Deletion and overexpression of the Aspergillus nidulans GATA factor AreB reveals unexpected pleiotropy

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The Aspergillus nidulans transcription factor AreA is a key regulator of nitrogen metabolic gene expression. AreA contains a C-terminal GATA zinc finger DNA-binding domain and activates expression of genes necessary for nitrogen acquisition. Previous studies identified AreB as a potential negative regulator of nitrogen catabolism showing similarity with Penicillium chrysogenum NreB and Neurospora crassa ASD4. The areB gene encodes multiple products containing an N-terminal GATA zinc finger and a leucine zipper motif. We deleted the areB gene and now show that AreB negatively regulates AreA-dependent nitrogen catabolic gene expression under nitrogen-limiting or nitrogen-starvation conditions. AreB also acts pleiotropically, with functions in growth, conidial germination and asexual development, though not in sexual development. AreB overexpression results in severe growth inhibition, aberrant cell morphology and reduced AreA-dependent gene expression. Deletion of either the DNA-binding domain or the leucine zipper domain results in loss of both nitrogen and developmental phenotypes.

INTRODUCTION

GATA transcription factors are a ubiquitous class of DNA-binding proteins in eukaryotes characterized by one or more highly conserved zinc finger motifs that recognize consensus WGATAR DNA sequences. The various members of this class of transcription factor play important roles in the regulation of cell metabolism, differentiation or development (for reviews see Lowry & Atchley, 2000; Scaccia, 2000; Park et al., 2006). In Aspergillus nidulans, there are multiple GATA factors that are known to function as global regulators of diverse biological processes, including nitrogen regulation (AreA), photosynthesis (AreB, AreC), iron homeostasis (SreA) and sexual development (NsD). AreA, the major nitrogen regulatory protein, contains a single C-terminal CX2CX17CX2C GATA zinc finger DNA-binding domain that is highly conserved in areA homologues identified in the genomes of filamentous fungi (see Wong et al., 2008a). AreA is required for the transcription of genes encoding permeases and catabolic enzymes for the breakdown of complex nitrogen-containing compounds, and loss-of-function areA mutations result in inability to grow on nitrogen sources except ammonium and glutamine (Kudla et al., 1990; Wilson & Arst, 1998). Studies have shown that AreA function is regulated in response to the nitrogen status of the cell by multiple mechanisms including regulation of areA transcript levels by autogenous control of areA transcription and differential nitrogen-source-dependent transcript stability (Langdon et al., 1995; Platt et al., 1996; Morozov et al., 2000, 2001). In addition, AreA activity is modulated by interaction with a co-activator, TamA, and a co-repressor, NmrA (Davis et al., 1996; Andrianopoulos et al., 1998; Small et al., 1999, 2001; Wong et al., 2007). The levels of NmrA expression, which in turn are dependent on the bZIP transcription factor MeaB, are an important determinant of AreA activation, and structural studies suggest that interaction of AreA with DNA and NmrA is mutually exclusive (Lamb et al., 2004; Wong et al., 2007; Kotaka et al., 2008). AreA activation potency is further elevated during nitrogen starvation compared with nitrogen limitation and this correlates with accumulation of AreA in the nucleus through a mechanism involving control of nuclear export (Todd et al., 2005).

In addition to the multiple controls that influence the level or activity of AreA under various nitrogen conditions, an additional member of the A. nidulans GATA family, AreB, was proposed to play a negative role in the regulation of nitrogen catabolism. The areB gene was first identified by

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Abbreviation: RIP, repeat-induced point mutation.
mutations involving chromosomal rearrangement at the areB locus that suppressed an areA null mutant (Tollervey & Arst, 1982; Arst et al., 1989, 1990). It was proposed that these gain-of-function areB mutations were due to fusion of a cryptic activation domain to the GATA DNA-binding region of AreB (Conlon et al., 2001). Putative areB null mutations resulting in premature truncation of AreB-cryptic-activation-domain fusions were isolated by selection for loss of this suppression (Conlon et al. 2001). These mutants showed derepression of ornithine transaminase under certain carbon limitation conditions (Dzikowska et al., 2003). Although these mutants are likely loss-of-function mutants, attempts to generate areB deletion strains were unsuccessful (Conlon et al., 2001). The areB gene is predicted to encode three protein products, designated AreBα, AreBβ and AreBγ, translated from four transcripts resulting from differential transcription start sites, translation initiation and splicing. The smallest transcript (encoding only AreBα) is expressed at higher levels under nitrogen-derepressed conditions and the three larger transcripts (potentially producing all three AreB isoforms) are optimally expressed in the presence of ammonium (Conlon et al., 2001). All three AreB variants contain an N-terminal GATA zinc finger domain and a C-terminal leucine zipper domain (Conlon et al., 2001). A similar class of GATA factors in Saccharomyces cerevisiae is encoded by the DAL80/UGA43 and GZF3/NIL2/DEH1 genes, which share 32 % and 25 % overall sequence identity, respectively, when compared with the full-length AreB protein (Conlon et al., 2001). The S. cerevisiae proteins are thought to act negatively to modulate nitrogen regulated gene expression by antagonizing the action of the positively acting GATA factors Gln3 and Gat1/Nil1 through competition for GATA DNA-binding sites (Coffman et al., 1997; Soussi-Boudekou et al., 1997; Cunningham et al., 2000a). By analogy with the action of the S. cerevisiae Dal80 and Nil2, AreB has been proposed to function negatively by competing with AreA for binding to the promoters of AreA-regulated genes (Conlon et al., 2001). Consistent with this, overexpression of nreB, the areB homologue in Penicillium chrysogenum, results in repression of the nitrate catabolic genes, and the DNA-binding domain of NreB fused to maltose-binding protein (MBP) was found to bind to the promoter of the nitrate assimilation gene cluster (Haas et al., 1997). Northern analysis demonstrated the presence of multiple nreB transcripts and, interestingly, loss-of-function nreB mutants could not be isolated (Haas et al., 1997). The homologous gene in Neurospora crassa was identified as Asd4 (Feng et al., 2000). Despite the obvious parallels between areB and nreB, a loss-of-function Asd4 mutant, obtained using repeat-induced point mutation (RIP), had no apparent nitrogen phenotype but revealed a major defect in ascospore production during sexual development (Feng et al., 2000).

Therefore, it was not clear whether the areB gene has an essential function and/or a role in the regulation of nitrogen metabolism in A. nidulans or whether its function is conserved across the filamentous fungi. To further define the role of the areB gene, we have deleted and over-expressed this gene in A. nidulans and investigated nitrogen-regulated gene expression in these strains. This study has led to the unexpected findings that AreB is a negative regulator of nitrogen metabolic genes during nitrogen limitation and starvation rather than under nitrogen-repressing conditions, and that it has additional novel roles.

**METHODS**

**A. nidulans strains, media, growth conditions and transformation.** The A. nidulans strains used in this study are shown in Table 1. Growth media and conditions were as described by Cove (1966). Genetic analysis was carried out using techniques described by Todd et al. (2007a, b). For homozygous areBα crosses, MH11284 or MH11285 conidia were point-inoculated on 1 % glucose minimal medium supplemented with 10 mM sodium nitrate as the nitrogen source, and grown at 37 °C for 2 days before the plate was sealed and further grown at 37 °C for 7 days. Outcrossing of the areB mutant (MH12257) with wild-type (A234) was carried out as described by Todd et al. (2007b). The strains carrying the xylPP/areB (MH12252), xylPP/areB-zypperΔ (MH12300) and xylPP/areB-GATAA (MH12331) fusion constructs at yA were crossed with the areB strain (MH12284) to give MH12384, MH12386 and MH12385, respectively. DNA transformations of A. nidulans were performed according to Andrianopoulos & Hynes (1988). Genomic DNA of A. nidulans was extracted from frozen mycelia as described by Lee & Taylor (1990). Colony growth (hyphal extension) (Wong et al., 2008b) and spore count (Todd et al., 2006) were determined as previously described. For determining percentage germination, conidia were inoculated on 1 % glucose liquid minimal medium containing 10 mM ammonium tartrate as the nitrogen source and grown at 37 °C. At 8 and 10 h of growth, a minimum of five pictures was taken from different fields of view using an Olympus IX70 inverted microscope equipped with a digital camera. The total number of germinated and non-germinated spores was manually counted and expressed as percentage of germination.

**Generation of the areBΔ mutant.** A PCR fragment containing the entire areB gene was amplified using areB-gamma-F (–863 5’- CAGGGAGCCAACCTTCTGACG-3’ –842) and areB-R (+1442 5’-CGAAGATGGACTGATCAAGAC-3’ +1422) primers from genomic DNA of MH1 and was cloned into pblluescript to give pCW7242. The Nco–EcoCI fragment of pCW7242 was replaced with an Nco–EcoCI fragment of pMT1612 carrying the glufosinate resistant marker Bar (Nayak et al., 2006) to give pCW7243. The Nde–Nco fragment of pCW7243 was deleted and the NdeI and Ncol sites were end-filled and ligated to give pCW7244, which contains the knockout cassette for removing the sequences encoding residues 17–257 of AreBz. A linear PCR fragment generated using areB-gamma-F and areB-R primers on pCW7244 was transformed into the MH11036 strain and transformants were selected on 1 % glucose minimal medium with 10 mM ammonium tartrate as the nitrogen source and grown at 37 °C. At 8 and 10 h of growth, a minimum of five pictures was taken from different fields of view using an Olympus IX70 inverted microscope equipped with a digital camera. The total number of germinated and non-germinated spores was manually counted and expressed as percentage of germination.
Construction of the areB overexpression strains. Two PCR fragments (areB and areB2) were generated using primer pairs areB-gamma and areB-R and areBalpha-F (−29 5’-TTTTCTCCCAT-CTATCTTCTGG-3’ −8) and areB-R, respectively, on genomic DNA of MH1. The former PCR product contains sequences encoding AreB, AreB and AreBz, while the latter one encodes only AreBz. The PCR products were introduced after the xylose-inducible promoter xylP(p) at the Smal site of the yA-targeting vector pCW7468. The resultant clones pCW7239 (xylP(p)areB) and pCW7236 (xylP(p)areB2), which could be targeted either to the yA locus as described previously (Wong et al., 2007) or to the areB locus, were transformed into MH11036. Two classes of PyrO⁺ transformants were obtained (Table 1) and Southern blot analysis was performed to confirm the site of integration (data not shown). For generating a C-terminally truncated form of AreB lacking the leucine zipper (residues 229−436 of AreB) (xylP(p)AreB-GATA) was generated by inverse PCR using the primers areB-GATATO-invR (+312 5’TATCGTCGCGTCGC-GTAAGATG-3’ +36) and areB-GATATO-invR (+45 5’-GGGCCT-GTAATTTAGGCGGTCTGTTG-3’ +22) on pCW7239, and the PCR product was treated with T4 polynucleotide kinase (New England Biolabs) and self-ligated to give pCW7240. pCW7240 and pCW7241 were checked by restriction digestion and sequencing for correct deletion of the respective sequences and transformed into MH11036. PyrO⁺ transformants were obtained and site of integration was confirmed by Southern blot analysis (data not shown).

β-Galactosidase assays. β-Galactosidase assays were performed as previously described and enzyme activities are expressed as units min⁻¹ per mg soluble protein (Davis et al., 1988). Protein was determined with the Bio-Rad Protein Assay reagent.

Microscopy. Conidia were inoculated into 1 % glucose liquid minimal medium containing 10 mM ammonium tartrate with or without 1 % xylose and grown at 25 °C for 14–16 h. For conidial germination experiments, images were captured using an Olympus IX70 inverted microscope equipped with a digital camera. DAPI staining of nuclei and immunostaining analysis of AreA⁺ and the associated microscopy were performed as described by Todd et al. (2005).

Sequence analysis. To search for the presence of additional orthologous sequences of AreB/NreB/ASD4 in A. nidulans and N. crassa, the protein sequences of AreB (accession no. AAG39531), NreB (accession no. AAC09045) and ASD4 (accession no. AAG5180) were used for BLASTp and tblASTX searches against their respective genome sequence databases available at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu). For identification of AreB orthologues in other ascomycetes, the sequence of AreB was the query in BLASTp and tblASTX searches (Altschul et al., 1997) of the genome database of Aspergillus terreus, Aspergillus clavatus, Neosartorya fischeri, Histoplasma capsulatum, Uncinocarpus reessii, Coccidioides immitis, Stagonospora nodorum, Magnaporthe grisea, Chetomium globosum, Fusarium verticillioides, Fusarium oxysporum, Fusarium graminearum, Botrytis cinerea, Scerotinia sclerotiorum (Broad Institute of MIT and Harvard), Aspergillus flavus (Payne et al., 2006), Aspergillus fumigatus (Nierman et al., 2005) (The Institute for Genomic Research, http://www.tigr.org) and Aspergillus oryzae (Machida et al., 2005) (National Institute of Technology and Evaluation, http://www.bio.nite.go.jp/dogan/Top). The DNA and annotated protein sequences of the putative AreB orthologues in various ascomycetes were obtained from their respective genome sequence databases. The gene identification numbers of the AreB orthologues are ATEG_0312 (A. terreus), ACA_027190 (A. clavatus), AFLG_06945 (A. flavus), Afu2g13380 (A. fumigatus), AO0900-26000315 (A. oryzae), NFIA_088570 (N. fischeri), HCAG_05973 (H. capsulatum), UREG_06515 (U. reesii), CIMG_05044 (C. immitis).

Table 1. A. nidulans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>A234</td>
<td>yA2 pabaA1, veA1</td>
</tr>
<tr>
<td>MH1</td>
<td>biA1, veA1</td>
</tr>
<tr>
<td>MH9046</td>
<td>yA1 pabaA1, fms-lacZ, niiA6, veA1</td>
</tr>
<tr>
<td>MH11036</td>
<td>pyroA4 nkuA::argB, veA1, riboB2</td>
</tr>
<tr>
<td>MH12247</td>
<td>areB::xylP(p)areB-A, fpyroA, pyroA6, veA1, riboB2</td>
</tr>
<tr>
<td>MH12249</td>
<td>areB::xylP(p)areB-A, fpyroA, pyroA6, veA1, riboB2</td>
</tr>
<tr>
<td>MH12251</td>
<td>areB::xylP(p)areB-A, fpyroA (multi-copy), pyroA6, veA1, riboB2</td>
</tr>
<tr>
<td>MH12252</td>
<td>ya::xylP(p)areB-A, fpyroA, pyroA6, veA1, riboB2</td>
</tr>
<tr>
<td>MH12257</td>
<td>areB::Bar, pyroA4 nkuA::argB, veA1, riboB2</td>
</tr>
<tr>
<td>MH12284</td>
<td>areB::Bar, yaA1 pabaA1, nkuA::argB, veA1</td>
</tr>
<tr>
<td>MH12285</td>
<td>areB::Bar, pyroA4 nkuA::argB, veA1</td>
</tr>
<tr>
<td>MH12289</td>
<td>areB::Bar, fms-lacZ, veA1</td>
</tr>
<tr>
<td>MH12300</td>
<td>ya::xylP(p)areB-A, fpyroA, pyroA4 nkuA::argB, veA1, riboB2</td>
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<tr>
<td>MH12302</td>
<td>areB::xylP(p)areB-A, fpyroA, gpd(p)areAHA fms-lacZ, nkuA::Bar, veA1</td>
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<td>MH12302</td>
<td>ya::xylP(p)areB-A, fpyroA, fms-lacZ, veA1</td>
</tr>
<tr>
<td>MH12311</td>
<td>ya::xylP(p)areB-GATAA-A, fpyroA, pyroA6, veA1, riboB2</td>
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<tr>
<td>MH12381</td>
<td>ya::xylP(p)areB-A, fpyroA, acul-lacZ, veA1</td>
</tr>
<tr>
<td>MH12382</td>
<td>ya::xylP(p)areB-GATAA-A, fpyroA pabaA1, fms-lacZ, veA1, riboB2</td>
</tr>
<tr>
<td>MH12383</td>
<td>ya::xylP(p)areB-GATAA-A, fpyroA, fms-lacZ, nkuA::Bar, veA1</td>
</tr>
<tr>
<td>MH12384</td>
<td>ya::xylP(p)areB-A, fpyroA areB, veA1</td>
</tr>
<tr>
<td>MH12385</td>
<td>ya::xylP(p)areB-GATAA-A, fpyroA areB, veA1</td>
</tr>
<tr>
<td>MH12386</td>
<td>ya::xylP(p)areB-zipperA-A, fpyroA areB, veA1, riboB2</td>
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SNOG_13332 (S. nodorum), MGG_06050 (M. grisea), CHGG_03821 (C. globosum), FVEG_06055 (F. verticillioides), FOXG_08613 (F. oxysporum), FGSG_05073 (F. graminearum), BCIG_15788 (B. cinerea) and SSIG_09784 (S. sclerotiorum). For phylogenetic analysis the protein sequences were aligned and an unrooted bootstrap neighbour-joining tree with random number generator seed of 111 and 1000 bootstrap trials was generated using CLUSTAL W 2.0.10 (Larkin et al., 2007). The phylogram was drawn using NJPlot (Perriere & Gouy, 1996). Multiple DNA or protein sequence alignment was performed by the CLUSTAL W program (Thompson et al., 1994) in Biomanager of the Australian National Genome Information Service (ANGIS, http://www.angis.org.au). The MacBoxshade 2.15E program (Marck, 1988). Manual annotation of genome sequences of orthologues of various fungi was performed when necessary with the DNA Strider 1.4f5 program (March, 1988).

RESULTS

A single AreB orthologue is present in filamentous fungi

A. nidulans AreB, P. chrysogenum NreB and N. crassa ASD4 share a highly conserved GATA DNA-binding domain and a conserved leucine zipper (Haas et al., 1997; Feng et al., 2000; Conlon et al., 2001). However, apparent differences in their function prompted us to analyse the fungal genome sequences that have become available since the initial comparisons to determine whether AreB, NreB and ASD4 are true orthologues. Searches using BLASTP and TBLASTX did not identify any additional orthologous protein coding sequences similar to AreB, NreB or ASD4 in A. nidulans or N. crassa genome sequences. Phylogenetic analysis revealed that AreB, NreB and ASD4 are evolutionarily more related to each other than to all the other GATA factors found in the respective genomes (Fig. 1A). Furthermore, a single protein coding sequence similar to AreB, NreB and ASD4 was identified in all other filamentous fungi analysed (see Methods). These orthologous sequences contain a highly conserved GATA DNA-binding domain and a conserved leucine zipper with the same structural arrangement as AreB/NreB/ASD4 (Fig. 1B). These findings indicate that AreB, NreB and ASD4 are orthologous and represent a discrete class of GATA factors within the ascomycetes. Interestingly, the leucine zipper of the Stagonospora nodorum AreB orthologue is one heptad repeat shorter than that of AreB, whereas the leucine zippers of ASD4 and the Fusarium fujikuroi orthologue are punctuated by an insertion.

Deletion of areB affects germination, growth and conidiation, but not sexual development

Previous attempts to inactivate areB were unsuccessful and the implication that AreB has a role in nitrogen regulation in A. nidulans came from analysis of areB translation mutants (Conlon et al., 2001; Dzikowska et al., 2003). Since these initial studies, A. nidulans nkuA gene targeting strains have been constructed to facilitate homologous integration (Nayak et al., 2006). In order to delete the areB gene in an nkuAΔ background, the coding sequences of areB and flanking regions were amplified by PCR and cloned (see Methods). Sequences encoding the entire DNA-binding domain and most of the leucine zipper of AreB (codons 17–257 of areB) were replaced with a selectable marker, Bar, conferring glufosinate resistance, and the deletion construct was transformed as a linear fragment into MH11036 (pyroA4 nkuA:: argB, riboB2) (Fig. 2A). Approximately 50 glufosinate-resistant transformants were obtained and for several of these inactivation of the areB gene was confirmed by Southern blot analysis (see Methods), indicating that areB is not an essential gene.

The areBΔ mutant, grown at 37 °C for 2 days on complete medium or minimal medium, had reduced colony size and poorer conidiation than wild-type (Fig. 2B). The previously identified areB403/901 translocation-loss-of-function mutant also formed smaller colonies than the wild-type (Conlon et al., 2001). Smaller colonies may be explained by slower growth rate or retarded germination. The growth rate of the areBΔ mutant as determined by hyphal extension from point-inoculum on solid complete medium was similar to that of the wild-type up to approximately 100 h after inoculation (Fig. 2C). Therefore the reduced colony size at 2 days did not result from retarded growth in the initial phases of colony formation. A gradual decline in growth rate was however observed for the areBΔ mutant after 100 h, while the wild-type showed continued linear growth. Effects of areBΔ on conidial germination were examined by microscopic analysis. The percentage germination of areBΔ mutant conidia was clearly reduced at 8 and 10 h post-inoculation at 37 °C when compared with wild-type (Fig. 2D). Furthermore, when spores of the areBΔ mutant were plated on complete medium at low density, non-uniform colonial growth was observed with colonies of various sizes, in which some were just becoming visible when others had already formed conidia (data not shown). This is consistent with heterogeneity in the degree of conidial germination delay. In addition to effects on conidial germination, quantitative analysis showed a twofold decrease in the number of conidia formed by the areBΔ mutant when compared with wild-type (Fig. 2D).

The N. crassa Asd4 RIP mutant fails to form viable meiotic progeny (Feng et al., 2000). To investigate whether areB has a role in the sexual cycle in A. nidulans, the behaviour of the areBΔ mutant in sexual crosses was examined. A. nidulans is homothallic, and in an outcross forms fruiting bodies (cleistothecia) containing viable ascospores from selfings of either parent and hybrid cleistothecia formed from both parents. The areBΔ mutant (MH12257: areB:: Bar, pyroAΔ, riboB2, nkuA:: argB, veA1) was crossed to an areBΔ strain (A234: yA2, pabaA1, veA1). In this cross, the areBΔ parent carried the riboB2 mutation, which results in self-infertility in a veA1 background (Oakley et al., 1987), and the areBΔ strain, as expected, participated only in the formation of hybrid cleistothecia. The hybrid cleistothecia contained viable ascospores and the areBΔ and areBΔ alleles segregated in the progeny according to

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Fig. 1. AreB, NreB and ASD4 are orthologues. (A) Phylogenetic relationships among GATA factors of *A. nidulans*, *P. chrysogenum* and *N. crassa* shown using an unrooted bootstrap neighbour-joining tree (see Methods). Bootstrap support following 1000 bootstrap trials with random number generator seed of 111 is indicated on the branch. Distance is represented on a scale of 0 (completely similar) to 1.0 (completely dissimilar). (B) Conserved sequence and structural arrangements of the DNA-binding domain and leucine-zipper domain of various AreB/NreB/ASD4 orthologues from *A. nidulans* (An), *P. chrysogenum* (Pc), *Uncinocarpus reesii* (Ur), *Coccidioides immitis* (Ci), *Botrytis cinerea* (Bc), *Sclerotinia sclerotiorum* (Ss), *Stagonospora nodorum* (Sn), *Fusarium fujikoroi* (Ff) and *N. crassa* (Nc). The ▲ symbol depicts the positions of the four cysteine residues that coordinate a zinc ion within the GATA zinc finger. The asterisk (*) highlights the expected positions for leucine residue in the heptad repeats of the leucine zipper domain of AreB.
Mendelian expectations. Among the areB\textsuperscript{D} segregants, variation in the timing of ascospore germination was observed, as some segregants formed bigger colonies than others after 2 days of growth at 37°C. In homozygous areB\textsuperscript{D} crosses using areB\textsuperscript{D} mutants carrying different nutritional markers [pabaA\textsuperscript{1} (MH11284) and pyroA\textsuperscript{4} (MH11285)], cleistothecia containing viable ascospores were formed. Furthermore, cleistothecia and viable meiotic progeny were obtained when these two areB\textsuperscript{D} mutants were allowed to self. Therefore, the areB\textsuperscript{D} mutant appeared fully competent in initiating and completing the sexual cycle. These results were in contrast to the effect of the Asd4 RIP mutant in N. crassa, in which fruiting body formation appeared normal but no viable ascospores were produced (Feng et al., 2000).

**AreB antagonizes AreA activation under nitrogen-limiting and nitrogen-starvation conditions:** evidence from deletion of areB

The role of areB in nitrogen regulation was analysed using growth tests on solid media containing a defined nitrogen source, which have been used to qualitatively assess AreA function (Arst & Cove, 1973; Wilson & Arst, 1998). No specific effect on nitrogen source utilization was detected using ammonium, proline, \(\alpha\)-aminobutyric acid, glutamate, 2-pyrrolidinone, alanine, nitrate or formamide at 10 mM as the sole nitrogen source (Fig. 2B and data not shown). Deletion of nmrA, a negative regulator of AreA activity, leads to partial derepression of various nitrogen catabolic genes in the presence of the repressing nitrogen source (Fig. 2B and data not shown). Deletion of nmrA, a negative regulator of AreA activity, leads to partial derepression of various nitrogen catabolic genes in the presence of the repressing nitrogen source.
ammonium, and consequently to sensitivity to toxic analogues of nitrate (chlorate), urea (thiourea) and asparagine (aspartate hydroxamate) (Andrianopoulos et al., 1998). Growth tests of the \( \text{areB}{\Delta} \) mutant on 10 mM ammonium with either 100 mM or 200 mM chlorate, 2.5 mM ammonium with 10 mM thiourea and 10 mM ammonium with 1 mM aspartate hydroxamate media, as well as the plate tests for derepression of extracellular protease activity (1% milk protein, 10 mM ammonium), provided no evidence for derepression of AreA-regulated gene expression in the \( \text{areB}{\Delta} \) mutant. The effect of \( \text{areB} \) deletion on AreA-dependent nitrogen regulation was also assessed by \( \beta \)-galactosidase assays of an \( \text{fmdS-lacZ} \) reporter gene (Fraser et al., 2001), which quantitatively measures AreA activation under different nitrogen conditions and is more sensitive than plate tests. A slightly elevated level of \( \text{fmdS-lacZ} \) expression when the \( \text{lacZ} \) gene (Fraser et al., 2001) provided no evidence for derepression of AreA-regulated gene expression in the \( \text{areB}{\Delta} \) mutant. The effect of \( \text{areB} \) deletion on AreA-dependent nitrogen regulation was also assessed by \( \beta \)-galactosidase assays of an \( \text{fmdS-lacZ} \) reporter gene (Fraser et al., 2001), which quantitatively measures AreA activation under different nitrogen conditions and is more sensitive than plate tests. A slightly elevated level of \( \text{fmdS-lacZ} \) expression was observed under nitrogen-limiting conditions (alanine) and a large increase in expression was found during nitrogen starvation (\(-N\)) as a consequence of \( \text{areB} \) inactivation (Fig. 2E). Consistent with the plate tests, there was no evidence of derepression of \( \text{fmdS-lacZ} \) expression when the \( \text{areB}{\Delta} \) mutant was grown under nitrogen-sufficient conditions (ammonium). This contrasts with the loss of the negative regulator NmrA, which leads to partial derepression of AreA activity under nitrogen-sufficient conditions (Andrianopoulos et al., 1998). Taken together, these results indicate that AreB negatively affects AreA activation specifically under nitrogen-limiting and nitrogen-starvation conditions.

**AreB antagonizes AreA activation under nitrogen-limiting and nitrogen-starvation conditions: evidence from \( \text{areB} \) overexpression**

Previous studies in *P. chrysogenum* indicated that overexpression of \( \text{areB} \) from the xylose-inducible promoter of \( \text{xyIP} \) resulted in repression of nitrate utilization genes (Haas et al., 1997). To investigate whether \( \text{areB} \) overexpression affected nitrogen-regulated gene expression in *A. nidulans*, sequences encoding \( \text{areB} \) were fused to the \( \text{xyIP} \) promoter (Zadra et al., 2000) to drive high-level expression (Fig. 3A). Two \( \text{xyIP}(p)\text{areB}{\alpha} \) fusions were made, one \( \text{xyIP}(p)\text{areB}{\alpha} \) (pcW7236) encoding only AreB{\alpha} and the other \( \text{xyIP}(p)\text{areB}{\gamma} \) (pcW7239) encoding AreB{\gamma}, AreB{\beta} and AreB{\alpha} (see Methods). The \( \text{xyIP}(p)\text{areB} \) fusions were present in a \( yA \)-targeting vector and integration of these constructs in single copy in MH11036 (\( \text{areB}{\alpha}^{+} \)) resulted in transformants in which the constructs are integrated at either \( \text{areB} \) or \( yA \) (see Methods). Two copies of \( \text{areB} \) are present in these transformants, with one copy driven by the wild-type promoter and the other under the control of the inducible \( \text{xyIP} \) promoter. Overexpression of either \( \text{areB}{\alpha} \) or \( \text{areB}{\gamma} \) on medium containing 1% xylose produced an identical phenotype regardless of the site of integration, resulting in the almost complete inhibition of growth (see below). All subsequent analyses, unless otherwise indicated, were performed using the strains that had the \( \text{xyIP}(p)\text{areB}{\gamma} \) fusion construct integrated at \( yA \).

In order to assess the effects of \( \text{areB} \) overexpression on nitrogen-regulated gene expression, transfer experiments were used to circumvent the detrimental effects of AreB overexpression on growth. Mycelia were grown overnight on glucose medium and transferred to 1% glucose-1% xylose medium for 4 h to induce expression of the \( \text{xyIP}(p)\text{areB} \) fusion gene or to 1% glucose medium as a control. Assays of \( \text{fmdS-lacZ} \) reporter gene expression revealed that AreA activation in nitrogen-limiting and nitrogen-starvation conditions was completely blocked when AreB was overexpressed (Fig. 3B). This lack of

**Fig. 3.** Overexpression of \( \text{areB} \) in *A. nidulans*. (A) Overexpression constructs of \( \text{areB}{\alpha} \), \( \text{areB}{\gamma} \), in-frame deletion of \( \text{areB}{\gamma} \) lacking the GATA zinc finger DNA-binding domain and truncation of \( \text{areB}{\gamma} \) removing the leucine zipper domain. The respective coding sequences were placed after a highly xylose-inducible promoter, \( \text{xyIP}(p) \) (Zadra et al., 2000), and were introduced into a \( yA \)-targeting vector and integrated at the \( \text{areB} \) or \( yA \) locus (see Methods). (B) Effects of \( \text{areB} \) overexpression on \( \text{fmdS-lacZ} \) expression. The wild-type (MH9046), \( \text{xyIP}(p)\text{areB}{\gamma} \) (MH12320), \( \text{xyIP}(p)\text{areB}{\gamma} \)-zipperΔ (MH12322) and \( \text{xyIP}(p)\text{areB}{\gamma} \)-GATAΔ (MH12383) strains were grown in 1% glucose minimal medium supplemented with 10 mM ammonium tartrate at 37 °C for 16 h. Mycelia were then washed with 1% glucose minimal medium and subsequently transferred to 1% glucose (−) or 1% xylose (+) minimal medium containing 10 mM ammonium tartrate (NH₄), 10 mM alanine (Ala) or no nitrogen source (−N) for an additional 4 h. \( \beta \)-Galactosidase assays were performed as previously described and enzyme activities are expressed as units min⁻¹ per mg soluble protein (Davis et al., 1988). Means and standard errors from at least three independent experiments are shown.
AreA activation during nitrogen starvation was not due to failure of AreA to enter the nucleus, as immunostaining analysis revealed that nuclear accumulation of AreAHA in response to 4 h of nitrogen starvation was not affected by overexpression of areB (data not shown). To eliminate the possibility that this was a consequence of the growth defect caused by AreB overexpression, expression of the AreA-independent acuJ-lacZ reporter (Hynes et al., 2006) was also analysed in a xylP(p)areB background. Basal and induced levels of this reporter gene were not affected by AreB overexpression (data not shown). Together with the elevated AreA-dependent gene expression observed with areB deletion, the loss of AreA-dependent gene expression during AreB overexpression strongly indicates that AreB has a role in nitrogen regulation to negatively modulate AreA activity.

**Overexpression of AreB leads to growth inhibition and morphological abnormality**

Overexpression of areB results in severe growth inhibition at the colony level. Conidia of the xylP(p)areB strain point-inoculated onto solid medium containing 1% xylose-1% glucose failed to form a visible colony (Fig. 4A). The growth of the xylP(p)areB strain was proportionally restored with decreasing xylose concentrations (Fig. 4A). Microscopic examination of xylP(p)areB conidia grown in 1% glucose-1% xylose minimal liquid medium at 37 °C showed no obvious reduction in germination after 1 day of growth compared with wild-type (Fig. 4B). However, the xylP(p)areB germings were short and somewhat swollen. Following an additional 1 day incubation, large irregular swollen structures were formed. These structures appeared to result from isotropic expansion of the germings, rather than the polarized apical growth of wild-type. DAPI staining revealed that these swollen structures were highly multinucleate (Fig. 4C).

The effect of areB overexpression on hyphal extension was confirmed by allowing point-inoculated conidia to germinate and initiate growth for 1 day on complete medium. Then 50 μl of 50% xylose solution was added to a well in the middle of the plate to create a xylose gradient by diffusion. Growth of the colony edge closest to xylose addition was severely impaired in both xylP(p)areBx and xylP(p)areBy strains and resistant sectors of growth were spontaneously formed, but no growth inhibition was observed in the wild-type control (Fig. 4D, right panel). There was no effect on growth of wild-type or xylP(p)areB strains when glucose was diffused into the medium (Fig. 4D, left panel). Therefore, high-level overexpression of AreB prevents vegetative growth of *A. nidulans*. Furthermore, the strain carrying multiple copies of xylP(p)areB showed slightly weaker growth than the wild-type in the absence of xylose induction, and the growth defect in the presence of xylose was more pronounced in this strain than the strain containing a single copy of xylP(p)areB (Fig. 4D). These results suggest that even a slight increase in AreB levels has a detrimental impact on the growth of *A. nidulans*.

**DNA-binding and leucine zipper domains are required for AreB function**

The ability of AreB lacking either the leucine zipper or the DNA-binding domain to complement the areBΔ mutant phenotypes was investigated. Strains that overexpress either a C-terminally truncated form of AreB lacking the leucine zipper (residues 229–436 of AreB)y (xylP(p)areBy-zipperΔ) or AreB with an in-frame deletion of the GATA-binding zinc finger (residues 140–228 of AreB)y (xylP(p)areBy-GATAΔ) were generated (Fig. 3A). These strains contain a single copy of the mutant construct integrated at the yA locus. For complementation analysis, we took advantage of the basal expression from the xylP promoter in the absence of xylose and the xylP(p)areB, xylP(p)areBy-zipperA and xylP(p)areBy-GATAΔ fusions were introduced into the areBΔ background by meiotic crossing (see Methods). While the full-length xylP(p)areB fusion construct complemented the conidiation and germination defects of areBΔ when expressed at basal level, the xylP(p)areBy-zipperΔ and xylP(p)areBy-GATAΔ constructs failed to complement the areBΔ phenotype (Fig. 2D). These results indicate that AreB requires both the DNA-binding and the leucine zipper domains for function.

We then sought to address whether the GATA DNA-binding domain and/or the leucine zipper domain of AreB are important for the detrimental effects of areB overexpression. The growth of these mutant strains was not inhibited by the addition of xylose (Fig. 4E), suggesting that both domains are required for the severe growth inhibition resulting from AreB overexpression. Furthermore, the hyphal morphologies and the number of nuclei of the xylP(p)areBy-GATAΔ and xylP(p)areBy-zipperΔ strains were similar to wild-type when these strains were grown for 2 days at 37 °C in the presence of xylose (data not shown). Significantly, overexpression of xylP(p)areBy-zipperΔ or xylP(p)areBy-GATAΔ also failed to prevent AreA activation of fndsS-lacZ (Fig. 3B). Therefore mutation of either of these motifs was sufficient to prevent the range of phenotypes associated with AreB overexpression, and both DNA-binding and dimerization functions are essential for the repressive role of AreB in nitrogen regulation.

**DISCUSSION**

Whole-genome comparisons have revealed the presence of multiple GATA factor families in the filamentous fungi (Park et al., 2006). The function of certain of these factors has been elucidated and it is clear that the GATA zinc finger as a DNA-binding motif has been harnessed into many diverse roles. In *A. nidulans*, the transcriptional activators AreA (nitrogen regulation), NsdD (sexual development), and LreA and LreB (photoregulation) contain a single C-terminally located GATA zinc finger,
**Fig. 4.** Overexpression of *areB* is detrimental. (A) The wild-type (MH11036) and *xylP(p)areB* (MH12320) strains were grown in solid minimal medium containing 10 mM ammonium tartrate, 1% glucose and the indicated xylose concentration for 2 days at 25 °C. (B, C) The wild-type (MH9046) and *xylP(p)areB* (MH12320) strains were grown in 1% glucose liquid minimal medium containing 10 mM ammonium tartrate with or without 1% xylose at 25 °C for 24–48 h and examined by microscopy. In (C) the nuclei of the wild-type (MH9046) and *xylP(p)areB* (MH12320) strains were stained with 4',6-diamidino-2-phenylindole (DAPI). DIC, differential interference contrast. Scale bars represent 20 μm. (D, E) Wild-type (MH11036) and strains containing *xylP(p)areB* (MH12247), *xylP(p)areB* (MH12249 (D)) and MH12320 (E), *xylP(p)areB* (multicopy) (MH12251), *xylP(p)areB* -zipperΔ (MH12322) and *xylP(p)areB* -GATAΔ (MH12382) were allowed to grow on complete medium for 1 day at 37 °C before addition of 50 μl of either 50% glucose or 50% xylose to a well cut in the middle of the plate. The strains were grown for an additional 2 days at 37 °C. Note that the *xylP(p)areB* overexpression constructs are targeted to *areB* in (D) and to *yA* in (E).
while the repressor SreA (iron acquisition) contains two N-terminal GATA zinc fingers (see Scanzocchio, 2000; Park et al., 2006). In comparison, AreB and its orthologues NreB and ASD4 contain a single N-terminal GATA zinc finger and a central leucine zipper motif.

The areB gene was initially identified through a selection strategy involving suppression of an areA loss-of-function phenotype. These mutations were associated with chromosomal rearrangements predicted to fuse the AreB GATA domain to cryptic activation sequences and indicated that the AreB DNA-binding domain could recognize AreA DNA-binding sites (Arst et al., 1990; Conlon et al., 2001). A further indication that AreB was likely to recognize similar DNA-binding sites to AreA but to function instead as a repressor came from the finding that NreB could prevent NRE activity in P. chrysogenum (Haas et al., 1997). A parallel situation has been described in S. cerevisiae, where opposing GATA factors regulate nitrogen catabolism and the positive regulators (Gln3, Nil1) contain conserved C-terminal GATA zinc fingers, while the negative regulators (Dal80/Uga43, Nil2/Gzf3) share a conserved N-terminally located GATA zinc finger and a leucine zipper motif (see Wong et al., 2008a). The possibility that A. nidulans areB and P. chrysogenum nreB null mutants were lethal and the loss of ascospore production associated with the N. crassa Asd4 mutant were not consistent with a simple role of these proteins in nitrogen control and cast doubt on the relationship between these factors. We have shown by phylogenetic analysis and gene sequence searches that AreB, NreB and ASD4 are orthologous. Furthermore, we have established that complete loss-of-function areB mutants can be readily generated by transformation and are viable.

Using both deletion and overexpression strains, we established that AreB plays a role in controlling nitrogen-regulated gene expression. Interestingly, this role lies in moderating the response to nitrogen limitation and nitrogen starvation rather than in mediating nitrogen metabolite repression. Loss of AreB function does not lead to derepression on ammonium and even with high-level overexpression, reporter gene activity is not affected on ammonium, where AreA activity is minimal. However, the areB deletion mutant exhibits elevated levels of AreA-dependent gene expression in response to nitrogen limitation and starvation and AreB overexpression inhibits these responses. These findings suggest that nitrogen-regulated genes such as fmdS are not normally expressed at maximal levels even under starvation conditions and that AreB acts to moderate these levels. A similar situation has been described by Cunningham et al. (2000b) in the nitrogen regulatory system in S. cerevisiae, producing ‘a highly responsive but equally highly buffered control circuit’. These authors suggest that as rich nitrogen sources are depleted, cells must synthesize proteins required to transport and degrade alternative nitrogen sources. However, this requirement occurs at a time when the cell can ill afford the expenditure of nitrogen resources. Therefore, as the GATA factors Gln3 and Gat1 activate nitrogen catabolic gene expression in response to nitrogen limitation, the negatively acting GATA factor Uga3/Dal80 begins downregulating this response to achieve finer control of GATA-factor-mediated expression (Cunningham et al., 2000b). Under nitrogen-starvation conditions, the effect of NnrA on AreA activity is minimal (Wong et al., 2007) and AreB would therefore provide an alternative means to dampen the starvation response.

The mechanism of AreB action is not known. However, AreB is neither acting at the level of transcriptional control of areA (Wong et al., 2007) nor preventing the nuclear accumulation of AreA during nitrogen starvation (data not shown). By analogy with the S. cerevisiae Uga43/Dal80 orthologue, which acts negatively under nitrogen-derepression conditions (Cunningham & Cooper, 1991; Coornaert et al., 1992; Coffman et al., 1997), we propose that AreB functions to antagonize AreA activity, by competition at the level of DNA binding, to finely modulate expression of nitrogen metabolic genes in response to nitrogen limitation and starvation. Both the zinc finger and the leucine zipper motifs are essential for AreB function. As dimerization may be a prerequisite for DNA binding, mutation of the leucine zipper region may also prevent the formation of a functional DNA-binding domain. Both motifs are also required for function in Uga43/Dal80 (Coornaert et al., 1992). It is also possible that AreB functions at other levels such as by direct interaction with AreA, possibly via their respective zinc fingers, to reduce AreA activity. It has not been established whether AreB is localized to the nucleus and, if so, under what conditions. The predicted protein contains a number of putative NLS sequences, including two classical NLSs and two (overlapping) canonical bipartite NLSs predicted with PSORTII as well as a putative Crm1-dependent NES sequence.

There was no indication from the phenotype of the areBΔ mutant that this gene has a role in sexual development. Interestingly, a new asd4 knockout mutant generated as part of the N. crassa systematic gene knockout project (Dartmouth College, Broad Institute) does not exhibit the sexual development defects observed in the original Asd4 RIP mutant (Feng et al., 2000). Inspection of the sequence annotation of the DNA fragment used to generate the Asd4 RIP mutant reveals, in addition to asd4 (NCU07039.3), another gene (NCU07038.3) and the 5’ portion of a flanking gene (NCU07037.3). The defect in sexual development is likely to be associated with RIP of one of these predicted genes of unknown function and not with RIP of asd4.

Our data indicate not only a role for AreB in nitrogen regulation, but pleiotropic action in vegetative growth and sexual development. The reduced growth and conidiation of the areBΔ mutant and severe growth inhibition and morphological effects of areB overexpression indicate that AreB participates in cellular processes outside the domain of AreA-dependent nitrogen regulation. Increasing expression of AreB led to a progressive reduction in growth and at high levels to a complete cessation of colony growth. The
basis of this effect is not known, but the growth and morphological phenotype of AreB overexpression may be complex, resulting from competition with several GATA factors and/or other proteins. There is no evidence that the negative effects of excess AreB are the result of a general effect on transcription, as illustrated by the AreA-independent acuJ-lacZ expression. It is predicted that AreB regulates the expression of non-nitrogen-related genes, and abnormal repression of these genes may have adverse effects on cell growth. Alternatively, excess AreB may compete, at the level of DNA binding, with another GATA factor(s). Phenotypes similar to those observed with areB overexpression have not been reported for any of the loss-of-function mutations for A. nidulans GATA-factor-coding genes (Kudla et al., 1990; Langdon et al., 1995; Haas et al., 1999; Han et al., 2001; Purschwitz et al., 2008). The presence of a leucine zipper motif in AreB raises the possibility that when in excess, AreB may sequester partner proteins required for normal cell growth. The ASD4 protein exists as a homotetramer in solution (Feng et al., 2000), and it is therefore possible that a multimeric protein structure dependent on the leucine zipper is required for AreB function. We have shown that deletion of the leucine zipper prevents the toxic effects of AreB overexpression. However, overexpression of the leucine zipper alone was not sufficient to cause AreB toxicity, suggesting that this is a less likely alternative. Interestingly, a recent publication has revealed that overexpression of nsdD, a GATA factor involved in sexual development, resulted in a colony phenotype that appears superficially similar to areB overexpression, with reduced growth and conidiation (Grosse & Krappmann, 2008). However, on microscopic examination, NsdD overexpression leads to an abnormal curling of the hyphae (Grosse & Krappmann, 2008), while we observed that AreB overexpression resulted in short swollen hyphae, so it is unlikely that the effects on colony morphology are related. Selection of suppressors of the overexpression phenotype may provide a means to identify the basis of this effect. This study has demonstrated a role for AreB in the modulation of nitrogen-regulated gene expression but has also highlighted the extent to which AreB may function beyond the realm of the AreA-dependent nitrogen regulation of gene expression.

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