Eur. J. Phycol. (2005), **40**: 345–352

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Regulation of nitrate reductase in *Chlamydomonas reinhardtii* by the redox state of the plastoquinone pool

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(Received 24 February 2005; accepted 24 August 2005)

In the chlorophyte alga *Chlamydomonas reinhardtii*, expression of the nuclear gene *NIA1*, encoding nitrate reductase, is regulated by light, but the signal transduction mechanism is poorly understood. Using inhibitors, mutants, and physiological manipulation, we searched for signals in the photosynthetic electron transport chain that potentially regulate *NIA1* expression. In the *NIA1*$^{+}$ wild-type clone CC-1692, nitrate reductase activity is strongly down-regulated when the reduction of plastoquinone is blocked by 3-(3′4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU), but unaffected or stimulated when the oxidation of plastoquinol is inhibited by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). Simultaneously, although DBMIB reduced *NIA1* expression by ~30% over a 6-h period relative to the control, DCMU inhibited expression of the gene by over 80%. A cross between *CC-1692* and a site-directed mutant, *CC-3388 A251I*, in which amino acid 251 in the PSII core protein, D1, was altered from alanine to isoleucine, thereby decreasing the binding affinity for Q_B, produced a cell with markedly reduced expression of *NIA1*. Our results indicate that expression of nitrate reductase is coupled to photosynthesis via a sensor related to the redox poise of the plastoquinone pool. When the pool is oxidized, carbon fixation is low and nitrate reductase is down-regulated; conversely, when the pool is reduced, carbon fixation is high and the gene and enzyme activity are up-regulated. These experimental observations suggest a model for the coupled light regulation of photosynthesis and nitrate assimilation.

Key words: *NIA1*, nitrogen, photosynthesis, plastoquinone, PQ pool, redox regulation

Introduction

Nitrogen assimilation and carbon fixation are highly coordinated in unicellular algae (see Huppe & Turpin, 1994; Beardall & Giordano, 2002; Giordano *et al*., 2003). Although many algae can store nitrate in vacuoles, once the nitrate is committed in a reduction pathway, it must be incorporated into a carbon skeleton or it may be lost. Algae cannot store nitrite or ammonium efficiently (with the exception of some Phaeophyceae with very acidic vacuoles; J.A. Raven, personal communication), nor can they reoxidize organic nitrogen back to nitrate. Consequently, the first step in the assimilation of nitrate begins with reduction to nitrite, catalyzed by the soluble, cytosolic enzyme nitrate reductase (NR). This enzyme is one of the most highly regulated enzymes in any biosynthetic pathway in unicellular algae (Berges, 1997; Fernandez *et al*., 1998; Gonzalez-Ballester *et al*., 2005).

In the unicellular chlorophyte alga, *Chlamydomonas reinhardtii*, the nuclear gene encoding NR, *NIA1* (previously *Nit1*; NCBI accession number AF203033) is part of a cluster of genes involved in the reduction and acquisition of NO_3^- and NO_2^- that share many regulatory features (Quesada *et al*., 1993; Fernandez *et al*., 1998). The gene product catalyzes a two-electron transfer, reducing NO_3^- to NO_2^- using NAD(P)H as the reductant. Subsequently, nitrite reductase reduces NO_2^- to NH_4^+ in a 6-electron transfer. NH_4^+ is then incorporated into glutamic acid to form glutamine (Zehr & Falkowski, 1988). In this nitrogen assimilation pathway, NO_3^- reduction is the rate-limiting step, and NR expression and activity is highly regulated. The factors that affect the activity of this enzyme include the availability of exogenous NH_4^+ and NO_3^-, carbon fixation,

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ISSN 0967-0262 print/ISSN 1469-4433 online © 2005 British Phycological Society
DOI: 10.1080/09670260500334263

and light (Fernandez *et al.*, 1989, 1998; Loppes *et al.*, 1999; Loppes & Radoux, 2001; Cheng *et al.*, 1992; Kamiya & Saitoh, 2002; Sherameti *et al.*, 2002; Song & Ward, 2004; Navarro *et al.*, 2005). The influence of light on *NIA1* expression is well established (see Fernandez *et al.*, 1998), but the signal transduction pathway is poorly understood.

Several experimental studies have established that the redox poise of photosynthetic electron transport components can transduce light signals to both chloroplast (Danon & Mayfield, 1994; Bruick & Mayfield, 1999; Barnes & Mayfield, 2003; Link, 2003; Pfannschmidt & Liere, 2005) and nuclear genes (Escoubas *et al.*, 1995; Pfannschimdt, 2003). For example, in *C. reinhardtii*, expression of the plastid-encoded *psbA* gene has been associated with both the thioredoxin/ferredoxin relay (Danon & Mayfield, 1994; Somanchi *et al.*, 2005) and the redox poise of the plastoquinone (PQ) pool (Trebitsh *et al.*, 2000). In the closely related chlorophyte algae, *Dunaliella tertiolecta*, genes encoding the chlorophyll *a*/*b* binding proteins of PSII appear to be regulated by the PQ pool redox state (Escoubas *et al.*, 1995). Sherameti and colleagues (2002), on the basis of responses elicited by different light regimes and inhibitors, proposed that NR expression in higher plants is stimulated by the oxidation of a component of the electron transport chain located after the PQ pool. However, the substantial physiological and biochemical differences in the regulation of NR (see Berges, 1997; Fernandez *et al.*, 1998), prevent extrapolation of these results to algae without experimental support. In this study, we tested the hypothesis that the PQ pool regulates *NIA1* expression in *Chlamydomonas reinhardtii*.

Materials and methods

Cultures

Chlamydomonas reinhardtii CC-1692 was cultured mixotrophically with acetate as a carbon source (TAP medium) or photoautotrophically on minimal (TP) medium (Harris, 1989) at 20°C with 4 mM of either KNO_3 or NH_4Cl as the sole N source. Cultures were maintained at 100 μmol photons $m^{-2} s^{-1}$, under continuous light and stirring, and were aerated with sterile air in 250-ml Erlenmeyer flasks containing 100 ml of algal suspension. Twenty-four hours prior to experimental manipulation, cells were washed and resuspended in fresh TP medium and transferred into 1-l flasks containing 400 ml of algal suspension; culture conditions were otherwise identical to those described above. Growth rates were determined by cell counts using a hemocytometer. Cell density at the beginning of each experiment was adjusted to 1×10^6 cells ml^{-1}.

Chlorophyll fluorescence measurements

The photosynthetic performance and redox status of the PQ pool were assessed using a fast repetition rate (FRR) fluorometer (Kolber *et al.*, 1998) with a modified detection unit (large area avalanche photodiode detector, Advanced Photonix). The instrument generates a train of short (0.6 μs) blue (470 nm) flashlets in the microsecond to millisecond timescale. Electron transport elicited by these light-pulses induces transient changes in Chl *a* fluorescence emission reflecting the redox and light acclimation status of the photosynthetic machinery. The FRR Chl fluorescence transient was first recorded in the dark, and the fluorescence parameters were determined as described previously (Kolber *et al.*, 1998). The photophysiological state of the cultures was checked before each experiment by determining chlorophyll variable fluorescence. Only cultures with F_v/F_m ratios at 0.65 or above were used. The efficacy of electron transfer inhibitors and the presence of the D1 mutation in the segregant strain (see below) was also determined by FRR fluorescence measurements, which provide information about the kinetics of electron transfer on the acceptor side of PSII (Kolber *et al.*, 1998; Lardans *et al.*, 1998).

Enzyme extraction and activity measurements

Cells were pelleted by centrifugation (1,000 *g*, 10 min), washed with an isosmotic NaCl solution, resuspended in the extraction buffer described by Campbell & Smarrelli (1986) and sonicated on ice (3 × 20 s cycles with 30-s intervals, 4 watts). NR activity in the crude extract was determined colorimetrically following the procedure described by Smarrelli & Campbell (1983).

NR from *C. reinhardtii* utilized either NADH or NADPH with comparable affinities ($K_m = 8.4$ and 8.9 μmol l^{-1}, respectively), however V_{max} was 1.86-fold higher with NADPH (3.89 ± 0.12 nmol $min^{-1} mg^{-1}$ protein) than with NADH (2.09 ± 0.1 nmol $min^{-1} mg^{-1}$ protein). For comparability with the existing literature, we assayed NR activity with NADH.

Proteins. Total proteins were measured with the Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Rockford, Ill.; Stoscheck, 1990), using BSA as a standard.

Inhibitors. The minimum concentration of 3-(3′4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU, 5 μM) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB, 1 μM) that gave a clear fully inhibitory fluorescence signature was used (Durnford *et al.*, 1998). The effectiveness of DCMU was ascertained from the inhibition of Q_A reoxidation after the single turnover flash. Control measurements proved that DCMU was effective for the entire course of the experiments. The inhibitory effect of DBMIB was assessed from the accelerated kinetics of the multiple turnover pulse as shown in Durnford *et al.* (1998). We observed, by both fluorescence and oxygen evolution measurements that, in irradiated

Chlamydomonas cultures, DBMIB was rapidly inactivated (half-life ~15–30 min). For this reason, the FRR fluorescence signature of DBMIB was checked every 30–60 min throughout the experiments; if necessary, a supplementary dose was given (on average a dose was given every 45 min). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used at a final concentration of 100 μM. All inhibitors were dissolved in dimethyl sulphoxide (DMSO), an equal volume of which was added to the controls. Samples were taken from each replicate immediately before the addition of inhibitors and then after 3 and 6 h.

Nucleic acid extraction, PCR reactions, Northern and Southern blots

The extraction and analysis of DNA/RNA was carried out using standard protocols (Sambrook *et al.*, 2001; Ausubel *et al.*, 1993). Cells were harvested by centrifugation and resuspended in lysis buffer (1.2% SDS, 30 mM EDTA, 50 mM Tris-HCl pH 8.0, 220 mM NaCl, 50 mM β-mercaptoethanol). Total RNA was fractionated by electrophoresis on a 1% agarose gel with 1 M formaldehyde. RNA was transferred to a charged nylon membrane (Nytran, Schleicher & Schuell BioScience, Dassel-Relliehausen, Germany) using a TurboBlottor System (S & S). Probes were labeled with α^{32}P-dATP using the Prime-A-Gene Labeling System (Promega Biosciences Inc., Madison, WI). Hybridization was performed using the PerfectHyb system (Ambion Inc., Austin, TX). The blots were washed twice with $0.2 \times$ SSC followed by 30 min incubation at 42°C before being exposed to Kodak BioMax MR/MS films. A pair of *C. reinhardtii*-specific primers (forward primer 5′-TAC ACG GTG TCA CAG CCCAA-3′; reverse primer 5′-CCA TAC ACA GGT CGC ACT CTC A-3′) was designed based on the published *NIA1* sequence (Zhang *et al.*, 1999, direct submission to Genbank). The subsequent amplification of a 998-bp *NIA1* fragment was obtained using either the JumpStart REDtaq kit (Sigma-Aldrich, St. Louis. MO) or the HotStart taq kit (Qiagen, Valencia, CA). The fragment was used as a template for a *NIA1* probe for both Northern and Southern assays. Specific PCR with the *NIA1* primers was also used to confirm the presence or absence of the *NIA1* gene in the genetic crosses. The amplified PCR products were further analyzed by a Southern assay probed with the specific *NIA1* fragment. For densitometric analysis, original Northern autoradiograms were scanned using an Epson 1680 Pro Scanner (1600×3200 dpi hardware resolution, 48-bit color with 3.6 Dmax optical density). NIH Image software (Scientific Computing Resource Center, National Institute of Health) was used to perform densitometric analysis of labeled bands.

Genetic crosses

In order to confirm the inhibitor results in a more 'natural' genetic background, crosses were made according to the procedure described by Harris (1989). The strains used were *CC-1692* wt mt$^-$ *NIA1*$^+$ and *CC-3388 A251I* mt$^+$. Strain *CC-3388* is derived from *CC-125* wild-type mt$^+$ (*137C*) (E. Harris, personal communication), that lacked functional *NIA1* and *NIT2* genes (and thus was able to grow only on NH_4^+); *NIT2* is a positive regulatory gene for nitrate assimilation (Fernandez & Matagne, 1986; Schnell & Lefebvre, 1993) and had a mutation in the Q_B binding site of the D1 reaction centre protein; the genotype and phenotype of this mutant are thoroughly described by Lardans *et al.* (1998). Since *Chlamydomonas* has uniparental maternal inheritance of chloroplasts, all crosses contained the chloroplast mutation (D1$^-$). However, the progeny would segregate *NIA1*$^+$. In order to select the appropriate segregants, cells were plated on solid media (TP + 1.5% agarose), with NO_3^- as the sole N-source. Colonies were then transferred into 4 ml of sterile liquid TP-NO_3^- medium and tested for the presence of D1 mutation by FRR measurement (Fig. 3). The clones with the D1 mutation were re-plated and the resulting colonies were tested again for the presence of the mutation, prior to further experimental manipulation. A cross was also generated from strain *CC-1692* wt mt$^-$ *NIA1*$^+$ and strain *CC-2964* that had a mutation in the *petA* protein of the cytochrome b_6/f complex; this cross was not viable. All strains were obtained from the *Chlamydomonas* Genetic Center, Duke University.

Statistical methods

All measurements were carried out on at least three different cultures. Analyses were replicated 4 times for each culture and the average of these determinations was used for further statistical treatments. The means and standard deviations presented are thus derived from measurements on separate, replicate cultures. The data were log-transformed for homogeneity of variance, before one-way ANOVA followed by a least significant difference (LSD) comparison of treatments.

Results

Effects of inhibitors on NR activity

To examine the potential effect of the redox state of the PQ pool on NR, we measured the enzymatic activity in cells grown with NO_3^- as the sole nitrogen source under photoautotrophic conditions. Within 6 h following the addition of DCMU, NR activity decreased by over 30%, while it increased in cells exposed to 1 μM DBMIB (Fig. 1). After 12 h, the DCMU-treated cells had lost over 70% of their activity, while those treated with DBMIB continued to have enhanced activity. The uncoupler CCCP had no effect on NR activity (data not shown). As a negative control, activity was measured on cells grown in either TAP or TP with NH_4^+; NR activity was below the level of detection ($0.006 +/- 0.0001$ nmol min^{-1} mg^{-1} protein). A similar effect was obtained by adding 1 mM of the NH_4^+ analogue, methylamine, to TP-NO_3^- cultures (data not shown).

Effects of inhibitors on NIA1 expression

Within 3 h following exposure to DCMU, the abundance of *NIA1* message decreased over 80%, and dropped to only 15% of the control after 6 h (Fig. 2). In contrast, there was no significant decrease in message levels in the presence of DBMIB (1-way ANOVA followed by LSD comparison of treatments, $p < 0.05$). In addition to inhibitors of electron transport, the effect of CCCP was tested, but it had no significant effect on the abundance of *NIA1* transcript. The variation in *NIA1* transcript abundance in control cultures between the initial time point and the 6-hour time point was not statistically significant (*t*-test, $p = 0.206$).

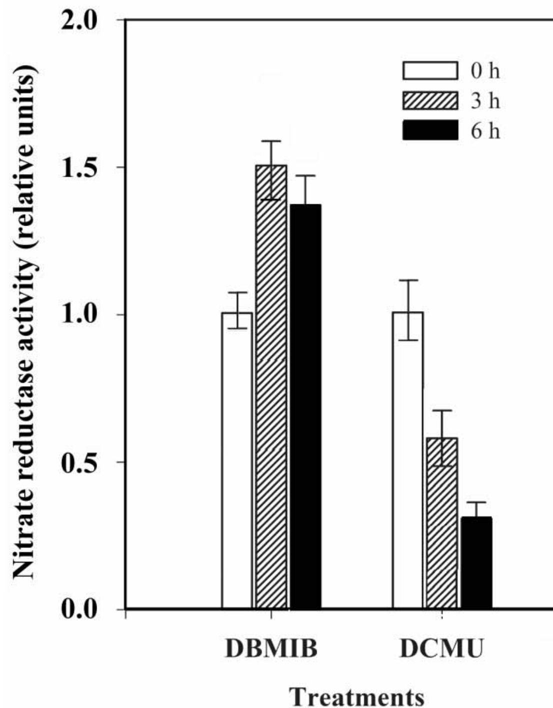


Fig. 1. Effect of DCMU and DBMIB on NR activity of *Chlamydomonas reinhardtii* cells cultured at 100 μmol quanta $m^{-2} s^{-1}$. Cells were incubated in the presence of 5 μM DCMU and 1 μM DBMIB for either 6 or 12 h, and enzyme activity in treated cells is shown relative to activity prior to addition of inhibitors (Time 0). Error bars indicate standard deviations ($n \geq 4$).

The cross between C. reinhardtii *CC-169 and CC-3388 A251I ($NIA1^+$ $D1^-$)*

The kinetics of electron transport on the acceptor side of PSII (Q_A reoxidation) were analyzed by following the decay in fluorescence using a custom-built FRR fluorometer (Lardans *et al.*, 1998). In *C. reinhardtii CC-1692*, the decay had two major components, a fast component with a half-time of *ca.* 350 μs and a slower component with a half-time of *ca.* 10 ms (Fig. 3A, wild-type *NIA1*). In a scrambled S-state, the former is the average time for electron transfer from Q_A^- to Q_B or Q_B^-, while the latter is the time constant for dissociation of the doubly reduced quinol from the binding pocket and its diffusion to cytochrome b_6/f. In *CC-3388 A251I* ($D1^-$ mutant), the fast component of electron transfer was virtually absent, and the kinetics were dominated by a single slow component with a half-time of *ca.* 10 ms (Fig. 3A). A segregant strain, derived from a cross between *CC-3388 A251I* ($D1^-$) and *CC-1692* (*$NIA1^+$*), was recovered from NO_3^- based TP medium. In this segregant, Chl *a* fluorescence emission kinetics was similar to that of the CC-3388 $D1^-$ strain (Fig. 3A), confirming that these cells had retained the defect in the Q_B binding pocket. The presence of the *NIA1* gene in this segregant was verified by

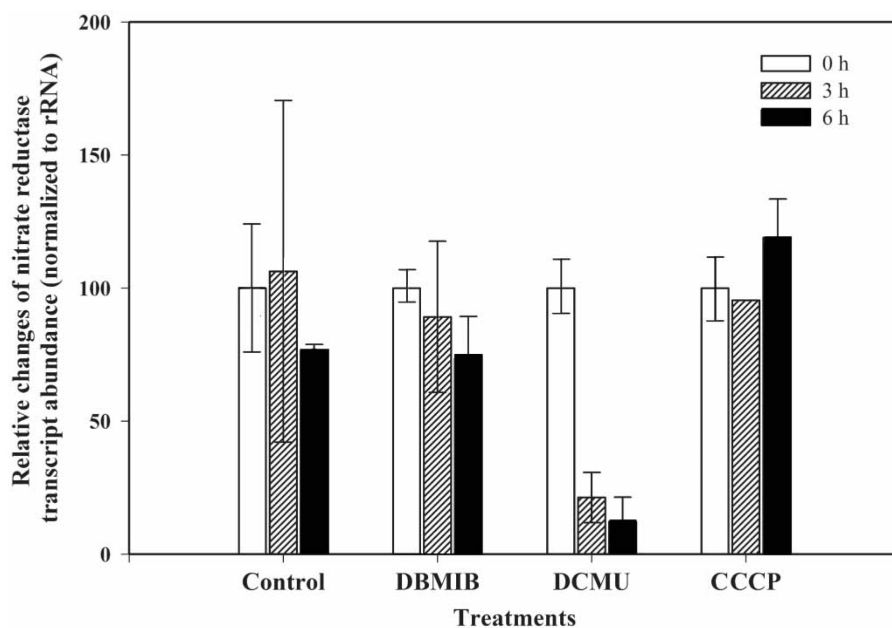


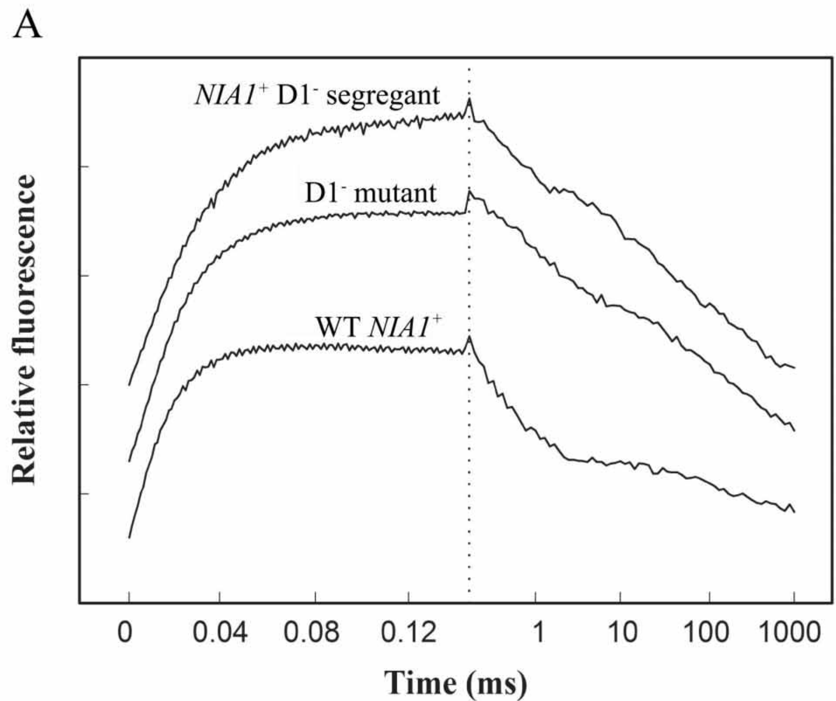
Fig. 2. Effect of DCMU (5 μM), DBMIB (1 μM) and CCCP (100 μM) on *NIA1* expression in *Chlamydomonas reinhardtii CC-1692* grown on TP-NO_3^-. Data expressed relative to the control and are means of three different northern gels that were normalized with respect to total RNA. Error bars indicate standard deviations ($n = 3$; but too small to be visible after 3 h in CCCP).

PCR amplification and a Southern blot carried out on the *NIA1* PCR fragments (Fig. 3B). The segregant strain was thus $NIA1^+$ $D1^-$.

Although the segregant could grow on NO_3^-, NR activity was barely discernable (data not shown). In order to test whether this was due to a lack of *NIA1* gene expression, northern blots were performed on total RNA extracts from these cells. The expression of *NIA1* in the segregant was approximately 30% that of the wild-type (Fig. 4, lanes 2 and 3) and was not up-regulated by the addition of DBMIB (Fig. 4, lanes 4 and 5).

Discussion

The results of these experiments suggest that *NIA1* expression and activity of NR are regulated by the redox state of the PQ pool. When reduction of the pool was prevented, either by the addition of DCMU (i.e. the photochemical equivalent of



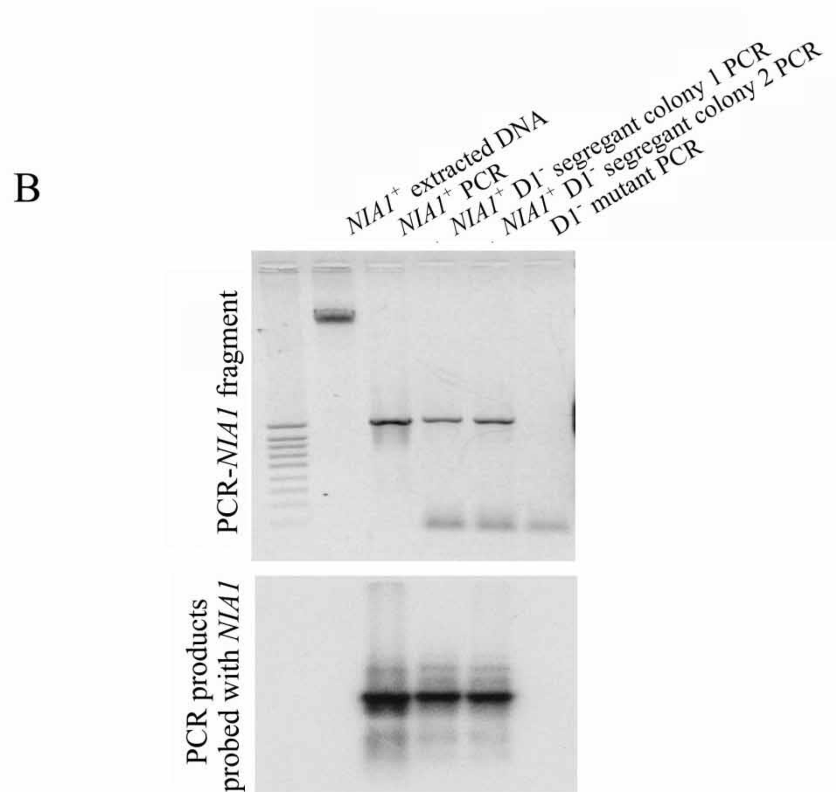


Fig. 3. A – FRR fluorescence trace of the $NIA1^+$ $D1^-$ segregant strain in comparison with the wild-type *CC-1692* and the $NIA1^-$ $D1^-$ mutant (*CC-3388*). B – upper gel: PCR amplification of *NIA1* fragments from $NIA1^+$ wild-type (*CC-1692*), 2 different isolates of the $NIA1^+$ $D1^-$ segregant (*CC-1692* + *CC-3388*), and $NIA1^-$ $D1^-$ mutant (*CC-3388*); lower gel: Southern blot carried out on the *NIA1* PCR fragments.

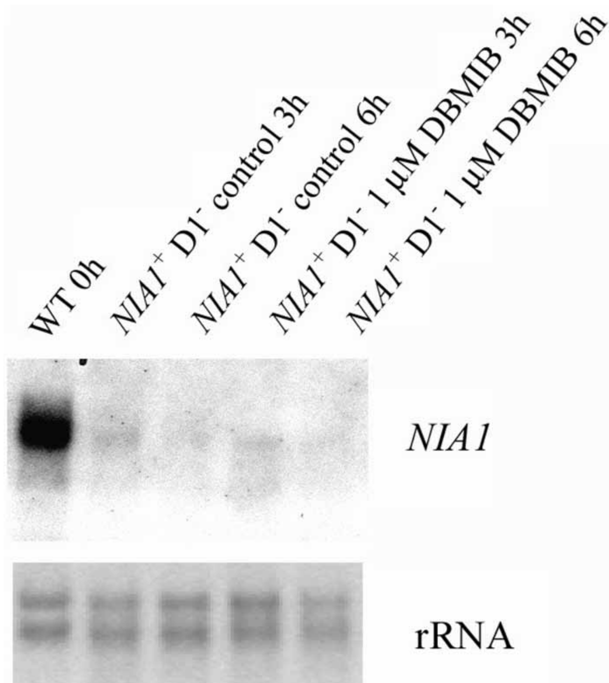


Fig. 4. Northern blot showing *NIA1* expression in *Chlamydomonas reinhardtii CC-1692/CC-3388 A251I* (*NIA1*$^+$ D1$^-$) cross incubated in TP in the presence of NO_3^- as the sole N source, and in the presence or absence of 1 μM DBMIB. For both controls and treated cells, samples were collected 3 and 6 h following addition of inhibitor. Upper panel: signal of the *NIA1* probe; Lower panel: ethidium bromide dyed northern gel. Equal amounts of total RNA (7.5 μg) were loaded on the northern gel.

low light) or by site-directed mutagenesis on the flanking region of the quinone binding pocket of the D1 protein, both *NIA1* transcription and enzymatic activity of the gene product were down-regulated. In contrast, when the plastoquinol binding pocket of the cytochrome b_6/f complex was inhibited with DBMIB, no effect was observed on gene expression, and enzyme activity was actually enhanced. The different responses to DBMIB of *NIA1* expression and NR activity suggest there are distinct transcriptional and post-translational regulatory mechanisms, both controlled by the redox state of the PQ pool. If a signal for regulation of *NIA1* expression were on either the donor side of PSII or downstream of the cytochrome b_6/f complex, DBMIB should have an effect comparable to DCMU. The efficacy of DBMIB is indisputable; FRR fluorescence measurements clearly indicated that the pool was largely reduced. The only electron transport component consistent with this pattern of expression is the PQ pool.

The basic pattern suggested by the experimental data and by the existing literature on NR light responses (Quesada & Fernández, 1994; Fernández *et al.*, 1998) is that both *NIA1* expression and NR activity are suppressed when the PQ pool is oxidized. This pattern is the reverse of that described for regulation of *LHCB1* gene expression (Escoubas *et al.*, 1995), but points to a similar underlying mechanism in which a photosynthetic electron transfer component within the plastid exerts control over a nuclear encoded gene. The reciprocal pattern between *NIA1* and *LHCB1* suggests that the redox state of the PQ pool may play a critical role in regulating nitrogen assimilation in relation to photosynthesis. In high light, *LHCB1* expression is low, since cells do not increase the effective absorption cross section of their reaction centres in order to harvest light (Durnford & Falkowski, 1997; Chen *et al.*, 2004). Under such conditions, the PQ pool is generally reduced, and carbon fixation is not limited by the rate of supply of reductants (Behrenfeld *et al.*, 1998). Carbon fixation provides skeletons for amino acid biosynthesis, and a source of ammonium becomes a potentially critical factor limiting cell growth (Falkowski *et al.*, 1989; Norici & Giordano, 2002). Under such conditions, if NO_3^- is the sole source of inorganic nitrogen, the rate of reduction of the substrate should be maximal. In contrast, under low irradiances the PQ pool is largely oxidized and *LHCB1* is up-regulated (Sukenik *et al.*, 1990), thereby increasing the photon capture cross section of the photosynthetic process (Falkowski & Raven, 1997). However, until the photoacclimation process is completed, low level expression of NR suffices to match the rate of synthesis of organic carbon skeletons. Interestingly, even if C-fixation is effectively eliminated by the addition of DBMIB or an uncoupler, the effect on *NIA1* expression is minimal.

Although there is increasing evidence of redox control of gene expression in eukaryotic photoautotrophs, the basic signaling factors remain unknown. Based on electromobility shift assays, Chen *et al.* (2004) identified several protein binding sites upstream of the start codon in *LHCB1* that appear to be subjected to redox control in the plastid. The DNA-binding proteins themselves have not yet been identified.

Our results suggest a working model for the transcriptional regulation of *NIA1*. The model points to the redox state of the PQ pool in the signal transduction pathway, but the PQ pool is itself embedded within the thylakoid membrane; there is no known protein on the stromal side of the thylakoid that directly accesses the pool. Our general model invokes a secondary messenger within the plastid that can be post-translationally modified (e.g. phosphorylated or reduced) by changes in the conformation of the cytochrome b_6/f complex. One candidate is Stt7, a chloroplast protein kinase that appears to be regulated by the redox state of the PQ pool (Depège *et al.*, 2003).

No suitable $D1^-$ mutant defective only in the *NIA1* gene is presently available. The selection of such a mutant could further clarify the mechanism of control for NR expression. This not withstanding, our results clearly show that the redox state of the PQ pool acts rather dramatically on NR expression, even if it is presently not possible to ascertain whether this effect is exerted directly on *NIA1* or via the regulatory gene *NIT2* (Fernandez & Matagne, 1986; Schnell & Lefebvre, 1993; Gonzalez-Ballester *et al.*, 2005). We were not successful in obtaining a viable genetic cross between $NIA1^+$ and a cytochrome b_6/f mutant; hence the exact role of the cytochrome b_6/f complex remains to be elucidated.

Our results unequivocally show a strong involvement of the redox state of the PQ pool in the regulation of NR. This control mechanism is probably a pivotal component of the complex regulatory network of nitrate assimilation that includes a variety of regulatory mechanisms distinct from the modulation processes mediated by the platoquinone redox state (see Fernandez *et al.*, 1998 and references therein; Gonzalez-Ballester *et al.*, 2005).

Acknowledgements

We thank Wilbur Campbell for his advice on the measurements of NR activity, Emilio Fernandez Reyes and John A. Raven for their comments to the manuscript, Mary Ann Tran and Carlos Gonzales for their participation in the experiments and Kevin Wyman for technical help.

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