Like many microalgae, *Chlamydomonas reinhardtii* forms lipid droplets rich in triacylglycerols when nutrient deprived. To begin studying the mechanisms underlying this process, nitrogen (N) deprivation was used to induce triacylglycerol accumulation and changes in developmental programs such as gametogenesis. Comparative global analysis of transcripts under induced and noninduced conditions was applied as a first approach to studying molecular changes that promote or accompany triacylglycerol accumulation in cells encountering a new nutrient environment. Towards this goal, high-throughput sequencing technology was employed to generate large numbers of expressed sequence tags of eight biologically independent libraries, four for each condition, N replete and N deprived, allowing a statistically sound comparison of expression levels under the two tested conditions. As expected, N deprivation activated a subset of control genes involved in gametogenesis while down-regulating triacylglycerol accumulation blocks by the glyoxylate cycle and gluconeogenesis but funneled directly into fatty acid biosynthesis. Additional fatty acids may be produced by membrane remodeling, a process that is suggested by the changes observed in transcript abundance of putative lipase genes. Inferences on metabolism based on transcriptional analysis are indirect, but biochemical experiments supported some of these deductions. The data provided here represent a rich source for the exploration of the mechanisms of oil accumulation in microalgae.

The search for sustainable sources of biofuels has led to renewed interest in microalgae as a potential feedstock and, consequently, a flurry of research has recently been initiated in microalgae (Wijffels and Barbosa, 2010). Microalgae accumulate large quantities of oils in the form of triacylglycerols (TAGs) when nutrient deprived, and a thorough analysis of the underlying molecular mechanism is currently in its infancy (Hu et al., 2008). At this time, *Chlamydomonas reinhardtii* is the premier microalgal molecular model for this analysis. As such, the formation of lipid droplets following nitrogen (N) deprivation has recently been documented in detail (Wang et al., 2009; Moellering and Benning, 2010). Although *C. reinhardtii* is not under direct consideration for the production of biomass as a biofuel feedstock, the analysis of its metabolism and physiology is expected to provide basic insights into mechanisms of TAG accumulation relevant to other microalgae at least of the green algal phylum.

The genome of *C. reinhardtii* is available (Merchant et al., 2007), and its annotation is currently at version 4 (http://genome.jgi-psf.org/chlamy/chlamy.home.html). At this time, a number of microarrays have been used to interrogate changes in response to environmental factors (Ledford et al., 2004, 2007; Jamers et al., 2006; Mus et al., 2007; Nguyen et al., 2008; Simon et al., 2008; Yamano et al., 2008; Mustroph et al., 2010). These microarrays could not cover all genes in the genome, but more recently, massively parallel cDNA sequencing approaches were applied to *C. reinhardtii*, overcoming the shortcomings of microarrays (Gonzalez-Ballester et al., 2010). Likewise, we have chosen a cDNA sequencing-based approach using 454 and...
Illumina technologies in parallel that allow the generation of large numbers of ESTs of varying abundance, which can be counted to obtain a measure of gene expression (Weber et al., 2007).

The goal of this study was to determine major changes in gene expression following N deprivation, the nutrient condition established in our previous analysis of lipid droplet formation and TAG accumulation in C. reinhardtii (Moellering and Benning, 2010). Comparison of the transcript levels of induced, N-deprived C. reinhardtii cultures with those of uninduced, N-replete cultures was expected to reflect the metabolic changes leading to TAG accumulation. Of course, making inferences about metabolism based on gene expression levels has its caveats, as gene expression does not necessarily directly translate into metabolic fluxes. To interrogate the meaningfulness of some of the transcript-level changes we observed with regard to metabolism, we also performed labeling experiments using acetate as the precursor. Acetate is a typical carbon source provided to C. reinhardtii for phototrophic growth enabling short doubling times, and it is readily incorporated into fatty acids, the main constituents of TAGs. Keeping in mind that these are clearly conditions optimized for an experimental laboratory system, we nevertheless expect to be able to make basic inferences that will be relevant to a broader understanding of the induction of TAG biosynthesis and lipid droplet accumulation in green algae.

RESULTS

Defining Conditions for N Deprivation of C. reinhardtii

Ideally, one would like to use finely spaced time-course experiments to distinguish rapid versus long-term changes in gene expression following N deprivation. However, because our resources were limited, we decided to focus on two conditions, N replete and N deprived. Independent biological replicates allowed for statistically sound interpretations of the data. To determine the time point for N deprivation most likely to provide an accurate snapshot of readjustment of transcript steady-state levels following N deprivation, we first used northern-blot hybridization to compare transcript levels in N-replete and N-deprived cells. For standardization, equal amounts of RNA were loaded and the 18S rRNA abundance was examined. Although C. reinhardtii ribosomes turn over following N deprivation (Siersma and Chiang, 1971; Martin et al., 1976), their abundance drops no lower than 50% (see below). AMT4 mRNA was absent from the uninduced cells and present at a high level in N-limited or N-deprived cells under the conditions tested. DGTT2 mRNA was present at low levels in all conditions. DGTT3 mRNA was present at low levels and increased slightly following N deprivation. N deprivation for 48 h showed the greatest difference in RNA levels compared with the N-replete cultures. Based on this basic analysis and our previous time-course study of lipid droplet formation and TAG accumulation (Moellering and Benning, 2010), a 48-h period of N deprivation was chosen to compare global transcript levels in N-replete and N-deprived cells.

Global Characteristics of C. reinhardtii Gene Expression following N Deprivation

To determine the differential expression of genes in C. reinhardtii under N-replete and N-deprived conditions, two sequencing approaches, 454 and Illumina, were applied. (The complete combined data set for both experiments is provided in Supplemental Spreadsheet S1.) Read length is longer for 454, but the number of reads per run is lower. As shown in Table I, 60- to 85-fold more sequence tags were generated with Illumina than with 454 sequencing. Among the 454 reads, 78% to 80% mapped to the C. reinhardtii genome. For the Illumina data, we mapped in three different ways, with varying stringency depending on whether 3’-end read quality and exon-spanning reads were considered. Without filtering reads, a substantially smaller proportion of Illumina reads (63%–68%) were mapped com-

![Figure 1. Transcript levels of specific genes. A, Cultures were grown in TAP medium that was N replete (10 mM NH₄⁺; 10), continual N limited (0.5 mM NH₄⁺; 0.5), or N deprived (0 mM NH₄⁺; 0) for 24 or 48 h. The expression levels of AMT4, DGTT2, and DGTT3 were measured by RNA-DNA hybridization, and rRNA was visualized as a loading control. B, Cultures were grown for 48 h in either N-replete or N-deprived conditions. The levels of PSBS1 and PSBS2 transcripts were measured using RT-PCR, and the constitutive IDAS5 gene served as a control.](image-url)
pared with 454 reads. Trimming low-quality 3′ regions of reads resulted in a further 2.7% decrease in the number of mapped reads. Despite the large number of unmapped Illumina reads, out of 16,710 C. reinhardtii gene models, 15,505 (92%) had one or more reads. In contrast, only 6,372 gene models (38.1%) were supported by the 454 transcriptome data set. In addition, nearly all genes covered by 454 were also covered by Illumina. Therefore, our sequencing data covered most annotated genes, enabling us to interrogate differential expression under normal conditions and following N deprivation. In addition, as expected, Illumina data provided a better coverage of the gene space than 454 sequences.

To determine differential gene expression following N deprivation, we modeled count data with a moderated negative binomial distribution (see “Materials and Methods”). Using thresholds of 5% or less false discovery rates and 2-fold or greater change for the Illumina data set, 2,128 and 1,875 genes were categorized as up- and down-regulated, respectively, following N deprivation. To see if fold changes inferred based on 454 and Illumina data sets were consistent, we determined the statistical correlation in fold change between these two data sets (Fig. 2) and found that it was rather weak (Pearson’s correlation coefficient, $r^2 = 0.10$, $P < 2.2 \times 10^{-16}$). There was an apparent anomaly, as 4,313 genes (out of 6,369 genes with one or more reads from both data sets) had a high degree of up- and down-regulation, which was observed with the 454 but not the Illumina data set (Fig. 2A). Most of these genes with extreme

Table 1. Summary of expression tags generated using two different sequencing methods

| Data Type | 454 Sequencing Methods | Illumina Sequencing Methods |
|-----------|------------------------|-----------------------------|
| Treatment |                        |                             |
| N R       | 2.51 $\times 10^{105}$ | 2.01 $\times 10^{105}$      |
| N D       | 1.69 $\times 10^{107}$ | 1.68 $\times 10^{105}$      |
| T1        | 1.83 $\times 10^{107}$ | 1.24 $\times 10^{107}$      |
| T2        | 1.78 $\times 10^{107}$ | 1.15 $\times 10^{107}$      |
| T3        | 1.77 $\times 10^{107}$ | 1.07 $\times 10^{107}$      |
| N1        | 1.79 $\times 10^{107}$ | 1.13 $\times 10^{107}$      |
| N2        | 1.52 $\times 10^{107}$ | 9.86 $\times 10^{106}$      |
| N3        |                       |                             |
| Mapped    | 2.01 $\times 10^{105}$ | 1.32 $\times 10^{105}$      |
| Genic     | 9.30 $\times 10^{104}$ | 9.43 $\times 10^{106}$      |
| Intergenic|                       |                             |

$^{a}$Treatment types: N R, T1, T2, and T3 represent N-replete growth; N D, N1, N2, and N3 represent N-deprived growth. For Illumina, there are three biologically independent replicates for each treatment (T1–T3 and N1–N3). $^{b}$Number of sequencing reads after filtering out low-quality reads based on 454 and Illumina base-calling methods. $^{c}$Number of sequencing reads after mapping to the C. reinhardtii version 4.0 genome. $^{d}$Number of mapped sequencing reads overlapping with C. reinhardtii version 4.0-filtered gene models. $^{e}$Number of mapped sequencing reads not overlapping with any C. reinhardtii version 4.0-filtered gene models.

Figure 2. Fold change correlation between Illumina and 454 data sets and impacts of Illumina length bias on differential expression call. A. Only genes with one or more 454 and Illumina reads under either N-replete (+N) or N-deprived (−N) conditions were plotted. Fold change is determined by the number of reads following N deprivation divided by the number of reads under N-replete conditions for each gene. Genes with 2$^{10}$-fold or greater or 2$^{-10}$-fold or lesser changes, the fold change values were set to 10. Blue circles (“high” 454 read genes) indicate genes with 10 or greater 454 reads (+N and –N combined) and one or more 454 reads in both +N and –N. Red circles (“low” 454 read genes) indicate genes that did not satisfy one or both of the above criteria. B. Each box plot depicts the numbers of reads for protein-coding genes (log base 10) in a protein sequence size bin (0–2,000 amino acids, bin size of 100 amino acids). All proteins of 2,000 or more amino acids are classified as 2,000 amino acids. Outliers are shown in black circles. C. Percentage of genes that are regarded as differentially expressed (DE) in each protein sequence size bin. The line indicates the linear fit, and the equation for the line is shown as well.
responses based on 454 had very low counts (less than 10 reads combined in both conditions or zero reads in one of the conditions; Fig. 2A, red data points). As a result, high and likely inaccurate fold change values were assigned to those genes. In fact, if we only considered 2,056 genes with 10 or more reads combined and one or more reads in both conditions, the correlation between Illumina and 454 data was substantially improved ($r^2 = 0.57, P < 2.2 \times 10^{-10}$; Fig. 2, blue data points).

One important consideration in identifying differentially regulated genes is that there is a considerable transcript length bias in Illumina data. A longer transcript tends to have more reads than a shorter transcript expressed at the same level (Oshlack and Wakefield, 2009; Bullard et al., 2010). Consistent with earlier findings, we observed a significant correlation between the number of reads assigned to a protein sequence and its length (Spearman's rank $\rho = 0.33, P < 2.2e-16$; Fig. 2B). Because of this length bias, longer transcripts may have more significant differences in their transcriptional regulation following N deprivation. These transfrags were joined into 1,828 “intergenic transcriptional units.” Most importantly, 287 of these intergenic transcriptional units were up-regulated and 176 were down-regulated following N deprivation. This finding indicates that, although length bias remains an issue, our differential expression call may not be as significantly affected as reported previously.

Approximately 7% to 14% of the Illumina reads mapped to the “intergenic regions” (Table I). We assembled Illumina reads into 42,574 transcribed fragments (transfrags). Among them, 17,095 transfrags did not map with, or within the vicinity of (1,855 bases, 99th percentile intron length), current gene models. With the same conservative criterion, transfrags were joined into 1,828 “intergenic transcriptional units.” Most importantly, 287 of these intergenic transcriptional units were up-regulated and 176 were down-regulated following N deprivation. These transfrags are unannotated genes that require further analysis to establish their authenticity.

Gene Ontology (GO) annotation was used to coarsely identify major categories of genes involved in particular biological processes to assess trends in their transcriptional regulation following N deprivation. We found multiple GO categories with significant enrichment in their numbers of differentially regulated genes (Table II). Particularly, genes associated with lipid metabolism tend to be up-regulated, while those involved in photosynthesis and DNA replication initiation tend to be down-regulated.

### Table II. GO categories significantly enriched in differentially regulated C. reinhardtii genes

| GO Category | Annotation | GO R | No GO R | GO U | No GO U | Reg | $P^6$ | $q^6$ |
|-------------|------------|------|---------|------|---------|-----|------|------|
| GO:0006270 bp | DNA replication initiation | 9 | 926 | 0 | 6,633 | Down | 6.48 x 10^{-9} | 4.51 x 10^{-6} |
| GO:0015979 bp | Photosynthesis | 23 | 912 | 0 | 6,633 | Down | 1.02 x 10^{-10} | 1.78 x 10^{-7} |
| GO:0005767 cc | Extracellular region | 11 | 924 | 0 | 6,619 | Down | 6.32 x 10^{-6} | 1.76 x 10^{-4} |
| GO:0009522 cc | PSI | 4 | 931 | 0 | 6,633 | Down | 2.32 x 10^{-4} | 5.94 x 10^{-3} |
| GO:0009538 cc | PSI reaction center | 5 | 930 | 0 | 6,633 | Down | 2.85 x 10^{-4} | 1.79 x 10^{-4} |
| GO:0009654 cc | Oxygen-evolving complex | 6 | 929 | 0 | 6,633 | Down | 3.51 x 10^{-3} | 7.32 x 10^{-3} |
| GO:0019988 cc | Extrinsic to membrane | 4 | 931 | 0 | 6,633 | Down | 2.32 x 10^{-4} | 5.94 x 10^{-3} |
| GO:003755 mfd | Peptidyl-prolyl cis-trans-isomerase activity | 21 | 914 | 32 | 6,601 | Down | 4.39 x 10^{-7} | 1.83 x 10^{-4} |
| GO:004600 mfd | Cyclophilin | 19 | 916 | 30 | 6,603 | Down | 2.29 x 10^{-6} | 5.98 x 10^{-4} |
| GO:0016851 mfd | Magnesium chelatase activity | 4 | 931 | 0 | 6,633 | Down | 2.32 x 10^{-4} | 3.23 x 10^{-2} |
| GO:0030051 mfd | FK506-sensitive peptidyl-prolyl cis-trans-isomerase | 19 | 916 | 30 | 6,603 | Down | 2.29 x 10^{-6} | 5.98 x 10^{-4} |
| GO:0042027 mfd | Cyclophilin-type peptidyl-prolyl cis-trans-isomerase activity | 19 | 916 | 30 | 6,603 | Down | 2.29 x 10^{-6} | 5.98 x 10^{-4} |
| GO:0006006 bp | Glc metabolic process | 5 | 944 | 1 | 6,618 | Up | 1.65 x 10^{-4} | 3.83 x 10^{-3} |
| GO:0006468 bp | Protein amino acid phosphorylation | 117 | 832 | 501 | 6,118 | Up | 2.37 x 10^{-6} | 2.47 x 10^{-3} |
| GO:0006629 bp | Lipid metabolic process | 18 | 931 | 38 | 5,581 | Up | 9.92 x 10^{-5} | 2.59 x 10^{-2} |
| GO:0004672 mfd | Protein kinase activity | 113 | 836 | 479 | 6,140 | Up | 2.08 x 10^{-6} | 2.47 x 10^{-3} |
| GO:0004713 mfd | Protein-Tyr kinase activity | 85 | 864 | 342 | 6,277 | Up | 8.04 x 10^{-6} | 4.20 x 10^{-3} |
| GO:0004674 mfd | Protein Ser/Thr kinase activity | 92 | 857 | 391 | 6,228 | Up | 2.50 x 10^{-5} | 7.46 x 10^{-3} |

$^a$GO R, Number of significantly up- or down-regulated (R) genes with the GO annotation in question. $^b$No GO R, Number of significantly up- or down-regulated genes without the GO annotation. $^c$GO U, Number of genes with no significant expression change with the GO annotation. $^d$No GO U, Number of genes with no significant expression change that do not have the GO annotation. $^e$Reg, Direction of regulation (N deprived compared with N replete). $^f$Fisher’s exact test $P$ value. $^g$The $q$ value is calculated using the R package qvalue. $^h$bp, Biological process. $^i$cc, Cellular component. $^mf$, Molecular function.
Induction of Gametogenesis and Sexual Reproduction

Because N deprivation triggers gametogenesis (Martin and Goodenough, 1975; Kurvari et al., 1998), we examined several genes known to be involved in mating-type plus (mt+) gamete differentiation or sexual fusion in C. reinhardtii as internal controls for the induction state of the cells following N deprivation (Supplemental Table S1). Following N deprivation, cells had substantial increases in the abundance of transcripts of four of the six genes considered. These genes encode FUS1, which is a glycoprotein required for sex recognition, SAG1 (the mt+ agglutinin gene), peptidase M gametolysin, which releases the gametes from the cell wall, and NSG13, which is a protein of unidentified function known to be expressed in gametes, as summarized by Harris (2009b). A second gametolysin gene (encoding peptidase M11) and GSP1, which encodes a gamete-specific transcription factor, did not show increased expression following N deprivation, but perhaps that is because only a single time point was examined.

Effects on Genes of N Metabolism and Protein Biosynthesis

Many genes involved in N import and assimilation are known to be induced following N deprivation (Schnell and Lefebvre, 1993; González-Ballester et al., 2004; Fernandez et al., 2009). Our analysis revealed greater than 2-fold up-regulation for several genes, including those that encode NO3− and NO2− transporters and reductases, as well as transport systems for NH4+ and organic N sources (Supplemental Table S2). Of the genes involved in assimilation of NH4+ by the Gln synthetase-Glu synthase cycle, only GLN3 was up-regulated. Similarly, most genes involved in amino acid biosynthesis did not show a greater than 2-fold change. Thus, transcript abundance suggests that following N deprivation, pathways for the acquisition of new N sources are strongly up-regulated, whereas biosynthetic pathways that utilize the assimilated N remain relatively unaffected.

Decades ago, N deprivation of C. reinhardtii was found to result in degradation and resynthesis of both cytoplasmic and chloroplast ribosomes (Siersma and Chiang, 1971; Martin et al., 1976). Both the rRNA and proteins of the ribosomes were turned over under the conditions of N deprivation that also induce gamete differentiation. Hence, we expected that the mRNAs for the ribosomal proteins might show different steady-state levels in the comparison of logarithmically growing cells and cells that have been N deprived for 48 h. Indeed, following N deprivation, the abundance of transcripts encoding proteins of the chloroplast ribosomes consistently decreased to 30% to 50% of their levels of expression in logarithmically growing cells (Supplemental Table S3).

A subset of the cytosolic 80S ribosomal protein genes has been identified in the version 4.0 genome data set. Among those that have been annotated, most are encoded by single-copy genes (Supplemental Table S4), although a few have two copies (e.g. L7, L10, L13, and L23). As these gene products are assembled into ribosomes, the respective genes have high levels of constitutive expression. The abundance of the transcripts in vegetative cells and N-deprived cells was fairly similar, although they are listed in the table starting with those that have the most elevated expression in the latter.

The RPL22 ribosomal protein of the cytosolic ribosomes is encoded by a multigene family in C. reinhardtii. Of the 37 RPL22 genes in version 4.0 of the genome data set, 13 appeared not to be expressed under either condition tested and six had barely detectable levels of transcripts. Of the 18 remaining genes, two gave rise to the most predominant transcripts (Supplemental Table S5, yellow highlighting), and their transcripts did not change markedly in abundance. Four of the 18 genes were moderately expressed, and their transcript levels doubled in the N-deprived cells (Supplemental Table S5, light blue highlighting). Six of the 18 genes had markedly lower levels of transcripts following N deprivation (Supplemental Table S5, medium blue highlighting). The RPL22 genes are scattered among at least six chromosomes, and no correlation was found between location and level of gene expression.

General Changes in Primary Metabolism

Changes in transcript abundance of genes encoding enzymes of primary metabolism are depicted in Figure 3 and summarized in Supplemental Table S6. Transcripts encoding key enzymes of the glyoxylate cycle, gluconeogenesis, and the photosynthetic carbon fixation cycle markedly decreased following N deprivation. Transcript abundance for the glyoxylate cycle enzymes isocitrate lyase and malate synthase decreased more than 16-fold. In addition, mRNA abundance of the cytosolic (predicted) phosphoenolpyruvate carboxykinase, which catalyzes the committed reaction of gluconeogenesis, dropped to 25% of the levels in N-replete cells, as did transcripts encoding enzymes involved in carbon fixation and reduction, ribulose-bisphosphate carboxylase, sedoheptulose 1,7-bisphosphate aldolase, and sedoheptulose-bisphosphatase. In contrast, there was a considerable increase in the transcript abundance of the cytosolic enzyme pyruvate phosphate dikinase. This is a key enzyme in the C4 photosynthetic pathway and is regulated by light. It has also been associated with suppressed phosphoenolpyruvate carboxykinase activity (Osterås et al., 1997) and salt stress (Fisslthaler et al., 1995). Recently, this enzyme has been shown to play an important role in N remobilization (Taylor et al., 2010). Likewise, the transcript abundances for enzymes of the pentose phosphate cycle predicted to be localized in the cyto-
sol, Glc-6-P 1-dehydrogenase and phosphogluconate dehydrogenase (decarboxylating), were increased under those conditions. The mRNA encoding for one of the pyruvate decarboxylase subunits represented in the data set was also increased in abundance following N deprivation. The pyruvate decarboxylase complex converts pyruvate to acetyl-CoA, which is a precursor of fatty acid biosynthesis. Genes for other enzymes of the glycolytic pathway, such as pyruvate kinase, did not show very drastic changes in response to the N deprivation.

To verify whether the changes observed in RNA abundance actually reflect changes in the activity of glyoxylate and gluconeogenic pathways, cells were grown in the presence of [U-13C]acetate. As the cells take up acetate as a carbon source, the distribution of the 13C in the cellular metabolites gives an insight into the activity of the pathways leading to them. The intracellular amino acids as well as the sugar units of the carbohydrates and RNAs were analyzed with gas chromatography-mass spectrometry (GC-MS) as described in “Materials and Methods” (Fig. 4). The natural abundance refers to the naturally occurring distribution of 13C in the molecule. The mass isotopomers M0, M1, M2, etc. refer to molecules having, respectively, zero, one, two, etc. atoms of 13C.

Cells grown in N-replete medium showed a higher degree of labeling in Ser and Gly than did N-deprived cells (Fig. 4). The fully labeled fraction (M3) accounted for almost 80% of the total Ser in the cells grown in N-replete medium. Hence, most of the Ser was derived from the gluconeogenic pathway, which incorporates the labeling of acetate into the glycolytic intermediate 3-phosphoglycerate, a precursor of Ser. There was also about 10% of M2 Ser. This probably derived from the reaction catalyzed by the reversible Ser hydroxymethyltransferase, which favors the production of Ser from Gly (Mattingly et al., 1976). The Gly in this reaction would mostly be fully labeled (M2; Fig. 4), largely from the glyoxylate cycle, hence giving rise to

Figure 3. Regulation of genes involved in primary metabolism. The figure indicates the central metabolic pathways of C. reinhardtii and gives the differential regulation of gene expression following N deprivation. Symbols represent log₂ fold change as follows: ++++, greater than 5; ++, greater than 2 and less than 5; +, greater than 1; ±, less than 1 and greater than −1; −, less than −1; − +, less than −2 and greater than −5; − − −, less than −5.
M2 Ser. In cells grown in N-deprived medium, we observed a markedly lower incorporation of the $^{13}$C atoms into the amino acids. Almost 80% of Gly was unlabeled (M0), indicating a very low activity of the glyoxylate cycle. Similarly, N-deprived cells had reduced label in carbohydrates and Rib. Since these molecules were formed essentially by the gluconeogenic pathway during growth in the medium employed, the N-deprived cells appeared to have much lower gluconeogenic activity. Thus, these biochemical data corroborated the transcript abundance data (Fig. 3) that suggested a down-regulation of the glyoxylate and gluconeogenic pathways in N-deprived cells.

No appreciable change in transcripts for genes encoding components of the mitochondrial respiratory pathway was noted following N deprivation. However, an 11-fold increase in the transcript abundance of an alternative oxidase gene ($AOX1$) was observed, while the $AOX2$ transcript was down-regulated 4-fold. These findings were consistent with previous observations of changes in gene expression of $AOX1$ and $AOX2$ (Baurain et al., 2003).

The candidate genes for peroxisomal β-oxidation showed an overall decrease in their transcript levels following N deprivation, with acyl-CoA oxidase and 3-oxoacyl-CoA thiolase ($ATO1$) transcript abundance decreasing most drastically (greater than 3-fold). The only exception was an enoyl-CoA oxidase/isoenzyme candidate gene ($ECH1$), which showed increased transcript levels (greater than 2-fold; Supplemental Table S8). An apparent down-regulation of fatty acid oxidation is in line with the accumulation of TAGs under these conditions.

Reduced Transcript Abundance for Most Photosynthetic Genes

In *C. reinhardtii*, photosynthetic efficiency decreases following N deprivation, at least partially due to a reduction in the abundance of light-harvesting complexes (Plumley and Schmidt, 1989; Peltier and Schmidt, 1991) and selective degradation of the cytochrome $b_{6}$f complex (Bulté and Wollman, 1992; Majeran et al., 2000). Likewise, the abundance of transcripts encoding photosynthesis-related proteins was substantially reduced following N deprivation. This regulation was not restricted to light-harvesting complexes and cytochromes but extended to the two photosystems as well (Supplemental Table S7). Following N deprivation, the steady-state level of all nucleus-encoded PSI genes decreased by at least 6-fold, while the abundance of transcripts from genes encoding the corresponding light-harvesting proteins was decreased even further, resulting in a 19- to 43-fold decrease relative to N-replete conditions. Only four of the cytochrome subunits are encoded by the nuclear genome, and three of them showed a considerable down-regulation (6-fold) following N deprivation. In contrast, the transcript levels of $PETO$ were weakly increased (2-fold). This observation supports the hypothesis that this protein may have a regulatory role as opposed to being a functional cytochrome $b_{6}$f subunit (Hamel et al., 2000), because the PETO protein is only loosely bound to the complex and its function is not required for the oxidoreductase activity. Expression of all nuclear genes encoding PSII components also decreased following N deprivation (Supplemental Table S7), although the two
least abundant transcripts decreased only slightly. The PSII light-harvesting complex encoding transcripts showed a comparable change in abundance. Most of the transcript levels were reduced, while the weakly expressed \textit{LHCB7} gene showed no alteration in transcript levels. The only two genes of the light-harvesting complex of PSII not following that pattern were \textit{PSBS1} and \textit{PSBS2}. Their transcript levels were strongly increased following N deprivation (119- and 52-fold, respectively). This result was confirmed by reverse transcription (RT)-PCR (Fig. 1B).

Specific Changes in Gene Expression Related to General Lipid Metabolism

\textit{N} deprivation has been demonstrated to lead to the accumulation of TAG in specialized organelles as well as to structural changes and breakdown of the intracellular membrane systems such as the thylakoids and the endoplasmic reticulum (ER; Martin et al., 1976; Moellering and Benning, 2010). Therefore, we expected this to be reflected in the expression of genes encoding enzymes of lipid metabolic pathways. However, changes in transcript levels of genes encoding fatty acid metabolism (Supplemental Table S8) were modest (Fig. 5). A 2-fold increase in transcript levels for ketoacyl-acyl carrier protein (ACP) synthetase was observed. This enzyme is part of the fatty acid synthase II complex that catalyzes the acyl-ACP-dependent elongation steps from C4 to C14 in higher plants. The gene for acyl-ACP thioesterase (\textit{FAT1}) also showed elevated transcript levels following \textit{N} deprivation (about 4-fold). Its reaction terminates fatty acid synthesis by cleaving the acyl chain from ACP. This reaction competes with the direct transacylation of ACP by glycerol-3-phosphate acyltransferases for the formation of phosphatidate. An increase in \textit{FAT1} activity, therefore, could be indicative of increased fatty acid export from the chloroplast to the ER, where TAG assembly occurs, as acyl-ACPs have to be hydrolyzed prior to export (Pollard and Ohlrogge, 1999).

A strong increase in transcript levels was observed for the gene encoding the committing step of TAG synthesis. Out of the five putative diacylglycerol acyltransferases genes identified in the version 4.0 genome data set, only four were expressed under either or both growth conditions (Supplemental Table S8). One of these genes (\textit{DGTT1}, PID 285889) was almost completely suppressed under \textit{N}-replete conditions but showed a large increase in transcript abundance following \textit{N} deprivation. Its reaction terminates fatty acid synthesis by cleaving the acyl chain from ACP. This reaction competes with the direct transacylation of ACP by glycerol-3-phosphate acyltransferases for the formation of phosphatidate. An increase in \textit{FAT1} activity, therefore, could be indicative of increased fatty acid export from the chloroplast to the ER, where TAG assembly occurs, as acyl-ACPs have to be hydrolyzed prior to export (Pollard and Ohlrogge, 1999).

Out of a total of 16 putative membrane-bound desaturase- and hydroxylase-encoding genes found in \textit{C. reinhardtii}, only three showed a change in transcript abundance that is greater than 2-fold. Transcript abundance for microsomal $\Delta_{12}$-desaturase was more than 3-fold higher following \textit{N} deprivation, as was that for the plastidic acyl-ACP-$\Delta_{9}$-desaturase, which introduces the first double bond in an acyl chain. Other microsomal desaturase-encoding transcripts, such as that encoding \textit{FAD13}, an $\omega_{13}/\Delta_{5}$-desaturase, were also slightly increased in abundance, whereas the plastid desaturase-encoding genes were not affected. Of all lipid-related genes, those encoding putative lipases showed the strongest differences in transcript abundance. Numbers indicate log$_2$ fold change of transcript abundance following \textit{N} deprivation. Enzymes labeled with an asterisk cannot be unequivocally assigned to a specific step in the metabolic pathway and are hypothetical.
abundance between the two conditions tested. By searching for “lipase,” “phospholipase,” or “patatin” through the version 4.0 genome sequence data, 130 proteins containing the GXSXG motif common to hydrolases were identified. Among the respective genes, 35 (27%) showed increased and 11 (8.5%) showed decreased transcript levels by 2-fold or more following N deprivation. Supplemental Table S8 lists these 46 most strongly differentially regulated lipase candidates. In addition, many potential lipases may be considered constitutively expressed. Seventy-four out of 130 (57%) lipase candidates were expressed at slightly higher levels following N deprivation. Some of these genes may encode lipases that are important for the turnover or replacement of membranes during cell growth or gamete fusion.

Changes in RNA Abundance for Transcription Factors

The Plant Transcription Factor Database (Pérez-Rodriguez et al., 2010) was used to identify 386 genes encoding putative transcription factors and transcriptional regulators in the C. reinhardtii transcript data set, which could be sorted into 53 families (Table III). Of the 368 genes, 83 showed a 2-fold or greater change in transcript abundance following N deprivation, with 46 being up-regulated and 37 being down-regulated.

To date, only a few of the putative transcription factors identified in the C. reinhardtii genome have a known function. Transcript abundance for the gene encoding NIT2, a transcription factor regulating nitrate metabolism, was increased 6-fold, while that for NAB1, a transcription factor regulating light-harvesting proteins, was decreased 16-fold, consistent with previously described physiological changes in response to N deprivation (Mussgnug et al., 2005; Camargo et al., 2007). The transcript level for the GSP1 mt+ gamete-specific transcription factor was decreased 3-fold at the 48-h sample point. When looking at the changes in RNA abundance of putative transcription factor genes, no obvious trends emerged. However, transcripts falling into the AP2-EREBP and bHLH families were generally more abundant following N deprivation, while those of the FHA family were generally decreased.

DISCUSSION

Microalgae such as C. reinhardtii undergo drastic changes in metabolism and ultimately development when N deprived. Some of the most remarkable changes involve gametogenesis (Harris, 2009b) and metabolic changes that lead to the accumulation of TAGs (Hu et al., 2008). The former aspect has been studied since the 1970s. However, focus on the latter has been largely motivated by the renewed interest in microalgae as biofuel feedstocks (Wijffels and Barbosa, 2010). As sequencing technology has become increasingly fast and affordable, comparison of transcriptomic changes under different experimental conditions by massive parallel sequencing of cDNA libraries is a viable first approach toward identifying genes that define changes in response to N deprivation or other nutrient stresses (González-Ballesta et al., 2010). With the goal of gaining a better understanding of the factors underlying or even controlling the process of TAG accumulation following N deprivation, the focus has to be on metabolism and genes that encode enzymes of relevant pathways or regulatory factors.

The validity of making inferences on metabolism from transcriptome data in this study has been verified in different ways. First, specific genes known to be induced following N deprivation, such as genes involved in gametogenesis or ammonium transport, were found to be expressed as described previously. Second, major metabolic changes predicted by transcript analysis, such as the redirection of sucrose from the glyoxylate cycle and gluconeogenesis to fatty acid biosyntheses following N deprivation (Fig. 3), were corroborated by labeling experiments (Fig. 4).

By and large, gross changes in transcript abundance in response to N deprivation follow expected themes: genes encoding enzymes directly involved in N metabolism or N compound uptake have to be induced, protein biosynthesis is reduced to adjust to the decreased availability of amino acids, and photosynthesis is down-regulated to adjust to the altered metabolic state of the cell. In cyanobacteria, N deprivation led to the degradation of the highly abundant phycobilip light-harvesting proteins so that they could be used as an N source for protein synthesis (Collier and Grossman, 1992). In C. reinhardtii, there is evidence that the cytochrome subunits are degraded not in response to the low concentrations of N per se but rather to the changed energy content of the cell. Thus, one possible advantage for the cells to decrease photosynthesis following N deprivation is to prevent the accumulation of reactive oxygen species (Bulté and Wollman, 1992). At the same time, genes encoding proteins of the respiratory chain in mitochondria were only moderately affected following N deprivation except for those encoding alternative oxidases, which showed elevated transcript abundance. These enzymes are induced under a number of stress conditions that affect the redox environment of the cell, but these effects can be quite indirect and are often difficult to causally connect to the applied stress, in this case N deprivation. It should also be pointed out here that only the expression of nuclear genes is probed in this study; the expression levels of genes for organelle-encoded proteins relevant to respiration or photosynthesis have not been examined.

A Role for PSBS following N Deprivation?

One particular surprise was the strong up-regulation of PSBS following N deprivation in C. reinhardtii. In Arabidopsis (Arabidopsis thaliana), PSBS has been shown to play a critical role in nonphotochemical
Table III. 

| PID  | Annotation        | TID   | T +N° | T −N° | Log₂FC | FDR   |
|------|-------------------|-------|-------|-------|--------|-------|
|      | Transcription factors |       |       |       |        |       |
| 115124 | SBP             | 115124 | 3     | 0     | −26.5  | 4.2 × 10⁻⁰ⁱ |
| 425069 | CPP             | 399885 | 332   | 5     | −5.9   | 5.1 × 10⁻²³ |
| 126810 | NAB1, involved in the light-regulated differential expression of the light-harvesting antenna, CSD | 126810 | 23,357 | 1,253 | −4.1   | 3.5 × 10⁻¹⁶ |
| 119948 | MYB-related     | 119948 | 50    | 3     | −4.0   | 1.5 × 10⁻⁰⁷ |
| 191829 | FHA             | 191829 | 221   | 15    | −3.8   | 1.2 × 10⁻¹⁴ |
| 147364 | CCAAT           | 147364 | 119   | 9     | −3.6   | 4.7 × 10⁻¹⁰ |
| 290169 | bZIP            | 290169 | 10,444 | 895   | −3.4   | 6.9 × 10⁻⁰⁸ |
| 154254 | FHA             | 154254 | 302   | 26    | −3.4   | 9.0 × 10⁻¹² |
| 119194 | MYB-related     | 119194 | 11    | 1     | −3.4   | 3.1 × 10⁻⁰² |
| 148404 | AP2-EREBP       | 148404 | 1,324 | 563   | −3.4   | 6.3 × 10⁻¹⁰ |
| 414856 | SBP             | 411130 | 1,520 | 6,689 | 2.2    | 2.8 × 10⁻¹² |
| 154243 | AP2-EREBP       | 154243 | 302   | 26    | −3.4   | 9.0 × 10⁻¹² |
| 119194 | MYB-related     | 119194 | 11    | 1     | −3.4   | 3.1 × 10⁻⁰² |
| 148404 | AP2-EREBP       | 148404 | 1,324 | 563   | −3.4   | 6.3 × 10⁻¹⁰ |
| 414856 | SBP             | 411130 | 1,520 | 6,689 | 2.2    | 2.8 × 10⁻¹² |
| 154243 | AP2-EREBP       | 154243 | 302   | 26    | −3.4   | 9.0 × 10⁻¹² |
quenching (Li et al., 2000, 2002), but previous studies in *C. reinhardtii* have not detected either of the two PSBS proteins in the thylakoids, and nonphotochemical quenching was shown to be independent of these proteins (Bonente et al., 2008). Only rarely have ESTs been found for PSBS transcripts, with the exception of the cDNA stress collection II, which contains RNAs from different stress treatments, including the switch from ammonium to nitrate (Shrager et al., 2003; Bonente et al., 2008). Our results indicate that PSBS expression in *C. reinhardtii* is induced by ammonium deprivation, as was observed previously for the expression of the gene for alternative oxidase, *AOX1* (Baurain et al., 2003).

**Recycling of Membrane Lipids or de Novo Synthesis of TAGs?**

The elevation of synthesis and export of fatty acids from the chloroplast following N deprivation could indicate that TAG is assembled from fatty acids that are synthesized de novo. This step would require the activation of the fatty acids by a long-chain acyl-CoA synthetase. In fact, increased abundance of RNA encoding a putative long-chain acyl-CoA synthetase was observed, and the respective protein has been identified in the lipid droplet proteome (Moellerling and Benning, 2010). However, another enzyme that could contribute to the changing spectrum of fatty acids is a putative phospholipid/glycerol acyltransferase, for which the transcript level decreased during N deprivation. Long-chain acyl-CoA synthetases are likely to play a key role in determining the fate of fatty acids in the cell (Shockey et al., 2002). Regulation of the respective genes could be a major factor in controlling the flux of fatty acids toward glycerolipid synthesis and their degradation by β-oxidation.

### Table III. (Continued from previous page.)

| PID  | Annotation                              | TID  | T +N  | T −N  | Log₂FC | FDR   |
|------|-----------------------------------------|------|-------|-------|--------|-------|
| 205642 | NIT2, nitrate-regulated transcription factor | 205647 | 1,366 | 7,924 | 2.6 | 7.5 × 10⁻¹⁸ |
| 117291 | MYB                                      | 117291 | 117   | 740   | 2.8 | 1.1 × 10⁻¹³ |
| 195838 | RWPK                                    | 195838 | 100   | 718   | 2.8 | 3.4 × 10⁻¹⁶ |
| 116658 | MYB-related                              | 116658 | 267   | 716   | 3.1 | 2.9 × 10⁻¹⁶ |
| 153934 | bHLH                                     | 153934 | 128   | 672   | 3.2 | 2.5 × 10⁻¹⁹ |
| 195891 | RWPK                                    | 195891 | 120   | 1,080 | 3.3 | 8.9 × 10⁻²⁵ |
| 424240 | CH1                                      | 424240 | 19    | 273   | 3.9 | 8.7 × 10⁻¹⁹ |
| 118761 | SBP                                      | 118761 | 1     | 2     | 5.5 | 2.4 × 10⁻⁰⁸ |
| 405949 | bHLH                                     | 405949 | 3     | 5     | 5.5 | 2.4 × 10⁻⁰⁸ |
| 177225 | bHLH                                     | 177225 | 7     | 1,666 | 8.0 | 1.2 × 10⁻⁰² |

| Transcriptional regulators |
|---------------------------|
| PID  | Annotation | TID  | T +N  | T −N  | Log₂FC | FDR   |
| 206670 | SNF2       | 206670 | 106   | 7     | −3.8  | 3.3 × 10⁻⁰⁹ |
| 143060 | PHD        | 143060 | 332   | 24    | −3.7  | 2.1 × 10⁻¹⁵ |
| 401818 | PHD        | 401818 | 72    | 2     | −1.7  | 3.5 × 10⁻⁰⁴ |
| 325701 | TRAF       | 325701 | 3,740 | 1,118 | −1.6  | 3.2 × 10⁻⁰⁷ |
| 146398 | TRAF       | 146398 | 372   | 113   | −1.6  | 5.6 × 10⁻⁰⁶ |
| 169174 | SNF2       | 169174 | 209   | 67    | −1.5  | 8.1 × 10⁻⁰⁵ |
| 287740 | PHD        | 287740 | 3     | 1     | −1.5  | 7.9 × 10⁻⁰¹ |
| 377090 | GNAT       | 377090 | 821   | 290   | −1.4  | 3.9 × 10⁻⁰⁴ |
| 151030 | TRAF       | 151030 | 406   | 156   | −1.3  | 4.1 × 10⁻⁰⁴ |
| 142283 | HMG        | 142283 | 11,467| 4,587 | −1.2  | 1.7 × 10⁻⁰³ |
| 142152 | GNAT       | 142152 | 189   | 80    | −1.1  | 4.7 × 10⁻⁰³ |
| 172711 | SET        | 172711 | 549   | 233   | −1.1  | 3.5 × 10⁻⁰³ |
| 115484 | SET        | 115484 | 39    | 71    | 1.0   | 5.8 × 10⁻⁰² |
| 193146 | TRAF       | 193146 | 1,032 | 1,899 | 1.0   | 2.5 × 10⁻⁰³ |
| 145759 | TRAF       | 145759 | 503   | 1,004 | 1.1   | 2.2 × 10⁻⁰³ |
| 154505 | SET        | 154505 | 1,304 | 2,760 | 1.2   | 4.5 × 10⁻⁰⁴ |
| 308637 | PHD        | 308637 | 20    | 49    | 1.4   | 1.3 × 10⁻⁰² |
| 188181 | TRAF       | 188181 | 265   | 723   | 1.5   | 3.5 × 10⁻⁰⁶ |
| 282628 | GNAT       | 282628 | 584   | 1,684 | 1.6   | 2.1 × 10⁻⁰⁶ |
| 192899 | HMG        | 192899 | 1,261 | 4,097 | 1.8   | 3.7 × 10⁻⁰⁸ |
| 205788 | GNAT       | 205788 | 215   | 782   | 2.0   | 1.4 × 10⁻¹⁰ |
| 423513 | HMG        | 423513 | 149   | 1,178 | 3.1   | 2.5 × 10⁻¹⁹ |
| 321619 | PHD        | 321619 | 0     | 2     | 26.0  | 1.0 × 10⁻⁰⁰ |
| 143723 | GNAT       | 143723 | 0     | 3     | 26.6  | 2.5 × 10⁻⁰¹ |

*PID and TID are the protein and transcript identifiers for each gene model for *C. reinhardtii* genome version 4.0. Annotation indicates gene name, family, or predicted function. T +N and T −N are the total number of hits for each gene model, from either N-replete or N-deprived conditions. Log₂FC is the log to base 2 of the difference in hits between the two conditions, for each gene model. FDR refers to the false discovery rate for each gene model.
Major intracellular changes occur following N deprivation, and these are likely accompanied by remodeling of membranes. Thus, fatty acids in membrane lipids might be recycled into TAGs. Consistently, some of the transcripts whose abundance changes the most were those encoding putative lipases. In general, lipases belong to a family of enzymes that deesterify carboxyl esters, such as TAGs and phospholipids. As TAGs accumulate following N deprivation, TAG lipases would be expected to be down-regulated. However, classifying lipases with selective substrate specificity based solely on their primary sequences is challenging, a fact that needs to be taken into consideration when interpreting our data set. A TAG lipase typically contains a Ser-Asp/Glu-His catalytic triad, with the Ser catalytic center located in a GXSXG motif (Brady et al., 1990; Winkler et al., 1990). Some recently characterized TAG lipases in animals, yeast, and plants contain a patatin-like, iPLA2 family Ser-Asp catalytic dyad (Zimmermann et al., 2004; Athisenstaedt and Daum, 2005; Eastmond, 2006; Kurat et al., 2006). Genes encoding lipases specific for membrane lipids would be expected to be up-regulated, as they might mobilize fatty acids from membrane lipids into TAGs. Moreover, signaling pathways involving lipid products generated by lipases, such as diacylglycerol, may also control steady-state TAG levels (Kanoh et al., 1993). Further biochemical characterization of some of the most regulated lipase candidate genes will be necessary to determine their role in TAG accumulation following N deprivation.

On the other hand, we recognize that lipase expression or activities may also be controlled at the posttranscriptional level, including translational regulation and posttranslational modifications of the encoded proteins. In mammals, a hormone-sensitive lipase is phosphorylated by protein kinase A upon cAMP elevation and consequently exhibits better accessibility to lipid droplets (Holm et al., 2000). Some of the C. reinhardtii TAG lipases may have a similar regulatory pattern and hence not show significant transcriptional changes when cells are N deprived. Reverse genetic studies on the lipase candidates and forward genetic screens for mutants with TAG deficiency phenotypes will disclose the bona fide TAG lipases and other lipases that impact TAG metabolism.

It should also be noted that during the analysis of the lipid gene data set, the annotation ambiguities of several fatty acid desaturases became obvious in the version 4.0 genomic sequence data set: the C. reinhardtii genome harbors four presumed paralogs of FAD5 (named FAD5a–FAD5d). Based on our current prediction analysis (Emanuelsson et al., 1999) and previous reports (Riekhof et al., 2005; Riekhof and Benning, 2009), FAD5a and FAD5b are presumed to be targeted to the chloroplast, whereas FAD5c and FAD5d are likely located in the ER membrane. However, experimental corroboration is still needed.

CONCLUSION

Our interpretation of this data set places emphasis on TAG metabolism and potential regulatory factors, which are undoubtedly not yet completely identified. We expect that others will be able to mine this data set, taking into account different biological processes pertaining to N deprivation. Cross-querying this data set with a lipid droplet proteomics data set (Moellering and Benning, 2010) should further narrow the possible candidates relevant for TAG accumulation. Likewise, a meta-analysis of this and other data sets, including those from other species, could facilitate the identification of genes most likely involved in TAG accumulation, as was recently done for low-oxygen stress (Mustroph et al., 2010). Thus, this study represents only a first step of many toward gaining a molecular understanding of TAG accumulation and other cellular changes triggered by N deprivation in C. reinhardtii.

MATERIALS AND METHODS

Strains and Growth Conditions

The Chlamydomonas reinhardtii strain used was dw15.1 (cw15, nit1, mt*), kindly provided by Arthur Grossman. The cells were grown in liquid cultures under continuous light (approximately 80 μmol photons m⁻² s⁻¹). For N-replete growth, TAP medium (Harris, 2009a) with 10 mM NH₄⁺ (TAP + N) was used. For preliminary experiments, N deprivation was applied by two methods: continuous growth in TAP with 0.5 mM NH₄⁺ or growth in TAP + N to 5 × 10⁶ cells mL⁻¹, followed by transfer to TAP with no NH₄⁺ (TAP – N) for an additional 24 or 48 h. For further experiments, N deprivation was defined as growth in TAP + N to 5 × 10⁶ cells mL⁻¹, followed by transfer to TAP – N for 48 h. For labeling studies, the cells were grown in 500-mL shaker flasks with a culture volume of 50 mL with continuous shaking. For the N deprivation experiment, cells were first grown in TAP medium with unlabeled acetate with at least five cell doublings to mid logarithmic phase to reach a biomass equivalent to 0.3 to 0.4 g cell dry weight L⁻¹. The cells were divided up and transferred to TAP medium containing [U-¹³C]acetate (Isotec), either TAP + N or TAP – N.

Sequencing Read Processing

To generate material for high-throughput sequencing, cells were grown in 100 mL of TAP + N to 5 × 10⁶ cells mL⁻¹. The cultures were split in half, and cells were collected by centrifugation, with one pellet being resuspended in 50 mL of TAP + N and the other in 50 mL of TAP – N. After 48 h, the total RNA was harvested using a Qiagen RNAeasy Plant Mini kit. The RNA samples were treated with Qiagen RNase-free DNase I during extraction.

For 454 sequencing, full-length cDNA pools were generated with the Clontech SMART cDNA library construction kit. cDNA was synthesized using a modified cDNA synthesis primer (5'-TAGAGACCGAGGCGCCGA-CATGTTTTGTTTTTTTTTCTTTTTTTTTTVN-3'). Full-length cDNA's were amplified by PCR and pooled to increase their concentration. An SfiI digest was performed, followed by size fractionation. Fractions with the highest intensity and size distribution were pooled and purified. The resulting cDNA pools were then submitted to the Michigan State University-Research Technologies Service Facility for sequencing on a 454 GSFLX Titanium Sequencer (454 Life Sciences). For Illumina sequencing, total RNA was submitted directly to the Michigan State University-Research Technologies Service Facility for sequencing on an Illumina Genome Analyzer II (Illumina).

Default parameters were used to pass reads using 454 and Illumina quality-control tools. The filtered sequence data were deposited in the National Center for Biotechnology Information Short Read Archive with the reference series number GSE24367 and subspecies numbers GSE24365.
and GSE24366 for the Illumina and the 454 data sets, respectively. The filtered 454 sequencing reads were mapped to the *C. reinhardtii* version 4.0 assembly from the Joint Genome Institute with GMAP (Wu and Watanabe, 2005). In GMAP, the maximum intron length was set at 980 bp, which is at the 95th percentile of annotated *C. reinhardtii* intron lengths. The Illumina reads were mapped with Bowtie (Langmead et al., 2009) using parameters as follows: two or fewer mismatches, sum of Phred quality values at all mismatched positions at 70 or less, and excluding reads mapped to one or more locations. Because the sequence qualities of Illumina reads degrades quickly toward the 3' end, an alternative mapping data set was generated with reads trimmed from the 3' end (until the 3'-end-most position with Phred-equivalent score was 20 or greater). Trained reads of less than 30 bp were excluded from further analysis. In addition to sequence quality issues, some reads may span two exons and would not be mapped by Bowtie perfectly. We used the TopHat (Trapnell et al., 2009) to identify these exon-spanning reads to generate another set of read mapping. The information from TopHat was used for assembling mapped reads into transfrags with Cufflinks (Trapnell et al., 2010). In Cufflinks, the maximum intron length was set at 1,855 bp (99th percentile of all the intron lengths), 5% minimum isoform fraction, and 5% pre-mRNA fraction. Transfrags within 1,855 bp of an existing *C. reinhardtii* version 4.0 gene model were regarded as potential missing exons of annotated genes. The rest were regarded as intergenic exons, and adjacent transfrags less than 1,855 bp apart were joined into “transcriptional units.”

**Northern-Blot Analysis and RT-PCR**

Total RNA was harvested from N-replete or N-deprived cells as described above, and 4 µg of each total RNA was separated on a 1% formaldehyde gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare). Probes were synthesized from cDNA and labeled with 32P using the Amersham MegaPrime labeling kit (GE Healthcare). The blots were hybridized with the labeled probes in Ambion ULTRAbase (Applied Biosystems/Ambion) at 42°C overnight. The blots were washed twice for 5 min with low-stringency buffer (1× SSC and 0.1% SDS) at 60°C and then twice for 5 min with high-stringency buffer (0.1× SSC and 0.1% SDS) at 60°C. The blots were exposed to a Molecular Dynamics phosphor screen (GE Healthcare) overnight and visualized with a Storm 820 phosphor imager (GE Healthcare). Probes were synthesized from cDNA for AMT4 (5'-GTATGCTCCTGATCTGC-3' and 5'-CTGGAATATGCTTAGG-3'), DGTT2 (5'-TAAAGCCACCGA-CAAATGTCG-3' and 5'-CATATGCCTGATCTGTTG-3'), and DGTT3 (5'-GCGGCTGCTCTCCTCTGA-3' and 5'-CCTGATACATCGGGC-3').

For RT-PCR, RNA was extracted from N-replete and N-deprived cultures using TRIzol reagent (Invitrogen) and subjected to DNase treatment with the Turbo DNA-free kit from Ambion. A total of 1 µg of DNA-free RNA was used for cDNA synthesis with the Invitrogen Moloney murine leukemia virus reverse transcriptase. A total of 0.5 µg of oligo(dT)12–18 primer (Invitrogen) and 0.5 µg of random hexamer primers (Promega) were added to the RNA, and the volume was adjusted to 20 µL final volume. After heating the samples at 70°C for 10 min, they were incubated on ice for 5 min. Twenty units of RNase inhibitor (Applied Biosystems), 20 nmol of deoxyxyribonucleotide triphosphates (Invitrogen), 4 µL of 1st-strand buffer, and 0.2 µmol of dithiothreitol were added to the reaction. The reaction mixture was incubated at 37°C for 10 min for primer annealing. A total of 200 units of Moloney murine leukemia virus reverse transcriptase was added, and the reaction was incubated at 37°C for 1 h followed by deactivation at 70°C for 10 min. A total of 1 µL of a 1:10 dilution of cDNA was used for PCR using GoTaq polymerase (Promega). The reaction mixture (25 µL) contained 1× buffer, 5 nmol of deoxyxyribonucleotide triphosphates, 12.5 pmol of each primer, and 1 unit of polymerase. PCR cycle conditions were 3 min of initial denaturation at 94°C, followed by 40 cycles of 30 s of denaturation, 30 s of annealing at 60°C, and 3 min of elongation at 72°C. Final elongation was performed at 72°C for 10 min. The PSBS-specific primers (5'-ATGCCCATGACCTGAC-3' and 5'-TTAGGCGCACTCTGTCG-3') amplify both PSBS1 and PSBS2. The IDAS gene (5'-GCCAGCTCTGCTCTGCG-3' and 5'-TACTCGAGCAAGCAGATCCA-3') served as a control.

**Analysis of Differential Gene Expression**

Differential expression between *C. reinhardtii* cultured in N-replete and N-depleted medium was determined using the numbers of mapped reads overlapped with annotated *C. reinhardtii* genes as inputs to EdgeR (Robinson et al., 2010). In the Joint Genome Institute database, multiple sets of *C. reinhardtii* version 4 gene models are available. We used the “filtered” gene models, which contain the best gene model for each locus. Genes were regarded as differentially expressed if they have 2-fold or greater change between N-replete and N-deprived samples and 5% or less false discovery rate. Differential expressed genes were regarded as up-regulated if their expression levels in N-deprived samples were significantly higher than those in N-replete samples. Conversely, down-regulated genes were those with significantly lower levels of expression following N deprivation.

In addition to EdgeR, we used three other methods to evaluate differential expression: Fisher’s exact test (Bloom et al., 2009), likelihood ratio test (Marioni et al., 2008), and a method based on intensity ratio and average intensity (MARS; Wang et al., 2010). All three methods were implemented in the DESeq package (Wang et al., 2010). We found that among 4,004 differentially expressed genes called by EdgeR, 99.7% to 100% were regarded as differentially expressed by the other three methods. On the other hand, EdgeR calls overlap with 96.6%, 94.7%, and 95.8% of calls by Fisher’s exact test, likelihood ratio test, and MARS, respectively. Our findings indicate that EdgeR is more conservative than the other methods, but the overall differential expression calls are highly similar among methods. We should note that methods other than EdgeR did not explicitly consider variance between replicates and, as a result, will likely have a higher false-positive differential expression call rate than that of EdgeR. Therefore, in all subsequent analyses, we used only EdgeR-based differential expression calls.

**GO annotation for the *C. reinhardtii* version 4.0 genome was acquired from the Joint Genome Institute. Enrichment of differentially regulated genes in each GO category was determined using Fisher’s exact test. To account for multiple testing, the *P* values from Fisher’s exact tests were adjusted (Storey, 2003) and a false discovery rate of 5% was used as the threshold for enriched GO terms.

**GC-MS Analysis**

To quantify 13C-labeling patterns such as mass-isotopomer distributions and fractional 13C enrichment, samples were analyzed using GC-MS using an HP 6890 GC apparatus (Hewlett-Packard) equipped with DB-5MS column (5% phenyl-methyl-siloxan-diphenylpolysiloxan; 30 m × 0.25 mm × 0.25 µm; Agilent) and a quadrupole mass spectrometer (MS 5975; Agilent). Electron ionization was carried out at 70 eV. The obtained mass spectrometric data were corrected for the natural abundance of the elements to give fractional 13C labeling.

**Sampling, Extraction, and Analysis of Intracellular Amino Acid**

Cells from TAP + N and TAP − N cultures were harvested after 24 h. This time point was chosen for these labeling experiments because at the required cell concentration (approximately 0.2 g cell dry weight L−1) for metabolite extraction, a more depletion occurs in cells grown in TAP + N at later time points due to the high initial inoculum. The harvested cells (approximately 25 mg cell dry weight) were centrifuged at 3,000g for 1 min, and the supernatant was removed and quenched with 5 mL of cold 100% methanol (Winder et al., 2008). The metabolites were harvested by vortexing the cells. A second extraction was performed with 5 mL of chloroform:methanol (1:2). The extracts were then pooled. Water was simply added to the pooled extracts for phase separation. The polar metabolites, which include the amino acids, were present in the aqueous phase. The aqueous phase was then dried under N2 and converted to its t-butyldimethylsilyl derivative using N-methyl-N-(t-butyldimethylsilyl)trimethylsiloxane (Mawhinney et al., 1986). The GC and MS conditions for this analysis were as described previously (Deshpande et al., 2009).

**Extraction and Analysis of Rib**

Rib for analysis was obtained from the RNA as described (Boren et al., 2003). RNA was extracted from cells (0.1–0.2 g L−1) at the same time point as the intracellular amino acids using the Tri reagent as described in the protocol (Molecular Research Center). The RNA was acid hydrolyzed to its monomers and dried under N. It was further analyzed using GC-MS by derivatizing it to its per-O-trimethylsilyl-O-ethyl oxime (MacLeod et al., 2001). The ions 481 to
486 (mass-to-charge ratio), corresponding to the whole carbon backbone of the Rib molecule (C$_{1}$-C$_{5}$), were monitored using single ion monitoring of the MS data.

**Extraction and Analysis of Carbohydrate**

The carbohydrates in the cells were acid hydrolyzed by 2 N HCl at 102°C, and the monomeric compounds were analyzed by GC-MS after the sample was dried under $N_2$. The sample was then converted to its di-O-isopropylidene acetate derivative for analysis by GC-MS (Hachey et al., 1999). The ions 287 to 293 (mass-to-charge ratio), corresponding to the whole carbon backbone of Glc (C$_{1}$-C$_{5}$), were monitored.

Sequence data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus under accession numbers GSE24367, GSE2466, and GSE2465.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Table S1.** Illumina analysis of transcripts related to gametogenesis.

**Supplemental Table S2.** Illumina analysis of transcripts related to N metabolism.

**Supplemental Table S3.** Illumina analysis of transcripts encoding chloroplast ribosomal proteins.

**Supplemental Table S4.** Illumina analysis of transcripts encoding cytosolic 80S ribosomal proteins.

**Supplemental Table S5.** Illumina analysis of transcripts encoding L22 ribosomal proteins.

**Supplemental Table S6.** Illumina analysis of transcripts encoding proteins of central metabolism.

**Supplemental Table S7.** Illumina analysis of transcripts related to photosynthesis.

**Supplemental Table S8.** Illumina analysis of transcripts related to lipid metabolism.

**Supplemental Spreadsheet S1.** Complete data set of all genes analyzed by 454 and Illumina.

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