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Eukaryotic nitrate and nitrite transporters

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Abstract. Nitrate transport is the key step controlling the amount of nitrate incorporated by the cells and subsequent of storage, reduction or export. Molecular, genetic and biochemical approaches to the study of eukaryotic nitrate/nitrite transporters allow an initial understanding of this step, which is much more complex and structured than previously suspected. At the plasma membrane level, two gene families, *Nrt1* and *Nrt2*, account for high- and low-affinity nitrate transporters. Functionality of NRT1 from *Arabidopsis* and NRT2 proteins from *Aspergillus* and *Chlamydomonas* has been demonstrated. However, redundancy of these systems makes it difficult to assign particular physiological roles to each. Data on genes involved in the regulation of nitrate transport and reduction are still scarce. Information on nitrite transporters to the chloroplast is biased by the belief that in vivo nitrous acid diffuses freely to this organellum. The recent progress on these aspects is discussed in this review.

Key words. Nitrate transporter; nitrite transporter; chlorate; *Nrt1*; *Nrt2*; chloroplast nitrite transport.

Introduction to eukaryotic nitrate/nitrite transporters

In photosynthetic eukaryotes, nitrate assimilation involves two membrane barriers: the plasma membrane, which delimits the cytosolic reduction of nitrate to nitrite, and the envelope of the chloroplast, where nitrite reduction to ammonium occurs [1]. In addition to its role as an essential nutrient, nitrate acts in plants as a signal molecule involved in root morphogenesis [2], shoot:root balance [3] and carbon metabolism adaptations [4], and in arresting the gametogenesis program in *Chlamydomonas reinhardtii* [5]. Although many aspects have still to be learnt about how nitrate is sensed and how the signal transduction mechanisms for nitrate-regulated genes operate, there exists a certain consensus that the nitrate transport (NT) step is key in controlling the efficiency of nitrogen assimilation.

Intensive efforts have been addressed to identifying and characterizing of nitrate transporters and reviewed recently [6–8]. The present review will focus on the functional characteristics of the plasmalemma nitrate/nitrite transporters in eukaryotes and nitrite transport to the chloroplast.

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Nitrate entry through the plasmalemma requires active transport systems, which are proton coupled and depend on a proton motive force generated by the H^+-ATPase [9, 10]. NT systems can be classified on the basis of physiological data (substrate affinity or induction) and gene sequence analysis [9, 11]. The physiological data show the existence of distinct NT systems: constitutive high-affinity (cHANT), inducible high-affinity (iHANT), constitutive low-affinity (cLANT) and inducible low-affinity (iLANT). The HANT systems operate at low external nitrate concentration (< 250 μM) and have different affinities and capacities to transport nitrate in plants. The LANT systems operate at high external nitrate concentration (> 1 mM), and thermodynamic evaluations and electrophysiology studies demonstrate that they also correspond to H^+-dependent active transport [11, 12]. The regulation of the individual components of the NT systems is complex, and both upregulation by nitrate and feedback repression by N-metabolites resulting from nitrate reduction have been shown for several transporters [13–16].

The identification of genes encoding nitrate transporters in eukaryotes started with the cloning of the *Aspergillus nidulans CrnA* gene in 1991 [17]. The *crnA* mutants from

A. nidulans are chlorate resistant and can use nitrate, though they are only deficient in N uptake in the conidiospore and young micelia stages [18]. Until now, at least 25 genes proposed to encode nitrate transporters have been identified, mainly in plants. According to their sequence analysis, nitrate transporter genes have been classified into two families: *Nrt1* and *Nrt2* [7, 8]. The nomenclature of these genes follows the rules proposed by the ISPMB [19]. Initially, *Nrt1* and *Nrt2* genes were related to LANT and HANT, respectively. However, the *Arabidopsis* NRT1;1, primarily proposed to be a LANT [12], is also involved in HANT [20, 21]. In addition, the NRT2;3 from *Chlamydomonas* is a LANT [22].

Molecular characteristics of NRT1 and NRT2 transporters

The NRT2 transporters, also named NNP family (for nitrate-nitrite porter), belong to the Major Facilitator superfamily (MFS), which includes sugar transporters from mammals, plants, yeast, and bacteria; bacterial drug-H^+ antiporters; metabolite-H^+ symporters; and eukaryotic phosphate-H^+ symporters, among 17 transporter families [23] (see also article by Moir and Wood). The NRT2 proteins have a typical membrane topology of 12 transmembrane domains arranged as two sets of six, connected by a cytosolic loop. NRT2 proteins share a conserved sequence motif, D/N-R-X-G-R-R/K, between transmembranes 2 and 3, and another, I-X_2-R-X_3-G-X_3-G, before and within transmembrane domain 4, which were present even among MFS members showing extensive divergence [23]. A consensus motif [FYK]-X_3-[ILQRK]-X-[GA]-X-[VASK]-X-[GASN]-[LIVFQ]-$X_{1,2}$-G-X-G-[NIM]-X-G-[GTA] within the fifth putative transmembrane domain has been proposed as the signature for the NNP family [23]. A portion of this sequence, A-G-W/L-G-N-M-G, present in the sequences from CRNA from *A. nidulans*, NRT2 from *Chlamydomonas* and *Hordeum vulgare*, and NARK from *Escherichia coli*, and absent in other MFS members, has been suggested as the substrate recognition motif [24].

The NRT1 transporters do not show significant conservation with members of the NRT2 family, and belong to the POT family, which includes numbers of H^+-dependent oligopeptide transporters from mammals, plants, fungi and bacteria [23, 25]. Although the POT family has sequence similarity to several members of the MFS, the small number of individuals and the short length of segments with significant similarity point to these two as distinct families [23]. NRT1 proteins are predicted to have 12 transmembrane domains, with a long loop containing many charged residues separating the first six transmembrane domains from the second six, and short N- and C-terminal ends [12, 26]. Interestingly, this secondary structure resembles those predicted for *A. nidulans* CRNA [17] and *Hansenula polymorpha* YNT1 proteins [27], and differs from plant and algal NRT2 proteins, which contain a long hydrophylic C-terminal domain [28, 29].

Functional and regulatory characteristics of NRT1 and NRT2 transporters

Even though many genes from both *Nrt1* and *Nrt2* families have been identified in eukaryotic organisms, the precise function for each transport system and their contribution to global N homeostasis remain to be elucidated. The functional and regulatory characteristics for some of these transporters are summarized in table 1.

The redundancy of nitrate transporters in many organisms results in a complicating factor when analysing the functionality of the transporters, which has been especially evident for AtNRT1;1. The *Arabidopsis Nrt1;1* (*Chl1)* gene was isolated from a T-DNA insertion allele (*chl1*) resistant to chlorate [12]. *AtNrt1;1* was initially proposed to encode a LANT since its heterologous expression in *Xenopus* oocytes showed characteristics typical of a H^+-coupled NT in the millimolar range [12], and the phenotype of the *chl1* mutant was consistent with a significant reduction in LANT activity but not in the NT at the high-affinity range (below 1 mM) [30, 31]. Recently, AtNRT1;1 has also been implicated in HANT [20, 21]. The confirmation that NRT1;1 has a role in HANT derives from measurements of nitrate depletion from the external media by *Nrt1;1*-injected *Xenopus* oocytes, resulting in a K_m of 50 μM [21]. The expression of *AtNrt1;1* transcript is upregulated by acid pH and by nitrate in the media at both low (μM) and high (mM) concentrations [12, 15]. This response to nitrate would agree with their proposed dual function in NT, which might also explain the reduced rate of uptake at both the low- and the high-affinity range in the *Arabidopsis chl1* mutant grown in ammonium nitrate [20, 21]. In contrast, *AtNrt1;1* expression is poorly sensitive to feedback regulation by N metabolites [15, 16].

The second *Nrt1* gene identified in *Arabidopsis* (*AtNrt1;2*, also named *NTL1*) corresponds to a cLANT system with no dual affinity for nitrite [26]. The *AtNtr1;2* is constitutively expressed in the absence of nitrate, and its functional analysis in *Xenopus* oocytes shows specificity for nitrate as a substrate (K_m 5.9 mM), but it is not able to transport dipeptides or histidine [26]. Transgenic plants containing an antisense *AtNrt1.2* also confirm its role as a LANT, since a decrease in NT

rates was observed at 5 mM but not at 500 μM [26]. Additional *Nrt1* homologous genes have been identified in *Arabidopsis* [32, 33] and tomato [34], but their function in NT is still unknown.

A gene phylogenetically close to *AtNrt1;1* has been identified in *Brassica napus* (*BnNrt1.2*) [35]. Both share the upregulation by low nitrate in the media, and activity for NT in the millimolar range when expressed in oocytes [35]. However, BnNRT1.2 is able to transport L-histidine with similar efficiency to nitrate and, in contrast to AtNRT1;1, does not transport chlorate [31, 35].

Nrt2 genes have been identified in fungi [17, 27], the alga *Chlamydomonas* [28, 36], and the plants barley [29], tobacco [37], *Arabidopsis* [15, 38] and soybean [39] (table 1). The *Arabidopsis* mutant *chl8* is defective in the cHANT, but the corresponding locus does not map to known *Nrt2* genes [40]. *Nrt2* genes have also been cloned in bacteria [41, 42]. NARK from *E. coli* appears to mediate the electrogenic nitrite export involved in anaerobic nitrate respiration [41], whereas NRTP from a marine cyanobacterium, *Synechococcus*, has an assimilatory function [42] (see also article by Moir and Wood).

In the eukaryotic green alga *Chlamydomonas*, nitrate assimilation genes are found in two gene clusters (fig. 1). A cluster of about 45 kb contains, among structural genes for nitrate (*Nia1*) and nitrite (*Nii1*) reduction, *Nrt2* genes for nitrate/nitrite transport [28, 43, 44]. A second cluster of about 15 kb contains a third *Nrt2* gene and a nitrate-regulated gene [36] which encodes a mitochondrial alternative oxidase [I. Gómez, A. Quesada and E. Fernández, unpublished]. Clustering of nitrate assimilation genes was previously reported in *A. nidulans* [45], and more recently in *H. polymorpha* [27] and *Arabidopsis* [38], which might represent a cell strategy to make the regulation of this important pathway efficient.

Molecular and physiological studies on mutant strains defective in several of the nitrate-clustered genes allow identification of four high-affinity nitrate/nitrite transporters in *Chlamydomonas* (fig. 1). System I corresponds to a bispecific HANT/HANiT encoded by *Nrt2;1*/*Nar2*, system II to a monospecific HANT encoded by *Nrt2;2*/*Nar2* [28, 46], system III to a bispecific HANiT and LANT, encoded by *Nrt2;3* [22, 36, 47], and system IV to a bispecific HANT/HANiT probably encoded by *Nrt2;4* [22, 47]. The existence of an *Nrt2;4* gene is based on its functional characteristics and on Northern and Southern blot hybridization analysis with *Nrt2;3* probes, but it needs to be further substantiated (fig. 1).

These transport systems are differentially regulated by the carbon and nitrogen source. Systems I, II and III are optimally expressed at high CO_2, and their activity is blocked by ammonium, whereas system IV is expressed optimally under limiting CO_2 and its activity is not inhibited by ammonium. In contrast to systems I, II and III, system IV activity is inhibited by CO_2, chloride and a chloride channel inhibitor [46, 47]. Concerning the function for each of these systems, mutants deleted in systems I and II and carrying functional systems III and IV are unable to grow efficiently in nitrate media [28].

Table 1. Properties of some nitrate/nitrite transporters from eukaryotes.

Gene name (other name)	Species	Substrate	Upregulation	Feedback repression	Function	References
Nrt1 Family						
AtNRT1.1(CHL1)	*A. thaliana*	NO_3^-, ClO_3^-	NO_3^-/low pH	No	HANT/LANT	16, 21
AtNRT1.2 (NTL1)	*A. thaliana*	NO_3^-	No	?	LANT	26
BnNRT1;2	*B. napus*	NO_3^-/his	NO_3^-	?	LANT	35
LeNRT1;1	*L. esculentum*	?	No	?	?	34
LeNRT1;2	*L. esculentum*	?	NO_3^-	?	?	34
Nrt2 Family						
AtNRT2;1	*Arabidopsis*	NO_3^-	NO_3^-	Yes	HANT	15, 38
AtNRT2;2	*Arabidopsis*	?	NO_3^-	Yes	?	38
CrnA	*A. nidulans*	NO_3^-, NO_2^-	NO_3^-	Yes	HANT/HANiT	17
CrNrt2;1 (Nar3)	*C. reinhardtii*	NO_3^-, NO_2^-	NO_3^-	Yes	HANT/HANiT	28, 46, 51
CrNrt2;2 (Nar4)	*C. reinhardtii*	NO_3^-	NO_3^-	Yes	HANT	28, 46, 51
CrNrt2;3	*C. reinhardtii*	NO_3^-	NO_3^-	Yes	LANT/HANiT	36, 47, 22
CrNrt2;4?	*C. reinhardtii*	NO_3^-, NO_2^-	Low CO_2/NO_2^-	No	HANT/HANiT	47, 22
GmNrt2;1	*G. max*	?	NO_3^-	?	?	39
HvNrt2;1 (BCH1)	*H. vulgare*	?	NO_3^-	?	?	29
HvNrt2;2 (BCH2)	*H. vulgare*	?	NO_3^-	?	?	29
NpNrt2;1	*N. plumbaginifolia*	?	NO_3^-	Yes	?	37
HpYNT1	*H. polymorpha*	NO_3^-	NO_3^-	Yes	HANT	27

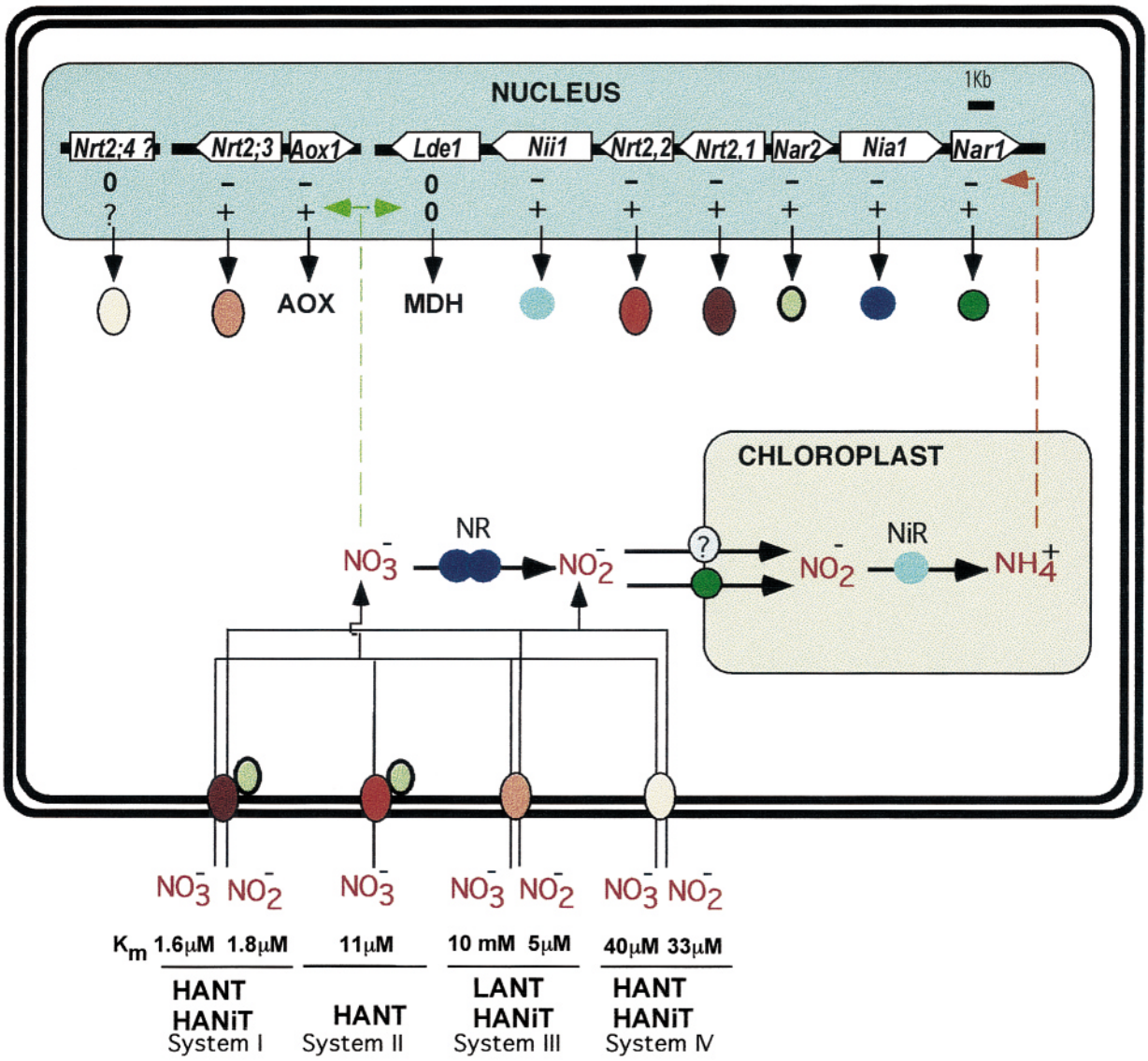

Figure 1. Scheme for the nitrate/nitrite transport systems in *Chlamydomonas*. Genes encoding transport systems I and II and the nitrite transporter to the chloroplast NAR1 are clustered in chromosome IX together with the NR and NiR genes. Location of the *Nrt2;3-Aox1* gene cluster and the putative *Nrt2;4* gene is unknown. Positive regulation by nitrate (+) and negative by ammonium (−) or no effect (0) is shown for each gene.

Thus, under sufficient CO_2, systems I and II have a primary function in the provision of nitrate for growth, and systems I and III in nitrite entry. Finally, system IV would allow nitrate and nitrite transport under limiting CO_2, even in the presence of ammonium. Under these conditions, ammonium cannot be incorporated efficiently into carbon skeletons, and nitrate and nitrite entry in the cells at amounts higher than those capable of being assimilated results in the excretion of ammonium [48, 49]. This activity of system IV might provide the cells with an alternative way to dissipate excess reducing power generated photosynthetically.

Deletion mutants lacking *Nar2*, *Nrt2;1* and *Nrt2;2* genes and having a functional *Nia1* gene recovered HANT activity and growth in nitrate when transformed with plasmids containing either *Nar2* + *Nrt2;1* or *Nar2* + *Nrt2;2*, but not with any of these genes alone [28]. Thus, systems I and II were proposed to require two protein components for activity [28, 46]. In addition, *Xenopus* oocytes injected with either *CrNrt2* messenger RNA

(mRNA) or *CrNar2* mRNA do not result in NT activity [50]. However, a HANT activity was measured in oocytes injected with the mixture of *CrNrt2;1* + *CrNar2* mRNAs, which is consistent with the participation of the two correponding proteins in the appropriate makeup of the transporter, as previously proposed. CrNAR2 appears to have a single transmembrane domain and might correspond to a membrane protein type Ia [P. L. Lefebvre, personal communication], which could interact with CrNRT2;1 to modify its function. The pH dependence of the nitrate-elicited currents by CrNRT2;1 + CrNAR2 expressed in oocytes is consistent with a H^+-cotransport mechanism [50]. Thus, the CrNRT2;1 and CrNRT2;2 proteins would be the first example of MFS proteins requiring two components for their functionality.

Nitrate at micromolar concentrations positively signals the expression of *CrNrt2;1*, *CrNrt2;2* and *CrNrt2;3*, *NpNrt2;1*, and *AtNrt2;1* [14, 15, 47, 51], and a negative feedback regulation by N-derivative metabolites from assimilated nitrate has also been reported for these genes [5, 14–16, 51]. In *Arabidopsis*, expression of *Nrt2;1* and *Nrt1;1* are also diurnally regulated in photosynthetically active plants, so that sucrose prevents the inhibition of the expression in the dark [16]. This regulation might be the result of the fluctuations of intracellular nitrate and reduced C compounds, which signals the pathway [16]. An scheme of characterized NT systems in *Arabidopsis* is shown in figure 2.

A regulatory role has been assigned to nitrate reductase (NR) itself as proposed in fungi, where mutations in nitrite reductase (NiR) or NT genes do not lead to the overexpression of nitrate assimilation genes observed in NR mutants [52, 53]. In algae and plants, NR mutants also overexpress NR, NiR and HANT gene transcripts, without the requirement of a positive signal of nitrate [51, 54]. NiR mutants from tobacco, produced by an antisense strategy [55], show a similar overexpression pattern to NR mutants, which suggests that the absence of reduced N metabolites is responsible for the observed effects [55]. However, in *Chlamydomonas* as in fungi, no overexpression of nitrate assimilation genes was found in NiR mutants, thus pointing to NR activity as the cause of the deregulation in NR mutants [22]. *Chlamydomonas* strains carrying the NR *Nia1* gene promoter transcriptionally fused to the algal arylsulfatase gene has been used to study this regulation by NR itself [56, A. Llamas, M. I. Igeño, E. Fernández and A. Galván, unpublished]. It has been demonstrated that the reason for deregulation in NR mutants is the activity of system I, which is able to scavenge submicromolar concentrations of nitrate present in N-free media and thus to keep

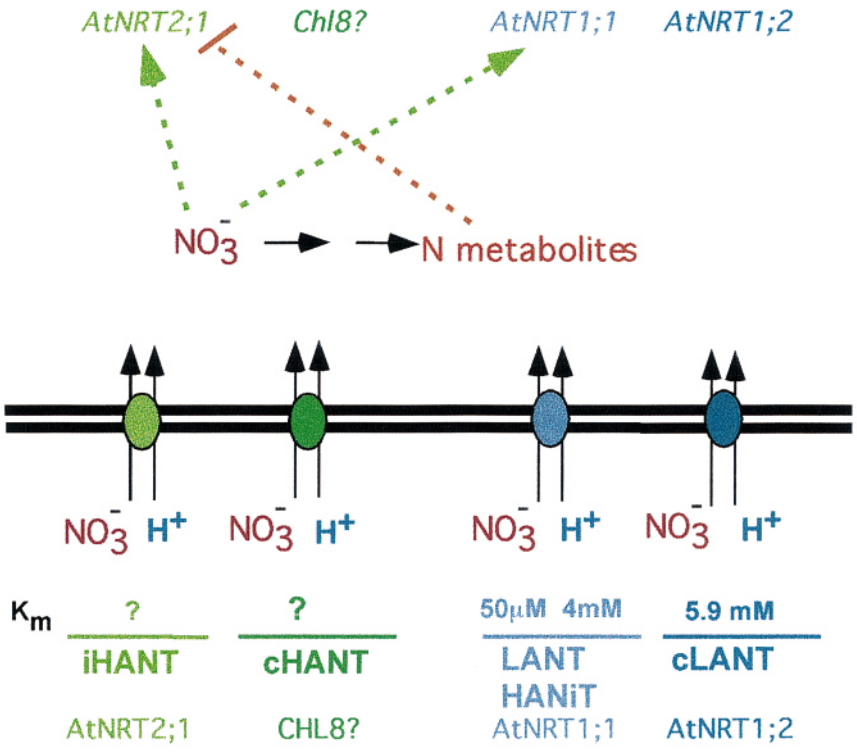

Figure 2. Scheme for functionality and regulation of some nitrate transport systems in *Arabidopsis*. Details are given in the text.

the NR promoter activity switched on [A. Llamas, M. I. Igeño, E. Fernández and A. Galván, unpublished]. The positive nitrate signaling on the NR promoter was also shown to depend directly on the activity of the NT systems.

Negative regulation of the *Nrt2* gene expression by NR activity is also evident in strains constitutively expressing the *Nia1* gene. Thus, expression of the *Nia1* gene under the *CabII-1* gene promoter in *Chlamydomonas* results in very low amounts of *Nrt2;1* and *Nar2* transcripts, which might be the result of a decrease in intracellular nitrate together with an increase in intracellular nitrite [57]. In agreement with this interpretation, transgenic *Nicotiana plumbaginifolia* plants carrying the *35S-Nia1* gene have a nitrate content in leaves significantly lower than normal plants [58]. A modulation by nitrite produced from nitrate and affecting expression of nitrate transporters has also been suggested [16, 22, 59, 60].

In addition to transcriptional regulation, ammonium has a immediate and strong negative effect on the activity of systems I, II and III from *Chlamydomonas* [46, 47]. The molecular mechanism of this regulation is unknown.

Chlorate resistance and nitrate/nitrite transporters

Chlorate as an analog of nitrate can be reduced by NR to produce chlorite, which is toxic for cells. Thus, mutants incapable of taking up chlorate or deficient in NR activity could be selected in chlorate media. This strategy has been widely used in fungi, algae and plants, and the mutants generated in this way were used to study the nitrate assimilation pathway [13, 52, 61]. Among mutants deficient in the structural genes of NR, genes for the biosynthesis of the molybdenum cofactor, essential for NR assembly and activity, and regulatory genes, mutants deficient in NT were also obtained in fungi [18], algae [43, 47] and plants [26, 30, 40].

Whether or not NR is responsible for chlorate toxicity has been evaluated by using *Chlamydomonas* mutants deficient in NR activity [62]. It has been shown that chlorate becomes toxic and causes mutagenesis in the cells by a process dependent on its transport and independent of NR activity [62]. Recently, *Chlamydomonas* strains deleted in genes for NR and HAN/NiT systems I and II, but possessing the HANiT system III, were found to be resistant to chlorate. However, activation of system III by a nitrate signal causes chlorate sensitivity [47], which supports the view that chlorate toxicity is independent of its reduction by NR in cells. Notwithstanding, it cannot be ruled out that minor amounts of chlorite present in the chlorate solution account for the observed effects. In fact, chlorite concentrations 10 times lower than chlorate produce toxicity in *Chlamydomonas* [A. Galván and E. Fernández, unpublished].

Chlorate transport mediated by a nitrate transporter is well established for the AtNRT1;1 system [12, 31], but not well documented for the NRT2 systems. In tomato roots, chlorate has been shown not to be a useful analog for HANT, which is much more selective for nitrate, though nitrate interferes with root chlorate uptake [63]. Expression of *AnCrnA* mRNA in oocytes shows that this bispecific nitrate/nitrite transporter cannot transport chlorate, though chlorite is efficiently transported [8]. These data suggest that nitrate transporters each with different specificity towards chlorate and chlorite might determine cell toxicity, depending on their expression under the experimental conditions.

Regulatory genes

In plants, regulation of NR, NiR and HANT gene expression is coordinately regulated with respect to the nitrogen source, the intracellular amounts of reduced-nitrogen compounds, light, hormones and carbon status [1, 7, 14, 61]. Identification of genes directly involved in regulation of the nitrate pathway is well characterized in fungi [64] but scarce in plants. These aspects have been reviewed recently [6, 8].

In *Chlamydomonas*, expression of nitrate assimilation genes is coregulated (fig. 1). These genes are subject to repression by ammonium, induction by nitrate and control of the positive-acting regulatory gene *Nit2* [13, 51]. *Nit2* has been cloned and shown to be subject to ammonium repression, which implies an additional level of control [65]. A second regulatory locus, closely linked to *Nit2*, is also required for expression of the *Nrt2;3* and possibly other nitrate-inducible genes [47].

The regulatory mechanisms in algae appear to be different from those in fungi [64]. In fact, at least two pathway-specific genes, *Nrg1* and *Nrg2*, mediate ammonium repression of NT and reduction genes [66]. Mutant strains defective in *Nrg* genes are partially insensitive to ammonium and their phenotype is additive, so that the threshold concentration of insensitivity to ammonium is increased in the double mutant. The existence of several genes mediating positive effects of nitrate and negative effects of ammonium, whose deficiency leads to partial phenotypes, might explain the difficulties in the genetic dissection of the regulation both in algae and plants.

Nitrite transport to the chloroplast

The nitrite transport step to the chloroplast has not been sufficiently studied, probably because there exists a general belief that nitrite can diffuse freely as nitrous

acid to the chloroplast compartment, and thus there is no need for a plastidic transporter [67]. However, the nitrite uptake in intact pea chloroplasts shows a saturation kinetics, a preference for alkaline pH and a sensitivity to protein modifiers, which favors the existence of a nitrite-mediated channel or transporter versus the permeation of the nitrous acid [68, 69]. Nitrite transport into pea chloroplasts has been reevaluated by determining fluorometrically H^+-linked transport of nitrite [70]. A rate of H^+-linked nitrite transport much higher than the measured NiR activity in chloroplasts would imply that a nitrite transporter is not needed in illuminated chloroplasts [70]. The nitrite concentrations used in these experiments are at the millimolar range, more than five times higher than those estimated for cytosolic nitrite in shoots [71]. In addition, nitrite concentrations have been shown to change significantly in roots of barley seedlings, depending on the nitrate and nitrite availability in the environment [72], and in spinach leaves during light-dark transitions [73]. These fluctuations in cytosolic concentrations of nitrite, which should be maintained low because of its cellular toxicity, might make inefficient the nitrite reduction step if it depended on a nonregulated nitrite diffusion. Thus, molecular characterization of the plastidic nitrite transport is needed.

In *Chlamydomonas*, the *Nar1* gene, clustered with other nitrate assimilation genes (fig. 1), encodes an integral protein of the chloroplast membranes, as deduced from the sequence of its putative transit peptide and immunodetection of NAR1 in the chloroplast envelope membranes with specific antibodies [74]. NAR1 sequence analysis predicts a protein with six transmembrane-spanning segments and significant homology to putative nitrite transport proteins from bacteria [75]. In isolated chloroplasts from *Chlamydomonas* strains, the expression of *Nar1* corresponds with HANiT activity (K_m in the μM range) which operates at basic pH. NAR1 is also required for optimal cell growth under conditions of limiting nitrate. In addition to NAR1, other plastidic nitrite transporters seem to operate in *Chlamydomonas* [74]. Similarly to plasma membrane nitrate/nitrite transport, the picture of nitrite transport to the chloroplast also appears to be complex.

Concluding remarks

Eukaryotic cells have provided themselves with many NT systems at the plasma membrane corresponding to two gene families, *Nrt1* and *Nrt2*. The redundancy of these systems in a single organism favors the multiplicity of functions needed to account for a regulated NT under changing environmental conditions, and for different requirements in plant tissues. Understanding the complex network of these transporters would require future efforts in i) elucidating the particular functions and regulation for each of the putative nitrate transporters identified, ii) molecular identification of chloroplast nitrite transporters in plants: would they correspond to the *Chlamydomonas Nar1* gene? iii) post-translational regulation of nitrate transporters which modify their activity according to nutritional and environmental conditions, and iv) how nitrate and N metabolites are sensed. Is nitrate sensed extracellularly? Which are the protein factors that transduce the positive and negative signals?

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1 Crawford N. M. (1995) Nitrate: nutrient and signal for plant growth. Plant Cell **7:** 859–868
2 Zhang H. and Forde B. G. (1998) An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. Science **279:** 407–409
3 Scheible W. R., González-Fontes A., Lauerer M., Muller-Rober B., Caboche M. and Stitt M. (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell **9:** 1–17
4 Scheible W. R., Lauerer M., Schulze E. D., Caboche M. and Stitt M. (1997) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. Plant J. **11:** 671–691
5 Pozuelo M., Merchán F., Macías M. I., Beck C. F., Galván A. and Fernández E. (2000) The negative effect of nitrate on gametogenesis is independent of nitrate assimilation in *Chlamydomonas reinhardtii*. Planta **211:** 287–292
6 Daniel-Vedele F., Filleur S. and Caboche M. (1998) Nitrate transport: a key step in nitrate assimilation. Curr. Opin. Plant Biol. **1:** 235–239
7 Crawford N. M. and Glass A. D. M. (1998) Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci. **3:** 389–395
8 Forde B. G. (2000) Nitrate transporters in plants: structure, function and regulation. Biochim. Biophys. Acta **1465:** 219–235
9 McClure P. R., Kochian L. V., Spanswick R. M. and Shaff J. E. (1990) Evidence for cotransport of nitrate and proton in maize roots. I. Effect of nitrate on the membrane potential. Plant Physiol. **93:** 281–289
10 Glass A. D. M., Shaff J. E. and Kochian L. V. (1992) Studies of the uptake of nitrate in barley. IV. Electrophysiology. Plant Physiol. **99:** 456–463
11 Aslam M., Travis R. L. and Huffaker R. C. (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley seedlings. Plant Physiol. **99:** 1124–1133
12 Tsay Y. F., Schroeder J. I., Feldman K. A. and Crawford N. M. (1993) A herbicide sensitive gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. Cell **72:** 705–713
13 Fernández E., Galván A. and Quesada A. (1998) Nitrogen assimilation and its regulation. In: Molecular Biology of *Chlamydomonas*: Chloroplast and Mitochondria, pp. 637–659, Rochaix J. D., Goldschmidt-Clermont M., Merchant S. (eds), Kluwer, Dordrecht

14 Krapp A., Fraisier V., Scheible W.-R., Quesada A., Gojon A., Stitt M. et al. (1998) Expression studies of *Nrt2:1Np*, a putative high affinity nitrate transporter: evidence for its role in nitrate uptake. Plant J. **14:** 723–731
15 Filleur S. and Daniel-Vedele F. (1999) Expression analysis of high-affinity nitrate transporter isolated from *Arabidopsis thaliana* by differential display. Planta **207:** 461–469
16 Lejay L., Tillard P., Lepetit M., Olive F. D., Filleur S., Daniel-Vedele F. et al. (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of *Arabidopsis* plants. Plant J. **18:** 1–11
17 Unkles S. E., Hawker K. L., Grieve C., Campbell E. I., Montague P. and Kinghorn J. R. (1991) *CrnA* encodes a nitrate transporter in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA **88:** 204–208
18 Brownlee A. G. and Arst H. N. (1983) Nitrate uptake in *Aspergillus nidulans* and involvement of the third gene of the nitrate assimilation gene cluster. J. Bacteriol. **155:** 1138–1146
19 Caboche M., Campbell W., Crawford N. M., Fernández E., Kleinhorf A., Ida S. et al. (1994) Genes involved in nitrate assimilation. Plant Mol. Biol. Rep. **12:** 45–49
20 Wang R., Liu D. and Crawford N. M. (1998) The *Arabidopsis* CHL1 protein plays a major role in high-affinity nitrate uptake. Proc. Natl. Acad. Sci. USA **95:** 15134–15139
21 Liu K. H., Huang C. Y. and Tsay Y. F. (1999) CHL1 is a dual affinity nitrate transporter of *Arabidopsis* involved in a multiple phases of nitrate uptake. Plant Cell **11:** 865–874
22 Navarro M. T., Guerra E., Fernández E. and Galván A. (2000) Nitrite reductase mutants as an approach to understanding nitrite assimilation in *Chlamydomonas reinhardtii*. Plant Physiol. **122:** 283–289
23 Pao S. S., Paulsen I. T. and Saier M. H. (1998) Major Facilitator Superfamily. Microbiol. Mol. Biol. Rev. **62:** 1–34
24 Trueman L. J., Onyeocha I. and Forde B. G. (1996) Recent advances in the molecular biology of a family of eukaryotic high affinity nitrate transporters. Plant Physiol. Biochem. **34:** 621–627
25 Paulsen I. T. and Skurray R. A (1994) The POT family of transport proteins. Trends Biochem. Sci. **18:** 404
26 Huang N. C., Liu K. H., Lo H. J. and Tsay Y. F. (1999) Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell **11:** 1381–1392
27 Pérez M. D., González C., Avila J., Brito N. and Siverio J. M. (1997) The YNT1 gene encoding the nitrate transporter in the yeast *Hansenula polymorpha* is clustered with genes *YNI1* and *YNR1* encoding nitrite reductase and nitrate reductase, and its disruption causes inability to grow in nitrate. Biochem. J. **321:** 397–403
28 Quesada A., Galván A. and Fernández E. (1994) Identificacion of nitrate transporters in *Chlamydomonas reinhardtii*. Plant J. **5:** 407–419
29 Trueman L. J., Richardson A. and Forde B. G. (1996) Molecular cloning of higher plants homologues of the high affinity nitrate transporters of *Chlamydomonas reinhardtii* and *Aspergillus nidulans*. Gene **175:** 223–231
30 Doddema H. and Telkamp G. P. (1979) Uptake of nitrate by mutants of *Arabidopsis thaliana* disrupted in uptake or reduction of nitrate II. Kinetics. Physiol. Plant. **45:** 332–338
31 Touraine B. and Glass A. D. M. (1997) NO_3^- and ClO_3^- fluxes in the *chl1-5* mutant of *Arabidopsis thaliana*. Plant Physiol. **114:** 137–144
32 Frommer W. B., Hummel S. and Rentsch D. (1994) Cloning of an *Arabidopsis* histidine transporting protein related to nitrate and peptide transporters. FEBS Lett. **347:** 185–189
33 Hatzfeld Y. and Saito K. (1999) Identification of two putative nitrate transporters highly homologous to CHL1 from *Arabidopsis* (Accession Nos. AJ011604 and AJ131464). Plant Physiol. **119:** 805–806
34 Lauter F. R., Ninnemann O., Bucher M., Riesmeier J. W. and Frommer W. B. (1996) Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc. Natl. Acad. USA **93:** 8139–8144
35 Zhou J., Theodoulou F. L., Muldin I., Ingemarsson B. and Miller A. S. (1998) Cloning and functional characterization of a *Brassica napus* transporter that is able to transport nitrate and histidine. J. Biol. Chem. **273:** 12017–12023
36 Quesada A., Hidalgo J. and Fernández E. (1998) Three *Ntr2* genes are differentially regulated in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. **258:** 373–377
37 Quesada A., Krapp A., Trueman L. J., Daniel-Vedele F., Fernández E., Forde B. G. et al. (1997) PCR-identification of a *Nicotiana plumbaginifolia* cDNA homologous to the high affinity nitrate transporters of the *crnA* family. Plant Mol. Biol. **34:** 265–274
38 Zhuo D., Okamoto M., Vidmar J. J. and Glass A. D. M. (1999) Regulation of a putative high affinity nitrate transporter (*Nrt2.1At*) of *Arabidopsis thaliana*. Plant J. **17:** 563–568
39 Amarasinghe B. H. R. R., Ranamalie B. H. R., de Bruxeles G. L., Braddom M., Onyeocha I., Forde B. G. et al. (1998) Regulation of *GmNRT2* expression and nitrate transport activity in roots of soybean (*Glycine max*). Planta **206:** 44–52
40 Wang R. and Crawford N. M. (1996) Genetic identification of a gene involved in constitutive, high-affinity nitrate transport in higher plants. Proc. Natl. Acad. Sci. USA **20:** 9297–9301
41 Rowe J. J., Ubbink-Kok T., Molenaar D., Konings W. N. and Driessen A. J. (1994) NarK is a nitrite-extrussion system involved in anaerobic nitrate respiration by *Escherichia coli*. Mol. Microbiol. **12:** 579–586
42 Sakamoto T., Inoue-Sakamoto K. and Bryant D. A. (1999) A novel nitrate/nitrite permease in the marine cyanobacterium *Synechococcus* sp. Strain PCC 7002. J. Bacteriol. **181:** 7363–7372
43 Quesada A., Galván A., Schnell R., Lefebvre P. A. and Fernández E. (1993) Five nitrate assimilation related loci are clustered in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. **240:** 387–394
44 Quesada A., Gómez I. and Fernández E. (1998) Clustering of the nitrite reductase gene and a light-regulated gene with nitrate assimilation loci in *Chlamydomonas reinhardtii*. Planta **206:** 259–265
45 Johnstone I. L., McCabe P. C., Greaves P., Gurr S. J., Cole G. E., Brow M. A. D. et al. (1990) Isolation and characterizarion of the *crnA-niiA-niaD* gene cluster for nitrate assimilation in *Aspergillus nidulans*. Gene **90:** 181–192
46 Galván A., Quesada A. and Fernández E. (1996) Nitrate and nitrite are transported by different specific transport systems and by a bispecific transporter in *Chlamydomonas reinhardtii*. J. Biol. Chem. **271:** 2088–2092
47 Rexach J., Montero B., Fernández E. and Galván A. (1999) Differential regulation of the high affinity nitrite transport systems III and IV: in *Chlamydomonas reinhardtii*. J. Biol. Chem. **274:** 27801–27806
48 Azuara M. P. and Aparicio P. (1983) In vivo blue-light activation of *Chlamydomonas reinhardtii* nitrate reductase. Plant Physiol. **71:** 286–290
49 Quiñones M. A., Galván A., Fernández E. and Aparicio P. J. (1999) Blue light requirement for the biosynthesis of a NO_2^- transport system in the *Chlamydomonas reinhardtii* nitrate transport mutants S10. Plant Cell Environ. **22:** 1169–1175
50 Zhou J. J., Fernández E., Galván A. and Miller A. J. (2000) A high affinity nitrate transport system from *Chlamydomonas* requires two gene products. FEBS Lett. **466:** 225–227
51 Quesada A. and Fernández E. (1994) Expression of nitrate assimilation related genes in *Chlamydomonas reinhardtii*. Plant Mol. Biol. **24:** 185–194
52 Cove D. J. (1979) Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. **54:** 291–327
53 Hawker K. L., Montague P. and Kinghorn J. R. (1992) Nitrate reductase and nitrite reductase transcript levels in various mutants of *Aspergillus nidulans* confirmation of autogenous regulation. Mol. Gen. Genet. **23:** 485–488
54 Pouteau S., Chèrel I., Vaucheret H. and Caboche M. (1989) Nitrate reductase mRNA regulation in *N. plumbaginifolia* nitrate reductase-deficient mutants. Plant Cell **1:** 1111–1120

55 Vaucheret H., Kronenberger J., Lépingle A., Vilaine F., Boutin J. P. and Caboche M. (1992) Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. Plant J. **2:** 559–569
56 Loppes R., Ohresser M., Radoux M and Matagne R. F. (1999) Transcriptional regulation of the *Nit1* gene encoding nitrate reductase in *Chlamydomonas reinhardtii*: effect of various enviromental factors on the expression of a reporter gene under the control of the *Nit1* promoter. Plant Mol. Biol. **41:** 701–711
57 Navarro M. T., Prieto R., Fernández E. and Galván A. (1996) Constitutive expression of nitrate reductase changes the regulation of nitrate and nitrite transporters in *Chlamydomonas reinhardtii*. Plant J. **9:** 819–827
58 Quilleré I., Dufossé C., Rouox Y., Foyer C. H. and Caboche M. (1994) The effect of deregulation of NR gene expression on growth and nitrogen metabolism of *Nicotiana plumbaginifolia* plants. J. Exp. Bot. **45:** 1205–1211
59 King B. J., Siddiqi Y. M., Ruth T., Warner R. L. and Glass A. M. D. (1993) Feedback regulation of nitrate influx in barley roots by nitrate, nitrite and ammonium. Plant Physiol. **102:** 1279–1286
60 Navarro M. T. (1998) Desarollo de estrategias moleculares para el estudio de la asimilación de nitrato en algas, PhD thesis dissertation, University of Córdoba
61 Hoff T., Truong H. N. and Caboche M. (1994) The use of mutants and transgenic plants to study nitrate assimilation. Plant Cell Environ. **17:** 489–506
62 Prieto R. and Fernández E. (1993) Toxicity and mutagenesis by chlorate are independent of nitrate reductase activity in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. **237:** 429–438
63 Kosola K. R. and Bloom A. J. (1996) Chlorate as a transport analogue for nitrate absorption by roots of tomato. Plant Physiol. **110:** 1293–1299
64 Marzluf G. A. (1997) Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. **61:** 17–32
65 Schnell R. A. and Lefebvre P. A. (1993) Isolation of the *Chlamydomonas* regulatory gene *nit2* by transposon tagging. Genetics **134:** 737–747
66 Prieto R., Dubus A., Galván A. and Fernández E. (1996) Isolation and characterization of two regulatory mutants for nitrate assimilation in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. **251:** 461–471
67 Enser U. and Heber U. (1980) Metabolic regulation by pH gradient: inhibition of photosynthesis by indirect proton transfer across the chloroplast envelope. Biochim. Biophys. Acta **592:** 577–591
68 Brunswick P. and Cresswell C. F. (1988) Nitrite uptake into intact pea chloroplasts. I. Kinetics and relationship with nitrite assimilation. Plant Physiol. **86:** 378–383
69 Brunswick P. and Cresswell C. F. (1988) Nitrite uptake into intact pea chloroplasts. II. Influence of electron transport regulators, uncouplers, ATPase and anion uptake inhibitors and protein binding reagents. Plant Physiol. **86:** 384–389
70 Shingles R., Roh M. H. and McCarty R. E. (1996) Nitrite transport in chloroplasts inner envelope vesicles. I. Direct measurement of proton-linked transport. Plant Physiol. **112:** 1375–1381
71 Siddiqi M. Y., King B. J. and Glass A. D. M. (1992) Effects of nitrite, chlorate and chlorite on nitrate uptake and nitrate reductase activity. Plant Physiol. **100:** 644–650
72 Aslam M., Travis R. L. and Huffaker R. C. (1993) Comparative induction of nitrate and nitrite uptake and reduction systems by ambient nitrate and nitrite in intact roots of barley (*Hordeum vulgare* L.) seedlings. Plant Physiol. **102:** 811–819
73 Riens B. and Heldt W. (1992) Decrease of nitrate reductase activity in spinach leaves during a light-dark transition. Plant Physiol. **98:** 573–577
74 Rexach J., Fernández E. and Galván A. (2000) The *Chlamydomonas reinhardtii Nar1* gene encodes a chloroplast protein involved in nitrite transport. Plant Cell **12:** 1441–1454
75 Peakman T., Crouzet J., Mayaux J. F., Busby S., Mohan S., Harborne N. et al. (1990) Nucleotide sequence, organisation and structural analysis of the products of genes in the nirB-cysG region of the *Escherichia coli* K-12 chromosome. Eur. J. Biochem. **191:** 315–323