EUKARYOTIC CELL, Oct. 2006, p. 1807–1819 Vol. 5, No. 10
1535-9778/06/$08.00+0 doi:10.1128/EC.00039-06

Role of the *Fusarium fujikuroi* TOR Kinase in Nitrogen Regulation and Secondary Metabolism

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Received 8 February 2006/Accepted 7 August 2006

In *Fusarium fujikuroi*, the biosynthesis of gibberellins (GAs) and bikaverin is under control of AreA-mediated nitrogen metabolite repression. Thus far, the signaling components acting upstream of AreA and regulating its nuclear translocation are unknown. In *Saccharomyces cerevisiae*, the target of rapamycin (TOR) proteins, Tor1p and Tor2p, are key players of nutrient-mediated signal transduction to control cell growth. In filamentous fungi, probably only one TOR kinase-encoding gene exists. However, nothing is known about its function. Therefore, we investigated the role of TOR in the GA-producing fungus *F. fujikuroi* in order to determine whether TOR plays a role in nitrogen regulation, especially in the regulation of GA and bikaverin biosynthesis. We cloned and characterized the *F. fujikuroi tor* gene. However, we were not able to create knockout mutants, suggesting that TOR is essential for viability. Inhibition of TOR by rapamycin affected the expression of AreA-controlled secondary metabolite genes for GA and bikaverin biosynthesis, as well as genes involved in transcriptional and translational regulation, ribosome biogenesis, and autophagy. Deletion of *fpr1* encoding the FKBP12-homologue confirmed that the effects of rapamycin are due to the specific inhibition of TOR. Interestingly, the expression of most of the TOR target genes has been previously shown to be also affected in the glutamine synthetase mutant, although in the opposite way. We demonstrate here for the first time in a filamentous fungus that the TOR kinase is involved in nitrogen regulation of secondary metabolism and that rapamycin affects also the expression of genes involved in translation control, ribosome biogenesis, carbon metabolism, and autophagy.

The rice pathogen *Fusarium fujikuroi* is well known for its production of gibberellins (GAs), a group of plant hormones causing the bakanae disease of rice seedlings (55, 67, 69). In addition to GAs, the fungus produces several other secondary metabolites, such as the red pigment bikaverin (44), fumonisins (54), and fusarin C (62). The biosynthesis of GAs and bikaverin, both nitrogen-free compounds, is strongly inhibited by high amounts of nitrogen in the culture medium (31). Recently, we have shown that this nitrogen regulation acts at the transcriptional level: six of the seven GA biosynthetic genes and the bikaverin-specific polyketide synthase gene, *pks4*, are repressed under nitrogen-sufficient conditions (44, 49). We demonstrated that AreA, the functional homologue of AreA/NIT2 in filamentous fungi (27, 40) and Gln3p in yeasts (14), is not only required for the expression of structural genes involved in nitrogen metabolism but also for the expression of those involved in the biosynthesis of secondary metabolites such as GAs (49, 68) and bikaverin (44). In *Neurospora crassa* and *Aspergillus nidulans*, the activity of NIT2/AreA is regulated by protein-protein interaction with the regulatory protein NMR1 (38) and NmrA (3), respectively. In *F. fujikuroi*, NMR does not play a crucial role in regulation of AreA activity. Deletion of the *nmr* homologue did not result in the expected deregulation of GA and bikaverin biosynthetic genes, although the gene fully complemented the *N. crassa nmr1* mutant (49).

On the other hand, we have shown that the *F. fujikuroi* glutamine synthetase (GS) is essential for the expression of the GA and bikaverin biosynthetic genes, as well as many other nitrogen-regulated genes. In contrast to our expectation, the transcript levels for these biosynthetic genes and several other nitrogen catabolite repression (NCR) genes were drastically reduced in the mutant despite the fact that the intracellular pool of glutamine, the major repressing compound, was dramatically reduced. Other genes, e.g., those involved in ribosome biogenesis and translation initiation/elongation, were highly upregulated in this mutant. The feeding of glutamine could restore the wild-type expression levels for most but not all of the genes (65), suggesting a role for GS itself in the signaling network of nitrogen regulation.

Apart from AreA, Nmr, and GS, not much is known of the regulatory network and signaling processes mediating nitrogen metabolite repression in filamentous fungi. Much more is known about components and their interactions in NCR in *Saccharomyces cerevisiae.* Thus, a number of transcription factors have been identified, including the AreA homologous GATA-type transcription activator Gln3p, which is responsible for the activation of NCR genes (reviewed in references 11, 12, 16, and 37). In recent years it has been shown that the activity of Gln3p is regulated by the target-of-rapamycin (TOR) proteins Tor1p and Tor2p (4, 6–8, 43). These highly conserved serine/threonine kinases are known to be inhibited by binding a complex consisting of the highly conserved FKBP12 protein and rapamycin (10, 11, 13, 18, 34). In yeasts, as in animals, they are key players of nutrient-mediated signal transduction pathways which control cell growth and proliferation (19, 23, 56, 57, 60). Both nitrogen starvation and inhibition of TOR by rapamycin, cause rapid dephosphorylation and nuclear accumulation of Gln3p, followed by expression of a wide range of NCR genes (4, 6, 7,15, 33). In the presence of good nitrogen sources,

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the repressor protein Ure2p complexes with phosphorylated Gln3p and sequesters it in the cytoplasm (4, 6, 7, 61). In addition to Gln3p, TOR was shown to mediate translocation between the cytoplasm and the nucleus of Rtg1/3, bHLH/Zip transcription factors involved in the regulation of several genes of the tricarboxylic acid cycle (39), and Msn2/4p, two zinc finger transcription factors involved in stress signaling (4; see references 16 and 37 for reviews).

Interestingly, the sequenced genomes of filamentous fungi, like those of animals and humans, contain only one *tor* homologue (50). Thus far, little is known about the functions of the TOR kinase in filamentous fungi or in which pathways this protein is involved. The antifungal activity of rapamycin against filamentous fungi, such as *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *Fusarium oxysporum*, and *Penicillium* sp. (18, 50, 70), as well as *Podospora anserina* (21), suggests functions for TOR in filamentous fungi that are similar to those in *S. cerevisiae* and humans. Recently, five genes encoding putative components of the TOR pathway have been identified in the genome of *A. nidulans*: *torA*, *fprA*, *jipA*, *sitA*, and *tapA* (26). However, there is no clear evidence that TOR signaling acts through AreA, and mutant phenotypes suggest that the TOR pathway plays only a minor role in regulating nitrogen metabolism. A detailed analysis of the impact of TOR on nutrient sensing, ribosome biogenesis, and autophagy by the TOR kinase is still lacking.

We investigate the nitrogen regulation network in the ascomycete *F. fujikuroi*, especially with respect to the biosynthesis of secondary metabolites such as GAs and bikaverin. After demonstrating an important role of AreA in GA and bikaverin biosynthesis, we wanted to identify the components acting upstream of AreA. Furthermore, we wanted to understand the mechanism by which this major regulator is activated and translocated to the nucleus upon nitrogen limitation and in particular whether TOR is involved in the regulation of AreA activity in *F. fujikuroi* in a way similar to the regulation of Gln3p activity in *S. cerevisiae.* Therefore, we cloned and characterized the *tor* gene from *F. fujikuroi.* We demonstrate that rapamycin can partially overcome nitrogen repression of GA and bikaverin gene expression, suggesting a role for TOR in AreA-mediated nitrogen regulation. Deletion of the *fpr1* gene resulted in rapamycin resistance of the mutants, confirming the expected link between the effect of rapamycin and TOR activity. We also show that TOR affects the expression of genes involved in ribosome biogenesis, translation, and autophagy.

MATERIALS AND METHODS

Fungal strains and culture conditions. Strain IMI58289 (Commonwealth Mycological Institute, Kew, United Kingdom) is a GA-producing wild-type strain of *F. fujikuroi*. The Δ*areA* and Δ*glnA* strains were described elsewhere (68, 65).

For all cultivations, the *F. fujikuroi* strains were first precultivated for 48 h in 300-ml Erlenmeyer flasks with 100 ml of Darken medium (20) with 2.0 g of glutamine/liter instead of $(NH_4)_2SO_4$, and 1 ml of this culture was used as the inoculum for cultivations in ICI medium (Imperial Chemical Industries, Ltd., United Kingdom) or complete medium (CM).

For DNA isolation, *F. fujikuroi* strains were incubated in 100 ml of CM (52) at 28°C on a rotary shaker at 200 rpm for 3 days or 18 h. For RNA isolation, the fungal strains were grown for 4 days in CM containing 2.0 g of glutamine/liter on a rotary shaker at 28°C. The washed mycelium was transferred into synthetic ICI medium (30) without nitrogen (0% ICI) for 6 h to induce nitrogen starvation and then transferred for 2 h into medium without nitrogen or with 100 or 10 mM $(NH_4)_2SO_4$, L-glutamine, L-glutamate, or L-arginine. Rapamycin (200 ng/ml; Calbiochem) was added 1 h after the shift for 1 h. Induction of the *alcA* promoter was achieved with the following culture conditions: the fungus was grown for 5 days in ICI medium as described above and then subcultured overnight in ICI medium containing 0.1% fructose or 3% lactose as a carbon source and 10 mM NH_4NO_3 as nitrogen source. Induction or repression was carried out by the addition of 50 mM ethanol or 2% glucose, respectively. The mycelium was harvested 2.5 h after addition of ethanol or glucose.

Bacterial strains and plasmids. *Escherichia coli* strain Top10F′ (Invitrogen) was used for plasmid propagation. Vector pUC19 was used to clone DNA fragments carrying the *F. fujikuroi tor* gene or parts of it. For *tor* gene replacement, a 1.3-kb KpnI/SalI fragment from the 5′-noncoding region and a 1.0-kb HindIII/SacI fragment from the 3′-noncoding region were cloned into the plasmid pUCH2-8 (1) carrying the hygromycin B resistance cassette. A KpnI/SmaI fragment of the resulting replacement vector, pΔtor, carrying both flanks and the hygromycin resistance cassette, was used for transformation. For overexpressing the *tor* gene, we amplified a 1.3-kb fragment, starting with the ATG start codon, with introduced HindIII and BamHI sites. This fragment was cloned HindIII/BamHI behind the *F. fujikuroi glnA* (65) or the *A. nidulans alcA* (48) promoters, respectively, which were cloned KpnI/ClaI into pUC19. A SacI/BamHI fragment carrying the promoter-*tor* fusion was then ligated into pUCH2-8 containing the hygromycin B resistance. The resulting vectors pglnA::tor and palcA::tor were transformed into the wild-type strain IMI58289. For *fpr1* gene replacement, a 0.8-kb SacII/XbaI-fragment from the 5′-noncoding region and a 0.65-kb SalI/XhoI fragment from the 3′-noncoding region were cloned into the plasmid pNR1 (47) carrying the nourseothricin resistance cassette. A SacII/XhoI fragment of the resulting replacement vector, pΔfpr, carrying both flanks and the nourseothricin resistance cassette, was used for transformation.

Screening of genomic library. About 40,000 recombinant phages of the *F. fujikuroi* genomic library were plated with *E. coli* strain XL1-Blue and screened by plaque hybridization as described previously (59). Plaques were blotted onto GeneScreen nylon membranes (DuPont) according to the manufacturer's instructions. A [^{32}P]dCTP-labeled 1.4-kb PCR fragment of the *F. fujikuroi tor* gene and the *fpr1* cDNA clone, respectively, were used as homologous probes. Hybridizations and washing were performed at 65°C. Putative positive phages were selected and screened by a second round of hybridization. Phage DNA was isolated as described previously (59) and used for restriction analysis.

cDNA library and macroarray construction. RNA isolation, cDNA library construction, and macroarray spotting were performed as described previously (65). Radioactive filters were exposed overnight on a PhosphorImager (Fuji BAS 2040) and visualized by using a Typhoon 9020 scanner (Amersham Biosciences, Germany). Quantitative analyses of spot intensity and expression comparison was carried out using Arrayvision (Imaging Research, Inc.).

Macroarray screenings. The macroarrays were differentially hybridized with cDNA probes from the wild-type cultivated in ICI medium as described above. The washed mycelium was transferred into ICI medium without nitrogen for 5 h and then shifted into ICI medium with 10 mM NH_4NO_3 for further 2 h. After 1 h, rapamycin was added, and the cultures were harvested after another 60 min of incubation. cDNA samples were prepared from poly(A)$^+$ mRNA isolated from total RNA samples by using an Oligotex mRNA kit (QIAGEN). Radiolabeled first-strand cDNA was synthesized from poly(A)$^+$ mRNA by using Superscript reverse transcriptase (Invitrogen) The reaction mixture contained 1.5 μg of mRNA, oligo(dT) as primer, and [α-^{33}P]dATP. The labeled cDNA was denatured in a heat block for 5 min, chilled on ice for 5 min, and added to the filters. The experiment was performed with two different filters for each probe. Clones that showed at least twofold up- or downregulation compared to the wild type were chosen for further analysis, including verification of differential expression patterns by Northern blots and sequence analysis.

DNA isolation. Lyophilized mycelium was ground to a fine powder with a mortar and pestle and dispersed (in the case of DNA for use in PCR) in extraction buffer as described previously (9). DNA for Southern hybridization experiments was prepared according to the protocol of Doyle and Doyle (25). Lambda DNA was isolated as described previously (59). Plasmid DNA was extracted by using a plasmid extraction kit (Genomed).

Southern blot analysis. For Southern analysis, genomic, plasmid, or phage DNA was digested to completion with appropriate restriction enzymes, fractionated in 1.0% (wt/vol) agarose gels, and transferred to nylon membranes (N$^+$; Amersham) by vacuum blotting. DNA probes were random labeled, and hybridizations were carried out overnight at 65°C. The blots were washed under hybridization conditions (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate at 65°C, followed by 0.1× SSC–0.1% sodium dodecyl sulfate).

RNA isolation. Total *F. fujikuroi* RNA was isolated by using the RNAgents total RNA isolation kit (Promega).

PCR and RT-PCR. PCRs contained 25 ng of DNA, 50 ng of each primer, 0.2 mM deoxynucleoside triphosphates, and 2 U of *Taq* polymerase (Red Taq; Sigma-Aldrich) in 50 μl. PCR was carried out at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min. For cloning a genomic fragment of the *F. fujikuroi tor* gene, the primers TOR-F1 (5′-TGG CTT GAG GTC ATA CCC CAG TTG ATC G-3′) and TOR-R1 (5′-ACA CTC TCC TTG TTC TCG CGC AAC ACC C-3′) were used. For reverse transcription-PCR (RT-PCR) with the Platinum Thermoscript One-Step RT-PCR kit (Invitrogen), 1 μg of total RNA as a template and the specific primers TOR-rec-F (5′-CAA CCT TCC CGG TGC TAT GCA CTT TC-3′), TOR-RT1 (5′-GAG CTG TGA TCC TGT TGT TGA CTG-3′), TOR-RT2 (5′-GTT CTG AAC GAG TTC GTC CGA GAT G-3′), TOR-RT3 (5′-CAA GGA AGG ACA CAC ACT CCA AGC-3′), TOR-RT6 (5′-CGA GAA CGT GAT GAG GGT TCT ACG-3′), and TOR-RT7 (5′-CCT ATG TAG TGC TGG CAC AGA TTC TCC-3′) (40 ng) were used. For the deletion of *tor*, the flanks were amplified with the primers TOR-KpnI (5′-CGG ATT GTA CGG TAC CGT TTG CGA TAC AGA GC-3′), TOR-SalI (5′-GTA TTT GAC GCC GAC GTC GAC GCC ATC GAA ATC G-3′), TOR-HindIII (5′-CCC CAA GCT TAT CAT GGC GCA AGC ACA GC-3′), and TOR-SacI (5′-CGA GCT CGT CGG GCG GAG AGC AAG GAG GAG G-3′) (40 ng). The diagnostic PCR for identification of transformants with homologous integration of the *tor* replacement cassette was performed with the primers dTOR-1 (5′-GGA AAC GTC TCG GCC GCA TTC ACA ACG-3′) and dTOR-2 (5′-CGA TCC GGT CTT TTG AGG ATT ACT CG-3′). For deletion of *fpr1*, the flanks were amplified with the primers (40 ng) FPR-SacII (5′-GGT CAT TAG ACC ACG CTG GAT CG-3′), FPR-XbaI (5′-GGT CTT CTG AAC ACC CAT TAT GG-3′), FPR-SalI (5′-CCA ATT ATC TAC CGC TAT CCT TCG-3′), and FPR-XhoI (5′-GGT TGC TAC CCT GAA AGC TAT CT-3′). The diagnostic PCR for identification of transformants with homologous integration of the *fpr1* replacement cassette was carried out with the primers dFPR-1 (5′-GCT ACC TTA CCT ACT AAG GTA CCT AG-3′) and dFPR-2 (5′-CTA AAG TAC ACA AGC TCA GCG TGG CAC AGC-3′).

Fungal transformations. Preparation of protoplasts of *F. fujikuroi* was carried out as described previously (66). A total of 10^7 protoplasts of strain IMI58289 were transformed with 10 μg of the KpnI/SmaI fragment of the replacement vector pΔtor, the SacII/XhoI-fragment of the vector pΔfpr1, or the circular vectors pglnA::tor and palcA::tor. For gene replacement experiments, transformed protoplasts were regenerated at 28°C in a complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% Casamino Acids) containing 120 μg of hygromycin B (Calbiochem)/ml or 100 μg of nourseothricin/ml, respectively, for 6 to 7 days. Single spore cultures for purification of the heterokaryons were established from the transformants with homologous integration of the replacement cassettes and used for DNA isolation and subsequent PCR and Southern blot analysis.

DNA sequencing and sequence homology searches. DNA sequencing of recombinant plasmid clones was accomplished with a LI-COR 4000 automatic sequencer (MWG Biotech). The two strands of overlapping subclones obtained from the genomic DNA clones were sequenced by using the M13/pUC forward (−40) and the M13/pUC reverse (−46) primers or specific oligonucleotides obtained from MWG Biotech. DNA and protein sequence alignments were performed by using Lasergene (DNASTAR, Madison, WI). Sequence homology searches were performed by using the NCBI database server. Protein homology was based on BlastX searches (2). For further analyses, the programs of DNASTAR, Inc. were used.

Plate assays. To study the rapamycin resistance of the wild-type and several mutants, the strains were cultivated on synthetic ICI agar with different nitrogen sources as indicated in the text and with 100 to 200 ng of rapamycin/ml.

RESULTS

Rapamycin causes drastic growth defects. As a first indication for the presence of a functional TOR protein in *F. fujikuroi*, the sensitivity of the wild-type strain to the specific TOR inhibitor rapamycin was assessed. Plate assays with different nitrogen sources and rapamycin concentrations showed sensitivity to the TOR inhibitor already at 100 ng/ml (Fig. 1A), suggesting the interaction of the rapamycin-FKBP12 complex with an active TOR homologue in *F. fujikuroi* as described for other organisms.

To get a better insight into the TOR-controlled signaling cascade, we compared the rapamycin resistance of the wild-type with that of several knockout mutants for genes involved in nitrogen metabolism. These included mutants with deletions of *areA* (68), the GS-encoding gene *glnA* (65), *nmr* (49), *tamA* (M. Wottawa and B. Tudzynski, unpublished data), *gdhA*, *gdhB*, and *gltA* (B. Tudzynski, unpublished data). Although most of the mutants were as sensitive as the wild type to rapamycin on all nitrogen sources (Δ*glnA* and Δ*areA* mutants grow only on glutamine as a nitrogen source), the Δ*nmr* mutant showed a slightly higher resistance (Fig. 1A). In contrast, the Δ*glnA* mutant is highly sensitive to rapamycin and does not grow at all on medium with glutamine and rapamycin (Fig. 1A).

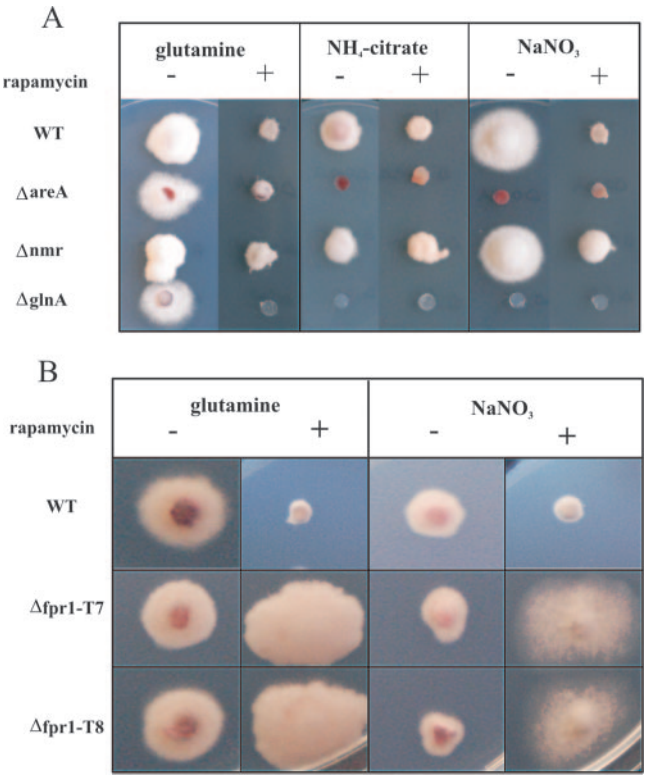

FIG. 1. Plate assays showing the growth of the *F. fujikuroi* wild-type (WT) and mutant strains on minimal medium containing 10 mM glutamine, ammonium citrate or sodium nitrate, respectively, with or without (+/−) rapamycin (100 ng/ml). (A) WT and Δ*areA*, Δ*nmr*, and Δ*glnA* mutants; (B) rapamycin resistance of two different Δ*fpr1* mutants compared to the rapamycin-sensitive WT.

Rapamycin inhibits TOR by binding to the FKBP12 protein. To demonstrate that the toxic effect of rapamycin is due to specific inhibition of TOR, we cloned and deleted the homologue of the *S. cerevisiae fpr1* gene encoding the rapamycin-binding protein FKBP12 (10, 34). Using a cDNA clone from the *F. fujikuroi* cDNA library (65) with high homology to the *S. cerevisiae* and *A. nidulans fpr1* genes as a probe, a genomic 2.6-kb SalI fragment comprising the complete coding sequence was isolated from the genomic library. The putative open reading frame (ORF), 603 bp in size (GenBank nucleotide sequence database accession number AM282587), encodes a 113-amino-acid protein with high identity to other FKBP12 proteins. Comparison between genomic and cDNA sequences revealed four introns with a size of 57 to 82 bp. The SacII/XhoI

fragment of the gene replacement vector pΔfpr1 (see Materials and Methods) carrying the nourseothricin resistance cassette as a selection marker was used to transform the wild-type strain IMI58289. Twelve of the seventeen transformants have integrated the replacement fragment at the *fpr1* locus (data not shown). Three transformants, Δ*fpr1*-T6, Δ*fpr1*-T7, and Δ*fpr1*-T8, were purified by single spore isolation. The homokaryotic Δ*fpr1* mutants revealed a high rapamycin resistance demonstrating that the inhibiting effect of rapamycin on the growth of the wild-type resulted from inactivation of TOR by binding the rapamycin-FKBP12 complex (Fig. 1B, shown for Δ*fpr1*-T7 and Δ*fpr1*-T8).

Cloning and sequencing of the *tor* gene. To study the role of TOR in growth, nitrogen regulation and secondary metabolism (GA and bikaverin biosynthesis) in *F. fujikuroi*, we cloned the *tor* homologue by a PCR approach. Primers (TOR-F1 and TOR-R1) were designed on the basis of sequence alignments between Tor1/2 of *S. cerevisiae* and the TOR homologues identified in the sequenced genomes of *Fusarium graminearum*, *Fusarium verticillioides*, *Neurospora crassa*, and *Aspergillus nidulans.* The 1.4-kb PCR product revealed 49% sequence identity at the amino acid level to *S. cerevisiae* Tor2p. The fragment was used as a probe to screen the genomic library of *F. fujikuroi*. Subsequent cloning and sequencing revealed an ORF of 7,241 bp. Comparison between the genomic and cDNA sequences of the *F. fujikuroi tor* homologue indicated the presence of three introns. The gene is predicted to encode a 2,423-amino acid protein with the highest degree of sequence identity to the putative TOR proteins of *F. graminearum* (95%; EAA71932), *N. crassa* (74%; EAA31334), and *A. nidulans* (61%; EAA57731). The nucleotide sequence of the *F. fujikuroi tor* gene can be accessed as accession number AM168274.

The *F. fujikuroi* TOR protein shows a conserved domain structure common to all TOR proteins (data not shown). The phosphatidylinositol 3-kinase domain displays the catalytic domain, whereas both the FAT and the FATC domains are proposed to mediate protein-protein interactions or to serve as a scaffold (63). N-terminal to the phosphatidylinositol 3-kinase domain, the TOR protein contains the conserved FKBP12-rapamycin binding domain. Missense mutations in the FKBP12-rapamycin binding domain at positions Ser1972 (Tor1p) and Ser1975 (Tor2p), respectively, conferred rapamycin resistance in yeast (63). The protein sequence of the *F. fujikuroi* TOR reveals a Ser at position 1973 between the FAT and the kinase domains, suggesting that this region functions as a rapamycin-binding domain. In addition, the TOR protein also contains a region with the typical tandemly repeated HEAT motifs at the N terminus which are thought to mediate protein-protein interactions (32, 42).

The TOR-kinase-encoding gene of *F. fujikuroi* is essential for growth. In contrast to *S. cerevisiae*, the genomes of filamentous fungi sequenced thus far contain only one *tor* homologous gene. To determine whether the *tor* gene is essential in *F. fujikuroi*, we constructed a *tor* replacement vector (pΔtor) carrying the hygromycin resistance gene. The replacement cassette was introduced into *F. fujikuroi* protoplasts, and the resulting hygromycin-resistant colonies were screened by PCR for homologous recombination at the *tor* locus (data not shown). Altogether, 34 hygromycin-resistant transformants from two independent transformation experiments revealed the expected diagnostic bands, indicating homologous integration of the replacement cassette into the *tor* locus, but still also contained nuclei with the wild-type gene copy (data not shown). Ten of them were purified four times by single spore isolation steps. However, it was not possible to obtain homokaryotic Δ*tor* mutants. The failure to obtain *tor* deletion mutants despite the high number of homologous integration events is a strong indication for the lethal effect of the *tor* knockout in *F. fujikuroi* as has been described for *tor1 tor2* double disruption, which confers G_1 arrest in *S. cerevisiae* (41).

Overexpression of the *tor* gene. Since we were not able to get knockout mutants, we wanted to overexpress the *F. fujikuroi tor* gene by using strong or inducible promoters. To avoid the difficulties of manipulating a vector containing the entire 7-kb ORF, a promoter replacement strategy was used, in which only the 1.3-kb 5′-region of the *tor* gene was linked to the strong *F. fujikuroi glnA* promoter (65) or to the alcohol-inducible *A. nidulans alcA* promoter (48), yielding vectors pglnA::tor (Fig. 2) and palcA::tor, respectively. These vectors were used to transform the wild-type strain in order to find transformants in which the integration at the *tor* locus would result in the replacement of the wild-type *tor* promoter with the two strong promoters. Hygromycin-resistant transformants were evaluated by PCR and Southern blot hybridization for homologous integration. Strains $glnA_{prom}$::tor-T8, -T11, and -T15, as well as strains $alcA_{prom}$::tor-T1, -T4, and -T10, showed the correct pattern of bands consistent with homologous integration (data not shown).

One transformant for each *tor* expression vector was chosen for transcription studies. Expression of the *tor* gene was significantly increased with both the *glnA* and the induced *alcA* promoters, whereas no *tor* transcript has been seen in the wild-type strain under all conditions tested (Fig. 3A). The strong *glnA* promoter mediates the highest transcription level of *tor* under nitrogen starvation conditions, due to the AreA-affected expression of the *F. fujikuroi glnA* gene. The *A. nidulans alcA* promoter-mediated *tor* transcription was drastically increased under induced conditions by the addition of ethanol, especially with high amounts of nitrogen. Repression of the *alcA* promoter by glucose revealed a much lower expression. However, the reduced transcript level with glucose is still higher than the wild-type expression level under all conditions. The overexpression of *tor* by both strong promoters resulted in a slightly higher resistance to rapamycin (Fig. 3B and data not shown).

Rapamycin causes gene deregulation. Previously, we have shown that the expression of GA and bikaverin biosynthesis genes depends on functional AreA (49) and GS (65) proteins. To determine whether TOR also affects the expression of these and other AreA- and/or GS-dependent genes (e.g., *40S26E* and *eIF4A* encoding a 40S ribosomal protein and a translation initiation factor, respectively), we examined the transcription level in medium with or without rapamycin. The addition of rapamycin to the wild-type mycelium resulted in significant alterations in the gene expression of the tested genes already after 1 h (Fig. 4). The GA-biosynthetic genes (e.g., *cps/ks* and *P450-1*) (69) and the bikaverin biosynthetic gene *pks4* (44), all positively regulated by AreA and strongly repressed by nitrogen, were partially derepressed by rapamycin with low concentrations of nitrogen (up to 10 mM). In contrast, the genes for

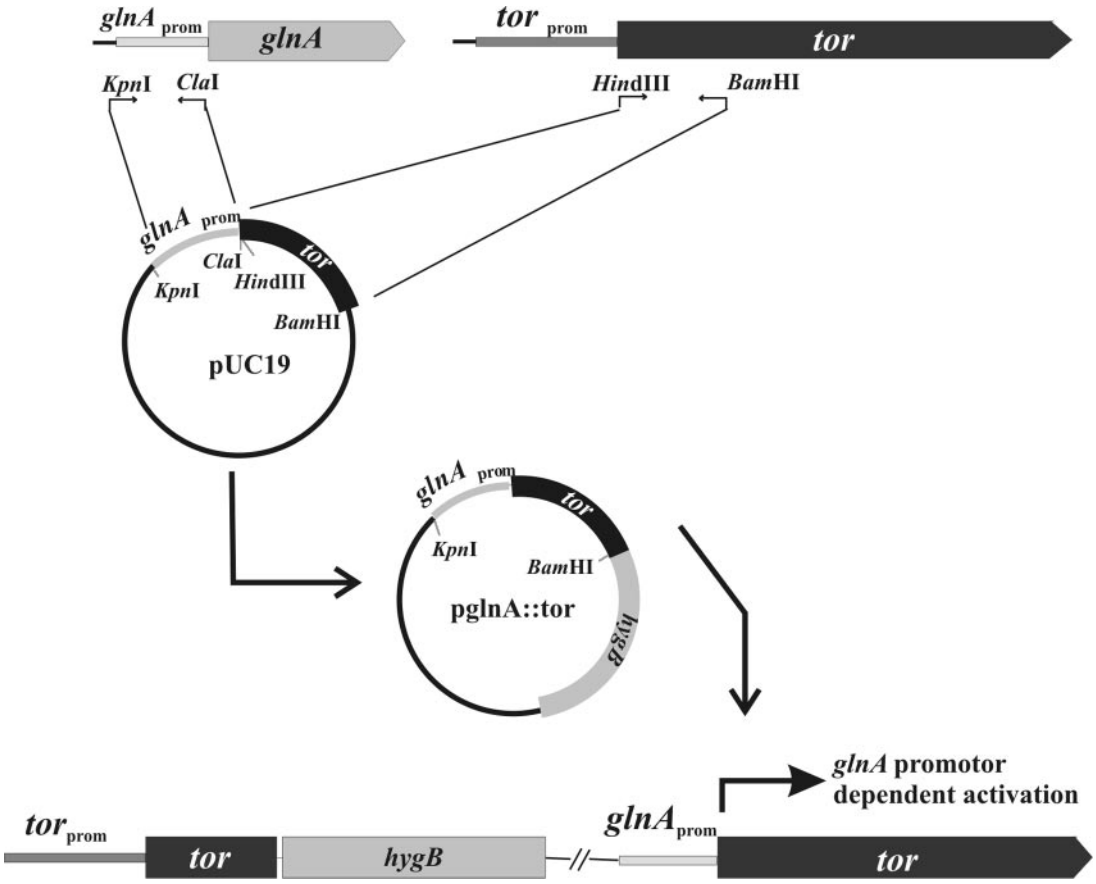

FIG. 2. Strategy for the construction of *tor* overexpression vectors. The first 1.3 kb of the *F. fujikuroi tor* gene was cloned behind the *F. fujikuroi glnA* or the *A. nidulans alcA* promoter (not shown). The vectors were transformed into the wild type, and transformants were screened for single-crossover events, resulting in a disrupted wild-type *tor* copy and a functional *tor* copy under the control of the introduced promoter.

the ribosome biogenesis protein 40S26E and the translation initiation factor eIF4A, which are weakly expressed under starvation conditions and highly expressed in media with nitrogen, are downregulated by rapamycin. As expected, rapamycin does not affect the expression of these genes in the Δ*fpr1* mutant (data not shown).

Interestingly, genes whose expression was shown to be affected in the *glnA* mutant or by the specific GS inhibitor L-methionine-DL-sulfoximine were also affected by rapamycin, though in opposite directions. Thus, the genes of ribosome biogenesis and translation control (e.g., *40S26E* and *eIF4A*) were upregulated in the Δ*glnA* mutant (65) but downregulated by rapamycin. Additionally, the expression of GA (e.g., *cps*/*ks* and *P450-1*) and bikaverin (*pks4*) biosynthesis genes was almost totally abolished in the Δ*glnA* mutant (65) but upregulated by rapamycin.

Determination of TOR target genes by use of macroarrays. To better understand the role of TOR in growth and development of *F. fujikuroi* and to identify more target genes of the TOR pathway, we used macroarrays spotted with 9216 cDNA clones from *F. fujikuroi* (65). These macroarrays were hybridized with cDNA probes from mycelia grown in synthetic ICI medium (10 mM ammonium nitrate) for 5 days and then transferred into the same medium with or without rapamycin. These experiments (three independent hybridizations) revealed a set of genes up- and downregulated by rapamycin treatment. The genes with the most significant expression patterns, their putative functions, and the effect of rapamycin on their expression are shown in Table 1. The genes can be accessed under accession numbers AM233688 to AM233739.

Among rapamycin-affected genes, we found those involved in stress response, ribosome biogenesis, translation initiation/elongation, autophagy, cross-pathway control, tricarboxylic acid and glyoxylate cycles, and secondary metabolism. Interestingly, genes belonging to the same functional group respond similarly to rapamycin. Thus, genes of ribosome biogenesis (e.g., *40S* and *60S*) and translation initiation (e.g., *eIF1A* and *eIF4A*), are downregulated by rapamycin, whereas genes involved in autophagy (homologues of the *P. anserina idi4* and *idi7* genes [22]), translation elongation (eEF1α), and protection against oxidative stress (the thioredoxin- and peroxiredoxin-encoding genes) are upregulated by rapamycin (Table 1). We also found the FKBP12-encoding gene *fpr1* among the genes upregulated by rapamycin.

Several genes upregulated by rapamycin have been recently identified as target genes of the GS that are dramatically downregulated in the *glnA* mutant (65). Two of them, *ddr48* (for DNA damage repair) and *cipC*, encode small proteins that are probably involved in stress response.

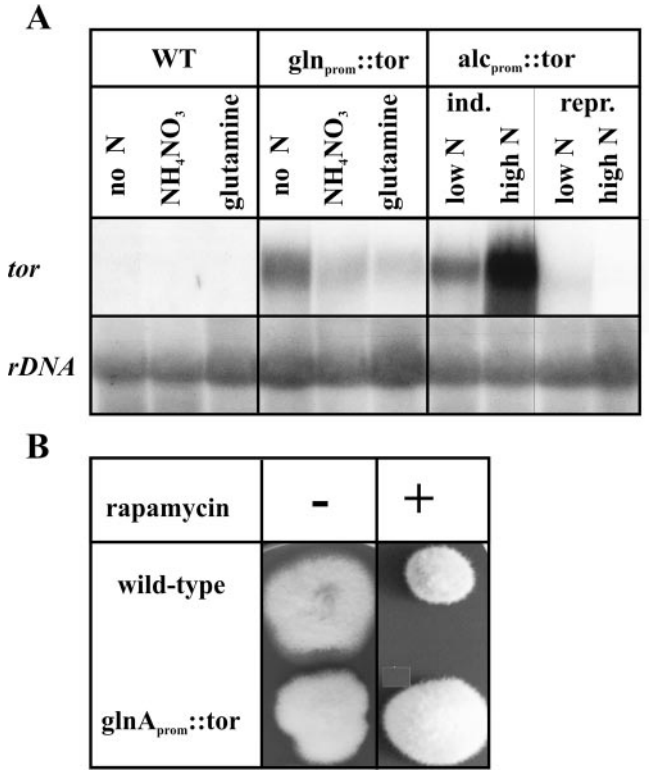

FIG. 3. Overexpression of the *F. fujikuroi tor* gene. (A) Expression of the *tor* gene in the wild-type and the glnA$_{prom}$::tor and alcA$_{prom}$::tor transformants. Strains were grown in synthetic ICI medium for 5 days. Mycelia of the wild-type and glnA$_{prom}$::tor transformants were transferred into media without (no N) or with 10 mM ammonium nitrate or glutamine. The transformants carrying the alcA$_{prom}$::tor were transferred into medium with low (10 mM) and high (100 mM) ammonium nitrate concentrations with 1% (vol/vol) ethanol (induction) or 20 g of glucose/liter (repression). A 3.1-kb *tor* cDNA fragment was used as a probe. (B) Plate assays with the wild-type and *tor* overexpression mutants carrying the glnA$_{prom}$::tor construct. The strains were grown on CM agar without or with 100 ng of rapamycin/ml. Mutants overexpressing *tor* revealed a slightly higher rapamycin resistance compared to the wild type.

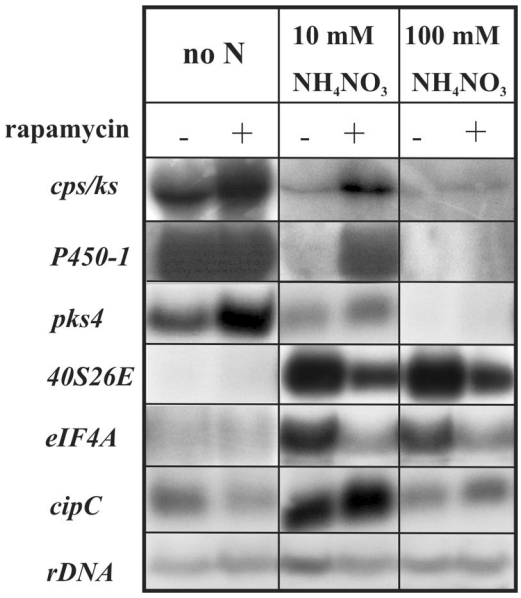

FIG. 4. Rapamycin-dependent expression of AreA and/or GS target genes, such as the GA biosynthesis genes *cps/ks* and *P450-1* (69), the bikaverin biosynthesis gene *pks4* (44), the ribosome biogenesis gene *40S26E*, the translation initiation factor *eIF4A*, and the gene *cipC* encoding a small protein of unknown function (65). The wild-type strain was grown for 4 days in synthetic ICI medium containing 20 mM ammonium nitrate and then transferred into nitrogen-free ICI medium. After a 5-h starvation period, 10 or 100 mM ammonium nitrate was added to two flasks each, whereas no nitrogen (no N) was added to the remaining two flasks. After 60 min, rapamycin (200 ng/ml) was added to one of the two identical flasks, and all cultures were incubated for a further 60 min.

Rapamycin-induced gene deregulation depends on the nitrogen source and concentration. We determined whether the rapamycin-induced alteration of target gene expression depends on the nitrogen source as found in *S. cerevisiae* (24). The expression of several genes identified by macroarray analysis was studied in media with 10 and 100 mM ammonium sulfate, glutamine, glutamate, and arginine, with or without rapamycin (Fig. 5). In most cases, the extent of deregulation by rapamycin depends on the nitrogen source. In medium with arginine and glutamate, rapamycin had no or only a low effect on the transcription level of most of the analyzed genes (Fig. 5). On the other hand, with ammonium and glutamine, the favorite nitrogen sources for *F. fujikuroi*, the effect of rapamycin on gene expression has been obvious. Thus, GA (e.g., *cps/ks* and *P450-1*) and bikaverin (e.g., *pks4*) biosynthesis genes, the putative regulator of autophagy *idi4*, ammonium transporters (e.g., *mepC*), the gene for the translation elongation factor eEF1α, and several GS target genes with an as-yet-unknown function (e.g., *ddr48*, *14-3-3*, and *cipC*) are upregulated by rapamycin to a gene-specific level. In contrast, genes involved in translation initiation (e.g., *eIF1A*, *eIF4A*, and *eIF5A*) and ribosome biogenesis (e.g., *40S26E* and *60SL2*) are downregulated by rapamycin in medium with ammonium or glutamine (Fig. 5 and data not shown). For the GA and bikaverin biosynthesis genes, the derepressing effect of rapamycin, especially in medium with glutamine, also depends on the nitrogen concentration. High amounts of glutamine (100 mM) abolished the derepressing effect of rapamycin. For the ammonium permease gene *mepC*, a clear effect of rapamycin has been obtained only with ammonium.

Does rapamycin affect gene expression in Δ*glnA* and Δ*areA* mutants? We determined whether rapamycin affects the expression of the same set of genes also in the Δ*areA* background and whether the lack of expression of several GS target genes (e.g., the GA and bikaverin biosynthesis genes, *cipC*, and *ddr48*) in the Δ*glnA* mutant can be overcome by rapamycin. The wild-type and the Δ*areA* and Δ*glnA* mutant strains were cultivated for 5 days in synthetic ICI medium with glutamine and then shifted into medium without nitrogen, with 10 mM glutamine or 10 mM ammonium and with or without rapamycin. As expected, the GA and bikaverin biosynthesis genes were weakly expressed in the Δ*areA* mutant, and rapamycin did not overrule the downregulation of these genes (Fig. 6), suggesting an AreA-dependent action of TOR. On the other hand, the genes involved in ribosome biogenesis and transla-

TABLE 1. Expression of EST clones down- or upregulated with rapamycin, as well as the expression of GS-regulated genes under the same conditions[a]

Cell function	Accession no.	Gene	Homology[b]	Expression[c]	
				Rapamycin	Δ*glnA*
Stress response	AM233688	*trx2*	Thioredoxin 2, *Podospora anserina*	+	−*
	AM233689	*TPx*	Peroxiredoxin/thioredoxin peroxidase, *Phanerochaete chrysosporium*	+	×
	AJ698900	*DDR48*	DNA damage-response protein, *Saccharomyces cerevisiae*	+	−
	AJ698914	*cipC*	Protein of unknown function, *Aspergillus nidulans*	+	−
	AM233690	*hsp70*	Heat shock protein 70-1, *Fusarium graminearum*	−	+*
	AM233691	*cph*	Cyclophilin, peptidyl prolyl isomerase, *Tolypocladium inflatum*	−	+*
Ribosome biogenesis	AM233692	*RPL29*	Ribosomal protein L29, *Aspergillus fumigatus*	−	+*
	AM233693	*RPS10*	Ribosomal protein S10, *Aspergillus fumigatus*	−	+*
	AM233694	*RPLS9*	Ribosmal protein S9, *Aspergillus fumigatus*	−	+*
	AM233695	*RPL14*	Ribosomal protein L14, *Aspergillus fumigatus*	−	+*
	AM233696	*RPL19*	Ribosomal protein L19, *Aspergillus fumigatus*	−	+*
	AM233697	*60L33-A*	Ribosomal protein L33-A, *Chaetomium globosum*	−	+*
	AM233698	*RPS18*	40S ribosomal protein S18, *Saccharomyces cerevisiae*	−	+*
	AM233699	*40S19*	40S ribosomal protein S19, *Fusarium graminearum*	−	+*
	AM233700	*40S10-b*	40S ribosomal protein S10-b, *Neurospora crassa*	−	+*
	AM233701	*ubi1*	Ubiquitin/rib. S27a fusion protein, *Neurospora crassa*	−	+*
	AM233702	*60SL2*	60S ribosomal protein L2, *Neurospora crassa*	−	+*
	AM233703	*60SL34*	60S ribosomal protein L34, *Neurospora crassa*	−	+*
	AM233704	*60SL30*	60S ribosomal protein L30, *Aspergillus fumigatus*	−	+*
	AJ698910	*40S26E*	Putative 40S ribosomal protein S26E, *Chaetomium globosum*	−	+
	AM233705	*60SL21*	60S ribosomal protein L21, *Aspergillus fumigatus*	−	+
	AM233706	*mrh*	Mitochondrial RNA helicase, *Saccharomyces cerevisiae*	−	+
Translation	AJ698906	*eEF1a*	Translation elongation factor	+	−
	AM233707	*eIF1A*	Translation initiation factor 1A putative, *Aspergillus fumigatus*	−	+*
	AM233708	*eIF4A*	Eukaryotic initiation factor 4A, *Neurospora crassa*	−	+*
	AM233709	*sui1*	Translation initiation factor SUI1, *Aspergillus fumigatus*	−	+*
Cross-pathway control		*cpc1*	Cross-pathway control protein	+	+
	AJ698905	*mbf1*	Cpc1 coactivator	+	+
Autophagy	AM233710	*idi4*	Autophagy related bZip factor, *Podospora anserina*	+	+
	AM233711	*idi7*	Idi4-dependent autophagy gene *idi7*, *Podospora anserina*	+	+
Secondary metabolism	Y15013	*cps/ks*	GA biosynthetic gene	+	−
	AJ41749	*des*	GA biosynthetic gene	+	−
	AJ278141	*pks4*	Bikaverin biosynthetic gene	+	−
	AM233713	*fum18*	FUM18, fumonisin biosynthesis, *Fusarium verticilloides*	−	+*
Nitrogen metabolism	AM168272	*MepA*	Ammonium permease	+	+
	AJ698901	*Uri*	Uricase	+/−	−
Transcription	AM233714	*C6*	Putative C2H2 Zn finger domain protein, *Aspergillus fumigatus*	+	+*
	AM233715	*Acr*	C6 finger Acr2, *Neurospora crassa*	+	−*
	AM233716	*C2H2*	Putative zinc finger, *Candida albicans*	+	−*
Cytoskeleton	AM233717	*Tropo*	Tropomyosin 1, *Aspergillus fumigatus*	+/−	−
Energy/respiration/ mitochondrial function	AM233718	*Aac*	ADP, ATP carrier protein, *Fusarium graminearum*	−	×
	AM233719	*Ndk*	Nucleosid-diphosphate kinase *Neurospora crassa*	−	×
	AM233720	*Cytc*	Cytochrome *c*, *Fusarium graminearum*	−	×
	AM233721	*cytb2*	Mitochondrial cytochrome b_2, *Aspergillus fumigatus*	+	×
	AM233722	*Phb*	Putative prohibitin, *Aspergillus fumigatus*	+	×
C-metabolism	AM233723	*FMN-Dh*	FMN-dependent dehydrogenase/lactate monooxygenase *Aspergillus fumigatus*	+	−*
	AM233724	*Pdc*	Pyruvate decarboxylase, *Aspergillus oryzae*	+	×
	AM233725	*Pgk*	Phosphoglycerate kinase, *Fusarium graminearum*	+	×
	AM233726	*hxt3*	Low-affinity hexose transporter, *Neurospora crassa*	−	×

Continued on following page

TABLE 1—*Continued*

Cell function	Accession no.	Gene	Homology[b]	Expression[c]	
				Rapamycin	Δ*glnA*
Sulfur metabolism	AM233727	*APSk*	Adenylyl-sulfate kinase, *Penicillium chrysogenum*	−	+
	AM233728	*hyp. protein*	Hyp. protein, Mo-co oxidoreductase dimerization domain, *Fusarium graminearum*	−	×
	AM233729	*CSase*	Cysteine synthase, *Fusarium graminearum*	+	×
Metabolism	AM233730	*Adh*	Alcohol dehydrogenase, *Neurospora crassa*	+	×
	AM233731	*EthD*	Unknown protein involved in ETBE degradation, *Rhodopseudomonas palustris*	+	×
TOR related	AM233732	*fpr1*	Gene encoding FKBP12, *Saccharomyces cerevisiae*	+	−*
	AM233739	*14-3-3*	14-3-3-like protein, *Hypocrea jecorina*	+	×
Drug related	AM233733	*MATE*	MATE efflux family protein, *Aspergillus fumigatus*	+	−*
Ca^{2+} signaling	AM233734	*CMD*	Calmodulin, *Fusarium graminearum*	+	+*
	AM233735	*calpain*	PALB, signaling protease, *Aspergillus nidulans*	+	+*
Proteolysis	AM233736	*Asp*	Aspartic protease, *Botryotinia fuckeliana*	+	×
Endocytosis	AM233737	*ypt51*	Putative RAB GTPase Ypt51, *Aspergillus fumigatus*	+	−*
Others	AM233738	*Bys*	BYS1 domain protein, *Aspergillus fumigatus*	+	+*

[a] Designations of expressed sequence tags are as submitted to the NCBI. Expressed sequence tag clones found already to be regulated by GS since GS target genes have been named according to the study by Teichert et al. (65).

[b] That is, the sequence similarity from BlastX (NCBI). The homologous gene of the accordant organism is also indicated.

[c] That is, the expression level determined by threefold analysis of macroarray using ammonium nitrate as nitrogen source. Most expression levels of EST clones identified by macroarray analysis have been verified by Northern analysis for most of the genes. *, Marked differential expression levels have only been demonstrated to be regulated by macroarray analysis. In addition, the expression level in the Δ*glnA* strain is indicated. Symbols: +, expression was upregulated by the addition of rapamycin or in the Δ*glnA* mutant; −, expression was downregulated by the addition of rapamycin or in the Δ*glnA* mutant; ×, expression was not differentially regulated in the Δ*glnA* mutant.

tion initiation were highly expressed when preferred nitrogen sources are available. Rapamycin treatment led to a downregulation in both the wild-type and the Δ*areA* mutant strains, suggesting an AreA-independent regulation by TOR. The low expression level of *40S26E* and *eIF4A* in the Δ*areA* mutant on ammonium is the result of the AreA-regulated expression of all three ammonium permease genes (S. Teichert and B. Tudzynski, unpublished data). In the Δ*glnA* mutant, rapamycin addition cannot restore expression of GA and bikaverin biosynthesis genes, suggesting that the GS is sufficient for the expression of these genes. On the other hand, genes involved in ribosome biogenesis and translation initiation are upregulated under starvation conditions (0% nitrogen) and downregulated with ammonium due to glutamine starvation. Glutamine, but not rapamycin, restored the wild-type expression level.

Interestingly, the expression of some genes in the Δ*glnA* mutant is still affected by rapamycin. Beside the ribosome biogenesis and translation initiation genes, *mepC* demonstrates a rapamycin-dependent expression in the Δ*glnA* mutant. This gene is expressed in a way similar to the GA and bikaverin biosynthesis genes in the wild-type and the Δ*areA* mutant but is upregulated in the Δ*glnA* mutant in medium without nitrogen or with glutamine. Rapamycin normalizes this upregulation. With ammonium as the substrate of MepC, the expression is downregulated compared to the wild type.

For some genes, e.g., *cipC* and *ddr48*, a rapamycin-dependent expression pattern has been demonstrated in the Δ*areA* mutant, although the pattern is in the opposite direction from that seen in the wild type (Fig. 6).

The gene *idi4* encoding a putative autophagy-related transcription factor (22) is weakly expressed in the wild type and slightly upregulated by rapamycin. In the Δ*areA* mutant, this gene is significantly upregulated in a rapamycin-independent manner, suggesting a repressing effect of AreA on this transcription factor. In the Δ*glnA* mutant, two transcripts with different sizes are produced. The addition of glutamine reduces the level of the second transcript.

The most obvious rapamycin-dependent expression pattern in the Δ*areA* mutant has been revealed for the hexose transporter-encoding gene *hxt3*. In the wild type, this gene is downregulated by rapamycin under starvation conditions, upregulated with ammonium, and not affected by rapamycin with glutamine (Fig. 6). In the Δ*areA* and Δ*glnA* mutant strains, the gene is almost completely downregulated. However, while the expression level can be restored by rapamycin under all conditions in the Δ*areA* mutant, the TOR inhibitor cannot overcome the almost complete loss of expression in the Δ*glnA* mutant. Therefore, neither rapamycin nor glutamine can overrule the effect of the *glnA* deletion on *hxt3* gene expression.

To better understand the role of GS in the AreA- and TOR-controlled pathways, we studied the expression of the *glnA* gene in the wild-type and the mutants with or without rapamycin. As previously shown (65), the *glnA* expression level is partially regulated by AreA. Now, we demonstrate that *glnA* is partially also regulated by TOR in an AreA-independent manner: the transcript level is significantly increased by rapamycin in the wild-type and in the Δ*areA* mutant (Fig. 6).

Therefore, TOR-dependent genes are not uniformly regulated by a common regulation system. Some genes are affected by rapamycin in an AreA-dependent manner, and others are not. The expression of genes that are almost completely downregulated in the Δ*glnA* mutant cannot be restored by rapamycin. On the other hand, several genes which are upregulated in the Δ*glnA* mutant are still affected by rapamycin.

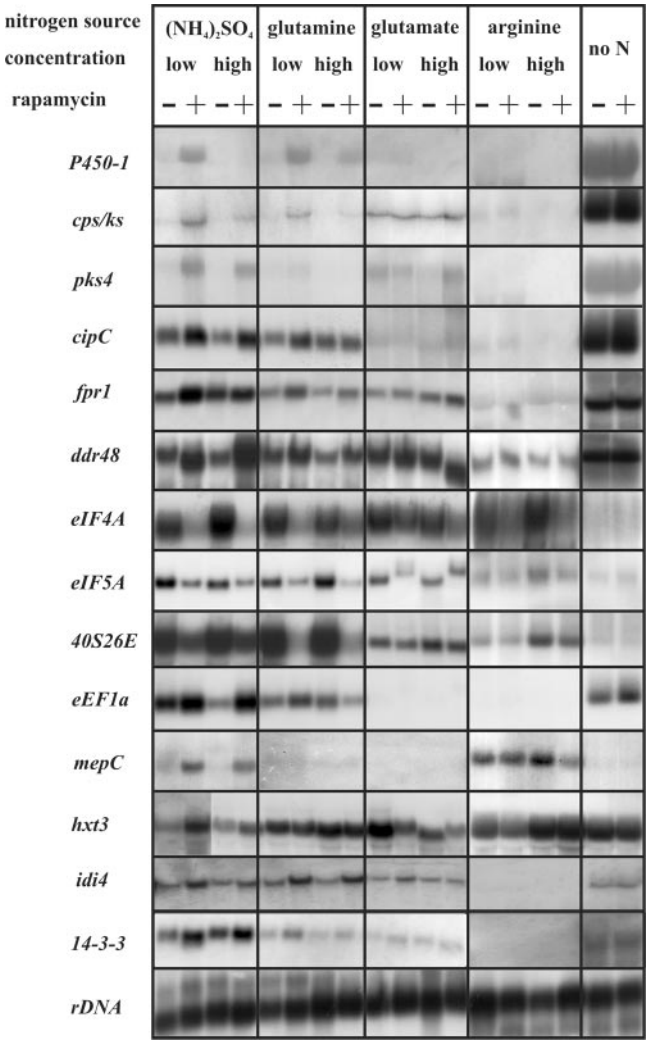

FIG. 5. Expression of rapamycin-affected genes identified by macroarray analysis (see Table 1) in medium without or with different nitrogen sources and concentrations in the wild-type strain. The cultivation conditions were as described in the legend of Fig. 4. Nitrogen sources were adjusted to that of 10 mM (low) and 100 mM (high) NH_4NO_3.

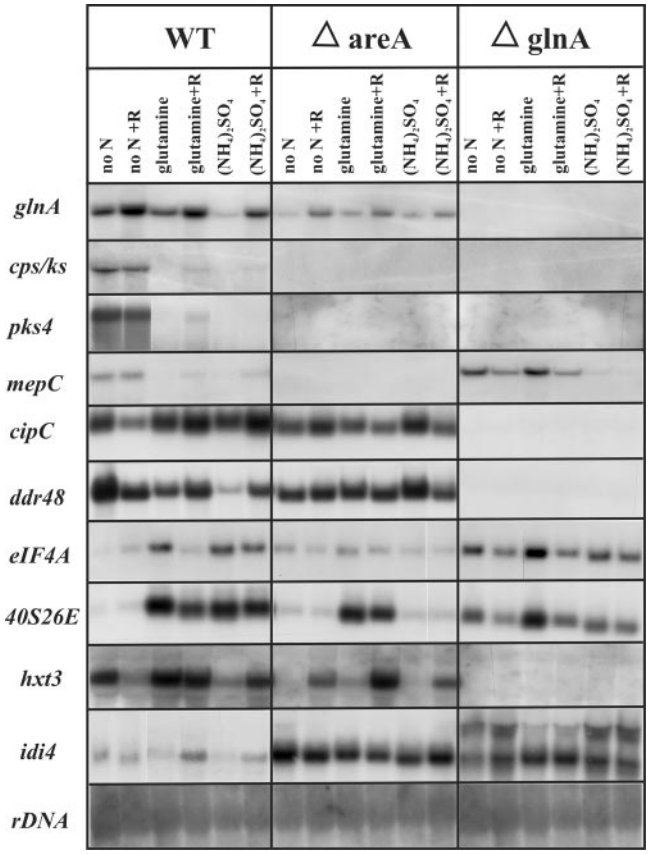

FIG. 6. Expression of some TOR target genes in the Δ*areA* and the Δ*glnA* mutants compared to that in the wild type. The cultivation conditions are described in Fig. 4.

DISCUSSION

Despite the fact that TOR kinases function in conserved signal transduction pathways in yeast and mammals, very little is known about the impact of TOR on cell growth regulation in filamentous fungi. Recently, several components of the TOR pathway were identified in *A. nidulans* (26), including the genes for the FKBP12 homologue and four putative proteins of the phosphatase 2A-complex acting downstream of TOR in *S. cerevisiae*. However, deregulation of genes subject to nitrogen metabolite repression in response to rapamycin treatment has not been detected in *A. nidulans* or any other filamentous fungus. The first results indicating functions of TOR in filamentous fungi similar to those in *S. cerevisiae* were obtained in *P. anserina*, where rapamycin treatment mimics heterokaryon incompatibility by autophagy induction (21, 22, 51).

We are interested in the nitrogen regulation of GA and bikaverin biosynthesis in the rice pathogen *F. fujikuroi.* Previously, we showed that both AreA and GS are essential for the expression of these biosynthetic genes (49, 65, 68). However, the components responsible for the activation and/or inactivation of AreA and the mode of action of GS as a putative key player in the nitrogen regulation network are not yet known. To get a deeper insight into the signaling pathway upstream of AreA, we determined whether TOR might act as a nutrient sensor as it does for the yeast. In contrast to *S. cerevisiae*, *F. fujikuroi* contains only one TOR-encoding gene in its genome. This gene encodes a 2,423-amino-acid protein with 46 and 47% sequence identity with Tor1p and Tor2p, respectively, of *S. cerevisiae*. The prediction of the same domains in an order identical to that in Tor1/2p in *S. cerevisiae* (32, 46) confirmed that *F. fujikuroi* TOR belongs to the TOR kinase family that is highly conserved from yeasts to humans. Targeted deletion of *tor* was not possible, probably as a result of inviability. In *S. cerevisiae*, the *tor1 tor2* double deletion is lethal and resulted in G_1 arrest, whereas the phenotype of Δ*tor1* mutants is much milder (35, 36).

To confirm that the observed inhibiting effect of rapamycin is due to a specific inhibition of TOR, we overexpressed the *tor* gene by using two strong promoters, the *F. fujikuroi glnA* and the inducible *A. nidulans alcA* promoters. In contrast to the wild type, the *tor* gene is highly expressed in transformants, with the transcriptional fusions resulting in a slightly higher

resistance to rapamycin. Furthermore, the specific inhibitory effect of rapamycin on TOR was also confirmed by targeted gene replacement of the FKBP12-encoding gene *fpr1*: all 11 Δ*fpr1* mutants revealed a high rapamycin resistance. Therefore, we demonstrated that the rapamycin-mediated changes in gene expression are due to inhibition of the TOR kinase in *F. fujikuroi* as it has been shown in *S. cerevisiae* where hundreds of genes revealed increased or decreased mRNA and protein levels after the addition of rapamycin (53).

We showed that the expression of GA and bikaverin biosynthesis genes which are strictly repressed under nitrogen-sufficient and derepressed under nitrogen starvation conditions in an AreA-dependent manner was only partially derepressed by rapamycin in media with ammonium and glutamine. Therefore, it is very likely that a second signaling pathway other than TOR controls the expression of AreA target genes. This second signaling cascade must be the major nitrogen sensing mechanism, leading to a strong repression of NCR genes. At high ammonium or glutamine concentrations (100 mM), the nitrogen repression of these genes cannot even partially be overruled by rapamycin-mediated TOR inhibition; this is probably due to an alternative nitrogen-sensing mechanism.

The same set of genes was previously shown to be drastically downregulated in the *glnA* mutant (65). This is in contrast to the situation in *S. cerevisiae*, where nitrogen starvation, rapamycin treatment, and deletion or inhibition of GS by L-methionine-DL-sulfoximine all generate the same outcomes, i.e., nuclear localization of Gln3p and increased Gln3p-mediated gene expression (17), although with diametrically opposite effects on Gln3p phosphorylation (64). There are two possible explanations for the opposite effects of rapamycin treatment and GS inactivation in *F. fujikuroi.* First, GS and TOR may act in different signaling pathways. However, this possibility is not very likely, since the same set of genes was affected by inhibition of TOR and by deletion of *glnA*, although in opposite directions. Second, GS might act in the same signaling pathway and interact with one or more components of the TOR signaling cascade. In the latter case, inhibition or deletion of GS would overrule the deregulating effect of rapamycin. This second possibility fits very well with our results for the genes that are downregulated in the Δ*glnA* mutant. Rapamycin cannot overcome the almost complete loss of expression of TOR target genes in the mutant in contrast to the wild type (see Fig. 6). These results support our previous suggestion that the GS might play an important regulatory role in the nitrogen regulation network, as has been recently postulated for *S. cerevisiae* (29).

Using a macroarray approach (with or without rapamycin), we identified more rapamycin-affected genes, presumably all regulated by TOR. The majority of downregulated genes function in ribosome biogenesis and translation initiation, whereas genes of translation elongation and autophagy were shown to be upregulated by rapamycin (see Table 1). Also, in yeast, *Drosophila*, and mammals, the inhibition of TOR results in a rapid and strong inhibition of ribosome biogenesis and translation initiation on the one hand and strong induction of autophagy on the other hand (reviewed in 16 and 37). Coregulation of genes of ribosome biogenesis and translation initiation upon rapamycin treatment has been shown in *S. cerevisiae* in a multitude of transcriptomic experiments (28, 53). The similar expression pattern of the same functional groups of genes in *F. fujikuroi* is probably based on a common regulation in a TOR-dependent manner in yeast (19, 58), filamentous fungi (the present study) and higher eukaryotes (reviewed in reference 16). Our results give one more example for the functional conservation of TOR regulating cell growth and proliferation on the basis of cellular energy levels and nutrient availability in all eukaryotes from yeast to mammals.

One of the central questions was whether the partial derepression of GA and bikaverin biosynthetic genes by inhibition of TOR is caused by activation of AreA as has been described for Gln3p in yeast. In contrast to the Δ*gln3* mutant in yeast, the Δ*areA* mutant is not rapamycin resistant, suggesting a different mode of regulation of AreA activity. Despite the difference in rapamycin resistance, we demonstrated that the partial derepression of several AreA target genes (e.g., GA and bikaverin biosynthetic genes, *mepC*) by rapamycin depends on AreA. In the Δ*areA* mutant, rapamycin did not even partially overrule the loss of gene expression caused by the deletion of the *areA* gene. In contrast to the AreA-dependent genes, the expression of genes for ribosome biogenesis and translation initiation factors is similar in the Δ*areA* and wild-type strains. Therefore, the TOR-mediated regulation of these genes is independent of AreA, suggesting that TOR regulates more transcription factors than AreA. In yeast, TOR controls some other transcription factors in addition to Gln3p. Thus, TOR inhibits the transcription of stress-responsive (STRE) genes by sequestering the general stress transcription factors Msn2p and Msn4p (Zn^{2+} transcription factors) in the cytoplasm (4). TOR also negatively regulates the heterodimeric bHLH/Zip transcription factors Rtg1p and Rtg3p (retrograde response transcription factors), which regulate the expression of tricarboxylic acid and glyoxylate cycle genes that are involved in de novo biosynthesis of glutamate and glutamine (17, 24). Therefore, it was not surprising to find several genes involved in sugar transport (e.g., *hxt3* [Fig. 5]) (6), glycolysis, and energy generation (see Table 1). Furthermore, the expression of a gene encoding a 14-3-3 protein is upregulated by rapamycin. In *S. cerevisiae*, it has been shown that the 14-3-3 homologues BMH1 and BMH2 are components of the TOR pathway. They associate with the TOR-regulated transcription factors Msn2/4p when glucose is available, whereas glucose starvation and rapamycin treatment cause a release of Msn2/4 from BMH2 (5). It is likely that 14-3-3 proteins in *F. fujikuroi* play a similar role binding to a yet-unknown transcription factor under carbon starvation or other stress conditions.

Several target genes of TOR, e.g., *hxt3*, *ddr48*, and *cipC*, show a rapamycin-dependent expression pattern in the Δ*areA* mutant, although in a way opposite to that observed in the wild type. We assume that AreA is part of a regulatory complex. Deletion of one of the components of this complex would lead to contrary outcomes of gene expression.

Based on these data we propose a first model for the TOR signaling network in *F. fujikuroi* (Fig. 7). This model also provides a possible explanation for the unexpected loss of AreA target gene expression (e.g., GA and bikaverin genes) in the *glnA* mutant even under nitrogen starvation conditions (65). Since these effects on gene expression could not be restored by rapamycin (see Fig. 6), the GS seems to be essential for the activation of AreA target genes under derepressing conditions.

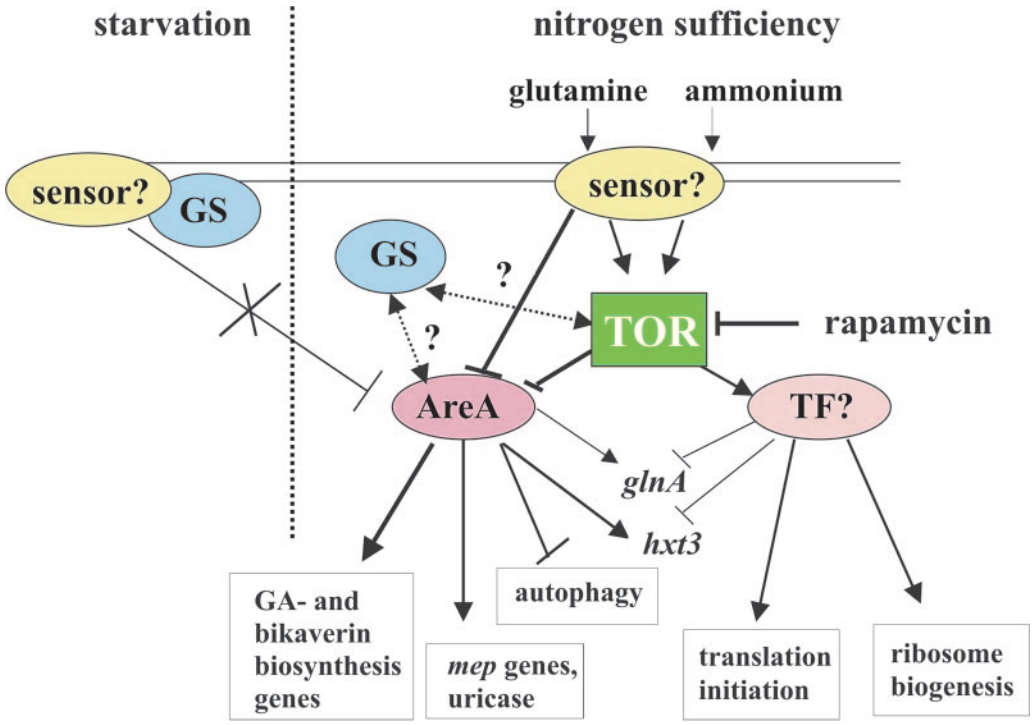

FIG. 7. Hypothetical model for the regulation of TOR target genes in *F. fujikuroi*. Some of the genes (e.g., GA and bikaverin biosynthesis genes) are partially regulated by TOR via AreA and additionally repressed by a proposed sensor under nitrogen-sufficient conditions. Genes involved in ribosome biogenesis and translation initiation are regulated by TOR in an AreA-independent manner, indicating at least one more TOR-regulated transcription factor (TF). Several genes are affected in both the Δ*areA* and the Δ*glnA* mutants, suggesting a regulatory role of the GS upstream of TOR. We postulate that the GS interacts with a second, yet-unknown nitrogen sensor under nitrogen starvation conditions. At nitrogen sufficiency, the GS dissociates from the sensor, which can then generate a repressing signal towards AreA. Thus, rapamycin treatment can only partially overcome the repression of NCR genes due to the existence of a second repressing signal affecting the activity of AreA.

The effect of inhibition or deletion of the GS is opposite to the effect of nitrogen starvation, which results in the strong derepression of AreA target genes. One possible explanation for these results would be the interaction of GS with a proposed second nitrogen-sensing system besides TOR (Fig. 7). Under starvation conditions, this interaction might prevent the transduction of a signal on an abundant supply of nitrogen. Under nitrogen sufficiency, the sensor is not bound to GS and thus confers nitrogen repression. A lack of GS would result in a lack of the signal-preventing complex and, therefore, lead to a permanently repressing signal despite the fact that the intracellular glutamine level is decreased. However, the regulation network seems to be much more complex, and we cannot yet explain the changed expression levels in the Δ*glnA* mutant for all genes. It is very likely that the GS can interact also with other proteins.

Thus far, we do not know the molecular mode of action of TOR and whether in *F. fujikuroi* TOR interacts with other proteins building TOR complex 1 (TORC1) and TOR complex 2 (TORC2) as has recently been shown for yeast (45). In the genomes of *F. graminearum* and *F. verticillioides*, two close relatives of *F. fujikuroi*, we found genes encoding proteins with high similarity to *S. cerevisiae* TORC1 proteins Lst8p (62% identity) and KOG1 (51% identity). Much less similarity (25 to 32% identity) was obtained for putative orthologs for the yeast TORC2 proteins AVO1, AVO2, and AVO3. It remains to be determined whether TORC1 and TORC2 exist and have conserved functions in filamentous fungi.

Thus, we showed here for the first time in a filamentous fungus that TOR affects the expression of a set of genes similar to that in *S. cerevisiae* and mammals. In addition to the target genes of TOR common in yeast and other eukaryotes, in *F. fujikuroi* the AreA-regulated GA and bikaverin biosynthesis genes are also under the control of TOR. However, the repression of these genes by nitrogen could not be completely overruled by rapamycin, suggesting that there must be an additional signaling pathway that senses nitrogen sufficiency and mediates a strong repressing signal toward the target genes of AreA.

ACKNOWLEDGMENTS

This study was supported by Deutsche Forschungsgemeinschaft grant Tu101/7.

We thank Sabine Richter for excellent technical assistance and Maria Cardenas, George Marzluf, and Michael Hall for critical reading of the manuscript.

REFERENCES

1. **Alexander, N. J., S. P. McCormick, and T. M. Hohn.** 1999. TRIII gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. Appl. Environ. Microbiol. **64:**221–225.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.

3. **Andrianopoulos, A., S. Kourambas, J. A. Sharp, M. A. Davis, and M. J. Hynes.** 1998. Characterization of the *Aspergillus nidulans nmrA* gene involved in nitrogen metabolite repression. J. Bacteriol. **180:**1973–1977.
4. **Beck, T., and M. N. Hall.** 1999. The TOR signaling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature **402:**689–692.
5. **Bertram, P. G., T. F. Zeng, J. Thorson, A. S. Shaw, and X. F. Zheng.** 1998. The 14-3-3 proteins positively regulate rapamycin-sensitive signaling. Curr. Biol. **8:**1259–1267.
6. **Bertram, P. G., J. H. Choi, J. Carvalho, W. Ai, C. Zeng, T. F. Chan, and X. F. Zheng.** 2000. Tripartite regulation of Gln3p by TOR, Ure2p, and phosphatases. J. Biol. Chem. **275:**35727–35733.
7. **Cardenas, M. E., N. S. Cutler, M. C. Lorenz, C. J. Di Como, and J. Heitman.** 1999. The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. **13:**3271–3279.
8. **Carvalho, J., P. G. Bertram, S. R. Wente, and X. F. Zheng.** 2001. Phosphorylation regulates the interaction between Gln3p and the nuclear import factor Srp1p. J. Biol. Chem. **276:**25359–25365.
9. **Cenis, J. L.** 1993. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res. **20:**2380.
10. **Chen, J., X.-F. Zheng, E. J. Brown, and S. L. Schreiber.** 1995. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc. Natl. Acad. Sci. USA **92:**4947–4951.
11. **Choi, J., J. Chen, S. L. Schreiber, and J. Clardy.** 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science **273:**239–242.
12. **Coffman, J. A., R. Rai, T. Cunningham, V. Svetlov, and T. G. Cooper.** 1996. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**847–858.
13. **Cooper, T. G.** 2002. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol. Rev. **26:**223–238.
14. **Couchesne, W. E., and B. Magasanik.** 1988. Regulation of nitrogen assimilation in *Saccharomyces cerevisiae*: roles of the URE2 and GLN3 genes. J. Bacteriol. **170:**708–713.
15. **Cox, K. H., J. J. Tate, and T. G. Cooper.** 2004. Actin cytoskeleton is required for nuclear accumulation of Gln3 in response to nitrogen limitation but not rapamycin treatment in *Saccharomyces cerevisiae*. J. Biol. Chem. **279:**19294–19301.
16. **Crespo, J. L., and M. N. Hall.** 2002. Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **66:**579–591.
17. **Crespo, J. L., T. Powers, B. Fowler, and M. N. Hall.** 2002. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proc. Natl. Acad. Sci. USA **99:**6784–6789.
18. **Cruz, M. C., L. M. Cavallo, J. M. Görlach, G. Cox, J. R. Perfect, M. E. Cardenas, and J. Heitman.** 1999. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in *Cryptococcus neoformans*. Mol. Cell. Biol. **19:**4101–4112.
19. **Cutler, N. S., J. Heitman, and M. E. Cardenas.** 1999. TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. Mol. Cell Endocrinol. **155:**135–142.
20. **Darken, M. A., A. A. L. Jensen, and P. Shu.** 1959. Production of gibberellic acid by fermentation. Appl. Microbiol. **7:**301–306.
21. **Dementhon, K., M. Paoletti, B. Pinan-Lucarré, N. Loubradou-Bourges, M. Sabourin, S. J. Saupe, and C. Clavé.** 2003. Rapamycin mimics the incompatibility reaction in the fungus *Podospora anserina*. Eukaryot. Cell **2:**238–246.
22. **Dementhon, K., S. J. Saupe, and C. Clavé.** 2004. Characterization of IDI-4, a bZIP transcription factor inducing autophagy and cell death in the fungus *Podospora anserina*. Mol. Microbiol. **53:**1625–1640.
23. **Dennis, P. B., S. Fumagalli, and G. Thomas.** 1999. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr. Opin. Genet. Dev. **9:**49–54.
24. **Dilova, I., S. Aronova, C. Y. C. Chen, and T. Powers.** 2004. Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1p/Rtg3p-dependent target genes. J. Biol. Chem. **279:**46527–46535.
25. **Doyle, J. J., and J. L. Doyle.** 1990. Isolation of plant DNA from fresh tissue. Focus **12:**13–15.
26. **Fitzgibbon, G. J., I. Y. Morozov, M. G. Jones, and M. X. Caddick.** 2005. Genetic analysis of the TOR pathway in *Aspergillus nidulans*. Eukaryot. Cell **4:**1595–1828.
27. **Fu, Y. H., and G. A. Marzluf.** 1990. *nit-2*, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein. Proc. Natl. Acad. Sci. USA **87:**5331–5335.
28. **Gasch, A. P., P. T. Spellman, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell **11:**4241–4257.
29. **Gavin, A. C., P. Aloy, P. Grandi, R. Krause, M. Boesche, M. Marzioch, C. Rau, L. J. Jensen, S. Bastuck, B. Dumpelfeld, A. Edelmann, M. A. Heurtier, V. Hoffmann, C. Hoefert, K. Klein, M. Hudak, A. M. Michon, M. Schelder, M. Schirle, M. Remor, T. Rudi, S. Hooper, A. Bauer, T. Bouwmeester, G. Casari, G. Drewes, G. Neubauer, J. M. Rick, B. Kuster, P. Bork, R. B. Russell, and G. Supert-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141–1477.
30. **Geissman, T. A., A. J. Verbiscar, B. O. Phinney, and G. Cragg.** 1966. Studies on the biosynthesis of gibberellins from (−)-kaurenoic acid in cultures of *Gibberella fujikuroi*. Phytochem. **5:**933–947.
31. **Giordano, W., J. Avalos, E. Cerda-Olmedo, and C. Domenech.** 1999. Nitrogen availability and production of bikaverin and gibberellins in *Gibberella fujikuroi*. FEMS Lett. **173:**389–393.
32. **Groves, M. R., P. Hanlon, O. Turowski, A. Hemmings, and D. Barford.** 1999. The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. Cell **96:**99–110.
33. **Hardwick, J. S., F. G. Kuruvilla, J. K. Tong, A. F. Shamji, and S. L. Schreiber.** 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc. Natl. Acad. Sci. USA **96:**14866–14870.
34. **Heitman, J., N. R. Movva, and M. N. Hall.** 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science **253:**905–909.
35. **Helliwell, S. B., P. Wagner, J. Kunz, M. M. Deuter-Reinhard, R. Henriquez, and M. N. Hall.** 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol. Biol. Cell **5:**105–118.
36. **Helliwell, S. B., I. Howald, N. Barbet, and M. N. Hall.** 1998. TOR2 is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae*. Genetics **148:**99–112.
37. **Inoki, K., H. Ouyang, Y. Li, and K.-L. Guan.** 2005. Signaling by target of rapamycin proteins in cell growth control. Microbiol. Mol. Biol. Rev. **69:**79–100.
38. **Jarai, G., and G. A. Marzluf.** 1990. Analysis of conventional and in vitro generated mutants of *nmr*, the negatively acting nitrogen regulatory gene of *Neurospora crassa*. Mol. Gen. Genet. **222:**233–240.
39. **Komeili, A., K. P. Wedaman, E. K. O'Shea, and T. Powers.** 2000. Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. J. Cell Biol. **151:**863–878.
40. **Kudla, B., M. X. Caddick, T. Langdon, N. M. Martinez-Rossi, C. F. Bennett, S. Sibley, and R. W. Davies.** 1990. The regulatory gene *are*A mediating nitrogen metabolite repression in *Aspergillus nidulans* mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. **9:**1355–1364.
41. **Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall.** 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell **73:**585–596.
42. **Kunz, J. R., U. Schneider, I. Howald, A. Schmidt, and M. N. Hall.** 2000. HEAT repeats mediate plasma membrane localization of Tor2p in yeast. J. Biol. Chem. **275:**37011–37020.
43. **Kuruvilla, F. G., A. F. Shamji, and S. L. Schreiber.** 2001. Carbon- and nitrogen-quality signaling to translation are mediated by distinct GATA-type transcription factors. Proc. Natl. Acad. Sci. USA **98:**7283–7288.
44. **Linnemannstöns, P., J. Schulte, M. M. del Prado, R. H. Proctor, J. Avalos, and B. Tudzynski.** 2002. The polyketide synthase gene *pks4* from *Gibberella fujikuroi* encodes a key enzyme in the biosynthesis of the red pigment bikaverin. Fungal Genet. Biol. **37:**143–148.
45. **Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Opplinger, P. Jenoe, and M. Hall.** 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell **10:**457–468.
46. **Lorenz, M. C., and J. Heitman.** 1995. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. J. Biol. Chem. **270:**27531–27537.
47. **Malonek, S., M. C. Rojas, P. Hedden, P. Gaskin, and B. Tudzynski.** 2004. The NADPH: cytochrome P450 reductase gene from *Gibberella fujikuroi* is essential for gibberellin biosynthesis. J. Biol. Chem. **279:**25075–25084.
48. **Mathieu, M., and B. Felenbok.** 1994. The *Aspergillus nidulans* CREA protein mediates glucose repression of the ethanol regulon at various levels through competition with the ALCR-specific transactivator. EMBO J. **13:**4022–4027.
49. **Mihlan, M., V. Homann, T.-W. D. Liu, and B. Tudzynski.** 2003. AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. Mol. Microbiol. **47:**975–991.
50. **Muthuvijayan, V., and M. R. Nmarten.** 2004. In silico reconstruction of nutrient-sensing signal transduction pathways in *Aspergillus nidulans*. In Silico Biol. **4:**606–631.
51. **Noda, T., A. Matsuura, Y. Wada, and Y. Oshumi.** 1995. Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **210:**126–132.
52. **Pontecorvo, G. V., J. A. Roper, L. M. Hemmonns, K. D. Mac Donald, and**

A. W. J. Bufton. 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. **141:**141–238.
53. **Preiss, T., J. I. Baron-Benhamou, W. Ansorge, and M. W. Hentze.** 2003. Homodirectional changes in transpcriptome composition and mRNA translation induced by rapamycin and heat shock. Nat. Struct. Biol. **10:**1039–1047.
54. **Proctor, R. H., R. D. Plattner, D. W. Brown, J.-A. Seo, and Y.-W. Lee.** 2004. Discontinuous distribution of fumonisin biosynthesis genes in the *Gibberella fujikuroi* species complex. Mycol. Res. **7:**815–822.
55. **Rademacher, W.** 1997. Gibberellins, p. 193–205. *In* T. Anke (ed.), Fungal biotechnology. Chapman & Hall, London, England.
56. **Rohde, J., J. Heitman, and M. E. Cardenas.** 2001. The Tor kinases link nutrient sensing to cell growth. J. Biol. Chem. **276:**9583–9586.
57. **Rohde, J., and M. E. Cardenas.** 2004. Nutrient signaling through TOR kinases controls gene expression and cellular differentiation in fungi. Curr. Top. Microbiol. Immunol. **279:**53–72.
58. **Rudra, D., Y. Zhao, and J. R. Warner.** 2005. Central role of Ifh1p-Fhl1p interaction in the synthesis of yeast ribosomal proteins. EMBO J. **24:**533–542.
59. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
60. **Schmelzle, T., T. Beck, D. E. Martin, and M. N. Hall.** 2004. Activation of the RAS/cAMP pathway suppresses a TOR deficiency in yeast. Mol. Cell. Biol. **24:**338–351.
61. **Shamji, A. F., F. G. Kuruvilla, and S. L. Schreiber.** 2000. Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. Curr. Biol. **10:**1574–1581.
62. **Song, Z., R. J. Cox, C. M. Lazarus, and T. J. Simpson.** 2004. Fusarin C biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*. Chem. Biochem. **5:**1196–1203.
63. **Stan, R., M. M. McLaughlin, R. Cafferkey, R. K. Johnson, M. Rosenberg, and G. P. Livi.** 1994. Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. J. Biol. Chem. **269:**32027–33230.
64. **Tate, J. J., R. Rai, and T. G. Cooper.** 2005. Methionine sulfoximine treatment and carbon starvation elicit Snf1-independent phosphorylation of the transcription activator Gln3 in *Saccharomyces cerevisiae*. J. Biol. Chem. **280:**27195–27204.
65. **Teichert, S., B. Schönig, S. Richter, and B. Tudzynski.** 2004. Deletion of the *Gibberella fujikuroi* glutamine synthetase gene has significant impact on transcriptional control of primary and secondary metabolism. Mol. Microbiol. **53:**1661–1675.
66. **Tudzynski, B., K. Mende, K.-M. Weltring, J. R. Kinghorn, and S. E. Unkles.** 1996. The *Gibberella fujikuroi niaD* gene encoding nitrate reductase: isolation, sequence, homologous transformation and electrophoretic karyotype location. Microbiol. **142:**533–539.
67. **Tudzynski, B.** 1999. Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects. Appl. Microbiol. Biotechnol. **52:**298–310.
68. **Tudzynski, B., V. Homann, B. Feng, and G. A. Marzluf.** 1999. Isolation, characterization and disruption of the *areA* nitrogen regulatory gene of *Gibberella fujikuroi*. Mol. Gen. Genet. **261:**106–114.
69. **Tudzynski, B.** 2005. Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Appl. Microbiol. Biotechnol. **66:**597–611.
70. **Wong, G. K., S. Griffith, I. Kojima, and A. L. Demain.** 1998. Antifungal activities of rapamycin and its derivatives, prolylrapamycin, 32-desmethylrapamycin, and 32-desmethoxyrapamycin. J. Antibiot. **51:**487–491.