

The Cell Surface Flocculin Flo11 Is Required for Pseudohyphae Formation and Invasion by *Saccharomyces cerevisiae*

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Submitted September 8, 1997; Accepted October 28, 1997

Monitoring Editor: David Botstein

Diploid yeast develop pseudohyphae in response to nitrogen starvation, while haploid yeast produce invasive filaments which penetrate the agar in rich medium. We have identified a gene, *FLO11*, that encodes a cell wall protein which is critically required for both invasion and pseudohyphae formation in response to nitrogen starvation. *FLO11* encodes a cell surface flocculin with a structure similar to the class of yeast serine/threonine-rich GPI-anchored cell wall proteins. Cells of the *Saccharomyces cerevisiae* strain Σ 1278b with deletions of *FLO11* do not form pseudohyphae as diploids nor invade agar as haploids. In rich media, *FLO11* is regulated by mating type; it is expressed in haploid cells but not in diploids. Upon transfer to nitrogen starvation media, however, *FLO11* transcripts accumulate in diploid cells, but not in haploids. Overexpression of *FLO11* in diploid cells, which are otherwise not invasive, enables them to invade agar. Thus, the mating type repression of *FLO11* in diploids grown in rich media suffices to explain the inability of these cells to invade. The promoter of *FLO11* contains a consensus binding sequence for Ste12p and Tec1p, proteins known to cooperatively activate transcription of *Ty1* elements and the *TEC1* gene during development of pseudohyphae. Yeast with a deletion of *STE12* does not express *FLO11* transcripts, indicating that *STE12* is required for *FLO11* expression. These *ste12*-deletion strains also do not invade agar. However, the ability to invade can be restored by overexpressing *FLO11*. Activation of *FLO11* may thus be the primary means by which Ste12p and Tec1p cause invasive growth.

INTRODUCTION

Diploid strains of *Saccharomyces cerevisiae* Σ 1278b switch from a single-cell form of growth to develop pseudohyphae, long-branched chains of elongated cells, in response to nitrogen starvation (for review, see Gimeno and Fink, 1992; Kron and Gow, 1995). These invasive filaments have been interpreted to be a foraging adaptation to nitrogen starvation. Haploid yeast do not form pseudohyphae when starved for nitrogen, but they invade agar when cultured on rich medium (Roberts and Fink, 1994).

Several elements of the mitogen-activated protein kinase (MAPK) pathway that controls mating pheromone signal transduction in haploid cells are also required for pseudohyphae development and invasion: Ste20p,

Ste11p, Ste7p, and Ste12p (Liu *et al.*, 1993; Roberts and Fink, 1994). This may be the most well studied of all mitogen-activated protein (MAP) kinase systems (for review, see Herskowitz, 1995; Levin and Errede, 1995). The pheromone receptor and G protein which mediate the initial steps in the mating signal transduction pathway are not required for pseudohyphae formation (Liu *et al.*, 1993). Instead, the G proteins encoded by *RAS2* and *CDC42* appear to control activation of the MAPK pathway leading to pseudohyphae development (Mosch *et al.*, 1996). The kinases Ste20p, Ste11p (MEKK), and Ste7p (MEK) are required for phosphorylation of the transcription factor Ste12p in response to mating pheromone in haploid cells (Elion *et al.*, 1993); these same proteins are required for invasion (Roberts and Fink, 1994) and pseudohyphae formation (Liu *et al.*, 1993). This pathway thus controls signal transduction in response to mating pheromones in haploid cells and pseudohyphae devel-

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opment in response to nitrogen starvation in diploid cells. Since pheromone-responsive genes are not detectably induced during haploid pseudohyphae formation (Roberts and Fink, 1994), Ste12p must have at least two functional states in the haploid cell. These two activities of Ste12p appear to be determined by association with an accessory transcription factor, Tec1p. A transcriptional reporter which is induced during filamentous growth and invasion but not during mating has been developed using regulatory sequences from the transposable element *Ty1* fused to *lacZ* (Mosch *et al.*, 1996). Transcription of this reporter is dependent on the cooperative binding of Ste12p and Tec1p to a filamentation and invasion response element (FRE) in the *Ty1* sequences (Madhani and Fink, 1997). To date, the only other gene reported to be regulated by a FRE is the *TEC1* gene itself (Madhani and Fink, 1997).

Surprisingly, although haploid cells invade rich agar, diploids do not (Roberts and Fink, 1994). Haploid invasiveness requires the same components of the MAPK pathway that are required for diploid pseudohyphal development, including the transcriptional activator Ste12p (Roberts and Fink, 1994). Invasiveness is distinct from formation of the pseudohyphal filaments in both haploids and diploids (Mosch and Fink, 1997; Roberts and Fink, 1994). Invasion does not require *BUD* gene functions, which regulate the budding patterns of haploid and diploid cells. Strains with mutations in *BUD1* and *BUD2* penetrate agar as efficiently as wild-type strains, even though these strains do not form ordered filaments (Roberts and Fink, 1994).

Investigations of pseudohyphae development to date have largely focused on regulators of the pseudohyphal pathway. These studies have revealed the involvement of transcription factors and the kinases and phosphatases characteristic of signal transduction pathways (Blacketer *et al.*, 1993, 1994; Liu *et al.*, 1993, 1996; Gimeno and Fink, 1994; Gavrias *et al.*, 1996; Ward *et al.*, 1995; Mosch and Fink, 1997; Roberts *et al.*, 1997). There must also be downstream genes that are regulated during pseudohyphae development that result in alterations of the cell surface, changes in cell shape and cell cycle control, and the ability to invade. We have recently characterized a gene (open reading frame YIR019c) that we have named *FLO11* which encodes a cell surface flocculin (Lo and Dranginis, 1996). In single-cell yeasts that are not developing pseudohyphae, *FLO11* expression causes cell-cell adhesion of the calcium-dependent type called flocculation. *FLO11* is identical to *MUC1*, a gene shown to be required for pseudohyphae formation by amylase-secreting strains of *S. cerevisiae* var. *diastaticus* in which both haploids and diploids switch to a pseudohyphal form of growth in response to carbon limitation (Lambrechts *et al.*, 1996). We report here that *FLO11* is essential for both invasion and filament formation by the Σ 1278b strain of *S. cerevisiae* that form pseudohy-

phae efficiently in response to nitrogen starvation and on which most of the studies of filamentous growth have been performed (Gimeno *et al.*, 1992). We place *FLO11* in the signal transduction pathway downstream of *STE12* and show that it is likely to be the only target of *STE12* required for invasion. Flo11p represents the first specific cell surface requirement for the formation of pseudohyphae and invasion, and connects the processes of cell adhesion, invasion, and pseudohyphae formation in yeast.

MATERIALS AND METHODS

Strains and Growth Conditions

Yeast strains used in this study are described in Table 1. They were derived from strains of the Σ 1278b genetic background (Gimeno *et al.*, 1992) generously provided by members of the laboratory of Gerald Fink. Strain YSL1015 contains the plasmids pRS423 and pRS424 and strain YSL1012 contains the plasmid pRS426 (Christianson *et al.*, 1992) solely to facilitate the construction of these strains as diploids. Standard yeast culture medium was prepared as described (Kaiser *et al.*, 1994). YEPD plates [2% yeast extract (Difco, Wilmington, DE), 4% Bacto peptone (Difco), 2% glucose, and 2% Bacto-agar (Difco)] were allowed to dry at room temperature before use. Low ammonia medium (SLAD) for pseudohyphal growth in diploids was prepared as described [6.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% glucose, 2% washed Bacto-agar (Difco), and appropriate auxotrophic requirements] (Gimeno *et al.*, 1992). Strains scored for pseudohyphal filament formation were streaked on SLAD plates and observed and photographed after 1 wk of growth at 30°C. For the invasive growth assay, strains were patched onto YEPD plates with a toothpick, with care being taken to avoid scratching the surface of agar. Patches were allowed to grow at 30°C for 3 d and incubated at room temperature for an additional 2 d. Cells that penetrated the agar were examined after washing the cells off the agar surface with deionized water (Roberts and Fink, 1994). Samples were photographed with Polaroid 65 film (Sigma, St. Louis, MO).

Plasmid Constructions

Plasmids used in this work are listed in Table 1. pRS315-a1 contains the 1.8-kb *XhoI*-*HindIII* fragment containing *MATa1* isolated from the *XhoI* linker insertion mutation matax 12 (Tatchell *et al.*, 1981) cloned into the polylinker of pRS315 (Sikorski and Heiter, 1989). pRS415-a2 contains the *MATa2* gene with its own promoter, amplified by polymerase chain reaction from base 1 to base 969 (Tatchell *et al.*, 1981) using primers with *SalI* and *SpeI* restriction sites at the 5' ends, cloned into the CEN-LEU2 vector pRS415 (Christianson *et al.*, 1992); it was a generous gift from Sharon Y. Roth. pSY2 is a 2 μ -LEU2 plasmid containing the *STE12* gene (Dolan and Fields, 1990) in the vector YEp13.

pYSL12 contains the entire *FLO11* gene (sequence number relative to initiating AUG: -870 to 4107) which was amplified by long polymerase chain reaction (Lo and Dranginis, 1996) as a *SalI*-*BglIII* fragment; the *FLO11* fragment was cloned into the LEU2-2 μ yeast shuttle vector pRS425 (Christianson *et al.*, 1992). pYSL13 is the same *FLO11* fragment cloned into the URA3-2 μ yeast shuttle vector pRS426 (Christianson *et al.*, 1992).

Clones used for the synthesis of ³²P-labeled riboprobes for Northern blots were as follows. The *FLO11* probe was made from pSL35, which contains a *HindIII*-*PvuII* fragment of *FLO11* (sequence number relative to initiating AUG of coding sequence of *FLO11*: 2669-3415) cloned into pGEM3Z(f+) vector (Promega, Madison, WI). The actin probe was made from pGEM-Act300, a gift from Kevin Wang, which contains a 300-bp *BalI* fragment from pYACT1 (Ng and Abelson, 1980).

Table 1. Strains and plasmid list

Strain or Plasmid	Genotype or Description	Reference Source Derivation
<i>S. cerevisiae</i> strain		
L5486	<i>MATa, ura3-52, leu2::hisG</i>	Roberts and Fink (1994)
L5487	<i>MATα, ura3-52, leu2::hisG</i>	Roberts and Fink (1994)
10560-4A	<i>MATa, ura3-52, leu2::hisG, trp1::hisG, his3::hisG</i>	Laboratory of G. Fink
10560-6B	<i>MATα, ura3-52, leu2::hisG, trp1::hisG, his3::hisG</i>	Laboratory of G. Fink
YSL1012	<i>MATa/MATα, ura3-52/ura3-52, LUE2/leu2::hisG [URA3]</i>	This work. L5487 \times YIY318 (Yamashita <i>et al.</i> , 1985) + plasmid pRS426 (Christianson <i>et al.</i> , 1992)
YSL1015	<i>MATa/MATα, ura3/ura3-52, leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG his3::hisG/his3::hisG [TRP1][HIS3]</i>	This work. 10560-4A \times 10560-6B + plasmid pRS423 + plasmid pRS424 (Christianson <i>et al.</i> (1992)
L5487flo11-4	<i>MATα, ura3-52, leu2::hisG, flo11::URA3</i>	This work
YSL12	<i>MATα, ura3-52, leu2::hisG, ste12::LEU2</i>	Derived from L5487 This work Derived from L5487
Plasmids		
pRS315-a1	CEN-LEU2 plasmid carrying <i>MATa1</i>	This work
pRS415-a2	CEN-LEU2 plasmid carrying <i>MATα2</i>	Sharon Y. Roth
pYSL12	2 μ -LEU2 plasmid carrying <i>FLO11</i>	This work
pYSL13	2 μ -URA3 plasmid carrying <i>FLO11</i>	This work
pSL35	pGEM3Z(f+) containing a 746-bp <i>HindIII</i> – <i>PvuII</i> <i>FLO11</i> fragment	This work
pGEM-ACT300	pGEM3Z containing a 300-bp <i>BalI</i> fragment from pYACT1	K. Wang; Ng and Abelson, 1980
pSUL16	<i>ste12::LEU2</i> -containing plasmid	Fields and Herskowitz, 1987
pSY2	2 μ -LEU2 plasmid carrying <i>STE12</i>	Dolan and fields, 1990

Gene Disruptions

Disruption of *FLO11* was performed as previously described (Lo and Dranginis, 1996). *STE12* was disrupted using the *ste12::LEU2*-containing plasmid pSUL16 (Fields and Herskowitz, 1987). All gene replacements and disruptions were confirmed by Southern blotting.

Northern Blot Analysis

Total RNA was prepared by a modification of published methods (Carlson and Botstein, 1982; Dranginis, 1989) as follows. Haploid and diploid yeast cells were grown in different media and harvested at different growth stages (time points: 0.5, 2, 4, 6, 8, and 24 h of growth). Cell density was estimated for each sample by measuring A_{600} (1OD₆₀₀ unit equals approximately 2×10^7 cells). The cells were collected by centrifugation, transferred in 1 ml of water to microfuge tubes, and collected again. To the cell pellet was added 0.4 g of washed glass beads (0.45–0.5 mm, Thomas Scientific, NJ), followed by 0.3 ml of cracking buffer (0.5 M NaCl, 0.2 M Tris-HCl, pH 7.4, 10 mM EDTA, and 1% SDS) and 0.3 ml of 25:24:1 phenol:chloroform:isoamyl alcohol. The samples were vortexed at top speed for 1 min. Another 0.3 ml of cracking buffer and 0.3 ml of phenol:chloroform:isoamyl alcohol was added, and the mixture was vortexed for 30 s. The samples were centrifuged 5 min and the aqueous phase was extracted with 0.3 ml of phenol:chloroform:isoamyl alcohol. The sample was precipitated with 1 ml of ethanol at -20°C and then centrifuged for 1 min. The pellet was washed with 70% ethanol and suspended in RNase-free water.

Ten micrograms of RNA per sample was run in 3-(*N*-morpholino)propanesulfonic acid-formaldehyde gels as described (Sambrook *et al.*, 1989). Gels were washed in the following way: 15 min

with distilled water, 30 min with 0.01 N NaOH, 15 min with 0.1 M Tris-HCl (pH 7.5), and 30 min with $0.1\times$ SSC. Transfer to Gene-Screen membrane (DuPont-New England Nuclear Research Products, Boston, MA) was performed by capillary blot using $10\times$ SSC. RNA was cross-linked to the membrane with a Stratalinker (Stratagene, La Jolla, CA) at 120,000 $\mu\text{J}/\text{cm}^2$.

Membranes were hybridized with ^{32}P -labeled riboprobes for *FLO11* and *ACT1*, produced from the plasmids described above by transcription with T7 RNA polymerase (New England Biolabs, Beverly, MA). The quantitation of the mRNA was performed using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA). *FLO11* RNA was normalized to the hybridization signal from the actin RNA (*ACT1*) which was used as a control for RNA loading. The *FLO11:ACT1* RNA ratio was plotted against OD₆₀₀ of the cell sample.

Photomicroscopy

Colonies were viewed through the bottom of the Petri dish with a Zeiss standard 16 microscope at $100\times$ total magnification and photographed using a 35-mm camera using TMAX 400 (Kodak) film.

RESULTS

FLO11 Is Required for Invasive Growth and Pseudohyphae Formation

We have recently described *FLO11*, a gene that encodes a cell surface flocculin (Lo and Dranginis, 1996). Expression of *FLO11* was examined by RNA hybridization analysis in several different yeast strain back-

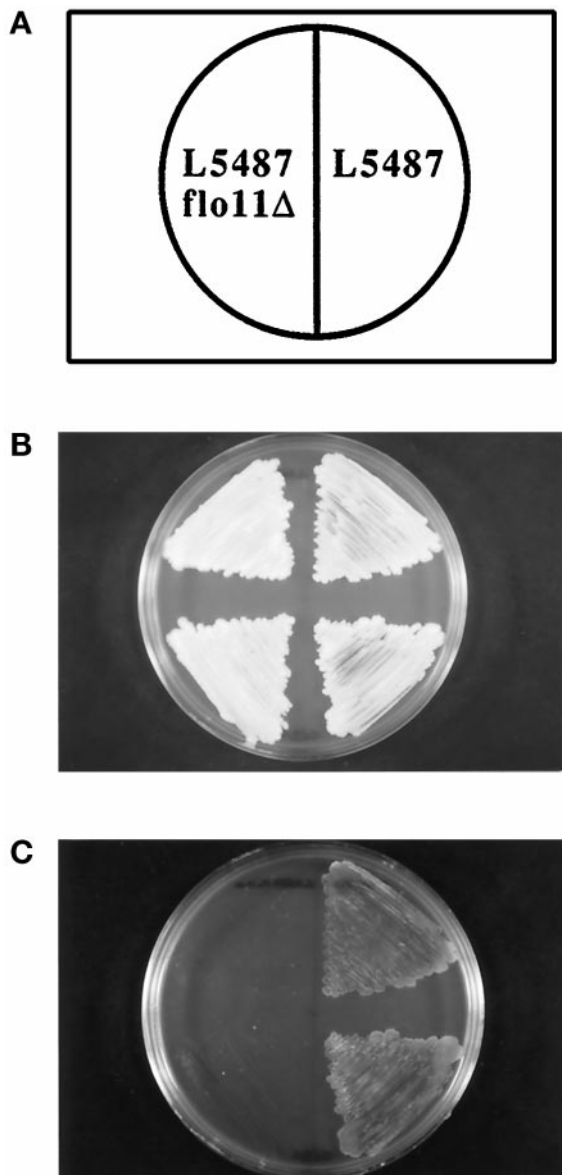


Figure 1. *FLO11* is required for invasiveness of haploid cells. Yeast were streaked on YEPD medium and grown for 3 d at 30°C followed by 2 d of growth at room temperature. As shown in the diagram in A, haploid α cells of strain L5487 are on the right half of the Petri dish while isogenic cells with a deletion of *FLO11* (L5487-*flo11*-4) are on the left half of the dish. (B) Total yeast growth on the surface of the agar, shown before the plate was rinsed with water. (C) The same plate after rinsing cells off the surface of the agar with a gentle stream of water. Invasive growth is demonstrated by cells that remain in the agar.

grounds. The 4.2-kb *FLO11* RNA was detected in the pseudohyphae-forming strain $\Sigma 1278b$ (L5486 and L5487; see Table 1) and in *S. cerevisiae* var. *diastaticus*, whose flocculation properties were previously studied (Lo and Dranginis, 1996), but *FLO11* transcripts were not detected in the laboratory strain S288C, which

does not form pseudohyphae (Gimeno *et al.*, 1992; Liu *et al.*, 1996; our unpublished results). To test the role of this cell surface molecule in invasion and formation of pseudohyphae, we constructed a gene deletion of *FLO11* in yeast strain $\Sigma 1278b$, on which many studies of filamentous growth have been performed. Figure 1 shows that *FLO11* is required for invasiveness of haploid cells. Cells were patched on rich medium (YEPD) and incubated at 30°C for 3 d and at room temperature for an additional 2 d (Figure 1B). Cells that penetrated the agar were examined after washing the cells off the agar surface with deionized water (Roberts and Fink, 1994). The wild-type cells on the right were able to invade the agar, but the isogenic strain with *FLO11* deleted was no longer able to penetrate the agar (Figure 1C).

We then tested the ability of diploids with a deletion of *FLO11* to form pseudohyphae in response to nitrogen starvation. Figure 2A shows that diploid cells form highly branched pseudohyphae when starved for nitrogen, but haploid colonies exhibit a smooth circular outline (Figure 2D). It has previously been shown that pseudohyphae formation in diploids is dependent on the $a1/\alpha 2$ repressor (Gimeno *et al.*, 1992; Lo *et al.*, 1998). Like the natural diploid, a pseudodiploid which is made by transforming α haploids with a single copy plasmid containing *MATa1* forms filaments efficiently (Figure 2B). The same pseudodiploid with a deletion of *FLO11* fails to form any filaments (Figure 2C). We have recently confirmed this result using true diploid cells homozygous for the *FLO11* deletion (our unpublished observations). *FLO11* is thus required for diploid cells to form pseudohyphae in response to nitrogen starvation.

FLO11 mRNA Is Induced in Diploids, But Not in Haploids by Nitrogen Starvation

The requirement for *FLO11* in the diploid to form pseudohyphae was puzzling, since we have previously shown that *FLO11* expression is repressed by the action of the $a1/\alpha 2$ repressor in diploids grown in rich media (Lo and Dranginis, 1996). One hypothesis is that *FLO11* expression, while repressed in rich media, is induced when diploids are transferred to nitrogen starvation media. Experiments with *STA1*, a related gene whose promoter has extensive sequence homology to the promoter of *FLO11*, support this hypothesis. *STA1* is repressed in a manner dependent on the $a1/\alpha 2$ repressor in diploids grown in rich media, but the RNA is induced when diploids are transferred to sporulation medium, which starves cells for nitrogen in the presence of acetate as a carbon source (Dranginis, 1989).

To test this hypothesis, we measured the expression of *FLO11* in both haploids and diploids in response to growth in rich medium and to nitrogen starvation by Northern blot analysis (Figure 3). The isogenic α

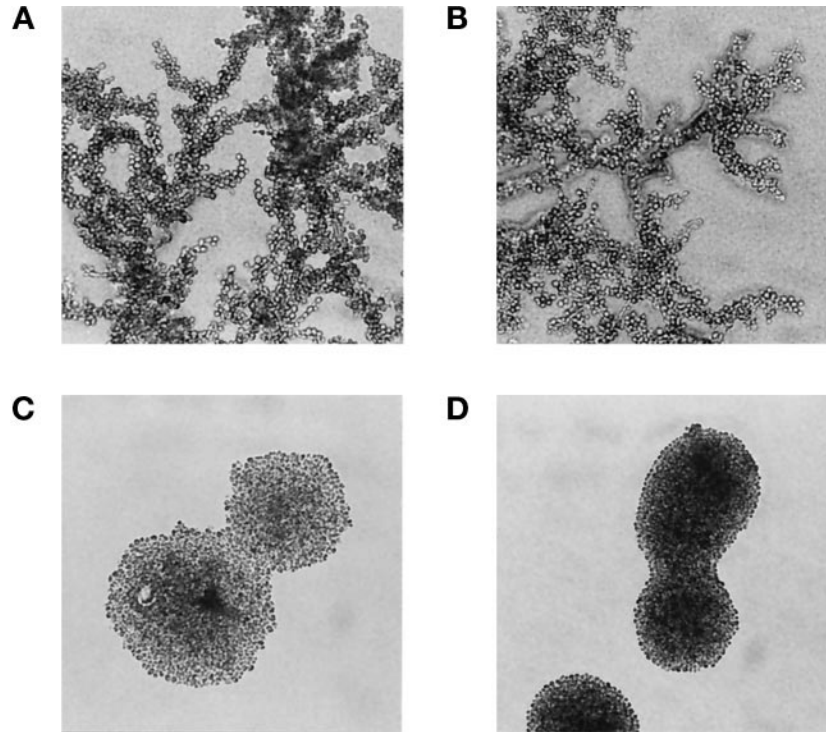


Figure 2. *FLO11* is required for pseudohyphae formation by diploids in response to nitrogen starvation. The morphology of yeast colonies grown on nitrogen starvation medium (SLAD) for 1 wk is shown. (A) Diploid strain YSL1012 (MATa/MATα). (B) "Pseudodiploid" constructed by transforming α haploid strain L5487 with a single-copy plasmid containing the *MATa1* gene (pRS315-a1). (C) *FLO11* genomic deletion in the pseudodiploid strain shown in B (strain L5487flo11-4 containing pRS315-a1). (D) Haploid α strain L5487.

haploid strain (L5487) and a/α diploid strain (YSL1015) were grown in YEPD liquid medium overnight, then diluted to an initial OD₆₀₀ of 0.10–0.125 into YEPD (rich) or SLAD (nitrogen-deficient) media. After incubation at 30°C, cells were harvested at different time points (0.5, 2, 4, 6, 8, and 24 h). The cell density of each sample was measured, and RNA was purified. RNA from each time point was run in formaldehyde gels and transferred to nylon membrane, and the *FLO11* and actin mRNA were detected by hybridization with riboprobes. Figure 3A shows the *FLO11* RNA from haploids (H) and diploids (D) which were cultured in YEPD media. The *FLO11* signal reaches its peak intensity after a 30-min incubation in both strains. However, the levels of *FLO11* RNA rapidly declined in the diploids, beginning with the 2-h sample and disappearing entirely after a 4-h culture (OD₆₀₀ ~0.4) whereas the expression of *FLO11* in haploids was still high (Figure 3A).

The blots were stripped and rehybridized with an actin probe to control for amount of RNA. To quantitate the expression, the band intensities were measured using a PhosphorImager and the *FLO11*:actin ratio was calculated and plotted as a function of cell growth (Figure 3). In rich media (YEPD), the ratio of *FLO11* to actin is maximum at early log phase for both haploid (*FLO11*/actin = 10) and diploid (*FLO11*/actin = 1.5). The *FLO11* signal in the haploid was initially higher than that in the diploid. The ratio of *FLO11* to actin RNA declined rapidly in the diploid

after a 4-h culture (*FLO11*/actin = 0.08) but in the haploid the ratio was still 0.9 after a 24-h culture (Figure 3A).

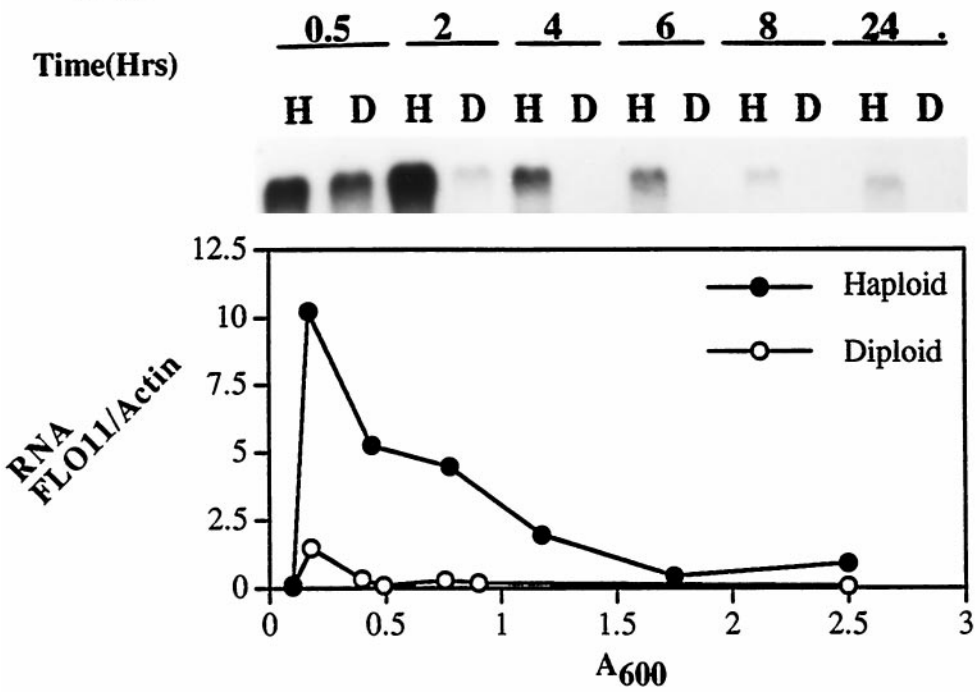
In contrast, when cells were transferred to nitrogen starvation media (SLAD) the *FLO11* signal declined very rapidly in haploids after 0.5 h and disappeared at 24 h (OD₆₀₀ ~1.5), but the expression of *FLO11* in diploids continued to increase in an overnight culture (Figure 3B). The *FLO11*:actin ratio climbed steadily in the diploids, reaching 1.7 at 24 h (Figure 3B). Similar curves were obtained when 28S rRNA was used as control for loading instead of actin (our unpublished observations).

These data indicate that *FLO11* expression is regulated by both mating type and nitrogen starvation. *FLO11* transcripts are induced in diploid cells upon starvation for nitrogen, but not in haploid cells. The pattern of *FLO11* expression may explain why diploids but not haploids form pseudohyphae when starved for nitrogen, whereas haploids but not diploids are invasive in rich medium.

Overexpression of FLO11 Induces Invasive Growth in Diploids

Could the inability of diploid cells to invade rich media be accounted for by the repression of *FLO11* in these circumstances? We tested this hypothesis by overexpressing cloned *FLO11* in a diploid and assaying for invasive growth on rich medium. A

A YEPD



B SLAD

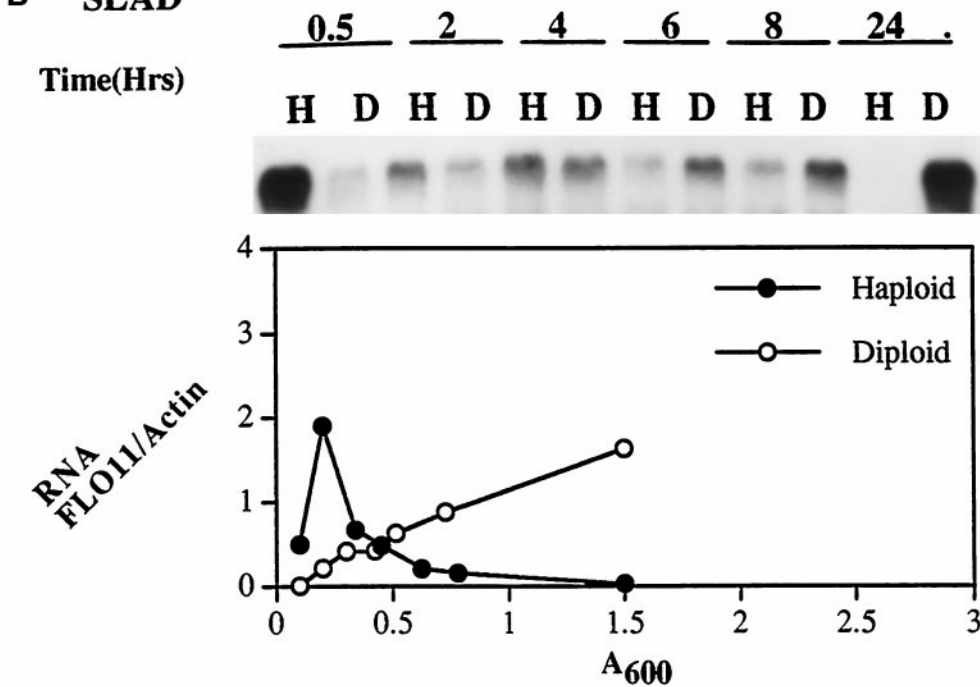


Figure 3. *FLO11* mRNA is induced in diploids, but not in haploids by nitrogen starvation. The isogenic haploid α strain (L5487) and diploid strain (YSL1015) were inoculated into YEPD and SLAD media, and RNA samples were prepared at the indicated time points (0.5, 2, 4, 6, 8, and 24 h). The Northern blots were hybridized with a 32 P-labeled *FLO11* riboprobe and then subsequently stripped and rehybridized with a 32 P-labeled actin riboprobe control as described. Lanes H indicate haploid samples; D indicates diploid. (A) RNA from cells grown in YEPD, hybridized with *FLO11*. (B) RNA from cells grown in low nitrogen medium (SLAD), hybridized with *FLO11*. Ratio of *FLO11* RNA to actin RNA at various growth stages (OD_{600}) of cells grown in YEPD and SLAD is plotted beneath the Northern blots. The RNA band intensities were determined with the PhosphorImager. Closed circles, haploid (L5487, Mata α); open circles, diploid (YSL1015, Mata α).

diploid strain (YSL1015) was transformed with a high-copy number vector containing the 2 μ origin of replication and the cloned *FLO11* gene (pYSL12)

(Figure 4, middle row of colonies). The same strain was transformed with the vector only (pRS425), without the cloned *FLO11* gene, as a control (Figure

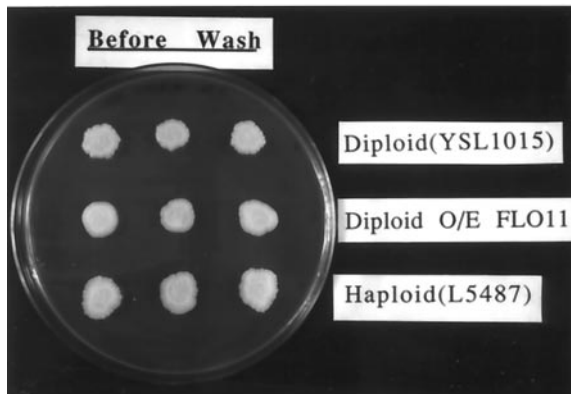
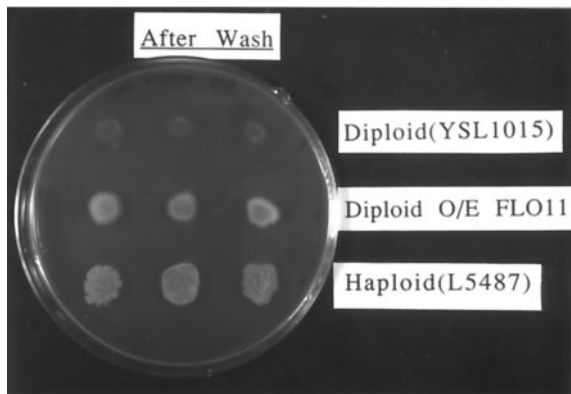
A Total growth**B Invasive growth**

Figure 4. Overexpression of *FLO11* in diploids growing on rich media enables them to invade agar. A diploid strain (YSL1015) was transformed with either the vector control pRS425 (first row of colonies) or with a multicopy plasmid carrying *FLO11*, pYSL12 (middle row of colonies). Invasive growth is compared with that of a wild-type isogenic haploid strain, L5487 (bottom row of colonies). Cells were patched onto YEPD medium and grown for 3 d at 30°C followed by 2 d of growth at room temperature. (A) The total growth on the surface of the agar, shown before the plate was rinsed with water. (B) The same plate after rinsing cells off the surface of the agar with a gentle stream of deionized water. Invasive growth is demonstrated by cells that remain in the agar.

4, top row of colonies). The lower row of colonies is the isogenic haploid (L5487), which is known to be invasive on rich agar. The plate was incubated for 3 d at 30°C followed by 2 d of growth at room temperature. Figure 4A shows the total growth on the surface of the agar before the plate was rinsed with water, and Figure 4B shows the same plate after rinsing cells off the surface of the agar with a

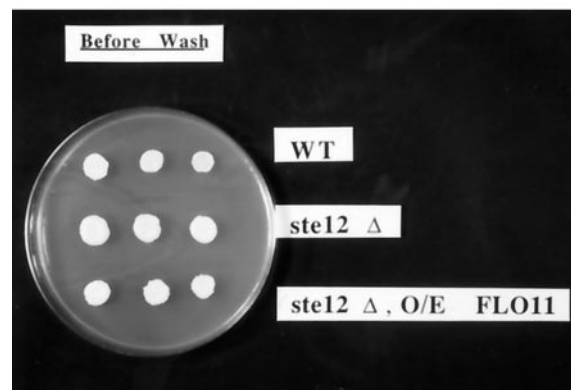
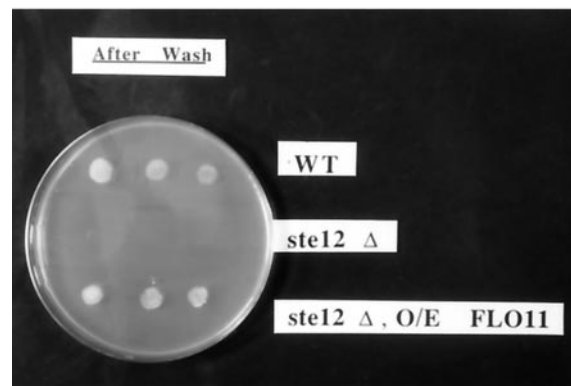
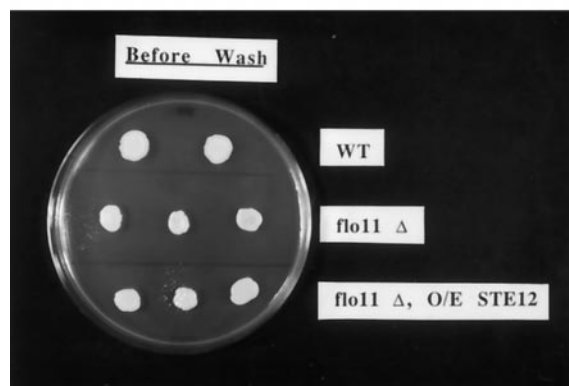
A Total growth**B Invasive growth**

Figure 5. Overexpression of *FLO11* restores invasiveness to yeast with a deletion of *STE12*. Invasiveness of the following strains is compared: Haploid strain L5487 (first row of colonies); strain YSL12 (L5487 with a deletion of *STE12*) transformed with the vector control pRS426 (middle row of colonies); and strain YSL12 (*STE12* deletion) transformed with pYSL13, a 2 μ -URA3 plasmid containing the *FLO11* gene (bottom row of colonies). Cells were patched onto YEPD medium and grown for 3 d at 30°C followed by 2 d of growth at room temperature. (A) The total growth on the surface of the agar, shown before the plate was rinsed with water. (B) The same plate after rinsing cells off the surface of the agar with a gentle stream of deionized water. Invasive growth is demonstrated by cells that remain in the agar.

gentle stream of deionized water. Invasive growth is indicated by cells that remain in the agar after washing. The diploid transformed with vector alone shows very weak invasive growth, but when the *FLO11* gene was present on a high-copy plasmid the diploid strain is as strongly invasive as the haploid. Therefore, expression of *FLO11* from a high-copy plasmid in diploids induces invasive growth on rich

A Total growth



B Invasive growth

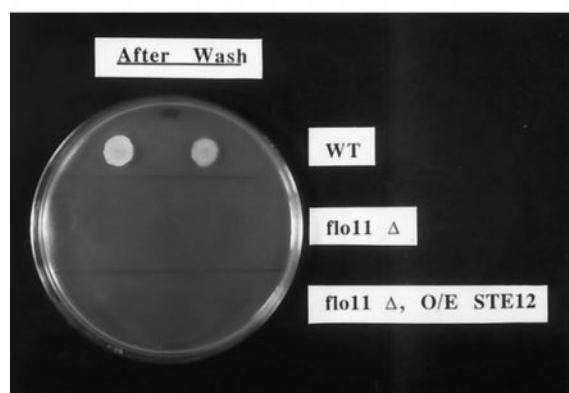


Figure 6. Overexpression of *STE12* does not restore invasiveness to yeast with a deletion of *FLO11*. Invasive growth of the following strains were compared: wild-type strain L5487 (first row of colonies); L5487flo11-4 (L5487 with a deletion of *FLO11*) transformed with the 2 μ vector control pRS426 (middle row of colonies); and L5487flo11-4 transformed with a multiple copy (2 μ) plasmid carrying *STE12* (pSY2). Cells were patched onto YEPD medium and grown for 3 d at 30°C followed by 2 d of growth at room temperature. (A) The total growth on the surface of the agar, shown before the plate was rinsed with water. (B) The same plate after rinsing cells off the surface of the agar with a gentle stream of deionized water. Invasive growth is demonstrated by cells that remain in the agar.

medium. Mating-type repression of *FLO11* suffices to explain the inability of diploid cells to invade.

Overexpression of *FLO11* Restores Invasiveness to Yeast with a Deletion of *STE12*

Ste12p is a critical transcription activator in the filamentous pathway in yeast (for review, see Kron and

Gow, 1995). Yeast with a mutation of *STE12* are defective for filamentous growth (Mosch and Fink, 1997). Using RNA blots, we have shown that yeast with a deletion of *STE12* do not express *FLO11* transcripts (our unpublished results), indicating that *STE12* is required for *FLO11* expression. To determine the role of *FLO11* in this signal transduction pathway, we performed epistasis studies to understand the relation between *FLO11* and *STE12*. A wild-type α strain and an isogenic *ste12*-deletion strain were transformed with a vector not containing *FLO11* (pRS426) and grown on the top and middle rows, respectively, of the agar dish shown in Figure 5A. The *ste12*-deletion strain transformed with *FLO11* cloned into the high-copy (2 μ) vector is shown as the bottom row of colonies in Figure 5A. The plate was incubated as described above before invasive growth was assayed by washing the plate (Figure 5B). Figure 5 shows that strains with a deletion of *STE12* cannot penetrate agar. The ability to invade can be restored by the presence of *FLO11* on a high-copy plasmid (pYSL13) in these *ste12*-deletion strains.

However, the inability to invade caused by a deletion of *FLO11* could not be overcome by overexpression of *STE12* (Figure 6). Ste12p is known to activate many genes in the mating pheromone signal transduction pathway (see for review, Sprague and Thorner, 1992) and to be required along with Tec1p for activation of a filamentation-specific reporter gene during the filamentous growth response (Madhani and Fink, 1997). Overexpression of *STE12* by expressing the gene from a 2 μ plasmid in this way has been shown to enhance filamentation and invasion (Mosch and Fink, 1997). The data presented here place *FLO11* downstream of *STE12* in the invasive growth pathway and furthermore suggest that *FLO11* may be the only target of *STE12* which is responsible for invasive growth.

The Upstream Region of the *FLO11* Promoter Contains a Filamentation and Invasion Response Element (FRE)

Although Ste12p can act alone to activate genes in the mating pathway, it has recently been reported that activation of the filamentous response requires the cooperative binding of Ste12p and Tec1p to gene promoters (Madhani and Fink, 1997). This combinatorial binding site has been called the filamentation and invasion response element (FRE) and it is comprised of a Ste12p-binding site (consensus: TGAAACA) and a Tec1p-binding site (consensus: CATTCC or CATTCT) in close proximity (Madhani and Fink, 1997). Examination of the sequence of the upstream region of *FLO11* revealed that it contains a sequence which is an almost exact match to the FRE between bases -725 and -699 with respect to the

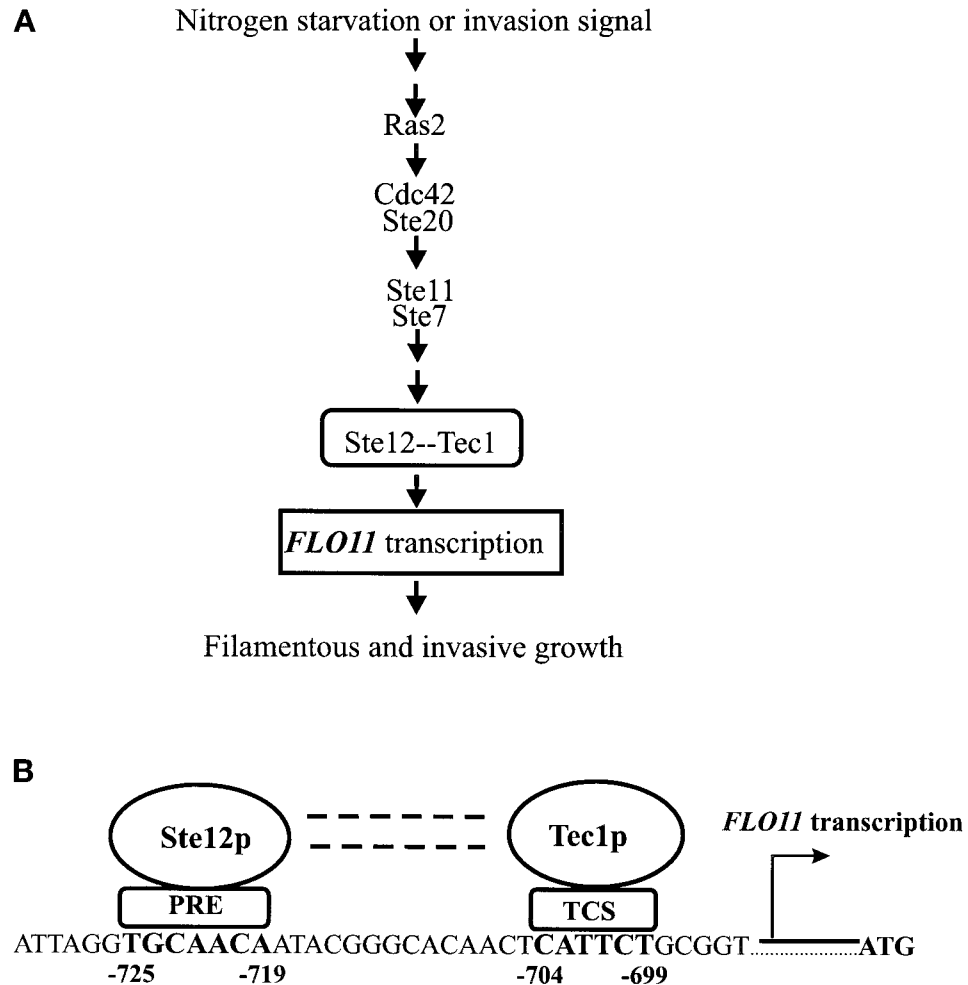


Figure 7. Models for *FLO11* action in the filamentous growth pathway. (A) In the filamentous signaling pathway Flo11p functions downstream of Ste12p and is the primary target of Ste12p to effect invasion. (B) The *FLO11* promoter region contains a filamentous response element (FRE), a site for the cooperative binding of the proteins Ste12p and Tec1p which transcriptionally activate genes during the filamentous response (Madhani and Fink, 1997).

start codon (Figure 7). There is only one base divergent from the FRE consensus sequence; this is at position -723 in the Ste12p-binding site where C is found instead of A. There are 14 bp separating the Ste12p and Tec1p sites in the *FLO11* sequence, which is identical to the spacing of these sites in the *Ty1* FRE (Madhani and Fink, 1997).

The only genes previously reported to have FRE sequences in their promoters are the *Ty1* transposable element and *TEC1* itself (Madhani and Fink, 1997). *FLO11* is the first downstream effector of the filamentous pathway reported to contain a FRE.

DISCUSSION

Studies of regulation of pseudohyphae development and invasiveness in yeast have resulted in the identification of genes encoding kinases, phosphatases, and transcription factors (Blacketer *et al.*, 1993, 1994; Liu *et al.*, 1993, 1996; Gimeno and Fink, 1994; Ward *et al.*, 1995; Gavrias *et al.*, 1996; Roberts *et al.*, 1997). Ulti-

mately, changes must occur at the cell surface to enable cells to invade substrate or to adhere to form a filamentous chain of cells. Flo11p has been previously identified as a flocculin, a cell surface molecule responsible for the calcium-dependent nonsexual aggregation known as flocculation (Lo and Dranginis, 1996). The work described here demonstrates that this flocculin is required for invasion and pseudohyphae development. Flo11p is the first cell wall protein shown to be involved in these processes. Recently, a screen for mutants defective in filamentous growth led to the identification of genes encoding structural proteins that interact with the cytoskeleton and genes responsible for bud site selection as well as previously identified components of the signal transduction pathway, but did not recover *FLO11* (Mosch and Fink, 1997). However, this screen also did not lead to the identification of several other genes known to be required for filamentous growth, suggesting that the screen was not saturated.

The transcriptional activator Ste12p is a downstream target of the pheromone-responsive MAPK cascade, elements of which are also required for the filamentous response (Liu *et al.*, 1993). Ste12p activates two different sets of genes, the mating genes and the filamentation genes, in response to different signals. A transcriptional reporter containing promoter sequences from the Ty1 element, FG(TyA)::lacZ, has been shown to be activated by Ste12p during filamentous growth, but not during the mating response (Mosch *et al.*, 1996). This filamentation-specific transcriptional induction was recently shown to be a consequence of the cooperative binding of Ste12p and Tec1p to a filamentation and invasion response element (FRE), which is comprised of a binding site for Ste12p adjacent to a binding site for Tec1p (Madhani and Fink, 1997). Two genes have thus far been described which have FRE elements: *Ty1* and the transcriptional factor *TEC1* itself, which seems to participate in a positive feedback loop (Madhani and Fink, 1997). *FLO11*, however, represents the first reported functionally important downstream gene in the filamentous response pathway which contains a FRE in its promoter.

We present evidence that *FLO11* is regulated by the transcriptional activator Ste12p. First, strains with a deletion of *STE12* do not express *FLO11* RNA. Second, the upstream region of *FLO11* contains a consensus FRE sequence. Finally, expression of *FLO11* on a high-copy plasmid overcomes the inability of the *ste12*-deletion strain to invade agar, indicating that activation of *FLO11* is the primary mechanism by which the transcriptional activator Ste12p promotes invasion.

It is possible that the adhesion, invasion, and filamentation functions of *FLO11* are related. One hypothesis is that *FLO11* is responsible for the adhesion of mother and daughter cells in the filamentous chain of cells of pseudohyphae. The adhesion properties of Flo11p might also account for its critical role in invasiveness. Adhesion to substrate may play a role in the mechanism of invasion or cell-cell adhesion may provide a stable filament of cells, allowing the force of cell division to drive invasion. A relationship between flocculation and pseudohyphae formation was recently suggested by the discovery that *PHD10*, an activator of pseudohyphae development, is the same gene as *FLO8* (Liu *et al.*, 1996), a putative transcriptional activator of the flocculin *FLO1* (Kobayashi *et al.*, 1996). A nonsense mutation in *FLO8* accounts for the inability of the common laboratory yeast strain S288C to form pseudohyphae (Liu *et al.*, 1996). It is possible that Flo8p also activates transcription of *FLO11*, and that this could account for the role of *FLO8* in pseudohyphae formation. Other flocculins such as Flo1p must not play the same role as Flo11p in Σ 1278b strains of yeast, since deletion of *FLO11* abolishes the ability to invade or to form pseudohyphae. Floccula-

tion per se does not cause invasiveness or filamentation, since we and others (Gimeno *et al.*, 1992) have observed flocculent strains that do not invade or form filaments.

We show that *FLO11* transcripts are induced in diploid cells upon starvation for nitrogen, but not in haploids. Conversely, in rich media *FLO11* transcripts persist in haploids but are not expressed in diploids. It may be that the diploid-specific repression of *FLO11* in rich media is accomplished through the activity of the FRE, since a *lacZ* reporter construct regulated by a FRE was shown to be expressed in diploids grown on rich media at less than one-tenth the level in haploids grown in rich media (Madhani and Fink, 1997). Interestingly, overexpression of *FLO11* in diploids on rich media enables them to invade, suggesting that the differential expression of *FLO11* accounts for the differences in the abilities of haploid and diploid cells to invade. The expression of the *FLO11* flocculin on the cell surface may represent a final common pathway of the transcription activators and other regulators of invasiveness and pseudohyphae development.

ACKNOWLEDGMENTS

This work was supported by an award from the Clare Boothe Luce Fund of the Henry Luce Foundation and by grant GM-51059 from the National Institutes of Health to A.M.D. The authors thank Diana Bartelt for the use of equipment and for critical reading of the manuscript, Kevin Wang for plasmids, Gerald Fink and members of his laboratory for generously sharing yeast strains, and two anonymous reviewers for thoughtful critiques.

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