Differential Regulation of the High Affinity Nitrite Transport Systems III and IV in *Chlamydomonas reinhardtii*

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Two high affinity nitrite transporters have been identified in *Chlamydomonas reinhardtii*. They have been named system III and system IV and shown to be differentially regulated by nitrogen and carbon supply. System III was induced under high CO₂ and required a micromolar nitrate signal for optimal expression, was inhibited by ammonium, and was not affected by either chloride or the chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino)benzoic acid. System IV was induced optimally under limiting CO₂ and did not require nitrate signal, was inhibited by chloride and 5-nitro-2-(3-phenylpropylamino)benzoic acid, but was not affected by ammonium. Two transcripts that shared the expression pattern of systems III and IV activities were detected with an Nrt2;3 gene probe. In addition, a mutant defective in both the activity of system III and the expression of Nrt2;3 gene has been isolated. Genetic crosses and in vivo complementation studies indicate that this mutant is defective in a locus that is closely linked to the regulatory gene NIT2.

The first and key step of the nitrate assimilation pathway is the entry of nitrate into the cells mediated by specific transporters (1, 2). Then, the sequential reduction of nitrate to nitrite, and nitrite to ammonium takes place in steps catalyzed by the enzymes nitrate reductase (NR) and nitrite reductase (NiR), respectively (3–5).

NR and NiR genes are single copy in many algae, fungi, and plants (2, 6, 7). However, physiological and molecular data support the existence of redundant nitrate transporters (1, 2). On the basis of nitrate affinity and expression conditions, transporters have been classified into constitutive or inducible high affinity transporters (CHANT, IHANT), and constitutive or inducible low affinity nitrite transporters (CLANT, ILANT). Molecular cloning of nitrate transporters from fungi, algae, and plants allows to classify them into two families (NRT1 and NRT2), according to sequence similarities (1). NRT1 belongs to the peptide transporter superfamily, and members of this family transport either nitrate or histidine with comparable efficiency (8). The Nrt2 genes belong to the major facilitator superfamily (1, 9).

The Chl1 gene from *Arabidopsis* is the first member identified in the NRT1 family, which was primarily proposed to encode for a LANT regulated at the transcriptional level by nitrate and acid pH (10). Inducible LANT Chl1 analogues have also been identified in tomato, LeNrt1;2, whose substrate specificity is unknown (11), and *Brassica napus*, BnNrt1;2 which can transport nitrate and basic amino acids (8). Other members of the NRT1 family such as the Arabidopsis AtNrt1 (NTL1) and the tomato LeNrt1;1 are expressed constitutively (12), so they could correspond to the CLANT.

Most of the Nrt2 gene family show a nitrate inducible expression: CrnA from *Aspergillus* (13); Nrt2;1, Nrt2;2, and Nrt2;3 from *Chlamydomonas reinhardtii* (14, 15); HoNrt2A from barley (9); AtNrt2;1 and AtNrt2;2 from *Arabidopsis* (1); GmNrt2 from soybean (16); NpNrt2;1 from *Nicotiana* (17); and YNT1 from *Hansenula* (18). Concerning the specificity and affinity for the NRT2 family transporters, CrnA, YNT1, CrNrT2, HoNrt2A and HoNrt2B have been shown to be IHANT (1). The *C. reinhardtii* Nrt2;1 is the only bisppecific high affinity nitrate/nitrite transporter reported until now (19). An *Arabidopsis* chlorate-resistant mutant called Chl8 has been identified and shown to be defective in CHANT (20). The Chl8 gene has not been cloned yet, and does not appear to map to the known Nrt genes from *Arabidopsis* (1).

Nitrate assimilation is a highly regulated pathway in which nutritional and environmental conditions are determinant for metabolic adaptation. In this context, transporters should play an important role in regulating the amount of nitrate going into the cell according to its capability to assimilate or accumulate it. To know in a single organism each of the transporters, its specificity, and its regulation is a challenge to understand the nitrate assimilation pathway.

In *C. reinhardtii*, two HANT have been identified. The bisppecific HANT/IHANT, named system I and encoded by Nrt2;1 and Nar2, and the specific HANT system II, encoded by Nrt2;2 and Nar2. Both are two-component systems which require for functionality a NAR2 protein (19, 21). A third gene Nrt2;3 has recently been cloned, but its function remains to be demonstrated. Since deletion mutants lacking system I and II are able to assimilate efficiently nitrate, it has been proposed that Nrt2;3 could be responsible for nitrate transport (15).

In this work, two HANT systems have been identified and shown to be differentially expressed depending on the nitrogen and carbon availability. Mutants deficient in nitrite transport activity and expression of the Nrt2;3 gene have been obtained. The possible regulatory role for the locus defective in one of these mutants is discussed.

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The abbreviations used are: NR, nitrate reductase; NiR, nitrite reductase; Chl, chlorophyll; kb, kilobase(s); NPPB, [5-nitro-2-(3-phenylpropylamino)-benzoic acid]; HANT, high affinity nitrite transporter; IHANT, high affinity nitrite transporter; CHANT, constitutive high affinity transporters; IHANT, inducible high affinity transporters; LANT, low affinity nitrite transporter; CLANT, constitutive low affinity transporter; ILANT, inducible low affinity transporter; Nit−, not growth in nitrate media.

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**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—C. reinhardtii wild type 6145c (mt−), Nit2 mutant 203 (mt−), and deletion mutant D2 (mt− ΔNrt2;2, Nrt2;1, Nar2, Nia1, Nar1) have been described elsewhere (14, 22).

Cells were routinely grown at 25 °C under continuous light and 5% CO2-enriched (v/v) air in minimal HS medium containing 7.5 mM ammonium chloride (22). Cells were collected at mid-exponential phase of growth by centrifugation (4000 × g, 5 min), washed twice with 50 mM potassium phosphate, pH 7.0, and then transferred to the indicated induction medium. Cultures were bubbled either with 5% CO2-enriched (v/v) air or with air washed through a saturated KOH solution. After the indicated times, cells were collected by centrifugation and processed immediately for enzyme assays, RNA extraction, or analytical determinations.

**Genetic Procedures**—Genetic crosses were carried out by the random spore plating method (23). Segregants analyzed for their ability to grow on media containing ammonium, nitrate, or nitrite. For *in vivo* complementation analysis, the mating mixture of gametes was plated in selective media containing 4 mM nitrate (24, 25).

**Enzyme Assays and Analytical Methods**—Ammonium-grown cells or cells induced in different media were transferred to media containing 100 mM nitrite, at a cell concentration of 10–30 µg Chl/ml. Samples were taken at different times, and nitrite concentration in the media was measured.

NiR activity was assayed as previously reported (26). The assay mixture contained 300 mM Tris-HCl, pH 8.0, 0.4 mM KNO₂, 0.8 mM methyl viologen, 16 mM dithionite, and toluene-permeabilized cells.

Nitrite transport was determined according to Snell and Snell (27), and phytofluor as detailed by Arnon (28).

**DNA and RNA Isolation from C. reinhardtii and Hybridization Analysis**—Isolation of genomic DNA and Southern transfer were performed as previously reported (29, 30). Conditions for hybridization were carried out according to Schloss et al. (31), and washes were performed at 65 °C, with 0.2× SSC and 0.2% SDS solution.

Isolation of total RNA was carried out according to previously reported methods (31). RNA (20 µg) was fractionated on 1.6% agarose gels containing 17.5% formaldehyde (31) and then transferred onto nylon membranes (Nytran-N2, Schleicher & Schuell) using 10× SSC buffer. Conditions for hybridization were previously reported (31), and washing was performed at 65 °C, with 0.2× SSC and 0.2% SDS solution.

Radioactive probes were labeled by the random primer method (32).

Probes used were: probe 1, a 1.1-kb KpnI-SacI fragment corresponding to the 5′ region of the Nrt2;3 gene; probe 2, a XbaI-BamHI fragment corresponding to the 3′ region of the Nrt2;3 gene; and probe 3, a KpnI-EcoRI fragment that contains the 5′ region of Nrt2;3 in addition to the Nar5 gene (Ref. 15 and Fig. 3A).

**RESULTS**

**Occurrence of Two Nitrite Transport Activities in Strain D2 from C. reinhardtii**—The C. reinhardtii strain D2 has a deletion at the Nia1 genome region including Nrt2;2, Nrt2;1, Nar2, Nia1, and Nar1 genes. This strain is not deficient at the NiR locus and assimilates nitrite efficiently (14). Thus, this strain is useful for the study of potential nitrite transporters without the background of system I, which is characteristic of the nitrate/nitrite transporters identified until now in C. reinhardtii. So, when cells are consuming either nitrate or nitrite, addition of ammonium immediately blocks their transport activities (19, 26, 34). However, 1 mM ammonium did not affect the system IV activity when added as sulfate and had a slight inhibitory effect when added as chloride. This inhibition was due to chloride, since at 10 mM amounts of either NaCl or KCl, inhibition of system IV activity was much stronger. In addition, the chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (35) inhibited system IV transport activity. In contrast, system III was slightly affected by chloride or NPPB. Systems III and IV were also regulated by CO2 in a different way. System III transport activity was partially inhibited when CO2 was removed from the medium. In contrast, system IV transport activity was blocked when cells were bubbled with 5% CO2.

Kinetic parameters were determined for both systems from the progress curves as previously reported (19, 33). System III had a *Km* for nitrite of 5 ± 2 µM and a *Vmax* of 19 ± 4 µmol nitrite/h mg Chl. System IV had a *Km* for nitrite of 33 ± 6 µM and *Vmax* of 10 ± 3 µmol nitrite/h mg Chl. The *Km* for system III fits with that previously reported in C. reinhardtii (19).

**Fig. 1. Induction of nitrite transport activities in the C. reinhardtii strain D2 under different nitrogen and carbon conditions.** Ammonium-grown cells were transferred to media containing 100 µM NO3 with nitrate at 0 (○), 10 µM (●), 50 µM (□), or 100 µM (△). Cultures were bubbled with CO2 (B), and nitrite in the media determined at the indicated times.
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Northern blot analysis was performed to study expression of the Nrt2;3 gene in strain D2. Total RNA was isolated from cells subject to different nitrogen and CO2 conditions. A KpnI-SaeI 1-kb fragment corresponding to the 5' region of Nrt2;3 gene was used as a probe (probe 1, Fig. 3A). Interestingly, this probe recognized two transcripts of 2.8 and 2.4 kb, respectively, which were differentially expressed in strain D2 (Fig. 3B). This result suggests that in addition to the Nrt2;3 transcripts (15), another gene sharing significant homology with the used probe was detected. The expression pattern of both transcripts was different. At high CO2 both transcripts were repressed in ammonium and optimally expressed when 100 µM nitrate was present in the medium. However, when CO2 was limiting the transcript of 2.8 kb was expressed under either nitrogen source, i.e. ammonium, nitrate, or without nitrogen, although optimally in nitrite (Fig. 3B). When a XbaI-BamHI 4-kb fragment corresponding to the 3' region of Nrt2;3 gene was used as a probe (probe 2, Fig. 3C), the 2.4-kb transcript was mostly observed and their expression pattern fits with that obtained with the probe 1. That the 2.4-kb transcript was optimally expressed at high CO2, required a nitrate signal, and was almost absent in nitrite medium without CO2 suggests a relationship between this transcript and system III transport activity. That the 2.8-kb transcript (named Nrt2;4) was optimally expressed under limiting CO2 in a nitrate signaling-independent manner suggests that it could correspond to that of system IV.

Southern blot analyses were performed. DNA from strain D2 was digested with SaeI, SmalI, and BamHI, and probed with the 5' region of Nrt2;3 KpnI-SaeI fragment (Fig. 4). The most intense bands corresponded to the expected sizes for the Nrt2;3 gene region (15). Additional faint bands were found in digests with the enzymes used, under high stringency conditions. The existence of these additional hybridization bands is in agreement with the presence of an Nrt2;3 analogue gene in strain D2.

Under high CO2 and 0.1 mM nitrite, both transcripts were expressed (Fig. 3B, lane 4), NIR activity was high (Fig. 2), but no nitrite transport activity was detected (Fig. 1A). This seems to indicate that nitrate and/or CO2 also have a regulatory role in the transporters at the post-transcriptional level. To gain more information, cells induced in the above medium during 5 h were processed in two ways. (a) Cells were kept with high CO2, and 0.1 mM nitrate or nitrate plus cycloheximide were added to the medium (Fig. 5A). Then, nitrite transport activity was observed only when cycloheximide was not present, and with a kinetics much faster than in an induction from ammonium-grown cells. (b) Cells were bubbled with air lacking CO2 in the presence or absence of cycloheximide, without nitrate signaling (Fig. 5B). Then, after CO2 removal, only cells without cycloheximide showed nitrite transport activity. Again, this transport activity was significantly faster than in an induction from ammonium-grown cells. These results indicate that protein synthesis is required for expression of system III and IV transport activity in response to signaling by nitrate and absence of CO2, respectively.

Isolation of Nitrite Transport Mutants from Strain D2—In C. reinhardtii, nitrate/nitrite transporters have been shown to be responsible for chlorate toxicity. Although NR has a role in mediating chlorate toxicity, the absence/presence of NR activity is not critical for chlorate resistance/toxicity (2, 36). Strain D2 is a chlorate-resistant mutant that lacks both HANT systems (I and II) and NR (14). When a functional NR is present in a D2 background, the resulting strain is also chlorate resistant (data not shown).

Since strain D2 contains other nitrite transporters, i.e. systems III and IV shown above, it could be possible to isolate nitrite transport mutants from this strain by selecting for chlorate-resistant colonies. However, strain D2 was resistant to 2 mM chlorate in medium containing a neutral nitrogen source such as urea. Notwithstanding, when 1 mM nitrate was present in the medium, strain D2 became sensitive to chlorate and spontaneous resistant colonies appeared in this medium at a ratio of about 1 × 10−6. Twenty-two chlorate-resistant mutants were analyzed, and all were defective in activities and/or kinetic parameters of nitrite transport. Only one was stable, and its phenotype was consistent with that of a nitrite transport mutant. This strain named DC2-III had the following characteristics. (i) It did not grow in nitrite medium despite having NIr activity (data not shown). (ii) It lacked nitrite transport activity at high CO2 (system III) (Fig. 6A). (iii) Under limiting CO2 conditions, DC2-III strain induced a nitrite transport activity (Fig. 6B) that was presumably due to system IV, since it was inhibited by chloride, strongly by CO2, but not significantly by ammonium (Fig. 6C).

Molecular and Genetic Characterization of the Nitrite Transport Mutant DC2-III—Mutant DC2-III seems to be affected in a gene(s) involved in expression of nitrite transport system III. Nit2 from C. reinhardtii is the only positive regulatory gene identified in photosynthetic eukaryotes (25, 37). Nit2 mutants have a Nit− phenotype and do not express NR or NR (37–39). The Nrt2;3 gene is clustered with another nitrate regulated gene Nar5 whose function and relationship with nitrate assimilation are to be solved out. Nar5 expression does not seem to be under the control of Nit2 (15).

Nar5 and Nrt2;3-related genes were analyzed for expression in the strain DC2-III. Total RNA was isolated from ammonium-grown or nitrate-induced cells for 3 h, under high CO2 conditions. A KpnI-EcoRI fragment, which contains fragments of Nrt2;3 and Nar5 genes (Fig. 3A; Ref. 15), was used as a probe. Results shown in Fig. 7 indicate that: (i) in contrast to the parental strain D2, both the 2.8-kb and 2.4-kb transcripts were almost undetectable in the strain DC2-III; and (ii) Nar5 expression was significantly decreased in DC2-III strain, when compared with that in the parental strain D2.

Since phenotype of mutant DC2-III was similar to that of Nit2 mutants, genetic analysis by in vivo complementation and genetic crosses was performed between these two strains. Diploid strains 203 (Nit2−) X DC2-III were able to grow in nitrate or nitrite-containing media. In addition, the genetic cross between strain 203 (Nit2−) and mutant DC2-III was analyzed from the growth of segregants in ammonium, and nitrate- or nitrite-containing media. The frequency of Nit+/Nit− and Nit+/Niit− segregants was 1:100, and 4:100, respectively. These results indicate that DC2-III was not defective at the Nit2 gene but in a locus that is closely linked to Nit2.
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The nitrite transport activity of systems III and IV were induced for 3 h in nitrate media and high CO2 and in nitrite media without CO2, respectively. Then, the nitrite transport activity was determined as indicated under “Experimental Procedures” by transfer of cells to fresh media containing 100 μM NaNO2 and the indicated compounds in the presence (+) or absence (-) of CO2. 100% activity corresponded to 16.6 ± 3.3 and 5.2 ± 1.1 μmol NO2/h mg Chl for systems III and IV, respectively.

| Condition          | System III NIT activity | System IV NIT activity |
|--------------------|-------------------------|------------------------|
| + CO2              | 100 ± 20                | - CO2                  |
| + CO2 + 1 mM NH4Cl | 3.0 ± 1.1               | - CO2 + 1 mM NH4Cl     |
| + CO2 + 0.5 mM (NH4)2SO4 | 1.6 ± 1.6              | - CO2 + 0.5 mM (NH4)2SO4 |
| + CO2 + 10 mM NaCl | 85.0 ± 0.2              | - CO2 + 10 mM NaCl     |
| + CO2 + 10 mM KCl  | 89.1 ± 1.0              | - CO2 + 10 mM KCl      |
| + CO2 + 100 μM NPPB| 88.4 ± 1.3              | - CO2 + 100 μM NPPB    |
| - CO2              | 49.0 ± 4.6              | + CO2                  |

**DISCUSSION**

In this work, the existence of two HANiT systems (III and IV), in addition to the previously reported bispecific HANT/S gene probe 1 indicated in Fig. 3. The nitrogen-transport activity of systems III and IV were regulated differentially by nitrogen and carbon signaling. Expression of system III transport activity occurs at limiting high CO2, and its activity is blocked by ammonium. In contrast, expression of system IV transport activity occurs at limiting CO2 independently of signaling by nitrate. System IV activity is not inhibited by ammonium, but it is blocked by CO2, and strongly inhibited by chloride, or the chloride channel inhibitor NPPB. Two transcripts of 2.4 and 2.8 kb were detected from strain D2 in Northern blots at high stringency, and by using Nrt2;3 probes. The 2.4-kb transcript corresponded to the Nrt2;3 gene, whose previously reported transcript size (15) has been reevaluated with probes corresponding to either 5’ or 3’ regions of the gene. The 2.8-kb transcript was mostly detected with a probe from the 5’ region of Nrt2;3. Sequence analysis of 5’ region shows that Nrt2;3 is a member of the Nrt2 gene family (15), so the 2.8-kb transcript could correspond to a fourth gene.
of this family in \textit{C. reinhardtii}, named \textit{Nrt2;4}. The existence of a \textit{Nrt2;4} gene was also supported by the Southern blot data.

Systems III and IV could be related with \textit{Nrt2;3} and \textit{Nrt2;4} genes, respectively, since their corresponding transport activities show a similar expression pattern to each of these transcripts. The expression of the 2.4-kb/\textit{Nrt2;3} transcript was clearly dependent on the micromolar nitrate signal, meanwhile the expression of the 2.8-kb/\textit{Nrt2;4} transcript occurs when CO$_2$ was limiting, even in the presence of ammonium. In addition, in nitrite-containing medium at limiting CO$_2$, where system IV activity is operative, the only transcript hybridizing to the \textit{Nrt2;3} probe was \textit{Nrt2;4}.

The \textit{C. reinhardtii} systems III and IV are HANiT with $K_a$ in the micromolar range. The characterization of \textit{C. reinhardtii} NiR mutants has shown that there exist a LANT activity with a $K_a$ for nitrate in the millimolar range which appears to correspond to system III.\textsuperscript{2} In addition, system IV in these NiR mutants shows a HANT activity with a $K_a$ for nitrate in the micromolar range.\textsuperscript{2} No HANT has been reported in higher plants until now. However, in \textit{C. reinhardtii}, it can be summarized that the nitrate and nitrite transport systems correspond to the bispecific HANT/HANiT systems I and IV; and the specific HANT system II, and HANiT system III (Ref. 19 and this work). Systems I and II are essential in \textit{C. reinhardtii} to support cell growth in nitrate-containing media (19, 21). \textit{C. reinhardtii} strains lacking systems I and II but having system III and IV are unable to grow in nitrate media, even though nitrate at low concentrations could be transported by system IV or at higher concentrations by system III (Refs. 14 and 19 and this work). Thus, the precise function of these systems III and IV in nitrate assimilation, other than transporting nitrite, remains to be solved. However, a regulatory role in controlling intracellular nitrate concentrations, according to nutritional and environmental conditions, could be suggested.

The nitrite transporter mutant DC2-III seems to be deficient in a locus, closely linked to \textit{Nit2}, and involved in the nitrate signaling required for expression of system III/\textit{Nrt2;3}. The selection strategy used to obtain this type of mutant is in agreement with this assumption. Parental strain D2 is chlorate-resistant in a neutral medium such as urea, since systems I and II are lacking (14), and systems III and IV are not operative under the high CO$_2$ conditions used for cell growth and selection. System IV is blocked by CO$_2$, and system III requires a nitrate signal for optimal expression. Thus, by providing a nitrate signal, the NR-deficient D2 strain became sensitive to chlorate, which indicates that chlorate entry by system III was responsible for cell toxicity. The phenotype of mutant strain DC2-III, \textit{i.e.} the absence of system III transport activity, the undetectable \textit{Nrt2;3} gene expression, and the significant decrease in the nitrate-dependent \textit{Nar5} gene expression, suggests that this strain is defective in nitrate signaling. Several hypotheses could explain these results. Mutant DC2-III could be defective in a regulatory gene mediating nitrate signaling by two different routes: one dependent on \textit{Nit2}, which would switch on expression of nitrate assimilation genes (2, 37), and another independent of \textit{Nit2}, which would switch on \textit{Nar5} gene expression (15). Alternatively, the mutation could have affected the \textit{Nrt2;3} gene itself and thus the activity of system III, which would be responsible for nitrate signaling. In this context, it has been observed that the presence or absence of specific transporters in \textit{C. reinhardtii} give different nitrate/nitrite signaling effects.\textsuperscript{3}

Interestingly, genetic data showed that mutant DC2-III is defective in a locus closely linked to \textit{Nit2}. Clustering of nitrate assimilation genes is appearing as a common feature in \textit{C. reinhardtii} and might represent a strategy for efficiency in regulatory common mechanisms under very changeable environmental conditions.

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