Cross-Species Hybridization with Fusarium verticillioides Microarrays Reveals New Insights into Fusarium fujikuroi Nitrogen Regulation and the Role of AreA and NMR

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The rice pathogenic fungus Fusarium fujikuroi belongs to the Gibberella fujikuroi species complex, which consists of about 60 species of genus Fusarium section Liseola of monophyletic origin (45, 47, 48, 85). Most Fusarium species are plant pathogens, with both wide and narrow host ranges represented. Fusarium species are also well known for synthesizing an extensive variety of sometimes toxic secondary metabolites. The synthesis of gibberellins (GAs) (56, 79) and the red pigment bikaverin genes (P. Wiemann and B. Tudzynski, unpublished data). AreA is a member of the GATA-type transcription factor family and a functional homologue of two positively acting nitrogen regulators in Saccharomyces cerevisiae, Gln3 (40) and Gat1 (Nil1) (69). AreA homologues have been isolated from several filamentous fungi and include the Aspergillus nidulans AreA (34), Neurospora crassa NIT2 (21), Penicillium chrysogenum NreA (26), Fusarium oxysporum Fnr1 (17), and Magnaporthe grisea Nut1 (20). Most of our knowledge on the molecular mechanism of AreA-dependent nitrogen regulation derives from studies of A. nidulans and N. crassa, where AreA activity is negatively affected by binding to the nitrogen metabolite regulators NmrA and NMR1, respectively, when rich nitrogen sources are available. The deletion of the nmr gene in either fungus resulted in a significant derepression of AreA target genes under nitrogen-sufficient conditions (2, 51). In contrast, for F. fujikuroi, the NMR homologue was shown to play only a minor role in nitrogen regulation, at least in the regulation of GA biosynthesis genes, despite fully complementing the N. crassa nmr-1 mutant (39). Therefore, we postulate that F. fujikuroi must contain additional regulatory proteins that affect AreA activity and nitrogen metabolite repression of its target genes. These additional proteins may be involved in protein interactions with AreA or in the poorly understood processes that modulate AreA activity via the rapid degradation of the areA transcript in response to intracellular ammonium and glutamine (7, 41). In A. nidulans, AreA accumulates in the nucleus during nitrogen starvation and is exported from the nucleus and complexes with NmrA when rich nitrogen sources are added to starving mycelia (78). In S. cerevisiae, the target of rapamycin (TOR) kinase cascade regulates the cellular response to the nutrient status of the cell. The TOR kinase is active under conditions of nitrogen sufficiency and is inactivated by nitrogen starvation conditions or by the addition of rapamycin. The transcriptional activators Gln3
and Gat1 (Nil1) are phosphorylated in a TOR-dependent manner under nitrogen-sufficient conditions and are restricted to the cytoplasm after complexing with the regulator Ure2. Nitrogen starvation or the addition of rapamycin inhibits TOR, resulting in the dephosphorylation of the transcriptional activators, their release from Ure2, and their translocation to the nucleus, where they activate their target genes (reviewed in references 13 and 60).

Recently, we have shown that the TOR protein kinase in *F. fujikuroi* plays a role in the regulation of genes involved in GA and bikaverin biosynthesis, ammonium transport, ribosome biogenesis, protein synthesis, and autophagy (76). In contrast to what is seen for the yeast model system *S. cerevisiae*, the inhibition of TOR by rapamycin resulted in only partial derepression of AreA-regulated nitrogen metabolite repression genes, which suggests that there must be additional factors which inactivate AreA under conditions of nitrogen availability (76).

One strategy to learn more about the role AreA may play as a global nitrogen regulator is to compare the expression profiles of the wild-type (WT) and areA mutant strains after growth in nitrogen-limited and -sufficient conditions. Microarrays are a powerful tool to investigate the genome-wide regulation of genes. The recent availability of genomic data from *Fusarium verticillioides*, a close relative of *F. fujikuroi* that is estimated to share ~93% nucleotide identity, provides an opportunity to explore gene expression changes in *F. fujikuroi* by a cross-species microarray analysis. Microarrays have already been successfully used for cross-species hybridization between different fungi. *S. cerevisiae* microarrays successfully identified differentially expressed genes of *Pichia pastoris* (63), *N. crassa* microarrays identified developmentally regulated genes and mating-type-dependent genes of *Sordaria macrospora* (46, 54), and finally, an *A. niger* macroarray identified growth-phenotype-related genes of *Aspergillus oryzae* (73).

The aim of this study was to discover a set of genes in *F. fujikuroi* which are significantly affected by nitrogen availability by cross-species hybridization using an oligonucleotide microarray derived from *F. verticillioides* expressed sequence tag (EST) data. By comparing the expression patterns of the WT and the areA mutant under conditions of nitrogen starvation and sufficiency, we were able to differentiate between AreA-dependent and non-AreA-dependent nitrogen-repressed and nitrogen-induced genes. The expression pattern of representative genes from each expression group was confirmed by Northern blot analysis and helped to provide an overview of the role AreA plays in nitrogen metabolite repression. Furthermore, we show that nitrogen-regulated genes can be differentiated into rapamycin (TOR)-dependent and -independent genes, confirming our previous suggestion (76) that TOR controls mainly AreA-independent genes (e.g., ribosome biogenesis and translation control genes) and only partially affects the expression of AreA target genes.

To further examine the role of NMR in nitrogen metabolism in *F. fujikuroi*, we compared the expressions of the newly identified, larger set of AreA target genes in the WT, nmr deletion, and nmr-overexpressing mutants and in areA deletion and areA-overexpressing strains. Although initial experiments did not indicate a significant effect of either deletion or overexpression of nmr on the expression of AreA target genes, we found that NMR has an inhibitory effect on AreA during early growth phases, as AreA target genes were upregulated in the areA mutant during the first 24 h of growth. We demonstrated by a yeast two-hybrid approach that AreA interacts with NMR, as has been shown previously for *N. crassa* and *A. nidulans* (35, 51), and that this interaction probably affects AreA activity mainly in early stages of growth.

### MATERIALS AND METHODS

#### Fungal strains and culture conditions.

Strain IM158289 (Commonwealth Mycological Institute, Kew, United Kingdom) is a GA-producing WT strain of *G. fujikuroi*, mating population C (anamorph, *F. fujikuroi*). The areA deletion (ΔareA-T19) and overexpression (glnA::areA-T19/66) strains were described before (81), as were the nmr deletion and overexpression strains, the Δnmr-T8 and glnA::areA:Δnmr-T1 strains (39).

For RNA isolation, the *F. fujikuroi* strains were first cultivated for 2 days in 500-ml Erlenmeyer flasks with 100 ml Darken medium (14) [but with 2.0 g/liter L-glutamine instead of (NH₄)₂SO₄] on a rotary shaker at 190 rpm at 28°C. One milliliter of this culture was then used to inoculate 100 ml of ICI medium containing 1.8 g/L glutamine/liter (23). Growth proceeded for 5 days, after which the mycelia were harvested, washed with deionized water, and added to fresh ICI medium without nitrogen for 4 h to induce starvation conditions. Then, glutamine was added to a final concentration of 9.2 g/liter to one half of the flasks, while the other flasks were kept under starvation conditions for a final 2 h of growth. For inhibition of the TOR kinase, rapamycin was added to a final concentration of 200 ng/ml after 1.5 h, with mycelia being harvested after an additional 30 min.

#### Bacterial strains and plasmids.

*Escherichia coli* strain Top10 F’ (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Vectors for the GAL4 yeast two-hybrid analysis were prepared as follows. Vector pAD-nmr, containing the 1.4-kb full-length *nmr* cDNA, was created by cloning the amplicon generated with primers nmr-AD-for and nmr-AD-rev into the SalI/XbaI-digested pAD-GAL4-2.1 vector (Stratagene, La Jolla, CA). Vector pBD-areA, containing the 2.9-kb full-length *areA* cDNA, was created by cloning the amplicon generated with primers areA-F3 and areA-RTR1 into the SrfI site of the pBD-GAL4 Cam vector. Vector pBD-areA-ZF was created by cloning a 0.92-kb amplicon generated with primers areA-ZF and areA-RTR1 into the SrfI site of the pBD-GAL4 Cam vector. The 0.92-kb sequence corresponds to amino acids 662 to 968 of AreA.

#### PCR and RT-PCR.

PCR mixtures contained 25 ng of template DNA, 50 ng of each primer, 0.2 mM deoxynucleoside triphosphates, and 1 U of Biotherm polymerase (Gencraft, Lüdinghausen, Germany). Reverse transcription-PCR (RT-PCR) was performed using the Superscript one-step RT-PCR with Platinum Taq kit (Invitrogen, Groningen, The Netherlands), 10 pM of oligo(dT) primer, and 1 µg of total RNA as the template.

#### Nucleic acid isolation and Northern blot analysis.

Plasmid DNA was extracted using the Qiagen Miniprep kit (Qiagen, Hilden, Germany) essentially as described by the manufacturer. Total *F. fujikuroi* RNA was isolated using the RNAgent total RNA isolation kit (Promega, Mannheim, Germany). Formaldehyde gel electrophoresis, transfer to nylon membranes (Hybond N+, GE Healthcare, Little Chalfont, United Kingdom), and subsequent hybridization were accomplished following the protocol of Sambrook et al. (62). 18S and 28S rRNA bands were visualized by illumination with UV light (λ = 254 nm) against the background of a silica thin-layer chromatography plate (Schleicher und Schuell, Dassel, Germany). PCR products using the primers pBS2 uni and pBS2 rev (Table 1) and templates derived from a plasmid cDNA library (75) or specifically created PCR products for genes not represented in this library were used as probes. The accession numbers of probe sequences are listed in Table 2.

#### Microarray hybridization and data analysis.

The development and initial characterization of an *F. verticillioides* Roche NimbleGen Systems, Inc. (Madison, WI) 24-mer oligonucleotide microarray comprising 16,500 probe sets have been described previously (R. Butcho and D. Brown, personal communication). Each probe set included up to 12 perfectly matching oligonucleotide probes and was designed based on an *F. verticillioides* EST library consisting of over 87,000 ESTs (5). Analysis of a recent annotation of *F. verticillioides* genome sequence data indicates that the microarray represents over 9,300 genes (4). The microarray data are available from the NCBI Gene Expression Omnibus (GEO) under the series accession number GSE10692.

*F. fujikuroi* mycelia were harvested from two individual cultures and RNA was extracted as described above. Microarray hybridization, data acquisition, and
TABLE 1. Oligonucleotides used in this study

| Primer name | Sequence |
|-------------|----------|
| area-F3     | CGTAAATTTGTATTTCTCCTGAGGACATCTGTGC |
| area-RTR1   | GTAAATTTGGCATTATGGTGCAGTGTGC |
| area-ZF     | CCACCAATATTACGCCGACGTGC |
| Asn-F2      | GTCACAGGGTGACTTCAACATCTG |
| Asn-R2      | GGAGAATCCGGAGGGGAGTCCAGGC |
| Nmr_AD_for  | CAGAGAGTGCAGCGCACTGCTATAGC |
| Nmr_AD_rev  | GCCAAGATTTGACCCGCTACTGAGT |
| pBS-rev2    | CGCTCTAGAACTAGTGGATC |
| pBS-un2     | CGACTCTACATAGGCAGCAATG |

* Enzyme restriction sites used for cloning are underlined.

Preliminary analysis of the microarray data with Acuity 4.0's self-organizing map function led to the identification of 1,241 probe sets that appeared to be differentially regulated either according to the nitrogen status or in the ΔareA mutant compared to the WT. Subsequent analysis of this subset with FiRe (22) identified 144 probe sets that differed significantly between the two experimental replicates and were not considered further. Genes for which expression was significantly up- or downregulated in response to nitrogen availability in both the WT and the ΔareA mutant were defined as AreA-independent nitrogen-regulated genes (Table 2, categories Ia and IIa), whereas genes for which expression was up- or downregulated by nitrogen in an AreA-independent manner were defined as AreA target genes (Table 2, categories Ib and Ic, and d). There were also genes which were differentially expressed in the ΔareA mutant compared to the WT, independent of nitrogen availability (categories III and IV).

Genes up- or downregulated in response to glutamine. We identified 300 probe sets representing 279 genes whose expression was upregulated (Table 2, category I; Fig. 1A) and 250 probe sets representing 223 genes whose expression was downregulated (Table 2, category II; Fig. 1B) by glutamine addition in both the WT and the areA mutant. Analysis of the biological function of the category I genes by use of the MIPS functional catalogue database (61) found that 58% (161 genes) are directly involved in de novo protein biosynthesis, whereas 36% (100 genes) play a role in ribosome biogenesis (predominantly encoding 40S and 60S ribosomal subunits) and 22% are involved indirectly in protein synthesis, e.g., genes coding for translation initiation and elongation factors (32 genes), general transcription (18 genes), RNA processing (5 genes), protein folding (4 genes), and protein targeting (2 genes) (Fig. 1A). Interestingly, we also identified two cross-pathway control (CPC) genes, cpc2 and cpc1. Cpc2 is a Gβ-like protein composed entirely of WD repeats first identified in N. crassa (42). The gene was shown to be expressed analogously to ribosome biogenesis genes (9, 82). Cpc1 is a bZIP transcription factor that acts as global regulator of amino acid biosynthesis genes (22). Beside these regulators, the induction of 22 amino acid biosynthesis genes, e.g., the argininosuccinate synthase gene arg1, of 6 nucleotide metabolism genes was observed (Table 2, category I).

Among the 223 genes downregulated in response to glutamine addition (Table 2, category II), 14% (31 genes) may play roles in carbohydrate metabolism and energy generation. For example, several genes for the basic generation of energy via glycolysis, the tricarboxylic acid cycle, and the pentose phosphate cycle were found, such as the genes coding for transketolases and transaldolases from the pentose phosphate cycle (17); malate dehydrogenase and an aconitate hydratase catalyzing the degradation of proline to glutamate (83); a choline oxidase, two xanthine oxidases responsible for the degradation of xanthine to urate (38, 65), and an ω-aminobutyrate transaminase catalyzing the formation of succinate semialdehyde and glutamate (57).
| Category                      | Gene Name | F. fujikuroi gene accession no. | F. verticillioides TGI ID | Best meaningful BLASTX hit of F. verticillioides TGI ID sequence | E value | Fold change^b | Expression pattern(s)^c |
|------------------------------|-----------|--------------------------------|---------------------------|---------------------------------------------------------------|---------|----------------|--------------------------|
| I: nitrogen-induced genes     |           |                                |                           |                                                               |         |                |                          |
| IA: AreA independent         | elf44*    | AM233708 TC26244               | Eukaryotic translation initiation factor (Neurospora crassa) | 0.0               | 4.32 | 3.67 | 1.35 | 1.13           |
|                              | rlp24*    | AM944647 TC31324               | 60 S ribosomal protein 24 (Batrosporia fuckeliana) | 4E-63             | 9.92 | 10.97 | 1.26 | 1.39           |
|                              | 40S S13*  | AM944648 FVHBH34TH             | 40 S ribosomal protein S13 (Neosartorya fischeri) | 5E-74             | 2.11 | 2.37 | 1.13 | 1.27           |
|                              | cpc2*     | AM944649 TC26288               | G-protein beta like WD repeat protein (Fusarium oxysporum) | 0.0               | 2.93 | 2.12 | 1.42 | 1.03           |
| IB: AreA dependent           | cpc1*     | AM944693 TC29814               | CPC transcription factor (Fusarium fujikuroi) | 5E-33             | 2.71 | 0.49 | 4.74 | 0.86           |
|                              | aux2      | AM944650 TC20756               | Asparagine synthetase (B. fuckeliana) | 0.0               | 2.57 | 0.16 | 11.36 | 0.56           |
|                              | arg1*     | AM944651 FVID122TH             | 40 S ribosomal protein S13 (Neosartorya fischeri) | 2E-106            | 4.89 | 0.36 | 6.66 | 0.43           |
| II: nitrogen-repressed genes  |           |                                |                           |                                                               |         |                |                          |
| IA: AreA independent         | ubi4*     | AM944654 TC26164               | Ubiquitin (Pichia guilliermondii) | 1E-147            | 0.13 | 0.17 | 1.05 | 1.39           |
|                              | mdbh1*    | AM944655 TC26209               | Malate dehydrogenase, NAD dependent (C. globosum) | 3E-103            | 0.15 | 0.32 | 0.69 | 1.50           |
|                              | mdbh*     | AM944655 TC26207               | Malate dehydrogenase, NAD dependent (C. globosum) | 7E-145            | 0.22 | 0.38 | 0.71 | 1.23           |
|                              | gad1*     | AM944656 TC29763               | Glutamic acid decarboxylase (N. crassa) | 2E-49             | 0.23 | 0.38 | 0.61 | 1.02           |
|                              | prc1      | AM944657 TC30180               | Pyruvate carboxylase (Aspergillus fumigatus) | 0.0               | 0.28 | 0.37 | 1.01 | 1.36           |
|                              | pep4      | AM944658 FVNBM36TH             | Serine endopeptidase (Hypocrea lutea) | 9E-101            | 0.36 | 0.39 | 0.87 | 0.86           |
|                              | sod*      | AM944659 TC26457               | Vacular protease A (Trichoderma atroviride) | 2E-174            | 0.47 | 0.41 | 1.11 | 0.98           |
| IB: partially AreA dependent | fpr1*     | AM233732 TC30045               | Rapamycin binding protein (F. fujikuroi) | 4E-57             | 0.24 | 0.72 | 0.65 | 1.95           |
|                              | 14-3-1    | AM233739 TC20195               | 14-3-1-like protein (Matrinella anysoplae) | 9E-111            | 0.23 | 0.5  | 0.69 | 1.49           |
|                              | zwf1      | AM944661 TC26263               | Glucose-6-phosphate 1-dehydrogenase (Gibberella zeae) | 0.0               | 0.27 | 0.64 | 0.74 | 1.68           |
|                              | aco1      | AM944662 TC26306               | Aconitate hydratase, mitochondrial precursor (Coccidioides immitis) | 0.0               | 0.40 | 0.72 | 0.68 | 1.22           |
|                              | codA      | AM944663 TC2948                 | Fructose-1,6-bisphosphatase (B. fuckeliana) | 3E-158            | 0.41 | 0.61 | 0.73 | 1.09           |
|                              | uqa1      | AM944664 TC26513               | Choline oxidase (A. fumigatus) | 0.0               | 0.36 | 0.64 | 0.77 | 1.37           |
|                              | Not cloned | AM944663 TC29266             | 4-Aminobutyrate aminotransferase (N. crassa) | 0.0               | 0.32 | 0.57 | 0.55 | 0.97           |
|                              | Not cloned | AM944664 TC31031             | Vacular protein sorting-associated protein VPS35 (Aspergillus terreus) | 3E-143            | 0.32 | 0.62 | 0.80 | 1.57           |
|                              | proc1     | AM944665 TC2948                 | Carbonylpyridoxal Y (Aspergillus niger) | 0.0               | 0.27 | 0.51 | 0.56 | 1.05           |
|                              | ygr7      | AM944666 TC25761               | Ral-related protein Rab7 (G. zeae) | 9E-105            | 0.39 | 0.79 | 0.71 | 1.43           |
|                              | rho1      | AM944667 TC29873               | Small GTPase-binding protein (F. oxysporum) | 2E-107            | 0.33 | 0.53 | 0.67 | 1.09           |
|                              | ypt12     | AM944668 TC30142               | Ras GTase Rab11 (N. fischeri) | 3E-96             | 0.36 | 0.62 | 0.86 | 1.49           |
|                              | snf4      | AM944669 TC31055               | Snf1 protein kinase complex subunit Snf4 (N. fischeri) | 4E-109            | 0.30 | 0.51 | 1.16 | 1.98           |
|                              | pkal*     | AM94498 TC32913                | Cycle AMP-Ser/Thr protein kinase PKA1 (F. verticillioides) | 1E-124            | 0.36 | 0.75 | 0.62 | 1.28           |
| Gene     | Accession | Type                        | Description                                                                 |
|----------|-----------|-----------------------------|-----------------------------------------------------------------------------|
| cpr      | CAE09055  | TC29748                     | Cytochrome P450 oxidoreductase (F. fujikuroi)                               |
| gldB*    | AM944670  | TC30762                     | Glycerol dehydrogenase (A. clavatus)                                       |
| mepA*    | AM16827   | TC30926                     | Ammonium permease (F. fujikuroi)                                            |
| mfl1*    | AM944671  | FVNA1997TH                  | Peptide transporter (Schizosaccharomyces)                                   |
| bkd*     | AM22867   | TC26531                     | O-Methyltransferase (F. fujikuroi)                                          |
| aap*     | AM944672  | TC26191                     | General amino acid permease (A. terreus)                                   |
| aap*     | AM944672  | TC26192                     | Amino acid permease Inda1 (T. atroviride)                                  |
| xdh2*    | AM944673  | TC28961                     | Xanthine dehydrogenase (N. crassa)                                         |
| mepB*    | AM16827   | TC30329                     | Ammonium permease (F. fujikuroi)                                            |
| gldB*    | AM944676  | TC29822                     | Glycerol dehydrogenase (A. clavatus)                                       |
| tal1     | AM944678  | TC26177                     | Transaldolase (Magnaporthe grisea)                                          |
| cm*      | AM23373   | TC30060                     | Calmodulin (G. zeae)                                                       |
| cmd*     | AM23374   | TC26924                     | Calmodulin transporter (S. sclerotiorum, Colletotrichum gloeosporioides)   |
| hsp70*   | AM944680  | TC26068                     | Heat shock protein 70-1 (Nicotiana tabacum)                                |
| meaB*    | AM944694  | TC29831                     | bZIP transcription factor (A. nidulans)                                     |
| sde1     | AM944681  | TC29810                     | Acyl coenzyme A desaturase (ApHymenaea capsulatus)                        |
| gxc2*    | AM944682  | TC32372                     | Glucan synthase catalytic subunit (F. oxysporum)                           |
| gluc1    | AM944683  | FVOS93TV                    | Endo-1,3(4)-beta-glucanase (N. fischeri)                                   |
| msf1     | AM944684  | FVGRS54TH                   | Transporter of the major facilitator superfamily (N. fischeri)            |
| cfas1    | AM944685  | TC30680                     | Cyclopropene-fatty-acyl-phospholipid synthase (A. fumigatus)               |
| idiA*    | AM233710  | TC30766                     | Autophagy bZIP transcription factor (Podpora anserina)                     |
| fxs2     | AM944666  | TC27298                     | Phenylnsalicyl-RNA synthetase alpha chain (S. sclerotiorum)                |
| mfb*     | AJ315471  | TC30348                     | Multigene bridging factor (F. fujikuroi)                                   |
| gdi*     | AM946805  | TC30098                     | NADP-specific glutamate dehydrogenase (F. fujikuroi)                       |
| tvt1*    | AM944687  | FVGA28TH                    | Branched-chain amino acid transferase (S. sclerotiorum)                    |
| Category | F. fujikuroi gene | F. fujikuroi gene accession no. | F. verticillioides gene | Best meaningful BLASTX hit of F. verticillioides TGI ID sequence | E value | Gln/no N (WT) | Gln/no N (ΔareA mutant) | ΔareA mutant/WT (no N) | ΔareA mutant/WT (Gln) | Expression pattern(s) |
|----------|-----------------|-------------------------------|------------------------|-------------------------------------------------|---------|---------------|------------------------|------------------------|------------------------|----------------------|
|          |                 |                               |                        |                                                 |         |               |                        |                        |                        | **WT**               |
|          |                 |                               |                        |                                                 |         |               |                        |                        |                        | **ΔareA mutant**      |
|          |                 |                               |                        |                                                 |         |               |                        |                        |                        |                      |
| **twt1** | AM944687        | TC29143                       | Branched-chain amino acid transferase (B. fuckeliana) | 2E-93    | 1.48          | 0.17                  | 4.84                  | 0.53                  |                        |                      |
| Not cloned | AM944688       | TC32276                       | Glnamyl-riRNA synthetase (A. terreus) | 7E-117    | 1.18          | 0.42                  | 2.72                  | 0.96                  |                        |                      |
| Not cloned | AM944689       | TC26669                       | Cytoplasmic asparaginyl-riRNA synthetase (A. fumigatus) | 1E-176    | 1.12          | 0.42                  | 2.65                  | 1.00                  |                        |                      |
| Not cloned | AM944690       | TC30345                       | Methionyl-riRNA synthetase (A. terreus) | 0.0       | 1.27          | 0.38                  | 3.87                  | 1.18                  |                        |                      |
| Not cloned | AM944691       | TC26672                       | Valyl-riRNA synthetase (N. fischeri) | 1E-175    | 1.18          | 0.34                  | 4.31                  | 1.26                  |                        |                      |
| Not cloned | AM944692       | TC29143                       | Prolyl-riRNA synthetase (A. clavatus) | 0.0       | 0.57          | 0.11                  | 6.21                  | 1.17                  |                        |                      |
| Not cloned | AM944693       | TC32276                       | Isoleucyl-riRNA synthetase (A. terreus) | 0.0       | 1.17          | 0.33                  | 4.22                  | 1.18                  |                        |                      |
| Not cloned | AM944694       | TC26669                       | Isoleucyl-riRNA synthetase (A. terreus) | 3E-93     | 0.98          | 0.33                  | 3.42                  | 1.15                  |                        |                      |
| Not cloned | AM944695       | TC29147                       | Aspartate-riRNA ligase (S. sclerotiorum) | 6E-72     | 0.88          | 0.21                  | 7.08                  | 1.70                  |                        |                      |
| Not cloned | AM944696       | TC30884                       | DEAD/DEAH box helicase (A. fumigatus) | 0.0       | 1.09          | 0.36                  | 4.02                  | 1.34                  |                        |                      |
| Not cloned | AM944697       | TC26672                       | Isocitrate lyase (F. fujikuroi) | 3E-129    | 0.86          | 0.12                  | 8.11                  | 1.11                  |                        |                      |
| Not cloned | AM944698       | TC29143                       | Mitochondrial peroxiredoxin (S. sclerotiorum) | 7E-106    | 0.47          | 2.29                  | 3.29                  | 15.34                 |                        |                      |
| Not cloned | AM944699       | TC29143                       | Malate synthase, glyoxysomal (G. zeae) | 5E-17     | 0.06          | 0.06                  | 2.76                  | 2.65                  |                        |                      |

* Listed are genes representative for each category. ΔareA-dependent genes are those showing expression patterns that differ significantly between the ΔareA mutant and the WT. *, Differentially regulated genes tested in Northern analyses.

b “Fold change” represents the expression value of one condition (e.g., Gln) divided by the value of another condition (e.g., no N). Genes with such changes of ≤0.5 or of ≥2 are regarded as differentially regulated under the specific conditions.

* , upregulated genes; ** , downregulated genes; --- , genes without significant expression changes.
Beside genes for the use of alternative nitrogen sources, we found genes with possible roles in the transport of external nitrogen sources or the degradation of nonessential proteins in the vacuole. Among the 14 genes (6%) involved in transport were those encoding all three ammonium transporters described for *F. fujikuroi*, namely, MepA, MepB, and MepC (74), an amino acid permease (Aap8) (B. Schöning and B. Tudzynski, unpublished data), a putative polypeptide transporter (Mtd1), and putative transporters for the secondary nitrogen sources allantoate and \( /H9253\)-aminobutyrate. We also noted genes with possible roles in protein degradation, such as proteasome component genes, genes involved in protein ubiquitination (e.g., that for the polyubiquitin Ubi4), and genes for proteases like the fungal vacuolar protease A (Pep4), the endopeptidase B (Prb1), and carboxypeptidase Y (Prc1), putatively involved in posttranslational modification and activation of multiple vacuolar proteases (32). In addition, we identified 12 genes probably involved in protein localization, e.g., a homologue of *VPS15*, which plays a role in vacuolar protein sorting in *S. cerevisiae* (68).

Another group of glutamine-repressed genes (7%) encode signal transduction proteins, including components of the TOR signaling pathway (Fpr1, 14-3-3), the catalytic and regulatory subunits of the cyclic AMP-dependent protein kinase (Pka1), the signaling pathway of alternative carbon source utilization (Snf4 and CreA), and small GTPases (Rho1, Rab7, Rab11), as well as calmodulin (Cmd1) and another calcium binding protein. In addition, nine stress-responsive genes were identified, such as heat shock protein-encoding genes (e.g., the *hsp70* gene) and the gene encoding the superoxide dismutase, *sod*. Finally, the following two glutamine-repressed genes are involved in secondary metabolism: *cpr*, encoding the NADPH-cytochrome P450 oxidoreductase donating electrons to the P450 enzymes from the GA biosynthetic pathway (37), and *bik3*, encoding the bikaverin-specific \(O\)-methyltransferase (Wiemann and Tudzynski, unpublished). A total of 33% share homology to genes with no known function.

The differential expression of several of these genes was verified in Northern blot experiments using probes derived from cDNA clones from an *F. fujikuroi* EST library (75) or PCR fragments generated from *F. fujikuroi* genomic DNA with primers derived from the *F. verticillioides* EST sequences (Fig. 2A and B; Table 2, categories I and II, respectively). The expression of all tested genes was consistent with the array data.

The role of *AreA* in nitrogen metabolite repression. AreA is known as a positively acting transcription factor that functions under nitrogen-limiting conditions to derepress a set of target genes involved mainly in the transport and metabolism of alternative nitrogen sources (41). However, the extent of AreA target genes among the nitrogen-repressed genes in filamentous fungi has not yet been studied in a genome-wide manner. Our microarray experiments revealed 225 genes showing at least a twofold reduction of expression by glutamine, of which we found only 62 genes (Fig. 3A) to be “classic” AreA target genes whose expression depends on both nitrogen sufficiency and the presence of AreA (category IIc). Thus, the expression

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\begin{align*}
\text{FIG. 1. Functional distribution of nitrogen-regulated genes.} & (A) \text{Pie chart representing the functions of genes upregulated by nitrogen addition.} \\
& (B) \text{Pie chart representing the functions of genes repressed by nitrogen addition. The division of genes into several functional categories was} \\
& \text{accomplished following the MIPS functional database catalogue (http://mips.gsf.de/proj/functDB).} \\
& \text{met., metabolism.}
\end{align*}
\]

\[
\begin{align*}
\text{FIG. 2. Genes with expression levels affected by nitrogen addition.} & (A) \text{Northern blot experiments showing genes upregulated by glutamine addition.} \\
& (B) \text{Northern blot experiments showing genes downregulated by glutamine addition. The WT was grown for 5 days in} \\
& \text{synthetic ICI medium containing 1.8 g/liter glutamine and then shifted} \\
& \text{into ICI medium without nitrogen (no N). After 4 h, glutamine was} \\
& \text{added to one half of the flasks to a final concentration of 9.2 g/liter} \\
& \text{(Gln), and the mycelia were harvested after 2 h. Probes used for} \\
& \text{hybridization are listed in Table 2. 28S and 18S rRNA was used as the} \\
& \text{loading control.}
\end{align*}
\]
of a larger group of nitrogen-repressed genes (161 genes) is not affected by the major regulator AreA (category IIa).

Prominent among the AreA-dependent nitrogen-repressed genes are those related to the transport of nitrogen sources, nitrogen metabolism, carbohydrate and energy metabolism, and secondary metabolism. Potential AreA-dependent transporters include the three ammonium permeases mentioned above and several amino acid and peptide transporters as well as permeases of other nitrogen compounds (Table 2, category IIc). Four genes whose products may be involved in nitrogen and carbon metabolism include the glutamine synthetase (GS)-encoding gene glnA (75); a gene coding for the xanthine dehydrogenase, which is responsible for the utilization of xanthine as an alternative nitrogen source (38, 65); and a glycerol dehydrogenase and a phosphatidyl transferase, probably involved in carbohydrate metabolism. Finally, this group also contained genes likely involved in secondary metabolite production. The expression pattern of several typical AreA target genes are shown in Fig. 4C.

The most surprising category of AreA-dependent genes were those that were nitrogen independent (Fig. 3A; Table 2, category III), which may suggest new roles for this transcription factor. The expression of 32 genes decreased in the areA mutant compared to the WT independent of the nitrogen status of the cell. Members of this group include a major facilitator-type transporter, two glucanases, a glucan synthase, a glycerol kinase, a stearyl coenzyme A desaturase, and a cyclopropane–fatty-acyl-phospholipid synthase. The most interesting member of this group was meaB, shown previously to be a negative regulator of AreA target genes in A. nidulans (55, 84). This observation suggests that MeaB might have additional roles outside the nitrogen regulation network (Table 2, category III). Although meaB expression level in the WT is almost unaffected by the nitrogen status of the cell, transcript sizes vary considerably (Fig. 4C). We are currently exploring what role this AreA-dependent change in transcript size may have in the function of meaB.

As mentioned above, the majority of nitrogen-repressed genes (161 of 223 genes) are AreA independent (Table 2, category IIa). These completely AreA-independent functional categories of genes include those with putative functions in protein degradation, modification, and localization, stress response, vitamin metabolism, and the utilization of alternative carbon sources and homeostasis (Fig. 3B).

Deletion of areA leads to the upregulation of amino acid biosynthetic genes. We also identified a set of 108 genes whose expression is upregulated by the deletion of areA under conditions of either nitrogen starvation or nitrogen abundance (Table 2, category IV). To this group belong amino acid biosynthetic genes, e.g., asn2, encoding asparagine synthetase, argl, encoding argininosuccinate synthase, hydroxynitrile lyase, which is known to link C and N metabolisms; the gene mfh (a homologue of the yeast multiprotein bridging factor Mbf1 [70]), and also the pyridoxine biosynthesis gene pdx1, a homologue of snz1 from S. cerevisiae, which has been reported to be under CPC in N. crassa, S. cerevisiae, and Candida albicans (77). In addition, this group also includes 11 tRNA synthetase-encoding genes for the synthesis of isoleucyl-, methionyl-, prolyl-, arginyl-, glutaminyl-, phenylalanyl-, threonyl-, aspartyl-, valyl-, and asparagyl-tRNAs and one gene encoding a DEAD/DEAH box helicase from the group of protein synthesis genes. Furthermore, 12 genes encoding enzymes involved in carbohydrate and energy metabolism are also up-regulated in the areA mutant; these genes include those for malate synthase and the glyoxylate cycle enzyme isocitrate lyase, which is known to link C and N metabolisms; the gene encoding the homologue of the Podospora anserina autophagy-specific transcription factor IDH4 (15); and the gdhA gene, encoding the NADP+-dependent glutamate dehydrogenase. The S. cerevisiae homologue gdh1 has also been reported to be under Gcn4 control (59). The expression pattern of an arbitrarily chosen subset of genes was confirmed by Northern blot analyses (Fig. 4B and data not shown).

Response of AreA target genes to rapamycin. Recently, we have shown that the F. fujikuroi TOR kinase is involved in the regulation of genes responding to changes in the nitrogen
status of the cell, though to a much lesser extent than in yeast. Among the genes partially derepressed by the addition of rapamycin were some AreA target genes (e.g., GA biosynthetic genes and the GS-encoding gene glnA). In contrast, the expression levels of several genes involved in processes of de novo protein synthesis, such as ribosome biogenesis and translation initiation, were significantly reduced by rapamycin (76). In order to further explore the role of the TOR kinase in F. fujikuroi, we transferred mycelia grown under standard conditions for 3 days into medium with or without rapamycin (200 ng/ml) and compared the expression levels of genes from all four categories (Table 2) under both conditions (see Fig. S2 in the supplemental material).

We found that both nitrogen-induced (category I) and repressed (category II) genes responded to rapamycin addition. Among the newly identified genes which are partially regulated
by rapamycin are some of the typical AreA target genes, e.g., the xanthine dehydrogenase-encoding gene xdh1 and the peptide transporter-encoding gene mitd1, but also several nitrogen-regulated AreA-independent genes, e.g., the glutamate decarboxylase gene gad1. The most pronounced effect observed for rapamycin was for the set of genes which are involved in protein synthesis and which were shown to be induced by glutamine as the nitrogen source with (+) or without (−) potassium chloride (10 mM). Pictures were taken after 2 (medium without chloride) or 4 (chlorate medium) days of incubation at 28°C. OE::areA, areA overexpression mutant (areA transcription regulated by the glnA promoter); OE::nmr, nmr overexpression mutant (nmr regulated by the glnA promoter).

**The role of NMR in AreA-dependent nitrogen regulation.** In *A. nidulans* and *N. crassa*, the deletion of *nmrA* and *nmr-1*, respectively, led to a significant derepression of AreA target genes (2, 30). In *F. fujikuroi*, the deletion of the *nmr* gene resulted in higher sensitivity against chlorate (39) and higher resistance toward the TOR inhibitor rapamycin (76), suggesting that NMR is involved in nitrogen regulation in some way. Likewise, the ability of the *F. fujikuroi* nmr gene to complement an *N. crassa* nmr-1 mutant indicates that the protein shares significant functional similarity to the *N. crassa* Nmr1 protein. However, the AreA-targeted genes involved in GA biosynthesis were not derepressed in the *nmr* mutant (39).

To further analyze the relationship between AreA and NMR in *F. fujikuroi*, we compared the growth behavior of the WT with that of the *areA or nmr* deletion mutants and strains overexpressing *areA or nmr* on plates with glutamine as the preferred nitrogen source and with or without chlorate. Under nitrogen starvation conditions, AreA activates the expression of the nitrate reductase-encoding gene *niaD* (39). This enzyme catalyzes not only the reduction of nitrate to nitrite but also that of chlorate to chlorite, a toxic and growth-inhibiting metabolite. In mutants with a defect in nitrogen metabolite repression, the toxic effect of chlorate should be stronger, whereas mutants with a defect in AreA-mediated target gene activation should be more chlorate resistant than the WT. We found that the *areA* overexpression mutant, the *glnAprom::areA* mutant, shows a strong growth inhibition similar to that of the *nmr* deletion mutant, indicating a derepression of the AreA target gene, *niaD*. The overexpression of *nmr* (*glnAprom::nmr* strain) and the deletion of *areA* resulted in higher chlorate resistance, presumably caused by the repression of the nitrate reductase gene (Fig. 5). These results indicate that nitrogen metabolite repression could indeed be regulated by balanced expression levels of the two antagonists AreA and NMR, as proposed for *A. nidulans* (84).

**AreA interacts with NMR.** NmrA and NMR1 have been shown to interact with AreA and NIT2, respectively, in their C-terminal regions, which include zinc finger (ZF) DNA binding domains (BD) (35, 51). As the role of NMR in *F. fujikuroi* remained ambiguous, we wanted to know whether NMR interacts with AreA as had been shown for *A. nidulans* and *N. crassa*. To achieve this, a GAL4 yeast two-hybrid experiment was designed using the HybriZAP two-hybrid system (Stratagene). Initially, we fused all of AreA to the DNA BD of the *S. cerevisiae* GAL4 transcription factor, creating vector pBD-AreA, while NMR was fused to the activation domain (AD), creating vector pAD-NMR. However, we found that the expression of the pBD-AreA construct alone led to the activation of the *his3* and *lacZ* reporter genes, a problem occasionally encountered in yeast two-hybrid assays using the transcriptional activation of genes as the reporter (Fig. 6, BD-AreA/AD). Therefore, we fused the AreA C-terminal fragment containing the ZF motif (amino acids 662 to 968) to the binding domain, creating vector pBD-AreA-ZF. On medium without histidine, yeast transformants containing pBD-AreA-ZF and pAD-NMR showed growth significantly better than that of

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**FIG. 5.** Nitrate reductase activity is affected in *areA* and *nmr* mutant strains. All strains were grown in media containing 9.2 g/liter l-glutamine as the nitrogen source with (+) or without (−) potassium chloride (10 mM). Pictures were taken after 2 (medium without chloride) or 4 (chlorate medium) days of incubation at 28°C. OE::areA, areA overexpression mutant (areA transcription regulated by the glnA promoter); OE::nmr, nmr overexpression mutant (nmr regulated by the glnA promoter).

**FIG. 6.** Yeast two-hybrid experiment showing that NMR interacts with the C terminus of AreA. Transformants were dropped in 10-fold dilution steps as indicated on SD medium without tryptophan and leucine (SD −W −L) and on SD without tryptophan, leucine, and histidine and with the addition of 50 mM 3-amino triazole (SD −W −L −H + 3-AT). The decreasing concentration is indicated by a triangle. The positive control was transformation with pBD-WT and pAD-WT (Stratagene). AD, transformation with pAD-GAL4-2.1 (Stratagene); AD-Nmr, transformation with pAD-Nmr, containing the full-length *nmr* cDNA; BD, transformation with pBD-GAL4 Cam (Stratagene); BD-AreA, transformation with pBD-AreA, containing the full-length *areA* cDNA; BD-AreA-ZF, transformation with pBD-AreA-ZF, containing the C-terminal ZF domain of AreA.
colonies transformed with the same plasmid and the empty AD vector (BD-AreA-ZF/AD) (Fig. 6). This observation lends significant support to the hypothesis that the F. fujikuroi NMR interacts with the C-terminal region of AreA.

**Expression of nitrogen-regulated genes in nmr deletion and overexpression strains.** In order to further explore the role of NMR, we examined the impact of nmr deletion or overexpression on the expression of a range of AreA target genes identified in this study. We previously found that neither the deletion of nmr nor its overexpression, using the strong glnA promoter, affected the nitrogen repression of GA production (39). This result was surprising, as NMR has been shown to play a critical role in both A. nidulans and N. crassa in the regulation of pathways subject to nitrogen repression.

The WT, ΔareA, glnA_{prom::areA} (areA overexpression [OE::areA]), Δnmr, and glnA_{prom::nmr} (OE::nmr) strains were cultivated as described in Materials and Methods, and the washed mycelia were transferred into media without nitrogen, with glutamine, or with nitrate. Nitrate (10 mM) was chosen as the alternative nitrogen source, as nitrate can be used only by strains with an active AreA due to the AreA-dependent expression of the niaD gene under starvation conditions (81).

We observed almost no effect of nmr deletion or overexpression on the expression of the secondary metabolism genes bik3 and cps/ks or on that of the gene cpc1, which is responsible for the regulation of amino acid biosynthesis genes. However, we did find that the transcript levels of glnA and meaB, both encoding proteins with a likely regulatory role in nitrogen metabolism, were significantly increased in the Δnmr mutant incubated with glutamine (Fig. 7).

The niaD gene, encoding the nitrate reductase, is slightly upregulated in the Δnmr and downregulated in the OE::nmr mutant with nitrate, while the niaD expression levels are almost identical under starvation conditions (Fig. 7). Interestingly, the expression of niaD depends on an active AreA but does not need nitrate as an inducing substrate, in contrast to what is seen for other fungi (Fig. 7).

To determine if there is any effect of the nmr mutation on AreA target gene expression at an early growth stage, we performed a time course experiment. For this, the WT and mutant strains were cultivated in ICI medium with glutamine (0.9 g/liter), and the cultures were harvested after 24 and 48 h of growth instead of after 5 days. Northern analysis revealed that several AreA target genes (e.g., the GA biosynthetic genes ggs2 and cps/ks and the nitrogen metabolism genes mtd1, mepB, and glnA, encoding a peptide transporter, an ammonium transporter, and the GS, respectively) were already expressed after 24 h of incubation in the nmr mutant when nitrogen was still available (Fig. 8). After 48 h, these differences in expression levels were no longer evident, indicating that nitrogen sources were depleted and nitrogen repression was relieved.
FIG. 8. The partial derepression of AreA target genes in the Δmrp mutant is time dependent. areA and mrp mutant strains (Fig. 5) were grown for 24 h and 48 h, respectively, in synthetic ICI medium containing 0.9 g/liter l-glutamine as the nitrogen source. Probes used for hybridization are listed in Table 2, except the gene coding for the geranylgeranyldiphosphate synthase, ggs2 (CAA75568), and the gene coding for the bifunctional ent-copalyl diphosphate/ent-kaurene synthase, cps/ks (Q9UVY5).

DISCUSSION

In this study, we performed a genome-wide search for F. fujikuroi genes responding to changes in nitrogen availability by use of an F. verticillioides microarray. The use of this heterologous system was very successful, as we observed significant expression changes for over 1,000 probe sets. The nitrogen source we focused on was glutamine, because of its strong repressing effect on nitrogen-regulated genes in F. fujikuroi (39, 76). In addition, glutamine is the only nitrogen source that allows comparable growth rates for the WT and the ΔareA mutant (see also Fig. 5), indicating that the uptake of glutamine by either the general amino acid permease or the glutamine-specific permease is not affected by the deletion of areA. In contrast to the A. nidulans ΔareA mutant, the F. fujikuroi ΔareA mutant is not able to grow on ammonium due the strict dependency of all three ammonium permease genes upon AreA (74).

Using this approach, we were able to delineate subsets of genes that were up- or downregulated in response to glutamine as a preferential nitrogen source or in the ΔareA mutant compared with what was seen for the WT. The differential expression of a representative subset of the genes has been confirmed by Northern blot analysis under the conditions used for the arrays.

Response of the cell to nitrogen-abundant conditions. Predominant among the group of genes upregulated in response to the addition of glutamine were genes involved in de novo protein synthesis: 61% of upregulated genes are involved in translation, ribosome biogenesis, amino acid and nucleotide biosynthesis, general transcription, RNA processing, and protein folding and targeting (Fig. 1A). Similar results were obtained for S. cerevisiae, where 67% of the genes highly expressed in response to nitrogen availability are also involved in amino acid and nucleotide metabolism, aminoacyl-tRNA synthetases among others (reviewed in reference 33). In N. crassa, 16 out of 20 tRNA synthetases were identified as Cpc1 target genes by a microarray approach (77). A similar set of genes was also identified in our studies under glutamine sufficiency conditions and also in the ΔareA mutant, suggesting that an excess of a single amino acid also leads to the activation of the CPC network and that this network is repressed by AreA in F. fujikuroi.

An external amino acid imbalance in S. cerevisiae has been shown to upregulate a homologous group of genes regulated primarily by the functional homologue of Cpc1, the transcription factor Gen4 (44). In contrast to what we found, glutamine did not induce the expression of Gen4 target genes. A recent microarray study using 20 different nitrogen sources revealed that only growth on leucine, isoleucine, methionine, threonine, tyrosine, or tryptophan as a sole nitrogen source led to the upregulation of the Gen4 target genes in yeast, whereas the expression of these genes is unaffected during growth on glutamine as well as on asparagine, serine, ammonium, alanine, arginine, and glutamate as nitrogen sources (24). In addition, Gen4 was identified in this study as a nitrogen catabolite repression (NCR) target, with its expression decreased under nitrogen-abundant conditions. A putative cross talk between CPC and NCR in S. cerevisiae remains ambiguous, although there are indications for a role for Gen4 in NCR by repressing Gln3 and for the repression of Gen4 translation under nitrogen starvation conditions (25, 67). This is in clear contradiction to our finding that cpc1 is upregulated by glutamine addition and in the areA mutant (Fig. 4B). For filamentous fungi, nothing is known about the coordination of nitrogen metabolite repression and CPC. The increase in cpc1 expression could be explained by the fact that the addition of glutamine as the sole nitrogen source necessitates the synthesis of the other amino acids required for protein synthesis during cell growth. The unexpected increase in cpc1 expression in the F. fujikuroi ΔareA mutant might be due to imbalances in the amino acid pool of the cell, as the downregulation of AreA target genes indirectly involved in amino acid metabolism could lead to a lack of single amino acids. The CPC in F. fujikuroi and its coherence with nitrogen metabolite repression are under investigation at the moment (Schöning and Tudzynski, unpublished).

Response of the cell to nitrogen starvation conditions. The biological functions of genes downregulated by glutamine addition are much more diverse than those of the upregulated genes (Fig. 1B). The most prominent subset of the 223 downregulated genes have functions in carbohydrate metabolism.
and energy generation (e.g., the pentose phosphate cycle, glycolysis, and the tricarboxylic acid cycle). For example, the expression of \textit{gdhA}, encoding the NADP⁺-depending glutamate dehydrogenase, is 2.5-fold reduced in response to glutamine. This enzyme plays a key role in providing cells with glutamate. Other important groups include genes involved in protein degradation, modification, and localization (14%). Under conditions of nitrogen starvation, an important internal nitrogen resource is the bulk turnover of nonessential proteins in the vacuole and their subsequent degradation by vacuolar proteases (71). Accordingly, we identified homologues of the yeast proteases Pep4, Prb1, and Pcr1 to be upregulated under conditions of nitrogen starvation.

Another protein degradation pathway is the turnover of proteins in the 26S proteasome, located in the nucleus and in the cytosol. It is elicited by environmental conditions like nutrient limitation, heat stress, and exposure to heavy metals (31) and is involved in the controlled degradation of key proteins of cell cycle control, signaling pathways, and general metabolism, where transcriptional regulators are degraded by the 26S proteasome (27). Various components of the ubiquitin/26S proteasome pathway, such as the polyubiquitin Ubi4, ubiquitin-conjugating and -activating enzymes, and catalytic and regulatory proteasome components, were shown to be upregulated under nitrogen starvation conditions in our study.

Nine genes encode proteins probably involved in stress response, like a \textit{hsp70} homologous gene and a gene coding for a manganese superoxide dismutase, \textit{sod}. Recently, an interesting link between nitrogen regulation and regulation of the reactive oxygen species (ROS) scavenger machinery has been uncovered for the strawberry pathogen 	extit{Colletotrichum acutatum}. Both ROS and nitrogen deficiency play important roles in the initial infection process. Consequently, a coordinated increase of proteins involved in nitrogen transport and utilization and protein degradation on the one hand and the generation and elimination of ROS on the other hand has been found by proteome analysis (29). This could also be the case for the rice pathogen \textit{F. fujikuroi}.

The group of transporter-encoding genes represents 6% of the genes that were downregulated under glutamine-sufficient conditions. Permeases play an important role in the utilization of different nitrogen sources. In plant pathogenic fungi, several groups of this gene have been described as pathogenicity-related genes. The conditions on the plant surface are similar to those of nitrogen starvation, as the fungus has to penetrate and overcome plant defenses before getting access to the plant’s carbon and nitrogen resources (11, 66, 72). In addition to nitrogen permease genes, other genes have been shown to be highly induced at the beginning of a plant infection in different fungi. In \textit{F. oxydysporum}, the genes encoding the amino acid permease Gap1 and the peptide transporter Mtd1 are induced (16). In \textit{M. grisea}, genes encoding the vacuolar serine protease Spm1, an orthologue of \textit{S. cerevisiae} Prb1, and the neutral trehalase 1, Nth1, are induced (18, 19). Homologues of these genes were also upregulated by nitrogen starvation conditions in our study. For some but not all phytopathogenic fungi, a role of AreA homologues in plant-fungus interaction has been shown, e.g., for \textit{Colletotrichum lindemuthianum}, \textit{F. oxysporum}, and \textit{M. grisea} (16, 20, 52) but not for \textit{Cladosporium fulvum} (53). The precise nature of the role AreA may play in pathogenesis in \textit{F. fujikuroi} will require further study.

**AreA regulates only a subset of nitrogen-repressed genes.** One of the most striking findings was that AreA controls only about 20% of nitrogen-repressed genes (Fig. 3A). Most of these AreA target genes whose products belong to the functional groups of permeases, catalytic enzymes needed to degrade poor nitrogen sources and secondary metabolism (Fig. 3B). One reason for the relatively low number of “classic” AreA target genes that we observed might be that genes involved in the utilization and metabolization of alternative nitrogen sources need the inducing substrate in addition to an active AreA to be expressed. Thus, the \textit{prn} cluster genes of \textit{A. nidulans} involved in proline utilization may be induced only when no preferred carbon (glucose) or nitrogen (ammonium or glutamine) sources are present in the medium and when proline is available (58).

In contrast to these AreA target genes, the genes of other functional groups, e.g., those involved in protein modification, degradation, and localization, are not affected by the \textit{areA} deletion. In \textit{S. cerevisiae}, the vacuolar proteasine \textit{A} (Pep4), vacuolar endoproteinase \textit{B} (Prb1), vacuolar aminopeptidase \textit{I} (Lap4), and vacuolar carboxypeptidase \textit{S} (Cps1) proteases are controlled by the activators Gln3 and Nil1 in response to nitrogen starvation, whereas vacuolar carboxypeptidase \textit{Y} (Prc1) is not affected by the deletion of the GATA factors or the nitrogen status of the cell (10). Homologues of Prb1, Pep4, and Prc1 were also part of the group of nitrogen-repressed genes in our study, although the expression of all of these genes does not depend on the GATA factor AreA, in contrast to their Gln3 dependence in \textit{S. cerevisiae}.

Previous work has shown that the biosyntheses of the secondary metabolites GA and bikaverin are repressed by nitrogen in \textit{F. fujikuroi}. The expression pattern of one gene in our microarray study supported this work. We found that the bikaverin biosynthesis gene \textit{bik3}, encoding a putative \textit{O}-methyltransferase with homology to \textit{Afl} from \textit{A. flavus}, is repressed by glutamine in an AreA-dependent manner, although the expression of bikaverin biosynthetic genes is affected by AreA to a lesser extent than are the GA genes. Recently, the \textit{F. oxysporum} homologue of \textit{bik3} has been identified as nitrogen starvation induced (16). The conservation of the bikaverin gene cluster between \textit{F. fujikuroi}, \textit{F. verticillioides}, and \textit{F. oxysporum} (Wiemann and Tudzynski, unpublished) appears to mirror a conserved strategy of regulation by nitrogen status. We also found the expression of numerous other genes with predicted functions in secondary metabolism (e.g., those encoding oxidoreductases, transporters of the major facilitator superfamily, and monoxygenases) to be repressed in a similar manner.

In \textit{S. cerevisiae}, two positively acting GATA factors, Gln3 and Gat1, are known to regulate the transcription of NCR-sensitive genes either alone or in combination with each other (69). By use of DNA microarrays, the expression profiles of a WT strain and of a \textit{gln3Δ gat1Δ} double mutant strain grown under different nitrogen conditions have been examined. Of the 392 genes whose expression was dependent on the quality of the nitrogen source, only 91 genes were activated in a GATA factor-dependent manner (64), which is consistent with our findings. No Gat1 homologue is known to exist in filamentous fungi.
tous fungi, indicating that the functions of Gln3 and Gat1 converge on AreA homologues in filamentous fungi.

The role of NMR in nitrogen regulation of *F. fujikuroi*. Previously, we have shown that NMR does not play a major role in regulating AreA activity in *F. fujikuroi*, at least with respect to GA biosynthetic genes. In contrast, NmrA and Nmr1 play essential roles in regulating AreA activity in *A. nidulans* and *N. crassa*, respectively. The ability of the *F. fujikuroi* NMR to fully complement the *N. crassa* nmr1 mutant (39) indicates that they share similar modes of action in both fungi. In this study, we wanted to examine the impact of the NMR protein on the expression of a larger set of genes and its possible interactions with AreA. We show that the *F. fujikuroi* NMR protein interacts with the C-terminal part of AreA (which includes the ZF domain) (Fig. 6), similarly to what is seen for *N. crassa* and *A. nidulans* (35, 51). We also found that the overexpression of NMR inhibits the expression of the AreA target gene *niaD* and is reflected by an increased chloride resistance (Fig. 5), as already shown in our previous work (39). Consistently, the AreA overexpression strains are more sensitive to chloride, while the areA deletion strains are more resistant (Fig. 5). Taken together, these results point toward a direct cooperation of these two regulatory proteins in nitrogen metabolite regulation in *F. fujikuroi* as well.

However, in contrast to the clear differences in chloride resistance between *areA* and *nmr* deletion mutants, the effect of *nmr* deletion or overexpression on AreA target gene expression is less obvious. For most of the AreA target genes, we did not observe a significant upregulation in the *nmr* deletion mutant in shift experiments. If NMR acted as the main inhibitor of AreA activity in *F. fujikuroi* in a manner similar to that seen for homologues NmrA and Nmr1 in *A. nidulans* and *N. crassa*, the expression of AreA target genes would be expected to be clearly upregulated in Δ*nmr* mutants and downregulated in *nmr*-overexpressing mutants. This was not the case, confirming our previous results (39), which had shown that the expression of the GA biosynthetic genes is not affected by the *nmr* deletion. Thus, NMR seems to play a minor role in the regulation of the GA biosynthetic genes is not affected by the nitrogen source to nitrogen-starved WT and *nmr* mutant mycelia indicates that in addition to NMR, another regulator(s) which switches off the expression of AreA target genes under nitrogen-sufficient conditions must exist. The nature of this repressor and its supposed interaction with AreA will need to be clarified in the future. One putative additional repressor of AreA target genes may be the MeaB BZIP transcription factor, as its deletion leads to a derepression of AreA target genes in *A. nidulans* (55, 84) as well as in *F. fujikuroi* (A. Schmeineck, D. Wagner, and B. Tudzynski, unpublished data). In addition, we recently cloned the gene encoding the GATA-type transcription factor AreB, the orthologue of the *S. cerevisiae* repressor Dal80 (49) and the *A. nidulans* AreB (12). Its role in nitrogen regulation and as a putative counterpart of AreA in *F. fujikuroi* is currently under investigation (P. Rengers and B. Tudzynski, unpublished data).

An interesting finding that distinguishes *F. fujikuroi* from *A. nidulans* is that the meaB gene is downregulated in the *areA* mutant and that the meaB transcript size depends on nitrogen availability in the WT (the transcript size is significantly smaller under nitrogen starvation conditions) and on AreA. In contrast, the expression of the *A. nidulans* meaB gene is almost constitutive, and no changes in transcript size were found under various nitrogen conditions. Furthermore, it has been shown that MeaB in *A. nidulans* induces the expression of *nmrA* under nitrogen-sufficient conditions, thereby affecting the activity of AreA (84). In *F. fujikuroi*, meaB itself appears to be an AreA target gene in terms of its effects on general expression level and transcript size (Fig. 4C). While the loss of AreA leads to the exclusive presence of the larger transcript, the mutation of *nmr* resulted in an increased expression of the smaller transcript, indicating that AreA and NMR act opposingly on the expression of the small transcript of *meaB*. Analysis of *F. verticillioides* and *F. fujikuroi* EST collections revealed that there exist two groups of EST clones with alternative transcription start points. The exact mechanism by which AreA and NMR are involved in the regulation of the two alternative transcripts remains to be elucidated.

In summary, we show the utility of the *F. verticillioides* microarray to detect global gene expression changes in *F. fujikuroi*. We were able to identify numerous new candidate AreA target genes and found that the loss of this transcription factor resulted not only in a dramatic downregulation of “classic” AreA target genes but also in the upregulation of a new set of target genes that mainly encode genes involved in amino acid biosynthesis. Studies with rapamycin indicated that, in contrast to what is seen for *S. cerevisiae*, only some AreA target genes, e.g., the GS-encoding gene glnA and the peptide permease-encoding gene *mtd1*, are derepressed by rapamycin, and this
only to some extent, whereas the global control of protein de novo synthesis functions in a TOR-dependent and AreA-independent manner in both organisms. We were able to show that NMR is a repressor of AreA target gene expression in a growth-phase-dependent manner and that it interacts directly with AreA. Our finding that the deletion of nmr did not prevent the strong repression of AreA target genes by glutamine suggests that additional regulators, e.g., MeaB and AreB, might play also a significant role in the regulation of AreA activity in *F. fujikuroi*.

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