Tagging Morphogenetic Genes by Insertional Mutagenesis in the Yeast *Yarrowia lipolytica*

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The yeast *Yarrowia lipolytica* is distantly related to *Saccharomyces cerevisiae*, can be genetically modified, and can grow in both haploid and diploid states in either yeast, pseudomycelial, or mycelial forms, depending on environmental conditions. Previous results have indicated that the *STE* and *RIM* pathways, which mediate cellular switching in other dimorphic yeasts, are not required for *Y. lipolytica* morphogenesis. To identify the pathways involved in morphogenesis, we mutagenized a wild-type strain of *Y. lipolytica* with a Tn3 derivative. We isolated eight tagged mutants, entirely defective in hyphal formation, from a total of 40,000 mutants and identified seven genes homologous to *S. cerevisiae* CDC25, RAS2, BUD6, KEX2, GPI7, SNF5, and PPH21. We analyzed their abilities to invade agar and to form pseudomycelium or hyphae under inducing conditions and their sensitivity to temperature and to Calcofluor white. Chitin staining was used to detect defects in their cell walls. Our results indicate that a functional Ras-cyclic AMP pathway is required for the formation of hyphae in *Y. lipolytica* and that perturbations in the processing of extracellular, possibly parietal, proteins result in morphogenetic defects.

The yeast-to-hypha morphological transition is typical of many fungi and seems to be important for the pathogenesis of fungi such as *Ustilago maydis* (44) and *Candida albicans* (23). Several groups have reported that strains of *C. albicans* that cannot form hyphae are avirulent in mice (16, 34, 41, 69). Thus, the characterization of the genes involved in dimorphism may lead to the discovery of new treatments for pathogenic fungi.

*C. albicans* lacks a sexual cycle and is a diploid organism (51); therefore, other yeasts are usually used as models because they are easier to manipulate. *Saccharomyces cerevisiae* was the first model used to unravel the mechanisms underlying the dimorphic transition in yeasts and remains the best model (25). Numerous genes involved in the regulation of pseudo-filamentous growth in *S. cerevisiae* have been identified (3, 15, 45, 52). These studies identified three pathways that couple afferent signals to cellular switches. The major pathway is the *STE* or mitogen-activated protein (MAP) kinase pathway, which mediates the mating pheromone response. In this pathway, at least four components participate in induction of filamentous growth of diploid cells and invasiveness of haploid cells (40). The second pathway is the cyclic AMP (cAMP) pathway (42), in which Ras2p and protein kinase A (Tpk2p) have prominent roles. The third pathway is less well understood and may involve the Rim101p zinc finger transcription factor (38). However, because *S. cerevisiae* does not display true hyphal growth, several issues could not be properly ad-

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**MATERIALS AND METHODS**

*Yeast strains and microbial techniques.* The *Y. lipolytica* isogenic strains used in this study were W29 [MATa] and P01a [MATα, ura3-302, leu2-270] (4). They were grown on YPD (4) or on YNB medium composed of 1.7 g of yeast nitrogen base (Difco) per liter without amino acids or ammonium sulfate. When required,
pools of mutagenized *Y. lipolytica* transposon that carries the randomly mutagenized in lines (unpublished observations). We used a W29 genomic library (ca. 2 kb) chosen for mutagenesis because its dimorphic phenotype is stronger than inbred efficiency of the library to 5(DMSO) solution (1:10 in water) was added to 200 μl acetate method with minor modifications (49): 2 release the transposed inserts. They were used to transform PO1a by the lithium Sequencer. The GCG package (Genetics Computer Group, University of Wisconsin 9600 was used for PCRs. Sequencing was carried out on an ABI 373 DNA MegaPrime kit (Amersham Pharmacia Biotech). A Perkin-Elmer Thermal Cy a cassette derived from the mutated locus and containing the copy of mTnYl1 in the mutants was checked by Southern blot analysis with mTnYl1 as a probe. In step 4, the wild-type locus was disrupted with a cassette derived from the mutated locus and containing the *YURA3* gene as a marker, and the phenotype was verified.

1 g of glutamate per liter, 10 g of glucose per liter, 50 μg of uracil per ml, and 50 μg of leucine per ml were added. Hyphal induction was tested with a mixture of 1% serum (horse serum; Sigma) and 2% agarose. Agar invasion tests were carried out as previously described (67). All cultures were grown at 28°C, except as otherwise stated. *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani medium supplemented with 100 μg of ampicillin per ml when required.

**Genetic techniques.** Standard molecular genetic techniques were used (58). Restriction enzymes and polymerases were supplied by Gibco BRL (France) or New England Biolabs. Genomic DNA was prepared from yeast transformants as previously described (4). DNA was digested with *Sac*I, separated on a 0.8% agarose gel, and transferred onto Hybond-N+ nylon membranes for Southern blotting (Amersham Pharmacia Biotech). Probes were labeled with the ECL (enhanced chemiluminescence) Direct Nucleic Acid Labeling and Detection blotting (Amersham Pharmacia Biotech) or with [*32P]*dCTP by use of the MegaPrime kit (Amersham Pharmacia Biotech). A Perkin-Elmer Thermal Cycler 9600 was used for PCRs. Sequencing was carried out on an ABI 373 DNA Sequencer. The GCG package (Genetics Computer Group, University of Wisconsin, Madison) was used for sequence analysis.

**Construction of *Y. lipolytica* mutants.** PO1a, a wild-type strain derivative, was chosen for mutagenesis because its dimorphic phenotype is stronger than inbred lines (unpublished observations). We used a W29 genomic library (ca. 2 kb) randomly mutagenized in *E. coli* by mTnYl1 (49, 57). mTnYl1 is a Tn5-based transposon that carries the *Y. lipolytica* gene *YURA3* as a selective marker. Four pools of mutagenized *Y. lipolytica* DNA were digested separately with *Sac*I to release the transposed inserts. They were used to transform PO1a by the lithium acetate method with minor modifications (49): 2 μl of a dimethyl sulfoxide (DMSO) solution (1:10 in water) was added to 200 μl of competent cells before addition of DNA. The addition of DMSO slightly increased the transformation efficiency of the library to 5 × 10^3 to 5 × 10^4 transformants per μg of DNA. Transformed cells were plated on YNB medium supplemented with 0.2% Casamino Acids (Difco), but without uracil.

**Isolation and characterization of disrupted loci.** Chromosomal fragments flanking mTnYl1 insertion sites were amplified by reverse PCR (68) on genomic DNA digested with *Sac*I, with either the *mnt1/juan1* or *mnt6/juan2* primers (Fig. 1) and the Expand Long Template PCR system (Boehringer Mannheim GmbH). The following PCR cycling conditions were used: 2 min at 94°C, followed by 10 cycles of 10 s at 94°C, 30 s at 56°C, and 10 min at 68°C; 20 cycles of 10 s at 94°C, 30 s at 56°C, and 10 min at 68°C with 15 s of ramping; and a final extension step of 15 min at 68°C. Each PCR product was sequenced with the *mnt1* and *mnt6* primers, and the sequence of the disrupted locus was assembled after trimming one of the 5-bp repeats created by transposition. The sequence on both sides of mTnYl1 was extended by primer walking on both strands. The following web pages were used for blastx analysis and to search for open reading frames (ORFs), respectively: http://www.ncbi.nlm.nih.gov/blast/blast.cgi and http://www3 .nci.nih.gov/gorf/gorf.html.

**Construction of a plasmid expressing the site-specific recombinase Cre.** The Cre recombinase mediates site-specific excision of DNA flanked by a pair of *loxP* sites (59). To excise the *YURA3* marker from mTnYl1 integrated in the *Y. lipolytica* genome (Fig. 1), we designed the replicative plasmid pRRQ2 carrying the *LEU2* marker and expressing the CRE gene. A hybrid promoter, hpl4d, was excised from pDNA1269 by digestion with KpnI and SalI (Table 1) and ligated into pDNA1053 that had been cut with *Kpn*I and *Sal*I and used to yield pRRQ1. Next, the CRE gene from pSH47 (kindly provided by J. H. Hegemann) was excised by digestion with KpnI and SmaI and ligated into pRRQ1 that had been digested with *Kpn*I and *Pml*I to yield pRRQ2.

**Other plasmid construction.** pCT246, carrying *HOY1* and *YLEU2*, pMGF1, carrying *YPHD1* and *YILEU2*, and p1.69, a pBluescript vector (Stratagene, La Jolla, Calif.) carrying *YPHD1*, were kindly provided by A. Dominguez. To construct pMR4 carrying *YPHD1* and *YURA3*, the *Apa*I-*Sac*I-digested fragment of p1.69, containing *YPHD1*, was ligated into pDNA240 that had been digested with *Apa*I and *Bgl*I by using an XhoI-*Bgl*II adapter. To construct pRRQ10, carrying *HOY1* and *YURA3*, the *HOY1* gene was obtained as two fragments by digesting pCT246 with BamHI-XhoI and EcoRI-XhoI, and both fragments were ligated into BamHI-EcoRI-digested pDNA444.

**Construction of disruption cassettes.** Different strategies were used. (i) In the case of Fil316, *YURA3* was inserted between the mTnYl1 flanking regions at the disrupted locus. The fragments were amplified by PCR with PO1a genomic DNA. Two oligonucleotides, 3161up and 3161dw (Table 1), were used to amplify a *Cla*I-ended 760-bp fragment. The *YURA3* gene was extracted from pKSURA (Table 1) by digestion with *Ava*I and *Cla*I. These three DNA fragments were ligated and amplified with 3161up and 3162dw to generate a single DNA fragment. The same strategy was used for Fil22 and resulted in a 2.5-kb fragment corresponding to *YURA3* flanked by the 710 bp and...
TABLE 1. Characteristics of plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pBluescript</td>
<td>CoeI lacZ bla</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pG209</td>
<td>1 kb of the YIGF7 gene in pGEM-T easy</td>
<td>This study</td>
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<tr>
<td>pG209URA</td>
<td>YURA3 gene in pG209</td>
<td>This study</td>
</tr>
<tr>
<td>pG354/CRE</td>
<td>1.3 kb of fil354 locus after mTnYl1 excision in pGEM-T easy</td>
<td>This study</td>
</tr>
<tr>
<td>pG354/CRE/URA</td>
<td>YURA3 gene in pG354/CRE</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T easy</td>
<td>ori F1 lacZ bla</td>
<td>Promega Corp., Madison, Wis.</td>
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<tr>
<td>pINA156</td>
<td>YURA3 in pUC13</td>
<td>This laboratory collection</td>
</tr>
<tr>
<td>pINA240</td>
<td>YLEU2 ARS68 in pBR322</td>
<td>This laboratory collection</td>
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<td>pINA444</td>
<td>YLEU2 ARS68 in pBR322</td>
<td>This laboratory collection</td>
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<td>pINA1056</td>
<td>YLEU2 ARS68 lacZ</td>
<td>This laboratory collection</td>
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<tr>
<td>pINA260</td>
<td>YLEU2 ARS68 PHO5 promoter XPR2 terminator</td>
<td>This laboratory collection</td>
</tr>
<tr>
<td>pICT246</td>
<td>YLEU2 ARS18; HOY1 with its own promoter</td>
<td>66</td>
</tr>
<tr>
<td>pKSURA</td>
<td>YURA3 in pBluescript</td>
<td>70</td>
</tr>
<tr>
<td>pMGFI</td>
<td>YLEU2, YPHD1 in pBluescript</td>
<td>A. Dominguez laboratory collection</td>
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<tr>
<td>pMR2</td>
<td>0.9-kb fragment of YURA3 gene containing I-SceI site in pGEM-T easy</td>
<td>This study</td>
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<td>pMR3</td>
<td>2.1-kb fragment containing YURA3 in pMR1 at I-SceI site</td>
<td>This study</td>
</tr>
<tr>
<td>pMR4</td>
<td>YPHD1 from p1.69 in pINA240</td>
<td>This study</td>
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<tr>
<td>pMR5</td>
<td>2.5 kb of the fil23 disruption cassette in pGEM-T easy</td>
<td>This study</td>
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<tr>
<td>pP209</td>
<td>0.8-kb fragment containing promoter from p1269 in pINA1053</td>
<td>This study</td>
</tr>
<tr>
<td>pPRQ2</td>
<td>1-kb fragment containing CRE from pSH47 in pPRQ1</td>
<td>This study</td>
</tr>
<tr>
<td>pPRQ10</td>
<td>HOY1 from pCTDin in pINA444</td>
<td>This study</td>
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<td>pSH47</td>
<td>YURA3 ARS4643664 CRE under GAL1 promoter and CYC1 terminator</td>
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<td>pUCURA</td>
<td>1.2-kb fragment of YURA3 from pINA156 in pNEB193</td>
<td>This study</td>
</tr>
<tr>
<td>p1.69</td>
<td>HOY1 gene in pBluescript</td>
<td>A. Dominguez laboratory collection</td>
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610 bp that flanked mTnYl1 in Fil23. (ii) In the case of Fil345, two primer pairs (3451upL/3451dwSceI and 3452upSceI/3452dwL) were used to amplify mTnYl1-flanking regions (630- and 300-bp DNA fragments, respectively). These were annealed through the I-SceI site and digested by PCR amplification. The resulting fragment was ligated into pGEM-T easy to yield pMR2. The YURA3 gene was excised from pKSURA by I-SceI digestion and ligated into pMR2 that had been digested with I-SceI to yield pMR3. pMR3 was digested with EcoRI to release the 2.1-kb disruption cassette. (iii) In the case of fil209, two primers, 209DEL1 and 209DEL2, were used to amplify a 1-kb DNA fragment of the PO1a genome containing the integration locus, which was ligated into pGEM-T easy to yield pG209. YURA3, rescued from pINA156 by digestion with XhoI and NotI, was inserted into the cloned locus at the naturally occurring pml1 and XhoI restriction sites, yielding pG209Ura. A 2.2-kb disruption cassette was generated from pG209Ura by use of 209DEL1 and 209DEL2. Similarly, in the case of fil246, two oligonucleotides, 246DEL1 and 246DEL2, were used to amplify a 2-kb fragment of PO1a that spanned the insertion site. The YURA3 gene was extracted from pINA156 by digestion with NotI, and was reinserted into pINA156 by digestion with PstI and EcoRI. These fragments were separately digested into the 2.4-kb PCR fragment that had been digested with pml1. The ligation products were amplified with 246DELI/URA3-A and 246DELI/URA3-B, digested with XhoI, which is unique within YURA3, ligated together, and amplified by PCR with 246DELI and 246DEL2 to yield the 3.2-kb disruption cassette. (iv) The fourth strategy involved transforming fil354 with pPRQ2 to excise most of mTnYl1 (Fig. 1), rendering the strain fil354/CRE Ura-. Two oligonucleotides, 354DEL1 and 354DEL2, were used to amplify a 1.3-kb fragment from fil354/CRE genomic DNA with unique BamHI and KpnI sites in the genomic DNA next to an mTnYl1 insertion. This fragment was ligated in pGEM-T easy to yield pG354/CRE. A KpnI-BamHI fragment carrying YURA3 was ligated into BamHI- and KpnI-digested pG354/CRE to yield pG354/CRE/URA. Amplification with 354DEL1 and 354DEL2 yielded a 2.5-kb disruption cassette.

These cassettes were used to transform PO1a by homologous recombination. All disruptions were confirmed by Southern blot analysis.

Staining procedures. Chitin was stained with Calcofluor white (1 mg/ml) in the dark at room temperature for 30 min on log-phase cells that had been washed twice in distilled water. After four washings, the cells were suspended in one drop of immunofluorescence mounting solution (100 mg of p-phenylenediamine in 10 ml of phosphate-buffered saline and 90 ml of glycerol) for observation.

Nucleotide sequence accession number. Sequences were deposited in the GenBank database under accession no. AF321446, AF321465, AF321466, AF321467, AF321468, and AF321469.

RESULTS

Isolation and preliminary characterization of mutants affected in morphology. PO1a was mutagenized by use of the mTnYl1 transposed-genomic DNA library of Y. lipolytica (see Materials and Methods). After 7 days, approximately 40,000 transformants were screened visually for smooth colonies; candidate mutants were rechecked after 2 weeks of growth on media.

TABLE 2. Oligonucleotide sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3161up</td>
<td>AATCTCACGTCGCGATGTCC</td>
</tr>
<tr>
<td>3161dw</td>
<td>AATAGCCATGGTCGTTTCATCTATCCTAGTGCC</td>
</tr>
<tr>
<td>3162up</td>
<td>AATAGCATCGAATAGTCCTATCACCTGCTTGCCG</td>
</tr>
<tr>
<td>3162dw</td>
<td>GTAGAAAGCCGATGAAATG</td>
</tr>
<tr>
<td>3451upl</td>
<td>GCTTACCGGTTATAAGTGC</td>
</tr>
<tr>
<td>3451dwScel</td>
<td>CATTACCGTTATJTCCTCCAATAGCATAAAGGTT</td>
</tr>
<tr>
<td>3452upSceI</td>
<td>CTAGGGATACAGGTTATTTGAGGCAGTGCC</td>
</tr>
<tr>
<td>209DEL1</td>
<td>TCGTAGCTGAC</td>
</tr>
<tr>
<td>209DEL2</td>
<td>TTGTTGTTGTTGGAGGCTGTGCAC</td>
</tr>
<tr>
<td>3161up</td>
<td>GACATAGTTAAGTCGGTTCCTGCC</td>
</tr>
<tr>
<td>3161sp</td>
<td>GATGCTTCTTGGAAAGAAGAAG</td>
</tr>
<tr>
<td>3161dw</td>
<td>CCCCTCATATCTCCTATCACCTAC</td>
</tr>
<tr>
<td>246DELI</td>
<td>CGATAGTCGACGCTACCTGGTCGTC</td>
</tr>
<tr>
<td>246DEL2</td>
<td>GGTCGTCGAGTTGGAAGAATAAGC</td>
</tr>
<tr>
<td>URA3-A</td>
<td>CCACCAAATAAGCTTCC</td>
</tr>
<tr>
<td>URA3-B</td>
<td>TGGTAGTACGTTGGTG</td>
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<tr>
<td>354DEL1</td>
<td>CACCCCTACAAACAAAATGAC</td>
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<td>354DEL2</td>
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<td>mtn2</td>
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<tr>
<td>juan1</td>
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<tr>
<td>juan2</td>
<td>CTCAAGCTCGTGCCAC</td>
</tr>
<tr>
<td>Adapter XhoI</td>
<td>CTTAGAGAACCCCCCTCG</td>
</tr>
<tr>
<td>Adapter PstI</td>
<td>TCTGGGGAAGCTTAG</td>
</tr>
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</table>

* Underlined segments represent restriction sites used for cloning.
YPD. A total of 99 mutants with defects in morphogenesis were retained. These mutants were screened a third time after 16 h on 1% serum solid medium; this caused PO1a to switch to the mycelial form immediately (Fig. 2A). Fifty mutants with a clear Fil2 phenotype were finally retained.

The mutants were analyzed in three steps (Fig. 1). First, we sequenced the fragments flanking the transposon after reverse PCR (see Materials and Methods). Second, to check whether the disruption cassette integrated by a single homologous recombination event, we designed primer pairs for each locus that annealed approximately 200 bp either side of the transposon integration site (Hup and Hdw [see Fig. 1]) and amplified the target locus from mutant and PO1a genomic DNA. If nonhomologous recombination had occurred, two fragments were amplified: a small fragment (400 to 500 bp) corresponding to the undisrupted wild-type locus and a larger fragment (>5 kb) corresponding to the disrupted locus. If homologous recombination occurred, a single fragment (>5 kb) was amplified (Fig. 1). Third, we checked whether a single copy of the transposon was present in the mutants, by using the transposon as a Southern blot probe.

The first step rejected 11 mutants because no PCR product was obtained for one or both sides of the transposon. The remaining 39 mutants were sequenced, and 7 were found to contain part or all of the plasmid pHSS6 integrated in the genome, suggesting illegitimate integration. Finally, two mutants were dismissed because inverted 5-bp repeats did not flank the transposon, indicating rearrangement during integration.

In the second step, PCR was used to test the 30 remaining mutants for homologous integration. Only 11 mutants yielded the expected pattern; Southern blotting showed that they all resulted from a single integration of mTnYl1 (data not shown).

Preliminary analysis of the sequences flanking mTnYl1 showed that two mutants resulted from independent disruptions of the XPR6 locus, i.e., the Y. lipolytica KEX2 homolog, which is involved in morphogenesis (18), and no ORF could be detected at the loci disrupted in the Fil34 and Fil78 mutants. These four clones were not analyzed further.

Identification of the disrupted genes. To confirm that the Fil2 phenotype resulted from an mTnYl1 insertion and not from a secondary mutation, we tried to redisrupt each of the six loci in PO1a. Different approaches involved either whole genomic DNA from the transposants or amplified mTnYl1-disrupted loci by PCR. These attempts resulted in a high frequency of nonhomologous integration events, so we finally constructed YlURA3 disruption cassettes (see Materials and Methods). At least five independent transformants resulting from homologous integration were analyzed in each case. The phenotypes of the original mutants and of the YlURA3 disruptants were identical for Fil23, Fil209, Fil316, Fil345, and Fil354, but not for Fil243, which was thus discarded. When we

FIG. 2. Mutant phenotypes. (A) Colony phenotype of PO1a and Fil2 mutants incubated for 2 days on serum medium at 28°C. (B) Cell phenotype of six mutants and PO1a incubated for 2 days on serum medium at 28°C. (C) Invasive phenotype of PO1a and of three mutants incubated for 2 days on YPD medium at 28°C. Results are shown for plates before and after being washed with sterile water.
tried to construct the YLURA3 disruption cassette in Fil51, the expected PCR products were not obtained, possibly because the corresponding ORF is part of a conserved gene family (see below). Although we could not confirm that its Fil\(^+\) phenotype was linked to mTNYl1, we tentatively retained Fil51.

Nucleotide sequences flanking mTNYl1 in Fil23, Fil209, Fil316, Fil345, Fil354, and Fil51 were obtained over 400 to 600 bp on both sides and assembled to reconstitute the disrupted ORFs. Preliminary analysis of these partial amino acid sequences suggested that mTNYl1 disrupted the following ORFs. Preliminary analysis of these partial amino acid sequences (Fig. 3D) showed that a coiled-coil region (amino acids 390 to 630) homologous to cytoskeletal proteins was highly conserved. Studies of Y. lipolytica BUD6 deletion mutants indicated that Bud6p is important for bipolar budding, from S. cerevisiae homologs: Bud6p for Fil23, Pph21p for Fil51, Gpi1p for Fil209, Snf5p for Fil316, Ras2p or Ras1p for Fil345, and Cdc25p or Sdc25p for Fil354. None of the disrupting events resulted in an in-frame fusion of green fluorescent protein (GFP), except for YLUBUD6, but no GFP expression could be detected in this case.

The results of all of the phenotypic tests undertaken with the six mutant strains (see below) are summarized in Table 3.

The Y. lipolytica BUD6 homolog (YLBUD6). We sequenced 1,980 nucleotides (nt) of the YLBUD6 gene corresponding to 660 amino acids in the N-terminal region of the protein. We found that the amino acid sequence displayed 37\% identity to the amino acid sequence of Bud6p, an actin-interacting protein required for bipolar budding, from S. cerevisiae (2, 71). Comparison of the predicted Y. lipolytica and S. cerevisiae amino acid sequences (Fig. 3D) showed that a coiled-coil region (amino acids 415 to 445 and amino acids 582 to 615) within a large domain (amino acids 390 to 630) homologous to cytoskeletal proteins was highly conserved. Studies of S. cerevisiae BUD6 deletion mutants indicated that Bud6p is important for the maintenance of cell shape, bud site location, and polarized growth (2, 61). Deletion of YLBUD6 results in the same morphological defects: cells were rounder than in the wild type, and there were no septa. Moreover, deleted cells form an aberrant pseudomycelium with many buds per cell on YPD medium and no pseudomycelium or hyphae on serum inducing media (Fig. 2B). Cultures lost the typical invasive growth phenotype on YPD medium (Fig. 2C). Diffuse chitin staining was observed in most mutant strains and especially in chain cell structures, but was not localized in the bud neck (Fig. 3A). Staining with rhodamine-phalloidin revealed few or no actin patches (not shown), suggesting that, like in S. cerevisiae, YLBUD6 is involved in actin localization. Also like in S. cerevisiae, mutant cells were thermosensitive (Fig. 3B) and sensitive to Calcofluor white on solid medium (Fig. 3C), indicating cell wall defects.

The Y. lipolytica RAS2 homolog (YIRAS2). The complete YIRAS2 sequence encodes a predicted protein of 249 amino acids displaying 43\% identity to Ras2p and 40\% identity to Ras1p of S. cerevisiae. The YIRAS2 gene contains a 348-bp intron inserted at nt 53 of the ORF, with typical Y. lipolytica splicing sites: 5’-GAGTAG-3’, 5’-TCTAACA-3’, and 5’-CA G-3’ for the donor, internal, and acceptor sites, respectively (54, 64). S. cerevisiae possesses two Ras homologs, RAS1 and RAS2. The complete predicted amino acid sequence for the disrupted ORF in Fil345 is slightly more similar to Ras2p than to Ras1p (see above) and is, therefore, tentatively referred to as Ras2p. The first 170 amino acids of the N-terminal domain of YIRas2p are nearly 90\% identical to Ras homologs from other organisms, and the last 4 amino acids, CAV1, conform to the CAAX consensus, which is crucial for the processing of Ras and its anchorage to the plasma membrane (33). The guanine nucleotide-binding site, GXXXXGK (residues 17 to 23 for Ras1p and Ras2p in S. cerevisiae), is also found (GEGG TGK) in YIRas2p between residues 15 and 21. Mutant cells disrupted for YIRAS2 display a complete lack of formation of pseudohyphae or hyphae on YPD or on serum inducing medium (Fig. 2B) and do not invade agar (not shown), but no particular pattern was seen after chitin staining. These cells were neither Calcofluor white nor temperature sensitive.

The Y. lipolytica CDC25 homolog (YICDC25). We sequenced 4,518 nt of YICDC25 corresponding to the 1,506 C-terminal amino acids of the protein, including the conserved GTP exchange factor (GEF) domain that was disrupted by mTNYl1. The predicted amino acid sequence of 1,506 amino acids is 33 and 31.5\% identical to Cdc25p and Sdc25p S. cerevisiae proteins, respectively. Although the gene nomenclature is somewhat arbitrary in the absence of the whole gene sequence, we chose YICDC25 because SDC25 mutations have no obvious phenotype in S. cerevisiae in the presence of a wild-type copy of CDC25 (9). Cells disrupted for YICDC25 do not undergo pseudohyphal or hyphal transition on YPD or on serum medium (Fig. 2) and are not invasive (data not shown). Chitin staining and Calcofluor white and temperature sensitivity were similar in the mutant and in the wild-type strain.

The Y. lipolytica SNF5 homolog (YISNF5). The complete sequence of YISNF5 encodes a predicted protein of 735 amino acids with 31\% identity to Snf5p (905 amino acids) of S. cerevisiae. The N terminus (bp 31 to 324) of Snf5p is rich in glutamine (42\%) and proline (11\%), whereas the N-terminal sequence of YISnflp (bp 1 to 222) is rich in glutamine (28\%), glycine (25\%), and methionine (21\%). Such domains may have a transcriptional activator role, although this region can be greatly reduced in size without loss of Snf5p function. Indeed, in S. cerevisiae, the N-terminal glutamine- and proline-rich region could be trimmed down from 125 Gln and 37 Pro residues to 15 Gln and 9 Pro residues, without any effect on Snf5p function (37). Comparison of Snf5p from S. cerevisiae, humans, and Y. lipolytica revealed that two imperfect repeat motifs spanning amino acids 389 to 436 and 477 to 536 in YISnflp were conserved. The roles of these repeat motifs have not yet been elucidated. In Y. lipolytica cells with YISNF5 deleted, neither pseudofilament or filament formation nor invasive growth could be observed on inducing medium (Fig. 2B). ΔYISnfl cells and wild-type strains had similar chitin staining patterns and temperature sensitivities. A slight sensitivity to Calcofluor white was reproducibly observed only at the highest concentration tested (Fig. 3C).
The *Y. lipolytica* PPH21 homolog (*YIPPH21*). The complete sequence of *YIPPH21* encodes a predicted protein of 380 amino acids with 74% and 72.5% identity to Pph21p and Pph22p of *S. cerevisiae*, respectively, and 82% identity over the last 250 amino acids. However, like in mammals or *Aspergillus fumigatus*, the N-terminal 60-acidic-amino-acid stretch is absent. Cells carrying Δ*Ylpph21* stay in the yeast form on inducing medium, but retain invasive growth (Fig. 2B and C). This indicates that
pseudomyceiliation and myceiliation are not necessary for agar invasion, as suggested for S. cerevisiae (46). No difference was observed between mutant and wild-type cells for chitin staining nor for Calcofluor white and temperature sensitivity.

The Y. lipolytica GPI7 homolog (YlGPI7). The complete sequence of YlGPI7 encodes a predicted protein of 860 amino acids with 33% identity with Gpi7p (830 amino acids) of S. cerevisiae. Like Gpi7p/LAS21, YlGPI7 is predicted to encode an integral membrane protein. Six to eight transmembrane domains (TDs) and three putative glycosylation sites are predicted, which are fewer than the numbers found in Gpi7p, which has 9 to 11 TDs and five putative glycosylation sites. No conserved motifs were identified. YlGPI7 deletion mutants are non-filamentous on YPD and serum medium and display a reduced invasive growth (Fig. 2B and C). Chitin and actin staining patterns and temperature sensitivities were similar in the mutant and wild-type cells. We noticed that ΔYlgpi7 cells are sensitive to Calcofluor white (data not shown), whereas conflicting data were reported for similar mutants in S. cerevisiae (5, 65).

Suppression of filamentation defects by HOY1 and YIPHD1. HOY1 and YIPHD1 are known to be involved in morphogenesis in Y. lipolytica. HOY1 is a homeo gene that is required for hyphal formation (66) on solid and liquid media. It appears to be a strong suppressor of morphogenesis defects in several Y. lipolytica mutants. YIPHD1 is homologous to PHD1 of S. cerevisiae (A. Dominguez, personal communication), which encodes a transcription factor that is involved in the regulation of filamentous growth (24) downstream of the cAMP pathway. Both genes were introduced into mutant strains on replicative, centromeric plasmids, which results in multicopy suppression in several instances in Y. lipolytica (7, 28). The introduction of YIPHD1 into ΔYlras2 mutant cells only restored pseudomyceiliation on inducing medium (Fig. 4). In agreement with the proposed role of these genes in S. cerevisiae, this may suggest that both genes act in the same pathway, although it does not prove it. Conversely, the introduction of YIPHD1 into cells carrying ΔYlsnf5, ΔYlbufd6, ΔYlcdc25, and ΔYlgpi7 did not affect morphogenesis on inducing medium. However, the introduction of HOY1 into Ylsnf5 and Ylgpi7 deletion mutants restored hyphal formation on inducing medium (Fig. 4), whereas it had no effect on other mutants.

DISCUSSION

Fungal dimorphism has received increasing attention because of its potential as a simple experimental model for eukaryotic cell differentiation and its implication in pathogenesis (44). Our current knowledge of the control of this morphological transition is limited to the molecular characterization of the main signaling pathways in yeasts and is mostly from studies of S. cerevisiae (25). Here, we investigated the yeast Y. lipolytica as an alternative model for dimorphism studies.

We used a transposon tagging approach to facilitate the identification of the genes involved in morphogenesis, because earlier studies experienced difficulties in sorting suppressors from cognate genes when morphogenetic mutants were complemented by genomic libraries (66; unpublished results). We screened 40,000 independent transformants, which is equivalent to two insertions per kilobase of genomic DNA if random mutagenesis occurs and given that the genome of Y. lipolytica is 20 Mb (13). We obtained 50 clones with clearly defective filament formation. This is equivalent to approximately 0.1%, which is similar to what was reported in a study performed with S. cerevisiae (56 mutants out of 100,000 transformants) (46). This indicates that many genes are involved in morphogenesis and that our current screen was not exhaustive. Accordingly, we found one case in which the same gene (XPR6) was independently interrupted twice. Identification of the disrupted loci by reverse PCR was difficult, because only 30 out of 50 mutants could be analyzed. Other approaches, such as rescuing flanking sequence after integration of an E. coli plasmid in mTnY1 (57), might be more efficient. Further tests showed that only 11 of the 30 mutants resulted from homologous integration. This high level of nonhomologous integration was unexpected in view of our previous results (4) and may reflect strain differences, locus dependence, or changes in the transformation protocol, such as the addition of DMSO to competent cells. Eight Fil– mutants out of the 11 analyzed identified seven different genes, whereas the mutation appeared to be unlinked to mTnY1 in three cases, which is similar to what was reported in S. cerevisiae (6 out of 45) (46). Seven different ORFs were thus identified, all with clear homologs in S. cerevisiae: RAS2, BUD6, SNF3, PPH21, CDC25, GPI7, and XPR6. Thus, transposon mutagenesis is a valuable tool in Y. lipolytica, but was complicated by the high frequency of illegitimate integration and the inefficient rescue of flanking sequences.

In S. cerevisiae, the dimorphic switch is controlled by at least three signaling pathways: the mating type or STE pathway (30, 43), the cAMP pathway (48), and the pH signaling or RIM101 pathway (38). C. albicans contains homologs of genes from...
each of these pathways, which all participate in cellular differ-
entiation (11), and other signaling pathways, such as osmosens-
ing (1) or sensing of microaerophilic conditions (62). Interest-
ingly, blocking a single pathway in either S. cerevisiae or C.

albicans never suppresses cellular differentiation under all con-
ditions. The situation appears to differ in Y. lipolytica. First,
unlike in S. cerevisiae, both haploid and diploid Y. lipolytica
forms can undergo the switch from yeast to hyphae and can
invade agar, and unlike C. albicans, they are not temperature
and pH sensitive (17). Second, previous analyses showed that
the RIM and STE genes are not required for agar invasion
or myceliation, contrary to what is found in both S. cerevisiae
and C. albicans (17, 67). Accordingly, we did not identify any
components from these pathways in our screen. Third, the
YIP1p transcriptional factor appears to act as a repressor of
the yeast form, like in C. albicans, but unlike in S. cerevisiae,
in which Tup1p is required for pseudomycelliation (10, 17).
Because two transcriptional factors (Hoy1p and Mhy1p) that do
not have any clear homologs in C. albicans or in S. cerevisiae
have been found to be essential for the dimorphic transition in
Y. lipolytica (32, 66), it was unknown whether a conserved
pathway controlling morphogenesis was conserved in the three
yeasts.

In this study, we identified seven genes: five that were al-
ready known to affect morphogenesis in S. cerevisiae and two
new ones, YISNF5 and YIGPI7.

The role of the cAMP pathway in Y. lipolytica morpho-
genesis had not been assessed previously. We identified two genes,
YIRAS2 and YICDC25, that are involved in this pathway. It
should be stressed that the gene nomenclature is arbitrary at
this stage, because we have no evidence for a second RAS
gene in Y. lipolytica or for a YISDC25 paralog. In S. cerevisiae,
Cdc25p is an essential GDP/GTP exchange factor (GEF) for
Ras2 (8). CDC25 null mutants are lethal in S. cerevisiae, but
homozygous CDC25 deletion mutants are viable in C. albi-
cans, although the strains have a partial defect in hyphal forma-
tion (19). Similar functions appear to be conserved in Y. lipolytica
and C. albicans, because the inactivation of YICDC25 prevents
invasion of the agar and formation of pseudomyelium and
hyphae. Activation of Ras2p of S. cerevisiae enhances pseudo-
hylal growth in the diploid and regulates invasive growth in
the haploid through both the STE and cAMP pathways (47,
48). Although there seems to be a single RAS gene in C. albi-
cans, homozygous deletion mutants are viable, unlike RAS1
RAS2 deletion mutants in S. cerevisiae, and are defective in
hyphal formation but not in pseudomyelium formation (22).
Y. lipolytica phenotypes are much stronger, because YIRAS2
deletion mutants cannot invade agar and do not form pseudo-
myelium or hyphae. Thus, our results with YICDC25 and
YIRAS2 indicate that the Ras2p-adenylate cyclase pathway is
a major pathway in Y. lipolytica morphogenesis.

Expression of the downstream transcriptional factor, YILP1p,
on a replicative plasmid led to partial suppression of the
YIRAS2 defect, restoring formation of pseudomyelium, but
not of hyphae. It had no effect in a strain in which the GEF
domain of YICdc25p was interrupted. This may reflect insuf-
sicient overexpression of YILPHD1, the existence of other path-
ways that are required for full activation of hyphal growth,
or both. Further work will thus be needed to confirm that
YILP1p is indeed a target of the cAMP pathway.

The switch from yeast to pseudomyelium is accompanied by
a switch in bud site selection in S. cerevisiae haploids and
diploids (46) and in C. albicans (29). In clear contrast, both
haploid and diploid cells of Y. lipolytica constantly bud in a
bipolar manner when in the yeast form or during germ tube
emission (29). One of our mutants was affected in a BUD6
homolog and exhibited severe defects in actin patch localiza-
tion, bud site selection, and cytokinesis and was unable to form
hyphae. BUD6 is required in diploid S. cerevisiae cells for
bipolar budding and interacts with the bud tip and neck during
spindle morphogenesis (60). Our results confirm that bipolar
budding is essential for correct cell division of haploids in both
the yeast and hyphal forms in Y. lipolytica. They also indicate
that bipolar budding and normal spindle organization are re-
quired for pseudomyeliation and for agar invasion.

Disruption of the YIXPR6 and YIGPI7 genes resulted in
marked deficiency of hyphal formation. Both gene products
modify exported proteins and are probably required for the
biogenesis of critical cell wall components. Consistent with this
hypothesis, KEX2 mutants show abnormal chitin deposition
(35). Both Y. lipolytica and C. albicans strains devoid of Xpr6p/
Kex2p activity have defects in pseudomyelium formation, but
still invade agar, whereas they completely fail to form hyphae
(18, 50). Possible targets are precursors of hypha-specific, cell
wall-associated proteins, such as Hwp1p (63), or proteins with
general cell wall biogenesis activities, such as exo-β-(1-3)-glu-
canases (14, 21), which all require Xpr6p/Kex2p processing.
Conversely, GPI7 is required to produce a functional glyco-
sphosphatidylinositol (GPI) anchor in S. cerevisiae (6), by
adding a side chain to the core structure (5). Over 58 GPI-
anchored proteins have been predicted in S. cerevisiae (12),
most of which are attached to the cell wall and some of which
are required for invasive growth and pseudomyeliation (27).
YIGPI7 mutated cells have defective cell wall biogenesis, as
suggested by their Calcofluor white sensitivity, and they show a
strong defect in formation of pseudohyphae and hyphae and a
partial defect in agar invasion. In Y. lipolytica, a YIGPI7 muta-
tion is partially suppressed by overexpression of the transcrip-
tional factor Hoy1p. This suggests that the expression of
members of GPI family genes through Hoy1p activation res-
cues YIGPI7 defects. Our results with YIXPR6 and YIGPI7
confirm that the biogenesis of extracellular proteins, possibly
cell wall associated, is critical for morphogenesis in yeasts.

The two remaining genes identified in our screen, YISNF5
and YIPPH21, probably exert even more pleiotropic effects.
S. cerevisiae Snf5p belongs to the SWI-SNF complex, which
is a large complex of 2,000 kDa and is highly conserved in all
eukaryotes (55). Snf5p is required for the functioning of a
variety of sequence-specific transcriptional factors (37), maybe
through remodeling of chromatin structure (31). Until now,
affinity of YIXPR6 and YIGPI7
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the situation appears to differ in Y. lipolytica. First,
unlike in S. cerevisiae, both haploid and diploid Y. lipolytica
defects in PPH21 did not result in the delocalization of actin patches, which are normally localized at bud and hyphal tips (36), or in increased sensitivity to Calcofluor white.

Taken together, our results strongly suggest that conserved elements control morphogenesis in distantly related yeasts. They also show that genes hitherto not recognized in S. cerevisiae as involved in morphogenesis are required in Y. lipolytica. Interestingly, both YIGPI7 and YISNF5 are partially suppressed by overexpression of HOY1, a putative transcriptional regulator without a clear homologue in S. cerevisiae (66). Understanding how these new players fit into the general picture may provide new light on fungal dimorphism.

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