

A Soluble Guanylate Cyclase Mediates Negative Signaling by Ammonium on Expression of Nitrate Reductase in *Chlamydomonas* ^W

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Nitrate assimilation in plants and related organisms is a highly regulated and conserved pathway in which the enzyme nitrate reductase (NR) occupies a central position. Although some progress has been made in understanding the regulation of the protein, transcriptional regulation of the NR gene (*NIA1*) is poorly understood. This work describes a mechanism for the ammonium-mediated repression of *NIA1*. We report the characterization of a mutant defective in the repression of *NIA1* and NR in response to ammonium and show that a gene (*CYG56*) coding for a nitric oxide (NO)-dependent guanylate cyclase (GC) was interrupted in this mutant. NO donors, cGMP analogs, a phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), and a calcium ionophore (A23187) repress the expression of *NIA1* in *Chlamydomonas reinhardtii* wild-type cells and also repress the expression of other ammonium-sensitive genes. In addition, the GC inhibitors LY83,583 (6-anilino-5,8-quinolinedione) and ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) release cells from ammonium repression. Intracellular NO and cGMP levels were increased in the presence of ammonium in wild-type cells. In the *cyg56* mutant, *NIA1* transcription was less sensitive to NO donors and A23187, but responded like the wild type to IBMX. Results presented here suggest that *CYG56* participates in ammonium-mediated *NIA1* repression through a pathway that involves NO, cGMP, and calcium and that similar mechanisms might be occurring in plants.

INTRODUCTION

Regulation of the nitrate assimilation pathway coordinates the incorporation of nitrogen with important processes of plant development, such as cell differentiation and photosynthesis. Genes and proteins of this pathway are induced by nitrate itself (Crawford, 1995; Daniel-Vedele et al., 1998; Forde, 2000; Llamas et al., 2002; Rexach et al., 2002) and are repressed by ammonium or by products of nitrate metabolism, such as Gln and Glu (Stitt, 1999; Crawford and Forde, 2002; Glass, 2003; Fan et al., 2006; Fernandez and Galván, 2007). A key component of the nitrate assimilation pathway is nitrate reductase (NR), which catalyzes the reduction of nitrate to nitrite. This enzyme and the gene encoding it are subject to complex regulation mechanisms: not only are gene expression and protein activity regulated by inorganic nitrogen compounds and metabolic derivatives, but they also respond to external cues, such as day and night cycles (Kaiser and Brendle-Behnisch, 1991; Galangau et al., 1998). In plants, NR activity is regulated by phosphorylation of a Ser residue that allows binding of 14-3-3 proteins and leads to subsequent degradation of NR (reviewed in Kaiser and Huber,

2001). Two kinases responsible for the phosphorylation of the Ser residue seem to be activated by calcium, whereas the third, a member of the SNF1 kinase family, is calcium independent (Douglas et al., 1997).

The green alga *Chlamydomonas reinhardtii* and higher plants share structural and regulatory elements for nitrate assimilation, making *Chlamydomonas* an interesting and useful model organism for studies of nitrogen metabolism (Galvan et al., 2006). The 14-3-3 proteins also appear to regulate nitrogen assimilation in *Chlamydomonas*; in fact, glutamine synthetase I is a target for these regulatory proteins (Pozuelo et al., 2001). However, redox regulation seems to be the major mechanism in regulating NR protein activity in the alga.

In *Chlamydomonas*, NIT2 is essential for nitrate assimilation by mediating transcriptional activation of *NIA1*, the gene encoding NR, in response to nitrate (Schnell and Lefebvre, 1993; Fernández et al., 1998; Camargo et al., 2007). This protein, containing GAF and RWP-RK domains, shows conservation with plant nodule inception-like proteins that might function similarly (Camargo et al., 2007; Castaigns et al., 2009). By contrast, the mechanism of negative regulation of *NIA1* expression is poorly understood.

In a previous study, we reported the generation of a *Chlamydomonas* insertional mutant library whose objective was to identify new regulators of *NIA1* expression (González-Ballester et al., 2005a). The genetic background of the parental strain 704 contained the *NIA1* promoter fused to the arylsulfatase (*Ars*) reporter gene, so that *NIA1* promoter activity was detected by ARS activity measurements in the conditions of the screen. A specific class of mutants, hereafter called ammonium insensitive

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(AI), was screened for *NIA1* promoter activity in medium containing nitrate and ammonium, a condition in which *NIA1* expression is not detected in the wild type. The tag was localized in the genome of the AI mutants, allowing us to identify a number of candidate genes involved in the repression of *NIA1* (González-Ballester et al., 2005a, 2005b). One of these genes showed significant homology with soluble nitric oxide (NO)-dependent guanylate cyclases (GCs) from animals.

An exponentially growing number of reports on the implication of NO and cGMP in physiological processes are accumulating in plants. NO was first demonstrated to function as a signal molecule in plant-pathogen interactions (Delledonne et al., 1998), and it has subsequently been shown to play an active role in regulating root elongation (Gouvea et al., 1997), abscisic acid-induced stomatal closure (Mata and Lamattina, 2001), flowering (He et al., 2004), senescence (Corpas et al., 2004), development of cell polarity (Salmi et al., 2007), and other processes. These functions have been extensively reviewed by Lamattina et al. (2003) who suggested the concept of NO functioning as a “synchronizing chemical messenger” to explain its implication in such diverse regulation pathways. In addition, cGMP signaling has been related to defense gene induction (Durner et al., 1998), cell polarity development (Salmi et al., 2007), or gene transcription and cation transport processes (Maathuis, 2006).

NR has been shown to synthesize NO using nitrite as a substrate (Dean and Harper, 1986, 1988; Yamasaki and Sakihama, 2000; Rockel et al., 2002; Sakihama et al., 2002), but little is known about the conditions regulating NO production in vivo. Mitochondrial electron transport was also found to contribute significantly to the nitrite-dependent NO production in plant cells (Planchet et al., 2005). In addition, nitrite is a known source of NO through nonenzymatic reactions (Bethke et al., 2004; Tischner et al., 2004). Interestingly, nitrite has been shown to inhibit transcription of *NIA1* in *Arabidopsis thaliana* and *Chlamydomonas* (Llamas et al., 2002; Loqué et al., 2003). Nevertheless, evidence of NO as a regulator of nitrate assimilation is restricted to NR activity in tomato (*Solanum lycopersicum*) roots depending on levels of nitrate supply (Jin et al., 2009).

In this work, we characterize *CYG56*, a GC gene related to the AI phenotype, and show that NO, cGMP, and calcium participate in a signaling pathway for ammonium repression in *Chlamydomonas*. We also propose a model signaling pathway that might also be operative in plants.

RESULTS

A GC Is Related to Ammonium Repression of *NIA1*

AI insertional mutants have been selected for derepression of *NIA1* expression using media containing nitrate and ammonium, and the location of the insertion tag in the genome of each of the 40 AI mutants was determined. This allowed us to identify candidate genes involved in negative regulation of *NIA1* transcription (González-Ballester et al., 2005a, 2005b). In one of these mutants, previously known as mutant 20.73, a gene annotated as *CYG56* (EU841916) was interrupted. Hereafter in

the text, mutant 20.73 will therefore be referred to as *cyg56*. AI phenotype of this mutant was determined by ARS activity assays (Ohresser et al., 1997) after growth on solid medium containing different sources of nitrogen as previously described (González-Ballester et al., 2005a).

In contrast with the parental wild-type strain 704, the expression of the ARS reporter under control of the *NIA1* promoter could be detected in *cyg56* mutant cells in media containing nitrate plus ammonium, though the intensity of the signal decreased with increasing amounts of ammonium in the medium (Figure 1A). The phenotype was confirmed in short-term NR activity assays in medium containing nitrate plus ammonium; NR activity could be detected in the mutant but not in the wild type (Figure 1C). In these conditions, NR activity in the mutant increased with time as ammonium concentration, still present,

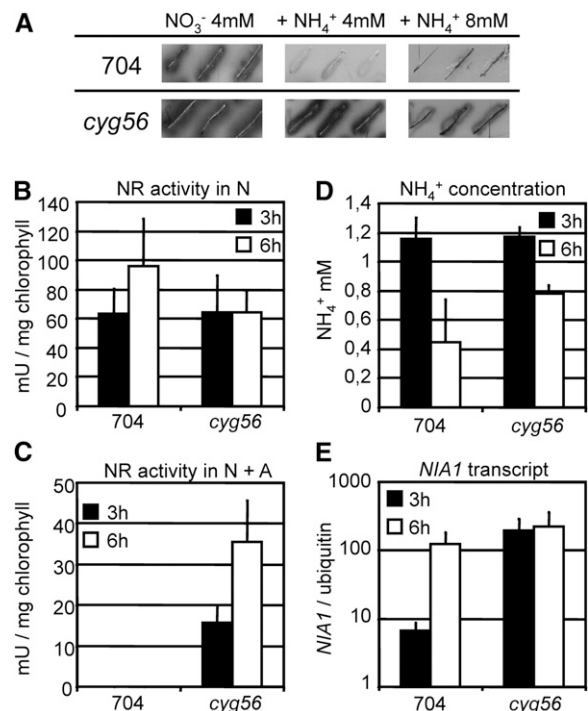


Figure 1. *NIA1* Expression and NR Activity Are Derepressed in the *cyg56* Mutant.

(A) ARS activity of *Chlamydomonas* wild-type strain 704 and mutant *cyg56* was assayed after 4 d of growth on solid media containing 4 mM NO_3^- , 4 mM $\text{NO}_3^- + 4 \text{ mM NH}_4^+$, and 4 mM $\text{NO}_3^- + 8 \text{ mM NH}_4^+$. Both strains contain the *Ars* gene under the control of the *NIA1* promoter in their genetic background, and ARS activity therefore reflects *NIA1* promoter activity.

(B) and **(C)** NR activity of strains 704 and *cyg56* was assayed in media 4 mM NO_3^- **(B)** and 4 mM $\text{NO}_3^- + 2 \text{ mM NH}_4^+$ **(C)** after incubation for 3 h (black bars) and 6 h (white bars).

(D) Remaining concentration of NH_4^+ at both time points of results shown in **(B)** and **(C)**, respectively.

(E) *NIA1* mRNA levels were measured by real-time PCR in the same conditions as in **(C)**. The error bars represent the SD of three biological replicates.

decreased (Figure 1D). Importantly, there was no change in NR activity between the strains in nitrate medium alone (Figure 1B).

Taken together, these results show (1) that the phenotype was not due to general activation of NR activity but to a reduced sensitivity of *cyg56* to ammonium, and (2) that *cyg56* shows an AI phenotype for NR activity still sensitive to increasing amounts of ammonium, consistent with the results of Figure 1A.

NIA1 transcript levels (Figure 1E) were then determined in the same conditions than Figure 1C. In agreement with the previous data, results showed that *NIA1* mRNA levels were higher in the mutant compared with the wild type at both time points, the difference being more pronounced after 3 h of induction. Detection of *NIA1* expression in the wild type was not surprising as it brought to light previously described posttranscriptional negative effects of ammonium on NR activity (Fernández et al., 1998).

Linkage between marker insertion in the *CYG56* gene and the AI phenotype in the mutant is supported by the following facts. First, a single insertion tag of the paromomycin marker was found in mutant *cyg56* by DNA gel blot and PCR analyses (González-Ballester et al., 2005b). Second, genetic crosses between strain *cyg56* paromomycin-resistant AI *mt+* and different *Chlamydomonas mt-* strains that are paromomycin sensitive and sense normally ammonium were performed. Even though crossing efficiency was very low, as reported for these cell wall-less mutants (Galvan et al., 2007), progeny were obtained and analyzed. A linkage between paromomycin resistance and the ammonium-insensitive phenotype (determined from *NIA1*-ARS activity) was found in all 27 segregants recovered that had the chimeric *NIA1*-ARS reporter. In these segregants, the intensity of the AI phenotype ranged from strong to weak, indicating that genomic background of particular segregants might modulate this phenotype. Third, the *cyg56* mutation was complemented by forming diploid cells. To do that, *cyg56* mutation was transferred to a 305*nia1-* mutant background by genetic crosses. The transfer of the AI phenotype to NR mutant background also resulted in a strong desensitizing of the *NIA1* reporter to ammonium (Figure 2, *cyg56.305mt-*), probably due to nitrate accumulation in the cells because of the lack of nitrate assimilation

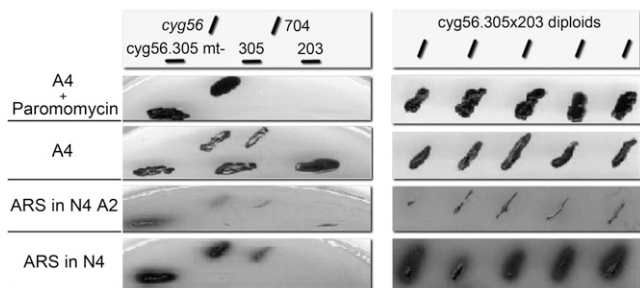


Figure 2. Complementation Analysis of the *cyg56* Mutation.

The mutation *cyg56* and the *Ars* marker were transferred to a 305*nia1* mutant background. Nit⁺ diploid strains were isolated by *in vivo* complementation between 305*nia1* and 203nit2 mutants and the AI phenotype of complemented diploids analyzed in medium with or without 4 mM nitrate (N4) + 2 mM ammonium (A2), 4 mM ammonium (A4), or 25 μ g/mL paromomycin. Phenotypes of the different strains used are shown in the medium indicated.

(Llamas et al., 2002). Then, Nit⁺ diploids (able to grow on nitrate) were recovered from mutant strains *cyg56.305* (*NIA1-* and *mt-*) with strain 203 (*Nit2-* and *mt+*) by complementation. All diploids (*cyg56.305x203*) obtained that were paromomycin resistant had recovered the normal ammonium sensitivity of wild-type 704 as assayed by ARS activity (Figure 2).

Insertional mutagenesis by marker DNA in *Chlamydomonas* causes results ranging from clean insertions to insertions involving deletions of several kilobases (Galvan et al., 2007). The cloning of the genomic regions flanking both sides of the insertion in mutant *cyg56* was performed, and it was shown that the DNA marker had integrated in chromosome 16 (JGI v4.0). As indicated in Figure 3A, the insertion caused a 21-bp deletion at the first exon (position +51 after the translation start site) and disrupted a putative GC protein sequence. The ATG shown in this figure has the optimal nucleotide context for translation initiation in *Chlamydomonas* (Silflow, 1998; Kozak et al., 2002) and was presumed to be position +1. As a consequence of an *rbcS2* promoter insertion, overexpression of the *CYG56* transcript was detected at levels of \sim 100 times those in the wild type (Figure 3B). This overexpression seems to result from the two enhancer elements of HSP70A promoter, a component of the transformation vector located at the vicinity of the *RBCS2* promoter inserted in mutant *cyg56*, as reported by von Gromoff et al. (2006).

RT-PCR amplifications (Figure 3C) using primers specific to the positions indicated in the scheme (Figure 3A) were performed to determine the 5' end organization of the transcript expressed in mutant *cyg56*, and the amplified bands were sequenced. The 5' end of the overexpressed *CYG56* transcript is deleted in the mutant in the region containing the ATG codon and corresponds to a chimeric mRNA containing at least 40 bp of the insertional marker DNA fused, based on evidence that RT-PCR amplifications did not generate bands with primers 1, 2, 3, 4, and 7. Clear bands are amplified with primers 5+7 and 6+7 due to the overexpression of the transcript. Efficiency of the RT-PCR in the wild type was small because of the low expression level of *CYG56* (Figures 3B and 3C), and this resulted in the appearance of nonspecific amplifications of high molecular weight bands when using primers 6 and 7.

Thus, these data indicate that a new transcription start site has been generated at the 3' end of the inserted sequence in mutant *cyg56* so that a partially deleted *CYG56* transcript is expressed. The analysis of the insertion site also showed that sequence elements essential for translation are absent in the *CYG56* chimeric transcript, ruling out the possibility that a functional *CYG56* protein could be translated in the mutant. If the overexpressed *CYG56* transcript was functional, the AI phenotype would have been obtained in diploids. This result of nonfunctional *CYG56* was further confirmed by the data presented hereafter in this work. In summary, the linkage and complementation tests suggest a scenario in which lack of *CYG56* functionality is responsible for the AI phenotype (i.e., *NIA1* expression in the presence of ammonium).

CYG56 Encodes a Soluble GC Regulated by Nitrogen

A full-length *CYG56* cDNA of 6111 bp was isolated and sequenced (EU841916). The sequence reveals an organization of

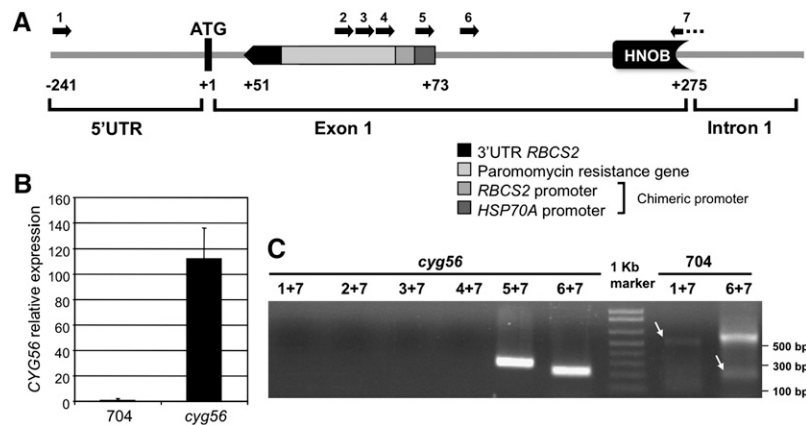


Figure 3. Analysis of CYG56 Expression Following Insertion of the Paromomycin Resistance Marker in the AI Mutant *cyg56*.

The marker DNA is inserted near the 5' terminal end of the CYG56 coding sequence.

(A) The vector pSI104 is inserted between positions 560341 and 560363 of scaffold 45 (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>) of the *Chlamydomonas* genome (corresponding to +51 and +72 of the coding region), just downstream of the initiation codon of CYG56 (annotated as +1) in the figure. Note that CYG56 is on the antisense strand of scaffold 45 in the *Chlamydomonas* genome. The parts of pSI104 marker are indicated.

(B) Expression of CYG56 was monitored by real-time PCR in the wild-type 704 and mutant *cyg56*. The data were obtained from a 3-h induction in 4 mM NO_3^- medium. Error bars represent the SD of three independent biological replicates.

(C) RT-PCR was performed in *cyg56* and wild-type 704 using different combinations of primers whose locations are represented in **(A)**.

23 exons and 22 introns (see Supplemental Figure 1A online) that shows important differences with the coding region annotated in the genome (EDO98900; Merchant et al., 2007). Exons vary in size from 7 to 1950 bp, with most of the small exons comprising the 5' half of the transcript. The cDNA has a long 3' untranslated region with the typical TGTA polyadenylation sequence that is common to other *Chlamydomonas* genes (Merchant et al., 2007).

Transcriptional regulation of CYG56 by specific nitrogen sources was analyzed (Figure 4). CYG56 transcript levels increased in response to ammonium compared with nitrate as the sole nitrogen source. Interestingly, CYG56 showed a peak of expression at 1 h when cells were transferred to ammonium or nitrate plus ammonium. This weak but consistent activation of CYG56 expression is in agreement with a role for CYG56 in ammonium repression of *NIA1*.

CYG56 encodes a protein (1721 amino acids) containing structural elements that are typical of animal NO-dependent GCs: HNOB (47 to 214), HNOBA (316 to 682), and GC catalytic domains (residues 692 to 822) (see Supplemental Figure 1B online). The HNOB domain is predicted to function as a heme-dependent sensor for gaseous ligands as NO and to transduce diverse downstream signals (Pellicena et al., 2004). HNOBA domain was recently shown to be involved in the dimerization of soluble guanylyl cyclase (Ma et al., 2008). This structure of globular N terminus extensions bearing HNOB and HNOBA domains before that of the catalytic GC seems to be an ancient conserved structure shared by animal soluble guanylyl cyclases and bacterial signaling proteins (Iyer et al., 2003; Nioche et al., 2004). In the deduced CYG56 sequence, a long C terminus of ~900 amino acid residues is also present that is unusual in animal GCs and contains no characteristic domains. His binding heme and the invariable sequence motif YxSxR (Pellicena et al.,

2004) are present in the HNOB domain (see Supplemental Figure 2A online). Some conserved Cys residues in HNOB and HNOBA domains that might be related to a strong activation of GC activity in animal GC (Fernhoff et al., 2009) are absent in *Chlamydomonas* GC (see Supplemental Figures 2A and 3 online).

The *Chlamydomonas* genome database contains 57 CYG genes, but only deduced proteins CYG11, CYG12, CYG15, CYG38, and CYG57 share the same domains structure with soluble CYG56 and GCs from animals (Nioche et al., 2004;

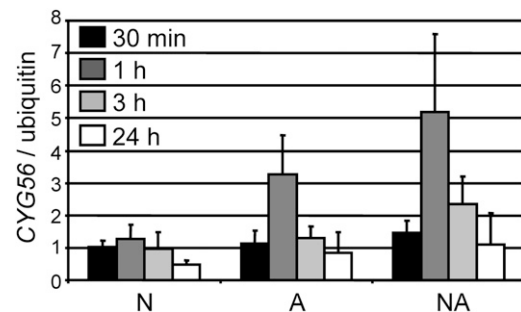


Figure 4. CYG56 Expression Is Activated by Ammonium in Wild-Type 704.

Wild-type 704 was grown in minimum medium containing 8 mM NH_4Cl . After 3 d of growth, cells were induced in media containing 4 mM NO_3^- , 8 mM NH_4^+ , and 4 mM $\text{NO}_3^- + 8$ mM NH_4^+ . Samples were taken at 30 min and at 1, 3, and 24 h (black, dark-gray, light-gray, and white bars, respectively). RNA was extracted and CYG56 transcript levels were monitored by real-time PCR and normalized using the level of the transcript encoding ubiquitin. The error bars represent the SD of three biological replicates.

Merchant et al., 2007), with an important degree of conservation (see Supplemental Figure 4 online). Scores of sequence identity obtained from comparisons of CYG56 with complete reported sequences range from 46% (with CYG57) and 24% (with CYG12) for *Chlamydomonas* proteins to 26% and 19% with proteins from animals. Interestingly, CYG11, CYG12, and CYG15 are linked within 50 kb at chromosome 7.

Both the full-length CYG56 protein and an N-terminal fragment of 957 amino acid residues were expressed in *Escherichia coli*, purified, and assayed for GC activity. The truncated protein was highly active and had a significant GC specific activity of 511 nmol·min⁻¹·mg protein⁻¹ in an assay with Mn²⁺ and 255 nmol·min⁻¹·mg protein⁻¹ using the physiological cation Mg²⁺ (see Supplemental Figure 5 online). However, it did not show any additional activation by the NO donors SNAP or DEA-NONOate described below. The full-length protein was partially purified and also showed a GC activity with a much lower activity assayed with Mg²⁺ relative to Mn²⁺ (see Supplemental Figure 5 online), and regulation by NO was not observed (data not shown).

The GC inhibitors ODQ, which is a selective inhibitor of NO-activated GC (Zhao et al. 2000), and LY-83,583 (LY) (Yang and Hatton, 1999) were used for in vitro assays with both the truncated and the full-length CYG56 proteins. ODQ showed a slight inhibition on GC cyclase activity assayed with Mg²⁺, but not with Mn²⁺, whereas LY caused a 60 and 45% inhibition in the activity of truncated and full-length CYG56 proteins, respectively, in assays with Mg²⁺, and much less with Mn²⁺ (see Supplemental Figure 6 online). The existence of long loops of amino acids disrupting the HNOBA domain in CYG56 might represent a difficulty for a proper folding in *E. coli*, resulting in a protein with a folded GC catalytic domain that is insensitive to signals from heme domains, and thus to possible activation by NO or inhibition by ODQ. Alternatively, another protein subunit could be required for assembly of an adequate functional complex that has specific NO control of GC activity. The lack of conserved Cys residues able to be nitrosylated (see Supplemental Figures 2 and 3 online; Fernhoff et al., 2009) might also result in weaker activation than in animal GCs. The presence of highly conserved HNOB and HNOBA and the series of experiments detailed below strongly suggest that CYG56 should be activated by NO in vivo.

NO and cGMP Repress *NIA1* Transcription in the *Chlamydomonas* Wild-Type Strain 704

To assess whether NO and cGMP indeed repress *NIA1* transcription, a set of pharmacological products that increase intracellular concentrations of these molecules were applied to *Chlamydomonas* cell cultures. The phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), the cGMP analog pCPT-cGMP, and NO donors SNAP and DEA NONOate were used, and their effects on *NIA1* expression were measured by real-time PCR. In the wild-type 704, IBMX inhibited *NIA1* transcription in a dose-dependent manner. Application of 1 mM IBMX led to a 75% decrease of *NIA1* transcript levels compared with the untreated control, and 2 mM IBMX led to almost full inhibition of gene expression (Figure 5A). The cGMP analog pCPT-cGMP at 100

μM had a minor effect; however, when combined with 200 μM IBMX, a concentration at which IBMX alone did not show any significant effect, this cGMP analog caused a strong inhibition of *NIA1* expression (Figure 5B). The NO donor SNAP also reduced wild-type *NIA1* transcript levels to 32% when used at 2 mM (Figure 5C). DEA NONOate, a more potent NO donor, caused a full inhibition of *NIA1* expression at concentrations as low as 10 μM, whereas 100 μM of the analog SulphoNONOate that does not produce NO had no effect. Nitrite derived from oxidation of NO (Ignarro et al., 1993) does not seem to be responsible for the observed effects of DEA NONOate since application of 100 μM nitrite had no effect (Figure 5D). Taken together, these results suggest that cGMP and NO are able to repress *NIA1* transcription.

Other Ammonium-Repressible Nitrogen Assimilation Genes Respond to NO and cGMP

NIA1 and *NRT2.1*, which encodes a component of a high affinity nitrate transporter, have in common that they are repressed by ammonium and are positively regulated by the nitrate-specific regulatory protein NIT2 (Llamas et al., 2002; Rexach et al., 2002). *AMT1.1* and *AMT1.2* encode ammonium transporters, and their

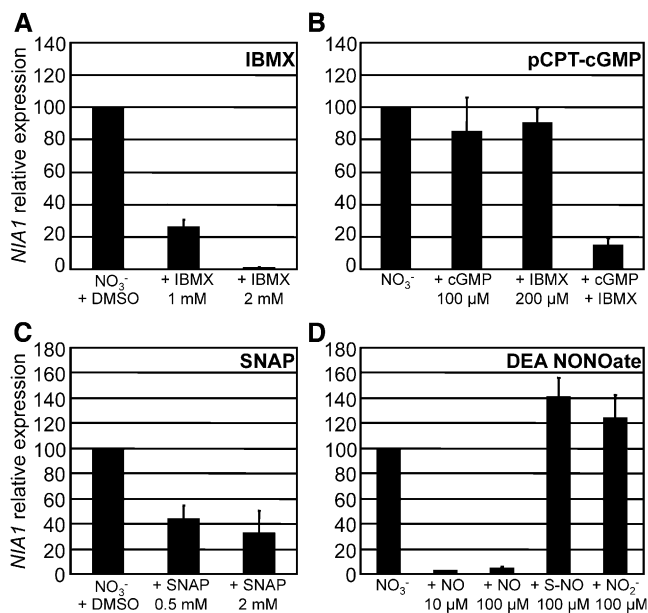


Figure 5. IBMX, pCPT-cGMP, SNAP, and DEA NONOate Repress *NIA1* Transcription in the Wild-Type 704.

The effect of IBMX (A), pCPT-cGMP (8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate) (B), SNAP (S-nitroso-*N*-acetylpenicillamine) (C), and diethylamine NONOate (DEA NONOate) (D) on *NIA1* transcription was determined by real-time PCR. The effect of DEA NONOate (NO) was compared with those of chemical control SulphoNONOate (S-NO) and nitrite at the indicated concentrations. The error bars represent the SD of at least three replicates, with each replicate corresponding to a different PCR run. As IBMX and SNAP are soluble in DMSO, DMSO was added in the untreated control. cGMP represents pCPT-cGMP.

expression levels are also significantly reduced in ammonium compared with nitrate. As for *NIA1* and *NRT2.1*, *AMT1.1* expression was shown to be regulated by NIT2 (González-Ballester et al., 2004). To assess whether the effect of NO and cGMP was specific for *NIA1* or whether they affected nitrate regulated genes in a more general way, we tested the effect of IBMX, pCPT-cGMP, and NO donors on the expression of *NRT2.1*, *AMT1.1*, and *AMT1.2*. As for *NIA1*, IBMX, pCPT-cGMP, SNAP, and DEA NONOate caused repression of *NRT2.1*, *AMT1.1*, and *AMT1.2* in a dose-dependent manner (Figures 6A to 6D). Again, 100 μ M pCPT-cGMP had a negative effect on the expression of those genes when combined with 200 μ M IBMX. Another cGMP analog, 8-bromo (8Br)-cGMP, had similar negative effects on expression of *NIA1*, *NRT2.1*, and *AMT1.1* as the Cl-derivative (i.e., minor when applied alone and significant in combination with 200 μ M IBMX). However, 8-Br-cGMP had no effect on expression of *AMT1.2* (see Supplemental Figure 7 online). The minor effects of cGMP analogs on nitrate assimilation gene expression can be explained by hydrolysis of these compounds by phosphodiesterases, that when inhibited resulted in the observed effects (Boss, 1989). This also shows that repression of *NIA1* by IBMX is most likely due to the upregulation of intracellular cGMP rather than cAMP.

DEA NONOate also proved to be a more potent NO donor than SNAP as concentrations as low as 10 and 100 μ M showed a

strong negative effect on the expression of those three genes (Figures 6C and 6D). Although the expression of the housekeeping gene ubiquitin ligase (see Methods) remained unaltered by these treatments, we tested the expression of actin as an additional control to check whether transcription was affected by DEA NONOate. The expression levels of actin remained unaltered after the treatments (data not shown). Taken together, these data suggest that cGMP and NO mimic the effects of ammonium on expression of these genes.

The GC Inhibitors, LY and ODQ, and the NO Scavenger cPTIO Release Repression by Ammonium and NO, Respectively, in the Wild-Type Strain

To support better that NO-dependent GC has a role on ammonium repression, we reproduced a deficiency of GC activity in the wild-type strain using the GC inhibitors LY and ODQ. The inhibitors were applied in medium containing NO_3^- and NH_4^+ (in which *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* transcript levels were low). As expected, expression of these four genes was derepressed by LY treatments in a dose-dependent manner (Figure 7A). Similarly, ODQ also caused derepression of the four genes in ammonium containing medium (Figure 7B). As shown in Figures 5 and 6, NO supplied by DEA-NONOate promoted a strong inhibition of nitrate induction for these genes. The effect of the specific NO scavenger cPTIO (Planchet and Kaiser, 2006) was determined in two different ways: first, by preincubating the cells with cPTIO during 15 min before adding DEA (protocol 1, Figure 7C) and by preincubating DEA and cPTIO together before applying them to the cells (protocol 2, Figure 7C). Both protocols were efficient in inhibiting the effect of DEA.

These data so far suggest that (1) repression of nitrogen assimilation gene expression by NH_4^+ at least partially requires an NO-dependent GC activity, and (2) NH_4^+ , NO, GC, and cGMP are components of a common regulation pathway that represses *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2*.

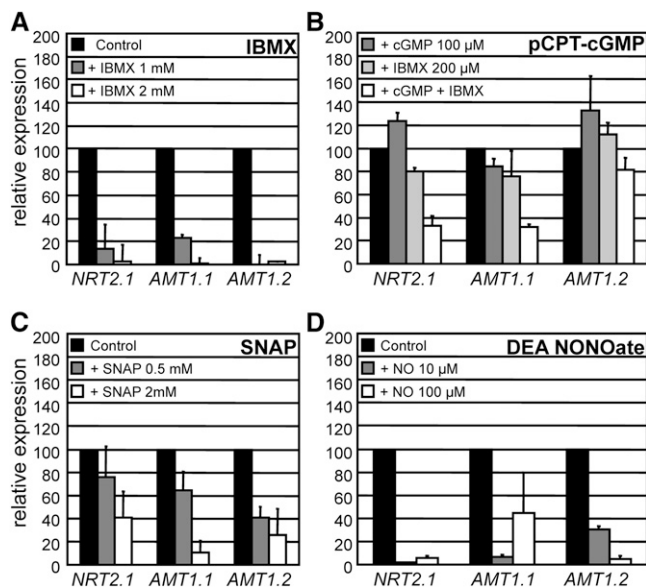


Figure 6. Effect of IBMX, pCPT-cGMP, SNAP, and DEA NONOate on Expression of Ammonium-Repressed Genes *NRT2.1*, *AMT1.1*, and *AMT1.2* in Wild-Type Cells.

The effect of IBMX (A), pCPT-cGMP (B), SNAP (C), and DEA NONOate (D) on the expression of *NRT2.1* and *AMT1.1* and *AMT1.2* was determined by real-time PCR. Conditions of the experiment were the same as in Figure 5 (see Methods). Black bars represent controls (set to 100%) without the added chemical; gray and white bars represent the concentrations of each chemical as indicated in each panel. The error bars represent the SD of three biological replicates.

Ammonium Enhances NO and cGMP Levels in *Chlamydomonas* Cells

If NO is implicated in the repression of *NIA1* by ammonium, then we predicted that ammonium would increase NO intracellular levels. To assess this, intracellular NO was quantified by fluorescence using the DAF-FM DA (4-amino-5-methylamino-2'7'-difluorofluorescein diacetate) dye (Kojima et al., 1998; Planchet and Kaiser, 2006). As shown in Supplemental Figure 6 online, DAF-FM DA only emitted fluorescence after entering the cells and fluorescence was directly proportional to the amount of applied DEA NONOate. NO production was measured after different nitrogen treatments of the cells (Figure 8). Cells transferred to media containing nitrate plus different amounts of ammonium showed that stimulation of NO production depended on the ammonium concentration (Figure 8A). NO production was higher in cells coming from a preculture in N-free medium for 24 h (see Supplemental Figure 8B online) than in cells coming from the standard ammonium medium (Figure 8A). These cells from N-free medium did respond slightly to increasing nitrate concentrations though much less than to ammonium (see

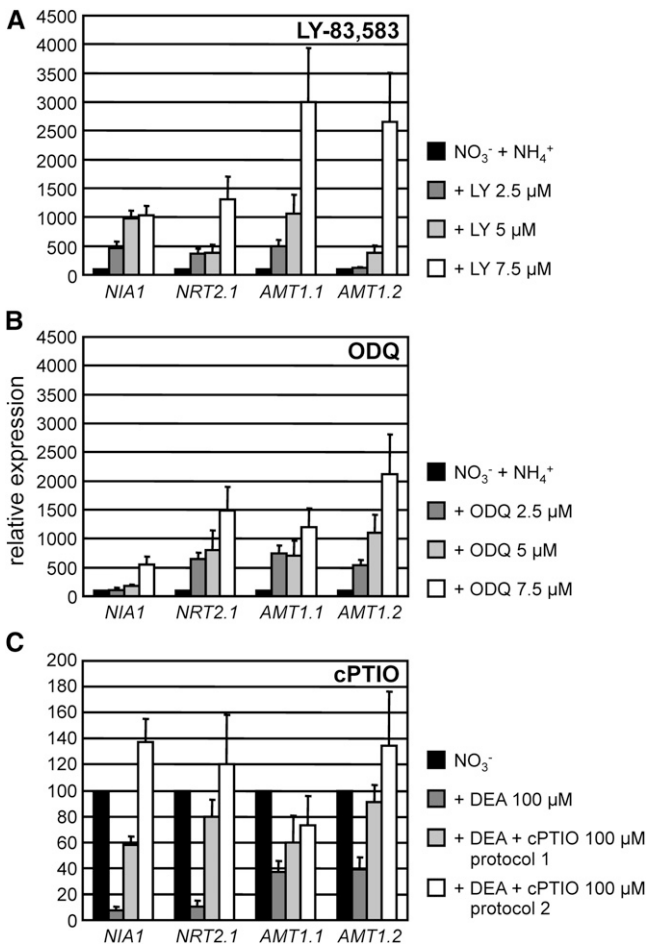


Figure 7. Treatment of Wild-Type Cells with GC Inhibitors LY and ODQ Leads to Partial Release of *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* Expression in Media Containing Nitrate and Ammonium: Effect of the NO Scavenger cPTIO.

The effect of LY83,583 (6-anilino-5,8-quinolinedione) and ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) on the expression of the indicated genes was determined by real-time PCR. Wild-type 704 was grown in minimum medium containing 8 mM NH₄Cl. After 3 d of growth, cells were induced during 1 h in minimum medium containing 4 mM KNO₃ + 1 mM NH₄Cl supplied LY (A) or ODQ (B) at the concentrations indicated. Alternatively (C), expression of these genes was determined in cells induced during 1 h in minimum medium containing 100 μM KNO₃ in the absence or presence of NONOate 100 μM (used as controls). The effect of 100 μM cPTIO was analyzed either after preincubating the cells 15 min with cPTIO previously to NONOate addition (protocol 1) or when added simultaneously with DEA NONOate at time 0 (protocol 2). The error bars represent the SD of three biological replicates.

Supplemental Figure 8B online). However, in media containing both 5 mM ammonium and increasing concentrations of nitrate, the observed NO production mostly resulted from ammonium with little appreciable effect of the high concentrations of nitrate (see Supplemental Figure 8C online).

Levels of cGMP in the cells were also determined in response to treatments with IBMX or ammonium. As shown in Figure 8B,

cGMP levels in cells incubated in 100 μM nitrate increased slightly due to the presence of ammonium 1 or 2 mM. However, these levels increased significantly after 1 h treatment with 2 mM IBMX. There was an insignificant effect of added ammonium, probably due to the fact that, from the high number of GCs in *Chlamydomonas* (Merchant et al., 2007), only a few of the GCs mediate ammonium effects. These results reinforce that ammonium effects are mediated by NO and cGMP.

Ammonium-Repressible Nitrogen Assimilation Genes Respond to Ca²⁺ and to L-NAME, an Inhibitor of NO Synthesis

In plants, synergistic effects of Ca²⁺, NO, and cGMP in various physiological processes, such as response to pathogen infection and development of cell polarity, have been reported (Sokolovski et al., 2005; Lanteri et al., 2006; Ali et al., 2007; Salmi et al., 2007). Implication of these second messengers in the same regulation pathways is often associated with Ca²⁺ being an activator of a possible nitric oxide synthase (NOS). We tested whether Ca²⁺ was also implicated in the regulation of *NIA1* and other

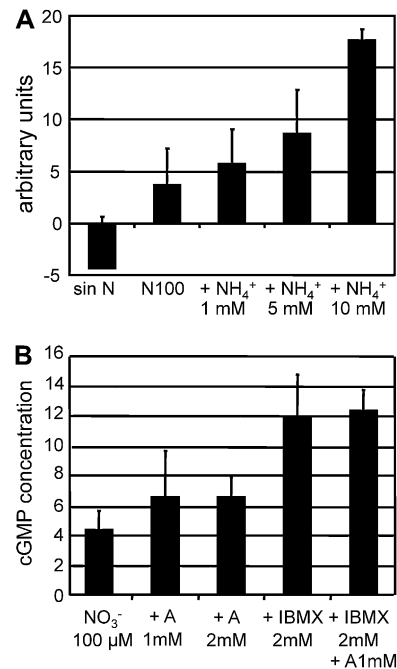


Figure 8. Ammonium Increases Intracellular Levels of NO and cGMP.

Fluorescence due to intracellular NO levels after incubation with the indicated nitrogen sources was determined using DAF-FM DA and is expressed as arbitrary units.

(A) Cells were grown in 8 mM ammonium medium, washed, and transferred to N-free medium and media containing 100 μM nitrate plus the indicated concentration of ammonium for 1 h, and then fluorescence was determined.

(B) cGMP concentration was determined from cells grown in 8 mM ammonium (A) medium, washed, and transferred for 1 h to the indicated medium. Amounts of cGMP are expressed as fmol/10⁶ cells. The error bars represent the SD of three biological replicates.

ammonium-repressible nitrogen assimilation genes by measuring the effects of the calcium ionophore A23187 on their transcript levels. Addition of A23187 reduced *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* expression in a dose-dependent manner, as 5 and 10 μM of A23187 resulted in a decrease in their transcript levels relative to untreated controls (Figure 9A). As for SNAP and IBMX, *AMT1.1* and *AMT1.2* were more sensitive to A23187. Though side effects caused by this calcium channel inhibitor cannot be ruled out, these data suggest that increases in intracellular Ca^{2+} levels also repress nitrogen assimilation gene transcription. On the other hand, L-NAME, an inhibitor of NO synthesis (Radomski et al., 1990), caused a partial release of the negative effects of ammonium on *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* expression (Figure 9B). Expression of actin, used as a control, was not affected by the presence of this inhibitor.

Interestingly, the Ca^{2+} ionophore promoted NO production in a dose-dependent manner (Figure 9C). L-NAME inhibited efficiently NO production resulting from either the treatment with ammonium or the calcium ionophore (Figure 9D). These results suggest that Ca^{2+} and a putative NOS could be involved in the negative regulation of ammonium regulated genes mediated by NO.

Mutant *cyg56* Is Altered in a Signaling Pathway That Involves Ammonium, NO, cGMP, and Ca^{2+}

Comparative studies between the wild type and mutant *cyg56* using effectors upstream and downstream of the GC enzyme in the signaling pathway were useful to better understand this pathway. Results of effectors are presented as matrices of gene expression where transcript levels are normalized to the untreated control and the level of repression is represented by a scale of shading (Figure 10; see Supplemental Table 1 online). Consistently, results showed that SNAP, an NO donor, did not affect expression of *NIA1* and *NRT2.1*, in mutant *cyg56* in contrast with the wild type. However, IBMX repressed *NIA1* and *NRT2.1* expression to a similar extent in both the mutant and the wild-type 704 (Figures 10A and 10B).

To strengthen these results, this experiment was performed with the NO donor sodium nitroprusside (SNP). The effects of SNP were compared with ferricyanide (FeCN) as a chemical control since both components contain cyanide in their molecular structure (five molecules of cyanide for SNP and six for FeCN). The difference between these two is the presence of a nitrous group responsible for the NO-generating property of SNP (Oh and McCaslin, 1995; Bethke et al., 2005). SNP strongly repressed *NIA1* and *NRT2.1* transcription in the wild type, but this effect was weaker in mutant *cyg56* (Figures 10A and 10B). Surprisingly, FeCN also repressed the expression of these genes, which suggested that cyanide acted negatively on *NIA1* and *NRT2.1*, but this effect was similar in 704 and *cyg56*. These results argued in favor of mutant *cyg56* being altered in a pathway specific for NO-mediated *NIA1* and *NRT2.1* repression that is independent of cyanide.

Unexpectedly, the strong NO donor DEA NONOate caused repression of *NIA1* and *NRT2.1* in both 704 and *cyg56* as de-repression of the genes in the mutant was very mild (see Supplemental Table 1 online). The repression of *NIA1* by DEA

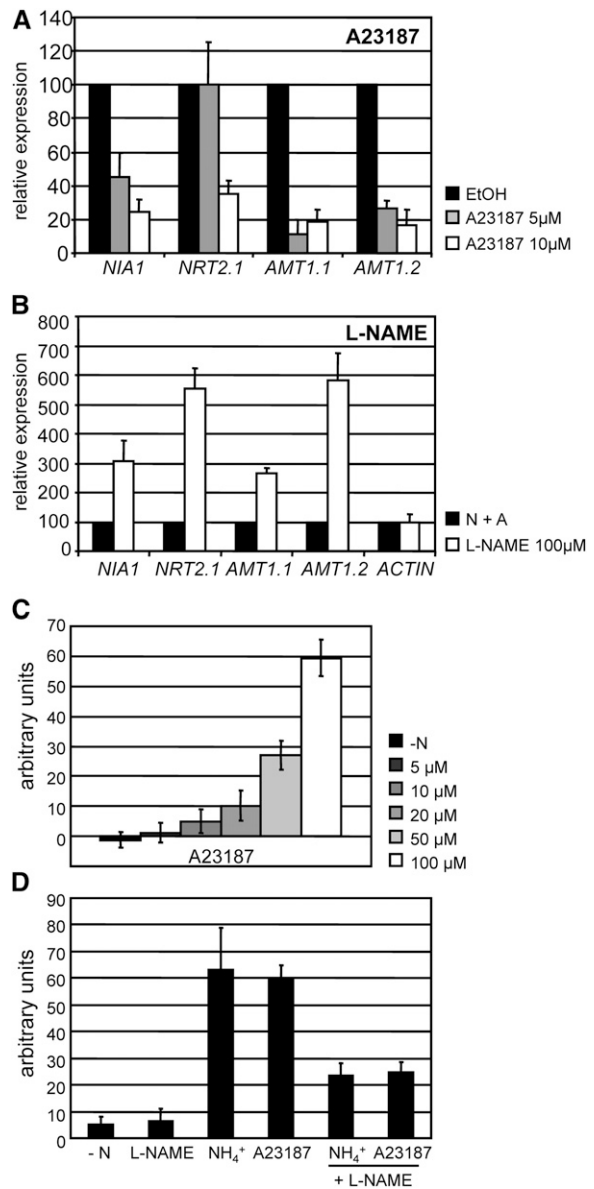


Figure 9. Effect of A23187 and L-NAME on Ammonium Repression of Gene Expression and on the Levels of NO.

The effect of A23187 (A) and L-NAME (B) on the expression of *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* was determined by real-time PCR. Wild-type 704 was grown in minimum medium containing 8 mM NH_4Cl . After 3 d of growth, cells were induced during 1 h in minimum medium containing 100 μM KNO_3 in (A) or 100 μM KNO_3 + 1 mM NH_4Cl in (B) and treated with the chemicals at the concentrations indicated. The effect of A23187 (C) and L-NAME (D) on the fluorescence levels due to intracellular NO determined using DAF-FM DA and expressed as arbitrary units. Cells preincubated for 24 h in N-free medium were transferred to the indicated N-free medium containing the indicated concentration of A23187 (C) or 5 mM ammonium, 100 μM A23187, and 100 μM L-NAME, as indicated (D). The error bars represent the SD of three biological replicates.

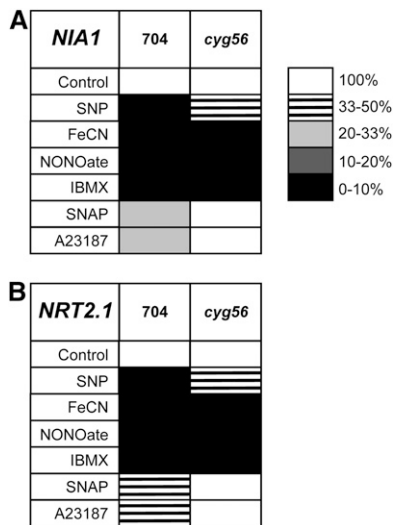


Figure 10. Expression of *NIA1* and *NRT2.1* Is Partially or Completely Derepressed in Mutant *cyg56* Cells When Treated with SNAP, A23187, and SNP but Not with IBMX and DEA NONOate.

Relative transcript levels of *NIA1* (A) and *NRT2.1* (B) were quantified by real-time PCR in strains 704 and *cyg56* when treated with the indicated pharmacological products. Concentrations were 1 mM SNP and FeCN, 10 μ M DEA NONOate, 2 mM IBMX, 2 mM SNAP, and 10 μ M A23187. Data are summarized in matrices of gene expression in which relative transcript levels are represented by a gray code. In both tables, each column corresponds to a strain and each row corresponds to the effect of a pharmacological product. Results from the treated samples were normalized to the untreated control for each PCR run. Conditions of the experiment were the same than in Figure 5. Raw data are presented in Supplemental Table 1 online.

NONOate in *cyg56* might be directly related to the higher potency of this NO donor, a more efficient release of intracellular NO possibly leading to activation of other GCs that are also implicated in *NIA1* repression. This idea is supported by the presence of >50 predicted GC domains in the genome of *Chlamydomonas* (Merchant et al., 2007) from which at least six are NO-dependent type GC as shown above.

Finally, calcium ionophore A23187 had a slight effect on expression of *NIA1* and *NRT2.1* transcripts in mutant *cyg56* in contrast with the wild type (Figures 10A and 10B; see Supplemental Table 1 online). Taken together, these results strongly suggest that NH_4^+ , NO, cGMP, and Ca^{2+} act in a common regulation pathway that represses *NIA1* and *NRT2.1* transcription and that this pathway is altered in mutant *cyg56*. Furthermore, opposing effects of A23187 and NO donors with IBMX in mutant *cyg56* suggest that Ca^{2+} and NO act upstream of cGMP in this pathway.

NO Represses *NIA1* and *NIA2* Transcription in *Arabidopsis*

Genes and mechanisms of the nitrate assimilation pathway are well conserved between *Chlamydomonas* and higher plants; thus, cGMP and NO could also be signal molecules for repression of nitrate assimilation genes in *Arabidopsis*. First, published microarray data (Maathuis, 2006) revealed that application of 10 μ M 8-Br-cGMP to *Arabidopsis* roots reduced *NIA2* expression to 35% relative to the untreated control 5 h after exposure. *NRT2.1* and *NRT2.2* mRNA levels were also reduced to 46 and 33%, respectively, in the same conditions. Moreover, *AMT1* was even more sensitive as expression levels of this gene dropped to 27% 2 h only after exposure to 8-Br-cGMP. These data suggested that cGMP represses nitrogen assimilation genes in *Arabidopsis*.

Here, we analyzed the effect of an NO donor on the expression of genes encoding NRs in *Arabidopsis* by applying DEA NONOate to 10-d-old seedlings. As shown in Figure 11, both *NIA1* and *NIA2* are repressed in a time-dependent manner by NO in comparison to control plants maintained in the medium with no additions. The chemical control SulfoNONOate did not affect expression of either of the two genes. These results show that *NIA1* and *NIA2* gene expression is sensitive to NO and suggest that, in *Arabidopsis*, mechanisms similar to those found in *Chlamydomonas* involving NO on the negative control of nitrate assimilation genes might be operative.

DISCUSSION

Regulation of nitrate assimilation involves the integration of positive signals (i.e., nitrate) and negative signals (i.e., ammonium

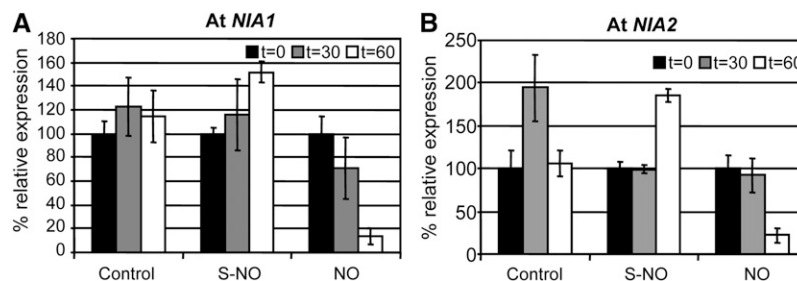


Figure 11. NO Represses *NIA1* and *NIA2* from *Arabidopsis*.

The effect of DEA NONOate and SulphoNONOate on At *NIA1* (A) and At *NIA2* (B) expression was tested by real-time PCR in 10-d-old *Arabidopsis* seedlings. DEA NONOate and SulphoNONOate (100 μ M) was applied directly on the seedlings as described in Methods. Plant material was harvested before treatment (black bars) and after 30 min (gray bars) and 1 h (white bars) as indicated. The error bars represent the SD of three biological replicates.

and ammonium derivatives), which result in appropriate use of the nitrogen source. In this work, we analyzed negative signaling by ammonium on *NIA1* expression using a *Chlamydomonas* wild-type strain and a mutant defective in a putative GC. The results allow us to conclude several points as described in the following sections.

Ammonium Repression of *NIA1* Appears to Be a Complex Process

Ammonium repression of *NIA1* is shared by the other genes for nitrate assimilation in *Chlamydomonas* (Fernandez and Galvan, 2007), and this negative regulation by ammonium assimilation derivatives is also observed in other plant systems (Stitt, 1999; Crawford and Forde, 2002; Glass, 2003; Fan et al., 2006). That this is a complex process can be deduced not only from data in the bibliography but also from results generated in *Chlamydomonas*. First, the screening of a *Chlamydomonas* insertional mutant library produced a set of mutants that showed different AI phenotype intensities and that were defective in different genes for ammonium sensing (González-Ballester et al., 2005a). Second, the AI phenotype depends on the concentration of ammonium in the medium, so that more than one mechanism might mediate ammonium sensing at diverse concentrations as previously proposed. Finally, of all the AI mutants isolated from that screen or from other previously reported screens (Prieto et al., 1996; Zhang and Lefebvre 1997; Perez-Alegre et al., 2005), none was fully insensitive to ammonium. One of the goals of this work was the analysis of one of those mutant strains that was affected in a putative GC, since that might reveal a relationship between regulation of nitrate assimilation and cGMP.

An NO-Dependent Guanylate Cyclase Is Responsible for the AI Phenotype of Mutant *cyg56*

Insertion of a marker gene in the AI mutant *cyg56* caused a critical deletion at the 5' end of the *CYG56* gene, fusing it to the sequence of the marker in a chimeric transcript that is overexpressed. These modifications are compatible with a loss of function of *CYG56* gene. This is also supported by the complementation of the mutant phenotype in diploids and the reproduction of an AI phenotype by the GC inhibitors LY and ODQ in the wild-type strain.

CYG56 expression was upregulated by ammonium plus nitrate medium, and the maximum level of expression was reached in 1 h, which indicates that upregulation of *CYG56* by nitrate plus ammonium could be transient. This regulation suggests a role of *CYG56* when both sources of nitrogen are present. In other terms, in ammonium plus nitrate medium, *CYG56* signaling on *NIA1* makes sense, since *NIA1* negative control by ammonium can only take place after expression has been switched on in nitrate (Camargo et al., 2007).

CYG56 is a NO-dependent GC containing the three domains typical of this kind of GCs that are present in animals and absent in plants (Nioche et al., 2004). The enzyme activity of *CYG56* as a GC was demonstrated using both the full-length protein and the N terminus protein fragment of 957 residues containing the GC catalytic domain and the domains for NO binding. However, the

expressed proteins already had a high GC activity so that additional NO activation could not be shown, possibly due to an inappropriate folding of this protein in the *E. coli* host that would release the GC domain from the control by NO. Additionally, another subunit might be required to make the GC domain further activated by NO as reported in other animal GCs (Koglin et al., 2001). Interestingly, *CYG56* lacks three Cys residues conserved in other GCs (see Supplemental Figures 2 and 3 online). Nitrosylation of Cys residues has been implicated in the strong activation by NO of animal GC versus the moderate activation caused by NO binding to the heme group (Fernhoff et al., 2009). The fact that at least six GCs from the *Chlamydomonas* genome (Merchant et al., 2007) have the same domain

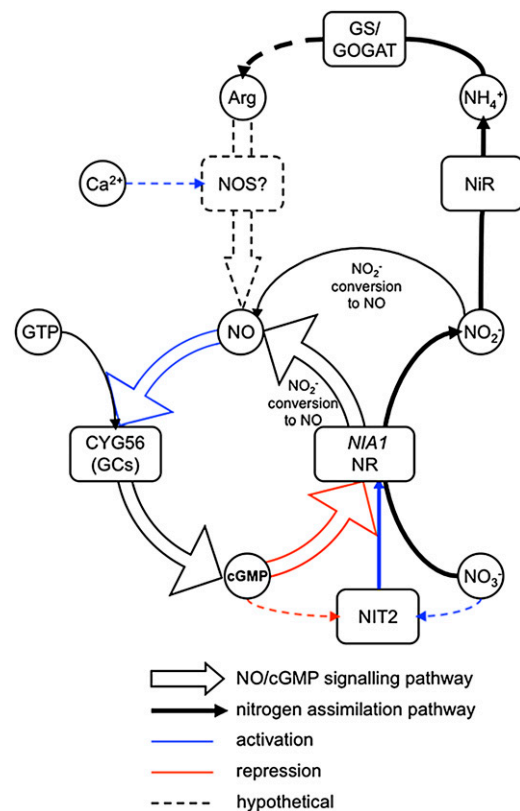


Figure 12. Proposed Scheme for *NIA1* Negative Regulation Involving Ammonium, Arg, Ca^{2+} , NO, cGMP, and Nitrite.

The regulation scheme involves the following steps: (1) Nitrate is reduced to nitrite and nitrite to ammonium by sequential action of NR and nitrite reductase (NiR). Ammonium can also be transported inside the cell from the extracellular medium. (2) The GS/GOGAT cycle integrates ammonium to the metabolism leading to the synthesis of amino acids. (3) In plant systems, Arg is converted to NO through Ca^{2+} dependent of a putative NOS activity, but the corresponding NOS gene is so far unknown, which is why this step is presented as hypothetical in the model. (4) NO activates *CYG56* and maybe other soluble GCs that catalyze the formation of cGMP. (5) cGMP represses *NIA1* transcription, maybe by interfering with NIT2 functionality. (6) NO can be generated by enzyme (NR activity or mitochondrial electron transport) or chemical conversion of nitrite.

structure as CYG56 could suggest a moderate activation of GCs, and this would be necessary for a more precise and fine regulation of gene expression. It is also interesting that of these six members of the gene family encoding NO binding GC, three (CYG11, CYG12, and CYG15) are clustered in the same chromosome 7 (CYG56 is in chromosome 16).

Thus, a signal transduction protein typical of animals has been selected in *Chlamydomonas* for regulating a metabolic pathway, nitrate assimilation, typical of plants and bacteria but not of animals. Guanylyl cyclases that are not well conserved with animal GCs have been described in higher plants (Ludidi and Gehring, 2003; Yuan et al., 2008) and proteins having a GC domain with a modular structure such as the brassinosteroid receptor described (Kwezi et al., 2007). However, NO and cGMP appear to regulate numbers of physiological processes in plants. A possibility is that proteins involved in sensing belong to diverse protein families or have particular combinations of domains to deal with similar regulatory circuits or strategies in the different organisms.

A Regulation Model for *NIA1* Transcription Involves Ammonium, Calcium, NO, and cGMP

This work provides pharmacological and genetic evidences that ammonium, calcium, NO, and cGMP act by a similar pathway to repress *NIA1* and *NRT2.1* and that this process requires GC activity. The facts supporting that NO is a component of the ammonium-CYG56-cGMP negative signaling pathway are (1) NO represses expression of *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2*. This was shown with the three NO donors SNAP, SNP, and DEA-NONOate. No effect was seen with the chemical

control SulfoNONOate. In addition, DEA-NONOate had no effect after preincubation with cPTIO. (2) CYG56 has a conserved NO binding domain. (3) Ammonium increases NO intracellular levels. (4) ODQ blocks the repression of *NIA1* by ammonium; and, most importantly, (5) the effect of SNAP and SNP on the expression of *NIA1* and *NRT2.1* does not take place in the *cyg56* mutant.

The facts supporting that cGMP is a component of the ammonium-CYG56-cGMP negative signaling pathway are (1) IBMX and ammonium increase intracellular levels of cGMP. (2) cGMP analogs (pCPT-cGMP and 8-Br-cGMP) cause repression of *NIA1* and *NRT2.1*. (3) Inhibition of the NO-dependent GC by LY or ODQ releases cells from ammonium repression of *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2*, reproducing the phenotype of mutant *cyg56*. Published microarray data from *Arabidopsis* suggested that this regulation pathway could be conserved in higher plants as application of 8-Br-cGMP repressed *NIA2*, *NRT2.1*, *NRT2.2*, and *AMT1* (Maathuis, 2006).

Based on results presented here and on data from the literature, we propose a regulation model for the repression of *NIA1* that integrates ammonium, calcium, NO, and cGMP (Figure 12). In this model, CYG56 has a central role. Ammonium is generated by sequential reduction of nitrate and nitrite through NR and NiR activities, respectively, or can be transported from the extracellular medium (Galvan et al., 2006). Ammonium is integrated into the structure of amino acids by the GS/GOGAT cycle, leading in turn to the synthesis of Arg (Crawford, 1995; Slocum, 2005). Hypothetically, Arg could be converted to NO and citrulline by a putative NOS activity, Ca²⁺ being involved in this process as an activator of the enzyme. NO would then activate one or several GCs that produce cGMP from GTP, and cGMP would in turn

Table 1. List of primers used

Primer	Sequence (5' to 3')	Use
Actinup	ACTGAGCACAATGTTAC	<i>Arabidopsis</i> ACTIN gene
Actinlw	GGTGATGGTGTGTCT	
AtNia1up	AGGCTACGCTTATTCTGGAGGA	<i>Arabidopsis</i> <i>NIA1</i>
AtNia1lw	GAACCGCGACGTCTTTAGCACTGAGCA	
AtNia2up	CGCCGACGAAGAAGTTGG	<i>Arabidopsis</i> <i>NIA2</i>
AtNia2lw	GGGTTGTGAAAGCGTTGATGGGAAGAAT	
Ubiupper	GTACAGCGGGCGGCTAGAGGCAC	<i>Chlamydomonas</i> (Cr) gene for ubiquitin
Ubilower	AGCGTCAGCGGGTTCAGGTATCT	
20.73RT1fw	GAGCGCTTCGAGGCAGAGACC	Cr CYG56 transcript
20.73RT1rev	CAGCAGTGCGTCAAACCTCGTTGTACA	
NRupper	CCGAGCGCTCCGGCTGTGGTACA	Cr <i>NIA1</i> transcript
NRlower	CTGGATCTGGCGGTCCCTTGCTGTA	
Nrt2.1upper	CGCCGTGGCAACTGACCCTGAG	Cr <i>NRT2.1</i> transcript
Nrt2.1lower	CGCCACCTCCTCCGCACTCCAC	
AMT1.1upper	GCACGGGAGGGCAAGAGGTTT	Cr <i>AMT1.1</i> transcript
AMT1.1lower	ATGTGCCGAGTCAAGAAGGATTT	
AMT1.2upper	GGCTCGCCACCTGCAAGAGACAAC	Cr <i>AMT1.2</i> transcript
AMT1.2lower	GATGGCACCGGCCTCGAGCATAGC	
Primer 1	ATGCAATTTCTTCTAGATGAACCCGT	see Figure 3
Primer 2	CCAGAGCTGCCACCTTGACA	
Primer 3	CCACCACCCCGAAGCCGATAA	
Primer 4	TCCTCCACAACAACCCACTCACAACC	
Primer 5	CGCCCTCATAGCCGCCAAAT	
Primer 6	TCCCGCTGCTTCTTGCTGCT	
Primer 7	CAGCCCCAGGATCTTGTA	

repress *NIA1* transcription. A similar process would be operative for *NRT2.1*. The positioning of NO and Ca^{2+} upstream of CYG56 is mainly supported by the A23187, SNAP, and SNP-mediated repression of *NIA1* being partially compensated in the *cyg56* mutant. In support of this model is the dose-dependent NO production promoted by Ca^{2+} ionophore and its inhibition by L-NAME, in a similar manner of the NO production promoted by ammonium.

The mechanism by which cGMP could regulate *NIA1* transcription is unclear. Putative cGMP-dependent protein kinases and their targets are not known. The presence of a GAF domain on the sequence of the *NIA1* positive regulator NIT2 suggests a possible role for cGMP in mediating NIT2 functionality (Camargo et al., 2007). This idea is supported by NIT2 being implicated in the regulation of *NIA1*, *NRT2.1*, and *AMT1.1*. As NO and cGMP repress the expression of all three genes, a possibility is that cGMP binds to NIT2, leading to its inactivation. The GAF domain was shown not to participate in DNA binding of NIT2 (Camargo et al., 2007), but it could be implicated in conformational changes of the protein. Interestingly, NO has also been shown to bind GAF domains of NorR protein (Busch et al., 2005). This signaling on NIT2 might occur at some other step.

An essential question that arises from the model and from our data is: By which mechanism NO is actually synthesized? Well-known sources of NO in plants and other organisms are Arg and nitrite. NOS activity dependent on Arg with citrulline formation has been observed in plant extracts, as its inhibition by Arg analogs (Cueto et al., 1996; Durner et al., 1998; Delledonne et al., 1998; Del Rio et al., 2004).

Arg is converted to NO through the activity of a possible NOS, a controversial enzyme that has not yet been cloned in plant systems (Guo et al., 2003; Zemojtel et al., 2006). The potential *Arabidopsis* NOS1 previously identified corresponds to a 561-amino acid protein having no sequence homology to the animal NOSs (Guo et al., 2003). At NOS1 has been renamed At NOA1 and shown to be a functional *Arabidopsis* member of the circularly permuted GTPase family (cGTPase) with no NO synthase activity (Moreau et al., 2008). In our experiments, evidence of NOS activity being implicated in the described model is given by the partial release of *NIA1* repression by L-NAME, an inhibitor of NOS activity. Moreover, the repression of *NIA1* by A23187 in the wild type but not in the mutant suggests the involvement of Ca^{2+} in *NIA1* regulation upstream of CYG56. These results support the implication of NOS in the model as Ca^{2+} is an activator of NOS in animals, and NOS activity has been shown to be Ca^{2+} dependent in plants (Delledonne et al., 1998; Courtois et al., 2008). Nevertheless, this evidence remains insufficient to demonstrate that NOS is the source of NO in the ammonium-mediated *NIA1* repression pathway as NOS activity has not yet been shown in *Chlamydomonas* and considering that the existence of this enzyme remains uncertain in plants. The presence of the enzyme in the model is therefore stated as hypothetical. Future experiments must address this question.

Nitrite can be converted to NO through either NR activity or the mitochondrial electron transport chain (Yamasaki and Sakihama, 2000; Bethke et al., 2004; Tischner et al., 2004; Planchet et al., 2005). Interestingly, nitrite was shown to repress transcription of *NIA1* (Loqué et al., 2003), raising the question of whether nitrite-mediated *NIA1* repression is due to nitrite conversion to NO. The

particular conditions regulating NR activity for nitrite conversion to NO in planta are not known. Lamattina et al. (2003) suggested that changes in NR activity could be modulated by nitrite availability. When the plant is photosynthetically active, nitrite produced by NR is immediately taken up by the chloroplast and reduced to ammonium (Rexach et al., 2000). However, nitrite accumulation can be expected when photosynthetic activity is low, so that NR-mediated conversion of nitrite to NO could take place in these conditions (Lillo et al., 2003; Lea et al., 2004) and NO could then repress *NIA1* transcription, the whole process forming a feedback loop. This negative feedback loop could be part of a mechanism for protection against nitrite toxicity, so that further accumulation of nitrite would be blocked.

Another important question that arises from our data is whether other GCs than CYG56 are implicated in the repression of *NIA1*. First, this idea is supported by the presence of >50 predicted GC catalytic domains in the genome of *Chlamydomonas* (Merchant et al., 2007). A report proposing the presence of a similar amount of GCs in the genome of *Arabidopsis*, although with limited sequence conservation (Kwezi et al., 2007), suggests a similar situation in higher plants. Second, the *cyg56* AI phenotype was mild in comparison with that of other mutants isolated from the initial screen (González-Ballester et al., 2005a). In *cyg56*, other GCs could still be repressing *NIA1* expression in response to ammonium, explaining why the phenotype was partial. Third, further evidence was given by the variety of responses obtained after treatment with different NO donors. SNAP was the less efficient NO donor tested, as the half-life of this compound is of several hours, whereas the half-life of DEA NONOate is of 17 min in controlled pH conditions. Consistently, application of DEA NONOate had drastic effects on *NIA1* expression, whereas the effect of SNAP was weaker. Moreover, release of *NIA1* repression in *cyg56* after treatment with SNAP was almost total and contrasted with the weak derepression observed with DEA NONOate. A possibility is that the stronger NO donor activates more efficiently other GCs implicated in the repression of *NIA1* that are not activated by SNAP. Taken together, this evidence suggests that various GCs being implicated in the ammonium-mediated repression of *NIA1* is a possible scenario that should also be addressed by future experiments.

In conclusion, we provide genetic evidence on that a NO-dependent GC of animal type is involved in the negative control of the nitrate assimilation pathway in *Chlamydomonas*. The effect of treatments with pharmacological compounds on gene expression allowed us to propose that ammonium, Ca^{2+} , NO, and cGMP act in a common pathway that represses *NIA1* and other ammonium-repressed genes. We also show that ammonium enhances the intracellular production of NO and cGMP. Evidence for a similar process to occur in plants is provided. A mechanistic approach is needed to understand how cGMP represses the transcription of ammonium-repressed genes.

METHODS

Strains and Culture Conditions

The *Chlamydomonas reinhardtii* strain 704 (*cw15 arg7+ NIA1:ArS mt+*) (Loppes et al., 1999) is the parental type of the AI mutant strain 20.73,

named here as *cyg56* (*mt+cw15 arg7+ NIA1:Ars rbsc2:AphVIII*) and obtained by insertional mutagenesis as previously detailed (González-Ballester et al., 2005a). Mutant strains 305 (*NIA1- mt-*) and 203 (*Nit2- mt+*) have been characterized elsewhere (Fernandez and Matagne, 1984). Cells were cultured under continuous light at 23°C in liquid or solid minimum medium (Harris, 1989) containing different concentrations of potassium nitrate depending on the experiment and were bubbled with 5% (v/v) CO₂-enriched air.

Arabidopsis thaliana Columbia-0 was grown on MS medium (Murashige and Skoog, 1962) in growth chambers providing controlled temperature (22°C) and a daylength regime of 16 h of light and 8 h dark per day. For the experiment shown in Figure 11, plants were grown in 12-well plates containing 2 mL of solid MS medium. Plant material harvested at different time points was grown in separate wells. Untreated, SulphoNONOate-treated and DEA NONOate-treated plants were grown in separate plates.

Genetic Analyses and Complementation Tests

Genetic crosses were performed using the random spore plating method (Harris, 1989). Segregants were analyzed for their ability to grow on medium containing nitrate by replica plating, for their resistance to 25 µg/mL paromomycin, caused by the inserted *APHVIII* gene from the marker, and for the Al phenotype as described (González-Ballester et al., 2005a). In vivo complementation was performed by isolating stable vegetative diploids according to the method of Ebersold (Fernández and Matagne, 1986; Harris, 1989).

ARS Activity

ARS activity was determined directly on the agar plates after removing cells from the agar surface with a razor blade and following the procedure previously reported (Ohresser et al., 1997). The reaction was stopped after 15 min.

NR Activity

NR activity was determined in vitro following the procedure previously described. Cells (100 µL) were lysed with 5 µL of 100% toluene (Fernandez and Cardenas, 1982). Benzyl viologen reduced with dithionite was the electron donor. The reaction was stopped by vortexing of the tubes, and nitrite, the product of NR activity, was determined as described by Snell and Snell (1949). Activity values were expressed as milliunits (mU), defined as the amount of enzyme catalyzing the transformation of 1 nmol of substrate per min, relative to the chlorophyll content, determined as described by Arnon (1949).

cGMP Determination and GC Expression and Activity

Both a full-length CYG56 protein and its N-terminal fragment of 957 amino acid residues were expressed in *Escherichia coli* using the vector pQE80 (Qiagen) that produces proteins labeled with a 6xhis tag (see Supplemental Figure 1B online). The protein was purified by metal affinity chromatography using Ni-NTA. Activity of the enzyme was assayed in 50 mM Tris-HCl buffer, pH 7.5, containing 4 mM MnCl₂, 0.2 mg/mL BSA, 1 mM GTP, 1 mM IBMX, 1% DMSO, and the enzyme. The assay was performed at 37°C for 10 min. Determination of cGMP was performed according to the immunoassay method (cGMP enzyme immuno assay kit direct; Sigma-Aldrich).

Determination of NH₄⁺ Concentration

NH₄⁺ concentration was determined using the Nessler reagent by adding 25 µL of reagent A and 25 µL of reagent B to 500 µL medium and

immediate measurement of OD at λ = 410 nm ($\epsilon = 1.67 \text{ mM}^{-1} \text{ cm}^{-1}$) according to Solórzano (1969).

Primers and PCR

All primers were designed using Primer Select (DNA Star version 4.05). For all the primer pairs used in the real-time PCR experiments, at least one of the two primers was designed so that its sequence would be overlapping two exons to avoid DNA contamination effects. Moreover, for each primer pair, the PCR product was run on a gel and sequenced to be sure of its specificity. Optimization of the primers was performed with an annealing temperature gradient and DMSO concentration gradient. In the real-time PCR experiments, the housekeeping gene corresponds to a ubiquitin ligase (EST references: BU648530, BU648531, BE237749, and BE237718) that shows constitutive expression under all the experimental conditions (González-Ballester et al., 2004). Primer sequences are given in Table 1.

Chemicals

Adequate amounts of products were weighed on the day of each experiment and resuspended just before application. DEA NONOate was resuspended in minimum medium for *Chlamydomonas* and liquid MS medium for *Arabidopsis*, and the pH of the solutions was adjusted to 7.2. After resuspension, DEA NONOate was applied immediately. In the experiment shown in Figure 11, 200 µL of DEA NONOate 100 µM was sprayed directly on the seedlings; the small size of the wells (2.5 cm diameter) allowed the liquid to stay in contact with the plants. Independent treatments were done in each well.

Isolation of RNA and cDNA Synthesis

Chlamydomonas cells were grown in minimum medium with 8 mM NH₄Cl, collected at mid-exponential phase of growth (4000g, 1 min) and transferred to medium containing the specified nitrogen source. RNA was extracted after lysis of the cells with 2% SDS in 100 mM Tris-HCl, pH 8.0, 400 mM NaCl, and 50 mM EDTA. Total RNA was extracted with phenol/chloroform as previously reported (Schloss et al., 1984).

Arabidopsis tissue was harvested after 10 d of growth in the previously described conditions. RNA was isolated from whole seedlings using the Qiagen RNeasy extraction kit, following the recommendations of the manufacturer. *Chlamydomonas* and *Arabidopsis* RNA was reversed transcribed using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen).

RT and Real-Time PCR

RT-PCR reactions were made in a 25-µL final volume with the following components: 0.2 pmol of each primer, 0.2 mM deoxynucleotide triphosphate, 0.5 units *Taq* DNA polymerase (BandM Labs), 2 mM MgCl₂, 1 µL cDNA (synthesized from 3 µg of total RNA), and 2.5 µL of the specific buffer. The standard thermal profile was 95°C, 5 min; 40× (95°C, 30 s; 60°C, 30 s; 72°C, 5 min). Real-time PCR was performed using the LightCycler Instrument (iCycler iQ real-time PCR detection system; BioRad) with SYBR Green I (Molecular Probes) as a fluorescent dye. Each individual reaction was made in a 25-µL final volume with the same components as for RT-PCR complemented with 1.25 µL of SYBR Green diluted 10⁻⁴ in DMSO. The LightCycler-run protocol was 95°C, 5 min; 40× (95°C, 30 s; 60°C, 30 s; 72°C, 15 s), and fluorescence measurements were done at 84°C for 10 s. The specificity of the PCR products was checked by a melting curve program and analyzed on a 2% agarose gel electrophoresis. The amplification rate of each transcript (Ct) was calculated by the PCR baseline subtracted method performed with LightCycler

software (iCycler iQ, Optical System Software, version 3) at a constant fluorescence level. Cts were determined over three repeats within the LightCycler and with three different runs.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed at the Servicio Central de Apoyo a la Investigación (University of Córdoba, Spain) with fluorescence-labeled terminators for an automatic sequencer (model ABI 377; Applied Biosystems, Perkin-Elmer) according to the manufacturer's instructions. Sequences were analyzed using the DNASTar software v. 4.05 (Lasergene Navigator), the Bioedit Sequence Alignment Editor v. 5.0.9 (Department of Microbiology, North Carolina State University), the National Center for Biotechnology Information BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the *Chlamydomonas* Joint Genome Initiative server (<http://genome.jgi-psf.org/Chlre4/Chlre4.home.html>). Domain predictions were analyzed with Expasy (<http://www.expasy.ch/cgi-bin/prosite/> and http://myhits.isb-sib.ch/cgi-bin/motif_scan) and Smart (<http://smart.embl-heidelberg.de/>) servers. Clustal comparisons were performed with ClustalW from the BioEdit program. Similarity scores were obtained with MegAlign from the DNASTAR package.

NO Quantification

Chlamydomonas cells were grown in minimum medium with 8 mM NH₄Cl, collected at mid-exponential phase of growth, and transferred to medium containing different nitrogen sources. DAF-FM DA (1 μM; Invitrogen Molecular Probes) (Kojima et al., 1998) was applied 30 min prior to the measurement. Cells were then washed and resuspended in 100 mM HEPES buffer, pH 7.5. Cells (200 μL) were transferred to a black optiplate 96F (Perkin-Elmer Life Sciences), and fluorescence was measured in a Multidetector microplate reader (Synergy 4; Biotek). Excitation and emission wavelengths were 495 and 515 nm, respectively. Three to eight technical replicates per condition were included on each plate.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers. Sequences from *Chlamydomonas* were as follows: CYG56 cDNA, EU841916; CYG56, ACJ05390.1; *NIA1*, AF203033; *NRT2.1*, Z25438; *AMT1.1*, AF479643; and *AMT1.2*, AF530051. Sequences from *Arabidopsis* were as follows: *NIA1*, P11832; and *NIA2*, P11035.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Genomic Structure of CYG56 and Domains of the CYG56 Protein.

Supplemental Figure 2. Sequence Comparison Annealing of HNOB and GC Domains.

Supplemental Figure 3. Sequence Comparison Annealing of HNOBA Domain.

Supplemental Figure 4. Degree of Identity and Divergence among Compared NO-Dependent Type GC Sequences.

Supplemental Figure 5. GC Activity Assayed with Mg²⁺ and Mn²⁺ of Truncated (pCYG56) and Full-Length CYG56 Purified Preparations.

Supplemental Figure 6. Effect of the Inhibitors ODQ and LY83,583 on GC Activity of Truncated and Full-Length CYG56 Purified Preparations.

Supplemental Figure 7. Effect of 8-Br-cGMP on Expression of *NIA1*, *NRT2.1*, *AMT1.1*, and *AMT1.2* in the Wild-Type 704.

Supplemental Figure 8. DAF-FM DA Fluorescence Is Proportional to NO Intracellular Levels.

Supplemental Table 1. Raw Data Used to Produce Figure 10.

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