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GPR1 Regulates Filamentous Growth through *FLO11* in Yeast *Saccharomyces cerevisiae*

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Cell growth and differentiation are regulated by nutrient availability in the yeast *Saccharomyces cerevisiae*. Under conditions of nitrogen limitation, diploid cells of *S. cerevisiae* differentiate to a filamentous growth known as a pseudohyphal growth, while haploid cells produce invasive filaments which penetrate the agar in nutrient-rich medium. We have found that *GPR1*, which encodes a putative G-protein-coupled receptor, is required for both pseudohyphal and invasive growth. Pseudohyphal growth was defective in Δ*gpr1*/Δ*gpr1* mutant strain and this defect was reversed by addition of cAMP. Also, haploid Δ*gpr1* mutant strain was defective in invasive growth. Northern blot analysis revealed that the transcriptional level of *FLO11*, which encodes a recently identified cell surface flocculin required for pseudohyphal growth, was reduced in Δ*gpr1* mutant strain. These results indicate that *GPR1* regulates both pseudohyphal and invasive growth by a cAMP-dependent mechanism. © 2000 Academic Press

On high glucose and low nitrogen containing plates, diploid cells of *Saccharomyces cerevisiae* undergo a dimorphic transition known as pseudohyphal differentiation (1). Haploid cells do not form pseudohyphae under nitrogen starved conditions, but they invade agar in nutrient-rich medium (2). Both pseudohyphal and invasive growth are regulated by cAMP dependent and cAMP independent mechanisms acting in parallel. With the cAMP independent mechanism, Ras2p/Cdc42p/mitogen-activated protein kinase (MAPK) pathway was shown to regulate the filamentous growth (3–6). This pathway consists of the kinases Ste20p, Ste11p, Ste7p, and Kss1p and the transcription factor Ste12p. Also, Tec1p transcription factor forms heterodimers with Ste12p and activates pseudohyphal growth (3). Whereas, in the cAMP dependent pathway, Ras2p has also been shown to regulate the pseudohyphal growth (7). A constitutive activated allele of *RAS2* as well as exogenous cAMP induces filamentous growth indicating that an increased intracellular cAMP level triggers the filamentous growth. Also Gpa2p, a heterotrimeric GTP binding protein α subunit, was shown to regulate pseudohyphal growth in a cAMP dependent manner (8, 9). In the cAMP signaling pathway, the cAMP-dependent protein kinase protein kinase A (PKA) was shown to play a key role in regulating pseudohyphal growth. Yeast PKA consists of a regulatory subunit, Bcy1p, and three catalytic subunits, Tpk1p, Tpk2p, and Tpk3p. At reduced intracellular cAMP levels, PKA forms an inactive tetramer in which two regulatory subunits bind to two catalytic subunits. When the intracellular cAMP level increases, cAMP binds to Bcy1p and active Tpk proteins are released. A recent study revealed that Tpk2p activates filamentous growth whereas Tpk1p and Tpk3p inhibit it (10, 11).

The *FLO11* gene, which encodes a cell surface flocculin, was shown to be required for both diploid pseudohyphal and haploid invasive growth (12). The MAPK and cAMP signaling pathways were shown to converge at the large *FLO11* promoter to regulate filamentous growth (13). The MAPK pathway regulates the transcriptional activation of *FLO11* through Ste12p/Tec1p, whereas *FLO8* is required for induction of *FLO11* transcription through the cAMP dependent pathway.

We have cloned *GPR1* which encodes a putative G-protein coupled receptor (14). Gpr1p was shown to interact with Gpa2p in a two-hybrid system (14–16). We also have found that Gpr1p regulates the cellular cAMP level in response to glucose as well as other fermentable sugars suggesting that Gpr1p is involved in cAMP signaling (17).

Here we show that Gpr1p regulates pseudohyphal as well as invasive growth. Our results suggest that *GPR1* as well as *GPA2* regulates *FLO11* transcription

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TABLE 1

Yeast Strains

Strain	Genotype	Source
MLY40	*MATα ura3-52*	Lorenz and Heitman (9)
MLY41	*MAT***a** *ura3-52*	Lorenz and Heitman (9)
MLY61	*MAT***a**/*α ura3-52/ura3-52*	Lorenz and Heitman (9)
HTY20	*MATα gpa2::kanMX ura3-52*	This study
HTY21	*MAT***a** *gpa2::kanMX ura3-52*	This study
HTY22	*MAT***a**/*α gpa2::kanMX/gpa2::kanMX ura3-52/ura3-52*	This study
HTY23	*MATα gpr1::kanMX ura3-52*	This study
HTY24	*MAT***a** *gpr1::kanMX ura3-52*	This study
HTY25	*MAT***a**/*α gpr1::kanMX/gpr1::kanMX ura3-52/ura3-52*	This study

Note. All strains were congenic to Σ1278b.

in a cAMP dependent manner and the switch to pseudohyphal and invasive growth.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions. Yeast strains used in this study are described in Table 1. All the strains are congenic to the Σ1278b background. The strains used for pseudohyphal growth experiments contain the plasmid pRS416. YEPD plates contain 1% yeast extract (Difco), 2% pepton, 2% glucose, and 2% Bacto-agar (Difco). Synthetic low ammonia dextrose (SLAD) medium, which contains 50 μM ammonium sulfate, 2% glucose, 0.17% yeast nitrogen base without amino acid and ammonium sulfate (Difco), and 2% washed Bacto-agar (Difco), was used to assay pseudohyphal growth (1, 9). The strains were streaked on same SLAD plates and a single cell of each was isolated and placed by a micro manipulator. Each strain was observed and photographed after 3 days or 1 week of growth at 30°C. For the haploid invasive growth assay, strains were patched onto the same YEPD plates and cells were grown for 3 days at 30°C and incubated at 25°C for an additional 2 days (12). Invasive growth was examined after washing the cells off the agar surface with a gentle stream of water. Before and after washing, samples were photographed.

Gene replacements were performed as described using PCR-derived cassettes containing the kanamycin resistance gene loxP-kanMX-loxP (18). The following primers were used to replace *GPR1*, and *GPA2* ORFs: 5′-ATCCGAAGTGTGACGAATAAAGCAAACT-CTCCAACTCAAAATGATACAGCTGAAGCTTCGTACGC-3′ and 5′-CCTTACTTTCCATTTTCAAACATCGCGATACAAAAAC-TTTATAATGGGCATAGGCCACTAGTGGATCTG-3′ (*GPR1*); 5′-TGTTACAGCACAAATCACGCGTATTTTCAAGCAAATATCAT-GGGTCAGCTGAAGCTTCGTACGC-3′ and 5′-GCATGCAGTT-TTGTCTCTGTTTTAGCTGTGCATTCATTGTAACACGCATAGGC-CACTAGTGGATCTG-3′ (*GPA2*).

RNA extraction and Northern blot analysis. Yeast total RNA was extracted as described (19), with a slight modification as follows. Cells were grown in YEPD liquid medium overnight, diluted to an initial OD_{600} of 0.1 in YEPD medium, and incubated at 30°C for 2 h with reciprocal shaking. Next, they were washed with ice-cold water, pelleted in microfuge tubes at 4°C, and suspended in 500 μl of lysis solution containing 0.3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA and 0.2% SDS, to which 500 μl of 25:24:1 phenol (water saturated):

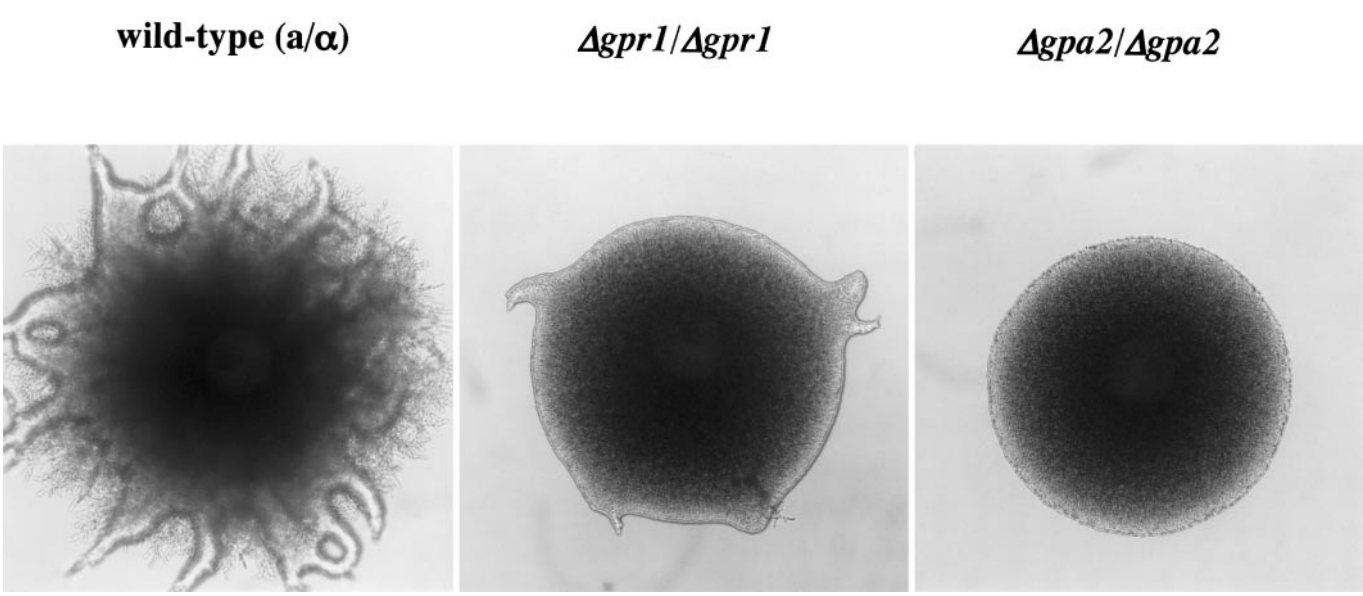

FIG. 1. The *gpr1* mutation reduces pseudohyphal growth. Homozygous wild-type (MLY61**a**/α) and Δ*gpr1*/Δ*gpr1* (HTY25**a**/α) and Δ*gpa2*/Δ*gpa2* (HTY22**a**/α) mutant strains containing the pRS416 plasmid were incubated on SLAD medium for 3 days at 30°C.

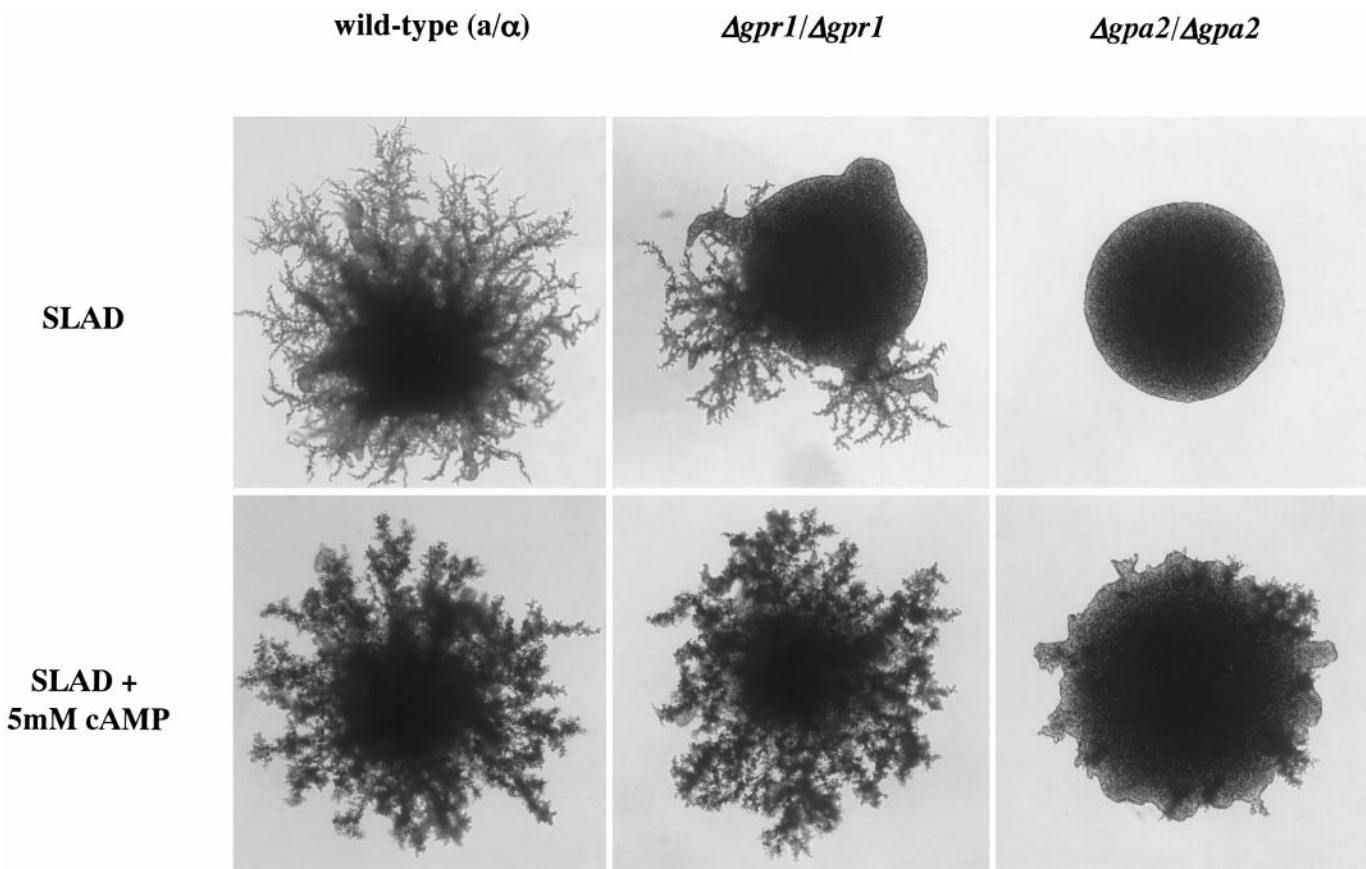

FIG. 2. Pseudohyphal defect in Δ*gpr1* mutant strain was suppressed by exogenous cAMP. Homozygous wild-type (MLY61**a**/α) and Δ*gpr1*/Δ*gpr1* (HTY25**a**/α) and Δ*gpa2*/Δ*gpa2* (HTY22**a**/α) mutant strains containing the pRS416 plasmid were incubated on SLAD medium with or without 5 mM cAMP for 1 week at 30°C.

chloroform:isoamyl alcohol and a 300-μl vol of acid-washed glass beads were then added. Samples were vigorously mixed with FastPrep FP120 cell disrupter (Savant Instrument Inc.) at speed 6 for 2 min. After centrifugation, the aqueous phase was extracted with 0.5 ml of phenol:chloroform:isoamyl alcohol. The sample was precipitated with 1 ml of ethanol at −70°C and then centrifuged for 5 min. The pellet was washed with 70% ethanol and suspended in RNase free water.

Ten micrograms of total RNA was subjected to formaldehyde-agarose gel electrophoresis followed by capillary transfer to Immobilon-Ny+ membrane (Millipore) and fixed with UV crosslinker. Hybridization with ^{32}P-labeled probe was done at 42°C for 16 h using ULTRAhyb hybridization solution (Ambion). A PCR product corresponding to bp 3,502–4,096 of the *FLO11* ORF was used to probe for *FLO11* message, and an 870 bp PCR product internal to the 3′ exon of *ACT1* was used to probe for *ACT1* message as a loading control. The quantitation of mRNA was performed using a Bio-imaging analyzer BAS2000 (Fuji Film). The amount of *FLO11* message was standardized to the amount of *ACT1* message in each strain and then normalized with respect to the wild-type strain.

RESULTS AND DISCUSSION

GPR1 Is Required for Pseudohyphal Growth

Ras2p has been shown to be involved in pseudohyphal regulation. Ras2p transduces signals through both the cAMP-PKA pathway and the MAPK pathway to regulate pseudohyphal development (7). Recently, *GPA2*, which encodes a putative heterotrimeric G-protein α subunit, was also shown to be required for pseudohyphal growth (8, 9). Although it has been suggested that Gpa2p transduced signals by a cAMP dependent mechanism, the signal transduction mechanism is still unclear.

We have cloned *GPR1*, which encodes a putative G-protein coupled receptor, using a two-hybrid system (14). Gpr1p interacts with Gpa2p, a heterotrimeric G protein α subunit, via its C-terminus as well as third cytosolic loop. We also reported that Gpr1p regulates the glucose-dependent cellular cAMP level (17). Since in part the pseudohyphal growth is regulated in a cAMP dependent manner, we examined if *GPR1* is required for pseudohyphal growth. We disrupted the gene encoding *GPR1* and *GPA2* against the Σ1278b back ground commonly used for studies of pseudohyphal growth. Isogenic wild-type, Δ*gpr1*/Δ*gpr1* and Δ*gpa2*/Δ*gpa2* mutant strains were assayed for pseudohyphal growth on SLAD plates.

After 3 days incubation, the wild type showed filamentous growth whereas the Δ*gpr1*/Δ*gpr1* mutant ex-

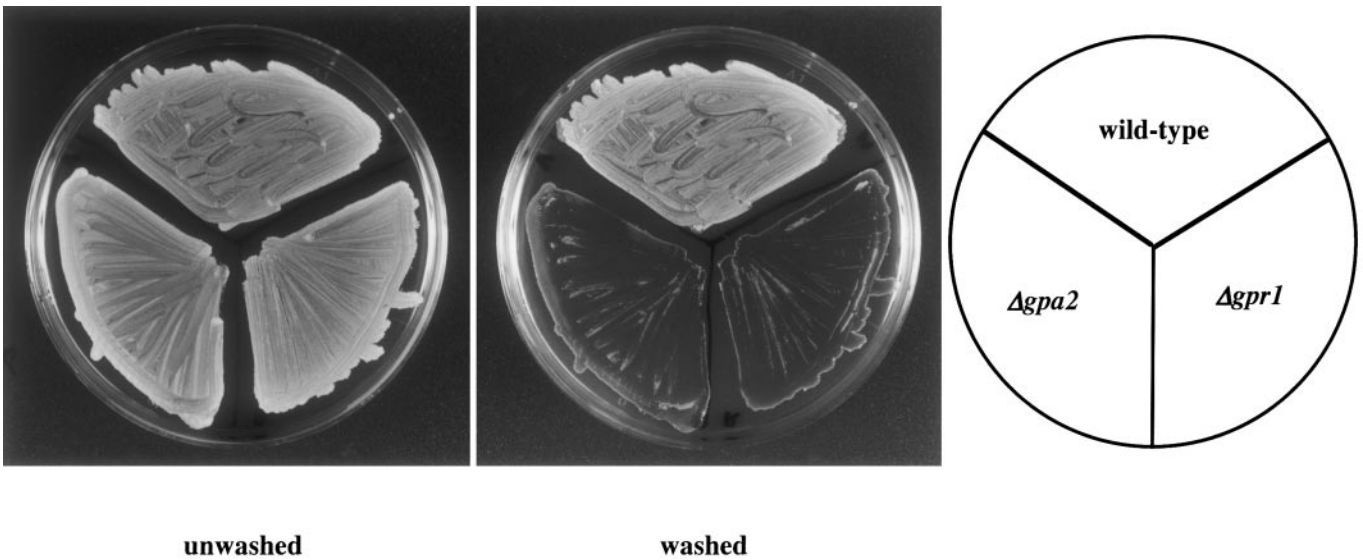

FIG. 3. The *gpr1* as well as *gpa2* mutation reduces invasive growth. Haploid wild-type (MLY40α), Δ*gpr1* (HTY23α) mutant, and Δ*gpa2* (HTY20α) mutant strain were patched onto YEPD plates and incubated for 3 days at 30°C and then at 25°C for an additional 2 days. Invasive growth was examined after the cells were washed off the agar surface with a gentle stream of water. Samples were photographed before and after washing.

hibited only partial pseudohyphal growth (Fig. 1). After 1 week incubation, Δ*gpr1*/Δ*gpr1* mutant strain showed filamentous growth whereas Δ*gpa2*/Δ*gpa2* mutant strain did not (Fig. 2). These results suggest that both *GPR1* and *GPA2* are required for pseudohyphal growth.

The reason the Δ*gpa2* mutant strain showed a more severe pseudohyphal defect than Δ*gpr1* mutant is unclear. But it is quite possible that there are other receptors coupled to Gpa2p, since *MEP2*, which encodes ammonium permease, was also shown to be required for pseudohyphal growth (20) but the signaling pathway has not been determined.

Defect of Pseudohyphal Growth in Δgpr1/Δgpr1 Mutant Strain Was Suppressed by cAMP

Since *GPR1* has been shown to regulate the glucose-dependent cellular cAMP level, we examined if extracellular cAMP can reverse the pseudohyphal defect in Δ*gpr1*/Δ*gpr1* mutant strain. The isogenic wild-type strain and Δ*gpr1*/Δ*gpr1* and Δ*gpa2*/Δ*gpa2* mutant strains were grown on SLAD medium with or without cAMP. After 3 days incubation, only the wild type strain formed filamentous growth (data not shown). After 1 week incubation, Δ*gpr1*/Δ*gpr1* mutant strain showed partial filamentous growth on SLAD medium without cAMP, and complete filamentation on SLAD medium containing cAMP (Fig. 2). From these results, Gpr1p was thought to regulate pseudohyphal growth in a cAMP dependent manner. This is consistent with our previous findings that Gpr1p regulates the glucose-dependent cellular cAMP level (17). Our results are also consistent with recent reports that pseudohyphal differentiation is stimulated by cAMP dependent protein kinase (10, 11).

The Δ*gpa2*/Δ*gpa2* mutant strain did not show filamentous growth on SLAD medium even after 1 week incubation, though it formed some filaments on SLAD medium containing cAMP (Fig. 2). Considering that exogenous cAMP reversed the pseudohyphal defect in both Δ*gpr1*/Δ*gpr1* and Δ*gpa2*/Δ*gpa2* mutant strains and that the C-terminal as well as the third cytosolic loop of Gpr1p interacts with Gpa2p, the Gpr1p-Gpa2p signaling pathway might regulate pseudohyphal growth by a cAMP dependent mechanism.

GPR1 and GPA2 Are Required for Haploid Invasive Growth

In contrast to pseudohyphal growth in diploid cells under nitrogen starved conditions, haploid cells produce invasive filaments which penetrate the agar in nutrient rich conditions (2). The invasive growth also requires Ras2p and the signaling pathway is thought to be the same as that for pseudohyphal growth.

Since *GPR1* and *GPA2* regulate pseudohyphal growth in diploid cells, we tested if they are also required for invasive growth in haploid cells. Isogenic wild-type, Δ*gpr1* mutant and Δ*gpa2* mutant strains were assayed for invasive growth on YEPD plates. Cells were patched on YEPD plates and incubated, first for 3 days at 30°C and then for 3 days at 25°C. The plates were washed with a gentle stream of water and invasive growth was observed. Both Δ*gpr1* and Δ*gpa2* mutant strains were washed off by the water (Fig. 3).

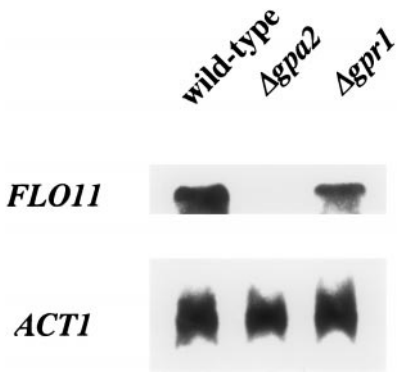

FIG. 4. *GPR1* regulates *FLO11* gene expression. Total RNA was prepared from wild-type (WT) (MLY40α) and Δ*gpr1* (HTY23α) and Δ*gpa2* (HTY20α) mutant strains. Total RNA (10 μg) was loaded onto 1% agarose gels, transferred to a nylon membrane, and probed with portions of the *FLO11* and *ACT1* genes.

These results indicate that both *GPR1* and *GPA2* regulate invasive growth in haploid strain.

Transcription of FLO11 Gene Is Reduced in Δgpr1 Mutant Strain

It has been suggested that *FLO11*, which encodes cell surface protein, is required for pseudohyphal growth (12). Recently *FLO11* was shown to be a target for both the MAPK and cAMP pathway (13). Since *GPR1* regulates the cellular cAMP level in response to glucose, we examined if *GPR1* is involved in cAMP signaling which increases *FLO11* gene expression. The isogenic wild-type and Δ*gpr1* and Δ*gpa2* mutant cells were cultured in YEPD medium for 2 h to OD_{600} = 1.0 and then subjected to RNA extraction followed by northern blot analysis. Northern blot analysis revealed that the level of *FLO11* mRNA was reduced about three-fold in Δ*gpr1* mutant strain and more than ten-fold in Δ*gpa2* mutant strain (Fig. 4). These results were also consistent with the result that Δ*gpr1* and Δ*gpa2* mutant strains were defective in invasive and pseudohyphal growth.

A recent study indicates that the MAPK and cAMP signaling pathways converge at a large promoter of the *FLO11* gene (13). Our results indicate that the Gpr1p-Gpa2p signaling pathway also regulates *FLO11* gene expression which leads to pseudohyphal growth in diploid cells as well as invasive growth in haploid cells. Whether *GPR1* regulates both the MAPK and cAMP pathway remains to be clarified.

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