Promoter-dependent disruption of genes: simple, rapid, and specific PCR-based method with application to three different yeast

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Abstract PCR product-based gene disruption has greatly accelerated molecular analysis of Saccharomyces cerevisiae. This approach involves amplification of a marker gene (e.g., URA3) including its flanking regulatory (promoter and polyadenylation) regions using primers that include at their 5' ends about 50 bases of homology to the targeted gene. Unfortunately, this approach has proved less useful in organisms with higher rates of non-homologous recombination; e.g., in the yeast Candida glabrata, desired recombinants represent ≤ 2% of transformants. We modified the PCR-based approach by eliminating marker-flanking regions and precisely targeting recombination such that marker expression depends on the regulatory sequences of the disrupted gene. Application of this promoter-dependent disruption of genes (PRODIGE) method to three C. glabrata genes (SLT2, LEM3, and PDR1) yielded desired recombinants at frequencies of 20, 31, and 11%, the latter representing a weakly expressed gene. For Candida albicans LEM3 and RHO1, specificity was 79–95% for one or both alleles, > sixfold higher than the published results with conventional PCR-based gene disruption. All 5 C. glabrata and C. albicans mutants had predicted phenotypes of calcifitor hypersensitivity (slt2Δ and rho1Δ), cycloheximide hypersensitivity (pdr1Δ), or miltefosine resistance (lem3Δ and lem3Δ/lem3Δ). PRODIGE application to the S. cerevisiae PDR5 gene in strains with and without the Pdr1–Pdr3 transcriptional activators of this gene confirmed that transformant yield and growth rate depend on promoter strength. Using this PDR5 promoter-URA3 recombinant, we further demonstrate a simple extension of the method that yields regulatory mutants via 5-fluoroorotic acid selection. PRODIGE warrants testing in other yeast, molds, and beyond.

Keywords Candida glabrata · Candida albicans · Saccharomyces cerevisiae · Gene deletion · Transformation

Introduction

Targeted gene disruption represents a cornerstone of molecular genetics, with application to a wide range of organisms. Gene disruption conventionally employs a cassette containing two regions of homology to the targeted gene flanking a selectable marker gene (nutritional or drug resistance) that includes promoter and polyadenylation regions required for marker expression. Multiple DNA cloning steps are generally required to generate these cassettes, incorporating flanking homology regions hundreds or thousands of bp long.

An important modification, now widely applied to the model yeast Saccharomyces cerevisiae, involves the use of PCR rather than cloning to generate disruption cassettes (McElver and Weber 1992; Baudin et al. 1993; Lorenz et al. 1995). The PCR primers typically include about 20 bases at their 5' ends for amplification of the marker gene including promoter and polyadenylation regions, along with 30–60 bases at their 3' ends that are homologous to the targeted gene. In S. cerevisiae, these short homology regions are adequate to achieve specificities of > 50% homologous recombinants versus total transformants (Manivasakam et al. 1995).

Unfortunately, in other organisms, PCR product-based gene disruption has proven much less useful, and this has been generally attributed to the need for longer regions of homology. In the diploid yeast Candida albicans, specificities of 4–21% (average = 13%) were achieved using 60 bp homology regions for alleles of
three different genes (Wilson et al. 1999, 2000). However, another laboratory reported that 100 bp of homology were required for efficient gene disruption in C. albicans, with 75 bp yielding <1% specificity (Gola et al. 2003). In the haploid Candida glabrata, homology regions of 50 bp yielded specificities of ≤ 2% (Cormack and Falkow 1999; Weig et al. 2001). Attempts have been made to address these limitations by using overlap or fusion PCR protocols designed to generate longer homology regions (Wach 1996; Willins et al. 2002); however, these methods require multiple PCR reactions and considerable optimization to generate the necessary yield and purity of DNA.

With any of the above methods, specificity is reduced because expression of the selection marker is largely independent of its integration site. We describe here a simple modification of conventional PCR product-based gene disruption: cassette are generated with a promoter-less marker gene such that its expression is dependent, following recombination, on the promoter of the targeted gene. Promoter-dependent disruption of genes (PRODIGE) dramatically enhanced specificity in C. glabrata and C. albicans, and is likely to be applicable to other organisms as well. Studies in S. cerevisiae with the URA3 marker suggest that only minimal levels of target gene expression are required; furthermore, PRODIGE can be readily extended to a selection system for regulatory mutants.

Materials and methods

Strains, plasmids, and media

Candida glabrata strains BG14 (ura3::Tn903NeoR; Cormack and Falkow 1999) and 200989 (ura3 his3::trp1::Kitada et al. 1995) were obtained from B. Cormack (Johns Hopkins University) and the ATCC (Manassas, VA), respectively. C. glabrata 8512-11 (ura3) was isolated in our lab as a spontaneous 5-fluoroorotic acid (5FOA)-resistant derivative of azole resistant clinical isolate 8512 (Vermily and Edlin 2004). C. albicans strain BWP17 (ura3Δura3Δ, his3Δ::his3Δ, arg4Δ::arg4Δ; Wilson et al. 2000) was obtained from A. Mitchell (Columbia University). S. cerevisiae nA1Δ 3Δ (MAAta3::ura3-52 his3-Δ200 leu2-Δ1 trpl-Δ63 pdr1::TRPI trp3::TRP1 pdr3::TRP1 his3) (Delaveau et al. 1994) was obtained from K. Krüger (University and Biocenter of Vienna) and IPY36 (MATa his3::leu2Δ1 ura3-52 trp1::HisG) was from S. Berger (Wistar Institute). Plasmids YEp24 and pRS414 (Christianson et al. 1992) were obtained from J. Nickels (Drexel University), pDBB57 (Wilson et al. 1999) was from A. Mitchell, and pFA-ARG4 (Gola et al. 2003) was from J. Wencel-Liotard (Friedrich-Schiller University). Routine cultures employed YPD medium (1% yeast extract, 2% peptone, 2% dextrose); this was supplemented with 80 μg/ml uridine for C. albicans BWP17 and its derivatives. Transformants were selected on synthetic defined medium with dextrose and appropriate supplements (DOB plus CSM-URA, CSM-TRP, or CSM-ARG; Qiogene/BIO 101) including uridine where indicated.

Primers

All primer sequences are presented in Table 1. Primers for amplifying disruption cassettes were 80 nucleotides in length and PAGE purified by the manufacturer (Integrated DNA Technologies), except for C. glabrata ADE2 disruptions which employed 60 nucleotide unpurified primers. The PRODIGE primers were designed following the strategy shown in Fig. 1a, with their’ 3’ 20–27 nucleotides (Tm ～ 60°C) derived from the termini of the marker gene coding sequence (e.g., URA3) and the remainder derived from the flanking sequences of the targeted gene. Marker gene coding sequences were obtained from pRS414 (S. cerevisiae URA3 and TRP1; GenBank accession number L09156 and U03448, respectively), pDBB57 (C. albicans URA3, AF173953), and pRS-ARGASspe (C. albicans ARG4; AF173956). S. cerevisiae gene and protein sequences were obtained from the Saccharomycyes Genome Database (http://www.yeastgenome.org), C. glabrata sequences from the Genolevures database (http://cbi.labri.fr/Genolevures/elt/CAGL), and C. albicans sequences from the Stanford database (http://sequence- www.stanford.edu/group/candida) or from CandidaDB (http://genolist.pasteur.fr/CandidaDB). Primers for PCR confirmation (Fig. 1b; Table 1) were generally derived from upstream (u) regions of targeted genes along with internal (i) regions of the target and marker genes; the C. albicans rhi3Δ confirmation primer derived from the downstream (d) region of the target gene.

PCR of disruption cassettes

Marker coding sequences were amplified with PRODIGE primers using the indicated plasmid DNA (purified by QIAprep spin, Qiagen) as template. Reactions (50 μl) included 0.2 mM dNTPs, 0.5 μM each primer, 1.5 units Ex-Taq (TaKaRa), 1 X Ex-Taq buffer, and about 20 ng of plasmid DNA. Amplification involved initial denaturation at 94°C for 2 min followed by 25–30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, and extension at 70°C for 1.5 min; the final extension at 70°C was 4–5 min. Four separate reactions were run to increase yield and decrease the effects of mutations. The reactions were pooled, a sample checked by gel electrophoresis, and the remainder purified using Wizard SV PCR Clean-up System (Promega) with elution in 20–30 μl water.

Transformation and screening

Both the lithium acetate transformation procedure (Ausubel et al. 1997) and the Frozen-EZ Yeast Trans-
formation II Kit (Zymo Research) were used, with the latter (using non-frozen cells) generating several-fold higher yields. In both cases, prepared cells were transformed with about 1 μg of PCR product (see above), and transformants were selected on appropriate medium with incubation at 30°C for 3-10 days. For PCR screening, genomic DNAs were prepared essentially as described (Ausubel et al. 1997). Briefly, isolated colonies from the transformation plate were cultured overnight in selective medium, pelleted, suspended in breaking buffer, glass beads, and buffer-saturated phenol and vortexed 2 min. Following centrifugation, the aqueous layer was reextracted with chloroform:isoamyl alcohol and the DNA ethanol precipitated. Following an ethanol wash and drying, the pellet was suspended in 100 μl water. The PCR reactions (25 μl) included 0.2 mM dNTPs, 1X reaction mix and 0.5 units Taq polymerase (Promega), 0.5 μM of indicated primers, and 1 μl of 1:10-diluted genomic DNA. Amplification conditions were as described above except that extension time was 1 min and cycle number was 25. Following this initial screen, selected transformants were streaked for isolation on YPD plates and the PCR screening of individual colonies was repeated.

Drug sensitivity assays

Drug sensitivities were examined by spotting about 500 cells from fresh cultures onto YPD plates with varying concentrations of calcifluor (Sigma-Aldrich), nystatin (Cayman), or cycloheximide (Sigma) followed by incubation at 30°C for 2-3 days. For the S. cerevisiae pdr5Δ::URA3 disruptant and its parent, growth rates of mid-log cultures were determined in DOB with CSM or CSM-URA broth at 30°C by measuring absorbance at 630 nm. Sensitivity to 5FOA (Zymo Research) was determined by broth microdilution assay (Vermitzky and Edlund 2004) in both YPD and synthetic defined medium at 30°C.

RNA blots and 5FOA-resistant mutants

The RNA was prepared, slot-blotted to nylon membranes, and hybridized as previously described (Vermitzky and Edlund 2004). Probes were amplified from S. cerevisiae genomic DNA using gene-specific primers and labeled with 32P by random priming. To isolate 5FOA-resistant mutants, 4x10⁷ cells from a fresh S. cerevisiae pdr5Δ::URA3 culture were spread on synthetic complete medium plates containing 0.05% 5FOA and incubated at 30°C for 5 days. Colonies were streaked for isolation on YPD plates, tested by spot assay for growth on DOB-ura and 5FOA media, and their RNA analyzed as described above.

Results

**PRODIGE method**

PCR primers were designed to generate a disruption cassette that, following recombination, would precisely replace the coding sequence of the targeted gene with the coding sequence of the marker (Fig. 1a). In contrast to the conventional gene disruption methods, expression of the promoter-less marker gene (i.e., colony formation on selection plates) should be largely limited to cells in which the desired homologous recombination occurred, thus expressing the marker from the targeted gene’s promoter. Consequently, while the yield of transfor-
<table>
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<th>Location^b</th>
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^a Sequence of primers used in this study.
^b Location is indicated relative to the start codon of targeted gene or marker gene (for PRODGE B primers, relative to stop codon of targeted gene).

See Fig. 1 for general diagram of primer location.

For PRODGE B primers, homology regions are labeled, start and stop codons of marker gene are underlined.
Fig. 2 Representative PCR confirmation gels and drug sensitivity assays for C. glabrata gene disruptions. a SLT2 disruption. Genomic DNAs were purified from isolated colonies and screened by PCR and gel electrophoresis. Loss of the CgLSTuF-CgLSTiR 456 bp product and formation of the CgLSTuF-ScURAbR 423 bp product (refer to Fig. 1b and Table 1 for primer locations and sequences) identified slt2Δ clones. CgLSTuF-CgLSTiR was used as a ura+ transformant that retained SLT2 and hence represents a non-specific recombinant. b About 500 cells were spotted on YPD plates with or without calcifluor and incubated for 2 days. c LEM3 disruption. Loss of the CgLEM3uF-CgLEM3iR 431 bp product and formation of the CgLEM3uF-ScURAbR 412 bp product identified lem3Δ clones. d Cells were spotted on YPD with and without miltefosine and incubated for 2 days. e Disruption of PDR1. Loss of the CgPDR1uF-CgPDR1iR 2806 bp product and formation of the CgPDR1uF-ScURAbR 2623 bp product identified pdr1Δ clones. f Cells were spotted on YPD with or without cycloheximide and incubated for 2 days.

The 3' end corresponding to marker sequence termini and 53–60 bases at the 5' end homologous to target gene flanking sequence. To minimize mutations to the marker coding sequence during PCR, a polymerase with relatively high fidelity was used along with moderate cycle numbers; the final extension was brief to minimize addition of non-templated residues. Following purification, the PCR products were used directly to transform yeast by standard methods.

d C. glabrata SLT2, LEM3, and PDR1 disruption

The haploid C. glabrata provided a stringent test of PRODIGE since, as noted above, short homology regions have yielded specificities of ≤ 2% (Cormack and Falkow 1999; Weig et al. 2001; our unpublished data). For our initial studies, we used the S. cerevisiae URA3 coding sequence (derived from YEp24) as the selection marker which has previously been shown to be functional in C. glabrata (Kitada et al. 1995). Three different C. glabrata ura3 host strains were used: BG14 (Cormack and Falkow 1999), ATCC 200989 (Kitada et al. 1995), and 8512-11 (a SFOA-resistant derivative of clinical isolate 8512) (Vermitsky and Edlin 2004).

The S. cerevisiae gene SLT2 encodes the MAP kinase of the cell integrity signaling pathway that confers protection from cell wall damaging agents such as chitin-binding agent calcifluor (Heinisch et al. 1999). A single, unambiguous SLT2 homolog (CAGLO005392) was identified within the recently released C. glabrata genome sequence database (http://cbl.labri.fr/Genolevures/slt/CAGL) (Dujon et al. 2004). Using the PRODIGE method, C. glabrata SLT2 was successfully disrupted in two strains with a specificity of 20% (3 of 15 and 5 of 25 transformants tested for strains BG14 and 200989, respectively). Representative PCR screening results with specific primer pairs (Table 1 and Fig. 1b) are presented (Fig. 2a). Specifically, the desired disruptants (labeled slt2Δ) show loss of the CgLSTuF-CgLSTiR product (i.e., product of SLT2 upstream forward primer paired with SLT2 internal reverse primer), while this product
was clearly observed with the C. glabrata parent (labeled CgP) and a representative non-specific transformant (labeled SLT2). Conversely, the desired disruptants generate the CgSLTuf2-ScURA1R product (SLT2 upstream forward primer paired with URA3 internal reverse primer) indicative of URA3 replacement of the SLT2 coding sequence. The C. glabrata slt2Δ mutants demonstrated the expected phenotype of calcofluor hypersensitivity relative to parent strains and non-specific transformants; representative agar spot assay results are shown (Fig. 2b).

Using the same approach, C. glabrata LEM3 (CAGL0D02442g) disruption mutants were generated in strain 200989 with a specificity of 31% (6 of 19 transformants tested); representative PCR screening results are shown (Fig. 2c). In S. cerevisiae, LEM3 encodes a glycoprotein involved in the membrane translocation of phospholipids and alkylphosphocholine drugs such as miltefosine (Hanson et al. 2003). As in S. cerevisiae, C. glabrata lem3Δ mutants demonstrated miltefosine resistance (Fig. 2d).

The C. glabrata PDR1 gene PDR1 (CAGL0A00451g) was similarly disrupted (Fig. 2e). Specificity was only 5–8% in strains 200989 and BG14 (1 of 20 and 1 of 12 transformants, respectively) but 20% in strain 8512-11 (4 of 20). These results probably reflect the generally weak but variable expression of this transcription factor gene (Vermitsky and Edlund 2004). C. glabrata PDR1 was previously implicated in transcriptional activation of the CDR1 multidrug transporter gene (Vermitsky and Edlund 2004), and consistent with this pdr1Δ mutants were hypersensitive to cycloheximide (Fig. 2f).

C. glabrata ADE2 disruption: different markers, shorter homology, and comparison to conventional method

Disruption of ADE2 in various yeast and molds results in the accumulation of a red-pigmented intermediate. We reasoned that this would provide a simple and sensitive screen, in lieu of PCR, with which to test different markers and shorter homology regions. Unpurified 60–63 nucleotide primer pairs with 40 nucleotides of C. glabrata ADE2 homology were used to amplify S. cerevisiae marker coding sequences. In two independent experiments with URA3, transformation of C. glabrata 200989 generated about 3,000 ura+ colonies, but only 0.3% were red (not shown). In contrast, using the TRP1 marker (from pRS414), about 600 trp+ colonies of varying size were obtained, of which 10% were red (Fig. 3). Attempts to similarly disrupt ADE2 with the bacterial hygromycin resistance gene hph coding sequence (from pAG32, EUROSCARF; selection on 250–500 μg/ml hygromycin) were unsuccessful (not shown).

To directly compare PRODIGE with conventional PCR-product gene disruption, primers with identical 40 nucleotide ADE2 homology regions were designed to amplify the TRP1 gene including its promoter and polyadenylation regions as incorporated into pRS414 (281 bp upstream and 47 bp downstream of the coding sequence, respectively; not shown). Following amplification, PRODIGE and conventional cassettes were compared in parallel transformations of strain 200989. In two independent experiments (500–1,000 total transformants), PRODIGE yielded 5–10% red colonies while the conventional cassette yielded <0.2%.

C. albicans LEM3 and RHO1 disruption

We initially tested the PRODIGE method in diploid C. albicans by targeting its LEM3 homolog (http://genolist.pasteur.fr/CandidaDB). The C. albicans URA3 coding sequence (derived from pDDB57; Wilson et al. 1999) was used as the initial selection marker with host strain BWPl7 (ara2Δ/ura3Δ, arg4Δ/arg4Δ, his1Δ/his1Δ; Wilson et al. 2000). Remarkably, LEM3 allele disruption was achieved with a specificity of 95% (19 of 20 transformants). Representative PCR screening results for a heterozygous LEM3/lem3Δ mutant and its parent are shown in Fig. 4a. To directly compare PRODIGE with conventional PCR-product gene disruption, a disruption cassette was generated with identical LEM3 homology regions but incorporating the URA3 promoter and polyadenylation regions from pDDB57 (411 bp upstream and 275 bp downstream of the coding sequence, respectively; not shown). Comparable yields of ura+ colonies (25–30) were obtained in parallel transformations; however, PCR screening revealed
However, it is likely that recombination of the URA3 marker into the leu3Δ::URA3 allele would dominate over the desired recombination into the LEM3 allele. The alternative approach involves using different markers. We initially tried C. albicans HIS1 (from pFA-HIS1; Gola et al. 2003), but no transformants were obtained in several attempts (not shown). Using C. albicans ARG4 (from pFA-ARG4), 79% (11 of 14) of the transformants on medium lacking both uridine and arginine showed disruption of both LEM3 alleles (representative result in Fig. 4a). As with C. glabrata leu3Δ (above), the C. albicans heterozygous and homozygous leu3Δ mutants demonstrated miltifosine resistance, in proportion to gene copy number (Fig. 4b).

RHO1 is an essential gene in C. albicans and other yeast that functions in cell integrity signaling upstream of the MAP kinase pathway (Heinisch et al. 1999; Smith et al. 2002). For this experiment, we used the S. cerevisiae URA3 coding sequence to test the general feasibility of using heterologous selection markers. This could prove useful in future studies of fungal species for which a cloned marker gene is unavailable, or in any strain where the marker is mutated but not deleted and hence remains a recombination target. Success seemed likely since marker expression in the PRODIGE method does not require recognition of a heterologous promoter. Indeed, 80% of transformants tested (8 of 10) demonstrated specific disruption of a RHO1 allele (Fig. 4c). Their growth on ura-plates was, however, very slow. S. cerevisiae URA3 includes one CTG codon which would be translated as Ser rather than Leu in C. albicans (Santos and Tait 1998), and this probably accounts for the slow growth since mutant and parent grew comparably on YPD. As expected, rho1Δ [RHO1] mutants were calcofluor hypersensitive (Fig. 4d).

S. cerevisiae PDR5 disruption: effects of variable marker expression

Expression of the S. cerevisiae PDR5 multidrug transporter gene is mediated by the PDR1 and PDR5 transcriptional activators, which upregulate PDR5 in response to drug treatment and other stresses (Kolaczowska and Goffeau 1999). Their double disruption results in greatly reduced basal and drug-induced PDR expression (Fig. 5a). Thus, pdr1Δ pdr3Δ mutant (nA1Δ 3Δ) and PDR1 PDR5 wild type (1P3Y36) strains were used to test the effects of transcriptional expression on PRODIGE-mediated PDR5 disruption with a URA3 marker. With the pdr1Δ pdr3Δ mutant, there were clear differences in the yield of transformants on ura-plates (tenfold lower) and growth rate in ura-broth (ninelfold slower); nevertheless, specificity of PDR5 disruption was ≥75% in both cases (Table 2). As expected, transformant sensitivity to 5FOA was proportional to the levels of PDR5 promoter-mediated URA3 expression (Table 2).

LEMS disruption in 11 of 11 PRODIGE (100%) compared to 2 in 11 (18%) conventional cassette transformants.

One approach to disrupting the second C. albicans LEM3 allele would involve using the same URA3 cassette and LEM3·lem3Δ::URA3 cells that have been subjected to 5FOA-selection to regenerate ura3 mutants.
Discussion

Our initial goal in developing PRODIGE was to modify conventional PCR product-based gene disruption as widely applied to S. cerevisiae such that it could be usefully applied to C. glabrata. In this important opportunistic pathogen, the specificity of conventional gene disruption methods employing flanking homology regions ≤100 bp is ≤2% (Cormack and Falkow 1999; Weig et al. 2001; our unpublished data). With such low specificity, extensive screening is required to distinguish random integrants from desired disruptants. With PRODIGE, the flanking regulatory regions of the marker are eliminated such that marker expression (i.e., colony formation) should be limited to the desired recombinants in which the marker coding sequence precisely replaces the targeted gene coding sequence. This simple modification yielded averaged specificities of 11, 20, and 31% for three C. glabrata genes tested. Remarkably, when applied to C. albicans, PRODIGE yielded specificities of 79–95%, a significant improvement over conventional PCR-based methods with comparable (60–75 bp) homology regions (Wilson et al. 1999, 2000; Gola et al. 2003). We anticipate that PRODIGE can be applied broadly within the fungal kingdom and quite possibly beyond, and it should be amenable to high-throughput gene disruption projects. Two different nutritional markers were shown to be PRODIGE-compatible in C. glabrata (URA3 and TRP1) and C. albicans (URA3 and ARG4), while the hygromycin-resistance gene hph did not show any promise.

A recognized potential limitation of PRODIGE is that it requires a minimal level of promoter activity in the targeted gene to permit marker expression. This was demonstrated in our studies of PDR5 disruption with URA3 in an S. cerevisiae pdr1Δpdr3Δ mutant: compared to the parent strain there were tenfold fewer colonies, and these formed only after extended incubation. It remains to be seen what fraction of genes are PRODIGE-refractory due to insufficient transcription. In our experience with five different genes and the URA3 marker this was not an issue; the disruption specificity for C. glabrata PDR1 was relatively low (5–11%, depending on strain) but still required only minimal screening for detection. Gene expression databases are now available for many organisms (e.g., http://db.yeastgenome.org/cgi-bin/expression/expression-Connection.pl); this data may be useful in predicting poor gene targets for the PRODIGE approach. A related concern is that variable expression of nutritional markers, particularly URA3, may compromise phenotypic comparisons of different PRODIGE mutants. This is dealt with by using adequately supplemented medium in vitro, but this would not be feasible in vivo. A similar concern applies to conventional gene disruption approaches using URA3 (Staab and Sundstrom 2003). Drug resistance markers provide a potential solution to this problem.

Fig. 5 a RNA slot blots comparing S. cerevisiae PDR5 expression in wild-type PDR1 PDR3 (wt) and pdr1Δ pdr3Δ (Δ3Δ) cultures, with or without miconazole treatment (15 μg/ml for 20 min). ACT1 serves as loading control. b RNA slot blots examining PDR5 promoter-dependent URA3 expression in S. cerevisiae pdr5Δ::URA3 cultures (PU) and SFOA-resistant mutants (1–16); mutants 9, 11, 12, and 15 show 8–10-fold decreased expression. Expression of URA3 RNA in the S. cerevisiae ura3-52 parent IPY36 (ScP) was not detected.

S. cerevisiae pdr5Δ::URA3: selection of regulatory mutants

Since the flanking regulatory elements of the disrupted gene remain intact in the PRODIGE method, we reasoned that SFOA could be used to select regulatory mutants of that gene when the marker is URA3. With respect to PDR5, for example, these could be cis mutations (within the PDR5 promoter) or trans mutations (within a transcription factor gene such as PDR1 or PDR3). The pdr5Δ::URA3 strain constructed above (4×10⁶ cells) was plated on 0.05% SFOA-containing medium; after 5 days, 16 mutant colonies were observed. As shown by RNA hybridization (Fig. 5b), 5 of these 16 mutants demonstrated eight to tenfold reduced URA3 expression, consistent with a regulatory mutation. All of these five were confirmed to be ura- (not shown). Of the remaining 11 SFOA-resistant mutants, six remained ura+, which is consistent with a mutation blocking SFOA uptake, and five were ura- but retained full URA3 expression consistent with a URA3 coding sequence mutation.

Table 2 Effects of pdr1Δ pdr3Δ mutation on PRODIGE-mediated PDR5 disruption in S. cerevisiae, and properties of transformants

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One assumption behind PRODGE is that 60 bp of flanking homology incorporated into the disruption cassette are insufficient to confer transcription of the marker. We are not aware of any yeast genes that do not fit this assumption, i.e., have functional promoters within 60 bp of the start codon. Where this is an issue, it can be addressed by reducing the homology regions; our studies of C. glabrata ADE2 disruption with the TRPI marker suggest that 40 bp homology regions may be adequate, which has the additional benefit of significantly reducing primer cost (by reducing both length and the need for purification). Further studies of homology length, marker, and target gene expression as variables in the PRODGE method are needed.

A relatively minor advantage of PRODGE versus conventional PCR product-mediated gene disruption is that the amplification products are about 500 or more bp shorter in the absence of marker promoter and polyadenylation regions. This can enhance the yield of the PCR reaction, an important consideration since some cells transform poorly and require large amounts of PCR product. Also, PRODGE is more amenable to the use of heterologous markers. For example, we successfully used the S. cerevisiae URA3 coding sequence to delete both C. glabrata and C. albicans genes. With conventional disruption methods this can be difficult because the less conserved flanking regulatory regions tend to confart species specificity. A heterologous marker is useful in host strains where the marker gene is not fully disrupted and consequently represents a site for undesired recombination events, or “marker reversion.” The heterologous marker would be less likely to undergo such events because of sequence differences.

Extension of the PRODGE method to the isolation of regulatory mutations appears to have much potential. The SFOA-resistant S. cerevisiae pdr3Δ::URA3 mutants that demonstrated decreased URA3 expression are in the process of being characterized (K. Henry, unpublished data). Similarly, we are currently applying PRODGE with URA3 and SFOA selection to the isolation of C. glabrata regulatory mutants.

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