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Amino Acid Starvation and Gcn4p Regulate Adhesive Growth and *FLO11* Gene Expression in *Saccharomyces cerevisiae*

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In baker's yeast *Saccharomyces cerevisiae*, cell-cell and cell-surface adhesion are required for haploid invasive growth and diploid pseudohyphal development. These morphogenetic events are induced by starvation for glucose or nitrogen and require the cell surface protein Flo11p. We show that amino acid starvation is a nutritional signal that activates adhesive growth and expression of *FLO11* in both haploid and diploid strains in the presence of glucose and ammonium, known suppressors of adhesion. Starvation-induced adhesive growth requires Flo11p and is under control of Gcn2p and Gcn4p, elements of the general amino acid control system. Tpk2p and Flo8p, elements of the cAMP pathway, are also required for activation but not Ste12p and Tec1p, known targets of the mitogen-activated protein kinase cascade. Promoter analysis of *FLO11* identifies one upstream activation sequence (UAS^R) and one repression site (URS) that confer regulation by amino acid starvation. Gcn4p is not required for regulation of the UAS^R by amino acid starvation, but seems to be indirectly required to overcome the negative effects of the URS on *FLO11* transcription. In addition, Gcn4p controls expression of *FLO11* by affecting two basal upstream activation sequences (UAS^B). In summary, our study suggests that amino acid starvation is a nutritional signal that triggers a Gcn4p-controlled signaling pathway, which relieves repression of *FLO11* gene expression and induces adhesive growth.

INTRODUCTION

Adherence of cells to one another and to surfaces is a prerequisite for the formation of multicellular structures. In the yeast *Saccharomyces cerevisiae*, cell-cell and cell-surface adhesion are required for many developmental processes that include mating (Roy *et al*., 1991; Cappellaro *et al*., 1994), haploid invasive growth (Roberts and Fink, 1994; Guo *et al*., 2000), biofilm formation (Reynolds and Fink, 2001), and diploid pseudohyphal development (Gimeno *et al*., 1992; Mösch and Fink, 1997). Each of these events is initiated by distinct signals that are coupled to the expression of specific cell surface proteins by the corresponding signaling pathways (for recent reviews, see Banuett, 1998; Lengeler *et al*., 2000). For instance, starvation for glucose causes invasive growth in haploid strains of *S. cerevisiae*, a developmental event that depends on expression of the cell surface protein Flo11p and that is under control of the mitogen-activated protein kinase (MAPK) and the cAMP pathways (Roberts and Fink, 1994; Mösch *et al*., 1999; Rupp *et al*., 1999; Cullen and Sprague, 2000). In diploid strains, starvation for nitrogen induces pseudohyphal growth, a morphogenetic development that also requires Flo11p and functional MAPK and cAMP signaling pathways (Liu *et al*., 1993; Lo and Dranginis, 1998; Robertson and Fink, 1998; Pan and Heitman, 1999).

FLO11 belongs to a gene family that encodes glycosyl-phosphatidylinositol (GPI)-linked glycoproteins of domain structure similar to the adhesins of pathogenic fungi (Lo and Dranginis, 1996). Flo11p is localized to the cell surface and is required for nutritionally induced cell-cell and cell-surface adhesion during invasive growth, biofilm formation, and pseudohyphal development (Lo and Dranginis, 1998; Guo *et al*., 2000; Reynolds and Fink, 2001). The unusually large *FLO11* promoter is complex and integrates multiple inputs from the cAMP pathway, the MAPK cascade, the mating type, and nutritional signals (Rupp *et al*., 1999). The transcription factor Flo8p is required for activation of *FLO11* by Tpk2p, the catalytic subunit of the cAMP-dependent protein kinase specifically involved in activation of invasive growth and pseudohyphal development (Liu *et al*., 1996; Pan and Heitman, 2002). Ste12p and Tec1p are transcription factors that are important for *FLO11* regulation and transmit signals from the MAPK to sites within the *FLO11* promoter that are distinct from the Flo8p target sites (Lo and Dranginis, 1998; Rupp *et al*., 1999; Köhler *et al*., 2002). The glucose-responsive protein kinase Snf1p and the transcriptional repressors Nrg1p and Nrg2p also regulate expression of *FLO11* (Kuchin *et al*., 2002).

In *S. cerevisiae*, starvation for a single amino acid induces a regulatory system known as the general amino acid control (Schürch *et al*., 1974; Hinnebusch, 1986), which activates transcription of numerous genes encoding enzymes involved in several amino acid biosynthetic pathways (Hinnebusch, 1992), amino acid tRNA synthetases (Meussdoerffer and Fink, 1983; Mirande and Waller, 1988), and enzymes of purine biosynthesis (Mösch *et al*., 1991). In the general amino acid control system, the sensor kinase Gcn2p phosphorylates the translation initiation factor eIF2 in response to amino acid starvation, an event that results in efficient translation

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
RH2648	*MAT***a** *ura3-52*	This study
RH2649	*MAT***a** *gcn2* Δ::*LEU2 ura3-52 leu2::hisG*	This study
RH2650	*MAT***a** *gcn4* Δ::*LEU2 ura3-52 leu2::hisG*	This study
RH2651	*MAT***a** *gcn4* Δ::*LEU2 gcn2*Δ::*kanR ura3-52 leu2::hisG*	This study
RH2652	*MAT***a** *flo8*Δ::*kanR ura3-52*	This study
RH2653	*MAT***a** *flo8*Δ::*kanR gcn2*Δ::*LEU2 ura3-52 leu2::hisG*	This study
RH2654	*MAT***a** *flo8* Δ::*kanR gcn4* Δ::*LEU2 ura3-52 leu2::hisG*	This study
RH2656	*MAT***a**/α *ura3-52/ura3-52 trp1::hisG/TRP1*	This study
RH2657	*MAT***a**/α *gcn2*Δ::*LEU2/gcn2*Δ::*LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/TRP1*	This study
RH2658	*MAT***a**/α *gcn4*Δ::*LEU2/gcn4*Δ::*LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/TRP1*	This study
RH2659	*MAT***a**/α *tpk2*Δ::*kanR/tpk2*Δ::*kanR ura3-52/ura3-52 leu2::hisG/LEU2 his3::hisG/HIS3 trp1::hisG/TRP1*	This study
L5627	*MAT***a**/α *ste12*Δ::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG	Liu *et al*., (1993)
HMC267	*MAT***a**/α *tec1::LEU2/tec1::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/TRP1*	Mösch and Fink (1997)
RH2660	*MAT***a**/α *flo8*Δ::*kanR/flo8*Δ::*kanR ura3-52/ura3-52 trp1::hisG/TRP1*	This study
RH2661	*MAT***a**/α *flo11*Δ::*kanR/flo11*Δ::*kanR ura3-52/ura3-52 trp1::hisG/TRP1*	This study
RH2662	*MAT***a** *flo11*Δ::*kanR ura3-52*	This study

of *GCN4* that encodes the transcription factor Gcn4p (Hinnebusch, 1997; Hinnebusch and Natarajan, 2002). Gcn4p activates transcription of target genes by direct promoter binding at sequence-specific Gcn4p-responsive elements (Hope and Struhl, 1985; Oliphant *et al*., 1989). Two recent studies using genome-wide transcriptional profiling showed that Gcn4p controls expression of >1000 target genes of diverse pathways and functional categories (Jia *et al*., 2000; Natarajan *et al*., 2001). These studies demonstrate that Gcn4p has much broader function as a master regulator of gene expression in yeast as previously anticipated. In the human pathogen *Candida albicans*, Gcn4p has recently been found to coordinate metabolic and morphogenetic reponses to amino acid starvation (Tripathi *et al*., 2002). However, whether amino acid starvation and Gcn4p in *S. cerevisiae* or *C. albicans* control cell-cell and/or cell-substrate adhesion by regulating expression of specific cell-surface proteins, e.g., Flo11p, has not been reported so far.

In this study, we show that amino acid starvation efficiently activates adhesive growth and expression of *FLO11* in both haploid and diploid strains in the presence of glucose and ammonium, known suppressors of adhesion. Starvation-induced adhesive growth requires Flo11p and depends on Gcn2p, Gcn4p, Tpk2p, and Flo8p, but not on Ste12p and Tec1p. We find that the *FLO11* promoter contains one upstream activation sequence (UAS^R) and one repression site (URS) that confer regulation by amino acid starvation. Gcn4p seems to be indirectly required to overcome the negative effects of this URS on *FLO11* transcription but is not required for regulation of the UAS^R by amino acid starvation. Gcn4p controls expression of *FLO11* by affecting two basal upstream activation sequences (UAS^B). We suggest that amino acid starvation is a nutritional signal that triggers a Gcn4p-controlled signaling pathway, which relieves repression of *FLO11* gene expression and induces adhesive growth.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

All strains used in this study are derivatives of the *S. cerevisiae* Σ*1278b* strain background (Table 1). Deletion mutants for *GCN2* (*gcn2*Δ) were obtained by using the *gcn2*Δ deletion plasmids pME1658 and pME1659 (Table 2). Plasmids pME1105 and pME1645 were used for constructing *gcn4*Δ and *tpk2*Δ mutant strains. *Flo8*Δ and *flo11*Δ mutants were obtained by using plasmids pME2155 and pME2156. Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al*., 1983). All gene deletions, integrations, or replacements were confirmed by Southern blot analysis (Ausubel *et al*., 1993). Standard methods for crosses were used and standard yeast culture medium was prepared essentially as described previously (Guthrie and Fink, 1991). For measurements of *FLO11* expression, strains were cultivated at 30°C in liquid synthetic minimal medium (YNB) supplemented with 10 mg/l uracile (Ura) and/or 40 mg/l arginine (Arg) where indicated, diluted into fresh medium, and cultivated for 6 h before assaying enzymatic activities or isolation of total RNA. For amino acid starvation, 3-amino-triazole (3AT) or 5-methyl-tryptophan (5MT) was added to cultures at the concentration indicated, and cells were incubated for 8 h before further assays. For nitrogen starvation, cells grown to logarithmic phase were washed with 2% glucose and incubated for 24 h in liquid YNB medium containing 50 μM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. For adhesive growth tests, strains were grown on solid (2% agar) YNB medium containing the indicated supplements and 3AT or 5MT at the respective concentrations to induce amino acid starvation. Qualitative pseudohyphal growth was assayed on SLAD plates (Gimeno *et al*., 1992).

Plasmids

Plasmids used in this study are listed in Table 2. Plasmid pME2155 carrying the *flo8*Δ:*kanR* deletion cassette was constructed by amplifying the plasmid backbone and sequences flanking the FLO8 open reading frame from plasmid pHL129 (Liu *et al*., 1996) by using the two primers OG33 (5′-GAAGATCTTCTACCACGGAATGCGTTTCC-3′) and OG34 (5′-GAAGATCTCTGACATTTCGCTAAATTTGGG-3′) to create a *Bgl*II restriction site, which was used to insert the *kanR* kanamycin resistance cassette of pME1765 (Grundmann *et al*., 2001). Similarly, deletion cassettes for *FLO11* (pME2156) and *TPK2* (pME1645) were created by replacement of the *FLO11* or *TPK2* open reading frames by *kanR* as selectable marker. Plasmid pME2519 carrying a functional *FLO11* gene was constructed by amplification of *FLO11* as an *Eco*RI-*Sal*I fragment (sequence number relative to the initiating AUG: −3043 to +4392) by using polymerase chain reaction (PCR) and primers FLO11-102 (5′-CCGGAATTCGTGGCGCGGTGCCAATACTACCGGTACTTG-3′) and FLO11-103 (5′-ACGCGTCGACCCCCAATTCAAGAATACAATTACTTAGCGTGG-3′) and insertion of the fragment into the *Eco*RI and *Sal*I sites of plasmid YCplac33 (Gietz and Sugino, 1988). Plasmid pME2212 was obtained by deletion of the 434-base pair *Xho*I fragment containing the endogenous UAS element of the *CYC1*-promotor region in plasmid pLG669Z (Guarente and Ptashne, 1981). To obtain plasmids pFLO11-5, pFLO11-6, pFLO11-9, and pFLO11-10, individual *FLO11* promoter fragments were amplified by PCR and cloned into pME2212 by using a restriction site (*Xho*I) introduced at the 5′ end of the PCR primers. The primers used have been described previously (Rupp *et al*., 1999) and were #5F and #5R to obtain pFLO11-5, #6F and #6R for pFLO11-6, #9F and #9R for pFLO11-9, and #10F and #10R for pFLO11-10.

Northern Hybridization Analysis

Total RNAs from yeast were isolated essentially as described previously from cultures grown in liquid media (Cross and Tinkelenberg, 1991). RNAs were separated on 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes by electroblotting. Gene specific probes were ^{32}P-radiolabeled with the HexaLable DNA labeling kit (MBI Fermentas, St. Leon-Rot, Germany). Hybridizing signals were quantified using a BAS-1500 phosphorimaging scanner (Fuji, Tokyo, Japan).

Table 2. Plasmids used in this study

Plasmid	Description	Source
pME1092	2.8-kb fragment containing *GCN4* in pRS314	Albrecht *et al.* (1998)
pME1098	2.8-kb fragment containing *GCN4m* in pRS314	Albrecht *et al.* (1998)
pME1105	Cassette for full deletion of *GCN4*-open reading frame (*LEU2*)	Albrecht *et al.* (1998)
pME1658	Cassette for full deletion of *GCN2*-open reading frame (*LEU2*)	Grundmann *et al.* (2001)
pME1659	Cassette for full deletion of *GCN2*-open reading frame (*kan*^{r})	Grundmann *et al.* (2001)
pME2155	Cassette for full deletion of *FLO8*-open reading frame (*kan*^{r})	This study
pME2156	Cassette for full deletion of *FLO11*-open reading frame (*kan*^{r})	This study
pME1645	Cassette for full deletion of *TPK2*-open reading frame (*kan*^{r})	This study
pME1765	pBluescriptKS$^+$ containing *kan*^{r}-cassette	Grundmann *et al.* (2001)
pME2519	7.43-kb fragment containing *FLO11* in YCplac33	This study
B3782	3-kb *FLO11*-promoter fragment in YEp355	Rupp *et al.* (1999)
p*flo11-1* to *pflo11-15*	200 bp deletions in B3782 from-1 to -201 bp, -202 to -401 bp until -2802 to -3001 bp	Rupp *et al.* (1999)
pME2212	pLG669Z-UASΔ	This study
p*FLO11*–2/1 to p*FLO11*-15/14	440 bp *FLO11* promoter sequence elements cloned into pLG669Z	Rupp *et al.* (1999)
pFLO11-5	239-bp *FLO11* promoter element cloned into pLG669Z	This study
p*FLO11*-6	239-bp *FLO11* promoter element cloned into pLG669Z	This study
p*FLO11*-9	247-bp *FLO11* promoter element cloned into pLG669Z	This study
p*FLO11*-10	239-bp *FLO11* promoter element cloned into pLG669Z	This study

β-Galactosidase Assay

Assays were performed with extracts of cultures grown in liquid media. Specific β-galactosidase activity was normalized to the total protein (Bradford, 1976) in each extract and equalized ($OD_{415} \times 1.7$)/(0.0045 × protein concentration × extract volume × time) (Rose and Botstein, 1983). Assays were performed for at least three independent transformants, and the mean value is presented. The SEs of the means were <15%.

Growth Tests and Photomicroscopy

Adhesive growth tests with haploid and diploid strains were performed essentially as described previously (Roberts and Fink, 1994). Strains were pregrown on solid YNB medium containing the indicated supplements for 20 h. Cells were then patched on fresh YNB containing supplements and 3AT or 5MT at the indicated concentrations and incubated at 30°C for 1 to 5 days. Plates were photographed and then carefully washed under a stream of water. The plates were photographed once again to document the remaining adhesive cells. Pseudohyphal growth was viewed with an Axiovert microscope (Carl Zeiss, Jena, Germany) and photographed using a Xillix microimager digital camera with the Improvision Openlab software (Improvision, Coventry, United Kingdom).

RESULTS

Amino Acid Starvation Activates Adhesive Growth and FLO11 *Gene Expression in the Presence of Glucose and Ammonium in* S. cerevisiae

We tested, whether starvation for amino acids activates adhesive growth and expression of *FLO11* in yeast. Haploid and diploid wild-type and *flo11*Δ mutant strains were tested for adhesive growth on solid high ammonium medium with or without the addition of 3AT, a histidine-analog that induces histidine starvation (Hilton *et al.*, 1965; Schürch *et al.*, 1974). Haploid strains are known to exhibit adhesive growth on high ammonium medium, but only after prolonged incubation of 4–5 d (Roberts and Fink, 1994). Therefore, we assayed invasive growth after 24 h, a time period that is not sufficient to induce substrate adhesion in haploids (Lo and Dranginis, 1996; Rupp *et al.*, 1999). As expected, haploid cells did not adhere significantly to the agar substrate after 24 h of growth on nonstarvation medium (Figure 1A) but became adhesive after prolonged incubation of 5 d (Figure 3A). In contrast, haploid cells became highly adhesive already after 24 h of growth on amino acid starvation medium (Figure 1A). Adhesive growth was dependent on *FLO11*, because a *flo11*Δ mutant strain was nonadhesive under all conditions tested, and adhesive growth on starvation medium could be restored by complementation of the *flo11*Δ mutant with a functional *FLO11* gene (Figure 1A). Similar results were obtained for diploid strains. Diploid strains were nonadhesive under nonstarvation conditions (even after 5 d), but they became highly adhesive when starved for amino acids even in the presence of high amounts of ammonium (Figures 1A and 4A). Deletion of *FLO11* blocked diploid adhesive growth under amino acid starvation conditions, and adhesion of a diploid *flo11*Δ/*flo11*Δ mutant was restored by complementation with *FLO11* on a plasmid (Figure 1A).

Expression of *FLO11* was measured in haploid and diploid yeast strains under different nutritional conditions, to determine the correlation between adhesive growth and expression of *FLO11*. When using a *FLO11-lacZ* reporter gene, a 3.6-fold increase in expression was found in nitrogen-starved haploids compared with nonstarved cells, and a 59-fold increase was measured in diploid cells (Figure 1B). An induction of *FLO11* expression by nitrogen starvation has been observed previously (Lo and Dranginis, 1998; Rupp *et al.*, 1999). Herein, we found that amino acid starvation led to an increase in the expression of *FLO11-lacZ* of 20-fold in haploid cells and 41-fold in diploid cells even when high amounts of ammonium are available (Figure 1B). Induction of *FLO11-lacZ* expression by the histidine-analog 3AT was partially reversible by addition of histidine (Figure 1B), suggesting that histidine starvation is the inducing signal for enhanced expression. The effect of amino acid starvation on transcript levels of *FLO11* was determined, to corroborate the data obtained with the *FLO11-lacZ* translational fusion. In haploid strains, amino acid starvation in the presence of high amounts of ammonium led to a 5.1-fold increase in *FLO11* transcript levels (Figure 2), correlating with *FLO11-lacZ* expression. In nonstarved diploid cells, very low *FLO11* transcripts were detectable in Northern hybridization experiments (Figure 2). In 3AT-treated diploid cells, the amount of *FLO11* transcripts increased to a level comparable to that found in nonstarved haploids (Figure 2), thus correlating with *FLO11-lacZ* expression. These results show that amino acid starvation not only enhances adhesive

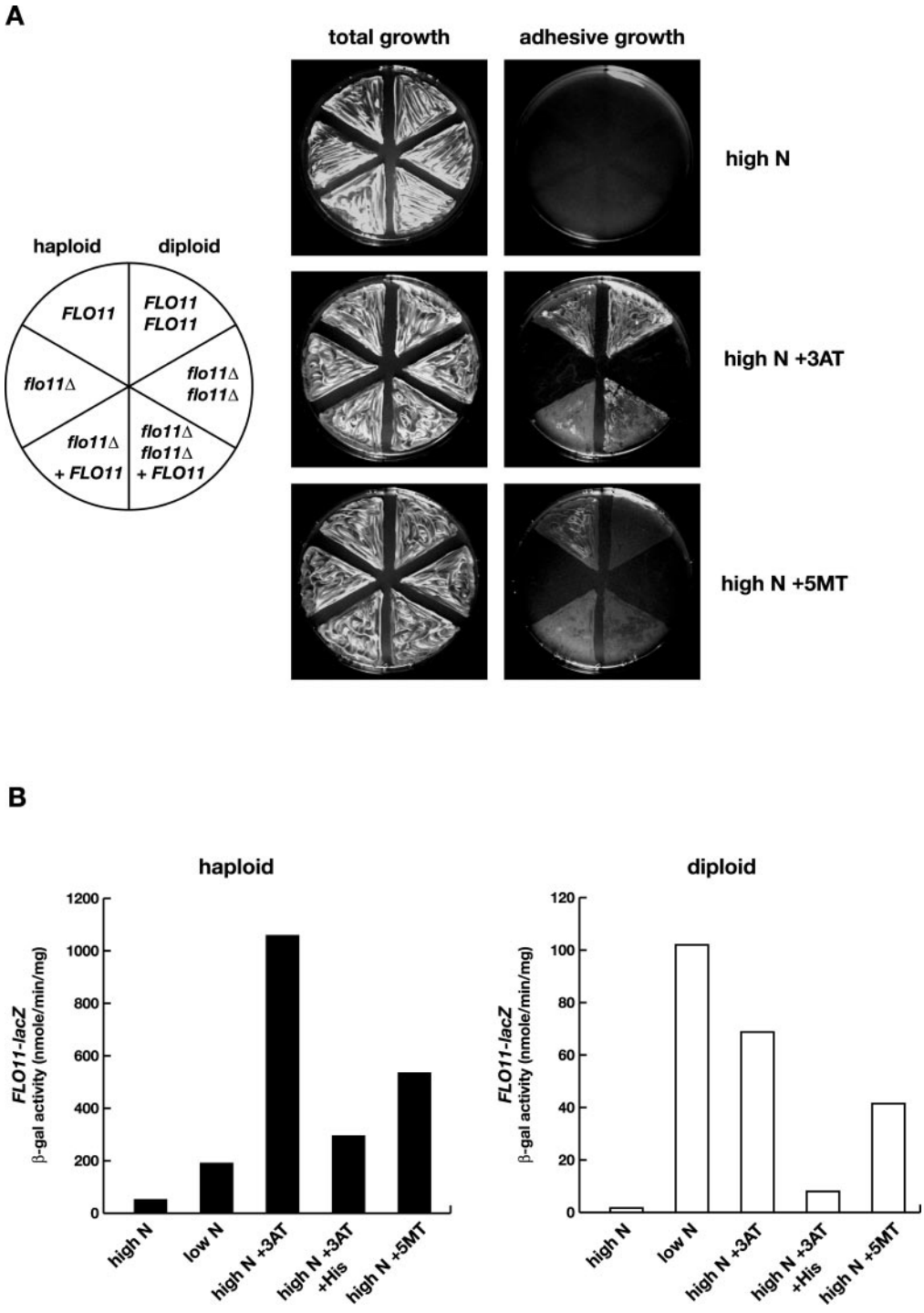

Figure 1. Amino acid starvation induces adhesive growth and *FLO11* expression in haploid and diploid *S. cereivisiae* strains. (A) Haploid strains RH2648 (*FLO11*) and RH2662 (*flo11*Δ), and diploid strains RH2656 (*FLO11/FLO11*) and RH2661 (*flo11*Δ/*flo11*Δ) carrying plasmid B3782 or pME2519 (+*FLO11*) were patched on YNB (high N), YNB + 5 mM 3AT (high N + 3AT) or YNB + 1 mM 5MT (high N + 5MT). Plates were incubated at 30°C for 24 h and photographed before (total growth) and after (adhesive growth) nonadhesive yeast cells were washed off the agar surface. (B) Expression of the *FLO11-lacZ* reporter gene was determined in yeast strains RH2648 (haploid) and RH2656 (haploid) carrying plasmid B3782 under different nutritional conditions. Cultures grown in YNB were used for assaying high ammonium conditions (high N, 50 mM ammonium sulfate). Nitrogen starvation (low N) was induced by growth in YNB with limited amounts of ammonium sulfate (50 μM) as sole nitrogen source. Amino acid starvation was induced by addition of 10 mM 3AT (high N + 3AT) or 1 mM 5MT (high N + 5MT). To revert histidine starvation, 1 mM histidine was added to YNB medium containing 10 mM 3AT (high N + 3AT +His). Units of specific β-galactosidase activities are shown in nanomoles per minute per milligram. Bars depict means of at least three independent measurements with a SD not exceeding 15%.

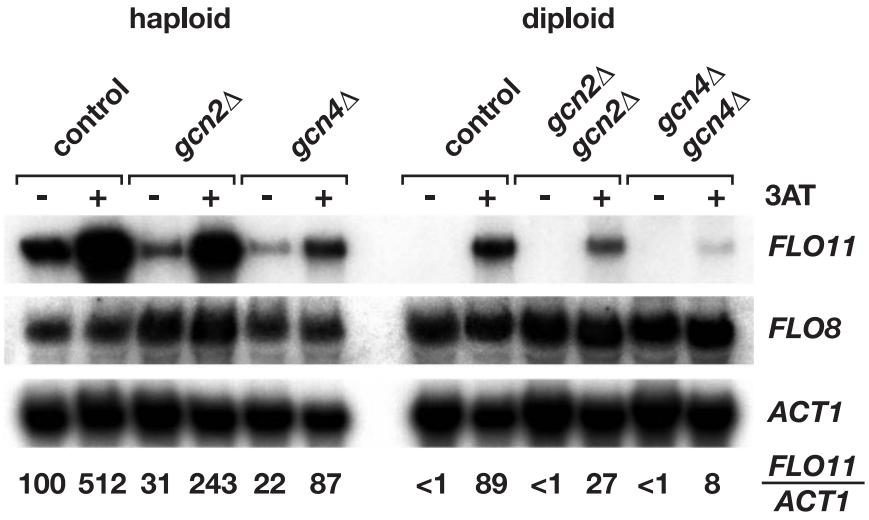

Figure 2. Transcript levels of *FLO11*, *FLO8*, and *ACT1*. Total RNAs were prepared from haploid yeast strains RH2648 (control), RH2649 (*gcn2*Δ), RH2650 (*gcn4*Δ), and the diploid yeast strains RH2656 (control), RH2657 (*gcn2*Δ/*gcn2*Δ), RH2658 (*gcn4*Δ/*gcn4*Δ) grown in high ammonium medium (YNB + Ura + Arg) to logarithmic phase before (−) and after (+) induction of amino acid starvation by addition of 10 mM 3AT. For measurements of *FLO11*, *FLO8*, and *ACT1* transcript levels, 10 μg of total RNA from each sample was subjected to a Northern hybridization analysis. Signals were quantified using a phosphorimaging scanner. Numbers given indicate relative expression levels of *FLO11* when compared with *ACT1* as internal standard with a value for nonstarvation expression of the haploid control strain set to 100. Values represent the average of three independent measurements.

growth of haploid and diploid cells in a *FLO11*-dependent manner but also causes a strong increase in the expression of *FLO11* in both cell types. However, *FLO11* expression does not seem to strictly correlate with adhesive growth, because nonstarved haploids are comparable with starved diploids with respect to *FLO11* transcript levels and expression of *FLO11-lacZ*, but not with respect to adhesive growth. This suggests that amino acid starvation-induced adhesive growth not only involves up-regulation of *FLO11* expression but also additional factors whose functions depend on Flo11p.

The tryptophan-derivative 5MT is a further amino acid analog known to induce amino acid starvation (Schürch *et al*., 1974). We also determined the effects of adding 5MT to high ammonium medium on adhesive growth and expression of *FLO11*. Results obtained by addition of 5MT were similar to that obtained by adding 3AT, although effects were less pronounced (Figure 1).

Haploid Adhesive Growth and Expression of FLO11 *Depend on* GCN2 *and* GCN4*, Elements of the General Amino Acid Control System*

The requirement of the sensor kinase Gcn2p and the transcriptional activator Gcn4p for adhesive growth and expression of *FLO11* was analyzed in haploid *gcn2*Δ, *gcn4*Δ, and *gcn2*Δ *gcn4*Δ mutant strains and compared with a *flo11*Δ strain. Under nonstarvation conditions, adhesive growth of haploid strains was significantly reduced in the absence of *GCN2* or *GCN4* (Figure 3A). The *gcn2*Δ *gcn4*Δ double mutant was indistinguishable from the single mutants. Amino acid starvation was induced by addition of either 1 mM 3AT or 10 mM 3AT to the growth medium, because addition of 10 mM 3AT inhibits growth of strains lacking *GCN2* or *GCN4* (Figure 3A). Addition of 1 mM 3AT was sufficient to enhance adhesive growth of a control strain, without significantly inhibiting growth of the *gcn2*Δ or *gcn4*Δ mutant strains (Figure 3A). Adhesive growth of haploid *gcn2*Δ, *gcn4*Δ, or *gcn2*Δ *gcn4*Δ mutants was reduced under both 1 mM and 10 mM 3AT starvation conditions, compared with the control strain.

Strains measured for adhesive growth were further assayed for expression of the *FLO11-lacZ* reporter gene and *FLO11* transcript levels. Under nonstarvation conditions, expression of *FLO11-lacZ* dropped from 52.1 U in a control strain to 11.5 U in the *gcn2*Δ, 11.5 U in the *gcn4*Δ, and 11.4 U in the *gcn2*Δ *gcn4*Δ double mutant strain, respectively (Figure 3B). The decrease in *FLO11-lacZ* expression in the *gcn* mutant strains correlated with a decrease in both *FLO11* transcript levels (Figure 2) and the resulting adhesive growth behavior (Figure 3A), although deletion of *GCN2* led to a less severe reduction of *FLO11* mRNA than observed for strains lacking *GCN4*. To induce amino acid starvation, 3AT was added to strains grown to logarithmic phase at concentrations of both 1 and 10 mM. Addition of 1 mM 3AT was sufficient to induce expression of *FLO11-lacZ* to levels (683 U) almost matching that obtained by the addition of 10 mM 3AT (1059 U) (Figure 3B). Expression of *FLO11-lacZ* significantly decreased by deletion of *GCN2* (71.5 U), *GCN4* (67.3 U), or both (66.5 U) under 10 mM 3AT conditions, corresponding to a roughly 16-fold drop in comparison to the expression measured in the control strain. These data suggest that Gcn2p and Gcn4p are required for full induction of *FLO11* expression by amino acid starvation. However, *FLO11-lacZ* expression and *FLO11* transcript levels measured in the starved *gcn* mutant strains were similar to levels measured in a control strain under nonstarvation conditions. Yet these strains were less adhesive under starvation conditions (Figure 3). This result indicates that Gcn2p and Gcn4p not only control expression of the *FLO11* gene but also might regulate Flo11p at a posttranslational level or expression of additional genes required for adhesive growth.

We further tested, whether high-level expression of Gcn4p is sufficient to induce adhesive growth and enhanced expression of *FLO11*. For this purpose, a mutant allele of *GCN4* (*GCN4m*) was expressed that carries point mutations inactivating all four μ open reading frames in the *GCN4* upstream leader and causes high expression of Gcn4p under nonstarvation conditions (Mueller and Hinnebusch, 1986). However, expression of *GCN4m* was not sufficient to induce adhesive growth and did not lead to enhanced expression of *FLO11-lacZ* (our unpublished data), indicating that Gcn4p might control expression of *FLO11* in concert with other transcriptional regulators or by an indirect mechanism.

In summary, our results show that Gcn2p and Gcn4p, elements of the general control system of amino acid biosynthesis, are required for adhesive growth and efficient expression of *FLO11* in haploid cells.

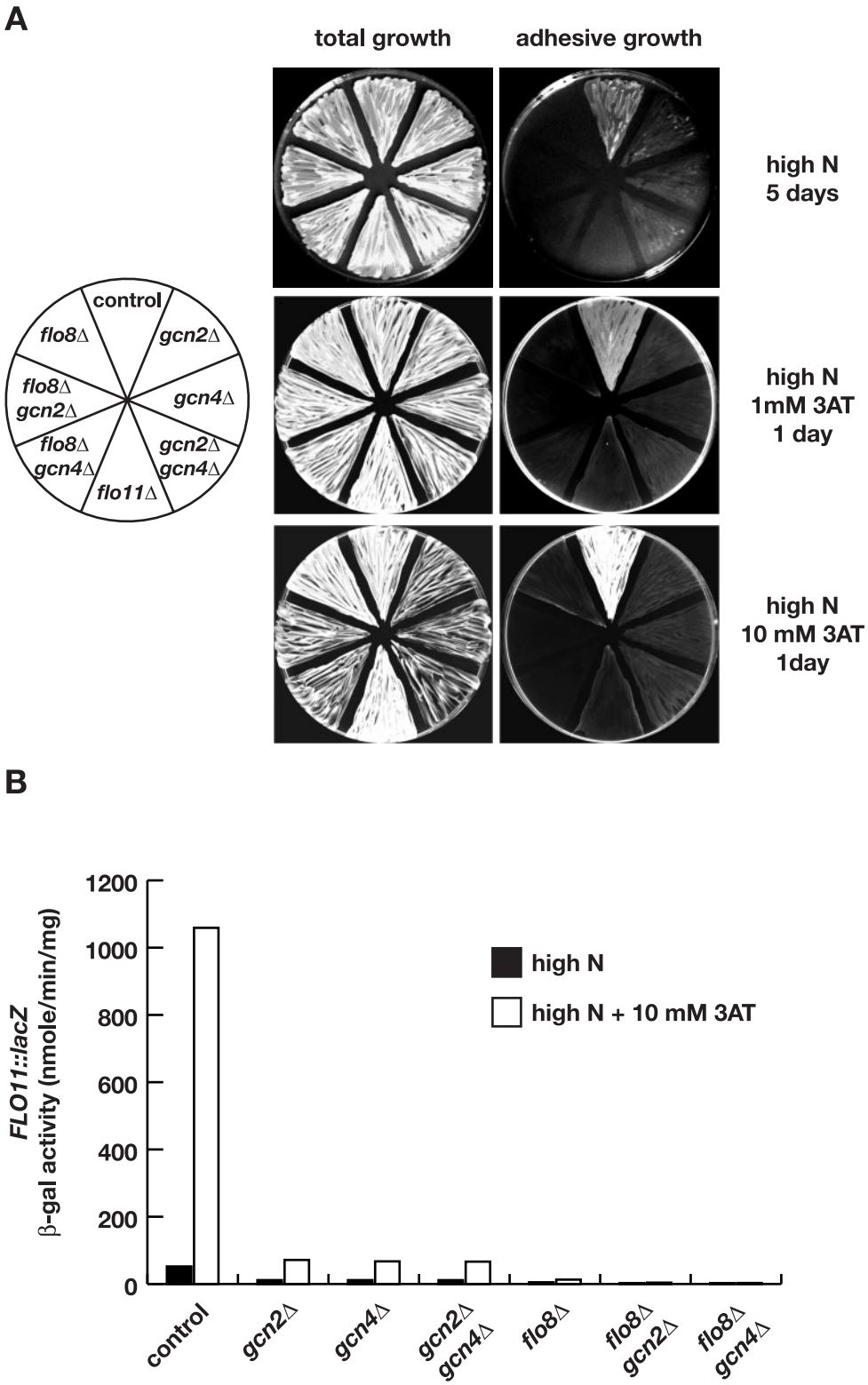

Figure 3. Requirement of *GCN2*, *GCN4*, and *FLO8* for amino acid starvation-induced adhesive growth and *FLO11* expression in haploid *S. cerevisiae* strains. (A) Haploid yeast strains RH2648 (control), RH2649 (*gcn2*Δ), RH2650 (*gcn4*Δ), RH2651 (*gcn2*Δ *gcn4*Δ), RH2662 (*flo11*Δ), RH2652 (*flo8*Δ), RH2653 (*flo8*Δ *gcn2*Δ) and RH2654 (*flo8*Δ *gcn4*Δ) carrying plasmid B3782 were patched on YNB +Arg medium (high N) and on YNB + Arg containing either 1 mM 3AT (high N + 1 mM 3AT) or 10 mM 3AT (high N + 10 mM 3AT). Plates were incubated at 30°C for 1 d (3AT-containing media) or 5 d (nonstarvation medium) and photographed before (total growth) and after (adhesive growth) nonadhesive cells were washed off the agar surface. (B) Expression of *FLO11-lacZ*. Strains used in A were grown to logarithmic phase in YNB +Arg medium (high N) or YNB +Arg medium containing 10 mM 3AT (high N + 10 mM 3AT) before specific β-galactosidase activities were measured. Bars depict means of at least three independent measurements of β-galactosidase activities with a SD not exceeding 15%. Units are nanomoles per minute per milligram.

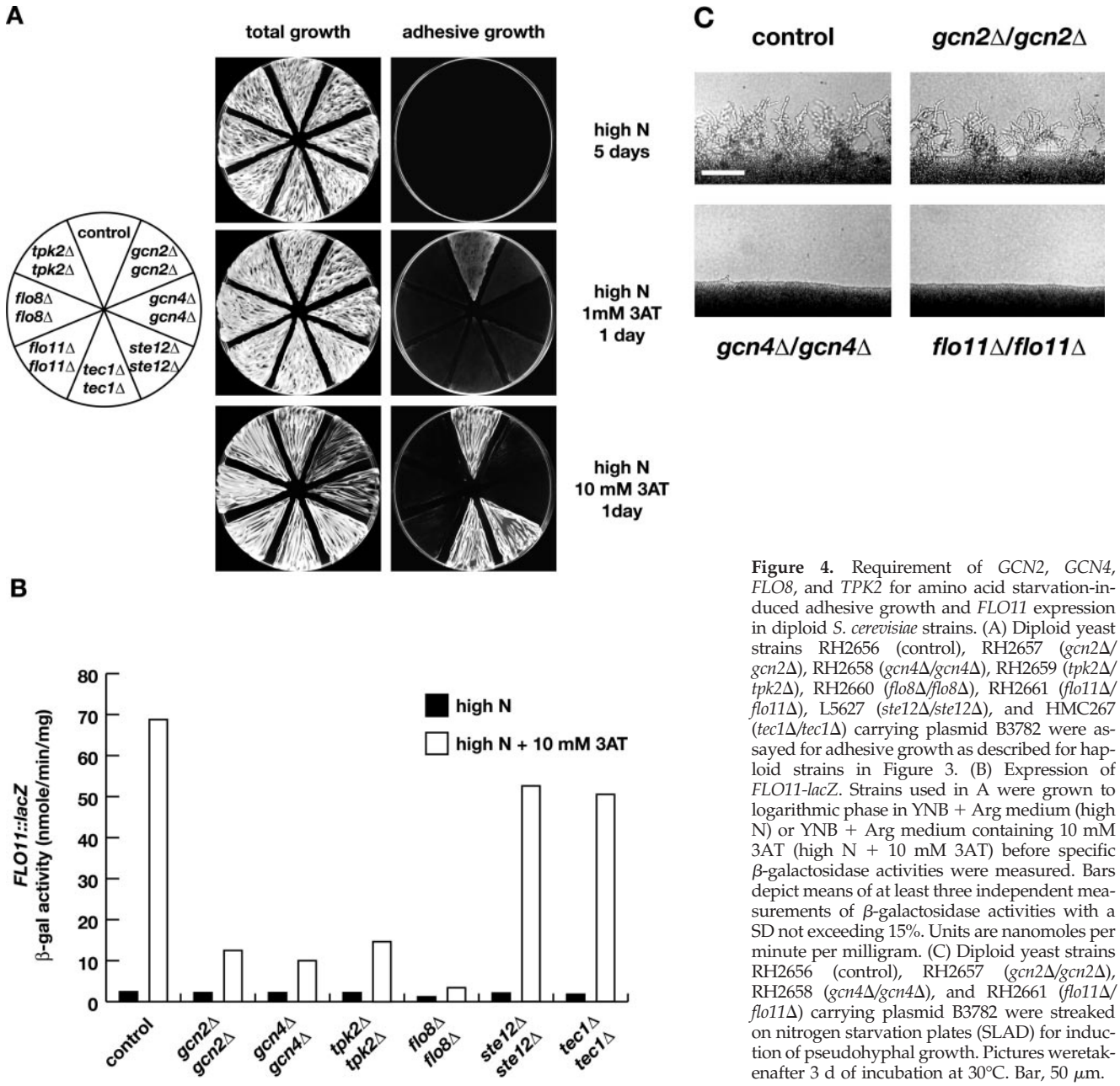

Figure 4. Requirement of *GCN2*, *GCN4*, *FLO8*, and *TPK2* for amino acid starvation-induced adhesive growth and *FLO11* expression in diploid *S. cerevisiae* strains. (A) Diploid yeast strains RH2656 (control), RH2657 (*gcn2*Δ/*gcn2*Δ), RH2658 (*gcn4*Δ/*gcn4*Δ), RH2659 (*tpk2*Δ/*tpk2*Δ), RH2660 (*flo8*Δ/*flo8*Δ), RH2661 (*flo11*Δ/*flo11*Δ), L5627 (*ste12*Δ/*ste12*Δ), and HMC267 (*tec1*Δ/*tec1*Δ) carrying plasmid B3782 were assayed for adhesive growth as described for haploid strains in Figure 3. (B) Expression of *FLO11-lacZ*. Strains used in A were grown to logarithmic phase in YNB + Arg medium (high N) or YNB + Arg medium containing 10 mM 3AT (high N + 10 mM 3AT) before specific β-galactosidase activities were measured. Bars depict means of at least three independent measurements of β-galactosidase activities with a SD not exceeding 15%. Units are nanomoles per minute per milligram. (C) Diploid yeast strains RH2656 (control), RH2657 (*gcn2*Δ/*gcn2*Δ), RH2658 (*gcn4*Δ/*gcn4*Δ), and RH2661 (*flo11*Δ/*flo11*Δ) carrying plasmid B3782 were streaked on nitrogen starvation plates (SLAD) for induction of pseudohyphal growth. Pictures weretakenafter 3 d of incubation at 30°C. Bar, 50 μm.

In Diploid Yeast Cells, GCN4 *Is Required for Amino Acid Starvation-induced Adhesive Growth and Nitrogen Starvation-induced Pseudohyphal Development*

Amino acid starvation-induced adhesive growth, *FLO11-lacZ* expression and *FLO11* transcript levels were further measured in diploid strains. Under nonstarvation conditions, diploid *gcn2*Δ/*gcn2*Δ and *gcn4*Δ/*gcn4*Δ mutant strains were indistinguishable from a control strain with respect to their nonadhesive growth behavior and the low expression of *FLO11-lacZ* or *FLO11* transcripts (Figures 2 and 4). When starved for amino acids, deletion of either *GCN2* or *GCN4* significantly suppressed the adhesive growth, which was induced in the control strain. This finding correlated with a decrease in expression of *FLO11-lacZ* of 4.6-fold in the *gcn2*Δ/*gcn2*Δ strain (15 U) and of 5.5-fold in the *gcn4*Δ/*gcn4*Δ mutant (12.5 U) in comparison with the induced levels measured in the control strain (Figure 4, A and B). Concomitantly, *FLO11* transcript levels decreased 3.3-fold in the absence of *GCN2* in comparison with the control strain and 11-fold, when *GCN4* was deleted. Thus, amino acid starvation-induced adhesive growth and expression of *FLO11* require *GCN2* and *GCN4* in diploid strains, corroborating the data obtained in haploids.

Cell-cell and cell-substrate adhesion are processes essential for the development of pseudohyphal filaments of diploid *S. cerevisiae* strains that have been starved for nitrogen (Lo and Dranginis, 1998). Diploid strains lacking *GCN2* or *GCN4* were tested for pseudohyphal development on nitrogen starvation medium. Only *gcn4*Δ/*gcn4*Δ mutant strains

Table 3. Expression of different *FLO11-lacZ* reporter constructs under nonstarvation and amino acid starvation conditions in haploid and diploid *S. cerevisiae* strains

Construct	Boundaries of deletion	*MAT*a			*MAT*a/α		
		YNB	YNB +3AT	3AT induction	YNB	YNB +3AT	3AT induction
FLO11	−none	52.1 (11.5)	1059 (67.3)	20.3 (5.9)	1.7 (1.9)	68.8 (12.5)	40.5 (6.9)
flo11-Δ1	−201 to −1	2.2	39.8	17.8	0.1	1.1	11.0
flo11-Δ2	−401 to −202	13.7	284	20.7	0.7	7.8	11.1
flo11-Δ3	−600 to −401	13.7	299	21.9	0.6	6.4	10.6
flo11-Δ4	−800 to −601	197	1693	8.6	5.7	208	36.5
flo11-Δ5	−1000 to −801	0.3	0.2	0.7	0.1	0.4	4.0
flo11-Δ6	−1200 to −1001	1.9	7.0	3.6	0.6	1.6	2.7
flo11-Δ7	−1400 to −1201	363 (5.2)	4597 (62.2)	12.7 (12.1)	13.0 (3.8)	380 (30.5)	29.2 (8.0)
flo11-Δ8	−1600 to −1401	375 (680)	397 (850)	1.1 (1.3)	132 (166)	98.0 (297)	0.7 (1.8)
flo11-Δ9	−1800 to −1601	30.8 (17.5)	680 (18.2)	22.1 (1.0)	1.0 (1.1)	28.2 (3.1)	38.2 (2.8)
flo11-Δ10	−2000 to −1801	51.4	1035	20.1	0.8	36.1	45.1
flo11-Δ11	−2200 to −2001	63.4	1328	20.9	0.8	54.2	67.8
flo11-Δ12	−2400 to −2201	65.4	1215	18.6	1.1	41.2	37.5
flo11-Δ13	−2600 to −2401	49.1	1003	20.4	1.2	54.0	45.0
flo11-Δ14	−2800 to −2601	108	1312	12.2	2.1	81.4	38.8
flo11-Δ15	−3000 to −2801	38.1	910	23.9	1.1	16.1	14.6

Expression of indicated *FLO11-lacZ* reporter constructs (Rupp *et al.*, 1999) was assayed in the haploid strain RH2648 (*MAT*a) and the diploid strain RH2656 (*MATa/α*) under nonstarvation conditions (YNB + Arg) and under amino acid starvation conditions (YNB +Arg +3AT). Values shown in parentheses were obtained by assaying constructs in *gcn4*Δ strains RH2650 (*MAT*a *gcn4*Δ) and RH2658 (*MAT*a/α *gcn4*Δ/*gcn4*Δ), respectively. Deleted segments in the individual constructs are numbered according to the numbering shown in Figure 5. Boundaries of deletions are indicated with respect to the translational start site of *FLO11* at position +1. Given numbers represent β-galactosidase activities in actual units nanomoles per minute per milligram. Values for 3AT induction represent the ratio of activities obtained for a given *FLO11-lacZ* construct in the presence or absence of 3AT, respectively. All values are means of at least three independent measurements with a standard deviation not exceeding 15%.

were suppressed for development of pseudohyphae comparable with *flo11*Δ/*flo11*Δ mutant strains (Figure 4C).

In summary, Gcn2p and Gcn4p are required for amino acid starvation-induced adhesive growth in diploids, but for pseudohyphal development, induced by nitrogen starvation, only Gcn4p is necessary.

Amino Acid Starvation-induced Expression of FLO11 *Requires the Transcription Factors Gcn4p and Flo8p, but Not Ste12p and Tec1p*

Adhesive growth and expression of *FLO11* are under control of the cAMP pathway and the MAPK pathway (Pan and Heitman, 1999; Rupp *et al.*, 1999). We tested whether amino acid starvation-induced adhesive growth and expression of *FLO11* requires Tpk2p or Flo8p, elements of the cAMP-regulated pathway, or the transcription factors Ste12p and Tec1p, elements of the MAPK pathway. Adhesive growth of diploid strains lacking *TPK2* or *FLO8* was suppressed to a degree comparable with a *flo11*Δ/*flo11*Δ control strain under amino acid starvation conditions (Figure 4A). Expression of *FLO11-lacZ* was reduced 4.3-fold in *tpk2*Δ/*tpk2*Δ strains (16 U) and 20-fold in *flo8*Δ/*flo8*Δ mutants (3.4 U). In comparison, deletion of *STE12* or *TEC1* did not suppress adhesive growth in the presence of 10 mM 3AT, and expression of *FLO11-lacZ* was reduced only 1.4-fold in both the *ste12*Δ/*ste12*Δ (52 U) and *tec1*Δ/*tec1*Δ (49 U) mutant strains (Figure 4B). Thus, efficient expression of *FLO11* under amino acid starvation conditions requires *FLO8* and *TPK2*, but not *STE12* and *TEC1*.

3AT-induced adhesive growth and expression of *FLO11-lacZ* was measured in haploid *flo8*Δ *gcn2*Δ and *flo8*Δ *gcn4*Δ double mutant strains and compared with *gcn2*Δ, *gcn4*Δ, and *flo8*Δ single mutants, to distinguish between a parallel and a linear configuration of the general control system and the transcription factor Flo8p. Under amino acid starvation conditions, both adhesive growth and expression of *FLO11-lacZ* is lower in the *flo8*Δ *gcn2*Δ (4 U) and *flo8*Δ *gcn4*Δ (2.9 U) double mutants than in the *gcn2*Δ (71.5 U), *gcn4*Δ (67.3 U), or *flo8*Δ (13 U) single mutants (Figure 3). The additive effects of the *gcn2*Δ and *flo8*Δ or *gcn4*Δ and *flo8*Δ mutations argue for independent functions of the general control system and Flo8p. This conclusion is supported by the fact that transcript levels of *FLO8* are not affected by amino acid starvation or by mutations in *GCN2* or *GCN4* (Figure 2).

In summary, amino acid starvation-induced expression of *FLO11* requires the combined action of the transcription factors Gcn4p and Flo8p but does not depend on Ste12p and Tec1p.

Identification of FLO11 *Promoter Elements Mediating Regulation by Amino Acid Starvation*

A set of 15 *flo11-lacZ* promoter deletion constructs spanning the region between the 3000 base pairs upstream of the *FLO11* initiation codon was used (Rupp *et al.*, 1999) to identify *FLO11* promoter elements that confer regulation of *FLO11* expression in response to amino acid starvation. Expression of this set of *flo11-lacZ* reporter constructs, each containing an individual 200-base pair deletion, was assayed in haploid and diploid strains under both nonstarvation and amino acid starvation conditions and compared with the intact *FLO11-lacZ* reporter (Table 3). A deletion was assigned to contain a UAS (upstream activation site) when leading to at least 50% reduced expression of *FLO11-lacZ*, and as a URS (upstream repression site) when causing at least threefold enhanced expression (Figure 5A).

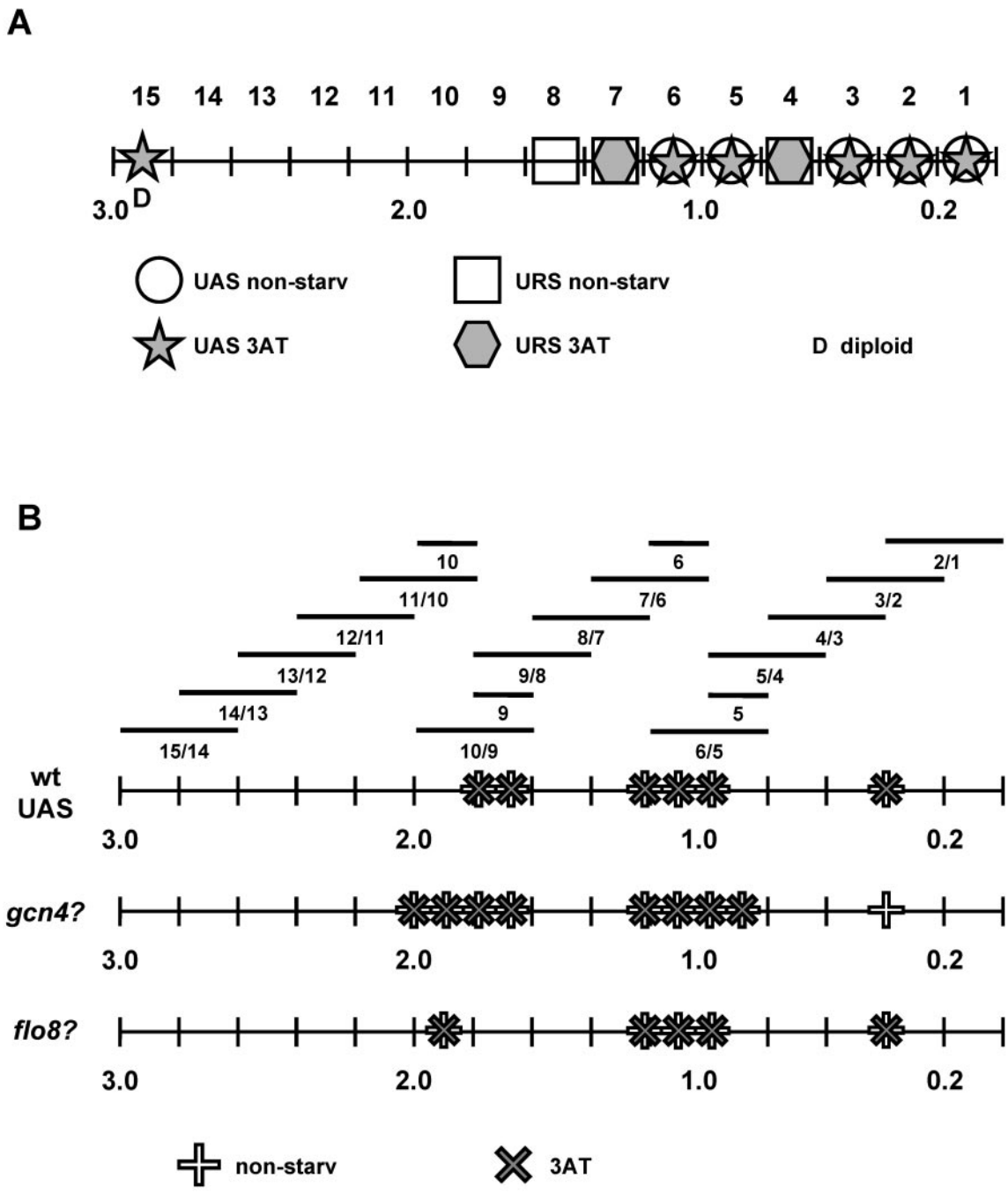

Figure 5. Sequence elements involved in regulation of *FLO11*. (A) The specific β-galactosidase activities of 15 yeast strains carrying individual 200-base pair deletions of the *FLO11* promoter region were compared with a yeast strain carrying the full-length 3-kbp wild-type promoter under nonstarvation (nonstarv) and amino acid starvation conditions (3AT) in haploid as well as in diploid yeasts (Table 3). Individual 200-base pair segments within the *FLO11* promoter region are numbered from 1 to15 and their relative position in kilobases is shown with respect to the translational start site. A ratio of specific β-galactosidase activity (deletion/3 kbp promoter) of three or higher was defined as URS. URS elements under nonstarvation conditions are marked by a square (URS nonstarv) and under starvation conditions by a hexagon (URS 3AT). At least 50% lower expression than the wild-type reporter is represented as an UAS. UAS elements under nonstarvation conditions are marked by a circle (UAS nonstarv) and under starvation conditions by a star (UAS 3AT). Elements, which occur only in the diploid strain are marked by a D. (B) Expression of 19 *CYC1-lacZ* reporter constructs carrying isolated segments of the *FLO11* promoter region was determined in haploid wild-type cells or in corresponding cells deleted for the transcription factors *GCN4* or *FLO8* under nonstarvation or amino acid starvation conditions (Table 4). The symbols (white cross, nonstarvation conditions, gray cross, starvation conditions) are placed on a line in a position that indicates, which of the fragments stimulated β-galactosidase activity. Each line represents the *FLO11* promoter in the indicated genetic background. The first row (wt UAS) denotes sequence elements showing a >5-fold elevation of the reporter over a plasmid without an insert. The next two lines (*gcn4*Δ and *flo8*Δ) represent sequence elements showing a >3-fold reduction of the β-galactosidase activity in the mutant (*gcn4*Δ or *flo8*Δ) compared with the activity of the element in the control strain.

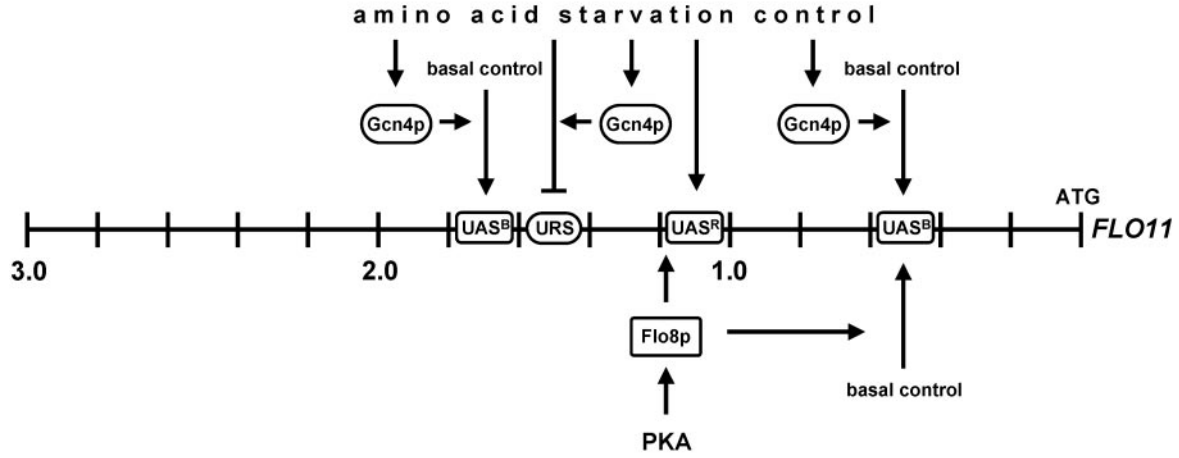

Figure 6. Model for regulation of *FLO11* by amino acid starvation, Gcn4p, and Flo8p. Shown is the promoter of *FLO11* and the regulatory elements that confer regulation by amino acid starvation (UAS^R and URS) or basal regulation under nonstarvation conditions (UAS^B). Gcn4p is postulated to control basal and amino acid starvation-regulated expression of *FLO11* indirectly, whereas control of *FLO11* expression by Flo8p can be indirectly or directly, as shown previously (Pan and Heitman, 2002). PKA, cAMP-dependent protein kinase.

The *flo11-1*, *flo11-2*, *flo11-3*, *flo11-5*, and *flo11-6* deletions identify at least five UAS elements. The UAS sites deleted in *flo11-1*, *flo11-2*, and *flo11-3* do not seem to confer amino acid starvation signals for the expression of *FLO11*, because their absence does not suppress induction of *FLO11* expression by 3AT in haploids. In contrast, the UAS sites defined by *flo11-5* and *flo11-6* might be involved in amino acid starvation-induced activation, because their deletion drastically reduced activation by 3AT in haploids and diploids. However, these UAS elements presumably have an additional more general activation function, because expression of *flo11-5* and *flo11-6* is already reduced under nonstarvation conditions. The *flo11-15* deletion defines a further UAS element in diploid strains that might be 3AT specific, because this deletion led to a 4.3-fold reduced expression of *FLO11* in the presence of 3AT.

Three URS elements are defined by the *flo11-4*, *flo11-7*, and *flo11-8* deletions. The URS sites in *flo11-4* and *flo11-7* do not seem to be regulated by amino acid starvation, because expression of both constructs is inducible by addition of 3AT (Table 3). In contrast, the URS element defined by the *flo11-8* deletion (base pairs −1400 to −1600) couples expression of *FLO11* to regulation by amino acid starvation, because its deletion led to a strong induction of *FLO11-lacZ* expression (7.2-fold in haploids and 77.5-fold in diploids) under nonstarvation conditions, but not in the presence of 3AT. Thus, the *FLO11* promoter contains at least one 3AT-responsive URS element in segment *FLO11-8* that might confer enhanced expression of *FLO11* in response to amino acid starvation (Figure 6). To test, whether derepressed *FLO11-lacZ* expression observed for deletion of segment 8 is Gcn4p dependent, expression of *flo11-8* was measured in strains lacking *GCN4* and compared with expression of *flo11-7* and *flo11-9*. We found that expression of *flo11-8* was not reduced in the absence of Gcn4p, neither in the absence nor presence of 3AT. In contrast, expression of *flo11-7* and *flo11-9* was strongly dependent on *GCN4*. This further corroborates that the URS element in segment *FLO11-8* is regulated by amino acid starvation and suggests that Gcn4p might be required indirectly to overcome the negative effects of this URS on *FLO11* transcription.

UAS elements that mediate regulation by amino acid starvation were identified in a second approach by using a series of 14 reporter constructs containing individual 400-base pair *FLO11* promoter fragments that overlap by 200 base pairs and are cloned in front of a *CYC1-lacZ* fusion gene (Rupp *et al*., 1999). This series of reporter constructs identified strong UAS elements in the segments *FLO11-3/2*, *FLO11-6/5*, *FLO11-7/6*, and *FLO11-10/9* (Table 4 and Figure 5B). These *FLO11* promoter elements increased expression of *CYC1-lacZ* at least fivefold compared with the reporter without any insert (Table 4). The elements present in *FLO11-3/2* (base pairs −620 to −182) and *FLO11-10/9* (base pairs −2020 to −1573) both confer similar activation in the absence and presence of 3AT, suggesting that they are basal UAS elements (UAS^B) that are not regulated by amino acid starvation (Figure 6). In contrast, activity mediated by segments *FLO11-6/5* (base pairs −1220 to −779) and *FLO11-7/6* (base pairs −1421 to −981) is at least 2.4-fold inducible by addition of 3AT, suggesting that these segments contain UAS elements that confer regulation by amino acid starvation (UAS^R). To better localize the strong basal UAS^B element in segment *FLO11-10/9* and the regulated UAS^R element in segment *FLO11-6/5*, expression of further transcriptional reporters was measured that carried individual segments *FLO11-5*, *FLO11-6*, *FLO11-9*, or *FLO11-10* in front of *CYC1-lacZ* (Table 4 and Figure 5). We found that *FLO11-9*, but not *FLO11-10*, conferred basal UAS activity, indicating that the basal UAS^B element is localized between base pairs −1820 to −1573 (Figure 6). Similarly, UAS activity and regulation by 3AT was conferred by*FLO11-6*, but not *FLO11-5*, indicating that the regulated UAS^R element is localized between base pairs −1220 to −981 (Figure 6).

FLO11 *Promoter Elements Mediating Regulation by Gcn4p and Flo8p in Response to Amino Acid Starvation*

Activation of *FLO11-lacZ* expression by 3AT is completely blocked when both Gcn4p and Flo8p are absent (Figure 4). To identify the regions of the *FLO11* promoter that are under control of Gcn4p and Flo8p, the set of *CYC-lacZ* reporter constructs was transformed into strains deleted for *GCN4* or *FLO8*. Deletion of *GCN4* led to a more than threefold reduction in the expression of *FLO11-3/2* under nonstarvation conditions, and of *FLO11-5*, *FLO11-6/5*, *FLO11-6*, *FLO11-7/6*,

Table 4. Expression of different *CYC1-lacZ* reporter constructs

Inserted segment	Boundaries of insert	YNB			YNB + 3AT			3AT induction		
		wt	*gcn4*Δ	*flo8*Δ	wt	*gcn4*Δ	*flo8*Δ	wt	*gcn4*Δ	*flo8*Δ
No insert		7.1	6.9	7.0	7.7	7.3	7.3	1.1	1.0	1.0
2/1	−421 to −1	3.3	2.2	3.0	2.6	3.1	3.0	0.8	1.4	1.0
3/2	−620 to −182	41.6	8.3	4.9	47.2	22.2	6.1	1.1	2.7	1.2
4/3	−820 to −381	5.4	1.9	4.4	9.8	4.3	6.0	1.8	2.3	1.4
5/4	−1018 to −581	4.5	4.2	2.6	7.7	3.9	2.7	1.7	0.9	1.0
5	−1018 to −779	3.4	0.3	1.4	14.1	1.1	4.7	4.2	3.7	3.4
6/5	−1220 to −779	159	15.2	3.8	525	51.1	25.1	3.3	3.4	6.6
6	−1220 to −981	187	58.2	61.5	584	188	193	3.1	3.2	3.1
7/6	−1421 to −981	126	20.8	8.0	299	61.7	27.5	2.4	2.9	3.5
8/7	−1620 to −1182	10.8	4.0	3.9	13.4	4.8	4.8	1.2	1.2	1.2
9/8	−1820 to −1381	8.6	5.0	21.0	18.4	8.4	13.3	2.1	1.7	0.6
9	−1820 to −1573	101	18.1	49.8	128	23.4	60.1	1.3	1.3	1.2
10/9	−2020 to −1573	194	32.7	157	235	42.9	186	1.2	1.3	1.2
10	−2020 to −1781	21.4	2.6	4.0	27.3	3.1	7.9	1.3	1.2	2.0
11/10	−2220 to −1781	22.4	1.0	15.1	36.8	1.2	25.1	1.6	1.2	1.7
12/11	−2420 to −1981	11.3	3.8	6.8	10.3	3.8	6.7	0.9	1.0	1.0
13/12	−2620 to −2181	2.1	1.2	1.5	2.0	1.8	2.2	1.0	1.5	1.5
14/13	−2820 to −2381	1.7	1.1	1.4	1.6	1.9	1.5	1.0	1.7	1.0
15/14	−2984 to −2581	2.1	1.3	2.0	1.7	2.1	2.3	0.8	1.6	1.2

Expression of indicated *CYC1-lacZ* reporter constructs (Rupp *et al*., 1999; Table 2) was assayed in the haploid strains RH2648 (wt), RH2650 (*gcn4*Δ) and RH2652 (*flo8*Δ) under nonstarvation conditions (YNB + Arg) and under amino acid starvation conditions (YNB +Arg +3AT). Segments of the *FLO11* promoter inserted upstream of *CYC1-lacZ* in the individual constructs are indicated and correspond to the numbering shown in Figure 5. Boundaries of insertions are indicated with respect to the translational start site of *FLO11* at position +1. pME2212 was used as a control plasmid carrying no insert upstream of *CYCl-lacZ*. Given numbers represent β-galactosidase activities in actual units nanomoles per minute per milligram. Values for 3AT induction represent the ratio of activities obtained for a given *CYC1-lacZ* construct in the indicated genetic background in the presence or absence of 3AT, respectively. All values are means of at least three independent measurements with a standard deviation not exceeding 15%.

and *FLO11-9*, *FLO11-10/9*, *FLO11-10*, and *FLO11-11/10* under both nonstarvation and starvation conditions. (Table 4 and Figure 5B). However, activation of *FLO11-5*, *FLO11-6/5*, *FLO11-6*, and *FLO11-7/6* by 3AT was not significantly reduced in the absence of Gcn4p. Together, these results suggest that Gcn4p controls expression of *FLO11* by affecting basal control mechanisms mediated by UAS^B elements, rather than by affecting UAS^R elements conferring regulation by amino acid starvation (Figure 6). However, regulation by Gcn4p is likely to involve further factors, because none of the Gcn4p-controlled segments of the *FLO11*-promoter identified herein contain a Gcn4p-binding site. In addition, none of these segments are able to bind Gcn4p protein present in yeast extracts or purified from *Escherichia coli* when tested in vitro (our unpublished data).

Deletion of *FLO8* had significant effects on expression of *FLO11-3/2*, *FLO11-6/5*, *FLO11-6*, and *FLO11-7/6* under both nonstarvation and amino acid starvation conditions (Table 4). These regions of the *FLO11* promoter were previously identified to be under control of Flo8p (Rupp *et al*., 1999). Results obtained herein indicate that Flo8p might not be involved in mediating amino acid starvation signals to these elements, because *FLO11-3/2* is not inducible by 3AT and because *FLO11-6/5*, *FLO11-6*, and *FLO11-7/6* were still inducible by amino acid starvation in the absence of *FLO8*.

DISCUSSION

Cell-cell and cell-substrate adhesion are morphogenetic events that are required for several developmental processes of bakers' yeast, including mating, invasive growth, biofilm formation, and filamentation. Each of these events depends on distinct intrinsic and extrinsic signals, corresponding signaling pathways, and specific cell surface proteins. The cell surface flocculin Flo11p is required for haploid adhesive growth in response to glucose starvation and for diploid filamentous growth in response to nitrogen starvation. Herein, we found that amino acid starvation is a further nutritional signal that activates adhesive growth in a Flo11p-dependent manner and that leads to induced expression of the *FLO11* gene, even in the presence of high glucose and ammonium. We have identified two distinct types of regulatory elements in the *FLO11* promoter that are involved in control by amino acid starvation. One UAS^R element was found that is located in the region −1000 to −1200 upstream of the *FLO11* open reading frame and that confers regulation by amino acid starvation (Figure 6). Regulation of this UAS^R, however, is independent of Gcn4p. In addition, one URS element was identified in the region −1400 to −1600 that confers repression of *FLO11* under nonstarvation conditions, but not under starvation conditions, suggesting that repression might be relieved by starvation (Figure 6). Moreover, Gcn4p might be required to overcome the negative effect of this URS on *FLO11* transcription. Previous studies have identified multiple *cis*-acting regulatory elements that confer regulation by cAMP, Flo8p, Ste12p, and Tec1p. Regulation by cAMP and Flo8p involves the region from −1000 to −1400, and Flo8p activates a further UAS element in the region from −400 to −600 (Rupp *et al*., 1999; Pan and Heitman, 2002). Ste12p has been reported to act on at least three elements located in regions −800 to −1200, −1600 to −1800, and −2000 to −2400, respectively, and control by Tec1p

involves three elements within regions −600 to −1000 and −1600 to −2400 (Lo and Dranginis, 1998; Rupp *et al*., 1999). However, although some of these regulatory elements are located within the same segments that harbor the regulatory elements conferring control by amino acid starvation and Gcn4p, our study indicates that neither of the transcription factors Flo8p, Ste12p, nor Tec1p is likely to be directly involved in amino acid starvation control. Future fine analysis must reveal the exact nucleotides within the 200-base pair segment of the *FLO11* promoter that confer regulation by amino acid starvation and other regulatory pathways that control *FLO11* expression.

What signaling pathways are involved in mediating the amino acid starvation signal to the regulatory elements in the *FLO11* promoter identified herein? We have found that Gcn2p and Gcn4p, elements of the general control system for amino acid biosynthesis, are required for amino acid starvation-induced adhesive growth and activation of *FLO11*. The general amino acid control system was previously unknown to regulate adhesive growth and expression of *FLO11* in budding yeast (Hinnebusch and Natarajan, 2002). In the human pathogen *C. albicans*, amino acid starvation and Gcn4p have been found to affect hyphal morphogenesis (Tripathi *et al*., 2002). However, whether cell-substrate adhesion and expression of specific cell-surface proteins is induced by amino acid starvation and depends on Gcn2p and Gcn4p has not been shown in *C. albicans*. Several observations suggest that expression of *FLO11* might not involve direct binding of Gcn4p to the *FLO11* promoter and that an increase in protein levels of Gcn4p per se is not sufficient for enhanced *FLO11* transcription. 1) Sequence analysis of the *FLO11* promoter does not predict any Gcn4p-responsive element sites. 2) Gcn4p protein does not bind to any region of the *FLO11* promoter when tested in vitro (our unpublished data). 3) High-level expression of Gcn4p in nonstarved cells is not sufficient to induce enhanced expression of *FLO11*. However, a scenario in which Gcn4p directly binds to the *FLO11*-promoter in combination with other transcriptional regulators cannot be ruled out by our data. We suggest two roles for Gcn4p in regulating expression of *FLO11* (Figure 6). First, Gcn4p regulates expression of *FLO11* by affecting activity of the two basal UAS^B elements present in the regions −400 to −600 and −1600 to −1800. This conclusion is based on the finding that *FLO11* expression drops significantly in the absence of Gcn4p and that both UAS^B elements require Gcn4p to mediate efficient activation. Gcn4p seems to control these UAS^B elements indirectly, because neither of them contains a consensus Gcn4p-binding site. Again, Gcn4p might directly regulate these elements in concert with other transcriptional factors by contacting yet unknown DNA sequence elements. The second role of Gcn4p suggested by our results is control of a pathway that confers relieve of URS-mediated repression of *FLO11* transcription in response to amino acid starvation. This conclusion is based on the finding that deletion of the URS located in the region −1400 to −1600 causes derepressed expression of *FLO11* independent of Gcn4p. The exact DNA sequence elements conferring repression and the pathway required for relieve of repression remain to be determined by future investigations.

We have identified two central elements of the cAMP pathway, Tpk2p and Flo8p, to be required for amino acid starvation-induced adhesive growth and activation of *FLO11*. Previous studies have shown that the protein kinase Tpk2p together with the transcriptional regulators Flo8p and Sfl1p confer regulation of *FLO11* in response to cAMP (Robertson and Fink, 1998; Pan and Heitman, 1999; Rupp *et al*., 1999; Pan and Heitman, 2002). Flo8p and Sfl1p are direct molecular targets of Tpk2p that antagonistically regulate expression of *FLO11* via a common promoter element located within base pairs −1400 to −1150 (Pan and Heitman, 2002). Herein, we found that Tpk2p and Flo8p are required for 3AT-induced expression of *FLO11*, which involves the UAS^R element located within base pairs −1200 to −1000, and the 3AT responsive URS element located within base pairs −1600 to −1400 (Figure 6). Flo8p is not likely to be involved in directly mediating the amino acid starvation signal to the *FLO11* promoter, because none of the 3AT-responsive elements in the *FLO11* promoter requires Flo8p for activation by amino acid starvation. Our data rather suggest that Flo8p is required for basal expression of *FLO11* and that absence of Flo8p cannot be compensated by amino acid starvation. Absence of Tpk2p is likely to cause repression of *FLO11* by efficient binding of Sfl1p to segment −1400 to −1150, a transcriptional block that cannot be relieved by amino acid starvation. Our study also disfavors involvement of the filamentous/invasive MAPK cascade in mediation of the amino acid starvation signal to *FLO11*, because neither Ste12p nor Tec1p were found to be required for activation by 3AT.

We have uncovered amino acid starvation as a nutritional signal that in *S. cerevisiae* efficiently activates adhesive growth and expression of *FLO11*, even when high amounts of preferred carbon and nitrogen sources are available. These growth conditions reflect the nutritional situation of yeast cells that grow on fruits, a natural habitat of *S. cerevisiae*. Fruits are rich in carbon sources such as saccharose or glucose and contain a variety of nitrogen sources at ample concentrations (Bisson, 1991). The content of different amino acids, however, is highly unbalanced in fruits. In grapes, an important natural substrate for *S. cerevisiae*, proline and arginine are the predominant amino acids, and their concentration often exceeds that found for histidine by 10–100 times (Huang and Ough, 1989). External amino acid imbalance is one of the signals that activate the general control system in *S. cerevisiae* (Niederberger *et al*., 1981). In conclusion, our study suggests that starvation for amino acids rather than a general lack of carbon or nitrogen sources might be the nutritional signal that activates cell-cell and cell-surface adhesion of yeast living on the natural habitat.

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