mg/ml glucose, third column). *ARG81Δ*-related phenotypes were highlighted on YNB with 1 mg/ml ornithine as sole nitrogen source (fourth column) and on canavanine selection medium (YNB with 80 mM ammonium, 20 mg/ml glucose, 200 μg/ml ornithine and 8 μg/ml canavanine, (fifth column).

GAL4Δ with the induction of genes belonging to the galactose pathway. Indeed, the transcriptional analysis by qRT–PCR has shown a significant decrease of *GAL1*, *GAL2* and *GAL10* expression in raffinose grown strains carrying pGint or pGVint plasmids after 1-h pulse with galactose (Figure 3A). Expression of genes encoding defective transcription factors unrelated to the galactose pathway did not significantly affect expression of *GAL* genes (transcriptional analysis of *GAL1*, *GAL2* and *GAL10* in *ARG81Δ:V5* expressing strain, data not shown) and growth on galactose (see below *ARG81Δ*).

By a similar approach, the *S. cerevisiae ARG81* coding sequence spreading from ATG to codon 180 (*ARG81Δ*), fused or not to the V5 epitope coding sequence, was placed under the control of *TDH3* promoter and cloned in an integrative plasmid. After transformation of BY4709 strain and selection of transformants harboring a single copy of these integrative plasmids, the production of Arg81pΔ was also verified by immunodetection with V5-specific antibodies (data not shown). The phenotype was verified by streaking cells on a minimal medium containing ornithine as sole nitrogen source. Growth of strains expressing *ARG81Δ* was inhibited in such medium (Figure 1), indicating that the production of truncated Arg81p is sufficient to establish a dominant-negative effect. The transcriptional analysis by qRT–PCR demonstrated a lower induction of catabolic *CAR1* expression and a reduced repression of anabolic *ARG3* expression in *ARG81Δ*-expressing strain when cells were grown in the presence of arginine, as compared to the wild-type strain (Figure 3B). This dominant-negative effect was also highlighted through enzymatic assays of the arginase and the ornithine transcarbamylase (data not shown). We also verified that production of truncated Arg81p does not significantly affect growth on galactose and that production of truncated Gal4p does not interfere with growth on ornithine, confirming the specificity of the dominant-negative effects (Figure 1).

Drug resistance due to dominant-negative effect of *GAL4Δ* and *ARG81Δ*

Various enzymes are able to convert specific prodrugs to toxic compounds and such property has been exploited for developing negative selection systems using particular mutant host strains (35,36). Such possibility was offered for the galactose pathway, and exploited in prototrophic host strains using *GAL4Δ*.

Resistance to 2DG, a glycolysis inhibiting prodrug whose toxic effect is supposed to require a permease (presumably Gal2p) and the Gal1p galactokinase (24,37), was evaluated. This required modification of the growth medium, as previous assays involved a wild-type strain and mutants constitutively expressing the *GAL* genes. Various assays were performed to define a medium allowing a *GAL4* strain to be intoxicated by 2DG while *GAL4Δ*-expressing strains being able to grow. A defined medium was optimized in view of selection of transformants carrying *GAL4Δ* (data not shown). The optimal medium contains 1 mg/ml galactose to induce the *GAL* genes (whose expression is required for 2DG

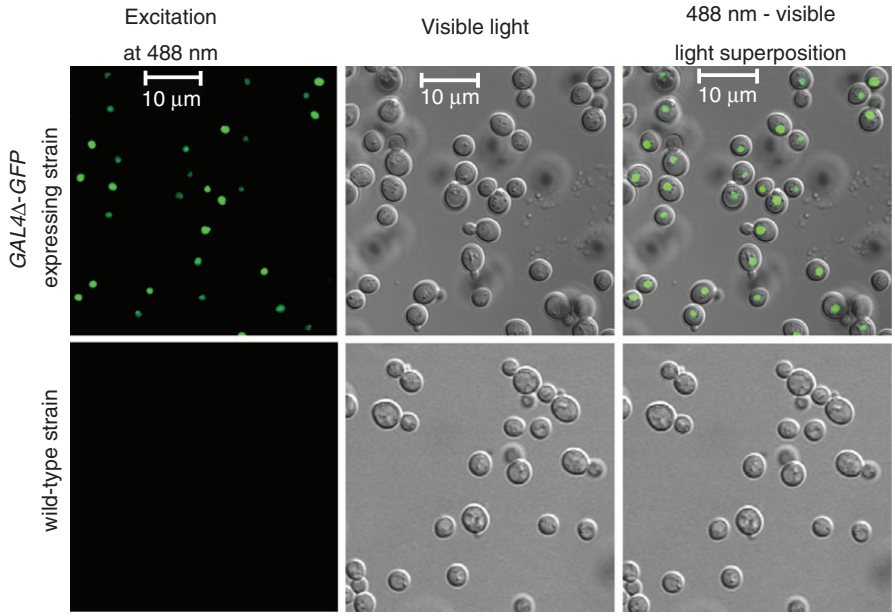

Figure 2. Subcellular localization of truncated Gal4p-V5 fused to GFP.

catabolism), a low concentration of glucose (0.2 mg/ml), 30 mg/ml glycerol (carbon sources for transformants carrying *GAL4*Δ) and 5 mg/ml casaminoacids (improving growth of transformants carrying *GAL4*Δ). The defined glucose concentration is apparently insufficient to impair the induction of *GAL* genes expression as well as galactose and deoxygalactose uptake. A reduced toxicity of this prodrug could be observed upon expression of *GAL4*Δ (Figure 1).

It was previously demonstrated that resistance to canavanine is encountered in *can1* and *argR* mutants (38). *ARG81*Δ-expressing strains were streaked on a selection medium (YNB, 80 mM ammonium, 20 mg/ml glucose, 200 µg/ml ornithine, 8 µg/ml canavanine) rendering evident the canavanine resistance of *argR* mutants (38). As compared to the *ARG81* control strain, *ARG81*Δ-expressing cells were resistant to the drug (Figure 1). *ARG81*Δ was then also potentially usable as positive selection marker.

Genetic engineering vectors for prototrophic *S. cerevisiae* strains

The selection through 2DG-resistance was assayed by transforming laboratory strains with a classical integrative plasmid carrying the *GAL4*Δ:*V5* selective marker (Supplementary Figure S2). After transformation of Σ1278b (haploid strain) and BY4700/9 (diploid strain), tiny colonies were obtained after 5–7 days on 2DG medium, picked with the aid of a binocular and purified by streaking on a fresh 2DG selection plate. The small size of the transformants was probably due to the low glucose concentration in the 2DG medium (only 0.2 mg/ml) and the residual metabolic activity of non-transformed cells. More than 50% of diploid colonies harbored the expected disruption of the targeted allele, as shown by PCR analysis of genomic DNA (data not shown) and expected 2:2 *leu2*:*LEU2* segregation was observed after sporulation. For Σ1278b, ~1% of 2DG-resistant clones appeared to carry the integrated vector. This lower efficiency probably results from a high frequency of spontaneous 2DG-resistant mutants (likely to be recessive, as not observed with the diploid). PCR-screening of the colonies should then be needed when transforming haploid strains. The efficiency of the negative selection system was demonstrated by selecting the vector excision event. Indeed, after growth on non-selective YPD medium and plating on minimal medium with galactose as sole carbon source, colonies showed a *GAL4*/*LEU2* phenotype.

To further evaluate the efficiency of the *GAL4*Δ selection marker, gene deletions were performed in wild yeast strains ('TR strains') (17). As most of the *S. cerevisiae* wild strains, they are homothallic diploids, which implies that *HO* needs to be deleted for generating stable genetically modified recombinant strains (otherwise haploids of both mating types should appear after sporulation, giving rise spontaneously to a diploid cellular population by mating). For such purpose, a vector including a *GAL4*Δ selective marker surrounded by upstream and downstream *HO* sequences and an appropriate small 30 bp directly repeated *HO* sequence for subsequent vector excision was constructed (Table 1 and Figure 4). BamHI restriction sites were created for allowing the generation of appropriate DNA fragments for gene-replacement by homologous recombination.

TR3, a *GAL* and 2DG-sensitive diploid strain, was first chosen for performing genetic modifications with the *GAL4*Δ system. The pGVdelHO BamHI-digested vector was introduced into TR3 after transformation and selection on the 2DG medium. After 5 days, colonies were picked and purified by streaking on a fresh selection plate. PCR-characterization of genomic DNA has shown

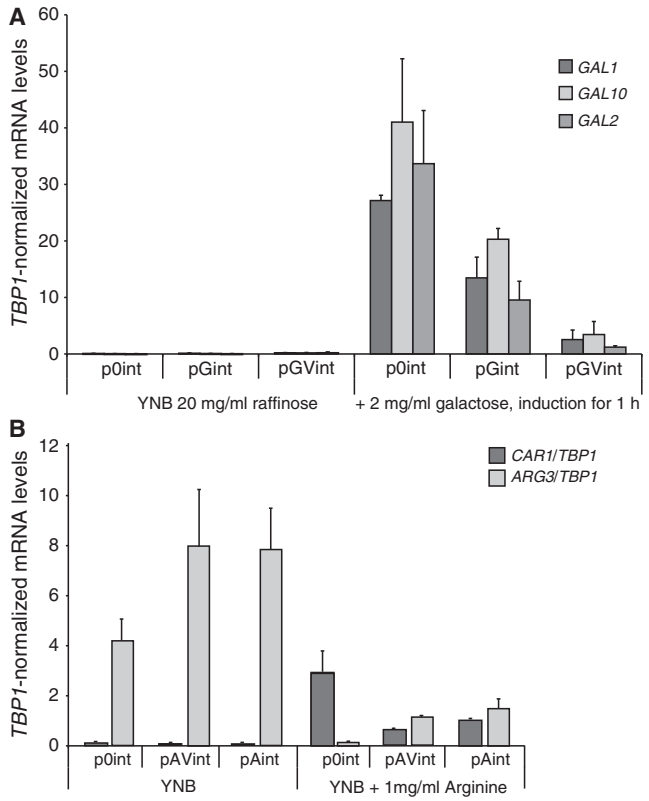

Figure 3. (**A**) Transcriptional analysis of *GAL1*, *GAL2* and *GAL10* expression in wild-type (carrying empty *CYC1* integrative plasmid p0int) and truncated Gal4p overproducing *S. cerevisiae* strains (harboring *CYC1* integrative pGint and pGVint vectors, enabling the expression of *GAL4*Δ and *GAL4Δ:V5* respectively). Strains were cultivated on YNB with 20 mg/ml raffinose (glucose repression relieved, no galactose induction) and, for inducing *GAL* gene expression, 2 mg/ml galactose was added for one hour. mRNA of *GAL1*, *GAL2* and *GAL10* were quantified by qRT–PCR and the values were normalized with *TBP1* mRNA. (**B**) Transcriptional analysis of *ARG3* and *CAR1* expression in wild-type (carrying empty integrative plasmid p0int) and truncated Arg81p overproducing *S. cerevisiae* strains (harboring *CYC1* integrative pAint and pAVint vectors, enabling the expression of *ARG81*Δ and *ARG81Δ:V5* respectively). Strains were cultivated either on YNB with 80 mM ammonium, 20 mg/ml glucose (expression of arginine biosynthesis genes, repression of arginine catabolic pathway genes) or on YNB with 80 mM ammonium, 20 mg/ml glucose and 1 mg/ml arginine (repression of arginine anabolic pathway genes, induction of arginine degradation genes). By qRT–PCR, mRNA of *ARG3* and *CAR1* were quantified and the values were normalized with *TBP1* mRNA.

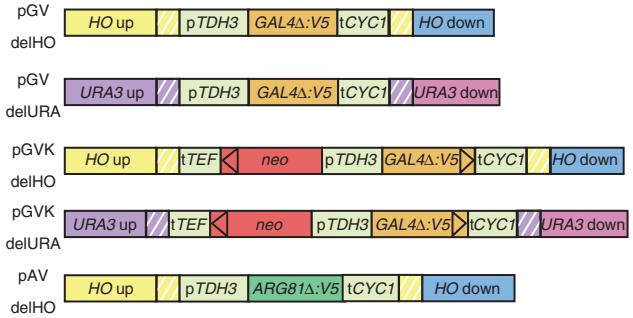

Figure 4. Scheme of integrative cassettes for the deletion of *HO* and *URA3*. All cassettes are excised from pUC19 plasmid by a BamHI double digest. Up sequences are identical to a sequence upstream of targeted ORF and down sequences are identical to a sequence downstream of targeted ORF. Dashed yellow and blue boxes are small 30 repeats of upstream *HO* and *URA3* sequences, respectively.

that >50% of the colonies exhibited the *GAL4Δ:V5* cassette integrated in *HO* locus. After dissecting tetrads, stable *ho* haploids deriving from TR3 were obtained (2:2 *HO*:*ho* segregation). The integrated cassette in *HO* locus was then excised by spontaneous homologous recombination involving the small directly repeated *HO* sequences (Figure 5), by selecting haploid *GAL* clones after growth on YPD medium as described in 'Materials and Methods' section (PCR characterization, Supplementary Figure S3). The cassette pop-out frequency was estimated at $3 \times 10^{-5} \pm 6.5 \times 10^{-6}$ from a set of three independent experiments. The expected accurate deletion of *HO* was confirmed by PCR-amplified genomic DNA sequencing. Among eight analyzed clones deriving from five independent transformants, seven carried the expected deletion (only one revertant showed an unexplained recombination event). Haploid strains of each mating type carrying the desired accurate *HO* deletion and devoid of any foreign DNA element were obtained and mated in order to generate a *ho*/*ho* diploid strain, TR3*ho*Δ.

Afterwards, *URA3* deletions were performed in TR3*ho*Δ strain to generate modified hosts that could be transformed with classical expression vectors. For this purpose, a vector carrying the *GAL4Δ:V5* cassette surrounded by appropriate *URA3* sequences was similarly constructed, pGVdelURA3 (Table 1 and Figure 4). Using the same approach, the deletion of *URA3* was successfully carried out starting from the diploid strain with a transformation efficiency of >50%. Haploids of both mating types carrying expected accurate deletions of *URA3*, as confirmed by genomic DNA sequencing, were obtained. Successive genetic modifications of strains could systematically be performed in the same way, by transforming diploids and performing classical genetics. Indeed, such procedure allows verifying expected segregation of the genetic modifications and selecting modified haploids of both mating types. In comparison, transformation of TR3*ho*Δ-derived haploids was undertaken and, as mentioned above with Σ1278b, extensive PCR-screening was required (transformation efficiency of 7.8 ± 4.3%), confirming that the diploid-transformation procedure is more convenient.

Surprisingly, some TR strains appeared to be naturally 2DG-resistant despite their *GAL* phenotype. One of these, the TR2 diploid, was transformed using a construct in which a *neo* marker was associated to *GAL4*Δ (bidirectional *TDH3*-driven expression cassette), designed for the deletion of *HO* ORF (pGVKdelHO; Table 1 and Figure 4). After selection of G418-resistant transformants and PCR characterization, we observed a *gal* phenotype. Therefore, the dominant-negative effect of *GAL4*Δ does occur in such 2DG-resistant wild strain. Using haploids derived from these TR2 transformants, stable *ho* haploids devoid of foreign DNA sequences were obtained after spontaneous vector excision at a frequency of $1.4 \times 10^{-4} \pm 6 \times 10^{-6}$ (based on three independent

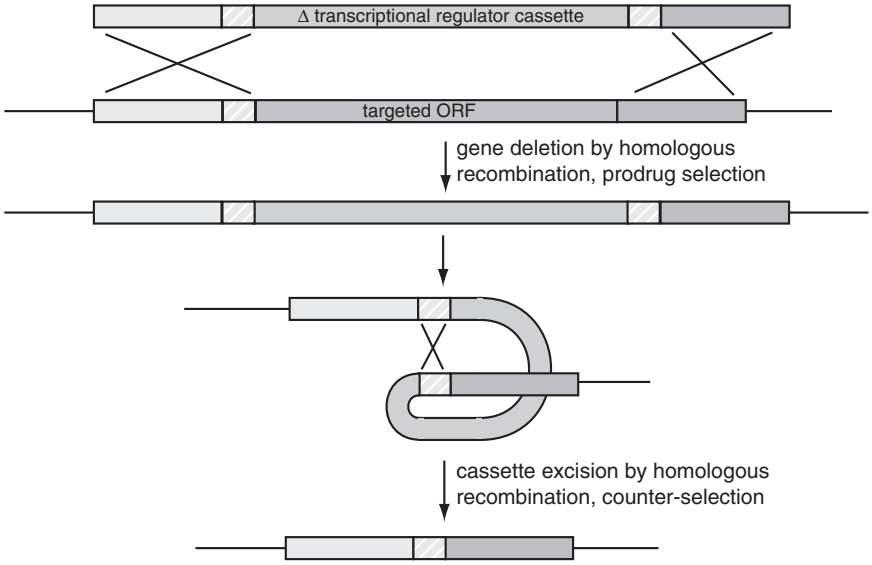

Figure 5. Scheme of gene deletion by homologous recombination between linearized integrative vectors and targeted genomic sequences, and subsequent excision by homologous recombination between the designed short (30 bp) directly repeated genomic sequences.

experiments). The *neo*/*GAL4*Δ expression cassette may then be convenient for modifying *GAL* strains that are not sensitive to 2DG (approach validated in BY4709 strain, data not shown). In addition, this vector provides an alternative for transformation of prototroph haploids (specifically in case only one mating type is available), as it avoids the PCR-screening of transformant candidates which was required with the 2DG selection due to the relatively high frequency of non-transformed 2DG-resistant haploid clones as mentioned above.

We also used the *ARG81*Δ marker for modifying the 2DG-resistant TR2 strain, with an optimized selective medium with canavanine (25 μg/ml). Using BamHI-digested pAVdelHO (Table 1 and Figure 4), deletion of *HO* was successfully carried out (data not shown). After sporulation, *ho* haploids of both mating types were obtained. The growth of these *ho* haploids on ornithine as nitrogen source was inhibited, as expected, allowing selection of vector excision after growth on non-selective YPD medium, at a frequency of $5.5 \times 10^{-5} \pm 1.6 \times 10^{-5}$. The expected accurate deletion of *HO* gene was confirmed by PCR-amplified genomic DNA sequencing. The *ARG81*Δ marker may then provide a useful alternative for modifying 2DG-resistant strains.

In order to compare efficiencies of the 2DG and canavanine positive selection systems, a series of transformation experiments were performed with the *HO* and *URA*3 deletion vectors in TR2 and TR3 strains (Table 2). These results demonstrate that the two selection systems have similar efficiencies. A set of convenient vectors harboring *GAL4*Δ and *ARG81*Δ was developed (see Figure 6 and plasmid sequences in Supplementary Data). With such plasmids, the introduction of homologous sequences to loci to be targeted can be easily carried out via two polylinkers flanking the selection cassette.

Table 2. Evaluation of transformation efficiencies for *HO* deletion in TR3 by pGVdelHO (2-deoxygalactose selection), *URA3* deletion in H2n1 (ho/ho diploid derived from TR3) by pGVdelURA (2DG selection) and *HO* deletion in TR2 by pAVdelHO (canavanine selection)

Strain	Vector/selection	Mean transformation efficiency (total screened colonies)	Standard deviation
TR3	pGVdelHO/2DG	63.3% (66)	±4.9%
TR3*ho*Δ	pGVdelURA/2DG	34.0% (79)	±8.7%
TR2	pAVdelHO/canavanine	91.7% (47)	±0.8%

Transformation efficiency is defined as the percentage of PCR-characterized-colonies harboring the deletion cassette at the expected locus. The amount of screened colonies is given between brackets. The mean calculation and standard deviation are based on three independent experiments.

CONCLUSION

The use of dominant-negative truncated transcription factors designed for interfering with specific regulatory pathways has been shown to allow performing precise genetic modification of wild strains of *S. cerevisiae*. This method should be convenient for performing any site-directed modification [including point-mutations, as described in ref. (6)] in the genome, and avoids side-alterations in the locus, which is useful for a number of functional studies. The approach is based on the use of a single expression cassette leading to both resistance to a toxic compound and inhibition of growth on a specific substrate. It is particularly attractive for specific applications, such as building of genetically modified strains for the food industry, as only specific genes derived from the host strain should be used (avoiding the utilization of foreign genes). The described method is also more powerful than previously described systems such as 5-fluoroorotic acid resistance of *ura3* strains.

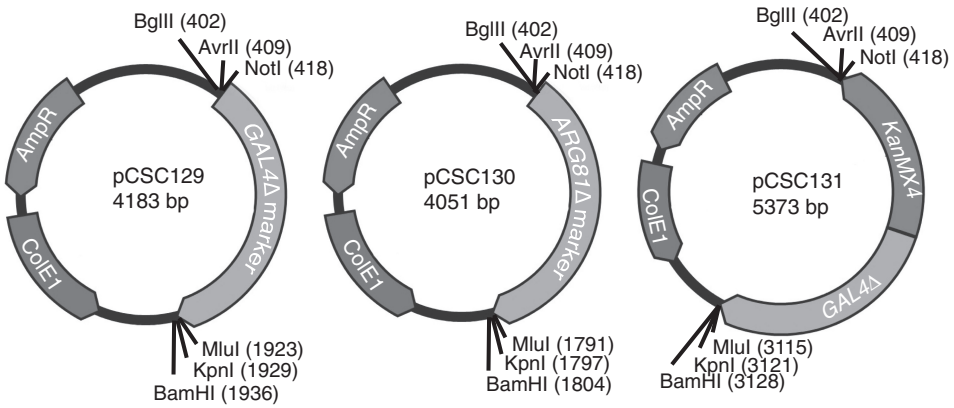

Figure 6. Plasmid maps of pCSC129, 130 and 131 containing *GAL4*Δ, *ARG81*Δ and *GAL4*Δ/*KanMX4* selection markers respectively. These vectors are derived from pUC19 and contain two convenient polylinkers, BglII-AvrII-NotI and MluI-KpnI-BamHI, to introduce homologous sequences in view of genetic modification.

Indeed, it does not require previous generation of a specific mutation or deletion. Moreover, in the specific case of the *Saccharomyces* wild strains, a preliminary deletion of *HO* is required for generating stable haploids, and the growth defect resulting from transcriptional interference appeared to be very convenient for subsequent vector recycling.

Resistance to a drug resulting from interference with a specific regulatory pathway is apparently not as systematically observed than metabolic alteration leading to growth inhibition. Indeed, not all the *GAL* wild strains appeared to be sensitive to 2DG in the various growth conditions assayed (data not shown). However, transformation of the 2DG-resistant diploid TR2 using a *neo* marker associated to the *GAL4*Δ cassette gave rise to transformants that were deficient for growth on galactose. Therefore, the transcriptional interference occurred, and the counter-selection was adequate for selection of subsequent vector excision on the galactose medium. We could not explain such resistance behavior on 2DG, as the toxicity of this prodrug through inhibition of glycolysis is not fully understood (37). Possibly, 2DG is not transported by the galactopermease or the derivative compound (2-deoxygalactose-1-phosphate and/or UDP-2-deoxygalactose) is not efficiently produced or else its toxic effect is not exerted in all *GAL* strains. In case of TR2, canavanine appeared to be toxic, and *ARG81*Δ was successfully used as positive/negative selection marker, which offers an useful alternative to *GAL4*Δ.

Based on the existing knowledge on metabolic pathways and regulatory genes, it should be possible to design a number of alternative dominant-positive/negative selection systems (see phenotypes database of *Saccharomyces* Genome Database). Systematic studies could be designed which should allow generating both useful knowledge on known or predicted transcription factors as well as identifying new selective markers of interest, by searching for dominant-negative phenotypes upon transformation with appropriate constructs involving the *neo* positive marker, for example.

The approach could be extended to other yeasts, but also to other microorganisms. Data generated in the context of genomic sequencing projects allow identifying an increasing number of candidate genes for generating such modified transcription factors. For genetic modification of diploid wild strains of *Saccharomyces*, the use of modified transcription factors appeared to be particularly efficient. In this respect, it should be noticed that diploidy or polyploidy has been observed in the case of various wild microorganisms (various lower eukaryotes, but also bacteria and archaea) (39–41).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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