

REVIEW

How *Chlamydomonas* handles nitrate and the nitric oxide cycle

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Abstract

The green alga *Chlamydomonas* is a valuable model system capable of assimilating different forms of nitrogen (N). Nitrate (NO_3^-) has a relevant role in plant-like organisms, first as a nitrogen source for growth and second as a signalling molecule. Several modules are necessary for *Chlamydomonas* to handle nitrate, including transporters, nitrate reductase (NR), nitrite reductase (NiR), GS/GOGAT enzymes for ammonium assimilation, and regulatory protein(s). Transporters provide a first step for influx/efflux, homeostasis, and sensing of nitrate; and NIT2 is the key transcription factor (RWP-RK) for mediating the nitrate-dependent activation of a number of genes. Here, we review how NR participates in the cycle $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^-$. NR uses the partner protein amidoxime-reducing component/nitric oxide-forming nitrite reductase (ARC/NOFNiR) for the conversion of nitrite (NO_2^-) into nitric oxide (NO). It also uses the truncated haemoglobin THB1 in the conversion of nitric oxide to nitrate. Nitric oxide is a negative signal for nitrate assimilation; it inhibits the activity and expression of high-affinity nitrate/nitrite transporters and NR. During this cycle, the positive signal of nitrate is transformed into the negative signal of nitric oxide, which can then be converted back into nitrate. Thus, NR is back in the spotlight as a strategic regulator of the nitric oxide cycle and the nitrate assimilation pathway.

Key words: *Chlamydomonas*, NO cycle, nitric oxide, nitrate reductase.

Introduction

Chlamydomonas is a model green alga that shares with plants the capability of assimilating different forms of nitrogen (N) compounds. Nitrate (NO_3^-) is the preferred nitrogen source used for the formation of amino acids, proteins, nucleic acid, and biomass. In addition, nitrate is an important signalling molecule in different plant processes (Crawford, 1995; Wang *et al.*, 2012; Krapp *et al.*,

2014; O'Brien *et al.*, 2016). Understanding how the nitrate assimilation pathway is regulated is key to improving nitrogen use efficiency and avoiding the adverse effects of N-fertilization, such as waste nitrogen and environmental contamination.

Nitrate assimilation includes (i) the transporters, (ii) nitrate reductase (NR), (iii) nitrite reductase (NiR), (iv) GS/GOGAT

enzymes, and (v) regulatory protein(s) involved in sensing and signalling of nitrate. Even though *Chlamydomonas* is a unicellular alga, it has developed a network of nitrate/nitrite (NO_2^-) transporters belonging to three different families: nitrate assimilation-related component 1 [NAR1; formate/nitrite transporter (FNT) family], nitrate transporter 2 [NRT2; nitrate nitrite porter (NNP)], and NRT1 (Fernandez and Galvan, 2007, 2008; Sanz-Luque *et al.*, 2015a). These transporters provide a first step for influx, homeostasis, and sensing of nitrate (Llamas *et al.*, 2002; Rexach *et al.*, 2002; Sanz-Luque *et al.*, 2015a). Although a number of regulatory factors have been related to nitrogen assimilation, the RWP-RK transcription factor NIT2 is crucial to mediate the nitrate-dependent transcriptional expression of a number of nitrate transporters and NR (Camargo *et al.*, 2007; Higuera *et al.*, 2014).

Once nitrate is in the cell, the cytosolic NR catalyses its reduction to nitrite, which is then transported into the chloroplast and converted to ammonium by the action of NiR. The nitrogen then continues up its assimilation pathway to be incorporated into amino acids/proteins (Fernandez and Galvan, 2007, 2008). Recently, NR was shown to be involved in nitric oxide (NO) production (Yamasaki and Sakihama, 2000), but not as the catalyser as was previously thought. However, NR is an essential partner protein of the amidoxime-reducing component (ARC), renamed NOFNiR (nitric oxide-forming nitrite reductase), which catalyses nitrite conversion to nitric oxide (Chamizo-Ampudia *et al.*, 2016). The truncated haemoglobin THB1 also uses NR as a protein partner for the conversion of nitric oxide into nitrate using its dioxygenase activity (Sanz-Luque *et al.*, 2015b, Sanz-Luque *et al.* 2015c).

In this work, we provide a view on how NR participates in the cycle $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^-$. The multifunctionality of NR depends on its partner proteins, and allows the conversion of nitrate, a positive signal for nitrate assimilation, into nitric oxide, which is a negative signal similar to ammonium. Both ammonium and nitric oxide inhibit the activity and expression of high-affinity nitrate/nitrite transporters and NR (de Montaigu *et al.*, 2010). During this cycle, the negative signal of nitric oxide can be converted back to the positive signal of nitrate. Thus, NR can additionally be considered a key regulator for nitrate assimilation through its involvement in the nitric oxide cycle.

Nitrate and nitrite transporters

Nitrate and nitrite transporters are the first step required for nitrogen entry into the cell. In a unicellular organism, such as *Chlamydomonas*, nitrate/nitrite transporters have particular roles for communicating with the surrounding environment and respond according to the nitrate/nitrite supply. This is important because nitrate is a positive signal for nitrate assimilation, but nitric oxide, which can derive from nitrate, is a negative signal.

In plants, nitrate transport is carried out by a sophisticated network of membrane proteins that mediate the sensing,

absorption, storage, and distribution of nitrate among the different tissues. Plant nitrate transporters belong to at least four main families: nitrate transporter 1/peptide transporter/nitrate peptide transporter family (NRT1/PTR/NPF), NRT2/nitrate nitrite porter (NRT2/NNP), chloride channels (CLC), slow anion channel-associated 1 homolog 3 (SLAC1/SLAH), and aluminium-activated malate transporters (ALMT), which have been extensively reviewed (Wang *et al.*, 2012; Krapp *et al.*, 2014; Krapp, 2015; O'Brien *et al.*, 2016; Sharma *et al.*, 2016).

Genes homologous to SLAC1/SLAH and ALMT are absent in the *Chlamydomonas* genome; it instead contains six CLC homologues (CLV1: Cre13.g57400; CLV2: Cre17.g729450; CLV3: Cre09.g404100; CLV4: Cre09.g402051; CLV5: Cre01.g038700; and CLV6: Cre01.g037150) (Phytozome v11.0). In *Arabidopsis*, AtCLCa is responsible for nitrate accumulation into plant vacuoles (De Angeli *et al.*, 2006) and its selectivity for nitrate is linked to the filter motif GXGIP, absent in the rest of the AtCLCs (Wege *et al.*, 2010). AtCLCe is postulated to be a chloride (Cl^-) or nitrite channel/transporter from the stroma into the thylakoid (Monachello *et al.*, 2009). AtCLCa and AtCLCe involve connected endomembrane systems that contribute to nitrate homeostasis in the plant cell. Whether the *Chlamydomonas* CLCs are involved in nitrate homeostasis is unknown. Notwithstanding, *Chlamydomonas* does not accumulate nitrate into a vacuolar compartment and its putative CLC/CLVs lack the nitrate selectivity filter motif.

Though *Chlamydomonas* is a unicellular organism, it also shows complexity for nitrate/nitrite transporters (Fig. 1), with a total of 13 transporters corresponding to the families NRT1 (one), NRT2 (six), and NAR1 (six), with NAR1 absent in higher plants (Sanz-Luque *et al.*, 2015a).

NAR1 transporters for nitrite and bicarbonate

NAR1.1 was identified in *Chlamydomonas* as a plastidic nitrite transporter (Rexach *et al.*, 2000). NAR1.1 belongs to the FNT family present in bacteria (FocA and NirC, in *Escherichia coli*), fungi (NitA in *Aspergillus nidulans*), yeast (NAR1 in *Hansenula polymorpha*), and some marine picocyanobacteria (NitM) and algae, but it is absent in plant genomes (Peakman *et al.*, 1990; Suppmann and Sawers, 1994; Maeda *et al.*, 2015; Sanz-Luque *et al.*, 2015a). Briefly, the roles proposed for the different NAR1/FNT/NirC are as follows: (i) in *E. coli*, NirC is involved in both nitrite uptake and efflux (Jia *et al.*, 2009); (ii) in *A. nidulans*, NitA mediates specific high-affinity transport of nitrite and also has some role in nitrite efflux (Wang *et al.*, 2008); (iii) in *H. polymorpha*, NAR1 mediates nitrate and nitrite efflux (Cabrera *et al.*, 2014); and (iv) in marine picocyanobacteria, NitM mediates specific nitrite uptake. Curiously, some NitM show a long C-terminus, proposed to inhibit/regulate nitrite uptake activity and avoid the toxicity caused by nitrite accumulation (Maeda *et al.*, 2015).

The highest number of NAR1/FNT proteins has thus far been found in *Chlamydomonas reinhardtii*, with three in the

plasma membrane (NAR1.3, NAR1.4, NAR1.6) and three in the chloroplast (NAR1.1, NAR1.2, NAR1.5) (Fig. 1). Like some NitM from picocyanobacteria, NAR1.3 and NAR1.6 have a long C-terminus, but its function is still unknown (Mariscal *et al.*, 2006). The plastidic transporter NAR1.1 is co-regulated with other key proteins for nitrate assimilation (NR, NiR, NRT2.1, NRT2.2, and NAR2) (Rexach *et al.*, 2000). NAR1.1 denotes a control step for nitrate assimilation at the chloroplast level. First, it is required for cell growth under limiting nitrate conditions, and second, it controls the

amount of nitrate assimilated by the cells under limiting carbon dioxide conditions (Rexach *et al.*, 2000, Mariscal *et al.*, 2004).

NAR1.2 also named LCIA (Low CO₂ Component A) is a chloroplast envelope transporter and bispecific for both nitrite and bicarbonate, which overexpresses under low CO₂ conditions (Mariscal *et al.*, 2006). NAR1.2 (LCIA) forms part of the carbon dioxide-concentrating mechanism (CCM) operating at low carbon dioxide concentrations and is regulated by the master gene for carbon *CIA5/CCM1* (Wang and

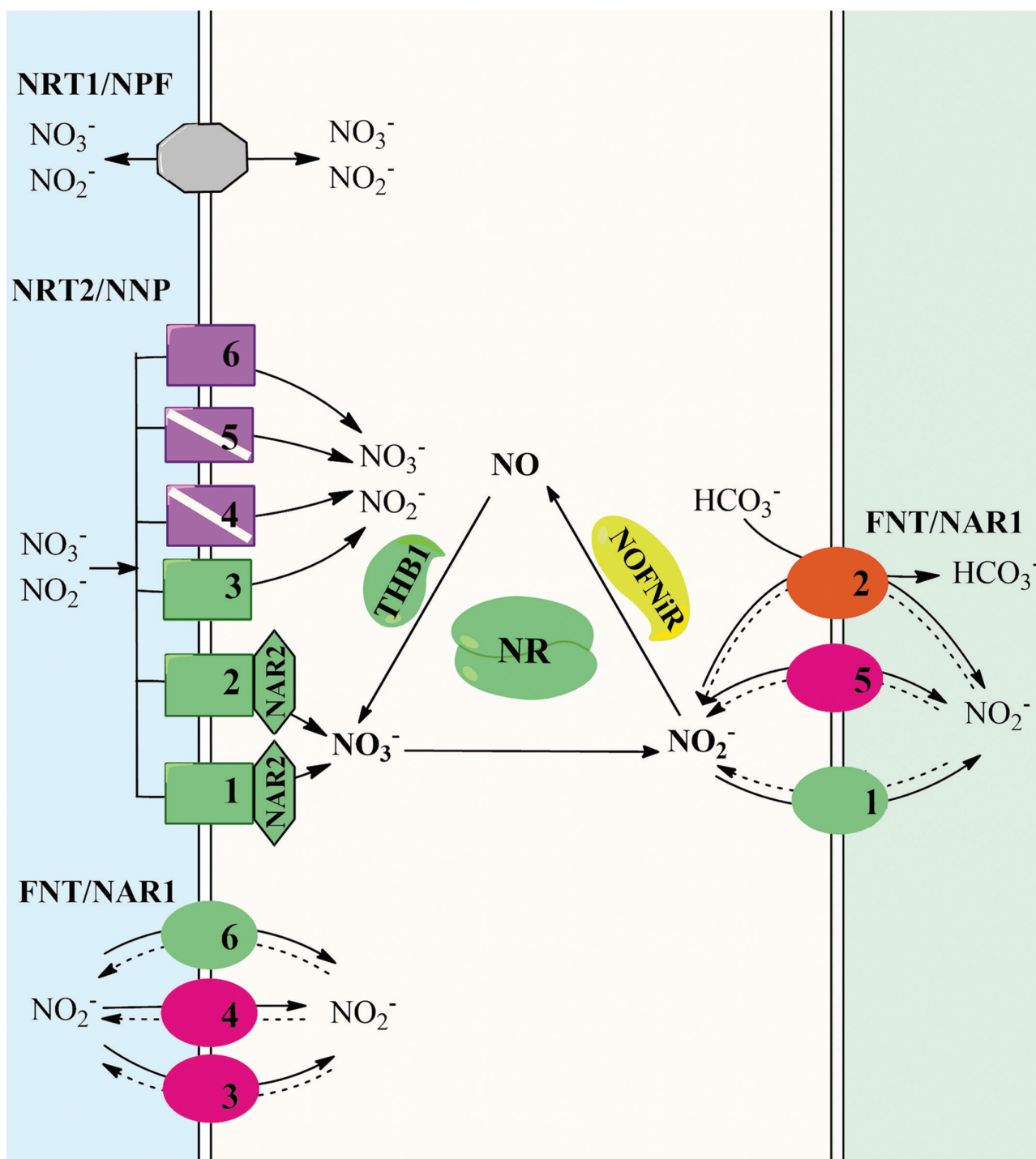


Fig. 1. Scheme for homeostasis of nitrate and nitrite in *Chlamydomonas* and the nitric oxide cycle. The transporters at the plasma and chloroplast membranes are shown as protein families. Those components under the control of the regulator NIT2 are shown in green.

Spalding, 2014; Yamano *et al.*, 2015). The CCM is essential in aquatic microalgae to accumulate carbon dioxide in the region close to the Rubisco for efficient photosynthesis. Accordingly, NAR1 transporters seem to adjust nitrate assimilation to carbon dioxide availability in *Chlamydomonas* (Mariscal *et al.*, 2004, 2006).

NRT2 are nitrate and nitrite transporters

An NRT2 was first identified in the fungus *Aspergillus nidulans* (Unkles *et al.*, 1991) and later in the alga *Chlamydomonas* (Quesada *et al.*, 1994) and in barley (*Hordeum vulgare*) (Trueman *et al.*, 1996). A relevant characteristic of these transporters is the requirement of a second protein component, NAR2 (NRT3), for most NRT2s from plants, with the exceptions of AtNRT2.7 from *Arabidopsis* that seems to be NAR2 independent (Chopin *et al.*, 2007) and OsNRT2.3b from rice (*Oryza sativa*) (Fan *et al.*, 2016). Only two of the six NRT2s from *Chlamydomonas* (NRT2.1 and 2.2) require NAR2 (Quesada *et al.*, 1994; Zhou *et al.*, 2000). The NRT2s from *Aspergillus* (NRTA, NRTB), *Hansenula* (YNT), and *E. coli* (NARK, NARU) do not require this second component for functionality. Another characteristic of NRT2s is their high affinity and specificity for the substrate. In *Arabidopsis*, the seven NRT2s have been characterized as influx and high-affinity nitrate transporters specific for nitrate (O'Brien *et al.*, 2016), whereas in fungi and yeast (AnNRTA, AnNRTB, HpYNT) as well as in *Chlamydomonas* (NRT2.1/NAR2) and *E. coli* (NARK, NARU), NRT2s are high-affinity nitrate and nitrite transporters (Galván *et al.*, 1996; Pérez *et al.*, 1997; Clegg *et al.*, 2002; Wang *et al.*, 2008; Akhtar *et al.*, 2015).

Six genes (*NRT2.1–6*) account for the *Chlamydomonas* NRT2 family. NRT2.1, together with NAR2, is a bispecific high-affinity nitrate and nitrite transporter (Galván *et al.*, 1996), and also has a role in nitrate signalling (Rexach *et al.*, 2002; Llamas *et al.*, 2002). NRT2.2/NAR2 is specific for nitrate. NRT2.1 and NRT2.2 account for all the high-affinity nitrate transport activity in the cell under high carbon dioxide conditions (Rexach *et al.*, 1999; Fernandez and Galvan, 2007). The rest of the transporters are NAR2 independent and very probably involved in nitrite transport. Recently, NRT2.4 and NRT2.5 were characterized as atypical NRT2 proteins because they are half-size transporters bearing six transmembrane domains, of which NRT2.4 was shown to have high-affinity nitrite transport activity (Higuera *et al.*, 2016). A significant fact in *Chlamydomonas* is that in spite of the high number of NRT2s, only one – NRT2.2 – is highly specific for nitrate, and the rest transport both nitrate and nitrite or only nitrite. This denotes the importance of regulating nitrite into the cell to avoid its toxicity. In fact, nitrite is not accumulated into *Chlamydomonas* cells under adverse conditions and is instead excreted outside the cell. These adverse conditions occur when cells are reducing nitrate owing to low carbon availability (Rexach *et al.*, 2000; Mariscal *et al.*, 2004), or when NiR is lacking (Navarro *et al.*, 2000).

NRT1 transporter

NRT1 was the first nitrate transporter identified in *Arabidopsis* and is known as CHL1/NRT1.1/NPF6.3 (Tsay *et al.*, 1993). NRT1 proteins are ubiquitously present in plants, animals, fungi, and bacteria, and a recently unified nomenclature renamed them as NPFs (Léran *et al.*, 2014). Plant genomes contain a high number of NPF genes, with 53 in *Arabidopsis* up to 139 in *Malus domestica* (Léran *et al.*, 2014). The NRT1/NPF transporters have been extensively studied and reviewed in *Arabidopsis*, in which different NPFs are involved in nitrate uptake by the root and its distribution throughout the plant tissues (Krapp *et al.*, 2014; Léran *et al.*, 2014; Krapp, 2015; O'Brien *et al.*, 2016). A key point for the large NPF family in *Arabidopsis* is their non-specificity for substrate. Most of the NPF studied are low-affinity nitrate transporters, but in addition to nitrate some transport nitrite, amino acids, peptides, glucosinolates, auxin, and abscisic acid (Léran *et al.*, 2014; O'Brien *et al.*, 2016).

CHL1/NRT1.1/NPF6.3 is a peculiar protein in the NPF family in *Arabidopsis*. It is a dual-affinity nitrate transporter, showing high-affinity transport activity when it is phosphorylated at T101, and low-affinity transport activity in the dephosphorylated form (Ho *et al.*, 2009). It is a bidirectional nitrate transporter involved in the influx and efflux of nitrate (Léran *et al.*, 2014) and is also a nitrate-regulated auxin transporter (Krouk *et al.*, 2010). This protein was proposed as a transceptor able to detect low and high nitrate concentrations (Ho *et al.*, 2009). Recently, in-depth knowledge of its characteristics has been derived from studies of its crystal structure (Parker and Newstead, 2014; Sun *et al.*, 2014), studies of the signal transduction pathway involving phospholipase C and calcium (Riveras *et al.*, 2015), and transcriptomic analyses that show multiple and differential gene expression depending on the phosphorylation state of NPF6.3 (Bouguyon *et al.*, 2015).

In contrast to higher plants, *Chlamydomonas*, as well as many other unicellular algae, contains a unique *NRT1/NPF* gene, but its precise role in the sensing, influx, and efflux of nitrate is still unknown (Sanz-Luque *et al.*, 2015a).

Regulation of nitrate assimilation: nitric oxide plays a role

In *Chlamydomonas*, the expression of nitrate-assimilation genes is regulated by ammonium (negative signal) and nitrate (positive signal) (Fernandez and Galvan, 2007). Nitrate is required to induce *NIA1*, *NII*, *NRT2.1–3/NAR2*, and *NAR1.1*. Ammonium, in contrast, represses all these genes (Fig. 1) (Quesada and Fernández, 1994; Fernandez and Galvan, 2007) and causes rapid inhibition of nitrate transport activity (Flores and Vega, 1983). Both positive and negative signalling are complex processes, as deduced from the high number of tagged genes involved (González-Ballester *et al.*, 2005). A noteworthy difference has emerged for the two types of regulation: none of the ammonium-insensitive mutants are completely released from ammonium repression, and the presence of nitrate is always required to make the

ammonium-insensitive phenotype evident. Notwithstanding, for the positive nitrate signal, the single *NIT2* gene is critical. Allelic *NIT2* gene mutants are unable to grow in nitrate and have null pNIA1-ARS activity in nitrate (Camargo *et al.*, 2007). Thus, *NIT2* is thus far considered to be the master regulator for nitrate assimilation genes and involved in the positive nitrate signal/signalling pathway. *NIT2* is a transcription factor containing GAF and RWP-RK domains that shows conservation with plant nodule inception-like proteins (NLP) (Camargo *et al.*, 2007; Castaings *et al.*, 2009; Konishi and Yanagisawa, 2013; Marchive *et al.*, 2013).

A number of transcription factors that regulate the nitrate response have been identified in *Arabidopsis*, including NLP, TGA1, TGA4, ANR1, bZIP1, LBD37, LBD38, TCP20, NAC4, SLP9 (reviewed in Chardin *et al.*, 2014; Vidal *et al.*, 2015; O'Brien *et al.*, 2016), and, recently, NRG2 (Xu *et al.*, 2016).

Concerning the *Arabidopsis* NLP family (which comprises nine proteins), NLP7 is an important regulator of the early nitrate response. It is translocated into the nucleus in response to nitrate to regulate gene expression, and moves from the nucleus to the cytoplasm when nitrate is removed (Marchive *et al.*, 2013). The nucleocytoplasmic shuttling seems to be a regulatory checkpoint for nitrate induction in both *Aspergillus nidulans* (NirA) (Berger *et al.*, 2006) and *Arabidopsis* (NLP7). The biochemical mechanism for this regulation is not completely resolved. However, a redox regulation mechanism underlines the activity of the fungal nitrate regulator NirA, in which a conserved methionine in the nuclear export sequence is key for its cellular location (Gallmetzer *et al.*, 2015). NRG2 has been shown to interact directly with NLP7, but this interaction has no effect on the nuclear retention of NLP7 (Xu *et al.*, 2016). NLP7 has also provided molecular evidence for nitrate-dependent regulation between nitrogen and carbon metabolism. In fact, NLP7 regulates several enzymes involved in carbon metabolism (Marchive *et al.*, 2013; Yu *et al.*, 2016) and the overexpression of NLP7 results in enhanced nitrogen and carbon assimilation and improved plant growth in both *Arabidopsis* and *Nicotiana tabacum* (Yu *et al.*, 2016). *Chlamydomonas* *NIT2* has also been reported to be involved in the regulation of carbon metabolism, including in acetate uptake, organic acid accumulation, and starch storage (Remacle *et al.*, 2014).

The negative ammonium signal and nitric oxide

In *Chlamydomonas*, ammonium and nitric oxide are considered to be negative signals that affect nitrate assimilation genes, at both the transcriptional and post-transcriptional levels (de Montaigu *et al.*, 2010; Sanz-Luque *et al.*, 2013, 2015b, 2016; Chamizo-Ampudia *et al.*, 2016).

Nitric oxide is a diffusible gas messenger that was first described in mammals through its role on blood pressure relaxation. Currently, nitric oxide is considered to be an ubiquitous signal molecule involved in many biological processes in bacteria, fungi, plants, and mammals. Nitric oxide has been postulated as perhaps the first signalling molecule to appear in biological systems (Feelisch and Martin, 1995). In plants, nitric oxide is involved in seed germination, senescence, root development, plant immunity, and abiotic stress (for reviews,

see Lamattina *et al.*, 2003; Yu *et al.*, 2014; Domingo *et al.*, 2015). Transcriptome profiling shows the multiple regulatory pathways mediated by nitric oxide in *Arabidopsis* (Hussain *et al.*, 2016).

In *Chlamydomonas*, nitric oxide has been related to the remodelling of chloroplast and thylakoid proteins upon nitrogen starvation (Wei *et al.*, 2014), the cell death induced by ethylene and mastoparan (Yordanova *et al.*, 2010), oxidative stress at very high light (Chang *et al.*, 2013), proline synthesis under copper stress (Zhang *et al.*, 2008), salt stress (Chen *et al.*, 2016), and regulation of nitrogen assimilation (de Montaigu *et al.*, 2010; Sanz-Luque *et al.*, 2013, 2015b, Chamizo-Ampudia *et al.*, 2016)

The first molecular evidence for nitric oxide regulating nitrogen metabolism in *Chlamydomonas* came from the identification of a defective form of the soluble nitric oxide-dependent guanylate cyclase CYG56 in an ammonium-insensitive mutant (de Montaigu *et al.*, 2010). This CYG56 enzyme participates in ammonium repression of *NIA1* through a pathway that involves nitric oxide, cyclic guanosine monophosphate, and calcium. In contrast to the wild type, the *cyg56* mutant is partially released from ammonium repression in media containing both ammonium and nitrate, which results in higher transcript levels of *NIA1*, *NRT2.1*, and the high-affinity ammonium transporters *AMT1.1* and *AMT1.2* than in the wild type (de Montaigu *et al.*, 2010). Besides the down-regulation of these genes expression, nitric oxide also has a post-transcriptional inhibition effect on high-affinity transport of nitrate, nitrite, and ammonium and on NR activity (Sanz-Luque *et al.*, 2013).

In *Chlamydomonas*, *NIT2* controls the expression of key players for nitrate assimilation in response to nitrate (Fig. 1). Two of these genes encode truncated haemoglobins, *THB1* and *THB2*, but *THB1* is also partially induced by nitric oxide (Sanz-Luque *et al.*, 2015b). *THB1* has been shown to connect nitric oxide metabolism and nitrate assimilation. Its dioxygenase activity is able to transform nitric oxide into nitrate under oxygenic conditions ($\text{NO} + \text{O}_2 + \text{e}^- \rightarrow \text{NO}_3^-$), but only when in a reduced form (bonded with Fe^{2+}). NR is able to reduce *THB1*, through its diaphorase activity, more efficiently than free cofactors (NADH and FAD), or cytochrome b5 reductase (Cytb5-R), which has high homology to NR (Sanz-Luque *et al.*, 2015c). The partners NR/*THB1* seem to result in two effects: first, the scavenging of nitric oxide to produce nitrate, transforming a negative signal into a positive one; and second, redirecting the electron flux from NR-diaphorase to *THB1*, which inhibits NR activity and thus moderates nitrite production (Fig. 2). The

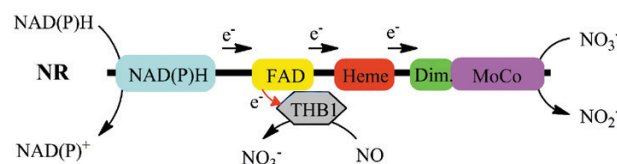


Fig. 2. Model for the dioxygenase activity of *THB1* involving NR.

The domains containing the prosthetic groups are shown in colour. The binding site domain of NADPH is indicated. The oxygenase reaction of *THB1* is proposed to receive electrons (red arrow) from the FAD domain of NR (Sanz-Luque *et al.*, 2015a).

major function of NR is to reduce nitrate to allow its assimilation, a task specific to plants-like organisms. However, nitrate reduction must be adjusted in line with the capacity to assimilate it into amino acids to avoid accumulating high concentrations of nitrite, which is both toxic and a substrate for nitric oxide production.

In maize (*Zea mays*), coordinated regulation of NR and non-symbiotic haemoglobins has also been described and proposed to be involved in nitric oxide homeostasis and signalling for root growth in response to nitrate (Manoli *et al.*, 2014; Trevisan *et al.*, 2014). NR and haemoglobins seem to be pieces of a complex puzzle for nitric oxide homeostasis. S-nitrosoglutathione, as a reservoir of nitric oxide, and S-nitrosoglutathione reductase, which catalyses the reduction of S-nitrosoglutathione into glutathione disulfide and ammonia, are also involved in nitric oxide homeostasis and the feedback regulation of nitrate assimilation (Frunghillo *et al.*, 2014), recently reviewed in plant (Frunghillo *et al.*, 2016). Interestingly, S-nitrosoglutathione reductase has been shown to be S-nitrosylated in *Arabidopsis* and *Chlamydomonas* (Frunghillo *et al.*, 2014; Chen *et al.*, 2016) as well NR in *Chlamydomonas* (Chen *et al.*, 2016). In summary, nitric oxide homeostasis seems to regulate nitrate uptake and reduction, but a key question in this puzzle is how nitric oxide is produced.

Nitrate reductase and the nitric oxide cycle

Chlamydomonas reinhardtii has no animal-type nitric oxide synthase

Although evidence has accumulated for more than 15 years showing that nitric oxide is a signal molecule in plants, the identification of the enzymes responsible for nitric oxide synthesis has remained a challenge (Gas *et al.*, 2009; Gupta *et al.*, 2011; Jeandroz *et al.*, 2016). In animals, nitric oxide synthase (NOS) converts L-arginine to L-citrulline and nitric oxide, via N-hydroxy-L-arginine as an intermediate. This enzyme has a typical molecular architecture containing an N-terminal oxygenase domain and a C-terminal reductase domain that are connected by a calmodulin-binding motif (Bredt and Snyder, 1990; Alderton *et al.*, 2001; Campbell *et al.*, 2014). Recently, a gene encoding functional NOS in the green alga *Ostreococcus tauri* was identified (Foresi *et al.*, 2010). However, genome and transcriptome analysis from 1087 land plants and 265 from algae show that land plants have no NOS. Interestingly, 15 algae species do contain a NOS with the complete multidomain structure of animals NOSs (Foresi *et al.*, 2010; Jeandroz *et al.*, 2016). As in land plants, the *Chlamydomonas reinhardtii* genome lacks a typical NOS.

Nitrate reductase and ARC synthesize nitric oxide

Many different pathways have been suggested for nitric oxide production in plants (reviewed in Lamattina *et al.*, 2003; Yu *et al.*, 2014). Depending on the nitric oxide precursors, two

pathways can be distinguished: the oxidative and the reductive routes. The oxidative route involves the production of nitric oxide from arginine, polyamines, or hydroxylamines. The reductive route uses nitrite as a substrate for nitric oxide production and includes NR, the plasma membrane-bound nitrite:NO reductase, or mitochondrial nitrite reduction. However, evidence from different organisms, including plants (reviewed in Lamattina *et al.*, 2003), the fungi *Aspergillus nidulans* (Marcos *et al.*, 2016), and the alga *Chlamydomonas reinhardtii* (Sakihama *et al.*, 2002) suggests that NR is a key enzyme in nitric oxide production. This role for NR has been debated on the basis that nitric oxide production *in vitro* by plant NR is inefficient, with only 1% of the normal nitrate reduction capability, and that this reaction occurs only in the absence of nitrate (an efficient inhibitor), a non-physiological condition for the plant cell (Rockel *et al.*, 2002; Planchet and Kaiser, 2006).

In humans, a novel type of molybdoenzyme, mitochondrial amidoxime-reducing component (mARC) has recently been shown to produce nitric oxide via a reductive route from nitrite and NADH and in anoxic conditions (Sparacino-Watkins *et al.*, 2014). mARC requires cytochrome *b5* and Cytb5-R to form an electron transfer chain from NADH to the substrate. Still, the physiological functions of ARC are not well known and a role in detoxifying N-hydroxylated bases has been suggested (reviewed in Tejada-Jiménez *et al.*, 2013). As in humans, *Chlamydomonas* also has this three-component complex (ARCO), which is able to reduce toxic compounds such as hydroxylamino-purine and artificial compounds such as benzamidoxime (Chamizo-Ampudia *et al.*, 2011). ARC is present in plant genomes, both monocotyledons and dicotyledons (Tejada-Jiménez *et al.*, 2013), and *Arabidopsis* ARC also produces *in vitro* nitric oxide from nitrite using dithionite as the electron donor (Yang *et al.*, 2015).

Interestingly, a significant conservation of structural organization and sequence homology with the three-component system complex ARCO can be observed in the molecular structure of the different domains in NR (Sparacino-Watkins *et al.*, 2014; Chamizo-Ampudia *et al.*, 2016, Fig. 3). This observation led to investigations in *Chlamydomonas* as to whether NR, the ARCO complex separately, or NR together with ARC were able to produce nitric oxide (Chamizo-Ampudia *et al.*, 2016).

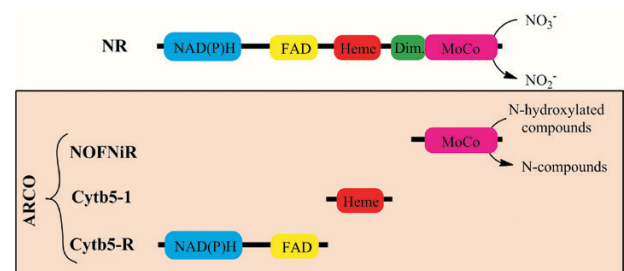


Fig. 3. Structural domains similarity of NR and the three components of the ARC complex. The ARC complex, named ARCO, comprises three proteins, two of which (Cytb5-R and Cytb5-1) have a high degree of conservation with the corresponding domains of NR.

The CrNR alone is able to produce NO in anaerobiosis from nitrite, though inefficiently, as previously reported (Yamasaki and Sakihama, 2000; Rockel *et al.*, 2002). The complex ARCO alone also inefficiently produces nitric oxide. However, the combination of NR and ARC results in an efficient machinery to synthesize nitric oxide under more physiological conditions: aerobiosis and the presence of both nitrate and nitrite (Fig. 4). *In vivo* experiments show that the dual system of NR and ARC is a major player in the production of nitric oxide in the cytoplasm in *Chlamydomonas*, and that the diaphorase-NR activity is required for supplying NAD(P)H electrons to nitrite.

Nitrate reductase and the nitric oxide cycle

Investigating the *Chlamydomonas* system, as a model of plants, has demonstrated the complete cycle of $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^-$, in which NR plays a central role. NR is required not only in the reduction step of $\text{NO}_3^- \rightarrow \text{NO}_2^-$ to allow for its assimilation, but also for the nitric oxide production step of $\text{NO}_2^- \rightarrow \text{NO}$ with ARC as a partner, and $\text{NO} \rightarrow \text{NO}_3^-$ with THB1 as a partner (Fig. 5). These new findings provide a novel function for a well-known enzyme, NR. This cycle allows for the control of nitric oxide metabolism and also for fine-tuning of nitrogen homeostasis by converting a positive signal, nitrate, for induction of nitrate assimilation genes (*NIA1*, *NRT2.1* *etc.*) into a negative signal, nitric oxide, that transcriptionally and post-transcriptionally inhibits NR and nitrate transport (de Montaigu *et al.*, 2010; Sanz-Luque *et al.*, 2013).

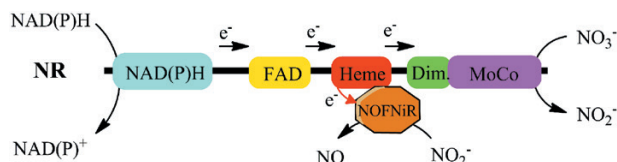


Fig. 4. Model for nitric oxide synthesis by the dual system of NR and ARC. NOFNiR (nitric oxide-forming nitrite reductase) corresponds to ARC, and receives electrons from the heme NR domain (Chamizo-Ampudia *et al.*, 2016)

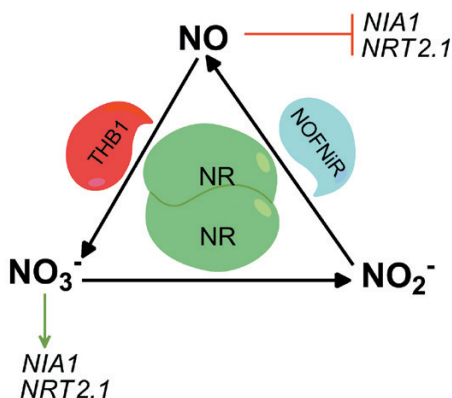


Fig. 5. NR and its central role in the nitric oxide cycle. The cycle $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{NO}_3^-$ is shown highlighting the transformation of the negative (red line) signal of nitric oxide into the positive (green line) signal of nitrate on the expression of *NIA1* and *NRT2.1* (de Montaigu *et al.*, 2010).

Concluding remarks and future prospects

Chlamydomonas, a plant model system, has provided insights into a new role of the well-known enzyme NR. In addition to reducing nitrate to nitrite, NR can also synthesize nitric oxide from nitrite when partnered with the molybdoenzyme ARC, and convert nitric oxide back into nitrate when partnered with the truncated haemoglobin THB1. These new properties seem to be derived from NRs diaphorase activity, which allows it to transfer electrons from NAD(P)H to its N-terminal domain or to other proteins like ARC or THB1. In addition to total NR activity, the diaphorase and terminal NR activities have been widely known for many years in plants, and the alga *Chlamydomonas*, several mutants of the alga, and *Nicotiana plumbaginifolia* all show *in vivo* and *in vitro* complementation, suggesting that electrons can be transferred from one NR-subunit to the other (Fernández and Cárdenas, 1982; Fernández and Matagne, 1986; Pelsy and Gonneau, 1991). Validation of this nitric oxide cycle in plants, as described in *Chlamydomonas*, is important to advance the study of nitric oxide metabolism and plant biology. A future direction for study will be to investigate the molecular mechanisms by which NR changes its partner and where the electron flow shunting occurs. Phosphorylation, sumoylation, and nitrosylation could be mechanisms to explore. In tobacco NR, the change of a phosphorylatable Ser 521 to Asp results in a permanently active enzyme, nitrite excretion, and nitric oxide emission in the dark (Lillo *et al.*, 2003; Lea *et al.*, 2004). In *Arabidopsis*, *NIA1* and *NIA2* are stimulated by the E3 sumo ligase AtSIZ1, and a conserved sumoylation site YKPE has been proposed (Park *et al.*, 2011). The mutant *siz1* results in low NR activity, low nitric oxide production, and high nitrate content.

Nitrate transporters constitute a complex network of different proteins families. An interesting characteristic of some transporters (*AtNRT1.1/NPF6.3* and *AtNRT2.1* or *CrNRT2.1*) is their role in nitrate sensing and signalling (O'Brien *et al.*, 2016). While the molecular transport mechanism for *AtNRT1.1* was recently unravelled, a future task is to understand the role of *NRT1* in organisms with a single copy of this gene, such as *Chlamydomonas*. Another question to be answered is whether cytosolic nitrate/nitrite is involved in sensing the nitrogen status in situations where the influx/efflux transporters have to play a role. This is of interest because changes in the nitrate/nitrite supply can result in changes in the concentration of nitric oxide signalling molecules.

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