The Opi1p Transcription Factor Affects Expression of FLO11, Mat Formation, and Invasive Growth in Saccharomyces cerevisiae†

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Mat formation in the bakers’ yeast Saccharomyces cerevisiae is a surface-associated phenomenon in which yeast cells spread over the surface of a low-density agar petri plate as a complex film. This spreading growth occurs by sliding motility and is dependent on the adhesion protein (adhesin) Flo11p. In order to identify molecular pathways that govern mat formation, whole-genome transcriptional profiling was used to compare cells growing as a mat to cells growing in a suspension culture (planktonic cells). This analysis revealed that S. cerevisiae upregulates a subset of genes in response to growth on a surface. These genes included the INO1 gene, which encodes the myo-inositol-1-phosphate synthase, which carries out the rate-limiting step in inositol biosynthesis. Further inquiry revealed that a transcription factor that controls INO1 expression, called Opi1p, participates in the regulation of mat formation. Opi1p appears to modulate mat formation by influencing the expression of FLO11. The opi1Δ mutant was found to exhibit reduced FLO11 levels. Consequently, the opi1Δ mutant perturbs the FLO11-dependent phenotype of invasive growth. The opi1Δ mutant’s defects in mat formation and invasive growth are dependent on the transcriptional activator Ino2p. These results indicate that Opi1p affects mat formation and invasive growth by participating in the regulation of FLO11.

Microorganisms in the laboratory are often studied in the context of growth as individual cells in liquid suspension cultures (planktonic growth). However, many microbes grow attached to one another as a community growing on a surface. This type of growth can be observed for colonies (43, 62), biofilms (70), and fruiting bodies (4, 29) and for some types of motility, such as sliding motility (42, 53). These “multicellular” behaviors are believed to reflect the state of many microbes in nature. Most work in this area has been done using bacteria. Some studies have been performed (14, 18, 28, 35, 43) using fungi, but much remains to be done in order to understand multicellular behavior in “unicellular” fungi such as the yeasts.

The bakers’ yeast Saccharomyces cerevisiae has been used as a powerful genetic model system for studying the yeasts. S. cerevisiae is able to generate a complex multicellular structure called a mat on the surface of low-density agar (0.3%) petri plates made with rich (yeast extract-peptone-dextrose [YEPD]) medium (called low-agar plates) (54). A mat is formed on low-agar plates through a process called sliding motility, where the cells spread as they grow over the surface of the medium (42, 53). As the mat grows, it spreads and generates patterns in the middle (called the hub), while the leading edge (called the rim) remains smooth in appearance (Fig. 1).

The morphological changes that lead to development of the rim and hub are dependent on the gene FLO11 (54). FLO11 encodes a large cell surface glycoprotein that is part of a superfamily of fungal cell surface adhesion proteins (adhesins) found in Saccharomyces and Candida species (37, 75). In S. cerevisiae, FLO11 is required for mat formation, adhesion to plastic, and filamentous growth (37, 54, 75). Haploid yeast undergoes filamentous growth when it grows as chains of cells that dig into the surface of solid 2% agar YEPD plates in a process termed invasive growth (55). Additionally, diploid yeast undergoes filamentous growth when it responds to nitrogen starvation by growing as chains of elongated cells that burrow into the agar in a process called pseudohyphal growth (21).

Multicellular behaviors of microorganisms appear to be dependent on both the nutrient environment and the surface properties of the substrate on which they are growing (36, 46). An important nutrient cue that drives mat formation in S. cerevisiae is glucose deprivation (T. B. Reynolds, A. Jansen, X. Peng, and G. R. Fink, unpublished results). In haploid bakers’ yeast, glucose deprivation also drives invasive growth on 2% agar YEPD plates (11) as well as biofilm formation on polystyrene (54).

The role that the surface plays in regulating multicellular behavior in S. cerevisiae remains to be explored. In this report, transcriptional profiling was used to compare cells grown in a mat to cells grown in liquid medium in suspension cultures (planktonic cells). This analysis revealed several genes that appear to be expressed in response to surface association. One of these genes is INO1, which is regulated by the inositol regulon. The inositol regulon consists of three transcription factors, Ino2p, Ino4p, and Opi1p, that control expression of INO1 and other phospholipid biosynthetic genes (3, 24, 31). This communication demonstrates that Opi1p is required for the full expression of FLO11. The effect of Opi1p on FLO11 expression is dependent on the transcriptional activator Ino2p.

MATERIALS AND METHODS

Yeast strains and media. All of the strains used in this study are from the yeast genetic background Σ1278b. They are all isogenic to the strain TBR1 (54). The strains and their genotypes are shown in Table 1. With the exception of the ino1Δ mutant, the mutants in this study were made by PCR-based gene disruptions using specific deletion mutants from the Yeast Knock Out Collection (Open
FIG. 1. *S. cerevisiae* forms a mat on low-agar plates. Wild-type yeast cells were inoculated on the center of YEPD plates containing 0.3% agar with a toothpick and grown at 23°C for 5 days.

BioSystems/Research Genetics) as templates (78). Each strain in the Yeast Knock Out Collection carries a gene disruption in which a particular open reading frame (ORF) is replaced by the kanMX4-disrupted ORF of interest plus ∼300 base pairs flanking either side of the ORF (Table 2). The resulting PCR product was then transformed into TBR1 by the lithium acetate transformation method (20), and transformants were selected on YEPD plates (71) containing 200 μg/ml G418 (54). PCR with primers that annealed outside of the disruption construct (Table 2) and inside the TEF promoter of the kanMX4 cassette (TR0369) (Table 2) (76) were used to confirm each construct. The *ino2Δ* mutant was made as described previously (22) using the primers listed in Table 2. The *ino2Δ opl1Δ* mutant was made by disrupting OPI1 in the *ino2Δ* mutant as described above. The *ino2Δ opl1Δ* double mutant was created by standard tetrad dissection techniques (63). Strains were maintained on YEPD plates (71), and experiments were performed on YEPD plates or in liquid YEPD medium (71) as indicated. Low-agar plates (54) (YEPD plates with only 0.3% agar) were used for mat assays, Northern blots, and microarray analysis as indicated.

Invasive growth assay. This method was performed essentially as previously described (57). Strains were streaked onto standard (2% agar) YEPD plates (71), grown at 30°C for 5 days, and photographed (25, 26). Tap water was used to wash noninvasive cells from the surface, and the plate was photographed again. Finally, under running tap water, a gloved finger was used to rub the plate and more thoroughly remove noninvasive cells, and the plate was photographed a third time.

Microarray analysis comparing mat and planktonic cells. Cells were isolated from mat cultures as follows. Mats were grown at ∼25°C on low-agar (0.3%) YEPD plates (54) for 7 days. Mats were collected from the plate by scooping up two whole mats per sample with a spoon and placing the cells in a 50-ml conical flask. Materials were inoculated on the center of YEPD plates containing 0.3% agar with a toothpick and grown at 23°C for 5 days. Wild-type yeast cells were inoculated on the center of YEPD plates containing 0.3%

Northern blotting. Northern blotting was performed as described in the work of Sambrook and Russell (59) by use of Chroma buffer for prehybridization and hybridization steps (8). A Technne HybriGene oven set to 65°C was used for all incubation and wash steps. For Northern blots of planktonically grown cells, the cells were grown in liquid YEPD medium (2% glucose) to logarithmic phase (OD<sub>600</sub> of between 0.7 and 1.0). The cells were collected by centrifugation, aspirated, flash-frozen in a bath of dry ice and methanol, and stored at −80°C. For Northern blots of mats, cells were collected with a spatula from mats grown for 5 days at 23°C and pelleted in a microfuge, and the agar was decanted. Total RNA was collected by acid-phenol extraction (32), and 10 μg of total RNA was subjected to Northern blotting. A PCR product (primers were TR0367, ATGCAAGAGCTTCTACTCG, and TRO368, TGCCAGAAGCCTTGATATTGAG) corresponding to the first 481 bp of the *FLO11* ORF was used to probe the total RNA. The probe was labeled with [α-<sup>32P</sup>]ATP using a Prime-It II random primer kit (Stratagene), and following probing, the blots were visualized on a Storm phosphorimager. The data were quantified using ImageQuant software. *FLO11* expression was normalized to the expression level of *ACT1*, which was probed on the same membrane. The *ACT1* probe was generated with the primers TRO480, GCCGGTTTGGCCGGTGACGAC, and TRO481, GCCGGTTTGGCCGGTGACGAC. The primer TRO481 was later discovered to contain two mismatches with the *ACT1* sequence; however, the resulting probe was sequenced and revealed to be specific for the yeast *ACT1* gene.

### Northern blotting

**Table 1. Yeast strains used in this study**

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| TBR1   | MATa ino3-52 leu2::hisG his3::hisG op1::kanMX4 | 54 |
| YTR138 | MATa ino3-52 leu2::hisG his3::hisG op1::kanMX4 | This study |
| YTR139 | MATa ino3-52 leu2::hisG his3::hisG ino2::kanMX4 | This study |
| YTR140 | MATa ino3-52 leu2::hisG his3::hisG ino4::kanMX4 | This study |
| TRX9-7b| MATa ino3-52 leu2::hisG his3::hisG ino2::kanMX4 op1::kanMX4 | This study |
| YTR147 | MATa ino3-52 leu2::hisG his3::hisG ino1::3paxMX6 | This study |
| YTR148 | MATa ino3-52 leu2::hisG his3::hisG ino1::3paxMX6 op1::kanMX4 | This study |

**Table 2. Primers used in this study**

| Primer | Purpose | Sequence |
|--------|---------|----------|
| TRO369<sup>a</sup> | Confirm disruptions | GCCAGTCGAAGCAGCTGCAAGG |
| TRO248 | Disrupt OPI1 | CGCATATATGTTGTCAGCCT |
| TRO249 | Disrupt OPI1 | TGGATCAAGAGGCGTTCCTCC |
| TRO250 | Confirm op1Δ | ACATGCACTGAGCCCTGAAAGT |
| TRO251 | Disrupt INO2 | CGCCTTTCCGTTGGAATCA |
| TRO252 | Disrupt INO2 | CGCAGTATATCTGTTGTTGAGAC |
| TRO253 | Confirm mo2Δ | CCAAATCTACGCCAGCTACAAAT |
| TRO240 | Disrupt INO4 | ATGGCGCCCTTGGGTCGCT |
| TRO241 | Disrupt INO4 | CGGGTAAAATGTTATGATTGTTG |
| TRO242 | Confirm moΔ | CCTTCACGGTCATTCGTA |
| TRO243 | Disrupt INO1 | ATGGCGCCCTTGGGTCGCT |
| TRO244 | Disrupt INO1 | GCCAGGCTTGCAGGAGACTG |
| TRO197 | Confirm moΔ | GCCGTAATAGTCAGCAGG |

<sup>a</sup>TRO369 hybridizes to the TEF promoter in the kanMX4 cassettes and served as a reverse primer in combination with the confirmation forward primers listed to confirm the mutations.
FIG. 2. Hierarchical clustering analysis revealed a unique transcriptional profile associated with mat growth on a low-agar plate. mRNA was collected from growing mats on low-agar plates or from liquid batch cultures (planktonic) at log or post-log phase. The mRNA was probed on Affymetrix yeast genome S98 chips, and ratios were generated by comparing data from mat and post-log-phase cultures to those from log-phase cultures. The ratios were subjected to hierarchical clustering via the programs Cluster and TreeView (http://rana.lbl.gov/EisenSoftware.htm) (15). See Materials and Methods for more details. Yeast systematic ORF names are shown to the right of clusters, and commonly accepted gene names are shown to the right of the systematic names where applicable. (A) A cluster of genes that was upregulated in both post-log-phase and mat cultures. (B) A cluster of genes that exhibited a large downregulation in post-log-phase cultures but not in mat cultures. (C) A cluster of genes that showed a large upregulation in mat cultures but not in post-log-phase cultures. The gene expression conditions shown are post-log phase (lanes 1), hub (lanes 2), rim (lanes 3), and whole mats (lanes 4). An asterisk marks genes that appear twice in a cluster due to more than one set of probes on the Affymetrix array for those select genes.
RESULTS

Gene expression in mat versus planktonic cells. In order to identify genes that were regulated in response to growing on the surface of the agar plate, cells growing as a mat were compared to cells growing planktonically in liquid suspension cultures by whole-genome transcriptional profiling. Transcriptional analyses of bacterial cells growing on surfaces as mature biofilms have shown that they resemble stationary-phase cells more than logarithmically growing cells (77). In an attempt to control for this, the yeast mats were compared to planktonic cells that were growing in log phase and post-log phase in liquid YEPD medium. Additionally, transcriptional profiles were generated from separated hub and rim populations within the mats. A direct comparison of the microarray data from the mat and from the rim is in preparation (T. B. Reynolds, A. Jansen, X. Peng, and G. R. Fink, unpublished results). The mat and post-log-phase data sets were compared to the log-phase data set as a common reference. The resulting ratios of gene expression were then compared to one another by hierarchical clustering using the programs Cluster and TreeView (15).

Cluster analysis revealed that there were 186 genes in the mat and post-log-phase cultures that exhibited changes in gene expression compared to the log-phase culture. The complete cluster analysis is available online (see Fig. S1 in the supplemental material).

One large cluster consisted of 99 genes that were upregulated in the mat and post-log-phase cultures compared to log-phase cultures (Fig. 2A). These genes largely overlapped (85%) with genes that have been shown to be upregulated during diauxic shift and stationary phase (12, 19). This overlap suggested that the gene expression profile of cells in a mat is similar to that of cells that are experiencing diauxic shift or entering stationary phase. Despite this similarity, the mat continued to expand, indicating that the cells in the mat, or at least those in the rim, continued to grow. The post-log-phase cells, conversely, experienced less than one doubling before arresting growth.

Another cluster of genes (29 genes) revealed a significant difference in gene expression between the mat and post-log-phase cells. This cluster consisted mostly of genes required for protein synthesis, and they were significantly downregulated in the post-log-phase cells but continued to be expressed in mat cells (Fig. 2B). Within this cluster, genes in the post-log-phase culture were downregulated by an average of 58-fold, while in the mat cultures they were downregulated by an average of only 2-fold. This cluster was dominated by genes involved in ribosomal assembly and/or protein translation. Consistent with our data, these genes have been shown in other studies to be downregulated as cells enter stationary phase or diauxic shift (12, 19).

There was a cluster of eight genes that were highly upregulated in the mat cultures but not in the post-log-phase cultures (Fig. 2C). These eight genes exhibited an average 38-fold increase in gene expression in the mat but an average 1.9-fold increase in the post-log-phase cultures. All eight genes except STR3 were disrupted, and the mutants were examined to determine if these mutations affected mat formation. None of these mutants exhibited a defect in mat formation compared to the wild-type strain.

The opi1Δ mutant exhibits a defect in mat formation and invasive growth. Three of the genes (INO1, PAU2, and DAN1) upregulated in the mats but not in the post-log-phase cultures (Fig. 2C) have been shown to be regulated by members of what this report refers to as the inositol regulon (3, 24, 31). This prompted a further investigation into the role of this regulon in mat formation. The inositol regulon consists of three transcription factors, Ino2p, Ino4p, and Opi1p, that control expression of several phospholipid biosynthetic genes (3, 24, 31). The most heavily regulated of these genes is INO1, which encodes the myo-inositol-1-phosphate synthase. Ino1p converts glu-
cose-6-phosphate into inositol-1-phosphate (13, 41). Ino2p and Ino4p form a heterodimeric transcriptional activator of INO1 expression (3), while Op1p acts antagonistically as a transcriptional repressor of INO1 (24).

Gene disruptions of INO2, INO4, and OP11 revealed that OP11 has a significant effect on mat formation, and INO2 and INO4 have minor effects. During mat formation, the wild-type strain exhibits development of a distinct hub and rim, forms spokes, and expands over the surface to a width of 41 ± 2.4 mm by day 5 (Fig. 1 and 3). The op11Δ mutant was retarded in morphological development and spreading compared to the wild-type strain (Fig. 3). This mutant did not develop spokes, had a poorly developed hub, and did not spread well, expanding to only 26 ± 2 mm by day 5.

The ino2Δ and ino4Δ mutants had only a slight defect in mat formation, producing mats that did not spread as well as the wild-type strain but had a similar morphology (Fig. 3). The ino2Δ and ino4Δ mutant mats spread to only 35 ± 1.2 mm and 35 ± 1.7 mm, respectively, by day 5. Development of the spokes in the ino2Δ and ino4Δ mutants appeared to precede that in the wild-type strain slightly but reproducibly (Fig. 3, 3 days).

The effects of the op11Δ mutant on mat formation seemed likely to be a consequence of a novel effect on FLO11 regulation. Northern blotting revealed that in liquid medium the op11Δ mutant had a significant reduction in FLO11 expression (Fig. 4). The defect in FLO11 expression shown by the op11Δ mutant in planktonic culture could also be seen when FLO11 expression was assessed in the mat (Fig. 5). Conversely, examination of the ino2Δ and ino4Δ mutants revealed that they exhibited modest increases in FLO11 expression compared to that seen for the wild-type strain when grown planktonically (Fig. 4) or as mats (Fig. 5).

The inositol regulon mutants’ defects in FLO11 expression suggested that they might exhibit defects in another FLO11-dependent phenotype, invasive growth. Wild-type cells growing as colonies on a solid (2% agar) YEPD plate adhered to the surface of the agar plate, even when washed with water, while most of the op11Δ mutant cells were removed (Fig. 6B). Conversely, the ino2Δ and ino4Δ mutants exhibited a slight increase in invasive growth compared to the that of the wild type. This could not be seen when the cells are washed from the plate with water alone (Fig. 6B). However, when the plate was rubbed with a gloved finger to more thoroughly remove noninvasive cells, it was revealed that the ino2Δ and ino4Δ mutants exhibited a level of invasive growth slightly greater than that of the wild type (Fig. 6C).

The op11Δ mutant’s defect in mat formation and invasive growth is dependent on INO2. Overexpression of INO1 by the op11Δ mutant is dependent on the Ino2p transcriptional activator. An ino2Δ mutant fails to overexpress INO1 in an op11Δ background (23). The op11Δ mutant’s failure to undergo invasive growth (Fig. 6) and mat formation (Fig. 3) was likewise dependent on INO2. An ino2Δ op11Δ double mutant behaved like an ino2Δ mutant rather than an op11Δ mutant in both mat formation (Fig. 7A) and invasive growth (Fig. 7B).

One hypothesis to explain the effect of the op11Δ mutant on mat formation and invasive growth was that increased production of INO1 and inositol in the op11Δ mutant might regulate these phenotypes (45). If this were the case, then an ino1Δ mutation should suppress defects in mat formation and invasive growth exhibited by an op11Δ mutant. However, analysis of
DISCUSSION

Growth on the surface of a low-agar plate appears to act as a stimulus that drives the expression of a unique transcriptional profile. This profile may reflect the effect of contact between the cells and the solid surface and/or represent a response to environmental conditions that are experienced by cells on the plate. There are two significant features of this profile. (i) Mat cells upregulate many stationary-phase genes upregulated by post-log-phase cells. However, they express protein synthesis genes at much higher levels than post-log-phase cells. (ii) Surface association correlates with the upregulation of eight genes, including some involved in inositol biosynthesis and anaerobic response. The observation that \textit{INO1} was upregulated led to the discovery that \textit{OPI1} affects \textit{FLO11} expression, mat formation, and invasive growth. These features are discussed below.

Mats exhibit a unique transcriptional profile. There was a large cluster of genes upregulated in the mat that were also upregulated in post-log-phase cells (Fig. 2A), and most of these genes are known to be upregulated in diauxic shift and stationary phase (12, 19). This suggests that compared to log-phase cells, cells throughout the mat are experiencing nutrient deprivation. However, in contrast to cells entering diauxic shift/stationary phase, mat cells did not experience a large downregulation of protein synthesis genes (Fig. 2B). One explanation for this discrepancy is that cells in the mat are continuing to actively grow or that at least a subpopulation of cells in the mat are clearly dividing as the mat continues to spread on the plate over the course of several days.
These results share some similarity with observations reported for the surface-associated phenomenon of biofilm formation in the fungus Candida albicans (18). A comparison of C. albicans biofilm cells to planktonic cells by transcriptional profiling revealed that the biofilm cells expressed higher levels of protein synthesis genes. The reasons for the upregulation of protein synthesis genes in C. albicans biofilms are not known. Upregulation of protein synthesis genes may be common to many biofilms, because it has been observed for Escherichia coli biofilms as well (18, 60).

One gene that was unexpectedly absent from the upregulated genes in the cluster analysis is FLO11. Examination of FLO11 expression in the microarray data revealed that it showed only a modest increase in post-log-phase cells (1.8-fold) or in mat cells (average of 2.0-fold) compared to log-phase cells, which explains why it was not included in the clusters. Robust FLO11 expression in log-phase cells grown in YEPD (Fig. 4) is most likely the reason for these small fold increases.

Surface-specific gene expression suggests an anaerobic environment in the mat. Eight genes were upregulated in a surface-specific manner (Fig. 2C), and among these were three genes (TIR4, DAN1, and PAU2) that encode secreted/cell wall proteins that are upregulated in response to anaerobic growth (1, 52). The upregulation of TIR4, DAN1, and PAU2 suggests that the cells in the mat are experiencing conditions that are more anaerobic than those experienced by planktonic cells. This is consistent with what has been observed for E. coli biofilms (51). The role that these anaerobic response genes have in mat formation is unclear, as disruption of PAU2, TIR4, or DAN1, or TIR4 and DAN1 simultaneously, did not have an effect on mat formation. There are many homologs for all three of these genes in S. cerevisiae that may be redundant in function (1, 52). However, disruption of the transcription factor UPC2, which controls expression of DAN1, TIR4, and their family members (2), had no effect on mat formation (T. B. Reynolds, unpublished data).

Opi1p interacts with several signal transduction pathways that regulate FLO11. A disruption of INO1 revealed no obvious role for this gene in mat formation (Fig. 8). Despite the lack of a defined function for INO1 or inositol in this phenotype, the OPI1 gene affects mat formation by activating FLO11 expression (Fig. 4 and 5). The role that Opi1p plays in activating FLO11 expression may be multifaceted, as Opi1p has been found to interact with components of several pathways that control invasive or pseudohyphal growth. These pathways include the protein kinase A (PKA) pathway (44, 48, 57), the Snf1 kinase pathway (11, 33, 34), and the unfolded protein response (UPR) pathway (5, 61, 67, 68).

The PKA pathway is required to activate invasive growth (44, 48, 57). Disruption of TPK2, a catalytic subunit of the PKA pathway, prevents cells from undergoing invasive growth because Tpk2p phosphorylates the transcription factors Flo8p and Sfl1p, which regulate FLO11 (49). Tpk2p phosphorylation of Flo8p, a transcriptional activator required for upregulation of FLO11, promotes Flo8p binding of the FLO11 promoter (49). Tpk2p phosphorylation of Sfl1p, a repressor of FLO11 transcription, inhibits Sfl1p binding of the FLO11 promoter (49). Opi1p has been shown to be a substrate for PKA phosphorylation, which enhances its activities as a repressor of...
INO1 (69). Thus, Tpk2p could act to enhance Opi1p activity, which may contribute to FLO11 upregulation. However, it is not clear which PKA subunit actually phosphorylates Opi1p in vivo.

The Snf1 kinase, like Opi1p, has been shown to regulate both INO1 and FLO11 transcription. Snf1p is required for upregulation of INO1, and it does so by regulating the binding of the TATA binding protein to the promoter of INO1 (64–66). Snf1p upregulates FLO11 by inactivating the transcriptional repressors Nrg1p and Nrg2p, thus derepressing transcription of FLO11 (33, 34). Microarray experiments have indicated that Opi1p can regulate the expression of the NRG2 repressor gene (30). It is possible that Opi1p contributes to the regulation of FLO11 by affecting NRG2 expression.

The UPR pathway regulates both INO1 and pseudohyphal growth (7, 9, 61). In the UPR pathway, the endoplasmic reticulum resident protein Ire1p senses accumulated unfolded proteins in the endoplasmic reticulum. Ire1p then catalyzes the splicing of an intron out of the HAC1 mRNA (HAC1*), to generate the spliced form of HAC1 (HAC1’). HAC1’ is translated into the transcription factor Hac1p, which upregulates UPR target genes (40). It has been reported that in hac1Δ/hac1α and ire1Δ/ire1α mutants, pseudohyphal growth is not properly repressed (61). In addition, hac1Δ and ire1Δ mutants do not express INO1 (7, 9). Hac1p has been shown to antagonize the function of the Opi1p repressor with regards to the repression of INO1 (5). It may have a similar relationship to Opi1p for regulating FLO11 and mat formation.

The position that Opi1p and the inositol regulon take in influencing FLO11 expression, invasive growth, and mat formation in relationship to these pathways is currently under investigation. In addition, it is possible that there are as-yet undiscovered connections to other pathways known to regulate filamentous growth (16, 17, 47, 74), such as the filamentous growth mitogen-activated protein kinase cascade (10, 55, 56, 74).

Opi1p may affect FLO11 expression by an indirect mechanism. Opi1p has been defined as a transcriptional repressor, so it was unexpected to find that it is required to activate FLO11 expression. This suggests a model in which Opi1p acts to repress another downstream repressor gene that acts directly on FLO11. Several known repressors of FLO11, including Nrg1p, Nrg2p, Sfl1p, and Sok2p (33, 34, 49, 50, 57), are attractive candidates. This, however, does not rule out the existence of a novel repressor. These possibilities are currently under investigation.

An alternative explanation is that Opi1p has a previously undefined role as a transcriptional activator. However, this seems unlikely, because the opi1Δ mutant requires a functional Ino2p transcriptional activator to perturb mat formation and invasive growth (Fig. 7). Genetic analysis of the ino2Δ and opi1Δ mutations revealed that the ino2Δ mutation is epistatic to the opi1Δ mutation for controlling mat formation and invasive growth (Fig. 7). The ino2Δ opi1Δ double mutant behaves like an ino2Δ mutant. This is similar to what has been observed for the regulation of INO1 by Ino2p and Opi1p. Previous studies have shown that an ino2Δ opi1Δ double mutant behaves like an ino2Δ single mutant and is unable to grow in the absence of inositol (23). Thus, Opi1p is mostly likely acting as a repressor of a gene upregulated by Ino2p. This target gene may encode a repressor of FLO11 or regulate a repressor of FLO11.

One possible candidate for this downstream gene is INO1. Overexpression of INO1 might influence intracellular signaling and affect FLO11 expression due to the production of large concentrations of inositol. Pseudohyphal growth in Candida tropicalis can be repressed by the addition of extracellular inositol (45). If this were the case, then a disruption of INO1 in the opi1Δ mutant should restore the wild-type phenotype. However, this was not the case, as the ino1Δ opi1Δ double mutant continued to behave like the opi1Δ mutant (Fig. 8).

The role of inositol regulon orthologs in other fungi. Homologs of Opi1p and other inositol regulon components are found in other fungi, including pathogens such as Candida albicans (27). This regulon may play an important role in controlling the expression of glycosylphosphatidylinositol-anchored adhesins in these fungi as well. Some glycosylphosphatidylinositol-anchored adhesins in the Candida species control biofilm formation and serve as virulence factors (6, 72, 73). Therefore, orthologs of inositol regulon components may play a role in virulence in these fungal pathogens.

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