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Pseudo-constitutivity of nitrate-responsive genes in nitrate reductase mutants

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ABSTRACT

In fungi, transcriptional activation of genes involved in NO_3^- assimilation requires the presence of an inducer (nitrate or nitrite) and low intracellular concentrations of the pathway products ammonium or glutamine. In *Aspergillus nidulans*, the two transcription factors NirA and AreA act synergistically to mediate nitrate/nitrite induction and nitrogen metabolite derepression, respectively. In all studied fungi and in plants, mutants lacking nitrate reductase (NR) activity express nitrate-metabolizing enzymes constitutively without the addition of inducer molecules. Based on their work in *A. nidulans*, Cove and Pateman proposed an "autoregulation control" model for the synthesis of nitrate metabolizing enzymes in which the functional nitrate reductase molecule would act as co-repressor in the absence and as co-inducer in the presence of nitrate. However, NR mutants could simply show "pseudo-constitutivity" due to induction by nitrate which accumulates over time in NR-deficient strains. Here we examined this possibility using strains which lack flavohemoglobins (*fhbs*), and are thus unable to generate nitrate internally, in combination with nitrate transporter mutations (*nrtA*, *nrtB*) and a GFP-labeled NirA protein. Using different combinations of genotypes we demonstrate that nitrate transporters are functional also in NR null mutants and show that the constitutive phenotype of NR mutants is not due to nitrate accumulation from intracellular sources but depends on the activity of nitrate transporters. However, these transporters are not required for nitrate signaling because addition of external nitrate (10 mM) leads to standard induction of nitrate assimilatory genes in the nitrate transporter double mutants. We finally show that NR does not regulate NirA localization and activity, and thus the autoregulation model, in which NR would act as a co-repressor of NirA in the absence of nitrate, is unlikely to be correct. Results from this study instead suggest that transporter-mediated NO_3^- accumulation in NR deficient mutants, originating from traces of nitrate in the media, is responsible for the constitutive expression of NirA-regulated genes, and the associated phenotype is thus termed "pseudo-constitutive".

1. Introduction

With few exceptions, like the yeast *Saccharomyces cerevisiae*, most fungi are able to use nitrate as an assimilatory nitrogen source and recent work showed that fungal nitrate assimilation significantly contributes to biogeochemical nitrogen cycling in nitrate-dominated agricultural soils (Gorfer et al., 2011). Bacteria, algae and plants also assimilate nitrate and in all systems NO_3^- needs to be reduced to ammonium in order to serve as nitrogen source for incorporation into amino acids. These sequential reaction steps are carried out by the enzymes nitrate reductase (NR, NO_3^- to NO_2^-) and nitrite reductase (NiR, NO_2^- to NH_4^+) (Cove, 1979). Nitrate assimilation in *Neurospora crassa* and *Aspergillus nidulans* served as early eukaryotic model systems to study adaptive enzyme formation due to the ease with which the enzymatic activity can be assayed in cell extracts (Cove and Coddington, 1965; Kinsky and McElroy, 1958). Genetic dissection of the pathway in *A. nidulans* resulted in the characterization of mutants affected in structural genes (Cove and Pateman, 1963; Pateman et al., 1967). Amongst others, NirA and one of the first eukaryotic regulatory mutations were identified using this model pathway (Cove, 1969). This work revealed that *de novo* synthesis of NR and NiR are subject to induction by nitrate or nitrite and to repression by ammonium (Cove, 1966; Kinsky, 1961). Both inducer molecules are internalized by active transport via the two nitrate permeases *nrtA* (*crnA*) and *nrtB*

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(*crnB*), also capable to transport nitrite (Brownlee and Arst, 1983; Unkles et al., 1991, 2001), whereas *nitA* encodes a specific nitrite transporter only (Unkles et al., 2011; Wang et al., 2008).

Today, the molecular basis of nitrate-responsive gene regulation in fungi is well understood. In *A. nidulans*, nitrate induction is mediated by the binuclear Zn-cluster protein NirA (Burger et al., 1991a,b; Cove, 1976) which accumulates in the nucleus in the presence of intracellular nitrate. Nuclear accumulation is the consequence of nitrate-or nitrite- mediated disruption of the interaction between the NirA nuclear export signal (NES) and the nuclear exportin KapK (Bernreiter et al., 2007). Subsequently, NirA binds to specific recognition sites in target promoters (Strauss et al., 1998). Transcriptional activation of most nitrate-responsive genes additionally requires interaction of NirA with AreA (Arst and Cove, 1973; Arst, 1982; Caddick et al., 1986; Kudla et al., 1990; Starich et al., 1998), a GATA-type transcriptional co-activator regulating genes involved in nitrogen metabolism. In the nitrate pathway AreA is required for *in vivo* DNA binding of NirA (Berger et al., 2006; Narendja et al., 2002), for recruitment of histone acetylation activities (Berger et al., 2008) and chromatin remodeling in nitrate-responsive promoters (Muro-Pastor et al., 1999, 2004). In such a way, NirA and AreA act synergistically to activate transcription of nitrate-responsive genes including the genes coding for NR (*niaD*) and NiR (*niiA*).

Cove and Pateman noted that mutants affected in NR activity constitutively synthesize NiR without the need of inducer addition and proposed an autoregulation model in which the NR holoenzyme would act as co-repressor of its own synthesis and that of NiR in the absence of nitrate or nitrite (Cove and Pateman, 1969). This hypothesis was further supported by findings that *cnx* mutants, unable to synthesize the molybdenum-containing cofactor of NR, are likewise constitutive for NiR activity (Mac Donald and Cove, 1974; Pateman et al., 1964). Interestingly, also the enzymes of the pentose phosphate pathway, later shown to be up-regulated in a NirA-dependent manner (Schinko et al., 2010), were found to be constitutively produced in *cnx* and *niaD* mutants (Hankinson and Cove, 1974). A molecular study confirmed the constitutive expression of both *niaD* and *niiA* in selected *niaD* and *cnx* mutants (Hawker et al., 1992).

However, an alternative way to explain the constitutive phenotype would be the intracellular accumulation of nitrate in NR-negative mutants from either external or internal sources. Such external trace amounts of nitrate may occur as contaminants of media components which might accumulate over time inside NR-mutant cells by nitrate transporter activity, and eventually lead to activation of NirA. In the yeast *Hansenula polymorpha* (Navarro et al., 2003) and in the algae *Chlamydomonas reinhardtii* (Llamas et al., 2002) this was shown to be the underlying mechanism of constitutive gene expression. In both organisms, constitutivity of the nitrate assimilatory genes observed in NR mutants was lost when nitrate transporters were non-functional. In *C. reinhardtii*, the authors showed also that intracellular levels of nitrate are detectable in NR mutants grown on "nitrate-free" medium. These results weaken the hypothesis that in these organisms NR itself possesses a regulatory function. However, from these studies it cannot be formally excluded that internal sources of nitrate – such as NO_2^- or NO_3^- derived from nitric oxide (NO) – artificially induce the system in cooperation with the transporters. In this alternative model nitrate transporters would additionally act as signalers, similar to what has been shown for *Arabidopsis thaliana* (Guo et al., 2003).

Although so far no clear evidence for the existence of mammalian-type classical NO synthases is available for fungi, algae and plants, NO can be generated in these organisms by the nitrate reductase enzyme itself as a by-product of the main enzymatic reaction (Besson-Bard et al., 2008; de Montaigu et al., 2010; Modolo et al., 2005; Rockel et al., 2002; Schinko et al., 2010; Wendehenne et al., 2001; Yamasaki, 2000; Yamasaki and Sakihama, 2000). Additionally, a variety of (bio)chemical pathways are known to generate NO in metabolically active cells (Nagase et al., 1997; Zweier et al., 1999). Notably, many processes have been shown to be regulated by NO in plants, e.g. stomatal closure, flowering, gravitropsim, and stress response (reviewed in Besson-Bard et al., 2008). NO is harmful to cells at higher concentrations causing proteins to become nitrosylated or nitrated impairing their proper function. Levels of NO are antagonized by spontaneous oxidation to nitrite and peroxinitrite and by enzymatic detoxification involving flavohemoglobins (*fhb*). These evolutionary conserved di-oxygenases have the ability to convert NO directly to NO_3^- and thereby efficiently remove excess NO (Gardner et al., 1998; Poole and Hughes, 2000). We have recently characterized two flavohemoglobin genes (*fhbA* and *fhbB*) in *A. nidulans* and cells lacking both enzymes show hypersensitivity to elevated environmental NO levels. FhbA was shown to be induced by nitrate in a strictly NirA-dependent manner but interestingly, and in contrast to all other known nitrate-responsive genes, *fhbA* expression does not require the function of the general nitrogen regulator AreA (Schinko et al., 2010).

To clarify whether in *A. nidulans* NR itself has a real regulatory role and if external or internal NO_3^- sources might evoke a "pseudo-constitutive" phenotype in NR loss-of-function mutants, we used mutant strains affected in nitrate transport and metabolism, and combined them with flavohemoglobin mutants lacking NO to NO_3^- conversion. Our results show that NR mutants accumulate significant levels of intracellular nitrate leading to NirA nuclear

Table 1
A. nidulans strains used in this study.

Strain name	Genotype	Reference
WT	*veA1 biA1 yA2*	Schinko et al. (2010)
ni*aD*Δ	*veA1 biA1 pyrG89 niaD*Δ *wA3*	Schinko et al. (2010)
nrtA$^-$ *nrtB*$^-$*	*veA1 biA1 pabaA1 argB2 crnA747 crnB110*	Ukles et al. (2001)
nrtA$^-$ *nrtB*$^-$	*veA1 biA1 pabaA1 argB* complemented *crnA747 crnB110*	Schinko et al. (2010)
nrtA$^-$ *nrtB*$^-$ *niaD*Δ	*veA1 biA1 pabaA1 niaD*Δ::*argB crnA747 crnB110*	Schinko et al. (2010)
*fhb*ΔΔ	*veA1 biA1 fhbA*Δ::*argB fhbB*::*argB yA2*	Schinko et al. (2010)
*fhb*ΔΔ *niaD*Δ	*veA1 biA1 fhbA*Δ::*argB fhbB*::*argB niaD*Δ *wA3*	Schinko et al. (2010)
nirA$^-$	*veA1 pabaA1 nirA637*	Schinko et al. (2010)
niiA$^-$	*veA1*, *biA1*, *niiA4*, *pyroA4*, *nkuA*Δ::*bar*	Schinko et al. (2010)
*niiA*Δ*:AFpyrG*	*veA1 nkuA*Δ::*argB niiA*Δ::AF*pyrG pyroA4 riboB2*	This study
*niaD*Δ *niiA*Δ	*veA1 biA1 niaD*Δ *niiA*Δ::AF*pyrG wA3*	This study
*nirA*Δ*:AFriboB*	*veA1 nkuA*Δ::*argB nirA*Δ::*AFriboB pyroA4 pyrG89*	This study
FLAG:nirA$_{cDNA}$*:GFP*	*veA1 nkuA*Δ::*argB* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$ *pyroA4 riboB2*	This study
*niaD*Δ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
nrtA$^-$ *nrtB*$^-$ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 crnA747 crnB110 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
nrtA$^-$ *nrtB*$^-$ *niaD*Δ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 crnA747 crnB110 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
TNO2A7	*veA1 nkuA*Δ::*argB pyroA4 pyrG89 riboB2*	Nayak et al. (2006)