Transcriptome analysis of nitrate assimilation in Aspergillus nidulans reveals connections to nitric oxide metabolism

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Summary

Nitrate is a dominant form of inorganic nitrogen (N) in soils and can be efficiently assimilated by bacteria, fungi and plants. We studied here the transcriptome of the short-term nitrate response using assimilating and non-assimilating strains of the model ascomycete Aspergillus nidulans. Among the 72 genes positively responding to nitrate, only 18 genes carry binding sites for the pathway-specific activator NirA. Forty-five genes were repressed by nitrate metabolism. Because nirA− strains are N-starved at nitrate induction conditions, we also compared the nitrate transcriptome with N-deprived conditions and found a partial overlap of differentially regulated genes between these conditions. Nitric oxide (NO)-metabolizing flavohaemoglobins were found to be co-regulated with nitrate assimilatory genes. Subsequent molecular characterization revealed that the strongly inducible FhbA is required for full activity of nitrate and nitrite reductase enzymes. The co-regulation of NO-detoxifying and nitrate/nitrite assimilating systems may represent a conserved mechanism, which serves to neutralize nitrosative stress imposed by an external NO source in saprophytic and pathogenic fungi. Our analysis using membrane-permeable NO donors suggests that signalling for NirA activation only indirectly depends on the nitrate transporters NtA (CrnA) and NtB (Crb).

Introduction

Nitrate (NO₃⁻) is the most abundant inorganic nitrogen (N) form in soils, and plants (Song et al., 2007), fungi (Joergensen and Wichern, 2008; Inselsbacher et al., 2009), bacteria (Klotz and Stein, 2008) and archaea (Erguder et al., 2009) all compete for this resource. Fungal biomass dominates in many soil types and although some fungi show denitrifying activities (Takaya, 2002; Fujii and Takaya, 2008; Kim et al., 2009) the presence of nitrate and nitrite assimilation genes in most fungal phyla (M. Gorfer et al., 2008) indicates a relevant ecological function of this metabolic system in soil nitrogen cycling. Provided sufficient carbon supply, fungi are able to efficiently utilize nitrate and nitrite for growth and incorporate this nitrogen source into biomass through reduction to ammonium and subsequent metabolism to glutamate and glutamine (Cove, 1979). Because of the high metabolic cost of nitrate reduction, negative feedback regulation by ammonium and glutamine prevents the expression of genes required for the uptake and assimilation of nitrate or nitrite (Caddick et al., 1994; Marzluf, 1997; Berger et al., 2008). Phenomenologically similar circuits also regulate nitrate assimilation in photosynthetic eukaryotes such as the algae Chlamydomonas reinhardii (Fernandez and Galvan, 2008) and plants (Crawford and Arst, 1993). However, because of additional constitutively expressed nitrate transporters, regulation of nitrate reductase (NR) by light and the function of nitrate as a developmental signal, nitrate regulation in plants is more complex (Daniel-Vedele et al., 1998; Zhang and Forde, 2000; Glass et al., 2002; Wang et al., 2003; Miller et al., 2007).

In Aspergillus nidulans, the transcriptional control of the assimilatory system is carried out by the synergistic action of the nitrate-specific activator NirA (Burger et al., 1991; Strauss et al., 1998) and the nitrogen status-sensing regulator AreA (Kudla et al., 1990). Nitrate and nitrite uptake is carried out via specific permeases (Brownlee
and Arst, 1983; Unkles et al., 1991; 2001; Wang et al., 2008) and subsequently, intracellular nitrate mediates accumulation of NirA in the nucleus. We have previously shown that nitrate functions to disrupt the interaction between the NirA nuclear export signal and the KapK–NplA nuclear export complex (Bernreiter et al., 2007). However, nuclear accumulation is required, but not sufficient for NirA activity but the exact mechanism by which nitrate acts to transform NirA into an active transcription factor has not been elucidated so far. Interestingly, in the simultaneous presence of external nitrate and ammonium NirA remains nuclear, but does not efficiently bind DNA. In vivo DNA binding of NirA requires, in addition to nitrate, an active, DNA-bound form of AreA (Narendja et al., 2002). The two transcription factors physically interact (Muro-Pastor et al., 2004; Berger et al., 2008) recruit chromatin remodelling complexes (Muro-Pastor et al., 1999; Berger et al., 2008) and act synergistically to activate transcription. The recruitment of chromatin remodelling complexes to the nitrate locus by AreA can be artificially triggered by extended nitrogen starvation (Berger et al., 2008), a condition in which very high levels of AreA accumulate in the nucleus (Todd et al., 2005). AreA is not only required for expression of nitrate genes, but positively regulates, in most cases in cooperation with the respective pathway-specific transcription factor, a range of permeases and catabolic enzymes required for the utilization of alternative, metabolically ‘costly’ nitrogen sources such as nucleotides, amino acids or primary and secondary amides (Caddick et al., 1994; Scanzocchio, 2000 and references therein). Several positive and negative feedback mechanisms operate to ensure that AreA is only active when available nitrogen is limited. Under nitrogen limitation conditions, AreA abundance and activity increases because of higher areA gene transcription (Langdon et al., 1995) and mRNA stability (Morozov et al., 2000; 2011; Caddick et al., 2006), and a preferential nuclear localization of the protein (Todd et al., 2005). Nitrogen sufficiency reduces AreA abundance and the activity of the protein is reduced by interaction with the negative regulatory protein NmrA (Andrianopoulos et al., 1998; Wong et al., 2007).

At the genomic level, the response to nitrate has been extensively studied in plants. Roughly 10% of the Arabidopsis thaliana detectable transcriptome responds to nitrate supply (primary response) and includes upregulation of genes involved in nitrate transport and assimilation. Plant nitrate assimilation is strongly influenced by light and the circadian rhythm, and responds to the carbon status, as well as hormonal and developmental signals (reviewed in Krouk et al., 2010). Because of this complexity, the nitrate response has been found to be highly context-dependent (genotypes, slight differences in experimental design) as only about 300 genes were identified in a meta-analysis of transcriptome datasets from different laboratories to be consistently nitrate-regulated (Gutierrez et al., 2007). It has been hypothesized that the high variability between different datasets might reflect a complex gene network providing the necessary nutritional adaptability of sessile organisms to fluctuating environmental conditions. Despite the enormous progress made in understanding the interface between nitrogen and other regulatory sub-networks using Systems Biology approaches, little is still known on transcriptional regulators of plant nitrate assimilation genes. Several regulatory proteins have been identified as responding to NO3−, such as the MADS-box NAR1 protein controlling lateral root development (Zhang and Forde, 2000), several protein kinases regulating the uptake system (Ho et al., 2009; Hu et al., 2009), and NLP7, a nuclear protein with putative regulatory functions in nitrate signalling and the starvation response (Castaings et al., 2009). NLP7 is similar to the C. reinhardtii NIT2 regulator, which was shown to be the pathway-specific activator for nitrate assimilation genes in this unicellular algae (Camargo et al., 2007). Initial protein–DNA interaction studies in the tobacco NR gene promoter showed a nitrate-inducible protection of GATA sites (Rastogi et al., 1997) reminiscent of AreA binding in Aspergillus nitrate-inducible genes (Muro-Pastor et al., 1999; Berger et al., 2008). Unfortunately, the involvement of putative plant GATA factors in nitrogen regulation was not substantiated by further studies leaving the nature of plant nitrate assimilation regulators still uncertain.

The work presented here shows that the fungal nitrate response reprograms about 1% of the A. nidulans transcriptome including genes for nitrate transport and metabolism. Several novel genes coding for putative regulators of the nitrate response were uncovered and we also found an inducible nitric oxide-detoxifying flavohemoglobin (FhbA) as target of the pathway-specific regulator NirA. Characterization of fhb genes provided evidence for an important physiological role of the enzymes under a variety of environmental conditions. Results using membrane-permeable NO donors suggest that signalling for NirA activation only indirectly depends on the nitrate transporters NtA/CmA and NtB/CmB.

Results

Experimental set-up and physiological responses to nitrate induction and N-starvation

Our interest was to identify A. nidulans genes that showed a short-term response to the presence of nitrate in the growth medium (induced conditions), as compared with growth conditions in which ammonium was supplied to the cultures (repressed conditions). In order to limit indirect effects from downstream metabolites of nitrate assimila-
tion, i.e. the formation of repressing nitrogen metabolites during continuous nitrate assimilation, we first performed a time series of nitrate induction. In these cultures we monitored the expression of marker genes, which are known to sensitively respond to the intracellular nitrogen status (Fig. 1A) and free amino acid pools (Fig. 1B and Table S1). Maximum niiA mRNA levels were reached after 40 and 50 min of induction, indicating maximum synergistic NirA–AreA activity. Maximum niiA levels coincided with a minimum of intracellular glutamine concentration. areA and gdhA levels responded roughly 20 min earlier and reached maximum transcript levels after 30 min. Up until 50 min of nitrate induction intracellular Gln levels continued to decline despite fully induced NR enzyme levels (Fig. S1). This indicates that the low Gln levels found at this time point are the result of increasing metabolic activity, which creates a higher demand for amino acids. In agreement with our previous results (Berger et al., 2008) Gln, but not Glu, appears to serve as intracellular amino acid storage and signalling pool (Fig. S2). Continuous NO₃⁻ assimilation (starting from 50 min onwards) produced more Gln than was utilized by metabolic activities and this led to a replenishment of the free Gln pool (Fig. 1B). Consequently, higher Gln levels led to partial AreA inactivation and thus to reduced transcriptional activity of the NirA–AreA activator complex and to a drop in amounts of niiA mRNA (Fig. 1A). Interestingly, areA and gdhA mRNA levels were continuously decreasing between 30 and 60 min of nitrate induction although the lowest levels of Gln were found at 50 min. This indicates that transcription and/or mRNA stability of areA and gdhA responds to additional metabolic signals related to the intracellular nitrogen or carbon status. The exact nature of such signals remains to be identified.

Growth conditions for transcriptome analysis

As a consequence of the time series experiments, we performed our transcriptome analysis with RNA derived from 30 min induced cultures because the low free Gln pool would prevent negative interference of assimilation products with the induction process. The 30 min induced cultures were compared with fully repressed cultures (e₁ = WT₆₃₇NH₄⁻_vs_WT₆₃₇NO₃⁻), i.e. cells that were transferred after pre-growth on ammonium again to ammonium-containing medium without an interim starvation period. In order to test which of the nitrate-regulated genes are dependent on the pathway-specific activator NirA, we performed an identical set of induction experiments with a nirA637 loss-of-function strain (e₃ = nirA637₆₃₇NH₄⁻_vs_nirA637₆₃₇NO₃⁻). It is important to note that nitrate uptake is only slightly impaired in nirA⁻ strains (Brownlee and Arst, 1983). This experiment allowed us to differentiate between nitrate-regulated genes that are a direct target of NirA and genes, which respond to nitrate induction independently of the transcription factor.

Physiologically, nitrate induction in a nirA loss-of-function strain is equivalent to strong nitrogen limitation, because the NR activities found during the induction period in the wild-type strain were reduced to very low levels in the nirA637 mutant (Fig. S1). Therefore, we also performed transcriptome analysis of wild-type cells subjected to continuous nitrogen starvation by incubation on nitrogen-free medium (–N conditions) for 60 min and compared this transcriptome with cells continuously grown on ammonium (e₂ = WT₆₃₇NH₄⁻_vs_WT₆₇). We reasoned that these conditions would enable us to differentiate between genes responding to nitrogen limitation and genes responding to nitrate in the absence of the transcription factor.

Overview of differentially expressed genes

Analysis of the microarray data revealed a total of 135 differentially expressed genes (DEGs) for all three experimental set-ups when thresholds of fourfold change (log₂ ≥ 2) and a t-test value of P ≤ 0.05 were applied (see Experimental procedures for details). Eighty-four genes responded to nitrate induction or nitrogen starvation by upregulation whereas 51 genes showed lower expression under these conditions. The Venn diagram in Fig. 2 shows a synopsis of DEGs from all experiments. Among the genes upregulated by at least fourfold 76 genes responded to NO₃⁻ (72 in WT and four in nirA⁻) whereas four genes were also upregulated by N-limitation (one in WT⁻ and three in nirA⁻NO₃⁻). An additional four genes positively responded to N-limitation in the wild type and three genes showed upregulation only in NO₃⁻-induced cultures in nirA⁻ strains. Also surprising is the comparatively low number of genes responding by at least fourfold upregulation to the limited time of nitrogen starvation (60 min total) applied in this experiment. The regulatory response during N-starvation (compare Tables S3 and S4) seemed to be less strong (5- to 10-fold upregulation), compared with the nitrate induction response (up to 60-fold upregulation). It should be noted, however, that our experimental conditions most probably do not represent full nitrogen starvation. A much more pronounced change in global gene transcription could be expected in this case, similar to what has been observed in other fungi (Matsuo et al., 2007; Schonig et al., 2008; Staschke et al., 2010).

A considerably high number of genes (45 DEGs) were downregulated in response to NO₃⁻ induction in the wild type. This negative response depended on NirA function and/or the nitrate assimilation process because no downregulated gene was detected in nirA637 under NO₃⁻ conditions. N-limited cells (WT-N) did not respond strongly
Fig. 1. A. Transcriptional profile of marker genes *niiA* (nitrite reductase), *area* (GATA TF) and *gdhA* (NADPH-glutamate dehydrogenase) during the nitrate induction process in the wild-type strain. mRNA levels were determined by RT-qPCR (see Experimental procedures). The normalized levels of specific transcripts are shown relative to *acnA* (actin) over a time period of 180 min. Aliquots were taken from the cultures at the induction starting point (0 min) and then after 10, 20, 30, 40, 50, 60, 90, 120 and 180 min following induction by 10 mM NaNO₃. Standard deviations are calculated from three independent biological replicates.

B. Schematic representation of experimental design and measurements of intracellular free amino acid levels. Cultures were grown in glucose minimal medium (GMM) in the presence of 10 mM NH₄⁺ as the sole N-source for 14 h (preculture NH₄), harvested, washed, resuspended in GMM lacking any nitrogen source and incubated under these conditions for 30 min (−N). Wild-type and *nirA*− cells subsequently received 10 mM NaNO₃ (NO₃), whereas starved wild-type cells were further incubated without nitrogen addition (−N).

Incubation proceeded for another 30 min until they were harvested for the microarray experiment (harvest point). For qPCR analysis and amino acid level determination cells were further incubated until 180 min. The graph shows intracellular free glutamine (Gln) levels measured immediately before the starvation period (−30), at the end of the starvation period (0) and then subsequently after 10, 20, 30, 40, 50, 60, 90, 120 and 180 min of NO₃⁻ induction (full lines) or after 30, 60, 90 and 150 min of continuous starvation (dotted lines) in wild-type cells. Values on the y-axis represent nmoles free Gln mg⁻¹ dry weight (DW), standard deviations are from three independent experiments.
by downregulation: only six genes were repressed by at least fourfold, but as in this case, like in upregulated DEGs, the limited time of N-deprivation might have restricted the response.

The function of NirA in the induction process

A vast majority (72 genes) of the total 80 genes positively responding to NO$_3^-$ induction in e1 (WT) and e3 (nirA$^{-}$) required the function of NirA. The central importance of NirA in the induction process is evident from the Venn diagram (Fig. 2) and functional assignments (Table 1 and Table S2), which shows that only seven genes responded to NO$_3^-$ in the nirA$^{-}$ mutant and three of these genes overlapped with the group of starvation-induced genes, making 95% of DEGs NirA-dependent for NO$_3^-$ induction. NirA requirement could be either direct, i.e. promoters carry functional 5’CTCCGHGG consensus target binding sites for NirA, or indirect, i.e. the upregulation response depends on nitrate or its metabolism. Bioinformatic analysis of 1 kb upstream regions of the 72 open reading frames revealed 16 promoters, which carry either confirmed or putative NirA binding sites (Table S3). Thus, for 51 NO$_3^-$- and NirA-dependent genes upregulation was indirect, most probably depending on the active nitrate assimilation process in which the transcriptionally active NirA–AreA complex would trigger secondary transcriptional activation events. In contrast, only seven genes positively responded to nitrate in a nirA637 loss-of-function mutant. Three of those genes are identical to genes responding to nitrogen starvation and thus are genes not requiring NO$_3^-$ for upregulation. Only one gene was commonly upregulated by NO$_3^-$ (but not under $-$N conditions) in the nirA$^+$ and nirA$^{-}$ strain and therefore should be classified as a gene directly responding to the presence of NO$_3^-$ in the cell. Interestingly, three genes were upregulated by NO$_3^-$ only when NirA is not functional suggesting a negative role for NirA and/or NO$_3^-$ metabolism in the transcriptional activation process of these genes. Because none of the three genes carries predicted

![Fig. 2. Differentially expressed genes in the nitrate and starvation response. The Venn diagram shows genes at least fourfold (log$_2 \geq 2$) upregulated (A) or downregulated (B) in response to different treatments. Numbers in the circles represent numbers of responding genes and numbers in overlapping sections represent genes that are commonly regulated by two or three conditions and/or in different strains. Circle 1 represents genes differentially regulated by NO$_3^-$ induction in the wild type (experiment e1. WT$^+$ vs. WTNO$_3^-$), circle 2 represents genes regulated by nitrogen depletion in wild-type cells (experiment e2. WT$^+$ vs. WT$^-$), whereas circle 3 represents genes of NO$_3^-$-treated nirA$^-$ cells that can not metabolize this nitrogen source (experiment e3. nirA$^{-}$NH$_4^+$ vs. nirA$^{-}$NO$_3^-$).]

| Main categories$^a$ | NO$_3^-$-induced WT | NO$_3^-$-repressed WT | $-N$-induced WT, nirA$^{-}$ | $-N$-repress. WT, nirA$^{-}$ |
|---------------------|----------------------|-----------------------|---------------------------|---------------------------|
| Total number of genes | 72                   | 45                    | 12                        | 6                         |
| NO$_3$ metabolism, direct NirA targets$^b$ | 16                   | 0                     | 0                         | 0                         |
| NirA targets, known functions | 6                   | 0                     | 0                         | 0                         |
| NirA targets, predicted or unknown function | 10                  | 0                     | 0                         | 0                         |
| Regulatory role$^c$ | 8                    | 8                     | 2                         | 2                         |
| Amino acid metabolism$^d$ | 11                   | 0                     | 8                         | 3                         |
| Carbon metabolism$^e$ | 5                    | 5                     | 1                         | 0                         |
| Lipid/secondary metabolism$^f$ | 11                  | 6                     | 0                         | 1                         |
| Hypothetical proteins, no known function$^g$ | 31                   | 25                    | 1                         | 0                         |

$^a$ Genes are grouped according to major metabolic categories, including a subcategory for genes putatively playing a role in nitrate assimilation. Genes can be grouped into more than one subcategory.

$^b$ Classification based on gene regulation (only induced in WT) and known or putative NirA binding consensus sites present in the 1 kb upstream region.

$^c$ Function in the metabolic pathways could be known or predicted based on similarity to proteins with known function.
NirA binding targets in their 1 kb upstream region, the negative function of NirA is most likely indirect.

Established and putative functions of genes positively responding to NO$_3^-$

Among the genes that most strongly responded to nitrate by 15- to 60-fold upregulation are the extensively characterized genes known to be involved in the nitrate assimilation process, such as niaD and niaA (coding for NR and nitrite reductase (NiR) respectively), the two nitrate transporter genes cmA/mrrA and cmB/mrrB, and the nitrate transporter gene nitA. A gene strongly upregulated (12-fold increase) but so far not known to respond to nitrate induction encodes a putative flavohaemoglobin (AN07169.1). This family of proteins is known to function in nitric oxide (NO) detoxification, converting the short-lived NO radical to nitrate (Gardner et al., 1998; Foster et al., 2009). The A. nidulans genome shows two highly conserved putative flavohaemoglobins and based on the known function of NO in signalling events, we studied these genes in more detail (see below). It is noteworthy that genes with a putative involvement in morphology determination and cytoskeleton function (putative chitin synthetase III, AN4367.3; putative hydrophobin gene AN7539.3; putative fluffy-determinant gene AN9451.3;) were upregulated by NO$_3^-$ induction, although these genes do not carry predicted NirA binding sites in their 5′UTR and thus the response is likely to be a consequence of the nitrate assimilation process. (Table S3). Several other novel genes that positively responded to nitrate encode proteins with a predicted function in the mobilization of extracellular and internal nitrogen sources. For example, we find genes coding for proteases and genes involved in the metabolism and transport of nitrogen-containing compounds such as amino acids, urea, allantoin and nucleotides (see Table S3). Our experimental set-up also revealed the activation of genes coding for putative signalling and regulatory proteins, such as kinases, GTP-binding proteins, guanylate kinase, helicase and transcriptional activators and repressors. Leucine biosynthesis and acetyl-coenzyme-A metabolism seem to occupy a special position in nitrate assimilation. Hypothetical isopropylmalate dehydrogenase (AN2793) and isomerase (AN5886) as well as dihydroxy-acid dehydratase (AN7358) genes are proposed to be involved in leucine biosynthesis and are upregulated by NO$_3^-$ as they do not contain predicted NirA binding sites within 1 kb of their 5′regions, the induction is indirect through the NO$_3^-$ assimilation process. Acetyl-coA metabolism (putative D-lactate dehydrogenase AN0628; enoyl-coA hydratase AN0180) and connected lipid metabolic genes (putative mitochondrial malic enzyme AN6933; lipase AN2602; esterase AN2834) also appear to be an overrepresented category. As two of these genes contain predicted NirA binding sites in their promoters (AN2834 and AN0628) the induction response might be directly performed by NirA. The connection of NO$_3^-$ and leucine/coenzyme-A metabolism might provide a link to secondary metabolism. It is known that NO$_3^-$ stimulates production of sterigmatocystin and penicillin in A. nidulans (Brakhage, 1998; Feng and Leonard, 1998) and acetyl-coA is a crucial precursor for the vast majority of fungal secondary metabolites including antibiotics (Keller et al., 2005). The elevated transcription of genes responsible for acetyl-coA, isopropylmalate and L-leucine biosynthesis (pathways connected to the penicillin precursors valine and α-L-aminoadipic acid) would be a logical hypothesis to explain the stronger production of secondary metabolites on nitrate as sole N-source in A. nidulans.

Genes positively responding to nitrogen limitation

Under the conditions (1 h nitrogen limitation) and thresholds (at least fourfold upregulation) applied in this study, only 12 genes were significantly upregulated by nitrogen limitation (Table 1 and Table S4). Four of these genes overlapped with the group of nitrate-induced DEGs and thus upregulation was most likely directly related to the intracellular glutamine level, which was basically identical between the nitrate-induced and the N-starved conditions. A strong response was obtained for genes involved in ammonium uptake and metabolism: meaA, the low-affinity transporter (AN7463), gdhA, the NADPH-dependent glutamate dehydratase (AN4376) and glnA, glutamine synthetase (AN4159). Also the recently confirmed major urea transporter similar to Dur3 (AN0418) (Abreu et al., 2010) responded positively to nitrogen limitation. The remaining four DEGs, which code for a peptide transporter (AN8903), a putative dipeptidyl-peptidase (AN2572), a putative Zn-cluster transcription factor (AN1927) and a conserved hypothetical gene (AN1404) did not overlap with the NO$_3^-$-induced group despite the fact that identical Gln levels were observed between these two conditions. We hypothesize that these four latter genes respond to additional signals, e.g. low metabolic activities. In this context it is interesting to note that concentrations of some free amino acids were significantly higher after 1 h starvation than after 30 min NO$_3^-$ induction. This might be due to re-assuming metabolic activity and demand for amino acids in the NO$_3^-$-induced cells, compared with lower activities in the N-starved cells. Table S1 shows a comparison of amino acid concentrations in the wild type between these two conditions and for Asp, Asn, Arg, Ala and Tyr the values in NO$_3^-$-induced cells were significantly lower than in the metabolically less active cells incubated under N-limitation.
Genes negatively responding to NO$_3^-$ and N-limitation

Addition of NO$_3^-$ resulted in downregulation of 45 genes (Table S5). None of these genes appeared to be repressed in the nirA- strain suggesting that the function of NirA is required directly or indirectly for this negative response. Only one out of the 45 genes, i.e. AN10059, which putatively encodes a fungal-specific C6 transcription factor, harbours a consensus NirA binding site. Therefore, all the other 44 genes most likely responded to the active nitrate assimilation process. The majority of repressed genes (25 genes) encode hypothetical or conserved hypothetical proteins with no predicted functions. As shown in Table S5, five genes encode proteins with similarities to enzymes involved in the breakdown of complex carbon sources (α- and β-galactosidase, mannanase and rhamnogalacturan-lyase) and the products of eight of these genes show similarities to proteins, which regulate cellular metabolism (e.g. putative signalling kinase, mRNA stability regulation, transcription of ribosomal genes).

Not overlapping with the NO$_3^-$-repressed genes was a group of six genes that were downregulated by 60 min N-limitation (Table S6). These genes putatively encode proteins functioning in diverse cellular processes with no preference to a specific pathway.

Flavohaemoglobin fhbA is co-regulated with the nitrate assimilation system

To gain further insight into the role of flavohaemoglobins during nitrate assimilation we studied these genes in more detail. The NO$_3^-$-inducible gene is most similar to yeast YHB1 and Cryptococcus neoformans FHB1 and was designated fhbA according to the A. nidulans nomenclature. The second putative flavohaemoglobin gene (designated fhbB) was only twofold induced by nitrate (data not shown) and the predicted protein carries a putative mitochondrial target sequence similar to fhbB in Aspergillus oryzae (Zhou et al., 2009). Both genes were deleted individually and in combination in order to study their function during the NO$_3^-$ induction process. Northern analysis (Fig. 3) of fhbA confirmed microarray data and showed that fhbA is co-regulated with the main assimilatory genes such as niaD and niaA. Strong nirA-dependent induction by NO$_3^-$ conditions and high constitutive transcript levels in a niaDΔ background (Cove and Pateman, 1969; Hawker et al., 1992) were observed for fhbA.

FhbA expression is AreA-independent

We tested if fhbA is regulated as other known nitrate-responsive genes, i.e. induced by NO$_3^-$ and subject to nitrogen metabolite repression by external ammonium (NH$_4^+$) mediated through the inactivation of the co-activator AreA. Figures 3 and 4 also show the expression profile of the niiA gene used as internal control. niiA was strongly induced by NO$_3^-$ and NO, dependent on AreA function, and subject to nitrogen metabolite repression when NH$_4^+$ was added to the medium simultaneously with NO. In contrast, expression of fhbA was independent of AreA, showing no ammonium-induced repression in the presence of NO and even a slight constitutive level under fully repressing conditions (NH$_4^+$ in WT and areA-). This is a surprising result, as all genes known so far to be regulated by NirA depend also on functional AreA. Moreover, four AreA consensus binding sites (5′WGATAR) are present within 500 bp of the fhbA promoter, in addition to a perfectly matching NirA site at position -225 from the ATG start codon. This sequence arrangement would suggest a canonical NirA–AreA synergism in fhbA activation although this is not the case. There are at least five other transcriptional regulators known in A. nidulans to contain functional GATA-type DNA-binding domains and some of these proteins, such as the iron-responsive repressor SreA (Oberegger et al., 2001) or the light-responsive regulators of sexual development LreA and LreB (Purschwitz et al., 2008), might bind to the fhbA promoter and participate in transcriptional regulation of this gene.

The role of NO in the regulation process

Treatment of the cells with the synthetic NO donor DetanNONOate led to a strong upregulation of all tested nitrate assimilation genes. The transcriptional response to NO was not dependent on FhbA or FhbB function, i.e. occurring equally well in the fhbAΔ fhbBΔ double mutant, making it unlikely that the induction by NO is only a consequence of NO$_3^-$ formation by flavohaemoglobin activities. DetanNONOate induction could also be triggered by formation of NO$_2^-$, which arises spontaneously from NO oxidation in the medium and inside the fungal cell (Yamasaki, 2000). To differentiate effects of NO and NO$_2^-$, we measured the intracellular concentrations of NO (Fig. S3) by electron paramagnetic resonance (EPR) spectroscopy (Xu et al., 2005; Pirker et al., 2008) and of NO$_2^-$ and NO$_3^-$ (Fig. 6) by a modified colorimetric assay (Inselsbacher et al., 2009). EPR is based on the trapping of NO radicals by the spin trap Fe$_2$-diethylthiocarbamate (Fe$_2$-DET) forming a Fe$_2$-DET–NO complex. This complex is detected as triplet in the EPR spectrum, which arises from the interaction of the unpaired electron with a nitrogen nucleus (spin I = 1) in the complex. Additionally, growth tests were performed (Fig. 5) using strains, which lack or have strongly reduced NO$_3^-$ and NO$_2^-$ transport respectively (Unkles et al., 2001; Wang et al., 2008). The growth tests (Fig. 5) showed that the transporter double mutant (lane crmA crnB) is impaired in NO$_3^-$ and NO$_2^-$.
utilization leading to a strongly reduced growth on these N-sources. The same strain grown on nitrate or nitrite plus NO (rows NO₃⁻ + NO and NO₂⁻ + NO) showed significantly improved growth, presumably because of formation of NO₃⁻ by flavohaemoglobins and of NO₂⁻ by intracellular spontaneous oxidation. The amine-containing Deta-moiety (diethylene-triamine) of DetaNONOate did not serve as a relevant nitrogen source in these growth tests as both niiA⁻ and nirA⁻ strains showed very poor growth on all DetaNONOate concentrations tested. This indicates that the extracellular and intracellular conversion of NO to NO₂⁻ and the intracellular generation of NO₃⁻ by FhbA and FhbB are the responsible mechanisms for DetaNONOate utilization as sole N-source. EPR measurements to determine intracellular NO levels (Fig. S3) showed that in all NO-treated cells high amounts of this free radical were
Northern blot analysis of NO3-treated cells (Fig. 6B) we found about 20 Fhb-generated NO3 simultaneously induced NR would effectively reduce the tent with these results is that, in the wild type, the aNONOate treatment. The simplest explanation consist-
ingly, the basal level expression of putative FhbB did not
subsequently accumulation, was not observed. Interest-
ingly, the wild-type and nirA- strains also showed no
detectable intracellular NO3 accumulation after Det-
aNONOate treatment. The simplest explanation consist-
tent with these results is that, in the wild type, the
simultaneously induced NR would effectively reduce the
Fhb-generated NO3 to NO2-. Indeed, a control strain
lacking NR activity (niaDΔ) showed very high NO3-
levels after NO addition whereas in nirA- strains, lacking
niaD and fhb expression, NO conversion into NO3- and
subsequently accumulation, was not observed. Interest-
ingly, the basal level expression of putative FhbB did not
result in sufficient activity that would allow the detection
of NO3- in a nirA- strain. In contrast, a niaDΔ strain
showed clearly detectable NO3- levels under non-
induced (arginine) growth conditions (see insert panel in
Fig. 6A) and these nitrate levels might be sufficiently high to promote expression of NirA target genes (niaA, fhbA) under non-induced conditions.

During these experiments we noted that the fhb double
deletion strain displayed considerably lowered intracellu-
lar NO3- levels (25% of WT) when grown on NO3- (Fig. 6A). This could be the consequence of reduced NO3-
transporters function similar to the nirA- strain (50% of
WT) or could be due to increased NR activities. We there-
fore analysed NR activities under all relevant conditions
(Fig. 7). We found that NR activities were not changed in
fhb double mutants during growth on NO3- but were dras-
tically reduced in the mutants when DetaNONOate was
added to the medium. These results provide evidence for
an important role of flavohaemoglobins in protecting NR
activity from damage directly by the NO radicals or indi-
rectly by NO-derived reactive nitrogen species. This regu-
ulatory role of flavohaemoglobins seems to be relevant
only at high concentrations of NO, as fhbAΔ single and
fhbAΔ/fhbBΔ double mutants grew normally on NO3- as
the sole N-source (Fig. 5). In contrast, stronger growth
suppression was seen in fhb mutants compared with the
wild type on NO3- + NO, consistent with considerably
reduced NR activities in the presence of the NO donor
DetaNONOate (Fig. 7).

A role for flavohaemoglobins in Aspergillus natural
environments?

Growth tests performed under a variety of conditions
(Fig. 5) provided support for the important function of the
inducible fhbA flavohaemoglobin in protecting A. nidulans
cells from nitrosative damage. The stronger toxic effect of
NO in fhbAΔ strains could indicate that not only NR, as
shown above by enzymatic assays, but also NiR activity is
sensitive to NO and that FhbA protects against this
damage. Growth tests performed at different ambient pH
values (Fig. 8) provided evidence that FhbA function is
necessary to allow optimal NiR activity. Whereas the wild-
type and fhbBΔ strains utilized NO3- over the whole range
of acidic pH values tested here (pH 6.8 to pH 4.5), the strain carrying fhbA deletions (fhbAD, fhbADfhbBD and fhbADfhbBdniADΔ) showed increasing sensitivity to 10 mM NO_2^– when ambient pH was reduced stepwise. As the growth reduction could also be due to generally higher NO stress susceptibility in fhbΔ strains and only indirectly related to NiR activity we tested growth in the presence of an additional nitrogen source, 3 mM L-arginine. When growth became independent from utilization of NO_2^– as the sole N-source and hence on functional NiR, the fhbΔ strains grew as well as the wild type over the whole range of pH conditions. These data support the results from growth tests using the NO donor Det-aNONOate (compare Fig. 6, row NO_2^– + NO) and strongly
suggest that NiR activity is sensitive to high levels of NO and that FhbA function is required for NiR activities under these conditions. Unfortunately, because of the significant spontaneous oxidation of NO to NO$_2^-$ we were not able to directly measure NiR activities under these conditions (data not shown). Finally, an interesting phenotype was obtained in these pH dependence tests in strains lacking either NiR (nia$\Delta$) or NirA (nir$A^{-}$, nirA$\Delta$) activity. In both genetic backgrounds the utilization of arginine was impaired in the presence of NO$_2^-$ already at the standard pH 6.8 of the medium and was almost totally inhibited as pH gradually decreased (Fig. 8, lanes nii$A^{-}$, nir$A^{-}$ and nirA$\Delta$: compare rows arg at different pH with rows NO$_2^-$/arg at different pH). These phenotypes indicate that high intracellular NO$_2^-$ levels (present in nii$A^{-}$ and nir$A^{-}$ strains incubated on NO$_2^-$/arginine; T. Schinko and J. Strauss, unpubl. obs.) negatively influence transporters, enzymes or regulators of the arginine catabolic pathway. At the moment it is not clear whether the utilization of other amino acids is also affected by NO$_2^-$ at low ambient pH.

As no typical NO synthetase producing NO and citrulline from L-arginine has yet been identified in fungi, it will be interesting in future to see if NO generating and detoxifying activities in A. nidulans may be related to arginine metabolism.

**Discussion**

We have shown here that the nitrate assimilation process in A. nidulans remodels central components of the nitrogen metabolism. As expected from previous studies, genes involved in uptake and reduction of nitrate to ammonium are induced by the main transcriptional activator NirA and all of these pathway-specific genes carry functionally char-
acterized or putative NirA binding sites (consensus 5′CTC-GHG). Although more than 800 genes in the genome show the presence of NirA consensus sites within 1 kb of their 5′ region, only roughly 80 genes are positively regulated by NO₃⁻. The vast majority of these genes require nitrate metabolism, and thus NirA, for this response, but only 19 of these NO₃⁻-responsive genes carry confirmed or predicted NirA consensus sites in their promoters.

We identified more than 50 novel genes, which were upregulated by nitrate and some of them encode enzymes that are required for the subsequent metabolization of the assimilation product (NH₄⁺), e.g. two types of glutamate dehydrogenases, glutamine synthetase, and three enzymes involved in leucine and coenzyme-A biosynthesis. Notably, the latter genes and some additional genes coding for amino acid transport and metabolism also responded to N-limiting conditions (60 min starvation). This regulatory response suggests that under our conditions (30 min NO₃⁻ induction after 30 min of N-starvation) the amount of NO₃⁻ channelled through the assimilatory system was still not producing sufficient amounts of assimilation products as to fully relieve the starvation response. This metabolic status was also seen in direct amino acid measurements. Despite induced NR activity on NO₃⁻ medium (Fig. S1), the intracellular level of glutamine, which is the molecule known to signal the intracellular nitrogen status (Margelis et al., 2001; Berger et al., 2008), continued to decline during the first 30 min of nitrate assimilation, identical to the falling Gln levels under N-starvation (Table S1). This apparent discrepancy could be explained by the increasing metabolic demand for amino acids in NO₃⁻-induced cells, which presumably resumed growth after NO₃⁻ supply. It was only after approximately 60 min of continuous assimilation that the pool of free Gln started to increase again reaching a steady-state level of balanced repletion/depletion after roughly 2 h. Because little is known about the eukaryotic nitrate signalling cascade, we were mainly interested in the short-term response to nitrate. Thus, our experimental set-up was generally designed to largely avoid the interference with nitrate metabolism, using short incubation times as well as a nirA⁻ mutant unable to turn on the assimilatory system. Doing so, we identified several putative regulatory components that might be involved in the signalling cascade, e.g. hypothetical proteins with similarity to guanylate kinases (AN10188), protein kinases (AN3181), transcription factors (AN7343, AN5405) or GTP-binding proteins (AN7222). On the other hand, several genes were repressed by the addition of NO₃⁻ (predicted transcription factor AN10059, kinase AN10082 or nucleotide-binding domain protein AN10344) and these putative regulators might also be involved in NO₃⁻-specific signalling.

A novel target of NirA was found to be a flavohaemoglobin-encoding gene (AN7169). This is a member of a gene family encoding evolutionarily conserved proteins, which appear to be widely distributed in bacteria and fungi. Their main role is to metabolize NO radicals, reactive nitrogen species implicated in protein nitrosylation, signalling and nitrosative stress. Flavohaemoglobins are known to catalyse NO di-oxgenation, the NADPH, FAD and O₂-dependent conversion of NO to NO₃⁻ (Gardner et al., 1998; Poole and Hughes, 2000). In

Fig. 7. Nitrate reductase (NR) activity was measured in A. nidulans wild type (WT), and in mutants lacking nitrate reductase (niaDΔ), both flavohaemoglobin genes (fhbAΔ fhbBΔ), or NirA function (nirA¹). Strains were pre-grown on arginine as non-inducing nitrogen source and harvested mycelia were starved prior to shift to media containing different nitrogen sources: 3 mM arginine (NI, shaded bars); nitrate-inducing conditions with 3 mM arginine + 10 mM nitrate (NO₃, white bars); or nitric oxide-inducing conditions with, 3 mM arginine + 1.5 mM DetaNONOate (NO, black bars). Data of two independent biological experiments were merged and the activities of the wild-type NR under nitrate-inducing conditions were arbitrarily given a value of 1 (100%). Bars and respective standard deviations show NR activities relative to wild type under NO₃⁻-induced conditions.
|         | WT | niaDΔ | niiAΔ | nirAΔ | fhaΔ | fhaΔ | fhhBΔ | fhhBΔ | niaΔ | niaΔ | crnAΔ | crnBΔ | niaDΔ |
|---------|----|-------|-------|-------|------|------|-------|-------|------|------|-------|-------|-------|
| **72 h**|    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NH₄⁺** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 6.8** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NH₄⁺** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NH₄⁺** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.0** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NH₄⁺** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 4.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 6.8** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.0** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 4.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 6.8** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.0** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 4.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 6.8** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.0** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 4.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻ / arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 6.8** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻ / arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻ / arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 5.0** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **NO₂⁻ / arg** |    |       |       |       |      |      |       |       |      |      |       |       |       |
| **pH 4.5** |    |       |       |       |      |      |       |       |      |      |       |       |       |
fungi they have been studied in several systems. *Saccharomyces cerevisiae* yhb1Δ (Liu et al., 2000), *Cr. neoforms* fhb1Δ (de Jesus-Berrios et al., 2003) or Candida albicans yhb1Δ (Hromatka et al., 2005) lack both significant aerobic NO metabolic activity and detoxification, and therefore are more sensitive to toxic effects of NO. Flavohaemoglobins also work as defence system during the host response to microbial pathogens and mutants show reduced virulence (Crawford and Goldberg, 1998; Idnurm et al., 2004). NO detoxification systems might be of significant importance to different fungal species in their natural environments. For pathogenic fungi such as *Aspergillus fumigatus*, detoxification of NO produced as defence molecule by macrophages during the infection process could be essential to counteract nitrosative stress and enhance the infection process. Such a defence system has already been observed for *Cr. neoformans* to be one of the pathogenicity factors (de Jesus-Berrios et al., 2003) and *Histoplasma capsulatum*, a pathogenic fungus not known to possess flavohaemoglobin, which uses a nitric oxide reductase to counteract the NO burst employed by infected macrophages as part of the antimicrobial defence system (Nittler et al., 2005). *A. nidulans* is not pathogenic but grows on a wide variety of organic substrates and is a common soil inhabitant. In such environments elevated NO concentrations can be released from NO$_2^-$ at acidic pH and it is known that a side-reaction of bacterial nitrification results in release of NO (Klotz and Stein, 2008). This reaction product or derivatives thereof such as nitrous oxide or peroxynitrite may impose nitrosative stress on the surrounding microbial community.

In aspergilli, flavohaemoglobin mutants have been biochemically characterized as NO dioxygenases (Zhou et al., 2009) with an additional, and unexpected function in promoting oxidative stress (Zhou et al., 2010). Phylogenetic analysis of fungal flavohaemoglobin proteins showed the presence of an *Aspergillus* sp.-specific group, which was proposed to have a function in mycelial branching (Te Biesebeke et al., 2010).

While the enzymatic activity of filamentous fungal Fhb enzymes has been confirmed in vitro (Zhou et al., 2009), their regulatory context and in vivo role has not yet been elucidated. Our characterization of the two flavohaemoglobin mutants during nitrate induction yielded novel insights not only into nitric oxide metabolism, but also nitrate and nitrite metabolism in *A. nidulans*. As expected, deletion of the NO-metabolizing genes resulted in higher susceptibility to nitrosative stress imposed by externally supplied nitric oxide. Enzymatic measurements and growth tests, however, provided solid evidence that both NR and NiR are protected by the inducible FhbA flavohaemoglobin from damage by high NO concentrations. As both NR and NiR carry Fe$^{3+}$-containing prosthetic groups (haem and Fe-S cluster respectively) this protective function of Fhb proteins might be relevant to nitrogen metabolism under special ecological conditions, such as high NO$_2^-$ concentrations at low external pH, which is known to spontaneously generate high NO concentrations. In plants, NO has been shown to act as a signalling molecule for growth, development and resistance to biotic and abiotic stress (Perazzolli et al., 2006). Although the synthesis of NO in plants by enzymatic activities of nitric oxide synthases still remains a matter of debate (Gas et al., 2009), intracellular NO generation from NO$_2^-$ by NR activity and spontaneous decomposition at low pH is well established (reviewed by Yamasaki, 2000). Plant flavohaemoglobin, termed non-symbiotic haemoglobins, have been shown to be induced by NO$_2^-$ in *A. thaliana* (Wang et al., 2000) and were shown to be responsive to NO$_3^-$, NO$_2^-$ and NO donors in cultured rice cells (Ohwaki et al., 2005). Interestingly, also in rice cells, the expression of flavohaemoglobin GLB1 genes is not repressible by nitrogen metabolites (Gln or Asn), identical to what we found for *A. nidulans* fhbA. Recently, NO has also been shown to mediate a negative function on the expression of several nitrate-responsive genes in *Chlamydomonas* and *Arabidopsis* (de Montaigu et al., 2010). Low concentrations of NO (10 μM; DetaNONOate) efficiently repressed NO$_3^-$ induced genes via a cGMP-mediated pathway, effectively mimicking nitrogen metabolite repression by ammonium. Whether or not low concentrations of DetaNONOate mediate a negative effect on nitrate assimilatory gene expression in *A. nidulans* is not yet known.

FhbA is the first reported AreA-independent NirA target thus escaping nitrogen metabolite repression. This regulation could reflect the important role of counteracting NO stress, regardless of the cellular nitrogen status. Using NirA as one of the principal transcriptional activators of FhbA seems to be a good choice as the presence of NO inside or outside the cell always results in spontaneous NO$_2^-$ generation and thus activation of NirA. In addition, intracellular NO may be converted to NO$_2^-$ by
Experimental procedures

Strains, growth conditions and genetic techniques

Genotypes of strains used in this study are listed in Table S7. Combinations of traits were obtained by sexual crosses and strains were grown under standard conditions on liquid or solid glucose minimal medium (GMM) with the appropriate supplements and pH adjusted to 6.8 as previously described (Pontecorvo et al., 1953). Final concentrations of nitrogen sources were as following: 5 mM di-ammonium tartrate (Fluka), 10 mM NaNO₃ (Roth), 10 mM NaNO₂ (Riedel-de Haen), 3 mM (L)-arginine (Sigma), 0.75–3 mM DetanONOate (Sigma). For growth experiments on solid GMM at low ambient pH (4.5; 5.0 and 5.5) the pH was adjusted prior to sterilization and agar concentration was increased to 3%. Plates were incubated at 37°C up to 72 h and scanned for documentation (300 dpi). Images were processed by using the Photoshop 8.0 software. Conditions for liquid cultures were generally as previously described (Narendja et al., 2002), a detailed description is provided in Supporting Information on Experimental procedures. Transcriptional analysis for selected genes (Northern analysis) followed our published procedures (Narendja et al., 2002), details for probes and isolation can be found in Supporting Materials. Analysis of intracellular nitrate and nitrite levels was performed as previously described for A. nidulans by our laboratory (Berger et al., 2008) with minor modifications according to Inselsbacher et al. (2009). Details are provided in Supporting Information on Experimental procedures.

Microarray experiment

Aspergillus nidulans strains used in the transcriptome study were pabaA1 (wild type) and the nirA loss-of-function strain nirA637 (Muro-Pastor et al., 1999) pabaA1 (nirA⁻). A full description of the experimental set-up is found in Supporting Materials and Methods. Briefly, strains were initially grown on GMM for 16 h with 5 mM di-ammonium-tartrate as nitrogen source and then switched to either ammonium (repressed conditions) or to N-free medium for 30 min to prepare the cells for nitrate induction (induced conditions) or for continuous nitrogen depletion (-N conditions). For the wild-type experiments comparing repressed versus induced conditions, seven biologically independent experiments were performed and analysed separately. For the experiments comparing wild type repressed versus N-starved and nirA⁻ comparing repressed versus induced, four independent experiments were each performed and analysed separately.

Analysis of microarray data. The experimental dataset with details on the microarray obtained from TIGR-PFGR (The Pathogen Functional Genomics Resource Center; http://pfgrc.jcvi.org/index.php/microarray/protocols.html) has been submitted to the GEO database with accession number GSE10475. For the three conditions, further data analysis was performed in the Biconductor R package (http://www.biconductor.org/) using the same criteria. Raw median intensity values were imported into R for statistical analysis using the Limma package. Data were normalized within each array using Loess normalization and between arrays using the constitutive FhbB protein also leading to NirA activation and subsequent upregulation of FhbA. This regulatory circuit, presented in Fig. 9, ensures an optimal counterstrike against internal and external NO stress and provides protection for the by-products of their enzymatic reactions. Furthermore, the use of membrane-permeable NO has provided strong evidence that in A. nidulans, the nitrate transporters do not seem to be involved in transmitting the induction signal to the pathway-specific activator NirA and the intracellular presence of NO, NO₃⁻ or NO₂⁻ is evidently sufficient to activate NirA. These results agree with earlier findings by Unkles et al. (2001) who reported induction of nirA and niaD by external NO₃⁻ in nrtA/cmaA, nrtB/cmbB double mutant cells. Although very young mycelium (germling state) was used in the experiments by Unkles and colleges and nitrate uptake kinetics are different between germings and the mature mycelium (14 h growth) used in our experiments, transporter-independent sensing for the NirA-mediated induction process remains a distinct possibility. This is in contrast to findings in Arabidopsis (Ho et al., 2009) and Chlamydomonas (Rexach et al., 2002) where nitrate transporters have been shown to participate in the signal transduction process.
the Aquantile normalization algorithm (Smyth and Speed, 2003). The duplicate correlation function was used to calculate replicate correlations (Smyth et al., 2005). The log-expression values for each probe were fitted to a linear model using lmFit (Linear Model for Series of Arrays) and moderated t-statistics were calculated by eBayes (empirical Bayes Statistics for Differential Expression) (Smyth, 2004). Genes were considered to be differentially expressed by each treatment if they satisfied both the P-value lower than 0.05 and at least a fourfold change.

**Functional enrichment in DEGs**

Proteins are functionally classified using the MIPS Functional catalogue (FunCat) (Ruepp et al., 2004). Genome-wide data were retrieved from the Pedant A. nidulans database (http://pedant.gsf.de/pedant3htmlview/pedant3view?Method=analysis&D=d=p3_p1130_Asp_nidulans) (Walter et al., 2009). To statistically assess functional enrichment of DEGs, the probability was calculated using the hypergeometric distribution. In the equation, \( m \) is the number of genes that contain the same FunCat in \( n \) selected DEGs, relative to \( M \) genes that contain the FunCat in all \( N \) genes of a genome. The functional enrichment of each FunCat was considered significant if the \( P \)-value was < 0.05.

\[
P - \text{value} = \sum \frac{\binom{M}{m} \binom{N-M}{n-m}}{\binom{N}{n}}
\]

NirA binding sites were identified in the genome using the ‘fuzznuc’ program from the EMBOSS package (Rice et al., 2000) for regular expression search in nucleotide sequences. The regular expression of the NirA binding site was defined as 5’-CTCCG[ATC]GG and both strands were searched.

**Construction of fhbA and fhbB deletion cassettes.** The two putative flavohaemoglobin genes designated flavohaemoglobin A (fhbA, AN7169.3) and flavohaemoglobin B (fhbB, AN3522.3), were deleted using overlapping chimeric polymerase chain reaction fragments produced according to published procedures (Yu et al., 2004) and described in detail in Supporting Information on Experimental procedures. The primers employed for fragment generation are described in Table S7.

**Nitric oxide (NO) quantification [(DETC)\(_2\)-Fe\(^{2+}\)–NO; EPR].**

Quantification of NO was performed according to Xu et al. (Xu et al., 2005). In brief, approximately 0.3 g of frozen sample powder was mixed with 800 µl phosphate buffer (100 mM) containing 0.32 M sucrose, 0.1 mM EDTA and 5 mM thioethyglycolic (pH 7.4). After centrifugation (13 000 g, 20 min, 4°C) the supernatant was incubated with 400 µl of the spin trapping reagent for 60 min. The stock solutions for the spin trap reagent consisted of 7.5 mM aqueous FeSO\(_4\) and 15 mM aqueous DETC containing 0.5 M Na\(_2\)S\(_2\)O\(_5\). FeSO\(_4\) and DETC solutions were mixed 1:1 just before addition to the sample solution. Ethyl acetate (800 µl) was subsequently added and the mixture was then shaken for 3 min. After centrifugation (13 000 g, 6 min, 4°C), the organic solvent layer was transferred into a quartz flat cell for EPR measurement.

Electron paramagnetic resonance spectra were acquired as first derivatives of the microwave absorption with a Bruker EMX CW spectrometer, operating at X-band frequencies (9 GHz) and using a high sensitivity cavity. Microwaves were generated by a Gunn diode and the microwave frequency was recorded continuously with an in-line frequency counter. Spectra were recorded using 20 mW microwave power, 100 kHz modulation frequency and 1 G modulation amplitude. Signal intensities were determined by double integration using the Bruker WINEPR software, and then were corrected for the dry weight of input material.

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**References**

Abreu, C., Sanguinetti, M., Amillis, S., and Ramon, A. (2010) UreA, the major urea/H\(^{+}\) symporter in Aspergillus nidulans. Fungal Genet Biol doi: 10.1016/j.fgb.2010.07.004.
Andrianopoulos, A., Kourambas, S., Sharp, J.A., Davis, M.A., and Hynes, M.J. (1998) Characterization of the Aspergillus nidulans nmrA gene involved in nitrogen metabolite repression. J Bacteriol 180: 1973–1977.
Berger, H., Basheer, A., Bock, S., Reyes-Dominguez, Y., Dalik, T., Altmann, F., and Strauss, J. (2008) Dissecting individual steps of nitrogen transcription factor cooperation in the Aspergillus nidulans nitrate cluster. Mol Microbiol 69: 1385–1398.
Berreiter, A., Ramon, A., Fernandez-Martinez, J., Berger, H., Araujo-Bazan, L., Espeso, E.A., et al. (2007) Nuclear export of the transcription factor NirA is a regulatory checkpoint for nitrate induction in Aspergillus nidulans. Mol Cell Biol 27: 791–802.
Brakhage, A.A. (1998) Molecular regulation of beta-lactam biosynthesis in filamentous fungi. Microbiol Mol Biol Rev 62: 547–585.
Brownlee, A.G., and Arst, H.N., Jr (1983) Nitrate uptake in...
Aspergillus nidulans and involvement of the third gene of the nitrate assimilation gene cluster. *J Bacteriol* **155**: 1138–1146.

Burger, G., Tilburn, J., and Scaccioch, C. (1991) Molecular cloning and functional characterization of the pathway-specific regulatory gene nirA, which controls nitrate assimilation in *Aspergillus nidulans*. *Mol Cell Biol* **11**: 795–802.

Caddick, M.X., Peters, D., and Platt, A. (1994) Nitrogen regulation in fungi. *Antonie Van Leeuwenhoek* **65**: 169–177.

Caddick, M.X., Jones, M.G., van Tonder, J.M., Le Cordier, H., Narendjia, F., Strauss, J., and Morozov, I.Y. (2006) Opposing signals differentially regulate transcript stability in *Aspergillus nidulans*. *Mol Microbiol* **62**: 509–519.

Camargo, A., Llamas, A., Schnell, R.A., Higuera, J.J., Gonzalez-Ballester, D., Lefebvre, P.A., et al. (2007) Nitrate signaling by the regulatory gene NIT2 in *Chlamydomonas*. *Plant Cell* **19**: 3491–3503.

Castaings, L., Camargo, A., Pocholle, D., Gaudon, V., Texier, Y., Boutet-Mercy, S., et al. (2009) The nodule incision-like protein 7 modulates nitrate sensing and metabolism in *Arabidopsis*. *Plant J* **57**: 426–435.

Cove, D.J. (1979) Genetic studies of nitrate assimilation in *Aspergillus nidulans*. *Biol Rev Camb Philos Soc* **54**: 291–327.

Cove, D.J., and Pateman, J.A. (1969) Autoregulation of the synthesis of nitrate reductase in *Aspergillus nidulans*. *J Bacteriol* **97**: 1374–1378.

Crawford, N.M., and Arst, H.N., Jr (1993) The molecular genetics of nitrate assimilation in fungi and plants. *Annu Rev Genet* **27**: 115–146.

Daniel-Vedele, F., Filleur, S., and Caboche, M. (1998) Nitrate transport: a key step in nitrate assimilation. *Curr Opin Plant Biol* **1**: 235–239.

Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M., and Verstraete, W. (2009) Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiol Rev* **33**: 855–869.

Feng, G.H., and Leonard, T.J. (1998) Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. *Appl Environ Microbiol* **64**: 2275–2277.

Fernandez, E., and Galvan, A. (2008) Nitrate assimilation in *Chlamydomonas*. *Eukaryot Cell* **7**: 555–559.

Foster, M.W., Liu, L., Zeng, M., Hess, D.T., and Stamler, J.S. (2009) A genetic analysis of nitrosative stress. *Biochemistry* **48**: 792–799.

Fujii, T., and Takaya, N. (2008) Denitification by the fungus *Fusarium oxysporum* involves NADH-nitrate reductase. *Biosci Biotechnol Biochem* **72**: 412–420.

Gardner, P.R., Gardner, A.M., Martin, L.A., and Salzman, A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc Natl Acad Sci USA* **95**: 10378–10383.

Gas, E., Flores-Perez, U., Sauret-Gueto, S., and Rodriguez-Concepcion, M. (2009) Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism. *Plant Cell* **21**: 18–23.

Glass, A.D., Britto, D.T., Kaiser, B.N., Kinghorn, J.R., Kroznucker, H.J., Kumar, A., et al. (2002) The regulation of nitrate and ammonium transport systems in plants. *J Exp Bot* **53**: 855–864.

Gutierrez, R.A., Gifford, M.L., Poultnay, C., Wang, R., Shasha, D.E., Coruzzi, G.M., and Crawford, N.M. (2007) Insights into the genomic nitrate response using genetics and the Sungear Software System. *J Exp Bot* **58**: 2359–2367.

Hawker, K.L., Montague, P., and Kinghorn, J.R. (1992) Nitrate reductase and nitrite reductase transcript levels in various mutants of *Aspergillus nidulans*: confirmation of autogenous regulation. *Mol Gen Genet* **231**: 485–488.

Ho, C.H., Lin, S.H., Hu, H.C., and Tsay, Y.F. (2009) CHL1 functions as a nitrate sensor in plants. *Cell* **138**: 1184–1194.

Hromatka, B.S., Noble, S.M., and Johnson, A.D. (2005) Transcriptional response of *Candida albicans* to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. *Mol Biol Cell* **16**: 4814–4826.

Hu, H.C., Wang, Y.Y., and Tsay, Y.F. (2009) AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. *Plant J* **57**: 264–278.

Idnurm, A., Reedy, J.L., Nussbaum, J.C., and Heitman, J. (2004) *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. *Eukaryot Cell* **3**: 420–429.

Inselsbacher, E., Ripka, K., Klaubauf, S., Fedosoyenko, D., Hackl, E., Gorfer, M., et al. (2009) A cost-effective high-throughput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface. *Plant Soil* **317**: 293–307.

de Jesus-Berrios, M., Liu, L., Nussbaum, J.C., Cox, G.M., Stamler, J.S., and Heitman, J. (2003) Enzymes that counteract nitrosative stress promote fungal virulence. *Curr Biol* **13**: 1963–1968.

Joergensen, R.D., and Wichern, F. (2008) Quantitative assessment of the fungal contribution to microbial tissue in soil. *Soil Biol Biochem* **40**: 2977–2991.

Keller, N.P., Turner, G., and Bennett, J.W. (2005) Fungal secondary metabolism – from biochemistry to genomics. *Nat Rev Microbiol* **3**: 937–947.

Kim, S.W., Fushinobu, S., Zhou, S., Wakagi, T., and Shoun, H. (2009) Eukaryotic nirK genes encoding copper-containing nitrite reductase: originating from the protomitocondrin? *Appl Environ Microbiol* **75**: 2652–2658.

Klotz, M.G., and Stein, L.Y. (2008) Nitrifier genomics and evolution of the nitrogen cycle. *FEMS Microbiol Lett* **278**: 146–156.

Krouk, G., Crawford, N.M., Coruzzi, G.M., and Tsay, Y.F. (2010) Nitrate signaling: adaptation to fluctuating environments. *Curr Opin Plant Biol* **13**: 1–8.

Kudla, B., Caddick, M.X., Langdon, T., Martinez-Rossi, N.M., Bennett, C.F., Sibley, S., et al. (1990) The regulatory gene *area* mediates nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. *EMBO J* **9**: 1355–1364.

Langdon, T., Sheerins, A., Ravagnani, A., Gielkens, M., Caddick, M.X., and Arst, H.N., Jr (1995) Mutational analysis reveals dispensability of the N-terminal region of the *Aspergillus* transcription factor mediating nitrogen metabolite repression. *Mol Microbiol* **17**: 877–888.
Takaya, N. (2002) Dissimilatory nitrate reduction metabolisms and their control in fungi. *J Biosci Bioeng* **94**: 506–510.

Te Biesebeke, R., Levasseur, A., Boussier, A., Record, E., Hondel, C.A., and Punt, P.J. (2010) Phylogeny of fungal hemoglobinins and expression analysis of the *Aspergillus oryzae* flavohemoglobin gene *fhbA* during hyphal growth. *Mycol Res* **114**: 135–143.

Todd, R.B., Fraser, J.A., Wong, K.H., Davis, M.A., and Hynes, M.J. (2005) Nuclear accumulation of the GATA factor AreA in response to complete nitrogen starvation by regulation of nuclear export. *Eukaryot Cell* **4**: 1646–1653.

Unkles, S.E., Hawker, K.L., Grieve, C., Campbell, E.I., Montague, P., and Kinghorn, J.R. (2001) *crnA* encodes a nitrate transporter in *Aspergillus nidulans* [published errata appear in Proc Natl Acad Sci USA 1991 May 15;88(10):4564 and 1995 Mar 28;92(7):3076]. *Proc Natl Acad Sci USA* **88**: 204–208.

Unkles, S.E., Zhou, D., Siddiqi, M.Y., Kinghorn, J.R., and Glass, A.D. (2001) Apparent genetic redundancy facilitates ecological plasticity for nitrate transport. *EMBO J* **20**: 6246–6255.

Walter, M.C., Rattei, T., Arnold, R., Guldener, U., Munsterkotter, M., Nenova, K., et al. (2009) PEDANT covers all complete RefSeq genomes. *Nucleic Acids Res* **37**: D408–D411.

Wang, R., Guegler, K., LaBrie, S.T., and Crawford, N.M. (2000) Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *Plant Cell* **12**: 1491–1509.

Wang, R., Okamoto, M., Xing, X., and Crawford, N.M. (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiol* **132**: 556–567.

Wang, Y., Li, W., Siddiqi, Y., Symington, V.F., Kinghorn, J.R., Unkles, S.E., and Glass, A.D. (2008) Nitrite transport is mediated by the nitrite-specific high-affinity NitA transporter and by nitrate transporters NrtA, NrtB in *Aspergillus nidulans*. *Fungal Genet Biol* **45**: 94–102.

Wong, K.H., Hynes, M.J., Todd, R.B., and Davis, M.A. (2007) Transcriptional control of *nmrA* by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in *Aspergillus nidulans*. *Mol Microbiol* **66**: 534–551.

Xu, Y., Cao, Y., Tao, Y., and Zhao, B. (2005) The ESR method to determine nitric oxide in plants. *Methods Enzymol* **396**: 84–92.

Yamasaki, H. (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo. *Philos Trans R Soc Lond B Biol Sci* **355**: 1477–1488.

Yu, J.H., Hamari, Z., Han, K.H., Seo, J.A., Reyes-Dominguez, Y., and Scaccia, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet Biol* **41**: 973–981.

Zhang, H., and Forde, B.G. (2000) Regulation of *Arabidopsis* root development by nitrate availability. *J Exp Bot* **51**: 51–59.

Zhou, S., Fushino, S., Nakanishi, Y., Kim, S.W., Wakagi, T., and Shoun, H. (2009) Cloning and characterization of two flavohemoglobins from *Aspergillus oryzae*. *Biochem Biophys Res Commun* **381**: 7–11.

Zhou, S., Fushino, S., Kim, S.W., Nakanishi, Y., Wakagi, T., and Shoun, H. (2010) *Aspergillus oryzae* flavohemoglobins promote oxidative damage by hydrogen peroxide. *Biochem Biophys Res Commun* **394**: 558–561.

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