Plant Physiology, January 2000, Vol. 122, pp. 283–289, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Nitrite Reductase Mutants as an Approach to Understanding Nitrate Assimilation in *Chlamydomonas reinhardtii*[1]

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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 CO_2, only strains carrying the above high-affinity nitrate transporter genes excreted stoichiometric amounts of nitrite from 100 μM nitrate in the medium. A double mutant, deficient in both the high-affinity nitrate transporter genes and NR, excreted nitrite at high CO_2 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 CO_2 conditions, the double mutant excreted nitrite 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 CO_2 and blocked by ammonium, whereas system IV is expressed optimally under limiting CO_2 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 (*Nia1* encoding nitrate reductase [NR], *Nii1* 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 *Nit2* (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.

[1] This work was supported by the European Union Biotechnology Program as part of the Project of Technological Priority 1997–2000 (no. BIO4–CT97–2231), the Dirección General de Investigación Científica y Técnica, Spain (grant no. PB96–055 4–CO–01), and the Junta de Andalucía, Spain (Plan Andaluz de Investigación grupo CVI–0128).

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MATERIALS AND METHODS

Strains and Growth Conditions

The *Chlamydomonas reinhardtii* strains used were: wild-type 6145*c*; 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) CO_2-enriched air (Sueoka et al., 1967). Cells were collected by centrifugation at the mid-exponential phase of growth (4,000*g*, 5 min), washed twice with 50 mM potassium phosphate, pH 7.0, and then transferred to medium containing ammonium chloride (4 mM) or potassium nitrate (at the indicated concentrations) or to nitrogen-free medium. After the indicated times, cells were collected by centrifugation and processed immediately for enzyme assays, RNA extraction, or analysis.

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-HCl 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). Electrotransfer 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-

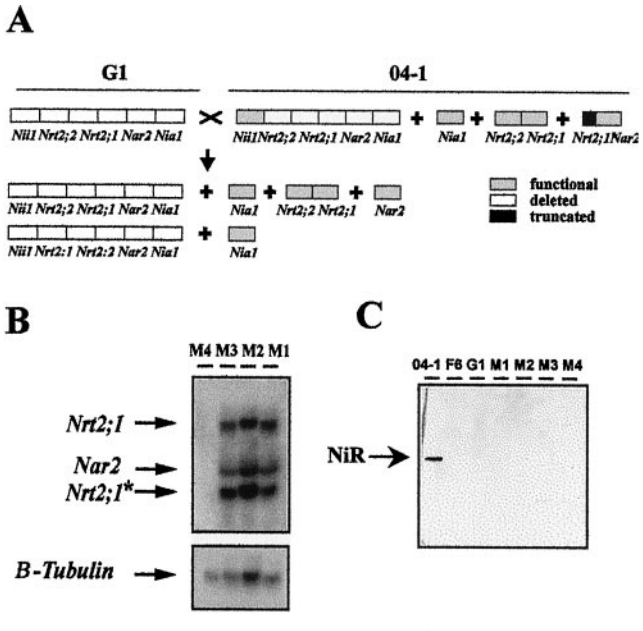

Figure 1. Strategy to obtain NiR mutants from *C. reinhardtii* by genetic cross between strains G1 and 04-1. A, Scheme of the genetic cross showing genotypes of parental strains and isolated segregants lacking NiR. Details on these strains are indicated in "Materials and Methods." B, RNA transfer analysis of NiR mutant strains induced in 4 mM nitrate medium over 3 h using DNA-specific probes for the indicated transcripts. C, Immunodetection of NiR in crude extracts (50 μg of protein) from the indicated strains induced in 4 mM nitrate medium after SDS-PAGE and using a NiR-specific antibody.

leted in the *Nia1* 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.

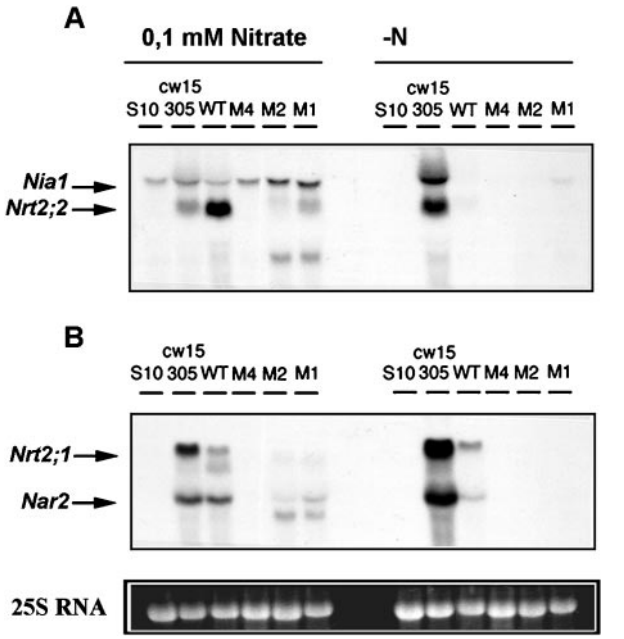

Figure 2. Expression of *Nia1*, *Nrt2;1*, *Nrt2;2*, and *Nar2* transcripts in the NiR mutants M1, M2, and M4, the NR mutant 305cw15, the HANT mutant S10, and the wild-type strain. Total RNA was extracted from the indicated strains after 1.5 h of incubation in 0.1 mM nitrate or nitrogen-free medium (−N). Total RNA (20 μg) was analyzed in RNA transfer hybridizations using the specific DNA probes indicated in "Materials and Methods" to detect *Nia1* and *Nrt2;2* transcripts (A) or these from *Nrt2;1* and *Nar2* (B).

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 CO_2-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

Table I. *NR activity in wild-type and mutant strains from C. reinhardtii in nitrogen-free and nitrate media*

BVH-NR activity of the indicated strains, grown in ammonium and incubated for 3 h in N-free (−N), 0.1 mM nitrate, or 5 mM nitrate media, was determined in situ. Data are means ± SD from four independent experiments.

Strain	BVH-NR Activity		
	−N	0.1 mM Nitrate	5 mM Nitrate
	milliunits/mg chlorophyll		
M1	4.3 + 4.1	135 + 57	170 + 64
M2	1.3 + 1.6	43 + 28	140 + 71
M3	6.0 + 7.0	132 + 52	185 + 48
M4	2.3 + 1.4	16 + 6	180 + 24
S10	4.0 + 3.3	15 + 7	83 + 18
305cw15	180 + 59	303 + 159	243 + 48
6145c (wild type)	68 + 35	98 + 38	167 + 49

concentrations to induce high levels of BVH-NR activity (Table I).

NiR Mutants as a Tool to Study Nitrate Transporters

NiR mutants were used as a tool to study the nitrate transporters in *C. reinhardtii* by measuring nitrite excreted to the media. *C. reinhardtii* wild-type cells and NiR mutants were grown in ammonium medium and transferred to medium containing 100 μM nitrate, and cells were maintained in conditions in which transporter systems I, II, and III were operative (i.e. bubbling cells with CO_2-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 *C. reinhardtii* and named systems III and IV. They have been identified and characterized in the strain D2 deleted in the *Nrt2;1*, *Nrt2;2*, and *Nar2* genes (Rexach et al., 1999). Systems III and IV are proposed to be encoded by *Nrt2;3* and *Nrt2;4*, respectively (Quesada et al., 1998b; Rexach et al., 1999). Both are HANiT, but they are differentially regulated by nitrogen and carbon conditions. Thus, system III is operative at high CO_2, whereas system IV is operative at limiting CO_2 (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 CO_2, 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 μM 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_s of 10 mM nitrate was estimated for this transporter. These results indicate that there exists a low-affinity nitrate transporter (LANT) in *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 CO_2, then transferred to fresh media containing different nitrate concentrations and low CO_2, and the nitrite excretion activity determined. Under these conditions, the M4 strain started to excrete nitrite from 25 μM nitrate in the medium, had a maximum activity at 1 mM nitrate, and concentrations higher than 10 mM had an inhibitory effect. A K_s of 40 μM was estimated for this transporter.

Nitrite excretion from micromolar nitrate under limiting CO_2 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 CO_2 (Rexach et al., 1999). Therefore, the effect of ammonium, CO_2 and chloride on the HANT activity in M4 strain was analyzed. As shown in Figure 6, the nitrite excretion activity from 100 μM nitrate under limiting CO_2 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% CO_2-enriched air.

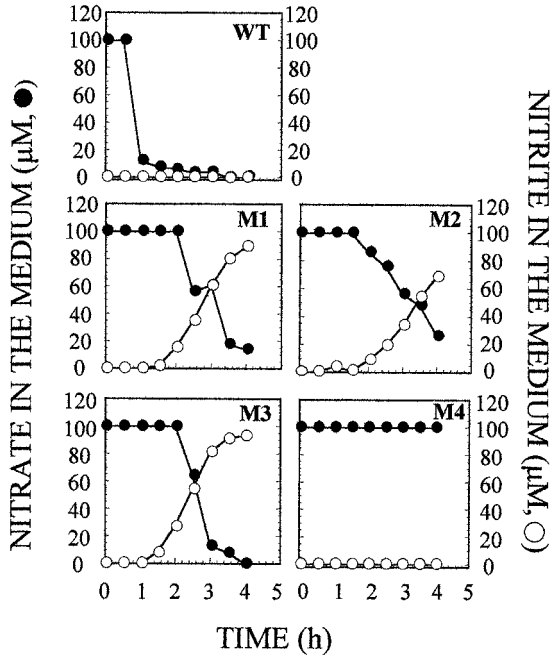

Figure 3. Nitrate uptake and nitrite excretion activity in wild-type (WT) and NiR mutant strains due to the HANT systems I and II. Strains M1, M2, M3, and M4, and the wild-type 6145c were grown in ammonium and then transferred to medium containing 100 μM nitrate at a cell concentration of about 15 to 25 μg chlorophyll/mL. The media were bubbled with CO_2-enriched air and, at the indicated times, nitrate (●) and nitrite (○) were determined.

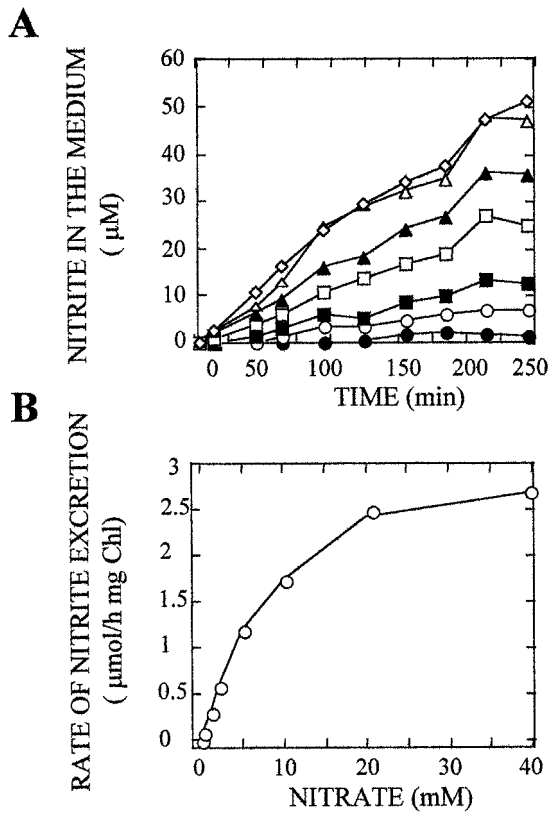

Figure 4. Nitrite excretion activity under high-CO_2 conditions by the NiR mutant strain M4. Cells from strain M4 were induced under 4% to 5% CO_2 in medium containing 4 mM nitrate for 4 h. Then cells were transferred to medium containing different nitrate concentrations from 0.1 to 40 mM (●, 0.1 mM; ○, 1.0 mM; ■, 2.0 mM; □, 5 mM; ▲, 10 mM; △, 20 mM; and ◇, 40 mM) and kept bubbling with 4% to 5% CO_2. 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.

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 *C. reinhardtii*, mutant strains deficient 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 Cárdenas, 1982; Galván et al., 1992; Quesada and Fernández, 1994). In contrast, the NiR mutants required nitrate for

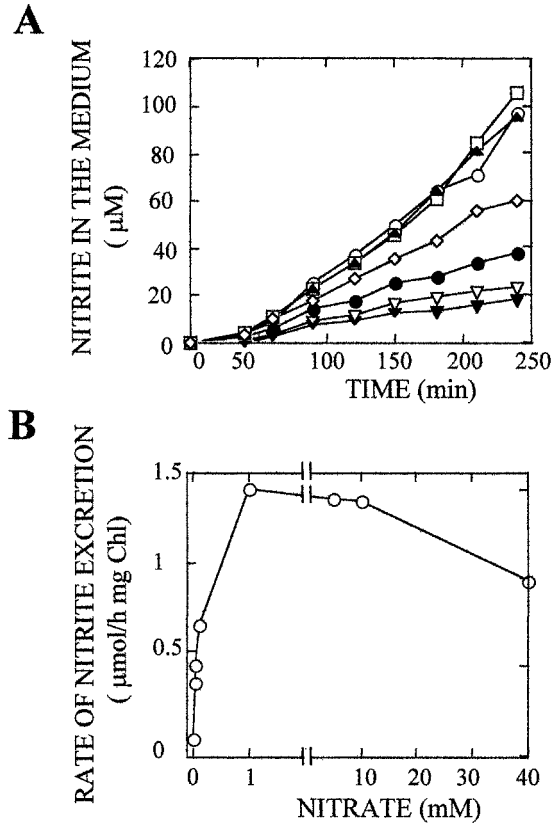

Figure 5. Nitrite excretion activity under limiting CO_2 conditions by the NiR mutant strain M4. Strain M4 was induced in media containing 4 mM nitrate for 4 h in cultures bubbled with air filtered through a CO_2 trap. Then cells were transferred to media containing different nitrate concentrations from 25 μM to 40 mM (▼, 25 μM; ▽, 50 μM; ●, 100 μM; ○, 1.0 mM; □, 5 mM; ▲, 10 mM; ◇, 40 mM) and kept under limiting CO_2 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.

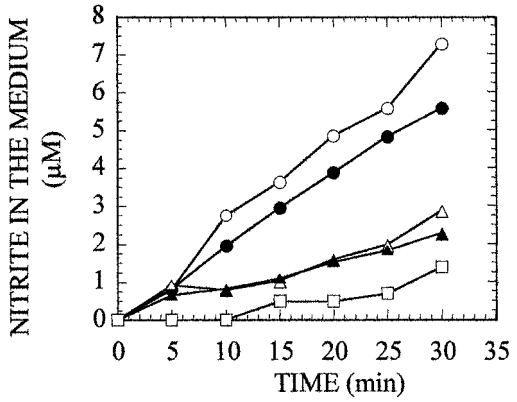

Figure 6. Effect of ammonium, chloride, and high CO_2 on the nitrite excretion activity induced under limiting CO_2 in the NiR mutant strain M4. Cells from strain M4 were induced as indicated in Figure 5, and transferred to medium containing 100 μM nitrate alone (○), plus 0.5 mM ammonium sulfate (●), 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.

optimum expression of these genes. These results indicate that in *C. reinhardtii*, a defective NR activity, and not the blocking of the NA pathway, is the cause for the observed deregulation, thus supporting the regulatory role of NR. These data are in agreement with those reported with *Aspergillus nidulans* (Hawker et al., 1992) and *Hansenula polymorpha* (Brito et al., 1996), for which NiR mutants were used to confirm the autogenous regulation of NR.

Ammonium and ammonium derivatives result in a negative regulation of genes for nitrate assimilation in *C. reinhardtii* (Fernández et al., 1998). Thus, if these negative factors were the major ones, one would expect that the blocking of the NA pathway at either the NR or NiR level would result in an overexpression pattern as proposed in higher plants (Vaucheret et al., 1992), which was not the case. We propose that the balance of regulatory elements both positive (i.e. nitrate) and negative (ammonium/derivatives, and possibly nitrite) would explain our results and the differences with higher plants. In NR mutants incubated in nitrogen-free medium, the positive signal from trace amounts of nitrate (which would accumulate in the cells and could not be assimilated) would prevail over negative ones. However, in NiR mutants, a signal of nitrite would prevail, since the low amounts of nitrate would be converted readily into nitrite. This nitrite signal could act either directly or indirectly by competing with nitrate and preventing its positive action.

The presence of a constitutive NR expressed from a *cabII-1* gene promoter during nitrate induction from ammonium-grown *C. reinhardtii* cells results in low levels of NR and *Nrt2;1*, *Nrt2;2*, and *Nar2* transcripts (Navarro et al., 1996, 1999). Thus, it has been suggested that NR provides some negative signal, which could be related to nitrite production (Navarro et al., 1999). Regulation of NA genes in NiR mutants isolated in the present study is in agreement with the reported regulatory role of NR in the pathway (Fernández and Cárdenas 1982; Galván et al., 1992; 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 nitrite 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-CO_2 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 K_s 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 CO_2 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 CO_2 (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.

ACKNOWLEDGMENTS

The authors thank M. Macías for technical support and C. Santos and I. Molina for secretarial assistance.

Received July 9, 1999; accepted September 24, 1999.

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