Nitrite Reductase Mutants as an Approach to Understanding Nitrate Assimilation in Chlamydomonas reinhardtii

María Teresa Navarro, Elena Guerra, Emilio Fernández*, and Aurora Galván

Departamento de Bioquímica y Biología Molecular and Instituto Andaluz de Biotecnología, Avda. San Alberto Magno, Facultad de Ciencias, Universidad de Córdoba, 14071–Córdoba, Spain

We constructed mutant strains lacking the nitrite reductase (NR) gene in Chlamydomonas reinhardtii. Two types of NR mutants were obtained, which either have or lack the high-affinity nitrate transporter (Nrt2;1, Nrt2;2, and Nar2) genes. None of these mutants overexpressed nitrate assimilation gene transcripts nor NR activity in nitrogen-free medium, in contrast to NR mutants. This finding confirms the previous role proposed for NR on its own regulation (autoregulation) and on the other genes for nitrate assimilation in C. reinhardtii. In addition, the NR mutants were used to study nitrate transporters from nitrite excretion. At high CO2, only strains carrying the above high-affinity nitrate transporter genes excreted stoichiometric amounts of nitrate from 100 μM nitrate in the medium. A double mutant, deficient in both the high-affinity nitrate transporter genes and NR, excreted nitrate at high CO2 only when nitrate was present at mM concentrations. This suggests that there exists a low-affinity nitrate transporter that might correspond to the nitrate/nitrite transport system III. Moreover, under low CO2 conditions, the double mutant excreted nitrate from nitrate at micromolar concentrations by a transporter with the properties of the nitrate/nitrite transport system IV.

In Chlamydomonas reinhardtii, at least four transporters are involved in the control of the nitrate or nitrite entry into the cell (Quesada et al., 1994; Galván et al., 1996; Fernández et al., 1998; Rexach et al., 1999). These transporters, named systems I, II, III, and IV, have been shown to have the following characteristics. System I is a bi-specific, high-affinity nitrate/nitrite transporter (HANT/HANiT) encoded by the Nrt2;1 and Nar2 genes (Quesada et al., 1994; Galván et al., 1996). System II is a specific HANT encoded by the Nrt2;2 and Nar2 genes (Quesada et al., 1994; Galván et al., 1996). System III is a HANiT that seems to be encoded by the Nrt2;3 gene (Quesada et al., 1998b; Rexach et al., 1999). System IV has been proposed to be a HANiT encoded by Nrt2;4, a fourth member of the Nrt2 gene family in C. reinhardtii (Rexach et al., 1999). These transporters are differentially regulated by the carbon and nitrogen supply. Systems I, II, and III are expressed optimally at high CO2 and blocked by ammonium, whereas system IV is expressed optimally under limiting CO2 and is not inhibited by ammonium (Galván et al., 1996; Rexach et al., 1999). Concerning the function for each of these transporters, the HANT-deficient mutants carrying systems III and IV are only complemented for nitrate growth and transport with the systems I or II (Quesada et al., 1994). Therefore, systems I and II have a primary function in the efficient entry of nitrate for growth, however, systems III and IV require further studies to understand their function.

The expression of nitrate assimilation genes (NiaI encoding nitrate reductase [NR], NiiI encoding nitrite reductase [NiR], and those for HANT) is co-regulated. In C. reinhardtii, these genes are subject to repression by ammonium, induction by nitrate and the control of the regulatory gene Nii2 (Quesada and Fernández, 1994; Fernández et al., 1998). In plants, the 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 the carbon status (Hoff et al., 1994; Crawford, 1995; Crawford and Glass, 1998; Krapp et al., 1998). In fungi, algae, and plants, mutants defective in the NR structural gene or in genes for the molybdopterin cofactor of NR overexpress NR, NiR, and HANT gene transcripts without the requirement for a positive signal of nitrate (Cove, 1979; Fu and Marzluf, 1988; Pouteau et al., 1989; Fauré et al., 1991; Galván et al., 1992; Hawker et al., 1992; Quesada and Fernández, 1994). A regulatory role of NR by itself was proposed in fungi, where mutations in NiR or nitrate transporter genes do not lead to the overexpression of nitrate assimilation (NA) genes observed in NR mutants (Cove, 1979; Fu and Marzluf, 1988; Hawker et al., 1992). NiR mutants have also been obtained from barley (Duncanson et al., 1993) and tobacco (Vaucheret et al., 1992). The tobacco NiR-deficient strains produced by an antisense strategy show a similar overexpression pattern as NR mutants, which has led to the proposal that the absence of reduced nitrogen compounds is responsible for the observed effects (Vaucheret et al., 1992).

In the present study, NiR mutants from C. reinhardtii have been constructed to address two points: (a) whether the blocking of the NA pathway at the level of nitrite reduction causes the same overexpression of NA genes as in plants, to provide answers to the regulatory role proposed for NR; and (b) whether NiR mutants could be used as a tool to study nitrate transporters.

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* Corresponding author; e-mail bb1feree@uco.es; fax 34–957–218606 (218591).
MATERIALS AND METHODS

Strains and Growth Conditions

The Chlamydomonas reinhardtii strains used were: wild-type 6145c; the Nia1- mutant strain 305; the mutant strain S10, which contains a functional copy of the NR gene but is deleted in the nitrate transporter genes Nar2, Nrt2;1, and Nrt2;2; the mutant strain 04-1, which has been obtained by transformation of S10 with the nitrate transporter genes Nar2, Nrt2;1, and Nrt2;2; and the NiR mutant strains F6 and G1, which have a deletion on the nitrate assimilation cluster (mt+ ac17, sr-1, Δ(Nar1, Nia1, Nar2, Nrt2;1, Nrt2;2, and Nii1)). All of these strains have been described and characterized elsewhere (Quesada et al., 1993, 1994, 1998a; Fernández et al., 1998).

Cells were grown at 25°C under continuous light in minimal liquid medium containing 7.5 mM ammonium chloride, with 5% (v/v) CO2-enriched air (Sueoka et al., 1978). All of these strains have been described and characterized elsewhere (Quesada et al., 1993, 1994, 1998a; Fernández et al., 1998).

Genetic Crosses

Genetic crosses were carried out by the random spore plating method according to the method of Levine and Ebersold (1960).

Preparation of Extracts, Enzyme Assays, and Immunodetection in Protein Blots

C. reinhardtii extracts were prepared by freezing and thawing in a 50 mM Tris-Cl buffer, pH 7.5, as previously reported (Fernández and Cárdenas, 1982). Reduced benzyl viologen (BVH) NR was determined in situ in 1 mL of cell culture permeabilized with 20 μL of toluene (Florencio and Vega, 1983), by determining nitrite enzymatically produced from nitrate and using BV chemically reduced with dithionite as an electron donor under previously reported conditions (Paneque et al., 1965). NiR activity was assayed according to previously reported methods (Galván et al., 1992) using reduced methyl viologen as an electron donor. SDS-PAGE was carried out as described by Laemmli (1970), using the low molecular weight protein markers from Sigma Chemical (St. Louis). Electrophoretic transfer of protein gels to nitrocellulose (0.45 μm) filters was carried out in a Tris (3 g/L)-Gly (14 g/L) buffer containing 20% (v/v) methanol, at 75 V, 4°C, during 3 h. Fd-NiR was detected by using a polyclonal anti-Fd-NiR antibody (Pajuelo et al., 1993), kindly supplied by Drs. E. Pajuelo and A. Márquez (University of Sevilla, Spain) and peroxidase-conjugated secondary antibody (Sigma).

DNA and RNA Isolation and Hybridization Analysis

Total RNA isolation, electrophoretic fractionation, and hybridizations were carried out according to previously reported methods (Schloss et al., 1984; Sambrook et al., 1989). Probes used were: B6a-6 to detect Nrt2;1 and Nar2 transcripts (Quesada et al., 1993), B6a-5.1 to detect Nia1 (Navarro et al., 1996), and the Nrt2;2 cDNA 1.1-kb insert (Quesada et al., 1994).

Analytical Methods

Nitrate was determined by HPLC as previously reported (Quesada et al., 1994). Nitrite was determined routinely according to the method of Snell and Snell (1949), chlorophyll as in Arnon (1949), and protein according to the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Construction of NiR Mutants from C. reinhardtii

We obtained mutants defective in NiR from a genetic cross between strain G1, having a deletion of the nitrate gene cluster (Nar1, Nia1, Nar2, Nrt2;1, Nrt2;2, and Nii1) (Quesada et al., 1993, 1998a), and strain 04-1 (Fig. 1A), which bears functional genes for NR (Nia1) and nitrate transporters (Nrt2;1, Nrt2;2, and Nar2) heterologously integrated (Quesada et al., 1994). Strain 04-1 is partially de-
ated in the NiaI genomic region and maintains a functional NiR gene Nii1 (Quesada et al., 1994). Genes encoding the NR, the HANT systems I and II, and the NiR segregated independently in the cross G1 × 04-1 (Fig. 1A). Segregation of this cross was analyzed from the growth of segregants in medium containing 2 mM nitrite or 4 mM nitrate, and corresponded to 55:45 Nii−:Nii+ and 16:84 Nit−:Nit+, where Nii and Nit represent growth in nitrite and nitrate media, respectively. Seven strains incapable of growing in both nitrate and nitrite media (Nit−Nii−) were randomly selected. Four of them (M1, M2, M3, and M4) showed NR activity and lacked NiR activity after incubation of cells in nitrate medium, and were selected for further analysis.

The presence of HANT genes (Nrt2;1, Nrt2;2, and Nar2) in strains M1, M2, M3, and M4 was determined from the analysis of transcript expression in RNA blots from cells induced in nitrate medium. As shown in Figure 1B, strains M1, M2, and M3 expressed the HANT transcripts corresponding to Nrt2;1 and Nar2. A transcript of about 1 kb, appearing in the blots below that of Nar2, corresponded to a nonfunctional and truncated Nrt2;1 gene (having about half of the coding sequence). This transcript is expressed from the integrated plasmid pB6a bearing the unlinked copy of the Nar2 gene (results not shown).

The presence/absence of NiR protein in these M mutants and parental strains was analyzed by immunodetection on nitrocellulose filters after transfer from SDS gels, and using anti-ferredoxin (Fd)-NiR antibody (Fig. 1C). This polyclonal antibody reacts specifically with the 63-kD NiR protein (Pajuelo et al., 1993). Crude extracts were obtained from nitrate-induced cells of parental strains 04-1 and G1, the NiR mutant F6, which has a genomic reorganization in the Nii1 region (Quesada et al., 1998a), and strains M1, M2, M3, and M4. Only the parental strain 04-1 showed NiR immunoprecipitate. According to the above data, strains M1, M2, and M3 appear to be NiR mutants, which have functional copies of the NR and HANT genes, and strain M4 a mutant deficient in both NiR and HANT I and II.

Transcript Levels in the Wild Type and Mutants Deficient in NR, NiR, or HANT I and II

The expression patterns for nitrate induction or derepression in nitrogen-free media of NA gene transcripts (Nia1, Nrt2;1, Nrt2;2, and Nar2) were analyzed in mutants affected at different levels of the route. Three strains were used as controls: the wild-type strain 6145c, the NR mutant 305cw15, which lacks functional NR and shows a deregulated expression of NA genes (Fernández and Cárdenas, 1982; Galván et al., 1992; Quesada and Fernández, 1994), and the strain S10, which has NR but lacks the HANT systems I and II (Quesada et al., 1994). As shown in Figure 2, after 1.5 h in N-free medium, only the NR mutant 305cw15 showed overexpression of Nia1, Nrt2;1, Nrt2;2, and Nar2 transcripts compared with the other strains. Wild-type cells showed much lower amounts of these transcripts than the NR mutant, in agreement with previously reported data (Quesada and Fernández, 1994). Almost undetectable amounts of these transcripts were present in the NiR mutants M1 and M2 after 1.5 h in N-free media. The NR transcript was also not expressed significantly in strains S10 (NiR−) and M4 (NiR−) in these N-free media. When induction of NA genes was performed in medium containing 100 μM nitrate, all strains analyzed expressed comparable amounts of Nia1 transcripts (Fig. 2A) and overexpression of NA transcripts was not observed in the NiR mutants.

NR Activity in Wild Type, NR Mutants, NiR Mutants, and Mutants Lacking HANT I and II

NR activity was also determined in these mutant strains defective in different steps of the NA pathway. Cells were grown in ammonium medium and then transferred to either nitrogen-free or nitrate-containing medium bubbled with CO2-enriched air to induce NR activity (Table I). As reported, the NR mutant 305cw15 overexpressed terminal NR activity in nitrogen-free medium (Fernández and Cárdenas, 1982; Galván et al., 1992). However, all other mutants with functional NR showed low levels of BVH-NR in nitrogen-free medium, and this activity was significantly increased by the presence of nitrate in the medium. By comparing the NiR mutants that bear the HANT I and II (strains M1, M2, and M3) with the NiR mutant M4, which lacks these transporters, significant differences were observed. Therefore, the NiR mutants having the HANT systems I and II responded to micromolar concentrations of nitrate to induce significant levels of BVH-NR activity, whereas the NiR mutant M4 required nitrate at millimolar
CO2, whereas system IV is operative at limiting CO2 (Rexach et al., 1999). Thus, system III is operative at high HANIT, but they are differentially regulated by nitrogen (Quesada et al., 1998b; Rexach et al., 1999). Both are proposed to be encoded by \textbf{Nrt2;3}.

\textbf{C. reinhardtii} and II. Nitrite accumulation within the NiR mutant cells did not take up nitrate at this concentration nor excrete metric excretion of nitrite to the media. However, strain M4 strains M1, M2, and M3 were able to take up nitrate at these nitrate from the medium at micromolar concentrations, but air) (Rexach et al., 1999). The wild-type cells consumed characterized in the strain D2 deleted in the \textit{Nar2} genes (Rexach et al., 1999). Systems III and IV are proposed to be encoded by \textit{Nrt2;3} and \textit{Nrt2;4}, respectively (Quesada et al., 1998; Rexach et al., 1999). Both are HANIT, but they are differentially regulated by nitrogen and carbon conditions. Thus, system III is operative at high CO2, whereas system IV is operative at limiting CO2 (Rexach et al., 1999). To determine whether systems III and IV were also able to transport nitrate, mutant M4 was analyzed for nitrite excretion activity from nitrate under conditions in which which system III or system IV were operative. System III was induced in strain M4 by incubation of cells in medium containing 4 mM nitrate at high CO2, then cells were transferred to medium containing different nitrate concentrations and the nitrite excretion activity evaluated. As shown in Figure 4, strain M4 did not excrete nitrite from nitrate at a 100 mM concentration, but excreted significant amounts of nitrite when nitrate in the medium was above 1 mM (1–40 mM). These amounts were comparable to those excreted by mutants M1, M2, and M3 (data not shown). At 40 mM nitrate, nitrite excretion activity was maximum (Fig. 4), and a \( K_m \) of 10 mM nitrate was estimated for this transporter. These results indicate that there exists a low-affinity nitrate transporter (LANT) in \textit{C. reinhardtii}.

M4 cells were also treated to induce system IV activity (Fig. 5). Cells were induced in the presence of 4 mM nitrate but under low CO2, then transferred to fresh media containing different nitrate concentrations and low CO2, and the nitrite excretion activity determined. Under these conditions, the M4 strain started to excrete nitrite from 25 \( \mu \)M nitrate in the medium, had a maximum activity at 1 mM nitrate, and concentrations higher than 10 mM had an inhibitory effect. A \( K_m \) of 40 \( \mu \)M was estimated for this transporter.

Nitrite excretion from micromolar nitrate under limiting CO2 conditions in strain M4 indicated that a HANT that could correspond to system IV was present in these cells. Nitrite transport activity of system IV has been reported not to be affected by ammonium, but inhibited by chloride and CO2 (Rexach et al., 1999). Therefore, the effect of ammonium, CO2 and chloride on the HANT activity in M4 strain was analyzed. As shown in Figure 6, the nitrite excretion activity from 100 \( \mu \)M nitrate under limiting CO2 conditions was almost unaffected by 1 mM ammonium, was inhibited significantly by 10 mM of either NaCl or KCl, and was inhibited strongly by 4% to 5% CO2-enriched air.

### Table I. NR activity in wild-type and mutant strains from \textit{C. reinhardtii} in nitrogen-free and nitrate media

<table>
<thead>
<tr>
<th>Strain</th>
<th>BVH-NR Activity (milliunits/mg chlorophyll)</th>
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<tbody>
<tr>
<td></td>
<td>(-N)</td>
</tr>
<tr>
<td>M1</td>
<td>4.3 + 4.1</td>
</tr>
<tr>
<td>M2</td>
<td>1.3 + 1.6</td>
</tr>
<tr>
<td>M3</td>
<td>6.0 + 7.0</td>
</tr>
<tr>
<td>M4</td>
<td>2.3 + 1.4</td>
</tr>
<tr>
<td>S10</td>
<td>4.0 + 3.3</td>
</tr>
<tr>
<td>305cw15</td>
<td>180 + 59</td>
</tr>
<tr>
<td>6145c (wild type)</td>
<td>68 + 35</td>
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Nitrite Mutants as a Tool to Study Nitrate Transporters

Nitrite mutants were used as a tool to study the nitrate transporters in \textit{C. reinhardtii} by measuring nitrite excreted to the media. \textit{C. reinhardtii} wild-type cells and NiR mutants were grown in ammonium medium and transferred to medium containing 100 \( \mu \)M nitrate, and cells were maintained in conditions in which transporter systems I, II, and III were operative (i.e. bubbling cells with CO2-enriched air) (Rexach et al., 1999). The wild-type cells consumed nitrate from the medium at micromolar concentrations, but no nitrite excretion was observed (Fig. 3). The NiR mutant strains M1, M2, and M3 were able to take up nitrate at these micromolar concentrations, which resulted in a stoichiometric excretion of nitrite to the media. However, strain M4 did not take up nitrate at this concentration nor excrete nitrite, as expected from its deficiency in HANT systems I and II. Nitrite accumulation within the NiR mutant cells was not detected (data not shown).

Nitrate/nitrite transport systems different from systems I and II have recently been shown in \textit{C. reinhardtii} and named systems III and IV. They have been identified and characterized in the strain D2 deleted in the \textit{Nrt2;1}, \textit{Nrt2;2}, and \textit{Nar2} genes (Rexach et al., 1999). Systems III and IV are proposed to be encoded by \textit{Nrt2;3} and \textit{Nrt2;4}, respectively (Quesada et al., 1998; Rexach et al., 1999). Both are HANIT, but they are differentially regulated by nitrogen and carbon conditions. Thus, system III is operative at high CO2, whereas system IV is operative at limiting CO2 (Rexach et al., 1999). To determine whether systems III and IV were also able to transport nitrate, mutant M4 was analyzed for nitrite excretion activity from nitrate under conditions in which either system III or system IV were operative. System III was induced in strain M4 by incubation of cells in medium containing 4 mM nitrate at high CO2, then cells were transferred to medium containing different nitrate concentrations and the nitrite excretion activity evaluated. As shown in Figure 4, strain M4 did not excrete nitrite from nitrate at a 100 mM concentration, but excreted significant amounts of nitrite when nitrate in the medium was above 1 mM (1–40 mM). These amounts were comparable to those excreted by mutants M1, M2, and M3 (data not shown). At 40 mM nitrate, nitrite excretion activity was maximum (Fig. 4), and a \( K_m \) of 10 mM nitrate was estimated for this transporter. These results indicate that there exists a low-affinity nitrate transporter (LANT) in \textit{C. reinhardtii}.

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DISCUSSION

The use of mutants defective in different steps of the nitrate assimilation pathway is a powerful tool to understand functional and regulatory aspects for different steps of this route (Cove, 1979; Hoff et al., 1994; Fernández et al., 1998). In the green alga Chlamydomonas reinhardtii, mutant strains defective at different levels of the NA pathway have been isolated and characterized, but none defective in the nitrite reduction step has been studied up to now. In this work, two kinds of NiR mutants have been constructed: strains M1, M2, and M3, which are only deficient in the NiR gene, and a double mutant, M4, which lacks both NiR (Nii1) and HANT (Nrt2;1, Nrt2;2, and Nar2) genes. The characterization of these NiR mutants has allowed us to: (a) confirm the regulatory role proposed for the NR enzyme in C. reinhardtii, and (b) show that these C. reinhardtii mutants can be used as a strategy to study nitrate transporters, suggesting that the HANiT systems III and IV correspond to a LANT and a HANT, respectively.

The regulatory role of NR was primarily proposed to account for the up-regulated NA gene expression in NR mutants from fungi, plants, and algae (Cove, 1979; Fu and Marzluf, 1988; Pouteau et al., 1989; Fauré et al., 1991; Galván et al., 1992; Hawker et al., 1992; Quesada and Fernández, 1994). In plants, the absence of ammonium-derived metabolites is considered to be responsible for this deregulation. Thus, in tobacco plants, the blocking of the NA pathway at the level of NiR by expressing an antisense Nii1 cDNA (Vaucheret et al., 1992) results in overexpression of the Nia1 gene, similar to the NR mutants (Pouteau et al., 1989). It has been proposed that Gln is the regulatory metabolite, since: (a) Gln levels show an inverse correlation with NR amounts along the circadian rhythm (Deng et al., 1991); (b) Gln synthetase inhibition by phosphinotricine prevents the decrease of NR mRNA during the diurnal phase (Deng et al., 1991); and (c) Gln treatment results in a decrease in the NR apoprotein (Shiraishi et al., 1992).

None of the C. reinhardtii NiR mutants showed the deregulation pattern for NA gene expression found in NR mutants in nitrogen-free medium (Fernández and Cardeñas, 1982; Galván et al., 1992; Quesada and Fernández, 1994). In contrast, the NiR mutants required nitrate for

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**Figure 4.** Nitrite excretion activity under high-CO2 conditions by the NiR mutant strain M4. Cells from strain M4 were induced under 4% to 5% CO2 in medium containing 4 mM nitrate for 4 h. Then cells were transferred to medium containing different nitrate concentrations from 0.1 to 40 mM (○, 0.1 mM; □, 1.0 mM; ■, 2.0 mM; □, 5 mM; ▲, 10 mM; △, 20 mM; and ◇, 40 mM) and kept bubbling with 4% to 5% CO2. A, The nitrite concentration in the medium was determined at the indicated times. B, The nitrite excretion rate activity was calculated and represented as a function of the initial nitrate concentration. Chl, Chlorophyll.

**Figure 5.** Nitrite excretion activity under limiting CO2 conditions by the NiR mutant strain M4. Strain M4 was induced in media containing 4 mM nitrate for 4 h in cultures bubbled with air filtered through a CO2 trap. Then cells were transferred to media containing different nitrate concentrations from 25 μM to 40 mM (▼, 25 μM; ▼, 50 μM; ○, 100 μM; □, 1.0 mM; □, 5 mM; ▲, 10 mM; ◇, 40 mM)) and kept under limiting CO2 conditions. A, The nitrite concentration excreted to the media was determined at the indicated times. B, The nitrite excretion rate activity was calculated and represented as a function of the initial nitrate concentration. Chl, Chlorophyll.
The medium was determined at the indicated times.

mM KCl (5, and transferred to medium containing 100 mM nitrate, plus 0.5 mM ammonium sulfate (Fernández et al., 1998). Since expression of a constitutive and functional NR in *C. reinhardtii* (Navarro et al., 1996) causes regulatory effects contrary to those of a mutant NR, we propose that the functionality of NR might be the key for the observed effects through the modification of nitrate/nitrite concentrations.

The NiR mutants were also used as a strategy for nitrate transporter studies. These *C. reinhardtii* NiR mutants excreted nitrite when incubated in nitrate medium, and no intracellular nitrite appeared to accumulate. The efficient nitrite excretion to the medium by NiR mutants has also been reported in *Hansenula polymorpha* (Brito et al., 1996) and *Aspergillus nidulans* (Cove, 1979), in contrast to plants where nitrate accumulated intracellularly (Duncanson et al., 1993). This capability to excrete nitrite could be related to the maintenance of nitrite levels below lethal concentrations and to the existence of an efficient nitrite export system. In fact, the *C. reinhardtii* NiR mutants were viable after long periods of time in media containing nitrate or nitrite at millimolar concentrations. Since nitrite was not accumulated in the algal cells, NiR mutants are a useful tool to evaluate the activity of both HANT and LANT in *C. reinhardtii* by an easy methodology.

Under high-CO₂ conditions, cells from mutants M1, M2, and M3 took up nitrate at concentrations lower than 100 μM by the HANT encoded by Nrt2:1, Nrt2:2, and Nar2 (systems I and II), and nitrite was excreted stoichiometrically. However, in strain M4, nitrate concentrations 10- to 50-fold higher were required for a significant nitrite production and an apparent Kₛ of 10 mM nitrate was estimated. These data indicated that a LANT system that accounts for the uptake of nitrate at the millimolar range is present in *C. reinhardtii*. LANT systems have been widely described in plants (Siddiqi et al., 1990; Tsay et al., 1993) and in the alga *C. reinhardtii* (Watt et al., 1995), and could correspond to either a modified HANT or a high-affinity anion transporter, which can use nitrate inefficiently. Since M4 strain lacks the Nrt2:1, Nrt2:2, and Nar2 genes, the interference of the HANT systems I and II would not exist and the LANT activity observed in this strain could be related to system III. This transport system III has been defined as a HANiT that is essential for nitrite growth and proposed to be encoded by the Nrt2:3 gene (Rexach et al., 1999). The G1 strain and the NiR mutants derived from them express the Nrt2:3 gene (Quesada et al., 1998b).

Finally, we have shown that there exists a HANT activity in the strain M4 that is operative under limiting CO₂ conditions. The functional characteristics of this transporter fit with those reported for system IV: (a) no inhibition by ammonium, (b) inhibition by chloride, and (c) strong inhibition of the transport activity by CO₂ (Rexach et al., 1999). System IV has been defined as a HANiT and proposed to be encoded by a fourth member of the *C. reinhardtii* Nrt2 gene family (Rexach et al., 1999). The data presented here suggest that system IV could also be a HANT, but its precise function is still unknown and further studies are required to address this question. However, previous data indicate that this transporter is not sufficient to allow an optimal nitrate transport and growth (Quesada et al., 1994; Galván et al., 1996), and so it could be involved in the balance of nitrate/nitrite taken up by the cells.

![Figure 6. Effect of ammonium, chloride, and high CO₂ on the nitrite excretion activity induced under limiting CO₂ in the NiR mutant strain M4. Cells from strain M4 were induced as indicated in Figure 5, and transferred to medium containing 100 μM nitrate alone (○), plus 0.5 mM ammonium sulfate (●), plus 10 mM NaCl (▲), plus 10 mM KCl (▲), or bubbled with 4% to 5%-enriched air (□). Nitrite in the medium was determined at the indicated times.](https://www.plantphysiol.org)
NITRITE REDUCTASE MUTANTS FROM CHLAMYDOMONAS REINHARDTI

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LITERATURE CITED


