An RNA interference knock-down of nitrate reductase enhances lipid biosynthesis in the diatom *Phaeodactylum tricornutum*

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SUMMARY

When diatoms are stressed for inorganic nitrogen they remodel their intermediate metabolism and redirect carbon towards lipid biosynthesis. However, this response comes at a significant cost reflected in decreased photosynthetic energy conversion efficiency and growth. Here we explore a molecular genetics approach to restrict the assimilation of inorganic nitrogen by knocking down nitrate reductase (NR). The transformant strain, NR21, exhibited about 50% lower expression and activity of the enzyme but simultaneously accumulated over 40% more fatty acids. However, in contrast to nitrogen-stressed wild-type (WT) cells, which grow at about 20% of the rate of nitrogen-replete cells, growth of NR21 was only reduced by about 30%. Biophysical analyses revealed that the photosynthetic energy conversion efficiency of photosystem II was unaffected in NR21; nevertheless, the plastoquinone pool was reduced by 50% at the optimal growth irradiance while in the WT it was over 90% oxidized. Further analyses reveal a 12-fold increase in the glutamate/glutamine ratio and an increase NADPH and malonyl-CoA pool size. Transcriptomic analyses indicate that the knock down resulted in changes in the expression of genes for lipid biosynthesis, as well as the expression of specific transcription factors. Based on these observations, we hypothesize that the allocation of carbon and reductants in diatoms is controlled by a feedback mechanism between intermediate metabolites, the redox state of the plastid and the expression and binding of transcription factors related to stress responses.

Keywords: *Phaeodactylum tricornutum*, nitrate reductase, lipids, RNAi, photosynthesis, GSE67449.

INTRODUCTION

When unicellular algae are grown under optimal conditions, carbon and nitrogen metabolism are tightly coupled and the fluxes of these two elements into macromolecules are largely pre-determined (Parsons et al., 1961; Orcutt and Patterson, 1975; Allen et al., 2011). While the first priority of an algal cell under balanced growth is to assimilate nitrogen into proteins (Myers, 1980), the carbon and reductants for these biosynthetic processes are primarily supplied by photosynthetic processes and the tricarboxylic acid (TCA) cycle. In diatoms, secondary eukaryotes that are ecologically prominent in present-day aquatic ecosystems, about 40% of the photosynthetically fixed carbon is incorporated into protein under optimal growth (Parsons et al., 1961; Badour and Gergis, 1965; Levitan et al., 2015). However, when cells are deprived of nutrients, the fate of carbon and nitrogen can be significantly altered. For example, when diatoms are stressed for nitrogen, only about 20% of the photosynthetically fixed carbon is allocated towards protein while more than 50% is allocated towards storage molecules that require little nitrogen, primarily lipids (Shifrin and Chisholm, 1981; Guerra et al., 2013; Levitan et al., 2015). How algal cells redirect carbon to specific end products is largely unknown. In this study, we used a reverse genetics approach to metabolically restrict the assimilation of inorganic nitrogen into the cells. Our primary goal was to elucidate how the intermediate metabolism of carbon...
and nitrogen are coupled and regulated in the fully sequenced, transformable model diatom *Phaeodactylum tricornutum* (Bowler et al., 2008).

In the 1960s and early 1970s careful laboratory studies revealed that the rate-limiting reaction in the assimilation of nitrate in unicellular algae is the reduction of the molecule to nitrite (Eppley et al., 1969, 1971; Eppley and Rogers, 1970; Berges and Harrison, 1995). This reaction is catalyzed by the molybdenum-containing protein nitrate reductase (NR; EC 1.7.1.1, protein ID 54983), which is coupled to the oxidation of NAD(P)H. In diatoms, the enzyme is encoded in the nucleus and, unlike in higher plants, its expression is repressed by ammonium or other reduced nitrogen sources (Cresswell and Syrett, 1979). Historically, NR activity in diatoms has been used as a diagnostic indicator for rapid assimilation (i.e. a ‘shift-up’) of nitrate (Dugdale et al., 1990; Smith et al., 1992; Allen et al., 2005). Using an RNA interference (RNAi)-induced silencing approach we generated 170 transformants and selected the one with the lowest enzymatic activity for extensive characterization (Levitan et al., 2015).

We previously reported that the knock-down transformant (NR21) had a level of NR mRNA, protein expression and enzymatic activity about 50% lower than the wild type (WT), but it simultaneously accumulated over 40% more lipids with a decrease in growth rate of only about 30% (Levitan et al., 2015). Based on the initial experimental results, we suggested a negative feedback mechanism that couples photosynthetic carbon fixation to lipid biosynthesis and is modulated by the nitrogen assimilation pathway (i.e. NR). Furthermore, we proposed that NR21 could be used as a model to elucidate the regulation of carbon and nitrogen metabolism. Here we present a comprehensive analysis of NR21, including a full physiological and biochemical characterization, carbon allocation data, transcriptomic analysis, a large set of measurements of the intermediate metabolite pool and the calculated quantum requirements for carbon, nitrogen, lipids, carbohydrates and proteins.

**RESULTS AND DISCUSSION**

**The response of nitrogen-starved WT and NR21 to nitrate induction**

To understand the differences in NR expression and activity of NR21 and the WT we nitrogen-stressed the cells for 48 h and then followed their responses to a saturating pulse (880 μM) of nitrate. At all time points during the first 12 h of recovery, NR expression and activity in NR21 were 40–70% of the WT values (Figure 1a). In both the WT and the transformant, the transcript abundance of NR declined by two orders of magnitude within the first 12 h, and was significantly different between the WT and NR21 at all time points (Figure 1b). The decline followed first-order kinetics with rate constants of 0.15 and 0.29 h⁻¹ for the WT and NR21, respectively. No other proteins appeared to be affected to this extent; for example, RbcL remained essentially constant over the 12-h recovery period, and the total amount of protein did not change between the WT and NR21 (Figure S1). These results clearly indicate that the RNAi construct, designed to match a sequence in the NADH-binding site in the enzyme, effectively knocked down expression and activity of NR. If NR is the rate-limiting step in the assimilation of nitrate, a constant decrease in its activity of about 50% should be reflected in the nitrogen-related physiology.

**Physiological and biochemical characteristics of NR21**

During the exponential phase at light saturation, the maximum growth rate of the NR21 transformant was only about 30% lower than that of the WT, yet the transformant had 43% more fatty acid (FA; measured as the methyl ester derivatives; Table 1). This difference was further reflected by a 7% increase in the allocation of photosynthetically fixed carbon into FA in NR21 (Figure 2). The increase in the allocation of carbon into FA in NR21 was accompanied by decreases of 5 and 8% in the allocation of the fixed carbon into carbohydrates and protein, respectively (Figure 2). In contrast to nitrogen-stressed WT, NR21 had virtually the
same maximum quantum efficiency of photochemistry ($F_{v}/F_{M}$), effective absorption cross-section of photosystem II ($\sigma_{PSII}$), efficiency of energy transfer between photosynthetic units (‘connectivity factor’ $p$) and rates of electron transport on the acceptor side of photosystem II (PSII) as the WT (Table 1). These results clearly show that, unlike the nitrogen-stressed WT cells (e.g. Levitan et al., 2015), the photochemical energy-conversion efficiency of PSII in NR21 is virtually identical to that of the WT.

Although nitrogen-stressed WT cells become chlorotic, the cellular chlorophyll content in NR21 was about 13% higher than in the WT; however, NR21 had a higher carbon content per cell. Consequently, the ratio of chlorophyll to carbon (by mass) was 15% lower than in the WT. Moreover, the optical absorption cross-section normalized to chlorophyll $a$ ($a^{*}$) (Falkowski and Raven, 2007) was about 30% lower in NR21 than in the WT. The decrease in $a^{*}$ in NR21 reflects an increased package effect in the plastid (i.e. a higher density of photosynthetic pigments per area of physical cross-section) (Berner et al., 1989). In unicellular algae, all other things being equal, a decrease in $a^{*}$ inevitably leads to an increase in the quantum requirement of photosynthesis (Falkowski and Raven, 2007). Indeed, the minimum quantum requirement for carbon fixation in NR21 was about 25% higher than the WT; however, photosynthesis at light saturation, which is independent of $a^{*}$, was only about 11% lower (Table 1).

The relatively subtle changes in photosynthetic reactions between the WT and NR21 were accompanied by profound changes in the allocation of absorbed light energy to intermediates in metabolism. On a bulk compositional level, changes in allocation of carbon were reflected in the elemental stoichiometry of the cell, with NR21 having 30% more total cellular carbon and an approximately 30% higher C:N ratio (Table 1). We compared the quanta allocated to specific end-products under exponential growth at the growth irradiance of 120 $\mu$ mol quanta m$^{-2}$ sec$^{-1}$. This light intensity is approximately equal to the $E_{k}$ value for growth (Frada et al., 2013; Guerra et al., 2013), which is the irradiance at which the rate of photon absorption matches the rate of electron output (Falkowski and Raven, 2007). This comparison revealed that NR21 had a 13% lower quantum requirement for total carbon assimilation and an 11% higher quantum requirement for nitrogen assimilation. Perhaps counter intuitively, despite the increase in the quantum requirement for nitrogen assimilation in NR21, total protein content was virtually unchanged in the transformant (Table 1, Figure S1).

Additionally, we examined how the redox state of the plastoquinone (PQ) pool varied under ambient light in both the WT and the NR21 transformant. This analysis revealed that in both WT and NR21 the PQ pool was fully oxidized in the dark and the PQ pool of the WT was reduced by about 50% only under high light (>500 $\mu$ mol quanta m$^{-2}$ sec$^{-1}$), However, the reduction of the PQ pool in NR21 occurred at much lower irradiance levels compared with the WT (Figure 3). As a result, at the optimal growth irradiance of 120 $\mu$ mol quanta m$^{-2}$ sec$^{-1}$ (Frada et al., 2013) the
PQ pool was >90% oxidized in the WT, yet was about 50% reduced in NR21. The sharp contrast in the reduction of the PQ pool between the WT and NR21 reveals that nitrate reduction plays a significant role in photosynthetic electron transport kinetics on the acceptor side of PSII. One of the key signal transduction pathways in unicellular algae that couples intermediate metabolites to gene expression is mediated by the redox state of the PQ pool (Escoubas et al., 1995; Pfannschmidt, 2003). For example, in Chlamydomonas under photoautotrophic growth, the expression of NR is strongly downregulated when the PQ pool is highly reduced (Giordano et al., 2005). Although such a retrograde signal may exist in diatoms, the redox state of the PQ pool is also potentially influenced by reductant exported from the plastid to the mitochondria (Bailleul et al., 2015). What are the rate-limiting step(s) and the sinks for the photochemically produced reductant?

Inspection of the calculated quantum requirements for various cellular pools reveals that FAs are the primary sink for the photosynthetically generated electrons in the WT (Table 1). In NR21, the increase in FA biosynthesis comes at the expense of nitrogen assimilation. Previously we used physiological measurements to calculate the fraction of reductants (NAD(P)H) used for FA biosynthesis (Falkowski et al., 1985; Levitan et al., 2015). Based in part on that calculation, we hypothesized that there could be an increased pool of NAD(P)H in NR21. Indeed, analyses of intermediate metabolites in NR21 indicated a 4.7-fold increase in NADPH and a 2.3-fold increase in malonyl-CoA (Table 2). Both of these intermediates are critical for lipid biosynthesis, but neither was enriched under nitrogen stress (Guerra et al., 2013). Although both the nitrogen-stressed WT and nitrogen-replete NR21 exhibit major increases in lipid content, when compared the latter has a quantum requirement for lipid biosynthesis that is about 50% lower, namely 317 mol quanta per mol carbon incorporated in to FAs (C FA) compared with 154 for nitrogen-replete NR21 (Table 1) (Guerra et al., 2013).

In the nitrogen-stressed WT, intermediate metabolism is remodeled and the nitrogen stored in proteins is scavenged by the urea and glutamine synthetase/glutamine 2-oxoglutarate aminotransferase pathways while the photosynthetic fluxes of carbon and reductants are simultaneously used to synthesize lipids (Allen et al., 2011; Levitan et al., 2015). The profile of intermediate metabolites in the nitrogen-replete NR21 was different from that of the nitrogen-stressed WT, or of the nitrogen-replete WT grown in the presence of tungsten, a chemical inhibitor of NR (Table 2) (Guerra et al., 2013). For example, under nitrogen stress, the 2-oxoglutarate pool in the WT increased by 18-fold relative to nitrogen-replete conditions. In contrast, the pool of this key intermediate was only 2-fold and 1.2-fold...
Knocking down NR enhances lipids in a diatom

To understand the effect of the knocking down NR on nuclear gene expression, we analyzed the transcript abundance (mRNA sequencing) between the nitrogen-replete and nitrogen-stressed WT and NR21 (Data S1, Figures S3–S6). We re-assigned Gene Ontology (GO) terms to all the annotated genes in *P. tricornutum*'s genome (http://genontology.org/) (Data S2) and performed enrichment analyses of the differentially expressed genes (Figures 4 and S2). Under nitrogen-replete conditions, 90% of the NR21 genes were non-differentially expressed (NDE) relative to the WT (Data S1, Figures S3–S6). However, differential expression was observed between nitrogen-stressed and nitrogen-replete conditions in both NR21 and the WT. In general, under nitrogen-stress, both the WT and NR21 exhibit downregulation of transcripts encoding genes related to lipid and carbohydrate metabolism, chlorophyll biosynthesis and photosynthesis (Figures 4 and S2, Data S1). This basic pattern is consistent with metabolic remodeling of intermediate metabolism around key hubs that redirect carbon and nitrogen, previously observed in the WT (Levitan et al., 2015). In contrast, under nitrogen-replete conditions, genes linked to lipid metabolism, organelle and signal transduction were upregulated in NR21 relative to the WT (Figures 4 and S4). The upregulation of lipid metabolism genes in NR21, together with the increased in the malonyl-CoA pool size, reveals that lipid metabolism fundamentally differs between nitrogen-replete NR21 and the nitrogen-stressed WT.

Analysis of transcript abundance of transcription factors

Changes in the expression of transcription factors (TFs) can often control the physiological responses of cells, including intermediate metabolism (Courchesne et al., 2009). Of the several hundred putative TFs in the *P. tricornutum* genome, 20 were differentially expressed (DE) between nitrogen-replete WT and NR21. For most of these genes, the expression pattern was similar for both nitrogen-stressed WT and nitrogen-repepete NR21. Four genes (43363, 49557, 14805 and 55070) were upregulated in the WT under conditions of nitrogen stress but were downregulated in nitrogen-repepete NR21. Three of these genes (43363, 49557, 55070) correspond to heat shock factors.

**Table 2** Abundance of key cellular metabolites for nitrogen-replete and exponentially growing NR21 relative to WT *P. tricornutum*. The molecular abundance was determined by liquid chromatography–mass spectrometry. Mean ± 1 SD (n = 3)

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Metabolite</th>
<th>NR21/WT fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Glutamate</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Pyr Nuc</td>
<td>NADP+</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>NAD+</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CEC</td>
<td>ATP</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>AMP</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>Sucrose</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>ADP-Glu</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>UDP-Glu</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>RuBP</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6PG</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Central carbon metabolism</td>
<td>R5P</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>X5P</td>
<td>1.6 ± 0.4</td>
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<tr>
<td></td>
<td>G1P</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>G6P</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>GAP</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>DHAP</td>
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</tr>
<tr>
<td></td>
<td>3PG</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PEP</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Citrate</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>AKG</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>0.9 ± 0.4</td>
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<tr>
<td></td>
<td>Oxaloacetate</td>
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</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>AcCoA</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>FA metabolism</td>
<td>MaCoA</td>
<td>2.3 ± 0.7</td>
</tr>
</tbody>
</table>

AA, amino acids; AcCoA, acetyl-CoA; AMP, ADP, ATP, adenosyl-(mono,di,tri)phosphate; CEC, adenylate cell energy charge; AKG, 2-oxoglutarate (=ketoglutarate); NAD+, NADH nicotinamide adenine dinucleotide; NADP+, NADPH, nicotinamide adenine dinucleotide phosphate; DHAP, dihydroxyacetone; FA, fatty acids; FBP, fructose bis-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; G1P, glucose-1-phosphate; GLN, glutamine; GLU, glutamate; MaCoA, malonyl-CoA; PEP, phosphoenolpyruvate; Pyr Nuc, pyridine nucleotides; RuBP, ribulose-1,5-bisphosphate; R5P, ribulose-5-phosphate; UDP-Glc, UDP-glucose; X5P, xylulose-5-phosphate; 3PG, 3-phosphoglycerate; 6PG, 6-P-gluconate.

Larger in nitrogen-replete NR21 and tungsten-enriched WT, respectively (Table 2). However, it seems that regardless of how we limit the nitrogen supply to the cells, there is an increase in the glutamate-to-glutamine (glu/gln) ratio in the cell. This phenomenon was found in nitrogen-stressed cells and in cells grown in the presence of tungsten, in which the glu/gln ratio was between 2- and 7-fold (Frada et al., 2013; Guerra et al., 2013). In agreement with these findings, genetically knocking down NR resulted in a 12-fold increase in the glu/gln ratio (Table 2). This glutamate can then be metabolized into 2-oxoglutarate that can fuel lipid production. These results suggest that the relative increase in the abundance of cellular glutamate (i.e. the glu/gln ratio) is a signal for remodeling intermediate metabolism. Thus, the 12-fold increase in glu/gln in exponentially growing NR21 is one potential signal that nitrogen assimilation (and hence protein biosynthesis) is restricted, resulting in a redirection of carbon towards lipids. How is this signal mediated in the transformant?
(HSFs), which are the most abundant family of TFs in the genome of this organism (Figure 5, Data S1 and S2) (Rayko et al., 2010). The HSFs are known to activate the expression of heat shock genes in response to stress by binding to elements found in the promoter region of the target genes. The downregulation of HSFs in nitrogen-replete NR21, versus their overall upregulation under nitrogen stress (Levitan et al., 2015). Six additional genes (490099, 41108, 47278, 39786, 32183 and 40994) from the bZIP, Myb and HSF families, were significantly upregulated in the nitrogen-replete NR21 in comparison with the nitrogen-replete WT (Figure 5). These genes were either downregulated or not differentially expressed in nitrogen-stressed NR21 compared with the nitrogen-replete NR21, meaning they are upregulated in the nitrogen-stressed NR21 when compared with the control WT treatment. Four of these seven genes were also upregulated when the WT was subject to nitrogen stress (Figure 5). The three most upregulated TFs (39786, 32183 and 40994) were also significantly upregulated in the nitrogen-stressed WT (Figure 5, Data S1). We suggest that an upregulation of these genes is linked to the accumulation of lipid regardless of the sensing of environmentally available inorganic nitrogen.

**Uncoupling of the carbon and nitrogen metabolic pathways**

Under nitrogen-stressed conditions, the abundance of NR mRNA increased in the WT relative to that of nitrogen-replete cells (Guerra et al., 2013; Yang et al., 2013; Levitan et al., 2015). A similar phenomenon was also found in NR21 (Figure 1, Data S1). While we note that transcript levels may not be correlated with those of proteins or with enzymatic activity, the phenotype resulting from the knock down of NR clearly had altered lipid metabolism. However, our observations imply that the allocation of photosynthetically fixed carbon towards lipid biosynthesis in NR21 fundamentally differs from that of nitrogen-stressed WT cells. Unlike in nitrogen-stressed WT cells, lipid accumulation in nitrogen-replete NR21 was not accompanied by a change in expression of the genes involved in the glutamate-glutamine-2-oxoglutarate hub or the urea cycle (Data S1) (Levitan et al., 2015). Rather, NR21 shunts more carbon and reductant towards lipid precursors, especially malonyl-CoA, and NADPH, and possibly also 2-oxoglutarate and succinyl-CoA (Table 2). These results suggest that NR transcript and/or the NR protein is potentially involved in the recruitment of TFs that influence intermediate metabolism.

Inspection of the 400-bp region upstream of the start codon of the NR gene reveals several putative binding sites for TFs based on homology with plants. These...
include: WRKI, known to act in the defense response, signaling and secondary metabolism; EIN3, known to respond to hormones; E2F, linked to cell cycle regulation; LEC1, a key regulator in FA biosynthesis; and bZIP, which can influence the omega-3 fatty acid content in seeds (Figure 6). Of the differentially expressed TFs three were annotated as bZIP TFs and one as an E2F (Figure 5).

**CONCLUSIONS**

Genetically knocking down NR gene expression and activity in *P. tricornutum* strongly influences intermediate metabolic pathways and leads to a redirection of photosynthetically fixed carbon towards lipids with a relatively low metabolic cost (Figures 1 and 2, Tables 1 and 2).
integrated analysis presented here clearly indicates that the accumulation of lipids in NR21 is mediated by a fundamentally different mechanism from that in nitrogen-stressed WT cells. In nitrogen-replete NR21 we detected differential expression of genes from pathways that are distantly related to nitrogen metabolism and specific TFs associated with stress responses (Figures 3–5 and S2; Data S1 and S2). In addition, we observed a much more reduced PQ pool and a significant change in the profile of intermediate metabolites, including a 12-fold increase in the glu/gln ratio in nitrogen-replete, exponentially growing, NR21. Based on this study (Table 2) and others (Frada et al., 2013; Guerra et al., 2013), it appears that an increase in the glu/gln ratio is a common response to nitrogen limitation, regardless of how the limitation is imposed. Thus, we hypothesize that both the glu/gln ratio and the redox state of the PQ (Figure 3), known to be related to intermediate metabolism and NR expression (Escoubas et al., 1995; Giordano et al., 2005; Pfannschmidt, 2003), potentially initiate a signaling cascade that results in shunting photosynthetically fixed carbon into lipids. We further propose that allocation of carbon and reductants in diatoms is controlled by a feedback mechanism between intermediate metabolites, the redox state of the plastid and the expression and binding of TFs to the promoter region of the NR gene. Although the precise mechanism of the phenomenon remains to be elucidated, our results clearly establish that knocking down a single gene in this model diatom can dramatically alter how carbon is distributed between the major pools of macromolecules.

EXPERIMENTAL PROCEDURES

Transformation and selection of the NR21 knock-down

A detailed description of the construction of the inverted repeat RNAi construct for silencing of the NR gene, the transformation protocol and the method for selecting the most suitable transformant (NR21) has been previously published (Levitan et al., 2015). Briefly, the RNAi construct was generated using standard molecular cloning methods for RNAi based on the pKS-Fa plasmid (De Riso et al., 2009). Our pKS-Sh ble nir-F vector was inserted into P. tricornutum using a PDS-1000/He Particle delivery system (Bio-Rad, http://www.bio-rad.com/) as previously described (Falcicatore et al., 1999). The insertion was verified by PCR of the antibiotic resistance marker. To select for the best transformant, survival curves were obtained by growing the transformants on 17 mM potassium chloride (KClO3) (Cove, 1976a,b; Levitan et al., 2015). NR21 was selected as the most suitable strain for further studies.

Cultivation and experimental planning

Cultivation of the P. tricornutum strain (accession Pt1, 8.6) (Martino et al., 2007), the NR21 transformant and nitrogen-stress experiments were done as previously described (Guerra et al., 2013; Levitan et al., 2015). To characterize the NR21 transformant we sampled optically thin cultures under exponential growth (with nitrate as their sole nitrogen source) under nitrogen-stressed conditions, nitrogen-replete conditions and at different time points upon recovery from nitrogen stress. Recovery from nitrogen stress was induced by spiking cultures, that were cultured in nitrogen-free media for 48 h, with 880 μM nitrate as the sole nitrogen source.

Analysis of physiological parameters

Growth rates of optically thin cultures were calculated from changes in their cell densities over time, \( u = \ln(N_t)/N_0/\Delta t \), where \( N_t \) and \( N_0 \) refer to the end and initial cell concentrations, respectively, and \( \Delta t \) is the time between sampling points. Cell numbers were determined using a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., https://www.beckmancoulter.com/). The cellular carbon and nitrogen content was measured from cells collected on pre-combusted 13-mm A/E filters (Pall Gelman, http://www.pall.com/) and analyzed on a CHN analyzer (Na 1500 series 2, Carlo Erba Instruments, CE Elantech Inc., http://www.ceelantech.com/).

For measuring total fatty acids (FAMES) 5 × 10^6 cells were filtered onto 25-mm GF/F filters (Whatman, http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryID=1050498&catalogId=10101&productId=1&storeID=11787&langId=-1) and subsequently extracted and transesterified in a single step. Analysis of the hexane fraction containing the FAMES was performed by gas chromatography (Rodriguez-Ruiz et al., 1998; Guerra et al., 2013).

Carbohydrate content was determined from about 5 × 10^6 cells collected on 1.2-μm pore size polycarbonate filters (Millipore, http://www.emdmillipore.com/). Total carbohydrate levels (intracellular reducing pentoses and hexoses) were measured by reaction with anthrone using a protocol adapted to diatoms (Trevelyan and Harrison, 1952; Post et al., 1985). For determination of the amounts of total protein subunits, P. tricornutum cells were spun for 8 min at 6000 × g using a Sorvall RC6 centrifuge (Thermo Scientific, http://www.thermoscientific.com/) set to 4°C. The samples were subsequently extracted as previously described (Guerra et al., 2013; Levitan et al., 2015) and the total protein concentration was measured using a modified Lowry assay (Bio-Rad DC protein assay kit, Bio-Rad, http://www.bio-rad.com/) using bovine serum albumin (BSA) as the comparative protein standard.

Chlorophyll a was measured spectrophotometrically from cells collected on 13-mm glass fiber filters (GF/F) (Whatman) and extracted in 90% acetone (Jeffrey and Humphrey, 1975). The optical absorption cross-sections normalized to chlorophyll a, referred to as \( a^* \), was measured in vivo using optically thin cultures on a SLM-Aminco DW-2000 spectrophotometer (Falkowski et al., 1985; Dubinsky et al., 1986). Biophysical characteristics of PSII were measured using a modified Lowry assay (Bio-Rad DC 500-0111) and a SpectraMax M3 microplate reader (Molecular Devices, http://www.moleculardevices.com/) at 750 nm. Bovine serum albumin (BSA) was used as the comparative protein standard.

Analysis of physiological parameters

Growth rates of optically thin cultures were calculated from changes in their cell densities over time, \( u = \ln(N_t)/N_0/\Delta t \), where \( N_t \) and \( N_0 \) refer to the end and initial cell concentrations, respectively, and \( \Delta t \) is the time between sampling points. Cell numbers were determined using a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., https://www.beckmancoulter.com/). The cellular carbon and nitrogen content was measured from cells collected on pre-combusted 13-mm A/E filters (Pall Gelman, http://www.pall.com/) and analyzed on a CHN analyzer (Na 1500 series 2, Carlo Erba Instruments, CE Elantech Inc., http://www.ceelantech.com/).

For measuring total fatty acids (FAMES) 5 × 10^6 cells were filtered onto 25-mm GF/F filters (Whatman, http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryID=1050498&catalogId=10101&productId=1&storeID=11787&langId=-1) and subsequently extracted and transesterified in a single step. Analysis of the hexane fraction containing the FAMES was performed by gas chromatography (Rodriguez-Ruiz et al., 1998; Guerra et al., 2013).

Carbohydrate content was determined from about 5 × 10^6 cells collected on 1.2-μm pore size polycarbonate filters (Millipore, http://www.emdmillipore.com/). Total carbohydrate levels (intracellular reducing pentoses and hexoses) were measured by reaction with anthrone using a protocol adapted to diatoms (Trevelyan and Harrison, 1952; Post et al., 1985). For determination of the amounts of total protein subunits, P. tricornutum cells were spun for 8 min at 6000 × g using a Sorvall RC6 centrifuge (Thermo Scientific, http://www.thermoscientific.com/) set to 4°C. The samples were subsequently extracted as previously described (Guerra et al., 2013; Levitan et al., 2015) and the total protein concentration was measured using a modified Lowry assay (Bio-Rad DC 500-0111) and a SpectraMax M3 microplate reader (Molecular Devices, http://www.moleculardevices.com/) at 750 nm. Bovine serum albumin (BSA) was used as the comparative protein standard.

Chlorophyll a was measured spectrophotometrically from cells collected on 13-mm glass fiber filters (GF/F) (Whatman) and extracted in 90% acetone (Jeffrey and Humphrey, 1975). The optical absorption cross-sections normalized to chlorophyll a, referred to as \( a^* \), was measured in vivo using optically thin cultures on an SLM-Aminco DW-2000 spectrophotometer (Falkowski et al., 1985; Dubinsky et al., 1986). Biophysical characteristics of PSII were measured using a custom built fluorescence induction and relaxation (FIRE) instrument (Kolber et al., 1998; Falkowski et al., 2004; Gorbonov and Falkowski, 2004). The kinetics of the single-turnover saturating flash were analyzed to obtain the maximum quantum efficiency of photochemistry (Fv/Fm), and the effective absorption cross-section of PSII (\( \alpha_{PSII} \)). Analysis of the relaxation kinetics of fluorescence yield following the single-turnover saturating flash provided the rates of electron transport on the acceptor side of PSII (i.e. rates of re-oxidation of the primary quine acceptor) (Kolber et al., 1988).

The redox state of the PQ pool was assessed from the analysis of fluorescence induction curves on a millisecond time scale in response to a saturating multiple turnover flash of 200-msec duration. To take into account irradiance-induced effects of non-photochemical quenching of chlorophyll fluorescence, the variable
component (Fv) of the induction curve was normalized to unity at each irradiance light level. The fraction of the reduced PQ molecules as a function of ambient irradiance was estimated from a reduction in the area above the normalized induction curve relative to the reference area measured for the fully oxidized PQ pool (Whitmarsh and Ort, 1984; Falkowski and Raven, 2007; Tóth et al., 2007). This reference area was calculated from the induction curve recorded in darkness following a short (about 2 sec) pre-exposure to far-red light (~700 nm).

Quantum requirement calculations

Quantum requirements for the incorporation of carbon, nitrogen and photosynthetically fixed carbon into lipid, protein and carbohydrate were calculated as previously described (Falkowski et al., 1985; Falkowski and Raven, 2007; Frada et al., 2013). The calculations followed the equation:

\[
\frac{1}{\Phi} = \text{[moles of photons absorbed per mole } X \text{ incorporated/produced]} = \left[ a' \times (\text{Chl } a/\text{biomass parameter}) \times E \times \text{unit conversion factor}/(\mu\text{mol photons } m^{-2} \text{ sec}^{-1}) \right]
\]

where \(a'\) is the spectrally integrated in vivo optical absorption cross-section (\(m^2 \text{ mg}^{-1} \text{ Chl } a\)); \(Chl \ a\) is the cellular chlorophyll content in pg Chl/cell; the biomass parameter, \(X\), represents the incorporated biomass units (C, N, CFA, CProtein, Ccarbohydrate) in pg cell\(^{-1}\); \(E\) is the point of saturating irradiance (\(\mu\text{mol photons } m^{-2} \text{ sec}^{-1}\)); the conversion factor is used to obtain units in moles and days (\(1.04 \times 10^{12}\) for C, \(1.21 \times 10^{12}\) for N, \(1.36 \times 10^{12}\) for CFA, \(2.36 \times 10^{12}\) for CProtein, \(2.36 \times 10^{12}\) for Ccarbohydrate); and \(\mu\) is the maximum growth rate as described in Analysis of physiological parameters. For the parameters CFA, CProtein and Ccarbohydrate the calculation of the conversion factors included the estimation that carbon is approximately 74, 44 and 40% of each macromolecule, respectively (Guerra et al., 2013; Levitan et al., 2015).

Protein quantification and NR characterization

The abundance of NR and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RbcL) were determined by Western blotting as previously described (Guerra et al., 2013; Levitan et al., 2015). Samples for quantitative polymerase chain reaction (qPCR) used for measuring the transcript abundance of the NR mRNA were analyzed as previously described (Guerra et al., 2013; Levitan et al., 2015). The enzymatic activity of NR was measured following Berges and Harrison (1995) and Levitan et al. (2015).

Intermediate metabolite determination

Determination of the concentration of intermediate metabolites was performed by liquid chromatography mass spectrometry (LC-MS/MS). Cells were filtered onto a 0.45-µm pore size nylon filter (Pall) and immediately cold extracted (Bennette et al., 2011). Samples were run on a 1200-series Agilent LC system coupled to an Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies, http://www.agilent.com/), using previously optimized selective reaction monitoring parameters (Bennette et al., 2011). For a detailed description of this method see Frada et al. (2013) and Guerra et al. (2013).

Messenger RNA sequencing

Samples for mRNASeq were harvested and extracted from nitrogen-replete and nitrogen-stressed cultures of both the WT and NR21 as described previously (Levitan et al., 2015). TruSeq RNA (Illumina, https://www.illumina.com/) was used to prepare mRNA libraries for each of the six samples according to the manufacturer’s instructions. The 50-bp single-ended libraries were multiplexed and sequenced on an Illumina MiSeq platform. The raw reads were trimmed for adaptor sequences and low-quality sequences and then aligned to the P. tricornutum version 2.0 set 10 402 filtered gene models (http://genome.jgi.doe.gov/Phatr2/Phatr2.info.html) using CLC Genomics Workbench v.6.02 (http://www.clcbio.com/), and filtered to retrieve uniquely aligned reads with no more than three mismatches. Gene counts were used for differential expression analysis carried out using the DESeq nBBIOCONDUCTOR package (Anders and Huber, 2010) which infers differential expression based on the negative binomial distribution. For this analysis we used a cutoff of 5% to control for false detection rate (FDR, false positives), and considered only genes that had a log2-fold change $\geq \pm 2$ and FDR < 0.05 to be differentially expressed.

Bioinformatics analyses of GO terms

We used BLAST2GO software to re-assign GO terms to all of the gene models found in the default analysis track of the P. tricornutum genome on the JGI database (http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Phatr2). This analysis took into consideration all the data currently available online regarding GO terms for genes from other organisms, thus providing a more complete understanding of the many hypothetical proteins in the P. tricornutum genome in comparison with the online databases (https://www.blast2go.com/). This was used to perform enrichment analysis for understanding the cellular processes and functions that were either up- or downregulated in the WT and the NR21 knock down under nitrogen-stressed and nitrogen-replete conditions.

Prediction of binding sites for regulatory elements

Prediction of possible binding sites in the 400-bp upstream to the NR gene coding region was done using the NSITE-PL online tool for recognition of PLANT regulatory motifs with statistics by ScirBerry (http://linux1.softberry.com/berry.phtml?topic=nsitep&group=programs&subgroup=promoter).

Accession numbers

The output of DESeq for all 10 402 genes was submitted to the Gene Expression Omnibus (GEO) under accession number GSE67449 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=odkhagsujlfhmnn&acc=GSE67449).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Comparison of the relative abundance of the large subunit (RbcL) of ribulose-1,5-bisphosphate carboxylase/oxygenase and total protein concentration between wild-type *Phaeodactylum tricornutum* and NR21 following the addition of 880 μM nitrate to nitrogen-stressed cultures.

**Figure S2.** Enrichment analysis results of differentially expressed genes based on the mRNA sequencing data and Gene Ontology terms for wild-type *Phaeodactylum tricornutum* and NR21 under nitrogen-replete and nitrogen-stressed conditions.

**Figure S3.** Changes in the relative transcript abundance of genes involved in central carbon and nitrogen biosynthesis for nitrogen-replete NR21/nitrogen-replete wild-type *Phaeodactylum tricornutum*.

**Figure S4.** Changes in the relative transcript abundance of genes involved in lipid metabolism for nitrogen-replete NR21/nitrogen-replete wild-type *Phaeodactylum tricornutum*.

**Figure S5.** Changes in the relative transcript abundance of genes involved in central carbon and nitrogen biosynthesis for nitrogen-replete NR21/nitrogen-stressed wild-type *Phaeodactylum tricornutum*.

**Figure S6.** Changes in the relative transcript abundance of genes involved in lipid metabolism for nitrogen-replete NR21/nitrogen-stressed wild-type *Phaeodactylum tricornutum*.

**Data S1.** Summary of the mRNA sequencing analysis of wild-type *Phaeodactylum tricornutum* and the NR21 knock-down transformant grown under nitrogen-stressed and nitrogen-replete conditions.

**Data S2.** Summary of the Gene Ontology term re-assignment of the 10 402 genes in the genome of *Phaeodactylum tricornutum*.

**REFERENCES**


