Dual positive and negative control of *Chlamydomonas* PII signal transduction protein expression by nitrate/nitrite and NO via the components of nitric oxide cycle

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**Abstract**

**Background:** The PII proteins constitute a large superfamily, present in all domains of life. Until now, PII proteins research in Chloroplastida (green algae and land plants) has mainly focused on post-translation regulation of these signal transductors. Emerging evidence suggests that PII level is tightly controlled with regard to the nitrogen source and the physiological state of cells.

**Result:** Here we identify that a balance of positive (nitrate and nitrite) and negative (nitric oxide) signals regulates *Chlamydomonas GLB1*. We found that PII expression is downregulated by ammonium through a nitric oxide (NO)-dependent mechanism. We show that nitrate reductase (NR) and its partner, truncated hemoglobin 1 (THB1), participate in a signaling pathway for dual control of GLB1 expression. Moreover, NO dependent guanilate cyclase appeared to be involved in the negative control of GLB1 transcription.

**Conclusion:** This study has revealed the existence of the complex GLB1 control at transcription level, which is dependent on nitrogen source. Importantly, we found that GLB1 gene expression pattern is very similar to that observed for nitrate assimilation genes, suggesting interconnecting/coordinating PII-dependent and nitrate assimilation pathways.

**Keywords:** *Chlamydomonas reinhardtii*, Nitrate, Nitrite, NO signaling, PII signal transduction protein, Truncated hemoglobin

**Background**

Inorganic nitrogen (N) acts as one of the most important mineral nutrients for all autotrophic organisms including plants. In natural ecosystems, the availability of nitrogen is often a limiting factor for plant growth. Plants have evolved highly efficient and selective systems for nitrogen acquisition to ensure an appropriate utilization of the scarce resources. In all domains of life [1–3] with representatives in most bacteria and in many archaea [4, 5] as well as in oxygenic eukaryotic phototrophs [6], regulation of N metabolism at various levels are coordinated by members of PII signal transduction proteins [7, 8]. PII proteins act as reporters of the metabolic state of the cell by interdependent binding of ATP/ADP and 2-oxoglutarate (2-OG) [9–11]. The conserved mode of PII function is based on the control of PII – target protein interactions via the effector molecules binding [12]. Furthermore, in plants, the cellular glutamine levels are additionally sensed via PII signaling [8, 12, 13].

A second, phylogenetically diverse regulatory mechanism is covalent modification of apical residues of the T loop in PII proteins that allows the integration of additional signals. In proteobacteria, actinobacteria and cyanobacteria PII proteins can be covalently modified by uridylylation, adenylylation and phosphorylation at the T loop residues, respectively [14–16].

However, in many other organisms, this second regulatory layer of covalent modification of the T loop is apparently missing, as in Archaea [7], *Bacillus* [17], and plant PII proteins [6, 18]. The lack of this regulatory
level can be partially compensated by control of PII-encoding genes at transcription level [19]. In Chloroplastida (green algae and land plants) PII-encoding GLB1 genes are nuclear-encoded and, in Rhodophyta they are coded by the plastid genome [6]. It is believed that regulation of PII in plants may be transcriptional [20–22]. However, unlike bacteria, the transcriptional control of plant PII expression remains poorly understood.

Chlamydomonas reinhardtii (Chlamydomonas in the following) is a model alga that shares with higher plants the capability of controlling by PII the activity of N-acetyl-L-glutamate kinase (NAGK) that leads to arginine formation [8]. Although Chlamydomonas efficiently uses nitrate and nitrite, ammonium is preferred N source and many genes involved in nitrate/nitrite assimilation are repressed in the presence of ammonium [23, 24]. It is also important to note that amino acids are extracellularly deaminated by Chlamydomonas and only ammonium enters the cells [25]. Given that ammonium depletion induces Chlamydomonas GLB1 up-regulation [21], we hypothesized that ammonium may play a role in such negative regulation and this gene may respond to a balance of negative and positive signals.

In Chlamydomonas, ammonium and nitric oxide (NO) inhibit the expression of high-affinity nitrate/nitrite transporters and nitrate reductase (NR) [24]. During the cycle \( \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^- \) the negative signal of NO can be converted back to the positive signal of nitrate. Recent publications have uncovered the function of NR in this cycle [26]. NR acts as an essential partner protein of the nitric oxide-forming nitrite reductase (NORNIR) that catalyzes the formation of NO from nitrite [27]. Furthermore, NR is a protein partner of truncated hemoglobin 1 (THB1) for the conversion of NO into nitrate [28]. In spite of the key role of nitrate as a major nutrient and signal molecule, its possible regulatory effects on GLB1 transcription have not been analyzed. In order to understand the processes PII is involved in, it is important to know how GLB1 gene expression is regulated and when the amount of this protein is increased.

This apparent gap in the information about plant PII control motivated us to investigate the role of the components of cycle \( \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^- \) in regulating GLB1 transcription in Chlamydomonas cells. In this work, we unveil that GLB1 expression responds to an extracellular \( \text{NO}_3^- / \text{NH}_4^+ \) balance. Moreover, we show that nitrate and nitrite induce GLB1, and NO represses this gene. Collectively, these results suggest that the NR and its partners, NOFNIR and THB1, participate in a signaling pathway for dual control of GLB1 expression.

### Methods

#### Algal strains, growth conditions and cell treatment

The following Chlamydomonas reinhardtii strains were used: wild-type cw15–325 (mt+, cw15, arg7), which was kindly provided by Dr. M. Schroda (University of Kaiserslautern, Germany) and transformants with reduced THB1 obtained from cw15–325 amiTHB1–11 (mt+, cw15), amiTHB1–14 (mt+, cw15) and amiTHB1–23 (mt+, cw15) [29]. The 305 mutant (mt nit1) affected in NAD(P)H-NR activity and without diaphorase-NR activity was originally obtained from the wild type 6145c (mt) [30]. The 305 and 6145 strains were kindly provided by Dr. E. Fernández (University of Córdoba, Spain).

Cells were grown mixotrophically in tris-acetate-phosphate (TAP) medium (https://www.chlamycollecion.org/methods/media-recipes/tap-and-tris-minimal) containing 7.5 mM NH₄Cl instead of NH₄NO₃ under continuous illumination with white light (fluence rate of 45 μmol m⁻² s⁻¹) at 22 °C with constant orbital agitation at 90 rpm. The TAP medium was supplemented with 100 mg L⁻¹ of arginine when required. Cells were collected at the midexponential phase of growth by centrifugation (4000 g, 5 min), washed twice with 10 mM potassium phosphate, pH 7, before being transferred to the induction media containing the different sources of nitrogen and chemicals. At each harvesting times the number of viable cells were counted microscopically with use of 0.05% (v/v) Evans blue (DIA-M, Russia) as described [31]. Non-viable (stained) and viable (unstained) cells were counted. Four-hundred cells from each sample were scored for three biological replicates.

Determination and calculations of total chlorophyll (μg/ml) were performed as previously described [29, 32].

The compounds DEA-NONOate [2-(N-diethylamino)--diazenolate 2-oxide sodium salt] and ODQ [1H-(1,2,4)]oxadiazolo(4,3-a) quinoxalin-1-one] are from Sigma-Aldrich.

#### Gene expression analysis

The total RNA was isolated with Trizol according to the manufacturer’s instructions (Invitrogen, USA). To remove genomic DNA, the RNA samples were treated with RNase-Free DNase I (Fermentas). Subsequently, RNA concentration and purity (260/280 nm ratio) was determined using spectrophotometer (SmartSpec Plus, Bio-Rad).

Revert Aid HMinus First Strand cDNA Synthesis Kit (Thermo Scientific) was used for reverse transcription reaction. The primer pairs for RTqPCR are given in Additional file 1: Table S1. RT qPCR was performed with a CFX96 Real-Time PCR Detection System (Bio Rad) using SYBR Green I according to [33]. Gene expression ratios were calculated with the ΔΔCt method [34]. The RACK1 (receptor of activated protein kinase C; Cre13.g599400) gene was
chosen as the control housekeeping gene. All reactions were performed in triplicate with at least three biological replicates. Significant differences between experiments were evaluated statistically by standard deviation and Student’s t-test methods.

Protein gel blot analysis

The protein content was determined with amido black staining and protein gel blot analysis was performed as described [33, 35]. After separation by SDS-PAGE on a 12% polyacrylamide gel (w/v), the proteins were transferred to nitrocellulose membranes (Carl Roth, Karlsruhe) with use of semidy blotting (Trans-blot SD BioRad). The dilutions of the primary antibodies used were as follows: 1:5,000 anti-CrPII and 1:2000 anti-HSP70B. As a secondary antibody, the horseradish peroxidase-conjugated anti-rabbit serum (Sigma) was used at a dilution of 1:5,000. After eliminating the cells by centrifugation at 3000 g, the proteins were transferred to nitrocellulose membranes (Carl Roth, Karlsruhe) with use of semidy blotting (Trans-blot SD BioRad). The dilutions of the primary antibodies used were as follows: 1:5,000 anti-CrPII and 1:2000 anti-HSP70B. As a secondary antibody, the horseradish peroxidase-conjugated anti-rabbit serum (Sigma) was used at a dilution of 1:5,000. The peroxidase activity was detected via an enhanced chemiluminescence assay (Roche). For quantification, films were scanned using Bio-Rad ChemiDocTM Imaging System, and signals were quantified using the Image LabTM software (version 5.1).

Nitrate determination

After eliminating the cells by centrifugation at 3000 g, nitrate concentrations in the medium were determined by dual-wavelength ultraviolet spectrophotometry as A\textsubscript{220} - A\textsubscript{275} using standard curve [36]. For the measurements, media with 4 mM nitrate were diluted 50-fold. Values were obtained from at least three biological replicates; each replicate was analyzed three times. Student’s t-tests were used for statistical comparisons. P-values of < 0.05 were considered as significant.

Measurement of NO

Cells were treated with DEA-NONOate or nitrite, then they were incubated with in the presence of 1 mM (4-aminono-5-methylamino-2′7′-difluorofluorescein diacetate) dye (DAF-FM DA, Sigma-Aldrich), at concentration of 45 μg/ml chlorophyll. After 15 min the cells were washed, resuspended in indicated medium and used for the fluorometric detection of NO. The supernatant was collected in a test tube and then used to detect NO in the medium. The measurement of NO was carried out with a microplate reader CLARIOstar (BMG) as described [29]. The excitation and emission wavelengths for the NO indicator were 483 ± 14 and 530 ± 30 nm, respectively. Fluorescence intensity was calculated as arbitrary units per chlorophyll or protein as described previously [29].

NO detection by confocal microscopy

Cells were treated as described above. Images were acquired with a Leica TCS-SP5 confocal microscope (Leica-Microsystems, Germany) as described [29]. All experiments were performed in triplicate.

Results

GLB1 is induced by nitrate

Ammonium and nitrate are the main nitrogen sources for Chlamydomonas [37, 38]. There is evidence that GLB1 transcript levels are rather low in cells grown in the presence of ammonium [21]. We wanted to investigate whether or not the expression of GLB1 is influenced by nitrate. In cw15–325, upregulation of GLB1 was detected in nitrate alone (Fig. 1a). Nitrate applied at concentration of 4 mM has been reported previously to induce NIT1 gene encoding nitrate reductase [24]. In 4 mM NO\textsubscript{3}−-exposed cells, the GLB1 transcript level was increased after 30 min and reached a maximum within 1–3 h of incubation. During further exposure to nitrate, the gene expression decreased again approximately 2.4 times higher than the control level. When Chlamydomonas cells were exposed to 100 μM nitrate, we observed the same expression levels as in 4 mM nitrate medium. Decreasing the nitrate concentration to 10 μM resulted in an even higher increase in the GLB1mRNA abundance (2.3 fold after 30 min and 4.5-fold after 1 h) (Fig. 1a). Importantly, during exposure of cw15–325 cells (arg7–8) to nitrate, arginine was added to the medium. Thus, the observed increase in GLB1 transcripts was not caused by nitrogen starvation. Moreover, nitrate remaining in the media during experiment was also assayed (Fig. 1b). The data suggest that that a saturating effect observed between 4 mM and 90 μM of nitrate.

Interestingly, GLB1 demonstrated a rather similar expression pattern to NIT1 (Fig. 1a, insertion). However, in contrast to GLB1, the higher levels of NIT1 up-regulation were reached when cells were exposed to 4 mM nitrate.

Next, we asked the question whether the nitrate-induced upregulation of GLB1 expression is accompanied by an increase in the PII protein. Compared to cells grown in ammonium, levels of PII were indeed higher in cells incubated in nitrate (Fig. 1c). The data suggest that the PII is induced by nitrate.

GLB1 transcription responds to the NH\textsubscript{4}+/NO\textsubscript{3}− balance

In Chlamydomonas, the NH\textsubscript{4}+/NO\textsubscript{3}− balance was shown to be sensed by cells modulating expression of the nitrogen pathway genes [39]. To test whether GLB1 transcription responds to a balance of NH\textsubscript{4}+ and NO\textsubscript{3}−, we monitored the accumulation of GLB1 transcripts in Chlamydomonas cells incubated in media containing different concentrations of NH\textsubscript{4}+ (1 mM or 7.5 mM) with 4 mM NO\textsubscript{3}−. In cells supplemented with 4 mM NO\textsubscript{3}− + 7.5 mM NH\textsubscript{4}+, GLB1 expression was fully blocked (Fig. 2a). However, GLB1 mRNA abundance was higher in 4 mM NO\textsubscript{3}− + 1 mM NH\textsubscript{4}+, although significantly lower than in
4 mM NO$_3^-$ medium. Therefore, like NIT1 (Fig. 2, insertion; [39]) GLB1 expression responds quantitatively to extracellular NH$_4^+$. Moreover, addition of 1 mM NH$_4^+$ to media containing different concentrations of NO$_3^-$ (4 mM, 100 μM and 10 μM) caused the more severe reduction in GLB1 transcription at the lowest nitrate concentration (Fig. 2b). These data additionally support the idea that and NH$_4^+$/NO$_3^-$ balance may play an important role in the regulation of the gene of interest.

**GLB1 is repressed by NO**

Stimulation of NO generation has been reported to be dependent on the ammonium concentration in the medium and as a consequence to control the expression of the nitrogen pathway genes in *Chlamydomonas* cells [24]. The above results (Fig. 2) suggest that NO may be involved in the control of GLB1 expression. To test this, the nitrate-induced cells were treated with DEA-NONOate as NO donor [40]. The concentrations of DEA-NONOate used (10 μM, 50 μM, and 100 μM) caused a significant inhibition of GLB1 expression after 30 min (Fig. 3a). Interestingly, the expression of this gene was largely recovered after 1 h and 3 h in the presence of 10 μM or 50 μM and 100 μM DEA-NONOate, respectively, hinting that NO may be converted in NO$_3^-$ for this period. DEA-NONOate also exhibited a negative effect on the transcription of NIT1 used as a control gene (Fig. 3a, insertion). These results are consistent with a balance of NO levels detected in cells and in culture broth (Fig. 3b). The combined real-time PCR and NO detection analysis suggested that mRNA levels of GLB1 were repressed by NO. We also propose that there is subtle regulation of the NO levels which control GLB1 expression, probably through fine tuning of the components mediating cycle NO$_3^-$ → NO$_2^-$ → NO →NO$_3^-$. 

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**Fig. 1** PII expression is increased by nitrate. a Time course of the GLB1 transcripts accumulation during incubation of illuminated cells in nitrate-containing medium. *Chlamydomonas* cells of cw15–325 strain were grown in ammonium-containing medium (Con, control) and transferred to media containing 4 mM, 100 μM, or 10 μM NO$_3^-$ in the light for 0.5 h, 1 h, 2 h or 4 h. Values are means ± SE of three biological replicates and three technical replicates and are given as expression level relative to a house-keeping gene RACK1 that has a value of 1. The increase in NIT1 transcript abundance was used as a positive control (insertion). b Changes in nitrate levels in the media. *Chlamydomonas* cells of cw15–325 strain were grown in ammonium-containing medium and transferred to media containing 4 mM, 100 μM, or 10 μM NO$_3^-$ in the light. At the indicated times, the amounts of nitrate present in the media were quantified. ND, not determined. Values are means ± SE of three biological replicates. c Time course of the PII protein accumulation during incubation of illuminated cells in nitrate-containing medium. PII levels were analyzed by western blotting in the same conditions as in (a). Each line corresponds to 20 μg of soluble proteins extracted from samples taken from cultures at the time points indicated. Quantitation of protein blots is given as proportion of signal in test variant to control variant. HSP70B signal served as a loading control.
Nitrite induces GLB1 gene and NR promotes NO-dependent GLB1 repression

In Chlamydomonas cells, NO appears as a consequence of nitrite accumulation [28]. To further explore the relationship between GLB1 expression levels and NO generation, two strains were assayed: the wild strain 6145c and its derivative mutant 305, which, affected in NAD(P) H-NR activity and without diaphorase-NR activity. Both strains were incubated in 10 mM nitrite. Unexpectedly, nitrite increased GLB1 transcript levels in cells (Fig. 4a). In addition, the nit1 mutant showed higher levels of GLB1 expression than WT (6145c), consistent with inability of the strain 305 to supply NAD(P)H electrons to nitrite [27].

Next, we asked the question whether the increased GLB1 mRNA levels correlate with a change in PII protein content in nitrite-induced cells. Although the kinetics of changes in GLB1 mRNA levels were not similar to the kinetics of changes in PII protein levels in both strains, the difference in PII protein abundance between parental strain 6145c and mutant 305 (Fig. 4b) was evident. The data suggest that GLB1 upregulation is dependent of nitrite. This result is in agreement with the fact that the NR mutant without diaphorase-NR activity did not show significant NO signal (Fig. 4c and d). In contrast to the mutant, very strong NO fluorescence appeared in the 6145c strain, supporting a correlation between NO generation and GLB1 mRNA abundance. Thus, these experiments allow nitrite to be added as a player in the control of GLB1 expression together with nitrate and NO. Taken together, the results strengthen the idea that control of GLB1 expression is regulated by a complex mechanism in which NO produced via NR/NOFNiR plays a crucial role.

THB1 controls GLB1 expression via the detoxification of NO

Previously, it was shown that NR uses the truncated hemoglobin THB1 in the conversion of NO to nitrate [28]. We therefore asked whether THB1 exerts any effect on the expression of GLB1. To test this, the cells of cw15–325 (WT) and THB1 knock-down strains were incubated in 4 mM nitrate with or without DEA-NONOate. Nitrate-induced expression of GLB1 was shown to be antagonized by NO (Figs. 3 and 4). As shown in Fig. 5, the downregulation of THB1 impaired the transcription of GLB1 in the presence of NO generator: compared with cw15–325, transcript accumulation for gene of interest in all THB1-amiRNA strains was reduced on average from 1.5-fold to 2.3-fold. These results are in agreement with the higher levels of NO measured in THB1 knock-down strains compared to parental strain (cw15–325). Collectively, these results suggest that reduction in THB1 expression allows NO concentration to increase, triggering down-regulation of GLB1 transcription.

NO-dependent GLB1 repression is released by guanylate cyclase (GC) inhibitor

Our analyses show that GLB1 is transcriptionally regulated by the same mechanisms than NIT1 gene that is
repressed by NO (Figs. 2, 3 and 4). Repression of NIT1 by NO is mediated by NO-dependent GC activity [24]. It would thus be interesting to test the GC inhibitor in order to better decipher how the GLB1 transcription is inhibited by NO. We found that ODQ, a selective inhibitor of NO activated GC [41], caused derepression of the GLB1 in ammonium containing medium when applied at concentrations 2.5 μM, 5 μM or 7.5 μM, whereas 1 μM of this inhibitor had no effect (Fig. 6). This result allows us to propose that, by sensing NO, GC activity could be involved in GLB1 repression.

Discussion

Plant chloroplasts contain cyanobacterial-like PII homologues [6]. However, unlike PII proteins from cyanobacteria, plant PII proteins seem not to be covalently modified [20, 21]. Furthermore, in cyanobacteria, PII signaling is involved in the regulation of nitrate assimilation and gene expression through co-activator of the global nitrogen control factor NtcA [5]. Importantly, no homologues of PipX and NtcA are conserved in plants [12]. In representative plants, PII proteins are regulated at the transcriptional level [20–22]. In this study, we demonstrate that expression of Chlamydomonas PII is under the complex control of positive signals (i.e., nitrate and nitrite) and negative signals (NO), and GLB1 gene expression pattern is very similar to that observed for nitrate assimilation genes.

Ammonium is the preferred nitrogen source for Chlamydomonas. In ammonium-containing medium Chlamydomonas PII is expressed at low levels [21]. Interestingly, the second major nitrogen source, nitrate, induces GLB1 transcription (Fig. 1). The cells might use this transcription regulation to limit PII levels under...
optimal nutritional conditions. It would thus be interesting to test whether GLB1 is strictly sensitive to ammonium or responds to a balance of ammonium and nitrate. We found that changes in the nitrate concentration modulate the response of GLB1 gene to ammonium (Fig. 2). Together these data stress the point that, a balance of positive and negative signals regulates GLB1.

A part of the response to ammonium/nitrate balance is a change in the intracellular concentration of NO [39]. We have shown herein that NO represses expression of GLB1 (Fig. 3). Interestingly, GLB1 exhibits a similar transcription pattern to NIT1 (Fig. 3a, insertion) and other genes from the nitrate assimilation cluster [24, 38, 42, 43], suggesting that the expression of PII is tightly controlled with regard to the nitrogen source and the physiological state of cells. Physiological studies of Arabidopsis suggested a role of PII in nitrite uptake [44]. In addition, GLB1 expression is up-regulated in the presence of nitrite (Fig. 4a). Together, these observations allow us to speculate that PII protein may also play some uncharacterized roles in control of nitrogen assimilation in Chlamydomonas cells.

In the cytoplasm, nitrite is converted into NO by NR that is partnered with NOFNiR [27]. Thus, NR modulates both the levels of NO and the amounts of nitrite available for metabolism. Importantly, the diaphorase-NR activity is required for supplying NAD(P)H electrons to nitrite [27]. We propose that GLB1 transcription is dependent on the dual system of NR and NOFNiR through fine tuning of NO levels. The fact that the diaphorase-NR activity is required to repress PII levels in the presence of nitrite (Fig. 4a; b) supports this idea. In agreement with these data, spectrofluorometric assays (Fig. 4c) and confocal microscopy (Fig. 4d) with DAF-FM DA allowed us to detect higher fluorescence levels in parental strain 6145c than nit1 mutant. More
generally we could propose that nitrite-dependent NO production plays role in the control of PII expression dynamics, ensuring possible interconnecting/coordinating PII-dependent and nitrate assimilation pathways.

As NO is toxic, plants have protective mechanisms to defend themselves. Like higher plants, Chlamydomonas cells use hemoglobins to convert NO into nitrate [28, 45, 46]. It has been previously shown that a truncated hemoglobin 1, THB1, has NO-dioxygenase activity [28]. In amiTHB1 strains, the nitrite-responsive accumulation of GLB1 transcripts is impaired (Fig. 5a). As expected, DEA-NONOate in nitrite-containing medium resulted to higher fluorescence levels in THB1-knockdown transformants than in parental strain (Fig. 5b). Taken together, these results strengthen the notion that NO acts as a signaling molecule for the transcriptional regulation of GLB1 gene, and THB1 is involved in this NO-dependent pathway.

The mechanism implicated in the repression of GLB1 by NO is not clear, although in Chlamydomonas, six proteins CYG11, CYG12, CYG15, CYG38, CYG56 and CYG57 share the same domains structure with mammalian NO sensors, the soluble guanylate cyclases [30, 47–49]. In animals, the GCs activity is usually stimulated by the binding of NO to their heme group [50]. Moreover, CYG56 participates in ammonium-mediated NIA1, NRT2.1, AMT1.1 and AMT1.2 repression through a pathway that involves NO and cGMP [24, 49]. Importantly, inhibition of the NO-dependent GC by ODQ releases cells from ammonium repression of not only genes from the nitrate assimilation cluster but also of GLB1 gene (Fig. 6). This correlation emphasizes that regulation of nitrate assimilation genes and GLB1 are closely related and
support the hypothesis that a soluble guanilate cyclase may also control GLB1 expression in NO-GC-dependent manner. Interestingly, only CYG56 is up-regulated in ammonium compared with nitrate-containing medium [24], hinting that ammonium-mediated repression of GLB1 might be regulated through CYG56-dependent signaling. However, which guanylate cyclase is a pivotal factor for the regulation of GLB1 in ammonium-containing medium has to be analyzed further. The experiments described here establish ammonium as a component of the signaling pathway for the negative control of the GLB1 transcription. However, they do not address how ammonium acts to repress GLB1 gene. Moreover, we cannot rule out that NO-dependent GLB1 repression by nitrite and ammonium may go through the various routes. We propose a model (Fig. 7) in which PII expression is under complex control of positive and negative signals depending on nitrogen source.

Conclusions
Our main conclusion is that PII level is tightly controlled with regard to the nitrogen source and the physiological state of cells. We provide evidence on that NO via the components of nitric oxide cycle is involved in the negative control of GLB1. On the other hand, nitrate and nitrite induce this gene transcription. Therefore, important regulatory layer in the PII-dependent signal transduction system in Chlamydomonas could be that the concentration of the PII protein must be balanced in order for the signaling mechanism to function properly – the system is fine-tuned.

Additional files

Additional file 1: Table S1. Primers sequence that were used for real time PCR analysis. (XLSX 10 kb)

Abbreviations
DAF-FM DA: 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DEA-NONOate: 2-(N,N-diethylamino)-diazenolate 2-oxide sodium salt; GC: Guanylate cyclase; NO: Nitric oxide; NOFNiR: Nitric oxide-forming nitrite reductase; NR: Nitrate reductase; ODQ: 1H-(1,2,4)oxadiazolo(4,3-a) quinoxalin-1-one; TAP: Tris-acetate-phosphate medium; THB1: Truncated hemoglobin 1

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
ZZ and LK performed all of the biological analyses. ZZ, LK and EE analyzed the data. EE designed the study and wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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