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Protistology

Sulfur deprivation-induced expression of THB1, a *Chlamydomonas reinhardtii* truncated hemoglobin, is mediated by nitrate reductase-dependent NO production

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Summary

During sulfur (S) deprivation, *Chlamydomonas reinhardtii* mediates a suite of specific responses to efficiently acquire S from various sources and remodel primary metabolism. Although the truncated hemoglobin 1 (THB1)-dependent regulation of a subset of S limitation-responsive genes has been elucidated, the mechanism through which *THB1* transcription is triggered in S-deprived cells is not known. Here, we show that signaling proteins and regulators associated with S deprivation specific responses did not control *THB1* expression. Following S depletion, rapid increase in nitrite concentrations is essential for *THB1* upregulation. These changes are consistent with NO accumulation. Increased nitrite, NO and *THB1* mRNA levels were not observed in mutant cells without diaphorase-NR activity. Our findings suggest that nitrate reductase - nitric oxide-forming nitrite reductase system plays a positive role in *THB1* transcription via generation of NO from nitrite in S-starved cells. NR-dependent NO production may be regarded as an early trigger, which contributes to *Chlamydomonas* adaptability to nutrient stresses.

Key words: nitrate reductase, nitric oxide, sulfur limitation responses, truncated hemoglobin 1

Introduction

Sulfur (S) is one of the essential macroelements that plays important role in plant nutrition (Giordano and Raven, 2014). Most algae and plants assimilate S as a sulfate and transport it to the plastids, where primary S metabolism takes place. Because S can be limiting in the natural environment, plants have evolved mechanisms to adjust to S deprivation conditions. Acclimation responses to S deficiency have been studied in various plants (Giordano and Raven, 2014; Pollock et al., 2005; Schachtman and Shin, 2007; Takahashi et al., 2011; Zhang et al., 2004) including the green alga *Chlamydomonas reinhardtii* (*Chlamydomonas* throughout). When experiencing S deprivation, *Chlamydomonas* cells increase the capacity for scavenging S from both internal and external resources (Aksoy et al., 2013; Davies et al., 1994). S-deprived *Chlamydomonas* exhibits upregulation of numerous proteins associated with sulfate uptake and assimilation, changes in metabolism and inter-

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nal S recycling (Saroussi et al., 2017; Zhang et al., 2004). Moreover, a truncated hemoglobin 1 is essential for proper cell response to S limitation (Minaeva et al., 2017).

In contrast to full length hemoglobins, truncated hemoglobins (THBs) tertiary structure share a characteristic helix arrangement folded in a 2-on-2 α-helical sandwich (Wittenberg et al., 2002). The key features of these proteins have been summarized in previous reviews (Nardini et al., 2007; Sarma et al., 2005). THBs are characterized by versatile biological functions in different organisms that are distinct from oxygen delivery and storage (Frey and Kallio, 2003). It is believed that THBs might be nitric oxide (NO) scavengers that protect cells from an accumulation of the radical molecule (Hunt et al., 2001; Milani et al., 2003). In addition, THBs have also been implicated to function as NO producers and thereby initiate NO-based signaling cascades (Milani et al., 2003).

In plants, NO is a widespread signaling molecule that functions in the modulation of several physiological processes during plant growth, development and nutrient assimilation (Jeandroz et al., 2016; Neill et al., 2002; 2008; Yu et al., 2014). NO has also been recognized as a player in various signaling pathways in *Chlamydomonas*, including cell death (Yordanova et al., 2010), remodeling of chloroplast bioenergetics upon nitrogen starvation (Wei et al., 2014), regulation of nitrate assimilation (Sanz-Luque et al., 2013), anaerobic acclimation (Hemschemeier et al., 2013) and salt stress (Chen et al., 2016). In this unicellular alga, NO is also found to act as a signaling molecule for the transcriptional regulation of several S-responsive genes upon sulfur deprivation (Minaeva et al., 2017; Zalutskaya et al., 2018). Therefore, NO production mechanisms are underlying for many processes throughout the plant life cycle.

In animals, NO is generated by proteins called NO synthases (NOS) (Alderton et al., 2001). Importantly, no animal NOS homolog has been identified in the *Chlamydomonas* or higher plant genomes sequenced to date. However, several other NO sources have been identified in plants (Domingos et al., 2014). Nevertheless, accumulating evidence suggests that nitrate reductase (NR) is the most potential enzymatic source of NO in plants (Desikan et al. 2002; He et al. 2004; Gupta et al., 2011; Santolini et al., 2016). Recent studies have uncovered in *Chlamydomonas* a dual system composed of the nitric oxide-forming nitrite reductase (NOFNiR) and NR that catalyzes the formation of NO from nitrite in aerobic conditions (Chamizo-Ampudia et al., 2016). Furthermore, THB1 is a protein partner of NR for the conversion of NO into nitrate (Sanz-Luque et al., 2015). In addition, *Chlamydomonas* THB1 is involved in the NO-dependent pathway during S starvation (Minaeva et al., 2017). However, the molecular mechanism of NO production in S-deprived cells was unknown. This prompted us to look for potential factors that mediate the NO synthesis upon S limitation and trigger *THB1* upregulation. We present evidence that specific regulators associated with S deprivation responses do not control *THB1* transcription. Our analysis demonstrates that NR-NOFNiR system plays a positive role in *THB1* expression via generation of NO from nitrite in S-starved cells.

Material and methods

Chlamydomonas strains and culture conditions

Chlamydomonas strains used are listed in Table 1. Cells were grown mixotrophically in tris-acetate-phosphate (TAP) medium containing 7.5 mM NH_4Cl instead of NH_4NO_3 (Sager and Granick, 1954) under continuous illumination with white light (fluence rate of 45 μmol m^{-2} s^{-1}) at 22 °C. The TAP medium was supplemented with 100 mg L^{-1} of arginine when required. To induce sulfur deprivation of the strains used, the cells grown in TAP medium were collected at the midexponential phase of growth by centrifugation (4000 *g*, 5 min), washed twice with sulfur-free medium (TAP-S) and then incubated in TAP-S. S free medium was prepared as reported previously (Davies et al., 1994). At each harvesting times the number of cells was measured employing a counting chamber and the viable cells were estimated microscopically with use of 0.0125 % (v/v) methylene blue (DIA-M, Russia) as described (Davies et al., 1996). Stained (non-viable) and unstained (viable) cells were observed and counted. 400 cells from each sample were examined for three biological replicates.

The chemical compounds L-NAME (N_ω-Nitro-L-arginine methyl ester hydrochloride), DEA-NONOate (2-(*N*,Ndiethylamino)-diazenolate 2-oxide sodium salt), cPTIO (2-phenyl-4, 4, 5,5-tetramethylimidazoline-1-oxyl 3-oxide), KCN, all from Sigma-Aldrich.

Table 1. *Chlamydomonas* strains used.

Strain	Genotype	References
cw15-325	*mt+, arg7*	Harris, 1989
6145c	*mt⁻*, wild type	Harris, 1989
21gr	*mt⁺*, wild type	Harris, 1989
CC124	*mt⁻, nit2, nit1*	Harris, 1989
305	*mt⁻nit1*	Sosa et al., 1978
CC4353	*mt⁻sac1*	Davies et al., 1994; 1996
CC4354	*mt⁺, sac3* (*snrk2.2*)	Davies et al., 1994
CC4355	*mt⁺snrk2.1*	Gonzáles-Ballester et al., 2008
CC4363	*mt⁺ars73a*	Aksoy et al., 2013

Gene expression analysis

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

Reverse transcription was performed with RevertAid HMinus First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific). The primer pairs used are listed in Table 2. Real time qPCR was performed on the Light Cycler Instrument (CFX96 Real-Time PCR Detection System, Bio Rad) using SYBR Green I as described (Minaeva et al., 2017). Gene expression ratios were normalized to *RACK1* (receptor of activated protein kinase C; Cre13.g599400) using the ΔΔCt method (Livak, Schmittgen, 2001). DEA-NONOate and cPTIO treatments had no effect on the accumulation of *RACK1* transcripts in *Chlamydomonas* cells. Controls without template or reverse transcriptase were always included. The accuracy and reproducibility of the real time assay was determined from low variation in C_T values across replicates. 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.

Determination of chlorophyll content and nitrite levels

Chlorophyll content was determined using ethanol extraction. 1 mL of culture was centrifuged and the pellet was resuspended in 1 mL ethanol to extract pigments. Cellular debris was pelleted by centrifugation and chlorophyll a and b levels were determined spectrophotometrically (SmartSpec Plus, BioRad) in the supernatant, by measuring optical absorbance at 645 and 663 nm. Calculations of total chlorophyll (μg mL^{-1}) were performed as previously described (Harris, 1989).

To determine intracellular nitrite accumulation, cells were collected by centrifugation at 3000 g, washed twice with ice-cold 75 mM potassium phosphate buffer, pH 7.0. Pre-frozen samples were thawed in ice, gently resuspended in 255 μL H_2O and then incubated at 99 °C for 3 min. The suspension was centrifuged at 30000 g for 5 min at 4 °C to yield a clear supernatant, and then 255 μL of the supernatant was mixed with 125 μL 1 % sulfanilamide in 2 N HCI and 0.2 % (w/v) N-(l-naphthyl)ethylenediamine. The absorption of the resulting violet color was measured at 540 nm against a blank. Nitrite contents were expressed as nM relative to the 10^6 cells.

Table 2. Primers for qRT-PCR analysis.

Target gene / Accession number	Primer name	Sequence (5´–3´)	Reference
THB1/Cre14.g615400	*THB1*F *THB1*R	ATGAAGAAGCAGCGCCGCAAAC ACCAGGTCAAAGTGGTGGTGGTTC	Sanz-Luque et al., 2015
NIT1/Cre09.g410950	*NIT1*F *NIT1*R	GCGCTGCCCTCCGTCACCTTCC CAGCCGCACGCCCGTCCAGTAG	This work
RACK1/Cre06.g278222	*RACK1*F *RACK1*R	CTTCTCGCCCATGACCAC CCCACCAGGTTGTTCTTCAG	Zalutskaya et al., 2015
SLT2/Cre10.g445000	*SLT2*F *SLT2*R	TGTCGCGATATCGTTCATCA TGCGACAAAGTCTGCCAAGT	Fang et al., 2014

Note: Sequences were obtained from Phytozome 12, *Chlamydomonas reinhardtii* v5.5

Measurement of NO

Chlamydomonas cells (45 μg mL^{-1} chlorophyll) were incubated in the presence of 1 μM (4-amino-5-methylamino-2'7'-difluorofluorescein diacetate) dye (DAF-FM DA, Sigma-Aldrich). After 15 min the cells were washed, resuspended in indicated medium and then incubated for an additional 30 min to allow complete de-esterification of the intracellular diacetates. Intracellular production of NO was measured using a microplate reader CLARIOstar (BMG). Excitation and emission wavelengths were set at 483±14 and 530±30 nm, respectively. Fluorescence intensity was expressed as arbitrary units per μg chlorophyll. Cell autofluorescence was subtracted from the total fluorescence obtained. Three technical replicates per condition were included on each plate and each experiment was performed three times independently.

NO detection by confocal microscopy

For confocal microscopy cells were grown and treated as described above (measurement of NO). Cells were visualized with a Leica TCS-SP5 confocal microscope (Leica-Microsystems, Germany) equipped with a HC PL APO ×63 oil immersion objective. Excitation was performed with a 488-nm argon laser. The signals arising from the DAF-FM DA were collected on the channel between 500 and 544 nm. Chlorophyll autofluorescence was monitored across a window of 600-680 nm. Images were collected and processed with the Leica confocal software LAS AF (Leica-Microsystems, Germany). The experiment was performed in triplicate.

Results

S deprivation regulators do not control the expression of *THB1*

As described previously, the sulfur-limitation responses in *Chlamydomonas* are regulated by complex mechanisms that involve some signaling proteins (SAC1, SNRK2.1 SNRK2.2) and the putative transcription factor ARS73a (Aksoy et al., 2013; Davies et al., 1994; González-Ballester et al., 2008; Pollock et al., 2005). To test, whether these regulatory components played any role in triggering *THB1* expression under S deprivation, we employed the mutants defective in genes encoding the proteins SAC1, SNRK2.1 SNRK2.2 and ARS73a (Fig. 1 A; Table 1). It was shown that the mutant *sac1* is defective for the specific and general S deprivation responses (Davies et al., 1996). As shown in Fig. 1 C, the gene *THB1* exhibited an increase of transcript level in S-starved *sac1* cells in comparison to S-replete cells. Furthermore, changes in the *THB1* transcript levels observed in S-deprived *sac1* cells were very similar to those observed in WT (compare Fig. 1 B, C). This analysis was supplemented by examining the accumulation of *SLT2* transcript (encoding the plasma membrane associated SO_4^{2-} transporter) that was highly affected in S-deprived *sac1* mutant (González-Ballester et al., 2008). Importantly, *sac1* cells used in these experiments were fully viable (Fig. S1).

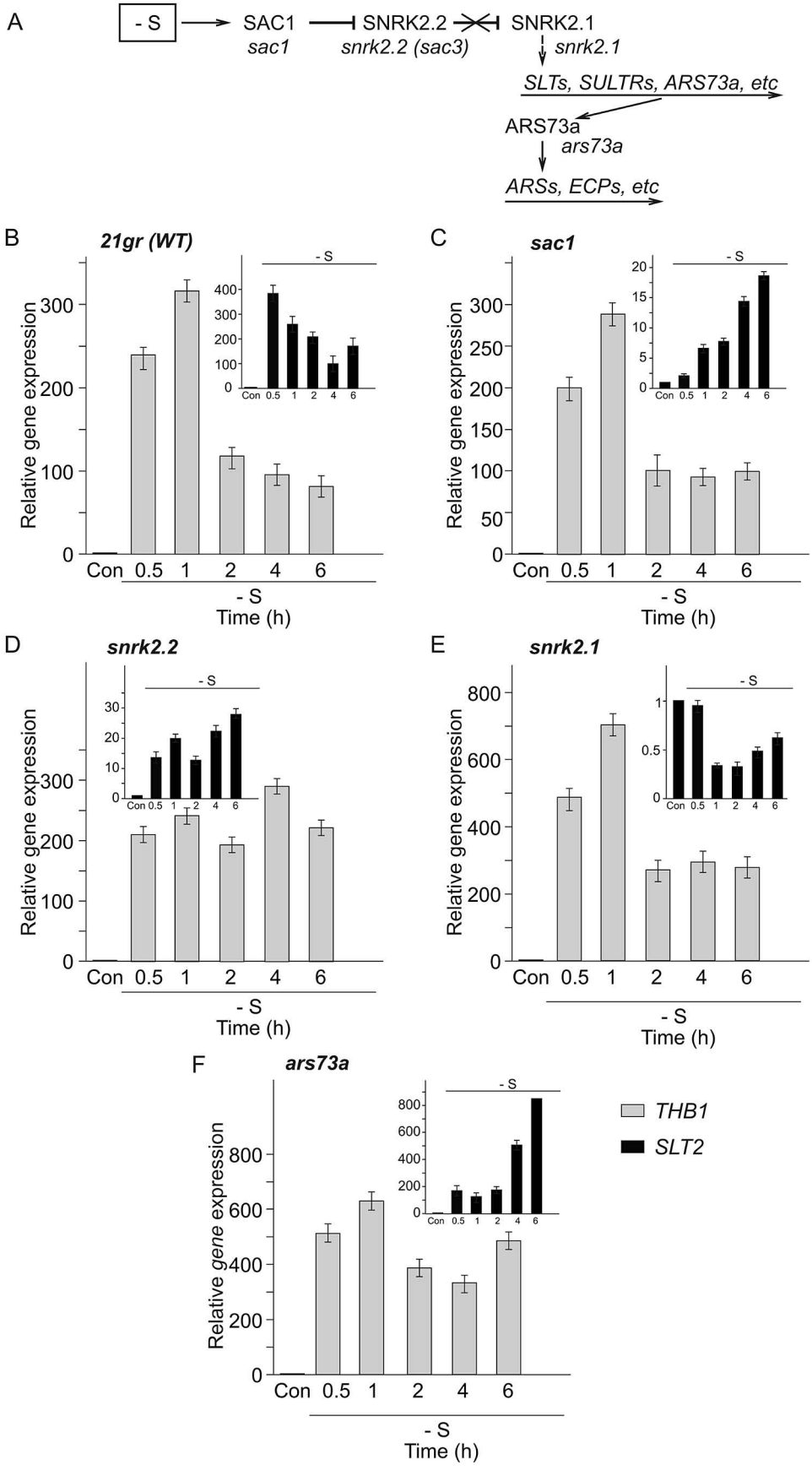
Also, the mutants *snrk2.1* and *snrk2.2*, known to be deficient in the expression of S-responsive genes (González-Ballester et al., 2008), did not fail to show the increase in *THB1* transcripts (Fig. 1 D, E), indicating that *THB1* expression does not depend on SNRK2 family Ser/Thr protein kinases, SNRK2.1 and SNRK2.2.

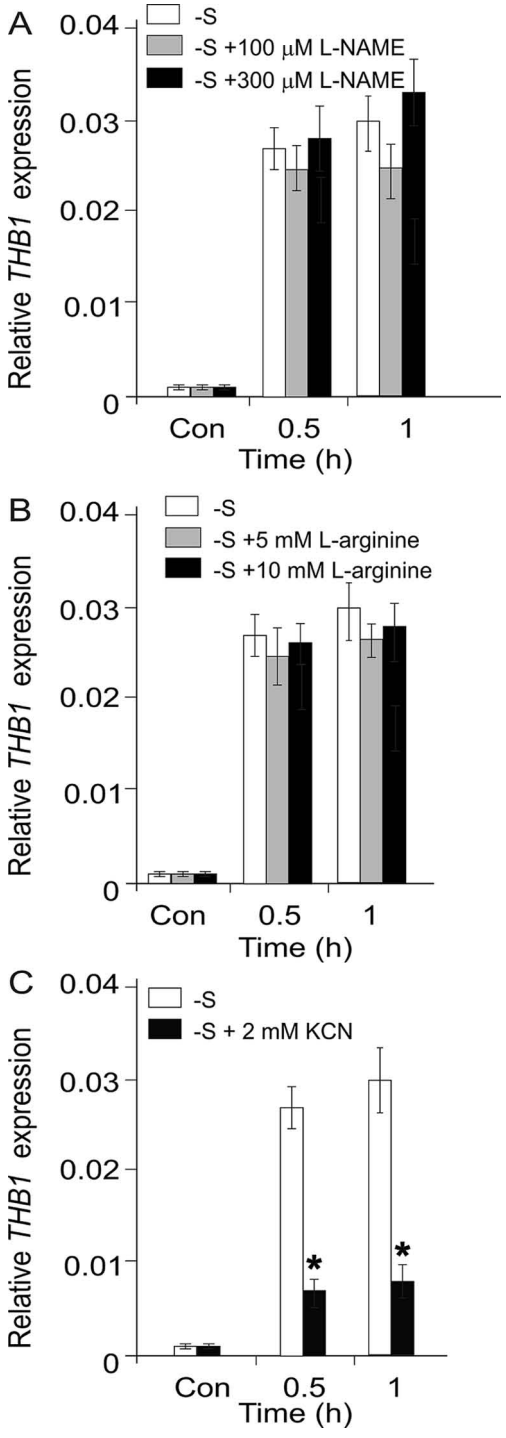
Additionally, the expression for these same genes was also analyzed in the mutant strain deficient in the ARS73a regulator (Fig. 1 F). The levels of the *THB1* transcripts were not diminished in the *ars73a* cells relative to the WT (Fig. 1 B, F). As expected, the induction of the control gene, *SLT2*, was not impaired after transferring the mutant strain to S-free medium. In summary, these data indicate that the THB1 expression is not subject to the same regulatory controls as many S-deprivation responsive genes.

S deprivation-induced *THB1* expression does not depend on NOS

How can sulfur deprivation lead to *THB1* induction? It has been shown previously that the *THB1*

Fig. 1. Expression changes of *THB1* gene in wild-type and mutants during sulfur deprivation. 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. (A) Scheme of key regulators involved in the sulfur-limitation responses in *Chlamydomonas*. Mutant names for all genes that encode the depicted proteins are shown in italics. (B) Relative expression levels of *THB1* gene in S-deprived cells of WT (21gr) and (C-F) of the mutants sac1, *snkr2.2, snkr2.1* and *ars73a*, respectively. Insets show the *SLT2* transcripts accumulation.


A
- S
SAC1
sac1
SNRK2.2
snrk2.2 (sac3)
SNRK2.1
snrk2.1
SLTs, SULTRs, ARS73a, etc
ARS73a
ars73a
ARSs, ECPs, etc
B
21gr (WT)
C
sac1
D
snrk2.2
E
snrk2.1
F
ars73a
Relative gene expression
Relative gene expression
Time (h)
Con 0.5 1 2 4 6
- S
THB1
SLT2



expression is upregulated by NO (Sanz-Luque et al., 2015). In animals, a key mechanism of NO formation is the deamination of arginine by NOS (nitric oxide synthase) to form NO and citrulline (Förstermann and Sessa, 2012). It was previously shown that NOS and NR are the potential enzymes to form NO in plants (Desikan et al., 2002, Gupta et al., 2011). To test the possible involvement of NOS in generation of NO, an analogue of arginine, L-NAME, has been widely used (Chen et al., 2016; Sakihama et al., 2002). L-NAME, did not affect upregulation of THB1 in S-deprived cells *Chlamydomonas* (Fig. 2 A). Moreover, arginine (the NOS substrate) did not induce *THB1* transcription (Fig. 2 B). This finding represents yet another line of evidence for non-involvement of NOS-like enzymes in NO generation in *Chlamydomonas*. In contrast, in the presence of KCN (used as the NR inhibitor), transcript levels of *THB1* were markedly diminished (Fig. 2 C), hinting at a possible role of NR.

NR is essential for *THB1* expression in S-starved cells

The potential involvement of NR in upregulation of *THB1* under S deprivation conditions was further investigated by examination of this gene transcription in the two strains: CC124 and 305. Remarkably, the strain CC124 failed to activate the expression of *THB1* upon S limitation (Fig. 3 A). However, *SLT2* transcripts were markedly increased in S-deprived CC124 cells (Fig. 3 A, insertion). It is known that this strain is defective in genes encoding NR and NIT2 transcription factor. In order to discriminate between these two proteins, we next examined the 305 mutant, affected in NAD(P)H-NR activity and without diaphorase-NR activity (Sosa et al., 1978). When the mutant 305 was incubated in the S-free medium, we obtained the same result as with the CC124 strain: no THB1 induction in S-starved cells (Fig. 3 B).

NR has been reported as the enzyme responsible for NO generation in *Chlamydomonas* (Sakihama et al., 2002). We proposed that *THB1* gene was not

Fig. 2. Effects of NOS- and NR-inhibitors and L-arginine on *THB1* expression during sulfur deprivation. 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*. (A) Relative expression levels of *THB1* gene in S-deprived cells of WT (21gr) exposed to L-NAME. (B) Relative expression levels of *THB1* gene in S-deprived cells of 21gr strain in the presence of NOS substrate, L-arginine. (C) Relative expression levels of *THB1* gene in S-deprived cells of 21gr strain exposed to KCN. Statistical significance of differences between the untreated and treated S-limited cultures is indicated by asterisks (* $P < 0.05$).

induced because the cells of both strains were unable to accumulate NO. To test this idea, these strains were incubated with NO donor DEA-NONOate (Floryszak-Wieczorek et al., 2006). Within 0.5 h and 1 h following the addition of DEA-NONOate, *THB1* transcript levels were significantly increased in both mutants (Fig. 3 A, B). However, the amount of *THB1* was severely reduced within 0.5 h and 1 h of incubation with cPTIO.

Intracellular nitrite is increased in S-deprived cells via NR

Intracellular nitrite is a limiting factor for NO production (Planchet et al., 2005). Strikingly, we found *THB1* upregulation in S-starved cells that were incubated in ammonium-containing medium (Minaeva et al., 2017; Figs 1 and 2). We therefore revisited this issue using 7.5 mM ammonium as a sole nitrogen source and could first determine nitrite in the cells following exposure to S limitation (Fig. 4). Significantly, in WT nitrite levels were found to be maximally increased after 30 min of S deprivation. In order to find out whether the observed increase in the intracellular nitrite during S deprivation was due to NR activity, nitrite levels were also compared in the 6145c (WT) and the mutant 305 (Fig. 4). Notably, there was no change in nitrite abundance upon S deprivation in cells affected in NR activity. Thus, our analysis reveals that nitrite levels were enhanced by exposure to S limitation, showing NR dependency.

S limitation induces NO production in Chlamydomonas via NR-NOFNiR system

As was shown by Chamizo-Ampudia et al. (2016) NO synthesis occurs in the cytosol and depends primarily on the enzyme NOFNiR, which requires electrons provided by NR. Hence, it appears likely that the expression of *THB1* in cells supplemented with ammonium upon S starvation is induced as a consequence of the nitrite-dependent accumulation of NO via NR activity. If this was the case, the amplitude of *THB1* expression in cells exposed to S deprivation in the presence of nitrite should be increased. To test this, cells were incubated in S-free media containing different concentrations of nitrite (Fig. 5 A). Notably, the increase of *THB1* transcripts was observed at 1mM of nitrite. Interestingly, supplementing S-deprived cells with higher nitrite concentrations did not result in the accumulation of higher levels of *THB1* (Fig. 5 A).

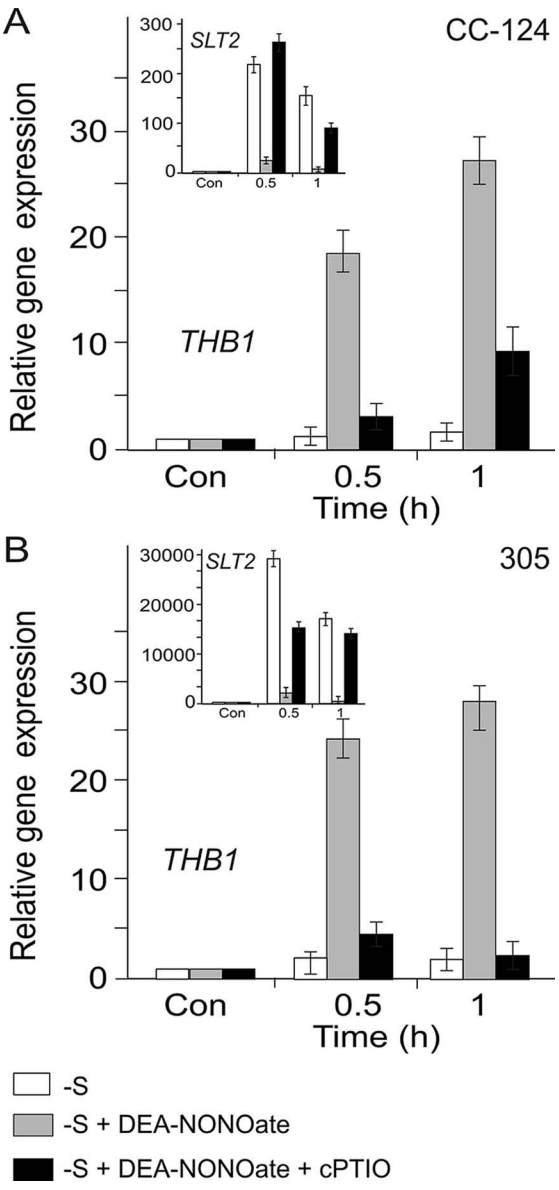


Fig. 3. Expression changes of *THB1* gene in nitrate reductase deficient mutants during sulfur deprivation. Values are means ± SE of three biological replicates and three technical replicates and are given as expression level relative to a house-keeping gene *RACK1* that has a value of 1. (A) Relative expression levels of *THB1* gene in S-deprived cells of CC124 strain. (B) Relative expression levels of *THB1* gene in S-deprived cells of 305 strain. Cells of both strains grown in TAP were washed in S-free-medium and incubated for 30 min or 1 h in the absence or presence of 100 μM DEA-NONOate. The effect of 300 μM cPTIO was analyzed when added simultaneously with DEA NONOate at time 0. Insertion show the *SLT2* transcripts accumulation.

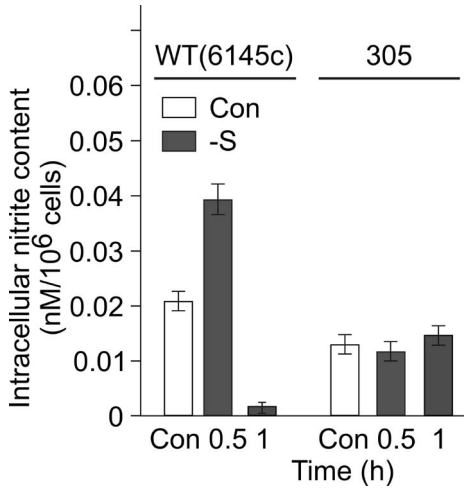


Fig. 4. Changes in nitrite levels in S-deprived cells. Nitrite concentrations in S-deprived cells of WT (6145c) and mutant 305 are expressed as nM per 10^6 cells. Values are means ± SE of three biological replicates.

In nitrite supplemented mutant cells without diaphorase-NR activity there was no increase in *THB1* mRNA abundance (Fig. 5 A). These data are in agreement with studies showing that NR is essential for *THB1* expression in S-starved cells (Fig. 3).

To explore the source of NO and its dependence on NR-NOFNiR activity, we measured NO production in WT and mutant cells during S limitation in the absence and presence of nitrite with the NO-specific dye DAF-FM DA. As show in Fig. 5 B, S deprivation markedly induced NO generation in WT cells following nitrite treatment. However, S limitation-induced NO formation was completely abolished in the 305 strain. These data also verified by confocal microscopy: in contrast to WT, this NR mutant showed no signal during S starvation both in the absence and in the presence of nitrite (Fig. 5 C). This finding indicates that S deprivation induces NO production in *Chlamydomonas* via NR-NOFNiR system. Together, the results demonstrated that the observed S limitation-induced NO plays role in *THB1* upregulation and excess levels of this signal molecule apparently are not required for full *THB1* increase to occur.

Discussion

During S deprivation, *Chlamydomonas* cells mediate a suite of specific responses to efficiently acquire S from various sources and remodel primary metabolism (Irihimovitch and Yehudai-Resheff, 2008). Some mechanisms of perception and response to S availability have been characterized (Aksoy et al., 2013; Zhang et al., 2004). *Chlamydomonas THB1* is a newly identified component that is involved in the control of proper response to S depletion (Minaeva et al., 2017; Zalutskaya et al., 2018). Although the THB1-dependent regulation of a subset of S limitation-responsive genes has been elucidated, how this truncated hemoglobin is controlled in S-deprived cells was largely unknown.

A plasma membrane protein SAC1 was previously shown to function as a sensor of S levels (Davies et al., 1996; Zhang et al., 2004). The expression analysis revealed that *THB1* gene was also positively regulated by S-limitation in *sac1* mutant (Figure 1 A, C). This implies that SAC1 is not involved in up-regulation of this target gene during S deprivation.

Upon S-deficiency, the serine-threonine kinase SNRK2.1 is activated and triggers transcriptional activation of many S-deprivation responsive genes (González-Ballester et al., 2008, Zalutskaya et al., 2018; Fig. 1 A). In S-replete medium, the action of SNRK2.1 is negatively controlled by another kinase, SNRK2.2. Furthermore, SNRK2.2 is repressed by protein SAC1 (Saroussi et al., 2017). We hypothesized that *THB1* expression is not controlled by signaling proteins and regulators associated with S deprivation specific responses. Indeed, SNRK2.1 and SNRK2.2 were both not engaged in *THB1* control (Fig. 1 D, E). Moreover, we found that *THB1* was not a regulatory target for transcriptional factor ARS73a (Fig. 1 F). This finding supports the hypothesis that other mechanism is required for *THB1* upregulation in S-free medium.

In *Chlamydomonas*, it has been reported that *THB1* is induced by nitrate (Johnson et al., 2014). Moreover, *THB1* is highly expressed in the presence of NO (Sanz-Luque et al., 2015). Given that NO is generated in S-deprived cells (Minaeva et al., 2017), the increase in *THB1* transcription detected during S limitation may reflect NOS activity. Recently, several algae were shown to contain NOS (Foresi et al., 2010; Jeandroz et al., 2016). This, however, did not appear to be the case in our experiments. *THB1* expression was not affected by the NOS inhibitor, L-NAME, or addition of NOS substrate, L-arginine (Fig. 2 A, B). Interestingly, no gene encoding functional NOS was identified in *Chlamydomonas* genome so far. As in this green alga, no NOSs have

been characterized in land plants as well (Fröhlich and Durner, 2011). KCN, in contrast, inhibited S depletion-induced *THB1* transcription (Fig. 2 C), in agreement with the main role of NR in NO generation in *Chlamydomonas* cells (Sakihama et al., 2002). In this study, we demonstrated that functional NR is essential for *THB1* expression in S-starved cells (Fig. 3).

More recently, Chamizo-Ampudia with coauthors (2016) found that a dual system formed by the NOFNiR and NR mediates NO synthesis from nitrite. Importantly, the mutant without diaphorase-NR activity failed to induce *THB1* gene upon S limitation (Fig. 3 B). Moreover, *THB1* upregulation in both *nit1* mutants was recovered after addition of NO donor. These results imply that, NOFNiR-NR-dependent NO formation may occur in the cells during S deprivation. A similar observation is previously reported that NR was mainly responsible for NO production in *Chlamydomonas* after salt stress (Chen et al., 2016). More generally we could suggest that NR-NOFNiR system plays a regulatory role in the control of NO generation dynamics, ensuring efficient shaping of this messenger by coordinating signal pathways upon responses to different stresses (Fig. 6).

As mentioned above, the system NR-NOFNiR is responsible for NO production from nitrite. However, it is clear that *Chlamydomonas* cells increased the *THB1* transcript levels in S-free ammonium-containing medium (Minaeva et al., 2017; Fig. 1 B). With the reported linkage between *THB1* upregulation and NR-dependent NO generation in S-starved cells (Figs 2 C; 3), it was logical to infer that alterations in the amount of intracellular nitrite would occur. Indeed, upon S limitation, an increase (2-2.5 fold) in nitrite concentrations was observed in WT (Fig. 4). It is important to note that this accumulation of nitrite in S-depleted cells was followed by increased production of NO, which appeared to lead to an increase in *THB1* expression (Fig. 5). Supplying only 1 mM nitrite to S-free medium triggered a change in *THB1* expression (Fig. 5 A). Notably, NOFNiR showed a Km for nitrite as 654.7± 98 μM (Chamizo-Ampudia et al., 2016). However, the gradual increase in *THB1* transcript levels was not observed when the higher concentrations of nitrite (up to 10 mM) were added to cellular suspension (Fig. 5 A), suggesting that this process depends on the threshold level of nitrite and NO formed from it.

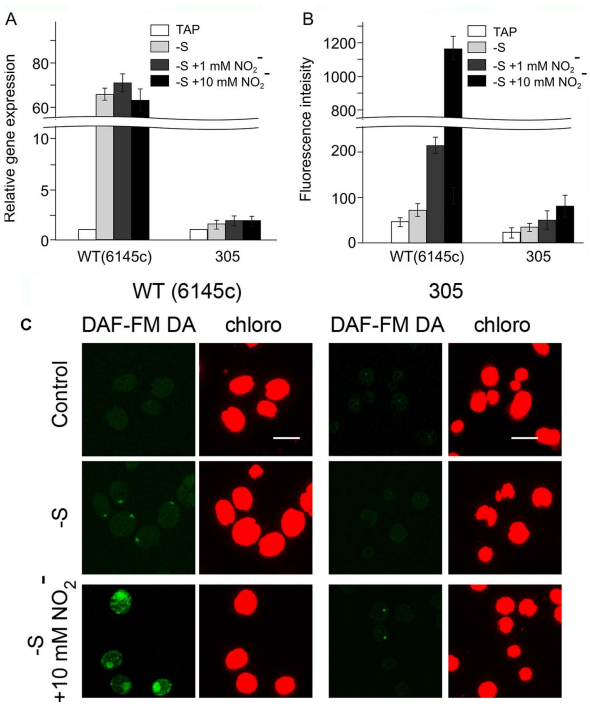


Fig. 5. Expression changes of *THB1* gene and nitric oxide production in nitrite supplemented S-deprived cells. (A) Relative expression levels of *THB1* gene in S-deprived cells of WT (6145c) and 305 strains. Cells of both strains grown in TAP were washed in S-free-medium and incubated for 1 h in the absence or presence of nitrite at indicated concentrations (1 mM or 10 mM). 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. (B) Detection of nitric oxide generation. Cells of the strains 6145c and 305 were grown in TAP medium and transferred to S-free medium in the absence or presence of nitrite in the light for 15 min. Fluorescence intensity due to intracellular NO was determined using 1 μM DAF-FM DA and was expressed as arbitrary units per chlorophyll. Cell autofluorescence was subtracted from the total fluorescence obtained. Values are means ± SE from three technical replicates of a representative experiment. (C) NO visualization by confocal microscopy. Images of cells grown in TAP (Con) or incubated in S-free (-S) or S-free supplemented with nitrite (-S+10 mM NO_2^-) for 15 min. The left-hand panels show DAF-FM fluorescence (green color) while the right-hand panels show Chl autofluorescence (red color). Scale bar equals 10 μm.

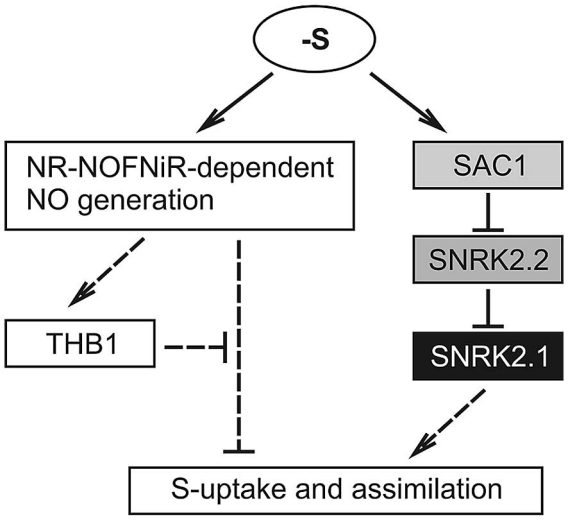


Fig. 6. Model depicting NR-dependent NO generation triggering early regulatory events in S-deprived cells. Solid lines indicate signaling pathways that control S limitation responses of *Chlamydomonas*. Dotted lines indicate transcriptional level of regulation. *Arrows* and blunted lines represent positive and inhibitory regulations, respectively.

Supporting this idea, the observed effects were not found in *nit1* mutant. It is consistent with the fact that under physiological conditions, the intracellular concentration of nitrite is low (Rexach et al., 2000). However, the other regulatory mechanisms that might be associated with alterations in nitrate/nitrite accumulation in S-deprived cells in ammonium-containing medium require further investigation.

In summary, characterization of the sulfur limitation-induced expression of *THB1* expands our understanding of the regulatory aspects of network during S deprivation beyond previous finding of the core components of S-specific responses (Saroussi et al., 2017). In this study, we find that NR-NOFNiR system plays a positive role in *THB1* upregulation by generation of NO in S-starved cells (Fig. 6).

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Supplementary materials

Fig. S1. The effect of sulfur deprivation on cell viability of WT (21 gr) the mutants *sac1, snkr2.2, snkr2.1* and *ars73a*. Values are means ±SD of three biological replicates.
(http://protistology.ifmo.ru/num12_2/fig_S1.pdf)

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