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Control of Ammonium Permease Expression and Filamentous Growth by the GATA Transcription Factors *GLN3* and *GAT1* in *Candida albicans*▽

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In response to nitrogen starvation, the human fungal pathogen *Candida albicans* switches from yeast to filamentous growth. This morphogenetic switch is controlled by the ammonium permease Mep2p, whose expression is induced under limiting nitrogen conditions. In order to understand in more detail how nitrogen starvation-induced filamentous growth is regulated in *C. albicans*, we identified the *cis*-acting sequences in the *MEP2* promoter that mediate its induction in response to nitrogen limitation. We found that two putative binding sites for GATA transcription factors have a central role in *MEP2* expression, as deletion of the region containing these sites or mutation of the GATAA sequences in the full-length *MEP2* promoter strongly reduced *MEP2* expression. To investigate whether the GATA transcription factors *GLN3* and *GAT1* regulate *MEP2* expression, we constructed mutants of the *C. albicans* wild-type strain SC5314 lacking one or both of these transcription factors. Expression of Mep2p was strongly reduced in *gln3*Δ and *gat1*Δ single mutants and abolished in *gln3*Δ *gat1*Δ double mutants. Deletion of *GLN3* strongly inhibited filamentous growth under limiting nitrogen conditions, but the filamentation defect of *gln3*Δ mutants could be rescued by constitutive expression of *MEP2* from the *ADH1* promoter. In contrast, inactivation of *GAT1* had no effect on filamentation, and we found that filamentation became independent of the presence of a functional *MEP2* gene in the *gat1*Δ mutants, indicating that the loss of *GAT1* function results in the activation of other pathways inducing filamentous growth. These results demonstrate that the GATA transcription factors *GLN3* and *GAT1* control expression of the *MEP2* ammonium permease and that *GLN3* is also an important regulator of nitrogen starvation-induced filamentous growth in *C. albicans*.

The yeast *Candida albicans* is a member of the microflora on mucosal surfaces of most healthy people, but it can also cause serious infections, especially in immunocompromised patients (20). Under various environmental conditions, e.g., an increase in temperature and pH, the presence of serum, or nutrient starvation, *C. albicans* switches from yeast to filamentous growth, and this capacity is considered important for the virulence of the fungus (15, 23, 25). Therefore, how *C. albicans* senses and transduces environmental signals that induce morphogenesis has been a major focus of research in the past years (5, 6, 14, 24).

One of the conditions that promote filamentation is growth on solid media in which nitrogen sources are present at limiting concentrations. In response to nitrogen starvation, *C. albicans* expresses the *MEP1* and *MEP2* genes, which encode ammonium transporters that enable growth of the fungus when ammonium at limiting concentrations is the only available nitrogen source (2). While deletion of either one of these two ammonium permeases does not affect the ability of *C. albicans* to grow at low ammonium concentrations, *mep1*Δ *mep2*Δ double mutants are unable to grow under these conditions. In addition to being an ammonium transporter, Mep2p is involved in the control of morphogenesis, as *mep2*Δ mutants do not switch to filamentous growth under limiting nitrogen conditions, while *mep1*Δ mutants behave like the wild type. The functions of the *C. albicans* ammonium permeases are similar to those of their counterparts in the model yeast *Saccharomyces cerevisiae*, which possesses three genes encoding ammonium permeases that mediate ammonium uptake into the cell (18). *S. cerevisiae* also differentiates into a filamentous, pseudohyphal growth form under nitrogen starvation conditions, which is thought to enable these nonmotile organisms to seek a preferable environment (9). Deletion of *MEP2*, but not of *MEP1* or *MEP3*, abolishes the pseudohyphal growth of *S. cerevisiae* in response to low ammonium concentrations, although *mep2*Δ mutants are not defective in ammonium uptake or growth under these conditions (16). The filamentation defect of *S. cerevisiae mep2*Δ mutants is limited to growth under low-ammonium conditions, and the mutants exhibit normal pseudohyphal growth when other nitrogen sources are limiting. In contrast, in *C. albicans*, Mep2p has a more central role in the control of morphogenesis, as *C. albicans mep2*Δ mutants have a filamentous growth defect under limiting nitrogen conditions, irrespective of the nature of the nitrogen source (2). However, *MEP2* is not required for filamentous growth of *C. albicans* in response to other inducing signals, e.g., in the presence of serum. It was found that the C-terminal cytoplasmic tail of Mep2p, which is dispensable for ammonium transport, has a specific signaling function, as its deletion abolished the ability of the ammonium permease to induce filamentous growth without affecting ammonium uptake. The ability of a hyperactive, overexpressed $MEP2^{\Delta C440}$ allele to overcome the filamentous growth defect of *cph1*Δ and *efg1*Δ single mutants, but not that of *cph1*Δ *efg1*Δ double mutants, suggested that Mep2p normally activates both the Cph1p-dependent mitogen-activated protein kinase pathway and the Efg1p-depen-

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dent cyclic AMP/protein kinase A pathway to induce filamentous growth in response to nitrogen starvation (2).

Among the distinguishing features of *MEP1* and *MEP2* are their expression levels. Both ammonium permeases are induced in response to nitrogen limitation, but Mep2p is expressed at much higher levels than Mep1p. Promoter-swapping experiments demonstrated that the differential expression levels are due to the stronger activity of the *MEP2* promoter than that of the *MEP1* promoter and presumably also to differential transcript stability (2). Expression of *MEP2* from the *MEP1* promoter resulted in reduced amounts of Mep2p, inefficient ammonium transport, and the loss of its ability to induce filamentous growth. In contrast, the expression of *MEP1* from the *MEP2* promoter resulted in increased Mep1p levels, which also conferred on it the ability to induce a weak filamentation. These results demonstrated that Mep1p is a highly efficient ammonium transporter that needs to be expressed only at low levels to support growth on limiting ammonium concentrations, while Mep2p is a less efficient ammonium transporter that is expressed at high levels, which in turn is a prerequisite for the induction of filamentation.

Since the control of *MEP2* expression is central to the regulation of nitrogen starvation-induced filamentous growth in *C. albicans*, we set out to identify the *cis*-acting sequences in the *MEP2* promoter as well as the *trans*-acting regulatory factors involved in the induction of *MEP2* expression and to elucidate their roles in morphogenesis.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C. Strains were propagated on synthetic dextrose (SD) agar plates (6.7 g yeast nitrogen base without amino acids [YNB; Bio101, Vista, CA], 20 g glucose, 15 g agar per liter). For routine growth of the strains, YPD liquid medium (20 g peptone, 10 g yeast extract, 20 g glucose per liter) was used. To support the growth of *ura3* strains, 100 $\mu g \cdot ml^{-1}$ uridine was added to the media. To observe *MEP2* expression, strains were grown overnight in liquid SD-Pro medium (which contains 0.1% proline instead of ammonium), diluted 50-fold, and grown for 6 h at 30°C in SD medium containing 100 μM ammonium or other nitrogen sources as indicated in the text. Growth of *mep1*Δ *mep2*Δ double mutants expressing *MEP2-GFP* from wild-type and mutated *MEP2* promoters was tested on SD 2% agar plates containing 1 mM ammonium. To study filamentation and Mep2p expression on solid media, the strains were grown as single colonies for 6 days at 37°C on SD 2% agar plates containing 100 μM ammonium or other nitrogen sources as described in the text. Filamentation was also tested on agar plates containing 10% fetal calf serum.

Plasmid constructions. (i) Plasmids containing a GFP-tagged or wild-type *MEP2* gene under the control of wild-type and mutated *MEP2* promoters. Plasmid pMEP2G6, which contains a green fluorescent protein (*GFP*)-tagged *MEP2* gene under the control of the wild-type *MEP2* promoter and served as the basis for the introduction of deletions and mutations in the *MEP2* regulatory region (see Fig. 1), was generated in the following way. A KpnI-XhoI fragment containing *MEP2* upstream sequences from positions −1478 to −5 with respect to the start codon was amplified from genomic DNA of *C. albicans* strain SC5314 with the primers MEP44 (5′-CAGCTATCTTGGTaCCTCATCAATCAATTGC-3′) and MEP24 (5′-CCAGACActcgaGTTATTAACTATTCAGAG-3′) (the lowercase letters here and below represent nucleotide exchanges introduced to create the underlined restriction sites [here, KpnI and XhoI]). The *MEP2* promoter fragment was then cloned together with an XhoI-BamHI fragment from plasmid pMEP2G5 (2) containing the *MEP2* coding region into KpnI/BamHI-digested pMEP2G2 (2). The cloned *MEP2* promoter fragment contained the polymorphic EcoRI site, which is present only in the *MEP2-1* allele (2). To construct pMEP2ΔP1, a distal *MEP2* promoter fragment (positions −1478 to −1015) was amplified with the primer pair MEP44 and MEP55 (5′-TCCTCGTTCTAgATACTAATGGTTGATACG-3′), and a proximal *MEP2* promoter fragment (positions −188 to −5) was amplified with the primer pair MEP51 (5′-GAAAAATCTagaAATCCCTATTGTGATTGG-3′) and MEP24. The PCR products were digested with KpnI/XbaI and XbaI/XhoI, respectively, and cloned together into KpnI/XhoI-digested pMEP2G6. To create pMEP2ΔP2, a proximal *MEP2* promoter fragment (positions −431 to −5) was amplified with the primer pair MEP52 (5′-AACCACtctAGATTTACCCCACTTCG-3′) and MEP24 and substituted for the XbaI-XhoI fragment in pMEP2ΔP1. Additional deletion constructs were made in an analogous fashion. Proximal *MEP2* promoter fragments from positions −620 to −5, −805 to −5, −287 to −5, and −217 to −5 were amplified with the primer pairs MEP53 (5′-GTGATGCTCtagATAAATACAATACCCAAACG-3′) and MEP24, MEP54 (5′-ATGATTTTCTaGaATATACCATGAAGTACCAAGC-3′) and MEP24, MEP61 (5′-TCTCAATTCTAgAATTATTCCTGTATATTGC-3′) and MEP24, and MEP62 (5′-TTTTTCTagaAAATTGTTTATCAGTGTGAAAAATC-3′) and MEP24, respectively, and substituted for the XbaI-XhoI fragment in pMEP2ΔP1 to generate pMEP2ΔP3, pMEP2ΔP4, pMEP2ΔP6, and pMEP2ΔP7. To construct pMEP2ΔP5, a distal *MEP2* promoter fragment (positions −1478 to −435) was amplified with the primer pair MEP44 and MEP60 (5′-GGGGTAAATCTagaGTGGTTAAAAGGATATCC-3′) and used to replace the distal *MEP2* promoter fragment in pMEP2ΔP1. For pMEP2MP1, in which the GATA sequence centered at position −208 is replaced by an XbaI site, a distal *MEP2* promoter fragment (positions −1478 to −210), amplified with the primers MEP44 and MEP64 (5′-GATTTTTCACACTctagAACAATTTCCCAG-3′), and a proximal *MEP2* promoter fragment (positions −205 to −5), amplified with the primers MEP63 (5′-CTGGGAAATTGTTctagAGTGTGAAAAATC-3′) and MEP24, were fused at the introduced XbaI site and substituted for the wild-type *MEP2* promoter in pMEP2G6. For pMEP2MP2, in which the GATA sequence centered at position −266 is replaced by a BglII site, a distal *MEP2* promoter fragment (positions −1478 to −269), amplified with the primers MEP44 and MEP66 (5′-GAAAAAAATATAGATctGCAATATACAGG-3′), and a proximal *MEP2* promoter fragment (positions −266 to −5), amplified with the primers MEP65 (5′-CCTGTATATTGCagATCTATATTTTTTTC-3′) and MEP24, were fused and substituted for the wild-type *MEP2* promoter in pMEP2G6. To introduce both mutations into the *MEP2* promoter, the same proximal *MEP2* promoter fragment was amplified from pMEP2MP1, fused with the distal *MEP2* promoter fragment, and substituted for the wild-type *MEP2* promoter in pMEP2G6 to create pMEP2MP3. pMEP2G7 was generated by substituting the *C. albicans*-adapted *SAT1* (*caSAT1*) marker (22) for the *URA3* marker in pMEP2G2 to allow the expression of the *GFP*-tagged *MEP2* gene in the wild-type strain SC5314 and the *gln3*Δ and *gat1*Δ mutants derived from it. Similarly, the *URA3* marker was replaced by the *caSAT1* marker in the previously described plasmid pMEP1G1 (2) to obtain pMEP1G4, which was used to express a *GFP*-tagged *MEP1* gene in the same strains. To express the wild-type *MEP2* gene from wild-type and mutated *MEP2* promoters, a PstI-SacI fragment from pMEP2K1 (2) was substituted for the PstI-SacI fragments in plasmids pMEP2G6, pMEP2ΔP5, pMEP2ΔP6, pMEP2MP1, pMEP2MP2, and pMEP2MP3 (see Fig. 1), thereby generating pMEP2K13, pMEP2ΔP5A, pMEP2ΔP6A, pMEP2MP1A, pMEP2MP2A, and pMEP2MP3A, respectively, in which *GFP*-tagged *MEP2* is replaced by wild-type *MEP2*.

(ii) Plasmids containing wild-type or hyperactive *MEP2* alleles under the control of the *ADH1* promoter. To express *MEP2* from the *ADH1* promoter in strain SC5314 and the *gln3*Δ mutants, an XhoI-BglII fragment from plasmid pMEP2K2 containing the *MEP2* coding region (2) was cloned between the *ADH1* promoter and the *ACT1* transcription termination sequence of plasmid pADH1E1, which contains the *caSAT1* selection marker and flanking *ADH1* sequences for integration into the *ADH1* locus (21), generating pMEP2E4. Similarly, an XhoI-BglII fragment from pMEP2ΔC2K2 containing the hyperactive $MEP2^{\Delta C440}$ allele (2) was inserted in the same way in pADH1E1 to obtain pMEP2ΔC2E2. A control plasmid, pADH1G4, contains the *GFP* gene instead of *MEP2* (Y.-N. Park and J. Morschhäuser, unpublished).

(iii) *MEP2*, *GAT1*, and *GLN3* deletion and reinsertion constructs. A *GLN3* deletion construct was generated in the following way. An ApaI-XhoI fragment containing *GLN3* upstream sequences from positions −204 to +6 with respect to the start codon was amplified from the genomic DNA of *C. albicans* strain SC5314 with the primer pair GLN1 (5′-ATAACgggCCCTACCTAGAGGAATAAGTTC-3′) and GLN2 (5′-GACTGACTATTCGctcgAGTCATTTGTCCC-3′). A SacII-SacI fragment downstream of *GLN3* from positions +2025 to +2293 was amplified with the primers GLN3 (5′-GGGGATTATAAGGccgcGGATTGGTTGAAG-3′) and GLN4 (5′-GTCGTTTAGGTCACgaGCTCCACGAGATG-3′). The *GLN3* upstream and downstream fragments were substituted for the *OPT1* flanking sequences in pOPT1M3 (22) to result in pGLN3M2, in which the *GLN3* coding region from positions +7 to +2024 (23 bp before the stop codon) is replaced by the *SAT1* flipper (see Fig. 3A). For reintegration of an intact *GLN3* copy into *gln3*Δ single or *gln3*Δ *gat1*Δ double mutants, an ApaI-BamHI

TABLE 1. *C. albicans* strains used in this study

Strain(s)	Parent strain	Relevant genotype or characteristic[a]	Reference or source
SC5314		Wild-type strain	8
CAI4	SC5314	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	7
CAI4RU1A and CAI4RU1B	CAI4	*ura3*Δ::*imm434*/*URA3*	This study
*mep2*Δ single and *mep1*Δ *mep2*Δ double mutants			
SCMEP2M1A	SC5314	*mep2-1*Δ::*SAT1-FLIP*/*MEP2-2*	This study
SCMEP2M1B	SC5314	*MEP2-1*/*mep2-2*Δ::*SAT1-FLIP*	This study
SCMEP2M2A	SCMEP2M1A	*mep2-1*Δ::*FRT*/*MEP2-2*	This study
SCMEP2M2B	SCMEP2M1B	*MEP2-1*/*mep2-2*Δ::*FRT*	This study
SCMEP2M3A	SCMEP2M2A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*SAT1-FLIP*	This study
SCMEP2M3B	SCMEP2M2B	*mep2-1*Δ::*SAT1-FLIP*/*mep2-2*Δ::*FRT*	This study
SCMEP2M4A	SCMEP2M3A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	This study
SCMEP2M4B	SCMEP2M3B	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	This study
MEP2M4A and MEP2M4B	CAI4	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	2
MEP2M4RU1A	MEP2M4A	*ura3*Δ::*imm434*/*URA3 mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	This study
MEP2M4RU1B	MEP2M4B	*ura3*Δ::*imm434*/*URA3 mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	This study
MEP12M4A and MEP12M4B	CAI4	*mep1-1*Δ::*FRT*/*mep1-2*Δ::*FRT mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT*	2
MEP12M6A	MEP12M4A	*mep1-1*Δ::*FRT*/*mep1-2*Δ::*FRT mep2-1*Δ::*FRT*/*mep2-2*Δ::*URA3*	2
MEP12M6B	MEP12M4B	*mep1-1*Δ::*FRT*/*mep1-2*Δ::*FRT mep2-1*Δ::*URA3*/*mep2-2*Δ::*FRT*	2
Strains expressing a *MEP2-GFP* fusion under the control of wild-type and mutated *MEP2* promoters in a *mep1*Δ *mep2*Δ double mutant background			
MEP12MG6A	MEP12M4A	*mep2-1*::P_{MEP2}-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2}-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP1A	MEP12M4A	*mep2-1*::$P_{MEP2\Delta1}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP1B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta1}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP2A	MEP12M4A	*mep2-1*::$P_{MEP2\Delta2}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP2B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta2}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP3A	MEP12M4A	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta3}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP3B	MEP12M4A	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta3}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP4A	MEP12M4B	*mep2-1*::$P_{MEP2\Delta4}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP4B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta4}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP5A	MEP12M4A	*mep2-1*::$P_{MEP2\Delta5}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP5B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta5}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP6A	MEP12M4A	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta6}$-*MEP2-GFP-URA3*	This study
MEP12MG6ΔP6B	MEP12M4B	*mep2-1*::$P_{MEP2\Delta6}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP7A	MEP12M4A	*mep2-1*::$P_{MEP2\Delta7}$-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6ΔP7B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta7}$-*MEP2-GFP-URA3*	This study
MEP12MG6MP1A	MEP12M4A	*mep2-1*::P_{MEP2M1}-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6MP1B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2M1}-*MEP2-GFP-URA3*	This study
MEP12MG6MP2A	MEP12M4A	*mep2-1*::P_{MEP2M2}-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6MP2B	MEP12M4B	*mep2-1*::P_{MEP2M2}-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6MP3A	MEP12M4A	*mep2-1*::P_{MEP2M3}-*MEP2-GFP-URA3*/*mep2-2*Δ::*FRT*	This study
MEP12MG6MP3B	MEP12M4B	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2M3}-*MEP2-GFP-URA3*	This study
Strains expressing *MEP2* under the control of wild-type and mutated *MEP2* promoters in a *mep2*Δ background			
MEP2MK13A	MEP2M4A	*mep2-1*::P_{MEP2}-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
MEP2MK13B	MEP2M4B	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2}-*MEP2-URA3*	This study
MEP2MK13ΔP5A	MEP2M4A	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta5}$-*MEP2-URA3*	This study
MEP2MK13ΔP5B	MEP2M4B	*mep2-1*::$P_{MEP2\Delta5}$-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
MEP2MK13ΔP6A	MEP2M4B	*mep2-1*::$P_{MEP2\Delta6}$-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
MEP2MK13ΔP6B	MEP2M4B	*mep2-1*Δ::*FRT*/*mep2-2*::$P_{MEP2\Delta6}$-*MEP2-URA3*	This study
MEP2MK13MP1A	MEP2M4A	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2M1}-*MEP2-URA3*	This study
MEP2MK13MP1B	MEP2M4B	*mep2-1*::P_{MEP2M1}-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
MEP2MK13MP2A	MEP2M4A	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2M2}-*MEP2-URA3*	This study
MEP2MK13MP2B	MEP2M4B	*mep2-1*::P_{MEP2M2}-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
MEP2MK13MP3A	MEP2M4A	*mep2-1*Δ::*FRT*/*mep2-2*::P_{MEP2M3}-*MEP2-URA3*	This study
MEP2MK13MP3B	MEP2M4B	*mep2-1*::P_{MEP2M3}-*MEP2-URA3*/*mep2-2*Δ::*FRT*	This study
*gln3*Δ mutants and complemented strains			
GLN3M1A	SC5314	*gln3-1*Δ::*SAT1-FLIP*/*GLN3-2*	This study
GLN3M1B	SC5314	*GLN3-1*/*gln3-2*Δ::*SAT1-FLIP*	This study
GLN3M2A	GLN3M1A	*gln3-1*Δ::*FRT*/*GLN3-2*	This study
GLN3M2B	GLN3M1B	*GLN3-1*/*gln3-2*Δ::*FRT*	This study
GLN3M3A	GLN3M2A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*SAT1-FLIP*	This study
GLN3M3B	GLN3M2B	*gln3-1*Δ::*SAT1-FLIP*/*gln3-2*Δ::*FRT*	This study
GLN3M4A	GLN3M3A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT*	This study
GLN3M4B	GLN3M3B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT*	This study
GLN3MK1A	GLN3M4A	*GLN3-SAT1-FLIP*/*gln3-2*Δ::*FRT*	This study
GLN3MK1B	GLN3M4B	*gln3-1*Δ::*FRT*/*GLN3-SAT1-FLIP*	This study
GLN3MK2A	GLN3MK1A	*GLN3-FRT*/*gln3-2*Δ::*FRT*	This study
GLN3MK2B	GLN3MK1B	*gln3-1*Δ::*FRT*/*GLN3-FRT*	This study
*gat1*Δ mutants			
GAT1M1A	SC5314	*gat1-1*Δ::*SAT1-FLIP*/*GAT1-2*	This study
GAT1M1B	SC5314	*GAT1-1*/*gat1-2*Δ::*SAT1-FLIP*	This study

Continued on following page

TABLE 1—*Continued*

Strain(s)	Parent strain	Relevant genotype or characteristic[a]	Reference or source
GAT1M2A	GAT1M1A	*gat1-1*Δ::*FRT*/*GAT1-2*	This study
GAT1M2B	GAT1M1B	*GAT1-1*/*gat1-2*Δ::*FRT*	This study
GAT1M3A	GAT1M2A	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*SAT1-FLIP*	This study
GAT1M3B	GAT1M2B	*gat1-1*Δ::*SAT1-FLIP*/*gat1-2*Δ::*FRT*	This study
GAT1M4A	GAT1M3A	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
GAT1M4B	GAT1M3B	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
*gln3*Δ *gat1*Δ double mutants and complemented strains			
Δ*gln3*GAT1M1A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*SAT1-FLIP*/*GAT1-2*	This study
Δ*gln3*GAT1M1B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT GAT1-1*/*gat1-2*Δ::*SAT1-FLIP*	This study
Δ*gln3*GAT1M2A	Δ*gln3*GAT1M1A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*GAT1-2*	This study
Δ*gln3*GAT1M2B	Δ*gln3*GAT1M1B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT GAT1-1*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1M3A	Δ*gln3*GAT1M2A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*SAT1-FLIP*	This study
Δ*gln3*GAT1M3B	Δ*gln3*GAT1M2B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*SAT1-FLIP*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1M4A	Δ*gln3*GAT1M3A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1M4B	Δ*gln3*GAT1M3B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1MK1A	Δ*gln3*GAT1M4A	*GLN3-SAT1-FLIP*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1MK1B	Δ*gln3*GAT1M4B	*gln3-1*Δ::*FRT*/*GLN3-SAT1-FLIP gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1MK2A	Δ*gln3*GAT1MK1A	*GLN3-FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*gln3*GAT1MK2B	Δ*gln3*GAT1MK1B	*gln3-1*Δ::*FRT*/*GLN3-FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
*mep2*Δ *gat1*Δ double mutants			
Δ*mep2*GAT1M1A	SCMEP2M4A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT GAT1-1*/*gat1-2*Δ::*SAT1-FLIP*	This study
Δ*mep2*GAT1M1B	SCMEP2M4B	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*SAT1-FLIP*/*GAT1-2*	This study
Δ*mep2*GAT1M2A	Δ*mep2*GAT1M1A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT GAT1-1*/*gat1-2*Δ::*FRT*	This study
Δ*mep2*GAT1M2B	Δ*mep2*GAT1M1B	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*FRT*/*GAT1-2*	This study
Δ*mep2*GAT1M3A	Δ*mep2*GAT1M2A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*SAT1-FLIP*/*gat1-2*Δ::*FRT*	This study
Δ*mep2*GAT1M3B	Δ*mep2*GAT1M2B	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*SAT1-FLIP*	This study
Δ*mep2*GAT1M4A	Δ*mep2*GAT1M3A	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Δ*mep2*GAT1M4B	Δ*mep2*GAT1M3B	*mep2-1*Δ::*FRT*/*mep2-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT*	This study
Strains expressing *MEP2-GFP* or *MEP1-GFP* fusions in wild-type and mutant backgrounds			
SCMEP2G7A	SC5314	*mep2-1*::P_{MEP2}-*MEP2-GFP-caSAT1*/*MEP2-2*	This study
SCMEP2G7B	SC5314	*MEP2-1*/*mep2-2*::P_{MEP2}-*MEP2-GFP-caSAT1*	This study
Δ*gln3*MEP2G7A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT mep2-1*::P_{MEP2}-*MEP2-GFP-caSAT1*/*MEP2-2*	This study
Δ*gln3*MEP2G7B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT MEP2-1*/*mep2-2*::P_{MEP2}-*MEP2-GFP-caSAT1*	This study
Δ*gat1*MEP2G7A	GAT1M4A	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP2-1*/*mep2-2*::P_{MEP2}-*MEP2-GFP-caSAT1*	This study
Δ*gat1*MEP2G7B	GAT1M4B	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT mep2-1*::P_{MEP2}-*MEP2-GFP-caSAT1*/*MEP2-2*	This study
Δ*gln3*Δ*gat1*MEP2G7A	Δ*gln3*GAT1M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP2-1*/*mep2-2*::P_{MEP2}-*MEP2-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP2G7B	Δ*gln3*GAT1M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP2-1*/*mep2-2*::P_{MEP2}-*MEP2-GFP-caSAT1*	This study
SCMEP1G4A and SCMEP1G4B	SC5314	*MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gln3*MEP1G4A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gln3*MEP1G4B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gat1*MEP1G4A	GAT1M4A	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gat1*MEP1G4B	GAT1M4B	*gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP1G4A	Δ*gln3*GAT1M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP1G4B	Δ*gln3*GAT1M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT gat1-1*Δ::*FRT*/*gat1-2*Δ::*FRT MEP1*/*mep1*::P_{MEP1}-*MEP1-GFP-caSAT1*	This study
Strains expressing wild-type and hyperactive *MEP2* alleles from the *ADH1* promoter or carrying a control construct in wild-type and *gln3*Δ backgrounds			
SCADH1G4A and SCADH1G4B	SC5314	*ADH1*/*adh1*::P_{ADH1}-*GFP-caSAT1*	This study
SCMEP2E4A and SCMEP2E4B	SC5314	*ADH1*/*adh1*::P_{ADH1}-*MEP2-caSAT1*	This study
SCMEP2ΔC2E2A and SCMEP2ΔC2E2B	SC5314	*ADH1*/*adh1*::P_{ADH1}-*MEP2*$^{\Delta C440}$-*caSAT1*	This study
Δ*gln3*ADH1G4A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*GFP-caSAT1*	This study
Δ*gln3*ADH1G4B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*GFP-caSAT1*	This study
Δ*gln3*MEP2E4A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*MEP2-caSAT1*	This study
Δ*gln3*MEP2E4B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*MEP2-caSAT1*	This study
Δ*gln3*MEP2ΔC2E2A	GLN3M4A	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*MEP2*$^{\Delta C440}$-*caSAT1*	This study
Δ*gln3*MEP2ΔC2E2B	GLN3M4B	*gln3-1*Δ::*FRT*/*gln3-2*Δ::*FRT ADH1*/*adh1*::P_{ADH1}-*MEP2*$^{\Delta C440}$-*caSAT1*	This study

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette.

fragment containing the complete *GLN3* open reading frame and upstream sequences was amplified with the primers GLN1 and GLN5 (5′-ATATTgga<u>TCC</u>TAGAGTTTGCAAACACGTAC-3′) and cloned together with a BglII-SalI fragment from pCBF1M4 containing the *ACT1* transcription termination sequence (3) into ApaI/XhoI-digested pGLN3M2 to create pGLN3K1 (see Fig. 3B).

To generate a *GAT1* deletion construct, an ApaI-XhoI fragment containing *GAT1* upstream sequences from positions −429 to −69 was amplified with the primer pair GAT1 (5′-CCGATAACAATAA<u>ggg</u><u>CCC</u>TCCCAATCAG-3′) and GAT2 (5′-TGTAGTGGCTGTG<u>ct</u><u>CGAG</u>TTAAGCTGC-3′), and a SacII-SacI *GAT1* downstream fragment from positions +2003 to +2559 was amplified with the primers GAT5 (5′-GAGGGTT<u>CCgcgg</u>CTAGTGGAGTCAATACATC-3′)

and GAT6 (5′-TGACAGAGGAGctcATGATTGGGTTGGATCTG-3′). The *GAT1* upstream and downstream fragments were substituted for the *GLN3* flanking sequences in pGLN3M2 to result in pGAT1M2, in which the *GAT1* coding region from positions −68 to +2002 (63 bp before the stop codon) is replaced by the *SAT1* flipper. To delete *MEP2* in the wild-type strain SC5314, the *caSAT1* selection marker was substituted for the *URA3* marker in the previously described pMEP2M2 plasmid (2), generating pMEP2M5.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (10) with gel-purified inserts from the plasmids described above. Uridine-prototrophic transformants were selected on SD agar plates, and nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg ml^{-1} nourseothricin (Werner Bioagents, Jena, Germany), as described previously (22). Single-copy integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (19). Ten micrograms of DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, and after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced-chemiluminescence-labeled probes was performed with the Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

RNA isolation and Northern hybridization. Total RNA was isolated from log-phase cultures of the *C. albicans* strains in liquid synthetic low ammonium dextrose (SLAD) medium by the hot acidic phenol method (1). The *MEP2* transcript was detected by Northern hybridization with an XhoI-BglII fragment from plasmid pMEP2K2 (2) containing the complete *MEP2* coding sequence as the probe. The probe was labeled with the Amersham Rediprime II random prime labeling system (GE Healthcare), and Northern hybridization was performed using standard protocols (1).

Fluorescence microscopy and flow cytometry. Fluorescence microscopy was performed with a Zeiss LSM 510 inverted confocal laser scanning microscope equipped with a Zeiss Axiovert 100 microscope. Imaging scans were acquired with an argon laser with a wavelength of 488 nm and corresponding filter settings for GFP and parallel transmission images. The cells were observed with a 63× immersion oil objective. Fluorescence-activated cell sorter analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm-band-pass filter. Twenty thousand cells were analyzed per sample and were counted at a low flow rate. Fluorescence and forward-scatter data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software. Strain SC5314, which does not carry *GFP*, was included as a negative control in all experiments, and the background fluorescence values of this strain (between 1.6 and 3.7 in the various experiments) were subtracted from those of the reporter strains.

RESULTS

Two putative GATA factor binding sites in the *MEP2* regulatory region mediate the induction of *MEP2* expression in response to nitrogen limitation. To elucidate how *MEP2* expression is regulated, we first identified the sequences in the *MEP2* promoter that mediate its induction under limiting nitrogen conditions. For this purpose, we used a *GFP*-tagged *MEP2* gene as a reporter and expressed it under the control of wild-type and mutated *MEP2* promoters in *mep1*Δ *mep2*Δ double mutants. This approach allowed us to monitor the expression of Mep2p by observing both the fluorescence of the cells and the capacity of the tagged ammonium permease to restore growth of the double mutants at low ammonium concentrations. The reporter constructs were designed such that they could be integrated at the original *MEP2* locus (Fig. 1), since it has been shown that the integration of *C. albicans* genes at a different genomic site may affect their expression (11). Replacement of the resident wild-type promoter by the mutated *MEP2* promoters was verified by Southern hybridization analysis (data not shown), and two independent transformants containing a single copy of the reporter construct were used for further analysis in each case (see Table 1 for strain descriptions).

As reported previously (2), expression of the *MEP2-GFP* fusion from the wild-type *MEP2* promoter resulted in strong fluorescence of the cells in SLAD medium and wild-type growth on plates containing limiting ammonium concentrations (Fig. 1 and 2A). Deletion of *MEP2* upstream sequences ranging from positions −1014 up to −288 with respect to the start codon (ΔP2, ΔP3, ΔP4, ΔP6) reduced *MEP2* expression by only about 50% and did not detectably affect growth at limiting ammonium concentrations, but deletion of additional sequences to positions −218 or −189 (ΔP1, ΔP7) abolished *MEP2* expression, and transformants carrying these fusions behaved like *mep1*Δ *mep2*Δ double mutants. A shorter deletion ranging from positions −434 to −189 (ΔP5) produced the same phenotype, suggesting that sequences within this region control *MEP2* induction in response to nitrogen limitation. Inspection of the DNA sequence of this region revealed that it contained two putative binding sequences for GATA transcription factors (GATAA) (17) located at positions −264 to −268 and −206 to −210 on the antisense strand. To test whether these sequences are involved in the regulation of *MEP2* expression, we changed the GATAA sequence centered at −266 to GATCT and the GATAA sequence centered at −208 to CTAGA in the full-length *MEP2* promoter. Whereas mutation of the GATAA sequence at −208 alone (MP1) had no effect, mutation of the GATAA sequence at −266 (MP2) strongly reduced *MEP2* expression, and transformants carrying this construct showed only weak growth at limiting ammonium concentrations. Mutation of both GATAA sequences (MP3) reduced *MEP2* expression almost to background values, and the corresponding transformants were unable to grow at low ammonium concentrations, like *mep1*Δ *mep2*Δ double mutants. These results demonstrated that these GATAA sequences are important for *MEP2* expression and that GATA transcription factors may be involved in the induction of *MEP2* under nitrogen starvation conditions. There are a number of additional GATAA sequences located at more distal sites in the *MEP2* upstream region (Fig. 1), but the results of our promoter deletion and mutation analyses indicate that these sequences are neither required nor sufficient for *MEP2* expression.

***MEP2* expression levels correlate with nitrogen starvation-induced filamentous growth.** Since we had previously shown that the higher expression levels of *MEP2* than those of *MEP1* are a prerequisite for normal filamentation in response to nitrogen limitation (2), we assessed the effect of reduced Mep2p expression from mutated *MEP2* promoters on filamentous growth. For this purpose, *MEP2* was expressed from various mutated *MEP2* promoters displaying different activities (wild type, ΔP5, ΔP6, MP1, MP2, and MP3) in a *mep2*Δ background, thus allowing normal ammonium uptake due to the presence of the *MEP1* gene (Fig. 2B). As expected, normal Mep2p expression levels, as from the MP1 promoter, resulted in wild-type filamentation. A slight reduction (by about 50%) of Mep2p expression, as from the ΔP6 promoter, also did not detectably affect filamentous growth. However, the strongly (circa eightfold) reduced Mep2p expression levels obtained from the MP2 promoter severely affected the ability of the

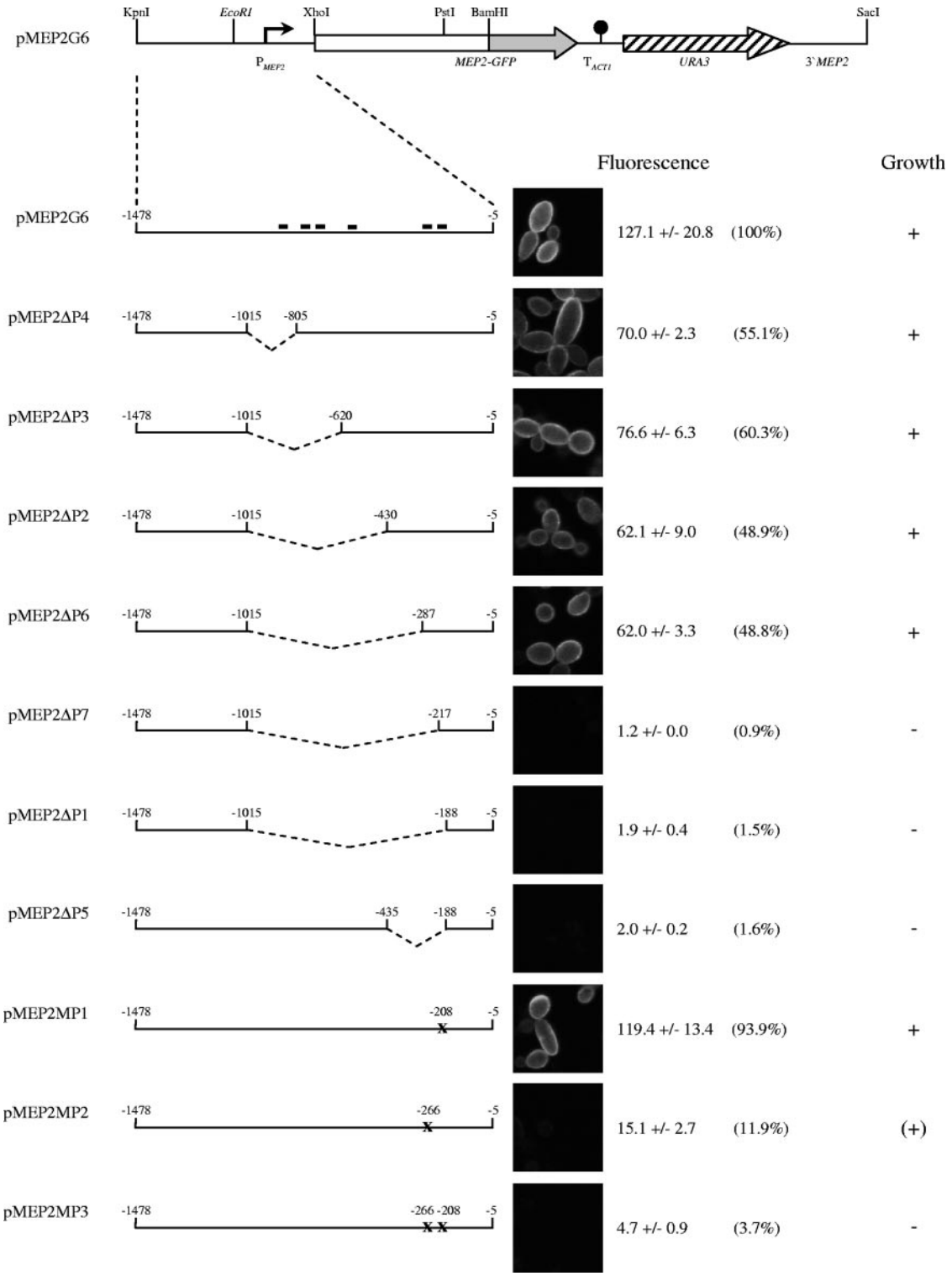

FIG. 1. *MEP2* promoter analysis. The structure of the insert of plasmid pMEP2G6, which contains a *GFP*-tagged *MEP2* gene under the control of the wild-type *MEP2* promoter, is shown on top. The *MEP2* and *GFP* coding regions are represented by the white box and the gray arrow, respectively, the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle, and the *URA3* selection marker by the hatched arrow. *MEP2* upstream and downstream sequences are represented by the solid lines, and the *MEP2* promoter (P_{MEP2}) is symbolized by the bent arrow. Relevant restriction sites are shown. The polymorphic EcoRI site, which is present only in the *MEP2-1* allele, is highlighted in italics. Enlarged representations of the *MEP2* regulatory region with the introduced deletions and mutations are shown below, and the names of the corresponding plasmids are indicated to the left. The extents of *MEP2* promoter sequences contained in the various plasmids are given. Internal deletions are indicated by the dashed lines. The locations of GATAA sequences within 1 kb upstream of the *MEP2* start codon are indicated by the short black bars in the wild-type promoter. The mutations of the GATAA sequences centered at positions −266 and −208 are marked by an

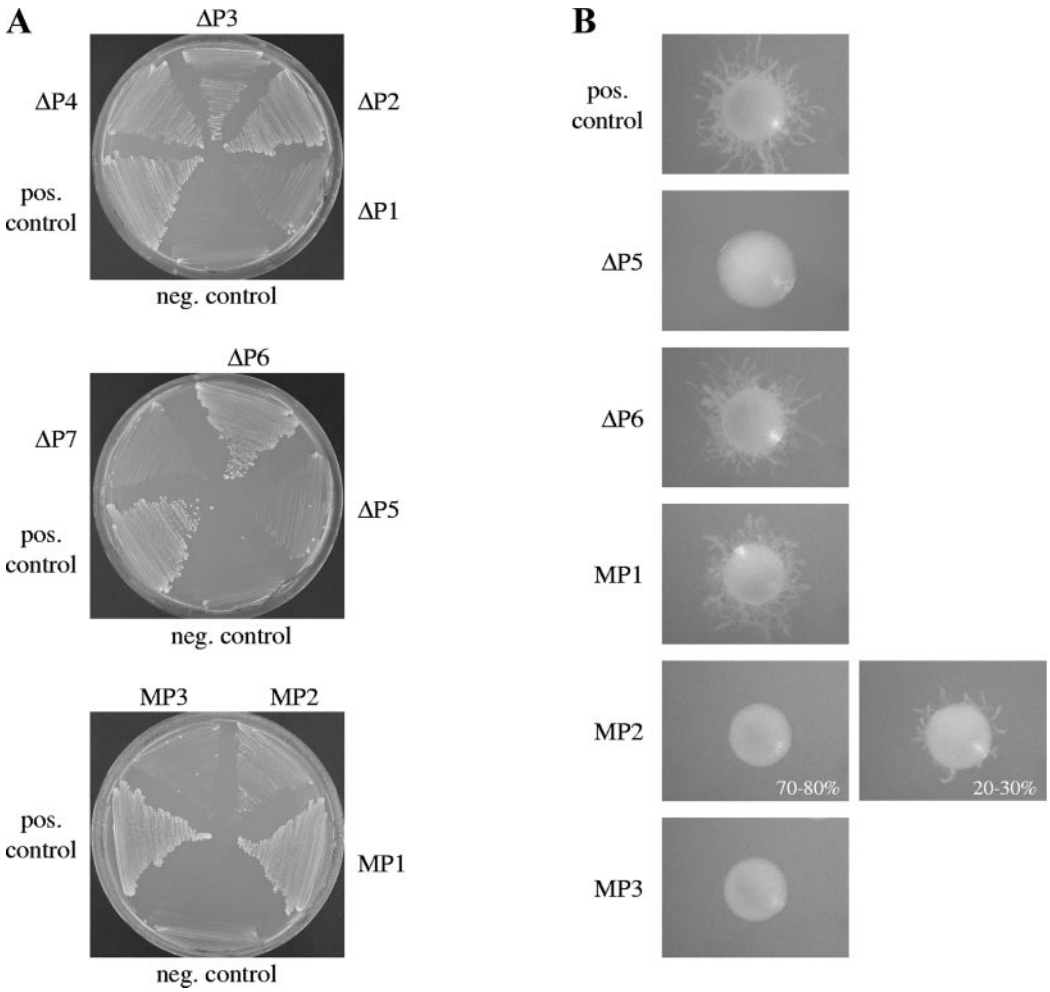

FIG. 2. (A) Growth of *mep1*Δ *mep2*Δ double mutants expressing a *GFP*-tagged *MEP2* gene from the wild-type *MEP2* promoter and derivatives containing the indicated deletions or mutations. The strains were grown for 4 days at 30°C on SD plates containing 1 mM ammonium. The following strains were used: MEP12M6A and -B (negative [neg.] control), MEP12MG6A and -B (positive [pos.] control), MEP12MG6ΔP1A and -B (ΔP1), MEP12MG6ΔP2A and -B (ΔP2), MEP12MG6ΔP3A and -B (ΔP3), MEP12MG6ΔP4A and -B (ΔP4), MEP12MG6ΔP5A and -B (ΔP5), MEP12MG6ΔP6A and -B (ΔP6), MEP12MG6ΔP7A and -B (ΔP7), MEP12MG6MP1A and -B (MP1), MEP12MG6MP2A and -B (MP2), and MEP12MG6MP3A and -B (MP3). The two independently constructed reporter strains behaved identically, and only one of them is shown in each case. (B) Filamentation of *mep2*Δ mutants expressing *MEP2* from the wild-type *MEP2* promoter and derivatives containing the indicated deletions or mutations. The strains were grown for 6 days at 37°C on SLAD plates, and individual representative colonies were photographed. The following strains were used: MEP2MK13A and -B (pos. control), MEP2MK13ΔP5A and -B (ΔP5), MEP2MK13ΔP6A and -B (ΔP6), MEP2MK13MP1A and -B (MP1), MEP2MK13MP2A and -B (MP2), and MEP2MK13MP3A and -B (MP3). Independently constructed strains behaved identically, and only one of them is shown in each case.

strains to produce filamentous colonies in response to nitrogen starvation. Most colonies were nonfilamentous, and a minority showed only a weak filamentation. Further reduction of Mep2p expression to nearly background levels, as from the ΔP5 or MP3 promoter, completely abolished filamentous growth. Therefore, nitrogen starvation-induced filamentous growth directly correlated with Mep2p expression levels.

***MEP2* expression is controlled by the GATA factors Gln3p and Gat1p.** In *S. cerevisiae*, *MEP2* expression requires at least one of the two GATA transcription factors Gln3p and Gat1p/Nil1p, which are involved in the transcriptional activation of many nitrogen-regulated genes (16, 18). *C. albicans* possesses homologues of *GLN3* and *GAT1* (4, 13), and we investigated whether these transcription factors are involved in the induc-

X. The phenotypes conferred by the various constructs upon integration into *mep1*Δ *mep2*Δ double mutants are shown to the right. Fluorescence of the cells was observed after 6 h of growth at 30°C in liquid SLAD medium. The fluorescence micrographs show representative cells, and the mean levels of fluorescence of the two independently constructed reporter strains as measured by flow cytometry are given. The percentages in parentheses are with respect to the activity of the wild-type *MEP2* promoter, which was set to 100%. Growth of the strains at limiting ammonium concentrations was as follows: +, wild-type growth; (+), weak growth; −, no growth (see also Fig. 2A).

tion of *MEP2* expression under limiting nitrogen conditions. For this purpose, we generated two independent series each of *gln3*Δ single mutants, *gat1*Δ single mutants, and *gln3*Δ *gat1*Δ double mutants from the *C. albicans* wild-type strain SC5314, using the *SAT1*-flipping strategy (22). The construction of the *gln3*Δ mutants and complemented strains is documented in Fig. 3, and inactivation of *GAT1* in strain SC5314 and in the *gln3*Δ mutants to create *gat1*Δ single and *gln3*Δ *gat1*Δ double mutants, respectively, was performed in an analogous fashion (data not shown).

To investigate whether *GLN3* and *GAT1* are involved in the induction of Mep2p expression in response to nitrogen limitation, we compared the expression of GFP-tagged Mep2p in the wild-type strain SC5314, the *gln3*Δ and *gat1*Δ single mutants, and the *gln3*Δ *gat1*Δ double mutants after the integration of a *MEP2-GFP* fusion into one of the *MEP2* alleles in these strains. The reporter strains were grown in liquid media containing limiting amounts (100 μM) of ammonium, glutamine, proline, or urea, and fluorescence of the cells was measured by flow cytometry. As can be seen in Fig. 4A, in all these media, Mep2p expression was strongly reduced in the *gln3*Δ and *gat1*Δ single mutants and was below the detection limit in the *gln3*Δ *gat1*Δ double mutants. These results, which were confirmed by Northern hybridization (Fig. 4C), demonstrated that both GATA transcription factors control *MEP2* expression in *C. albicans*, similarly to their counterparts in *S. cerevisiae*.

To test whether the GATA transcription factors also regulate *MEP1*, the expression of GFP-tagged Mep1p in the wild-type strain SC5314 and the *gln3*Δ, *gat1*Δ, and *gln3*Δ *gat1*Δ mutants was monitored under the same conditions (Fig. 4B). In agreement with our previous results (2), Mep1p was expressed at much lower levels than Mep2p in the wild-type background (roughly 20-fold less as measured by flow cytometry). Compared with that of the wild type, Mep1p expression was slightly reduced in the *gln3*Δ mutants but elevated in the *gat1*Δ mutants. Only very low levels of Mep1p expression were observed in the *gln3*Δ *gat1*Δ double mutants. Therefore, both Gln3p and Gat1p also regulate *MEP1* expression.

The GATA factor Gln3p regulates nitrogen starvation-induced filamentous growth in *C. albicans*. We then tested the growth of the strains on agar plates containing limiting concentrations of different nitrogen sources (Fig. 5A). All mutants grew as well as the wild-type strain SC5314 on SLAD plates, indicating that the reduced Mep1p expression levels in the *gln3*Δ *gat1*Δ double mutants still allowed sufficient ammonium uptake for normal growth. However, the *gln3*Δ mutants showed delayed and strongly reduced filamentation on SLAD plates as well as on plates containing 100 μM urea, proline, glutamate, glutamine, or histidine as the sole nitrogen source, conditions under which *MEP2* is required for filamentous growth (2). While wild-type colonies started to produce filaments after about 3 days of incubation, no filamentous colonies of *gln3*Δ mutants were seen at day 4, and fewer and shorter filaments than in the wild type were observed after 6 days (Fig. 5A and data not shown). In contrast, deletion of *GAT1* did not affect filamentation on these plates, and *gln3*Δ *gat1*Δ double mutants behaved like the *gln3*Δ single mutants, except on urea plates, on which no filamentation was observed in the double mutants. Reintroduction of *GLN3* into the *gln3*Δ single and the *gln3*Δ *gat1*Δ double mutants restored filamentation to wild-type levels, confirming that the filamentous growth defect of the mutants was due to *GLN3* inactivation. No filamentation defect of the mutants was observed on plates containing serum, indicating that *GLN3* is specifically required for normal filamentous growth in response to nitrogen limitation.

To investigate whether the filamentation phenotype of the mutants correlated with Mep2p expression on solid media, we observed the expression of GFP-tagged Mep2p in the corresponding reporter strains on SLAD plates. As in liquid SLAD medium, Mep2p expression was strongly reduced in both the *gln3*Δ and *gat1*Δ single mutants and was undetectable in the *gln3*Δ *gat1*Δ double mutants also on filamentation-inducing solid medium (Fig. 5B). Therefore, the reduced *MEP2* expression in strains lacking *GLN3* correlated with their filamentation defect. In contrast, deletion of *GAT1* did not affect filamentous growth under these conditions, despite the fact that *MEP2* expression was similarly reduced in *gln3*Δ and *gat1*Δ mutants.

Inactivation of *GAT1* activates *MEP2*-independent filamentation pathways. The ability of the *gat1*Δ mutants to filament under limiting nitrogen conditions despite strongly reduced Mep2p expression levels suggested that nitrogen starvation-induced filamentous growth may not depend on the Mep2p ammonium permease in the prototrophic wild-type strain SC5314, in contrast to its auxotrophic derivative CAI4, which was the parent of our *mep2*Δ mutants (2). To exclude the possibility that inappropriate *URA3* expression levels at the *MEP2* locus were responsible for the filamentation defect of the *mep2*Δ mutants, we inserted the *URA3* gene back at its original locus in the two independently constructed *mep2*Δ mutants MEP2M4A and MEP2M4B as well as in our copy of strain CAI4. The resulting *mep2*Δ mutants MEP2M4R1A and MEP2M4R1B exhibited the same filamentation defect as our previously constructed *mep2*Δ mutants, whereas the wild-type control strains CAI4R1A and CAI4R1B showed normal filamentation (data not shown). As a further test for the requirement of Mep2p for nitrogen starvation-induced filamentous growth in *C. albicans*, we deleted *MEP2* in the prototrophic wild-type strain SC5314 using the *SAT1*-flipping strategy. Two independent *mep2*Δ mutants were generated, and both exhibited the same filamentous growth defect on SLAD plates as our previously constructed *mep2*Δ mutants (Fig. 6, top panels). These results confirmed that Mep2p controls nitrogen starvation-induced filamentous growth in *C. albicans*.

We hypothesized that the absence of a functional Gat1p protein might result in the activation of filamentation-inducing signaling pathways that do not require high *MEP2* expression levels or that are even independent of Mep2p. To test this hypothesis, we deleted *GAT1* from the two *mep2*Δ mutants constructed from strain SC5314. Strikingly, both independently generated *mep2*Δ *gat1*Δ double mutants regained the ability to produce filaments under nitrogen starvation conditions, albeit not to wild-type levels (Fig. 6). Therefore, the ability of the *gat1*Δ mutants to produce filaments normally despite strongly reduced Mep2p expression levels can be explained by the activation of additional signaling pathways which can induce filamentation in response to nitrogen starvation, even in the absence of Mep2p.

Forced *MEP2* expression bypasses the requirement of *GLN3* for filamentous growth. If the failure to express *MEP2* at ap-

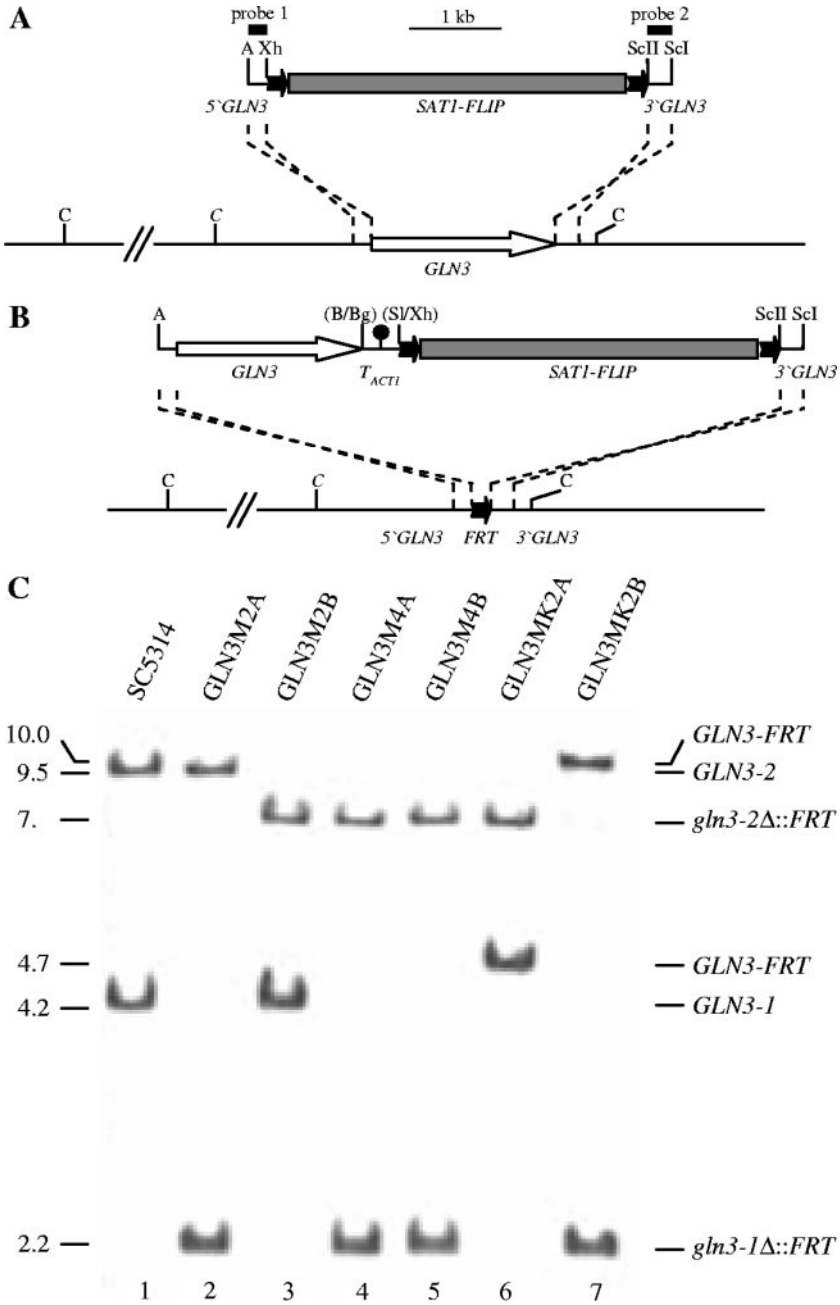

FIG. 3. Construction of *gln3*Δ mutants and complemented strains. (A) Structure of the deletion cassette from plasmid pGLN3M2 (top), which was used to delete the *GLN3* open reading frame, and genomic structure of the *GLN3* locus in strain SC5314 (bottom). The *GLN3* coding region is represented by the white arrow and the upstream and downstream regions by the solid lines. Details of the *SAT1* flipper (gray rectangle bordered by *FRT* sites [black arrows]) have been presented elsewhere (22). The 34-bp *FRT* sites are not drawn to scale. The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. (B) Structure of the DNA fragment from pGLN3K1 (top), which was used for reintegration of an intact *GLN3* copy into one of the disrupted *GLN3* loci in the *gln3*Δ single and *gln3*Δ *gat1*Δ double mutants (bottom). Only relevant restriction sites are given in panels A and B, as follows: A, ApaI; B, BamHI; Bg, BglII; C, ClaI; ScI, SacI; ScII, SacII; Sl, SalI; and Xh, XhoI. Sites shown in parentheses were destroyed by the cloning procedure. The ClaI site marked in italics is present only in the *GLN3-1* allele. (C) Southern hybridization of ClaI-digested genomic DNA of the wild-type strain SC5314 and *gln3*Δ mutants and complemented strains with the *GLN3*-specific probe 1. The sizes of the hybridizing fragments (in kb) are given on the left side of the blot, and their identities are indicated on the right. Insertion of the *SAT1* flipper into either of the two *GLN3* alleles of the parental strain SC5314 (lane 1) and subsequent FLP-mediated excision of the cassette produced the heterozygous mutants GLN3M2A and GLN3M2B (lanes 2 and 3). Insertion of the *SAT1* flipper into the remaining wild-type *GLN3* alleles, followed by recycling of the *SAT1* flipper cassette, gave rise to the homozygous *gln3*Δ mutants GLN3M4A and GLN3M4B (lanes 4 and 5). An intact *GLN3* copy was reintroduced into the *gln3*Δ mutants with the help of the *SAT1* flipper, which was subsequently excised again to produce the complemented strains GLN3MK2A and GLN3MK2B (lanes 6 and 7).

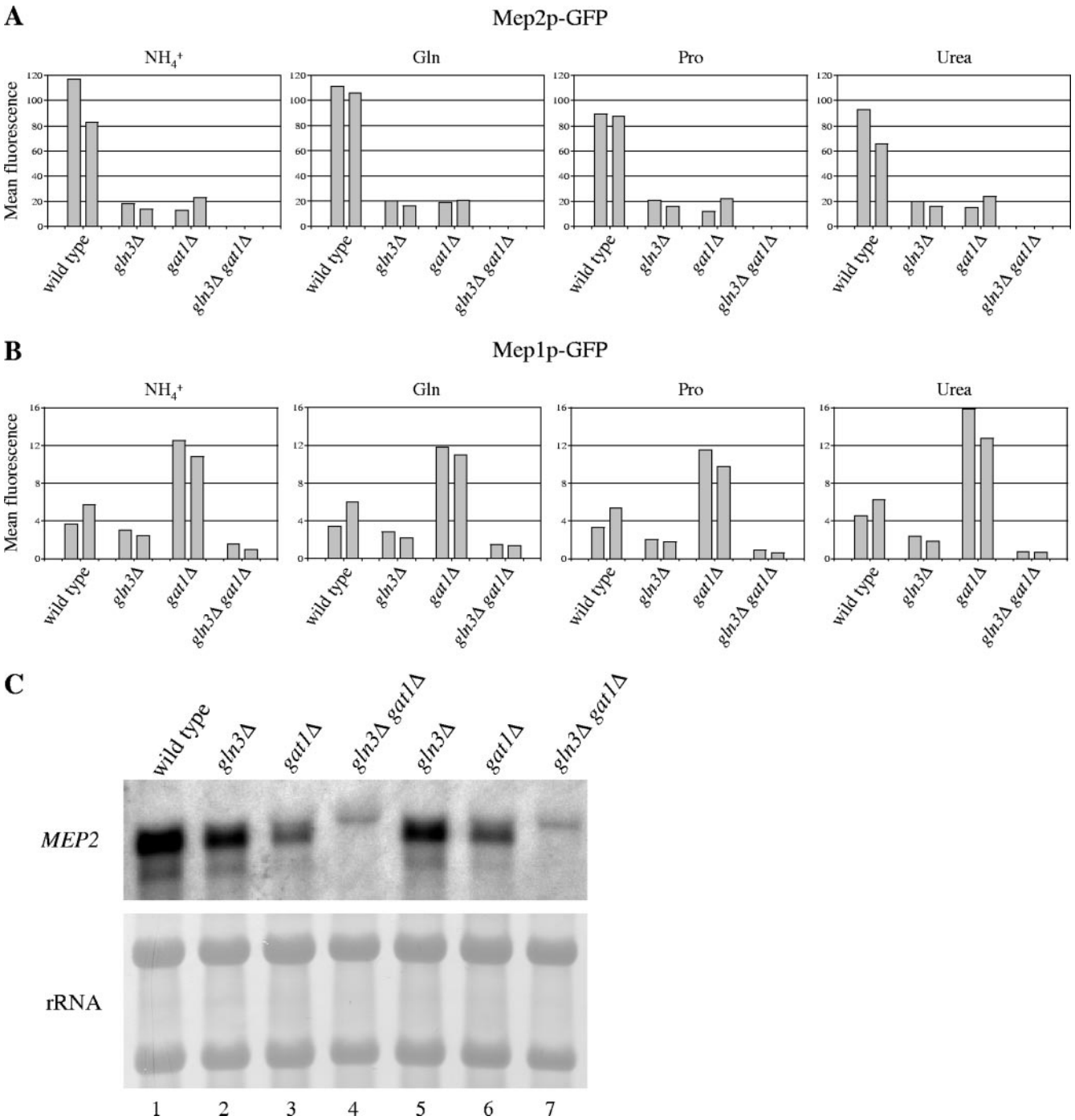

FIG. 4. *GLN3* and *GAT1* control ammonium permease expression in *C. albicans*. (A and B) Expression of GFP-tagged Mep2p (A) and Mep1p (B) in the wild type, *gln3*Δ and *gat1*Δ single mutants, and *gln3*Δ *gat1*Δ double mutants in liquid media containing limiting concentrations (100 μM) of the indicated nitrogen sources. Overnight cultures of the reporter strains in SD-Pro medium were diluted 50-fold in the test media and grown for 6 h at 30°C. The fluorescence of the cells was measured by flow cytometry. The first columns show the results obtained with the A series, and the second columns show the results obtained with the B series of the reporter strains (see Table 1). Note that the scales of the *y* axis are different for Mep2p-GFP and Mep1p-GFP. (C) Detection of *MEP2* mRNA by Northern hybridization with a *MEP2*-specific probe. Overnight cultures of the wild-type strain SC5314 (lane 1), the *gln3*Δ mutants GLN3M4A (lane 2) and GLN3M4B (lane 5), the *gat1*Δ mutants GAT1M4A (lane 3) and GAT1M4B (lane 6), and the *gln3*Δ *gat1*Δ double mutants Δ*gln3*GAT1M4A (lane 4) and Δ*gln3*GAT1M4B (lane 7) in SD-Pro medium were diluted 50-fold in liquid SLAD medium, and RNA was isolated after 6 h of growth at 30°C. The *MEP2* transcript is absent from the *gln3*Δ *gat1*Δ double mutants; the faint band of slightly higher molecular weight than that of *MEP2* seen in these and all other strains represents a cross-hybridizing transcript.

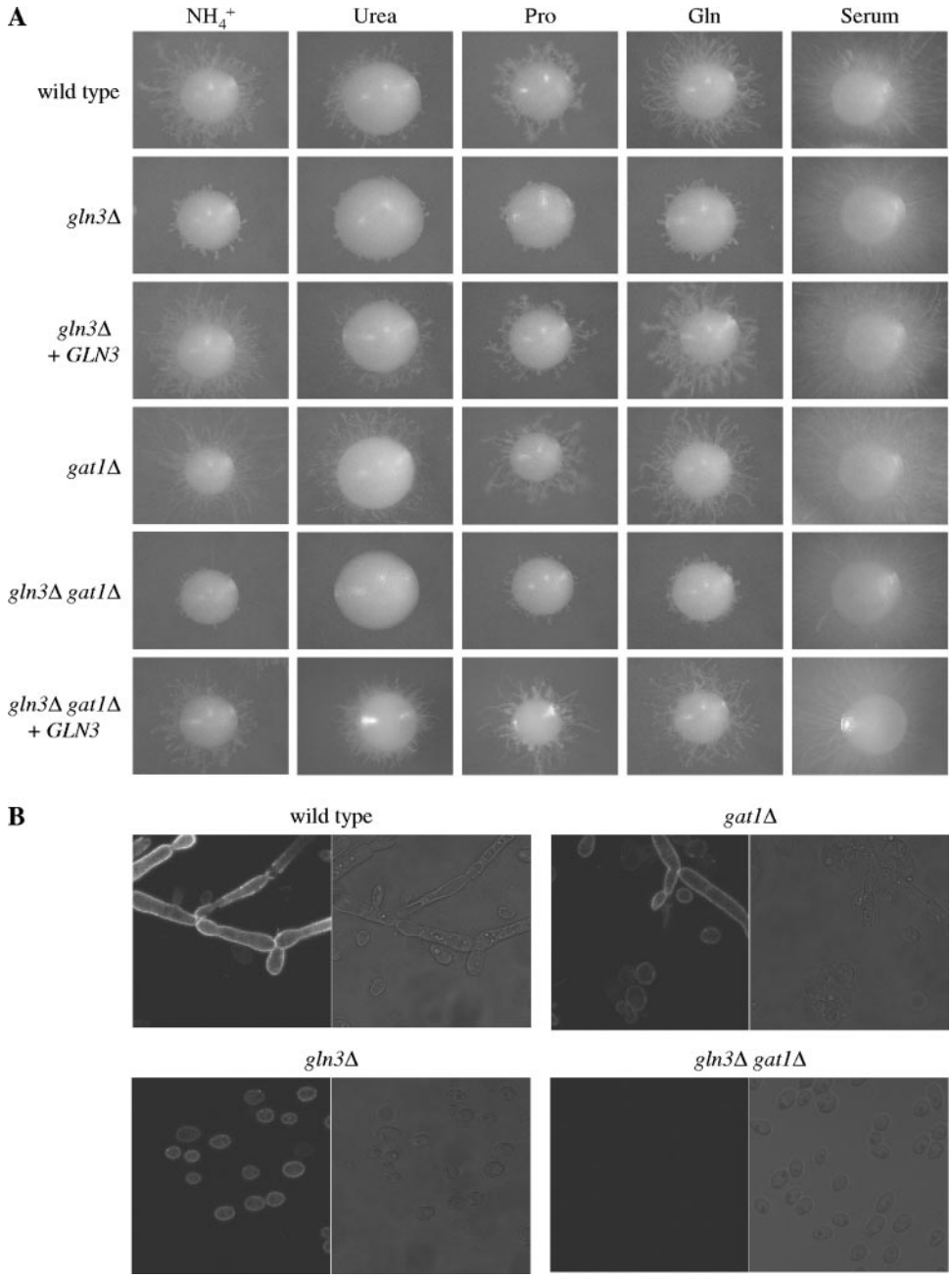

FIG. 5. (A) *GLN3* is required for the normal filamentous growth of *C. albicans* in response to nitrogen limitation. YPD precultures of the strains were appropriately diluted and spread on SD agar plates containing the indicated nitrogen sources at a concentration of 100 μM or on agar plates containing serum as an inducer of filamentous growth. Individual colonies were photographed after 6 days of incubation at 37°C. The following strains were used: SC5314 (wild type), GLN3M4A and -B (*gln3*Δ), GLN3MK2A and -B (*gln3*Δ + *GLN3*), GAT1M4A and -B (*gat1*Δ), Δ*gln3*GAT1M4A and -B (*gln3*Δ *gat1*Δ), and Δ*gln3*GAT1MK2A and -B (*gln3*Δ *gat1*Δ + *GLN3*). Independently constructed mutants and complemented strains behaved identically, and only one of them is shown in each case. (B) Expression of GFP-tagged Mep2p in the wild-type (strains SCMEP2G7A and -B), *gat1*Δ (strains Δ*gat1*MEP2G7A and -B), *gln3*Δ (strains Δ*gln3*MEP2G7A and -B), and *gln3*Δ *gat1*Δ (strains Δ*gln3*Δ*gat1*MEP2G7A and -B) backgrounds on SLAD plates. The pictures show fluorescence and corresponding phase-contrast micrographs of cells taken from colonies of the reporter strains grown for 6 days at 37°C.

propriate levels was the cause of the filamentous growth defect of the *gln3*Δ mutants, forced expression of *MEP2* from a different promoter should restore normal filamentation. To test this hypothesis, we expressed *MEP2* under the control of the *ADH1* promoter in the wild-type strain SC5314 and in the *gln3*Δ mutants. In addition, we also expressed the hyperactive $MEP2^{\Delta C440}$ allele, which is strongly overexpressed, presumably due to enhanced transcript stability (2), from the *ADH1* promoter. As a control, the *GFP* gene was integrated instead of *MEP2* in an identical fashion.

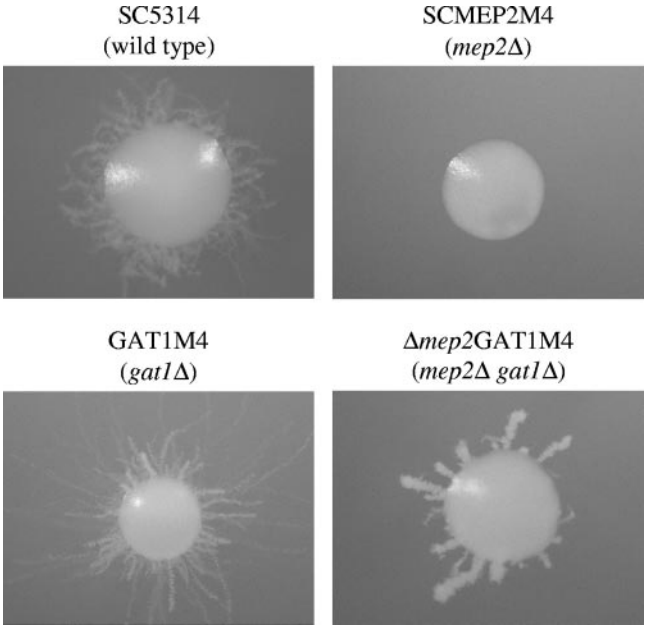

FIG. 6. Inactivation of *GAT1* results in *MEP2*-independent filamentation. The indicated strains were grown for 6 days at 37°C on SLAD plates, and individual colonies were photographed. Independently constructed mutants behaved identically, and only one of them is shown in each case.

Expression of an additional *MEP2* copy from the *ADH1* promoter had no detectable effect on filamentation of the wild-type strain SC5314, but expression of the hyperactive $MEP2^{\Delta C440}$ allele from the *ADH1* promoter in the same strain resulted in a hyperfilamentous phenotype, as previously reported for strain CAI4 expressing the $MEP2^{\Delta C440}$ allele from the native *MEP2* promoter (2). Forced expression of *MEP2* from the *ADH1* promoter partially rescued the filamentation defect of the *gln3*Δ mutants, and expression of the hyperactive $MEP2^{\Delta C440}$ allele resulted in the same hyperfilamentous phenotype as in the wild-type background (Fig. 7). Therefore, forced expression of *MEP2* overcomes the filamentous growth defect caused by *GLN3* inactivation.

DISCUSSION

The ammonium permease Mep2p plays a major role in nitrogen starvation-induced filamentous growth of *C. albicans*. An important aspect of the ability of Mep2p to stimulate filamentation in response to nitrogen limitation is the much higher expression levels of Mep2p than those of Mep1p, since reduced *MEP2* expression from the *MEP1* promoter abolished filamentous growth (2). The filamentous growth defect of strains expressing *MEP2* from the *MEP1* promoter in a *mep1*Δ *mep2*Δ background was not caused simply by the slower growth of these strains on SLAD plates due to inefficient ammonium uptake, since the defect was also observed when the P_{MEP1}-*MEP2* fusion was expressed in *mep2*Δ single mutants, which can grow at wild-type rates because of the presence of an intact *MEP1* allele (K. Biswas and J. Morschhäuser, unpublished results). A correlation between filamentous growth and Mep2p expression levels was also found in the present study when *MEP2* was expressed from mutated *MEP2* promoters with different levels of activity. Obviously, expressing Mep2p at appropriate levels is a prerequisite for *C. albicans* to be able to induce the switch from yeast to filamentous growth in response to nitrogen starvation. Therefore, we set out to investigate how the expression of *MEP2* is regulated. We found that two putative GATA factor binding sites in the *MEP2* promoter are essential for the upregulation of *MEP2* under limiting nitrogen conditions, pointing to the involvement of GATA transcription factors in the regulation of *MEP2* expression. While mutation of the GATAA sequence centered at position −208 alone had no significant effect, the same mutation almost completely abolished *MEP2* expression when combined with a mutation of the GATAA sequence centered at position −266, which by itself already reduced *MEP2* expression very strongly. Since a single GATAA sequence is essential but not adequate for nitrogen regulation in *S. cerevisiae* (17), GATAA sequences located further upstream or other regulatory sequences may also act together with the −266 site.

Our results demonstrate that both of the GATA transcription factors Gln3p and Gat1p are required for full *MEP2* expression, since deletion of either *GLN3* or *GAT1* strongly reduced *MEP2* expression under all conditions tested and no *MEP2* expression was detected in the absence of both transcription factors. In *S. cerevisiae*, both Gln3p and Gat1p also promote high-level expression of *MEP2*, but the contribution of each factor depends on the available nitrogen source (18).

The *gln3*Δ mutants were also severely impaired in filamentous growth under nitrogen starvation conditions, a phenotype that correlated well with the reduced *MEP2* expression levels in these mutants. Therefore, the inability of the *gln3*Δ mutants to induce *MEP2* expression at appropriate levels may be responsible for their filamentation defect, and we hypothesized that forced *MEP2* expression from a different promoter should bypass the filamentous growth defect of *gln3*Δ mutants. In fact, when a *MEP2* copy was expressed from the *ADH1* promoter in the *gln3*Δ mutants, filamentation was partially restored, and forced overexpression of a hyperactive *MEP2* allele completely rescued the filamentation defect of the *gln3*Δ mutants and resulted in a hyperfilamentous phenotype, as in a wild-type background. *S. cerevisiae gln3*Δ mutants are also defective in pseudohyphal differentiation, but expression of *MEP2* from a heterologous, inducible promoter did not restore filamentation, indicating that Gln3p has additional targets that are critical for pseudohyphal growth in *S. cerevisiae* (16). Our results do not exclude the possibility that, in *C. albicans*, Gln3p has targets in addition to *MEP2* that are normally required for the induction of filamentous growth in response to nitrogen starvation, but the expression of the hyperactive *MEP2* allele bypasses the need to activate these pathways. A similar observation was made previously, when we found that expression of the hyperactive *MEP2* allele from its own promoter overcame the filamentation defect of *cph1*Δ and *efg1*Δ single mutants but not that of *cph1*Δ *efg1*Δ double mutants, although both transcription factors, which are at the end of a mitogen-activated protein kinase cascade and a cyclic-AMP-dependent signaling pathway, respectively, are normally required for nitrogen starvation-induced filamentous growth of *C. albicans* (2).

Considering that *MEP2* expression in the *gat1*Δ mutants was reduced to levels similar to those in *gln3*Δ mutants, it was

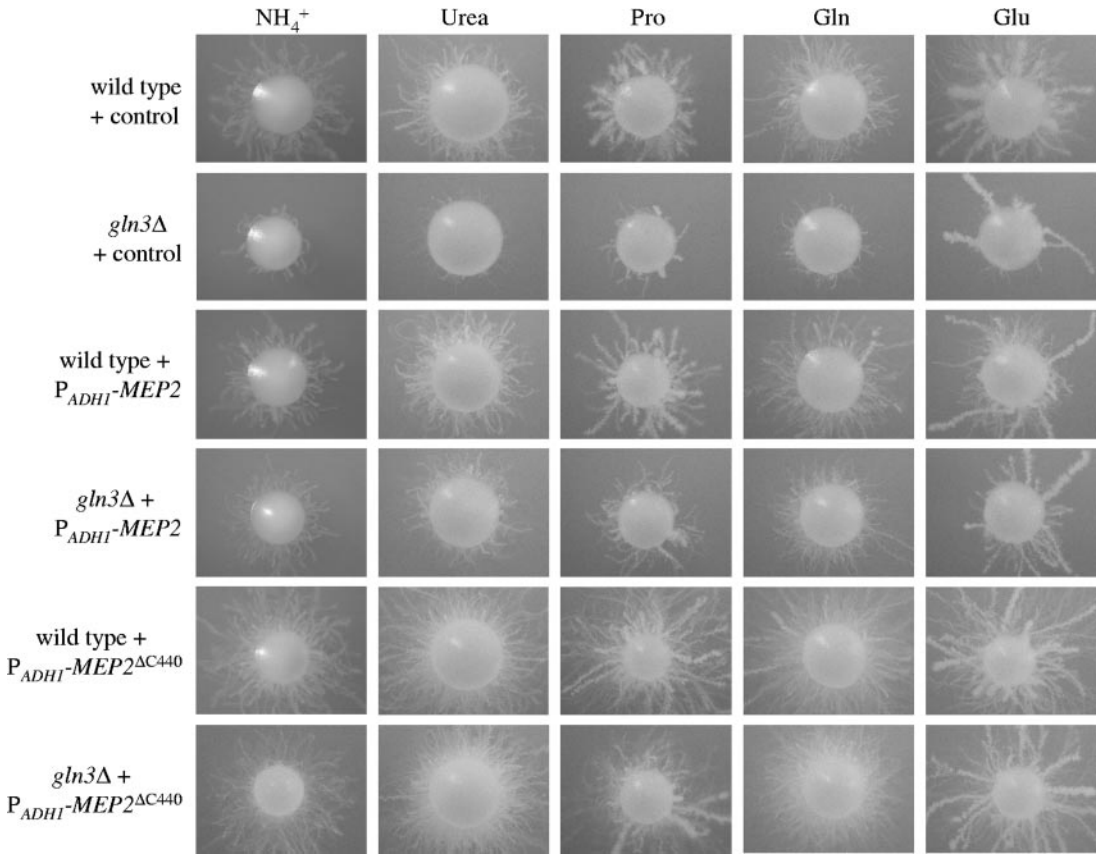

FIG. 7. Forced expression of *MEP2* overcomes the filamentous growth defect of *gln3*Δ mutants. YPD precultures of the strains were appropriately diluted and spread on SD agar plates containing the indicated nitrogen sources at a concentration of 100 μM. Individual colonies were photographed after 6 days of incubation at 37°C. The following strains were used: SCADH1G4A and -B (wild type + control), Δ*gln3*ADH1G4A and -B (*gln3*Δ + control), SCMEP2E4A and -B (wild type + P_{ADH1}-*MEP2*), Δ*gln3*MEP2E4A and -B (*gln3*Δ + P_{ADH1}-*MEP2*), SCMEP2ΔC2E2A and -B (wild type + P_{ADH1}-*MEP2*$^{\Delta C440}$), and Δ*gln3*MEP2ΔC2E2A and -B (*gln3*Δ + P_{ADH1}-*MEP2*$^{\Delta C440}$). Independently constructed mutants and complemented strains behaved identically, and only one of them is shown in each case.

surprising that *gat1*Δ mutants had no obvious filamentation defect. However, this result is in agreement with an independent study in which it was reported that the deletion of *GAT1* from *C. albicans* did not affect filamentation (13). It is possible that the reduced *MEP2* expression levels seen in both mutants are still sufficient to induce filamentation, but other targets which are also required for filamentous growth are affected by the inactivation of *GLN3* but not of *GAT1*. In fact, we found that the expression levels of GFP-tagged Mep2p in the *gln3*Δ and *gat1*Δ mutants were still higher (roughly twofold) than those in strains expressing the *MEP2-GFP* fusion construct from the *MEP1* promoter, which were not sufficient for filamentation (data not shown). In addition, we found that, in the absence of the Gat1p transcription factor, *C. albicans* activates signaling pathways that can partially bypass the requirement of Mep2p for filamentation. Wild-type filamentation in the *gat1*Δ mutants still depends on Gln3p activity, as *gln3*Δ *gat1*Δ double mutants exhibited the same strong filamentation defect as *gln3*Δ single mutants. Interestingly, work in Bill Fonzi's group has recently demonstrated that *GLN3* expression is negatively regulated by Gat1p in *C. albicans* (12). Increased expression of *GLN3* in *gat1*Δ mutants may therefore be responsible for the increased *MEP1* (but not *MEP2*) expression levels seen in these mutants and may also result in the activation of Gat1p-independent pathways that induce filamentation despite reduced *MEP2* expression.

In summary, our results demonstrate that, by placing *MEP1* and *MEP2* under the control of the GATA transcription factors Gln3p and Gat1p, *C. albicans* ensures that the expression of these ammonium permeases is induced when the preferred nitrogen source, ammonium, is present at low concentrations or absent. The signaling activity of Mep2p then also induces morphogenesis when ammonium levels remain limiting, despite transporter-mediated uptake, allowing the fungus to adjust its growth mode to environmental conditions.

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