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Nitrate Reductase Regulates Expression of Nitrite Uptake and Nitrite Reductase Activities in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

In *Chlamydomonas reinhardtii* mutants defective at the structural locus for nitrate reductase (*nit-1*) or at loci for biosynthesis of the molybdopterin cofactor (*nit-3, nit-4,* or *nit-5* and *nit-6*), both nitrite uptake and nitrite reductase activities were repressed in ammonium-grown cells and expressed at high amounts in nitrogen-free media or in media containing nitrate or nitrite. In contrast, wild-type cells required nitrate induction for expression of high levels of both activities. In mutants defective at the regulatory locus for nitrate reductase (*nit-2*), very low levels of nitrite uptake and nitrite reductase activities were expressed even in the presence of nitrate or nitrite. Both restoration of nitrate reductase activity in mutants defective at *nit-1, nit-3,* and *nit-4* by isolating diploid strains among them and transformation of a structural mutant upon integration of the wild-type *nit-1* gene gave rise to the wild-type expression pattern for nitrite uptake and nitrite reductase activities. Conversely, inactivation of nitrate reductase by tungstate treatment in nitrate, nitrite, or nitrogen-free media made wild-type cells respond like nitrate reductase-deficient mutants with respect to the expression of nitrite uptake and nitrite reductase activities. Our results indicate that *nit-2* is a regulatory locus for both the nitrite uptake system and nitrite reductase, and that the nitrate reductase enzyme plays an important role in the regulation of the expression of both enzyme activities.

Nitrate assimilation is a highly regulated process that involves the transport of nitrate into the cell, its subsequent reduction to nitrite and ammonium, and the incorporation of ammonium into carbon skeletons (20). NR,[2] NiR, and proteins involved in nitrate and nitrite transport have been reported to be inducible systems under favorable conditions (12, 20, 22, 28, 30). Nitrate seems to play an essential role in turning on the expression of the proteins involved in the pathway, but the precise mechanism by which nitrate effects this response is not completely understood.

NR (5, 18) and NiR (2) expression appears to be regulated at the transcriptional level by nitrate, but no regulatory genes have been described in higher plants. In the plant kingdom, only the *nit-2* locus of *Chlamydomonas reinhardtii* has been shown to be an NR regulatory gene (13). In fungi, nitrate seems to operate through regulatory genes specific to the pathway, *nit-4* in *Neurospora crassa* and *nirA* in *Aspergillus nidulans,* that turn on the expression of genes encoding NR, NiR, and NU (4, 17, 28). It is also believed that fungal NR itself regulates its own expression and that of NiR and NU (4, 17, 27, 28).

In the haploid, unicellular green alga *C. reinhardtii,* the expression of the only structural gene for NR, *nit-1,* seems to be controlled by ammonium at both transcriptional (13, 16) and posttranscriptional levels (16). Nitrate does not seem to be required for NR transcription in *C. reinhardtii,* but it could participate at a posttranscriptional level (16). It has been shown that NR plays an important role in the regulation of its own enzyme activity levels by means of a reversible interconversion process (9, 15). In the absence of nitrate, NR would undergo reversible inactivation and would be subsequently degraded, but the presence of nitrate would stabilize the enzyme in its active form, which is much more stable than the inactive one, and thus would keep it at high levels (15).

Previous reports indicated that NU and NiU, in *C. reinhardtii,* are driven by two different systems, and that NiU and NiR show different kinetic properties (3). Maximal expression of NiU and NiR activities occurs after induction in nitrate, and NiU repression depends primarily on ammonium and repression of NiR on both ammonium and ammonium derivatives (19).

In the present report, we have analyzed the role of the NR enzyme on the expression of NiU and NiR activities by studying NR-deficient mutants of *C. reinhardtii.* Our data support the hypothesis that expression of NiU and NiR activities is under control of the regulatory locus *nit-2,* and that the NR enzyme itself plays a regulatory role on the expression of NiR and the nitrite transporter.

MATERIALS AND METHODS

Strains

Chlamydomonas reinhardtii 6145c (wild type) was supplied by Dr. Ruth Sager (Sidney Farber Cancer Center, New York). Mutants 102, 104, 305, 301, 307, and 203 have been characterized elsewhere (10, 11). The nit-2–137c mutant was isolated

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[2] Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; NU, nitrate uptake; NiU, nitrite uptake.

from 137c, and 307d was derived from the cross 307(−) × 21gr(+) as reported previously (10). Transformants Tx1, Tx2, Tx3, Tx5, Tx6, Tx7, Tx8, Tx10, Tx12, Tx13, and Tx14 were obtained by Kindle *et al.* (21) from the NR-deficient mutant 305 by the microprojectile approach and the plasmid pMN 24 containing the complete NR gene (13). Diploids 305(−) × 307(+), 305(−) × 104(+), and 307(−) × 104(+) were isolated from the original haploid strains as reported elsewhere (11).

Culture and Induction Conditions

Cells were grown at 25°C under saturating white light (50 W m^{-2}) in minimal medium (26) with 4% (v/v) CO_2-enriched air and containing 8 mM NH_4Cl as the nitrogen source. Cells were harvested at the exponential phase of growth, thoroughly washed with 25 mM potassium phosphate buffer, pH 7.0, and transferred to minimal medium containing either 4 mM KNO_2, 4 mM KNO_3, or no nitrogen source. After 6 h, cells were harvested, thoroughly washed, and divided into two portions, one for NiU measurement and the other for NiR activity determination.

In some experiments, to ensure that no traces of nitrate or nitrite were present in nitrogen-free media, nitrate-grown wild-type cells (A_{660} = 1.0–1.5) were incubated for 6 h to deplete the possible nitrogenous traces. Then, cells were removed by centrifugation, and the resulting supernatant was used after sterilization. The absence of nitrate and nitrite in the media was checked by HPLC according to the method of Romero *et al.* (24) on an HPLC Nucleosil 100–10 SB 10 μm column (250 × 4 mm) equilibrated with 125 mM potassium phosphate buffer, pH 4.0, at a flow rate of 2 mL min^{-1}.

Nitrite Uptake Rate Determination

Cell pellets (10–30 μg Chl/mL) were resuspended in minimal medium containing 50 to 100 μM KNO_2 and then maintained under the conditions of temperature, light, and CO_2 indicated above. Samples were taken at 1- to 2-min intervals over a 15-min uptake period and centrifuged, and the nitrite concentration in the supernatant was measured. NiU rate was calculated from the linear phase of the nitrite consumption curve and expressed as nmol NO^{-}_2 min^{-1} mg^{-1} Chl.

NiR Activity Determination

NiR activity was assayed as reported previously (19). The *in situ* assay was carried out with cells treated with 0.01% toluene to increase cell permeability (14). The reaction mixture contained 300 mM Tris-HCl, pH 8.0, 0.4 mM KNO_2, 0.8 mM methyl viologen, 16 mM dithionite, and an adequate amount of toluene-treated cells.

Analytical Methods

Nitrite was estimated colorimetrically according to Snell and Snell (25), and Chl according to Arnon (1).

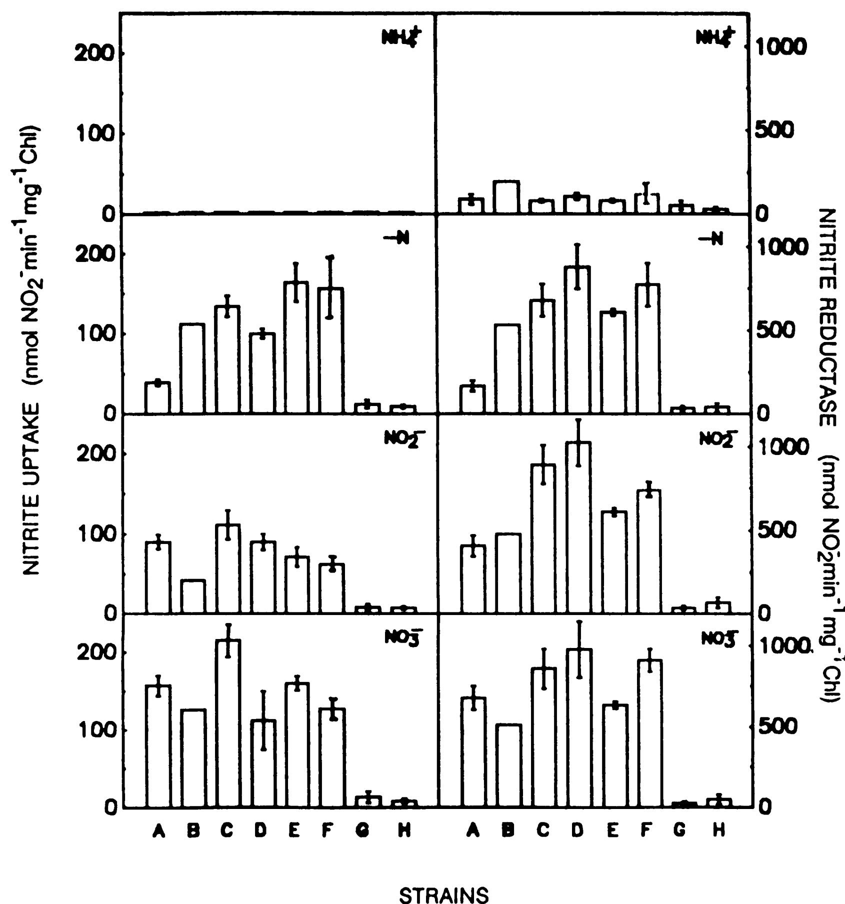

Figure 1. Nitrite uptake and nitrite reductase activities in wild-type and mutant strains of *C. reinhardtii*. Ammonium-grown cells were thoroughly washed and incubated in the indicated media for 6 h, and NiU and NiR activities were determined. Strains analyzed were: A, 6145c (wild type); B, 301 (*nit-1*); C, 305 (*nit-1*); D, 307 (*nit-3*); E, 104 (*nit-4*); F, 102 (*nit-5, nit-6*); G, 203 (*nit-2*); and H, *nit*-2–137c (*nit-2*). Data are expressed as mean values (columns) ± SE (bars). The number of independent experiments was 11, 2, 5, 4, 3, 3, 3, and 4 for the strains listed above, respectively.

RESULTS

In wild-type cells of *C. reinhardtii*, both NiU and NiR activities were repressed in ammonium-grown cells and required nitrate for maximum expression (19) (Fig. 1, lane A). The expression of both activities has been analyzed in mutant strains from *C. reinhardtii* defective at the structural locus *nit-1* for NR, alleles 305 and 301, or at any of the loci for the molybdenum-cofactor component of NR (*nit-3, nit-4, nit-5,* and *nit-6*), alleles 307, 104, and 102, which possess a nonfunctional NR (12). As shown in Figure 1 (lanes B–F), both NiU and NiR activities were repressed in all the mutants incubated in ammonium medium. As shown in wild-type cells (19), a low basal NiR activity was also evident in the mutants. In contrast with the wild-type cells, nitrate was not required for maximum expression of NiU and NiR activities in the structural locus and molybdenum-cofactor loci defective NR-mutants, which showed significant levels of these two activities when derepressed in nitrogen-free medium. On the other hand, NiR and NiU for two different mutant alleles defective at the regulatory locus *nit-2* for NR were not markedly derepressed in any of the media tested, which indicates that *nit-2* is also involved in the regulation of the expression of both activities (Fig. 1, lanes G and H).

NR activity can be restored in the structural mutant 305-*nit*-1 after introducing an intact *nit-1* gene by integrative transformation (21). In the analyzed transformants, the expression pattern for NiU and NiR activities of the original 305-*nit*-1 mutant was changed (Table I), becoming instead like that of wild-type cells (Table I), which required nitrate for maximum expression of both activities. In addition, functional NR was also restored in the structural mutant 305-*nit*-1 and in some molybdenum-cofactor-deficient mutants by constructing diploids among them by *in vivo* complementation. The regulatory pattern of NiU and NiR activities of the corresponding haploid strains was also changed in the diploids (Fig. 1, Table II), which became similar to that in wild-type cells (Table I). All of these results suggest that in *C. reinhardtii* an active NR is involved in the regulation of NiU and NiR activity levels.

NR can be inactivated *in vivo* in wild-type cells by growing them in a medium containing sodium tungstate instead of molybdate (29). Tungstate-treated wild-type cells behaved like NR-deficient mutants and showed maximum levels of both NiR (Fig. 2) and NiU (Table III) activities in the nitrogen-free, nitrite, and nitrate media. Significantly, in nitrite medium, tungstate is not toxic and has no effect on the growth of cells (20).

DISCUSSION

In *C. reinhardtii* wild-type strain, NR activity is repressed in ammonium-grown cells and requires nitrate induction for maximal expression (9). Expression of NiU and NiR activities follows the same regulatory patterns as NR (19).

The absence of molecular probes for NiR and the NiU system from *C. reinhardtii* has precluded an evaluation of the regulation of these enzymes at the mRNA level. However, the reliable measurement of these enzyme activities under the different conditions studied in wild-type and mutant algal strains indicate that, as in fungi (4, 17), NR also plays an important role in controlling the expression of NiR and NiU activities. The following observations support our proposal: (a) mutants defective in NR structure show high levels of NiU and NiR activities in nitrogen-free media, without the requirement of nitrate for induction (Fig. 1); (b) by restoring a functional NR, either by transformation of a structural mutant (Table I) or by construction of diploids (Table II), nitrate inducibility is regained in mutants, even though nonfunctional hybrid molecules of NR exist (11, 21); and (c) inactivation of NR in wild-type cells by tungstate treatment caused the loss of nitrate inducibility of NiU (Table III) and NiR

Table I. *Nitrite Uptake and Nitrite Reductase Activities in Transformants of C. reinhardtii*

Nitrite uptake and NiR activities were determined in ammonium-grown cells (NH_4^+) and after a 6-h derepression in media lacking nitrogen (−N) or containing 4 mM NO_2^- or NO_3^- Transformants were from Kindle *et al.* (21). Data for 6145c (wild type) and *nit-1*-305 are from Figure 1.

Strain	Nitrite Uptake Rate in Culture medium				Nitrite Reductase Activity in Culture medium			
	NH_4^+	−N	NO_2^-	NO_3^-	NH_4^+	−N	NO_2^-	NO_3^-
	nmol NO_2^- min^{-1} mg^{-1} Chl				*nmol NO_2^- min^{-1} mg^{-1} Chl*			
6145c	0	39 ± 4	90 ± 9	157 ± 13	88 ± 30	166 ± 31	408 ± 63	677 ± 74
305	0	134 ± 13	111 ± 18	215 ± 21	79 ± 1	679 ± 9	897 ± 117	859 ± 124
Tx1	0	60	113	164	58	232	592	905
Tx2	0	18	37	101	73	234	397	477
Tx3	0	61	92	127	26	158	376	583
Tx5	0	7	50	254	101	350	404	589
Tx6	0	15	93	164	47	264	553	671
Tx7	0	82	57	107	23	225	398	600
Tx8	0	33	64	161	62	233	650	991
Tx10	0	117	66	151	54	290	365	770
Tx12	0	67	54	96	27	225	404	496
Tx13	0	33	65	200	26	198	476	748
Tx14	0	54	53	162	9	198	363	960

Table II. *Nitrite Uptake and Nitrite Reductase Activities in Diploid Strains of C. reinhardtii*

Diploids were obtained from crosses corresponding to the indicated strains (11). Nitrite uptake and NiR activities were determined in ammonium-grown cells (NH_4^+) and after derepression for 6 h in media lacking nitrogen (−N) or containing 4 mM NO_2^- or NO_3^-. nd = Not detectable.

Diploids	Nitrite Uptake Rate in Culture medium				Nitrite Reductase Activity in Culture medium			
	NH_4^+	−N	NO_2^-	NO_3^-	NH_4^+	−N	NO_2^-	NO_3^-
	nmol NO_2^- min^{-1} mg^{-1} Chl				*nmol NO_2^- min^{-1} mg^{-1} Chl*			
305(−) × 307_d(+)	0	36	72	99	nd	62	230	396
305(−) × 104(+)	0	46	88	100	56	193	312	471
307(−) × 104(+)	0	34	62	107	77	171	244	360

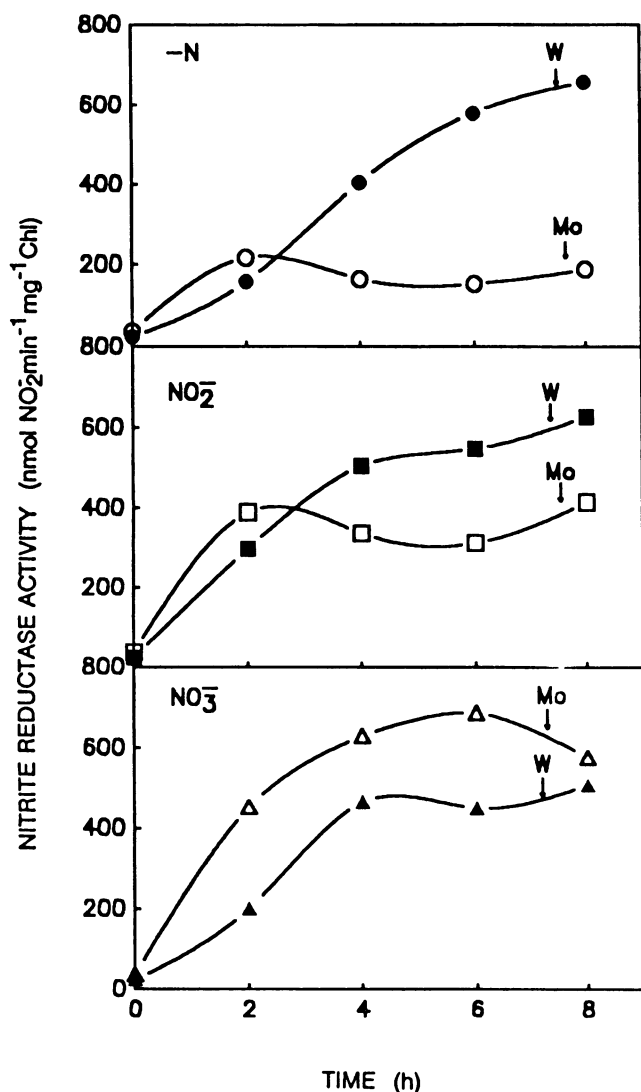

Figure 2. Effect of tungstate on the expression of nitrite reductase activity in wild-type cells of *C. reinhardtii*. Ammonium-grown cells were derepressed in the indicated media (either 4 mM in nitrogen source or lacking nitrogen, −N). Mo, standard medium (26); W, 1 mM sodium tungstate substituted for molybdate in both the ammonium and the derepression media.

activities (Fig. 2). Although it is difficult to rule out completely that traces of nitrate could accumulate in cells with nonfunctional NR incapable of metabolizing it, removal of possible traces of nitrate or nitrite from nitrogen-free medium, as indicated in "Materials and Methods," had no effect on the levels of NiU and NiR activities expressed.

In *Nicotiana tabacum,* tungstate deregulates the expression of NR by increasing both NR mRNA and protein levels (7). The effect is interpreted in terms of a blockage of nitrate reduction, which may limit the content of nitrogen metabolites, such as nitrite, ammonium, or glutamine, to low levels and results in overexpression of the NR structural gene (7). Under our experimental conditions of the tungstate treatment in nitrogen-free or nitrite media (Fig. 2, Table III), it is unlikely that tungstate affects the cellular nitrogen metabolite content in those media in which either no nitrogen can be assimilated or nitrite is bypassing the requirement for an active NR for nitrogen assimilation. We propose that NR enzyme, as a primary producer of nitrite and metabolites generated from its reduction, does not regulate NiU and NiR expression, but the NR enzyme itself in a functional form. A possible working hypothesis would be that the reversibly inactive form of NR would inhibit its own expression and that of NiR and the NiU system. Because mutant or tungsten-inactivated forms of NR are not reversibly inactivated under our experimental conditions (15), this inactivation would not take place and nitrate would not be required for maximum expression. Alternatively, another enzyme activity related to

Table III. *Effect of Tungstate on the Expression of Nitrite Uptake Activity in C. einhardtii Wild-Type 6145c*

Experimental conditions as in Figure 2. Nitrite uptake rate was assayed after a 6-h derepression.

Nitrogen Source	Nitrite Uptake Rate	
	Mo[a]	W[b]
	nmol NO_2^- min^{-1} mg^{-1}Chl	
NH_4^+	0	0
−N	41	114
NO_2^-	56	110
NO_3^-	117	137

[a] Standard medium. [b] 1 mM tungstate substituted for molybdate in both the ammonium and the derepression media.

the active NR could also account for the regulatory effects we observed. Perhaps a nitrite reduction activity similar to that of the molybdenum-containing portion of constitutive NAD(P)H-NR of soybean could account for these regulatory effects (6).

The fact that mutants of *Nicotiana plumbaginifolia* with defective NR apoenzyme retain nitrate inducibility of NR expression points to a regulatory mechanism in higher plants somehow different from that reported in fungi (23). Our data indicate that, in *C. reinhardtii,* regulation of expression of both NiR and the NiU systems seems to be mediated by the same factors as that of NR, which include the NR enzyme itself.

On the other hand, the *nit-2* locus has been shown to be involved in NR expression (13) and not related to expression of xanthine dehydrogenase (8). Our data suggest that the regulatory locus *nit-2* is also involved in NiU and NiR expression (Fig. 1). *nit-2,* which has been suggested to act positively on the expression of *nit-1* (11), appears to be similar to the pathway-specific regulatory genes *nit-4* and *nirA* of *N. crassa* and *A. nidulans,* respectively (4, 27, 28).

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