Algal Research 79 (2024) 103458

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Comparative transcriptome study highlights the versatility of nitrogen metabolism in *Chlamydomonas*

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ARTICLE INFO

Keywords:
Chlamydomonas
Nitrate-deplete
Nitrate-replete
Transcriptome analysis
Urea metabolism

ABSTRACT

Nitrogen is an essential macronutrient and nitrate is one of the main forms of this macronutrient available for plants and microbes. Nitrate is not only the substrate for the nitrate assimilation pathway, but also a crucial signal for the regulation of numerous metabolic, developmental, and cellular differentiation processes. In the present study, two species of the *Chlamydomonas* genus, *Chlamydomonas reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216 were used to investigate the versatility of nitrate metabolism in green microalgae. Quantification of nitrate removal efficiency showed that *Chlamydomonas* sp. MACC-216 strongly outperforms *C. reinhardtii* cc124. Transcriptional changes occurring under nitrate-replete and nitrate-deplete conditions were specifically investigated in the selected species of *Chlamydomonas*. Whole transcriptome analysis revealed that the genes playing a role in nitrate assimilation did not show differential expression in *C. reinhardtii* cc124 under changing nitrate conditions (only 45 genes exhibited differential regulation), while in *Chlamydomonas* sp. MACC-216 a large set of genes (3143) showed altered expression. Furthermore, genes responsible for urea metabolism, like *DUR3A* gene corresponding to urea transport, were found to be upregulated in *Chlamydomonas* sp. MACC-216 under nitrate-deplete condition, while the same gene showed elevated expression level in *C. reinhardtii* cc124 under nitrate-replete condition. The present study indicated the diverseness of nitrate metabolism among species within the *Chlamydomonas* genus.

1. Introduction

Nitrogen plays a key role in the life cycle of all organisms. It is a necessary macroelement for the growth and development of plants and algae. Various nitrogen sources like nitrate, nitrite, ammonium, and organic nitrogen compounds like urea can be utilized by microalgae [1]. Most of these nitrogen sources are first reduced to ammonium and are then assimilated in the form of amino acids. Initially, ammonium was considered the preferred nitrogen source due to its lower energy cost for assimilation compared to nitrate/nitrite and because genes involved in nitrate/nitrite assimilation are negatively regulated in the presence of ammonium. However, recent studies have shown that many microalgae prefer nitrate over ammonium as a primary nitrogen source [2–7]. Nitrate is the preferred nitrogen form for growth even in plants, especially crop herbaceous plants, in combination with ammonium, as it also serves as an efficient signal for affecting various cell activities, including root development, root-shoot balance, and stomatal opening [8–12].

Understanding the mechanism behind the regulation of the nitrate assimilation pathway is crucial in improving nitrogen use efficiency and avoiding the negative effects of nitrogen fertilisation, such as environmental contamination and waste nitrogen. *Chlamydomonas reinhardtii*, a unicellular green alga, has been established as a prominent model organism for investigating photosynthesis and other plant-specific physiological processes [13]. This is primarily due to the abundance of essential resources, such as convenient heterologous gene expression, genetic maps, and access to multiple strains through the *Chlamydomonas* Genetics Center and other repositories [13]. Notably, the availability of an annotated genome sequence in Phytozome facilitates gene-based comparative research [14].

Nitrate assimilation pathway in microalgae consists of two transport steps (nitrate and nitrite transport) and two reduction steps. First nitrate is transported into the cell, where a cytosolic nitrate reductase (NR) catalyzes the reduction of nitrate into nitrite, which is subsequently transported into the chloroplast; nitrite is further reduced to ammonium by the action of the enzyme nitrite reductase (NiR) in the chloroplast [15–17]. Chloroplast is the main site for ammonium incorporation into

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https://doi.org/10.1016/j.algal.2024.103458
Received 8 November 2023; Received in revised form 16 February 2024; Accepted 1 March 2024
Available online 2 March 2024
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carbon skeletons by glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) or glutamate synthase cycle [15–17]. Despite being a unicellular organism, *Chlamydomonas* exhibits a notable level of complexity in its nitrate/nitrite transporters, with a total of 13 transporters. These transporters can be categorized into three families: NRT1 (consisting of one transporter), NRT2 (comprising six transporters), and NAR1 (comprising six transporters) [18]. The regulatory gene *NIT2* is well-studied and known to be associated with positive regulation of nitrate [19,20]. It has been shown that *NIT2* defective mutants were unable to grow in the presence of nitrate as the sole nitrogen source [21]. To date, only one regulatory gene has been identified to interact with *NIT2*, known as *CrNZF1* (Nitrate zinc finger 1) [22]. *CrNZF1* encodes a CCCH-type tandem zinc finger protein, a class of proteins conventionally associated with RNA binding, thereby influencing RNA cleavage, degradation, polyadenylation, and even export processes. Intriguingly, mutants with defective *CrNZF1* displayed varying lengths of polyadenylated *NIT2* transcript and exhibited reduced upregulation of nitrate assimilation genes. Conversely, the wild-type strains showcased the longest forms of polyadenylated *NIT2* transcript and exhibited the expected levels of expression of nitrate assimilation genes.

In addition to its role as an essential nutrient, nitrate can also lead to an induction in growth and lipid accumulation or impede gametogenesis in *C. reinhardtii*. Various studies have explored the effects of nitrogen deprivation on microalgae through transcriptome analysis [13,23–27]. Nitrogen deprivation in microalgae leads to the accumulation of triacylglycerol lipids and the production of hydrogen [13,23,25]. In contrast to glycerolipids found in membranes, triacylglycerols predominantly function as a means of storing carbon and energy to help prepare the microalgae for environmental stress situations, demonstrating active involvement in the stress response. Although many studies have been performed on the effect of nitrogen deprivation on microalgae, most of these studies talk about the effect of nitrogen deprivation on lipid accumulation inside microalgae [13,23,26,28]. The existing literature lacks an adequate exploration of the differences in nitrate metabolism between microalgae proficient in utilizing nitrate as a growth substrate and those that lack this capability. This knowledge gap is particularly pronounced concerning the genes and regulatory elements responsible for nitrate sensing.

The objective of the current study was to conduct a comparative analysis of nitrate metabolism in two distinct microalgal species, namely *Chlamydomonas* sp. MACC-216 and *Chlamydomonas reinhardtii* cc124. *Chlamydomonas* sp. MACC-216 can happily grow in the presence of nitrate as the sole source of nitrogen, whereas *C. reinhardtii* cc124 is unable to grow under the same conditions. Both species of *Chlamydomonas* were cultivated under either nitrate-deplete or nitrate-replete conditions for six hours. For the present study, a six-hour time point was chosen as we were interested in identifying the early responses in the transcriptomes of both microalgae. After six hours of cultivation in the respective media, transcriptome analysis was performed to identify the differences in the nitrate metabolism pathways between the two species.

2. Materials and methods

2.1. Microalgae and growth conditions

Chlamydomonas sp. MACC-216 and *C. reinhardtii* cc124 microalgae used for the present study were provided by the Mosonmagyaróvár Algae Culture Collection (MACC) and were maintained on TAP (Tris-acetate-phosphate) medium. TAP medium was prepared as mentioned in our previous study, Rani and Maróti [29]. The microalgae were maintained at 25 °C under white light with a light intensity of 50 μmol m^{-2} s^{-1} with continuous shaking at 180 rpm in a regime of 16:8 light-dark periods.

For further experiments, two different kinds of modified TAP media were prepared: TAP-N0 (nitrate-deplete) and TAP-N15 (nitrate-replete). TAP-N0 did not contain any nitrogen source. TAP-N15 was prepared by replacing ammonium chloride in normal TAP with 15 mM (1.27 g L^{-1}) of sodium nitrate. Also, 0.001 g L^{-1} $(NH_4)_6Mo_7O_{24}{\bullet}4H_2O$ was replaced with 0.006 g L^{-1} of $Na_2MoO_4{\bullet}2H_2O$ in TAP-N0 and TAP-N15 media so that there is no other nitrogen source present. *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124 were grown in TAP medium for three days. After three days, both microalgae cultures were centrifuged at 3220 *g* for 5 min. After centrifugation, each microalga was resuspended in 10 mL of TAP-N0 and TAP-N15 media to a final cell density of 6×10^6 cells mL^{-1} followed by further incubation at 25 °C under white light with a light intensity of 50 μmol m^{-2} s^{-1} with continuous shaking at 180 rpm for six hours. At the end of this period, samples were collected from each culture for nitrate estimation and RNA isolation.

2.2. Nitrate estimation

Nitrate estimation was done at 0 and 6 h by the salicylic acid method [30]. Briefly, to 10 μL of each sample, 40 μL of 5 % (*w/v*) salicylic acid in concentrated H_2SO_4 was added. Then samples were kept at room temperature for 30 min and then 950 μL of 8 % (w/v) NaOH in water was added followed by cooling down of samples to room temperature. The absorbance was determined at 410 nm using a Hidex microplate reader.

The nitrate removal efficiency was calculated using the following equation:

$$R = \left(1 - \frac{Cf}{Ci}\right) \times 100 \quad (1)$$

where R is the nitrate removal efficiency (%), Ci and Cf are the initial and final concentrations of nitrate (mM) in the growth medium.

2.3. RNA isolation and sequencing

RNA isolation was performed using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) by following manual instructions. The purified RNA was quantified using the Qubit RNA Assay. Zymo-Seq RiboFree Total RNA-Seq Library Kit was used for the depletion of ribosomal RNAs. Illumina Nextseq550 was used to generate 150 nt (nucleotide) paired-end reads.

2.4. Transcriptome analysis

Sequenced reads were first processed using rcorrector v1.0.5 [31], a kmer-based error correction method for RNAseq data. The error-corrected reads were then trimmed using Trimmomatic v0.39 [32] to remove adapter sequences while keeping a quality score of 25 over 5 nucleotides (nt) sliding window. Reads shorter than 50 nt were discarded from further analysis. The trimmed reads were then mapped to reference *Chlamydomonas reinhardtii* v5.6 transcripts downloaded from Phytozome using Kallisto v0.46.1. Transcripts were retained in the analysis only if each transcript had >10 reads mapping back to it, across 4 samples. This prevented genes with low counts from being included in the analysis. Genes with a log2 fold change (FC) of ±1 and adjusted *p*-value ≤0.05 were identified as differentially expressed genes (DEGs). Gene ontology (GO) enrichment was carried out using the TopGO v2.46.0 package in Rstudio using ontology IDs retrieved from the biomart v2.50.3 package. Only terms with Benjamini & Hochberg adjusted *p*-value ≤0.05 or smaller were considered enriched terms. The transcriptome data was deposited in the NCBI SRA (National Institutes of Health Sequence Read Archive) database and can be accessed by the accession number PRJNA1027627.

2.5. Quantitative reverse transcription polymerase chain reaction

RT-qPCR, short for quantitative reverse transcription polymerase chain reaction, is a technique employed to detect and quantify RNA. The

process involves the initial transcription of either total RNA or mRNA into complementary DNA (cDNA). The cDNA is subsequently used as the template for the quantitative PCR (qPCR). cDNA was prepared from the isolated RNA in Section 2.3 using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) by following manual instructions. Primers were designed by obtaining sequences of the selected genes from the Phytozome using gene identifiers (Table 1). Sequence for the endogenous reference gene beta 1 tubulin was obtained from NCBI database. Quantitative polymerase chain reaction (qPCR) was performed with 50 ng of cDNA, 500 nM each of forward and reverse primer, 3 μL of SYBR™ select master mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and nuclease-free water to a final volume of 6 μL in Bio-Rad CFX384 C1000 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Analysis of results obtained by qPCR was done using $2_T^{-\Delta\Delta C}$ method [33]. TAP-N0 condition was selected as a control for the analysis. The expression of each gene was achieved by normalization of a target gene in each condition to an endogenous reference gene.

2.6. Statistical analysis

Rstudio version 1.2.5019 was used to perform the statistical analyses. All statistical analyses in the current study were performed using one-way analysis of variance (ANOVA) followed by Tukey's test which was used to detect significant differences among the treatments, where p-value ≤0.05 was considered a significant difference. All experiments were carried out in the replicates of 3.

3. Results

3.1. Nitrate removal efficiency

The short-term changes in gene expression in response to various nitrate conditions were studied by incubating *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124 for 6 h in TAP-N0 and TAP-N15 growth media. The initial cell density of both microalgae at the start of the incubation period was 6×10^6 cells mL^{-1}. *Chlamydomonas* sp. MACC-216 showed ~22 % nitrate removal efficiency from the TAP-N15 medium, whereas *C. reinhardtii* cc124 showed only ~4 % nitrate removal efficiency after 6 h incubation period (Table 2).

Table 1
List of genes and their primers.

Gene	Gene identifier	Primer (5′ → 3′)	
DUR1	Cre08.g360050	Forward	CGGGCAATTTGACATCGAGA
		Reverse	CACCAGCATCTTGTCCATCG
DUR2	Cre08.g360100	Forward	CTACTTGAAGCGGGCGGTAT
		Reverse	TACCACCAGAGATGAACCGG
DUR3A	Cre08.g360200	Forward	CAACGGCAAGCAGATGATCA
		Reverse	CAGAGACACCTGGAGCTTCA
DUR3B	Cre08.g360250	Forward	CGTGTATGCTGCTTCCTTCC
		Reverse	AATCACCGAAACGTTCAGCC
DUR3C	Cre17.g703800	Forward	GCGACGTGTACCAACTTGTT
		Reverse	AATGAACCAACCCAGTCCCT
NRT1.1	Cre04.g224700	Forward	AGGCTCTGCCCCTGATAGA
		Reverse	CCTCCCATCACATTGCAGA
NRT2.1	Cre09.g410850	Forward	TGAGAAGCCAGCCACAGTAA
		Reverse	AAGCAAATCCAGGACAGGTG
NRT2.2	Cre09.g410800	Forward	CCATCTTCGGCCTTATGAAC
		Reverse	CGTTAGCGAGTTGCTGACCT
NIT1	Cre09.g410950	Forward	AGCCGTTGACTTTGACCATG
		Reverse	GCATGTTCTCCTCCTTGCG
NAR1.5	Cre12.g541250	Forward	TAACGTGCCCTCCGCACT
		Reverse	GCGTCGACGTCTGCACAT
NAR1.6	Cre01.g012050	Forward	ATGACCCAGGCCTCTACAAC
		Reverse	TGTACAGATCCGAGCCCATC
NII1	Cre09.g410750	Forward	ACAGCTCGCGTACTCGCTAC
		Reverse	ACTTGTTGGCCTTCTTGTCG
GLN1	Cre02.g113200	Forward	TGTGCCGAGTATGTGTGGAT
		Reverse	GAGCCGTCGTAGTTCCAGTG
GLN2	Cre12.g530650	Forward	CGTGGTGTTACCGCTAAGG
		Reverse	GAGCGCATCTCGTTGAAGAT
NIT2	Cre03.g177700	Forward	TTCTCCACGGACGTCATGAA
		Reverse	CACCTGCTTGTCGTACTTCTG
Beta 1 tubulin	M10064[a]	Forward	CGCATGATGCTGACCTTCT
		Reverse	GTCCAGGACCATGCACTCAT

[a] NCBI GenBank number.

3.2. Reads trimming and mapping

RNA sequencing generated paired reads of 150 nt size. Raw paired reads were error-corrected to obtain a total of 39 million read pairs from TAP-N0 and 35 million read pairs from TAP-N15 samples in *C. reinhardtii* cc124. A total of 33 million read pairs from TAP-N0 and around 35 million read pairs from TAP-N15 were obtained from *Chlamydomonas* sp. MACC-216 samples. After trimming using Trimmomatic v0.39, around 30 million read pairs from TAP-N0 and 27 million read pairs from TAP-N15 were obtained in *C. reinhardtii* cc124, whereas approximately 26 million read pairs from TAP-N0 and 27 million read pairs from TAP-N15 were obtained in *Chlamydomonas* sp. MACC-216.

3.3. Variation between the two growth conditions

Principal component analysis (PCA) of transcript abundances for all samples was used to visualize similarity in gene expression patterns. All replicates from TAP-N0 and TAP-N15 conditions used for *C. reinhardtii* cc124 clustered together which indicated no major transcriptional reorganization. However, in *Chlamydomonas* sp. MACC-216, replicates from each TAP-N0 and TAP-N15 conditions clustered together but were separated by conditions, indicating significant transcriptional changes between the two conditions (Fig. 1).

3.4. Differential gene expression and gene ontology enrichment analysis

Differential gene expression analysis was conducted by using TAP-N0 as the control and TAP-N15 as the treatment condition. Only 45 genes were identified to be differentially regulated with a log2 fold change of >1 and an adjusted p-value ≤0.05 in *C. reinhardtii* cc124. Of these 45 genes, 23 were found to be upregulated under the TAP-N15 condition, and 22 were found to be downregulated (Fig. 2, Table S1). In *C. reinhardtii* cc124, upregulation of genes involved in nitrate transport and metabolism was not observed. Instead, genes involved in urea transport were found to be upregulated which was interesting as urea was not provided in the growth medium to the cultivated microalga. As nitrogen limitation or starvation is linked with increased accumulation of lipids, differential regulation of genes involved in this process was also searched, but no differential expression of such genes was observed.

Gene ontology (GO) enrichment studies were performed on the upregulated and downregulated genes to identify important groups differentially expressed under the TAP-N15 condition. Fig. 3a and b show enriched terms in upregulated and downregulated gene lists. In the upregulated gene list, GO terms such as ammonium transmembrane transporter, thiamine biosynthetic process, protein processing, proline biosynthetic process, glycerol 3-phosphate catabolic process, glycerol-3-phosphate dehydrogenase (NAD^+) activity, and branched-chain amino acid biosynthetic process were identified. In the downregulated GO terms list, voltage-gated potassium channel activity, ionotropic glutamate receptor activity, and potassium ion transport were identified.

Table 2
Nitrate removal efficiency under TAP-N15 growth condition after 6 h. Values are represented as mean ± SD.

Microalgae	Nitrate removal efficiency (%) after 6 h
C. reinhardtii cc124	4.21 ± 1.87
Chlamydomonas sp. MACC-216	21.52 ± 2.09

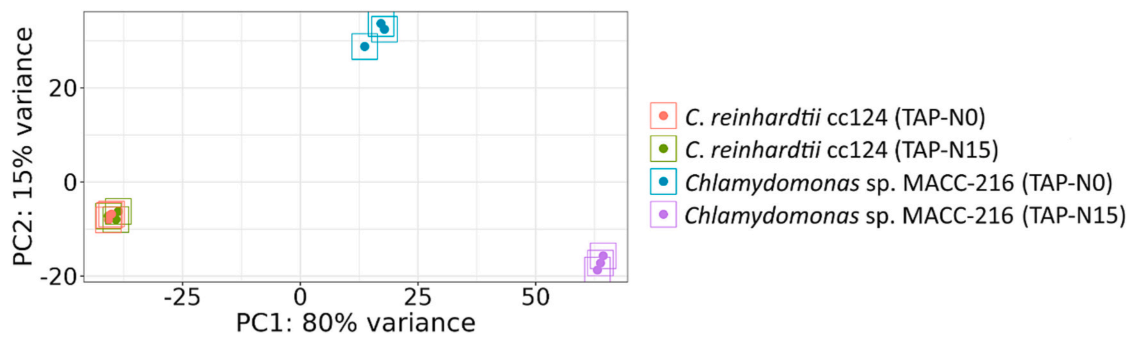


Fig. 1. Variance between samples in a dataset. PCA plot showing the variation between samples from TAP-N0 and TAP-N15 conditions in *C. reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216.

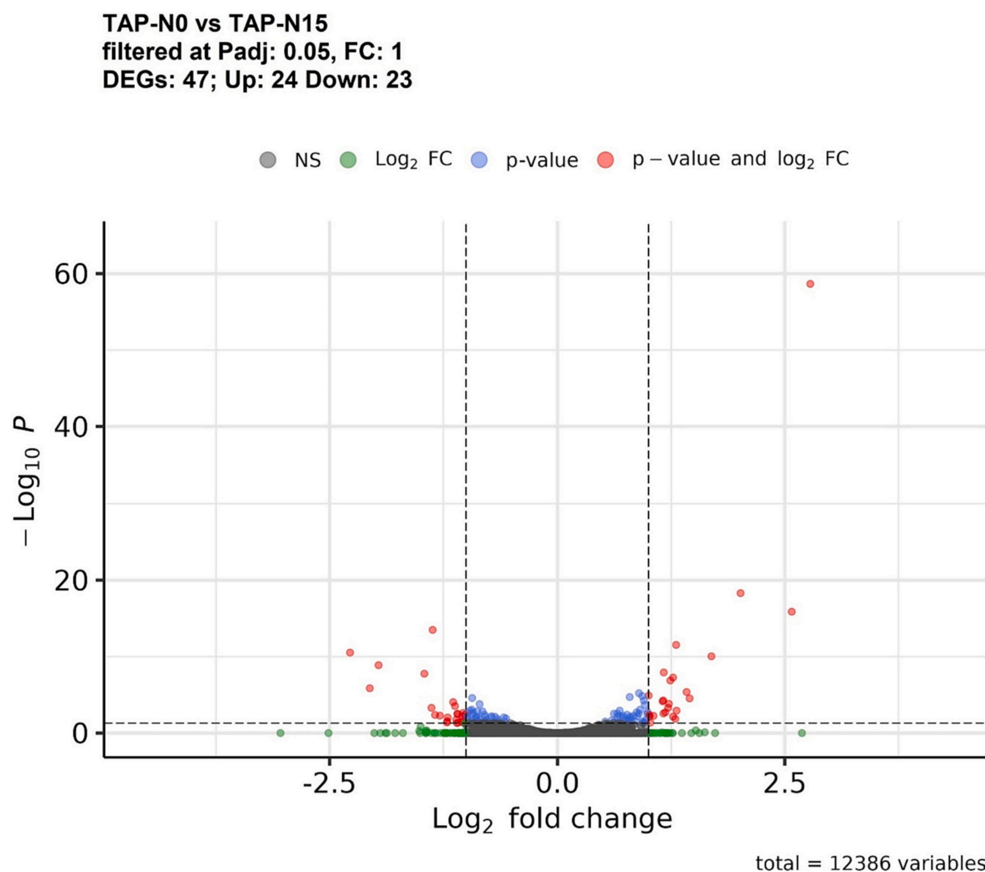


Fig. 2. Differential expression of genes in *C. reinhardtii* cc124. Volcano plot showing differentially expressed genes in *C. reinhardtii* cc124 under TAP-N0 vs TAP-N15 conditions. The genes were recorded as differentially expressed at a significance level of *p*-value $\leq$ 0.05 with a log2 fold change (FC) of 1. Red dots represent significantly differently expressed genes at log2 FC of 1. DEGs: differentially expressed genes. NS: Non-significant. Padj: Adjusted p-value $\leq$ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In *Chlamydomonas* sp. MACC-216, differential expression analysis was also performed by using TAP-N0 as control and TAP-N15 as treatment condition. A total of 3143 genes were differentially regulated at a log2 fold change of 1 and adjusted p-value $\leq$0.05. 1604 genes were upregulated, while 1539 genes were downregulated under the TAP-N15 condition (Fig. 4, Table S2). Genes coding for nitrate transporters, accessory proteins, nitrate metabolism, and nitrite metabolism were observed to be upregulated under nitrate-replete conditions. Nitrate transporters NRT2.1, NRT2.2, and NRT2.4 encoded by gene identifiers Cre09.g410850, Cre09.g410800, and Cre03.g150101, respectively were upregulated, whereas nitrate transporters NRT2.3, NRT2.5, NRT2.6 encoded by gene identifiers Cre09.g396000, Cre03.g150151, and Cre02.g110800, respectively, were downregulated. Furthermore, gene identifiers coding for nitrate reductase (Cre09.g410950) and nitrite reductase (Cre09.g410750) were also upregulated. Gene identifiers coding for nitrite transporters were downregulated except gene identifiers Cre12.g541250 and Cre01.g012050 which coded for nitrite transporters NAR1.5 and NAR1.6, respectively.

GO enrichment analysis produced a comprehensive list of upregulated and downregulated GO terms in comparison to *C. reinhardtii* cc124. GO enrichment analysis indicated GO terms related to photosynthesis and mitochondrial respiration to be enriched in the upregulated gene list under nitrate-replete condition (Fig. 5a). Furthermore, GO terms related to the biosynthesis of certain amino acids (methionine, leucine,

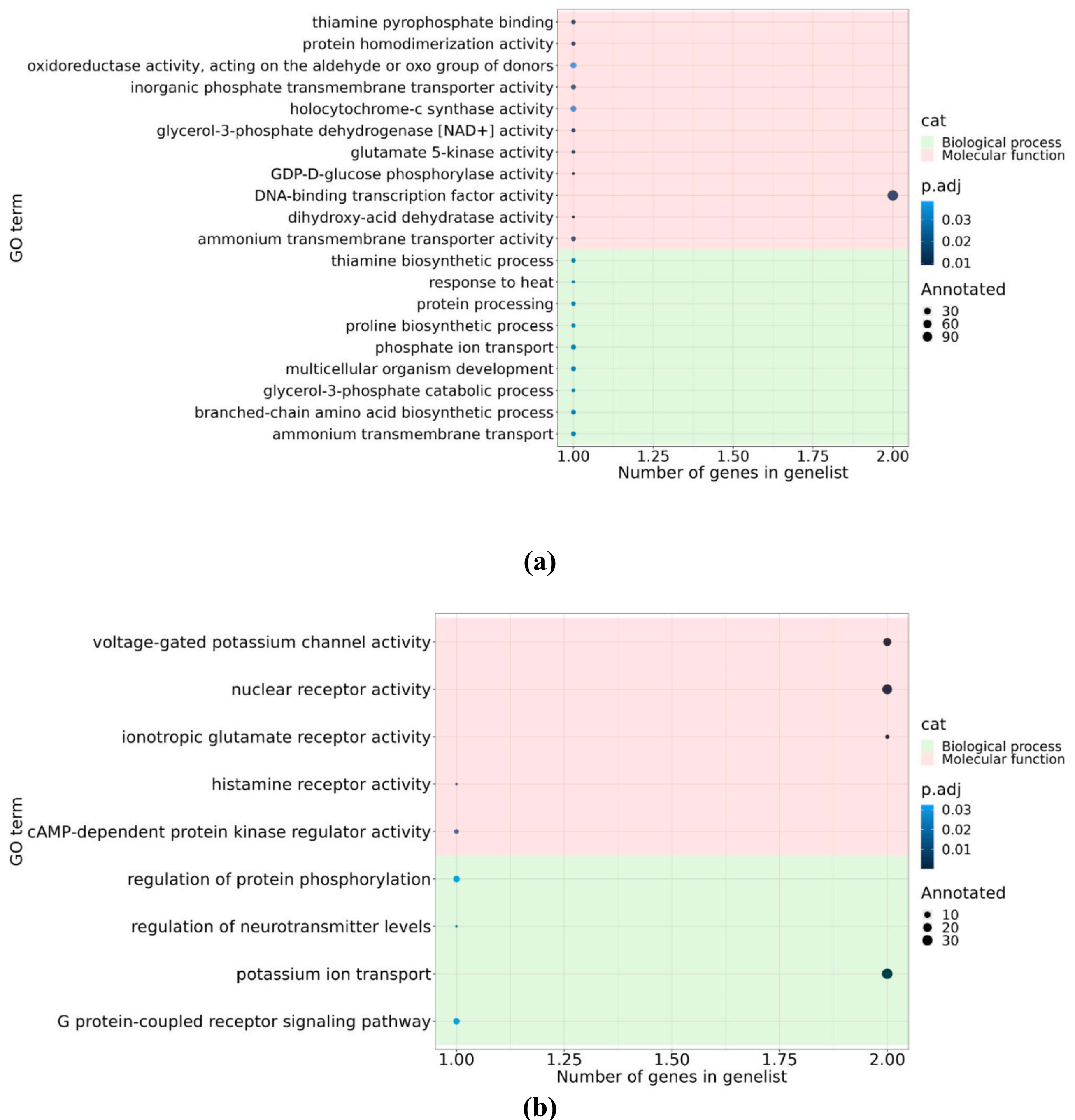


Fig. 3. Annotation enrichment in *C. reinhardtii* cc124. Enriched GO terms in upregulated (a) and downregulated (b) gene lists for *C. reinhardtii* cc124. Genes were systematically classified into two categories by GO analysis, including biological process, and molecular function. X-axis represents the number of the differentially expressed genes corresponding to a GO term. Y-axis represents GO terms. P.adj defines the *p*-value of significance. Size of the dot defines the number of genes associated with a particular GO term.

histidine), biosynthesis of tetrapyrrole, biosynthesis of fatty acids, and acetyl-CoA carboxylase complex were enriched in nitrate-replete samples. Acetyl-CoA carboxylase (ACCase) complex is known to play a role in the synthesis and elongation of fatty acids.

All mRNAs encoding the different ACCase subunits were upregulated under nitrate-replete condition. One gene identifier (Cre17.g699100) related to the hydrolysis of triacylglycerols was observed to be upregulated. Finally, two gene identifiers coding for *RHP1* and *RHP2* were also found to be upregulated. These genes are hypothesized to function as channels for carbon dioxide gas transport.

In the downregulated list (Fig. 5b), GO terms such as zinc ion binding, ammonium transmembrane transporter activity, and ion transport were enriched terms. Gene identifiers Cre14.g62992, Cre13.g569850, Cre09.g400750, Cre02.g111050, and Cre12.g531000 coding for ammonium transporters AMT2, AMT4, AMT5, AMT7, and AMT8, respectively were downregulated except gene identifier Cre07.g355650 which codes for AMT6 which was found to be upregulated. Moreover, stress-related response i.e., response to freezing was also noticed to be downregulated.

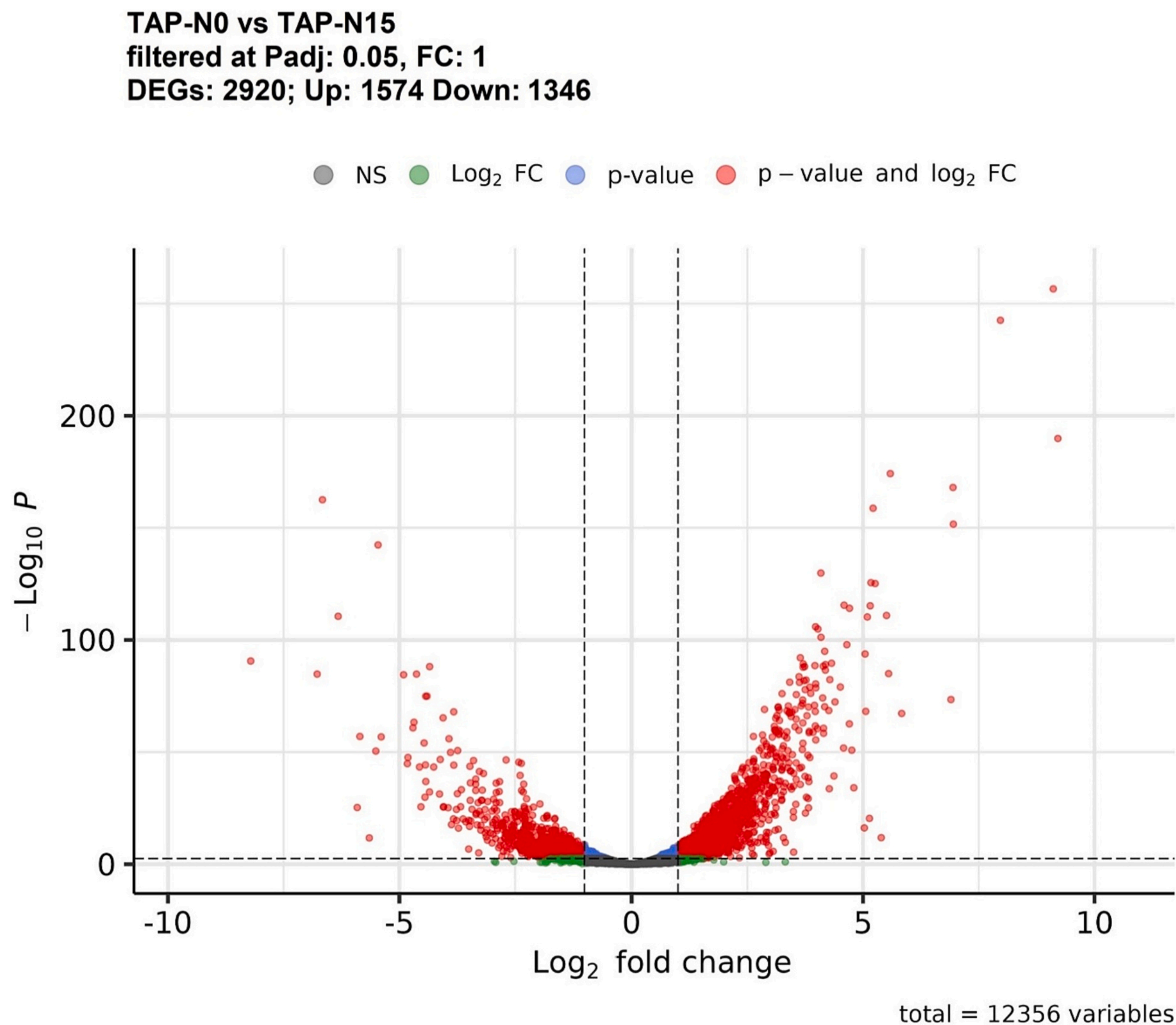


Fig. 4. Differential expression of genes in *Chlamydomonas* sp. MACC-216. Volcano plot showing differentially expressed genes in *Chlamydomonas* sp. MACC-216 under TAP-N0 vs TAP-N15 conditions. The genes were recorded as differentially expressed at a significance level of p-value ≤ 0.05 with a log2 fold change (FC) of 1. Red dots represent significantly differently expressed genes at log2 FC of 1. DEGs: differentially expressed genes. NS: Non-significant. Padj: Adjusted p-value ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Cross-species analysis of differentially expressed genes

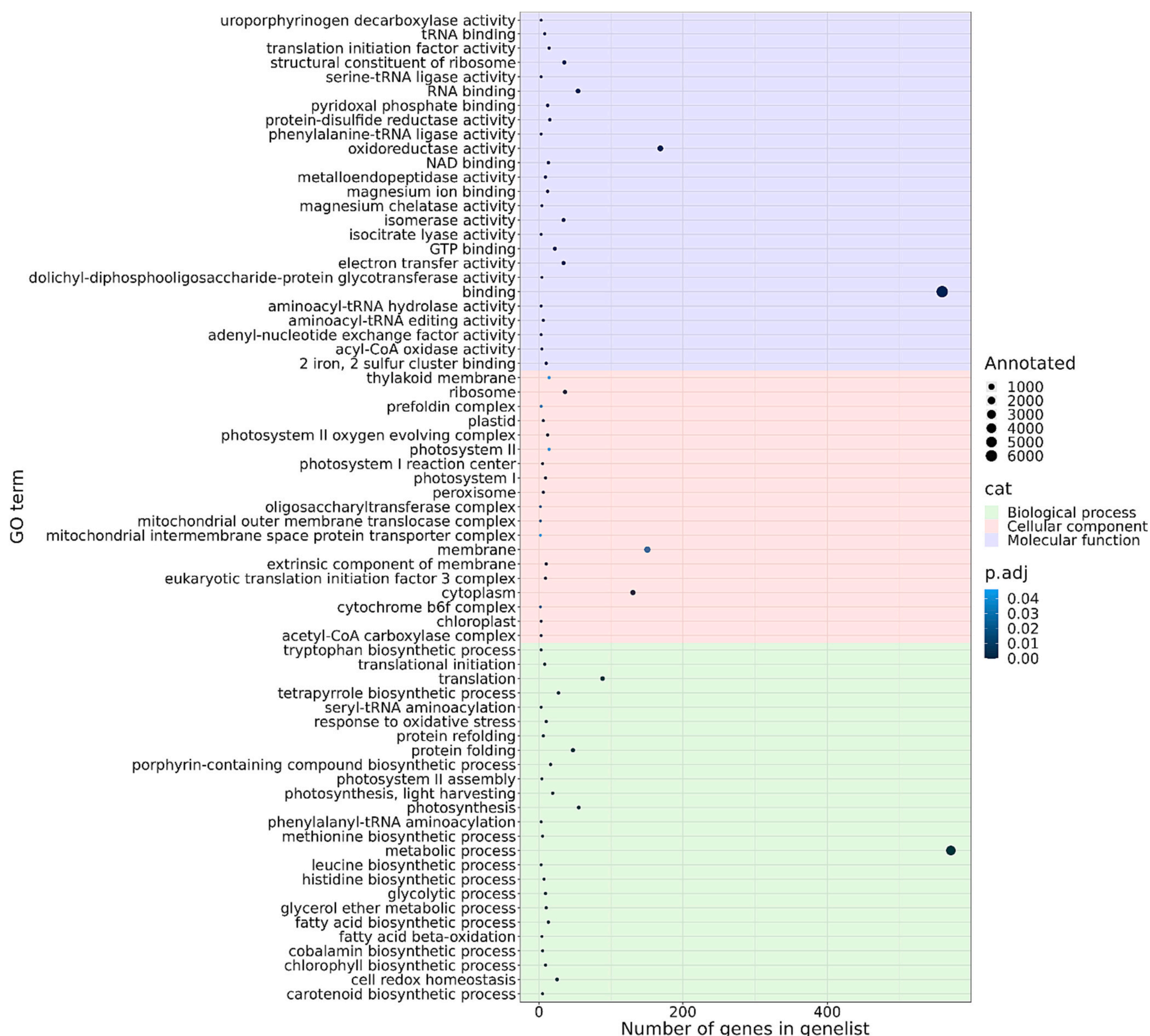
A heat map was generated to illustrate the differential expression of 28 nitrate metabolism-related genes under TAP-N0 and TAP-N15 conditions in both microalgae (Fig. 6 and Table 3). Upregulation of *NIA1*, *NII1*, *NRT2.1*, *NRT2.2*, *NRT2.4*, *NAR1.5*, *NAR1.6*, and *NAR2* genes was observed under TAP-N15 condition in *Chlamydomonas* sp. MACC-216, whereas downregulation of these genes was observed in *C. reinhardtii* cc124 under the same condition (Fig. 6).

Furthermore, it was noted that the gene associated with urea transport, *DUR3A*, exhibited a substantial upregulation under the TAP-N15 condition in *C. reinhardtii* cc124. In contrast, a marked downregulation was observed for genes *DUR1*, *DUR2*, *DUR3A*, *DUR3B*, and *DUR3C* under the TAP-N15 condition in *Chlamydomonas* sp. MACC-216. Notably, all the aforementioned genes displayed elevated expression levels in *C. reinhardtii* cc124 under both nitrate-replete and nitrate-deplete conditions, while they exhibited heightened expression solely in the nitrate-deplete condition for *Chlamydomonas* sp. MACC-216 (Table S3). From the heatmap, it was evident that gene identifiers related to nitrate assimilation and metabolism were not highly expressed in *C. reinhardtii* cc124, and this microalga behaved more or less in a similar manner whether it was cultivated in TAP-N0 or TAP-N15 media. This was in stark contrast to the expression of nitrate assimilatory genes in *Chlamydomonas* sp. MACC-216.

3.6. Quantification of gene expression

For the validation of transcriptome data, RT-qPCR was performed for the selected genes (Table 1). In the case of *C. reinhardtii* cc124, it was observed that genes responsible for nitrate transport (*NRT1*, *NRT2.1*, and *NRT2.2*) did not show any significant variation in gene expression under the TAP-N0 and TAP-N15 conditions (Fig. 7a). Similar observations were also observed for the gene responsible for nitrate reduction (*NIT1*). Genes participating in urea metabolism such as, *DUR3A*, and *DUR3B*) showed elevated expression under the TAP-N15 condition in comparison to the TAP-N0 condition. For *DUR1* and *DUR3C* genes, no significant variation was observed in expression between the two conditions.

In the case of *Chlamydomonas* sp. MACC-216, genes responsible for nitrate transport and nitrate reduction (*NRT2.1*, *NRT2.2*, *NIT1*, *NAR1.6*, *NII1*, and *GLN2*) showed increased expression under TAP-N15 condition (Fig. 7b). Especially, *NRT2.2* which showed highest expression among all the other selected genes under TAP-N15 condition. *NRT1* gene was expressed more under the TAP-N0 condition in comparison to the TAP-N15 condition. Even the regulatory gene *NIT2* was expressed more under the TAP-N0 condition. All of the genes related to urea transport



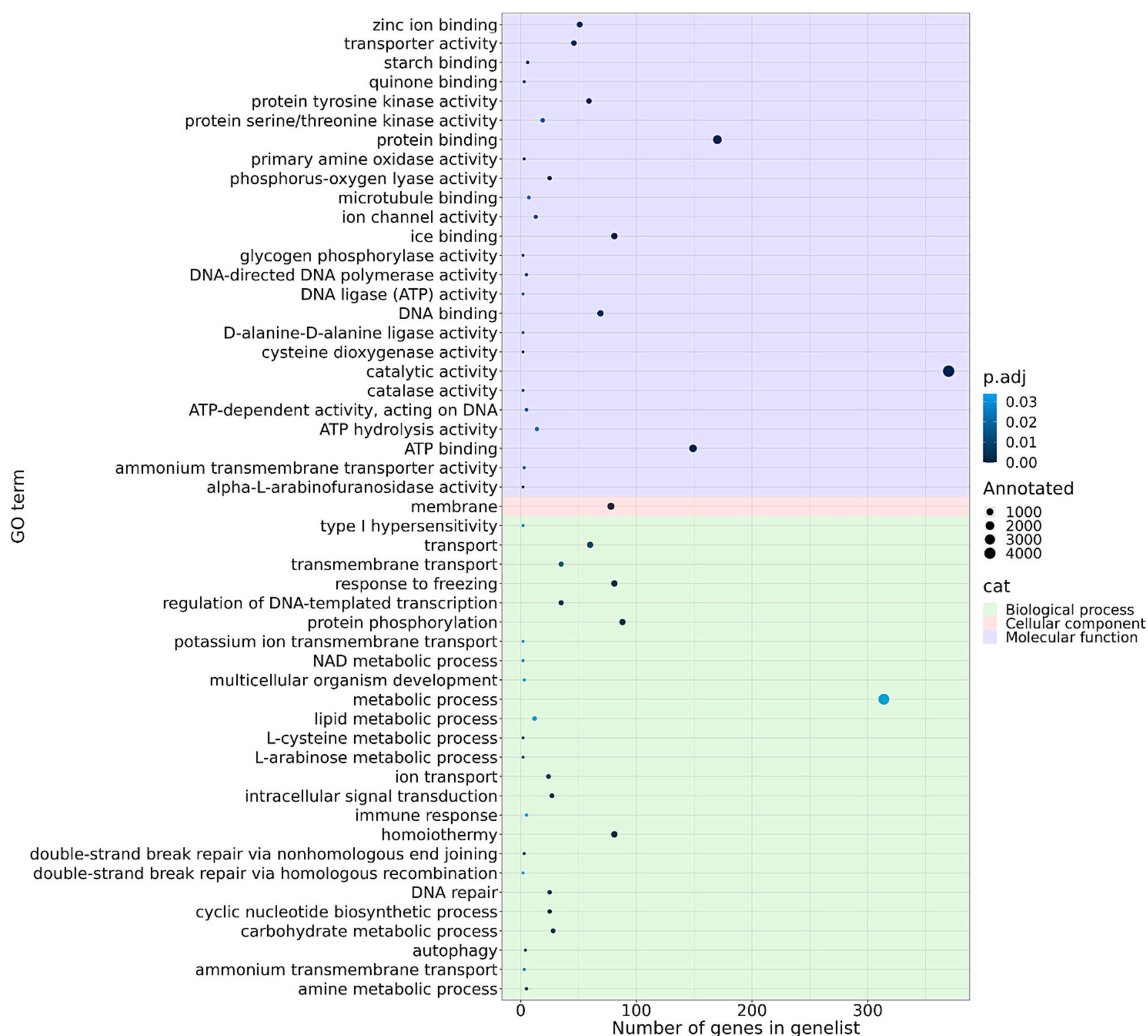
(a)

Fig. 5. Annotation enrichment in *Chlamydomonas* sp. MACC-216. Enriched GO terms in upregulated (a) and downregulated (b) gene lists for *Chlamydomonas* sp. MACC-216. Genes were systematically classified into three categories by GO analysis, including biological process, cellular component, and molecular function. X-axis represents the number of the differentially expressed genes corresponding to a GO term. Y-axis represents GO terms. P.adj defines the *p*-value of significance. Size of the dot defines the number of genes associated with a particular GO term.

and metabolism were upregulated under the TAP-N0 condition.

4. Discussion

The present study investigated the differences between the transcriptome of *C. reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216 under nitrate-deplete and nitrate-replete conditions. Previous experiments and literature data indicated that *C. reinhardtii* cc124 struggles to grow in TAP medium when nitrate is present as the sole nitrogen source [34,35] (Fig. S1a, c). At the same time, *Chlamydomonas* MACC-216 showed normal growth in media containing nitrate as the sole nitrogen source (Fig. S1b, d). Therefore, we aimed to examine the differences in nitrate metabolism in these two closely related species of *Chlamydomonas* microalgae. As a high-throughput approach, we investigated the initial changes occurring in the transcriptome of both microalgae after short-term exposure to two growth conditions (TAP-N0 and TAP—N15). Nitrate analytical measurements revealed that *C. reinhardtii* cc124 - as expected - did not remove a significant amount of nitrate from the TAP-N15 medium, while *Chlamydomonas* sp. MACC-216 efficiently removed 3.22 mM nitrate within 6 h (approximately 22 % of the total nitrate). This inability to remove nitrate within the first six hours further confirmed that *C. reinhardtii* cc124 had difficulties with the



(b)

Fig. 5. (*continued*).

uptake and metabolism of nitrate.

PCA plot of transcriptome samples showed that there was extremely low variability between the samples of *C. reinhardtii* cc124 grown under TAP-N0 and TAP-N15 conditions. This suggests that there is no significant reorganization in the transcriptome of *C. reinhardtii* cc124 between the two conditions, and this may be due to the inability of *C. reinhardtii* cc124 to sense nitrate in the TAP-N15 medium. Additionally, no enriched GO terms related to nitrate assimilation were observed in *C. reinhardtii* cc124, even when it was cultivated in the TAP-N15 medium. Carbonic anhydrase (CAH1) was upregulated in nitrate-replete samples of *C. reinhardtii* cc124. Previous studies have identified the upregulation of mitochondrial carbonic anhydrase (CAH5) as an early-stage response to nitrogen starvation [26]. Glycerol-3-phosphate dehydrogenase (GPDH) activity and glycerol-3-phosphate catabolic processes were enriched GO terms in the upregulated gene list; these two terms are associated with the production of glycerol. NAD^+-dependent Glycerol-3-phosphate dehydrogenase (GPDH) is known for catalyzing the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) by using NADH as an electron donor [36]. G3P is subsequently converted to glycerol by the action of a G3P phosphatase (GPP) or perhaps via the reversible reaction of a glycerol kinase [37]. G3P plays a crucial role in osmotic stress response and is a major metabolite in triacylglycerol production [36–38]. However, in our results, upregulation of GO term related to lipid accumulation in nitrate-replete samples was not observed, so it was assumed that G3P is playing a role in the stress response as *C. reinhardtii* cc124 does not grow well in TAP-N15 medium. The accumulation of intracellular glycerol has been shown to provide osmotic adjustment to salt stress in various organisms such as *Saccharomyces cerevisiae*, *Dunaliella* spp., *C. reinhardtii* [37–42]. Glutamate 5-kinase was an upregulated gene in *C. reinhardtii* cc124 under TAP-N15 condition, it catalyzes the ATP-dependent phosphorylation of L-glutamate to L-glutamate 5-phosphate during the biosynthesis of proline from glutamate [43]. Upregulation of this enzyme might explain the identification of proline biosynthesis as an

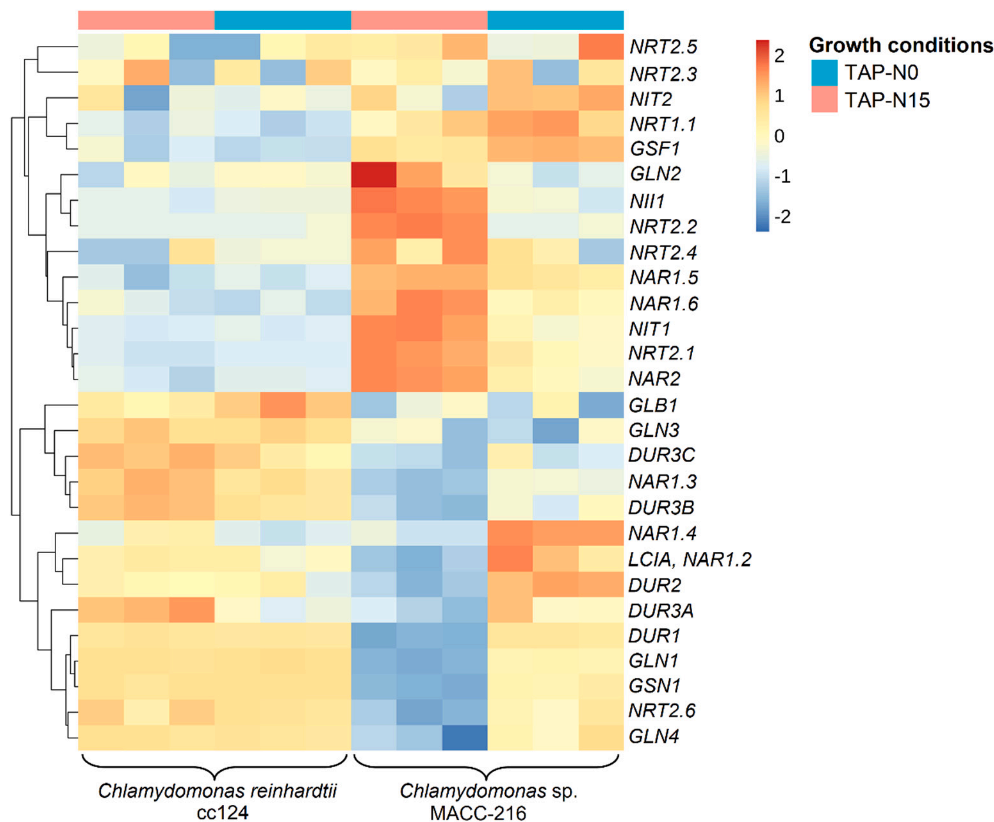


Fig. 6. Visualization of gene expression data. Heatmap representing nitrate metabolism-related differentially expressed genes in *C. reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216. Upregulated genes are coloured in red, while downregulated genes are shown in blue colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enriched GO term in the upregulated gene list of *C. reinhardtii* cc124 under TAP-N15 condition. Proline accumulation in stressed plants is linked with reduced damage to membranes and proteins [44,45]. Currently, it is uncertain if proline plays a similar role in improving *C. reinhardtii* cc124 survival under nitrate-replete or -deplete condition, future experiments involving exogenous proline supplementation are planned to test this theory. Upregulation of the branched-chain amino acid (BCAA) biosynthetic process was also observed. Leucine, isoleucine, and valine possess a common branched aliphatic chain and that is why they are known as branched-chain amino acids. Degradation products of these BCAAs include an acetyl-CoA which is a potential substrate for the de novo synthesis of fatty acids [46]. BCAAs are also known to play a role as structural and signalling compounds/molecules besides acting as a respiratory substrate [46,47]. Additionally, ammonium transmembrane transporter was one of the enriched upregulated GO terms under TAP-N15 condition in *C. reinhardtii* cc124; this GO term is probably associated with the detection of any trace of ammonia in the surroundings. GO terms such as ionotropic glutamate receptor activity, voltage-gated potassium channel activity, and potassium ion transport were downregulated for *C. reinhardtii* cc124 cultivated under TAP-N15 condition. Glutamate-like receptors (GLRs) of plants are homologs of mammalian ionotropic glutamate receptors (iGluRs), which were found more than a decade ago, and are speculated to function as possible amino acid sensors in plants [48]. Several studies have shown the involvement of GLRs in many biological processes such as abscisic acid (ABA) biosynthesis and signalling [49], innate immune responses [50,51], carbon and nitrogen balance or metabolism [52]. Hence, it can be assumed that GLRs may play a role in conveying nitrogen status and triggering the appropriate response. Schmollinger et al. [24] observed a high upregulation of GLR1 (Cre16.g685650) under nitrogen deplete condition in three strains of *C. reinhardtii*. Through our study, we also observed an upregulation of two GLRs (Cre12.g532850 and Cre12.g532950) under nitrate-deplete condition whereas downregulation of these two receptors was observed under nitrate-replete condition which explains a certain role of GLRs during nitrogen starvation. Upregulation of KCN11 potassium channel activity during nitrogen starvation has been shown to be essential in maintaining cell cycle activity and osmoregulation [53]. The lack of this particular protein is connected to loss in growth rate, inhibition of chlorophyll, and triacylglycerol accumulation.

The PCA plot of *Chlamydomonas* sp. MACC-216 samples showed a clear separation between the samples from TAP-N0 and TAP-N15 conditions, indicating major transcriptional reorganization between the two conditions. In *Chlamydomonas* sp. MACC-216, much more upregulated and downregulated enriched GO terms were obtained in comparison to *C. reinhardtii* cc124 (Fig. 5a, b). GO terms related to photosynthesis and mitochondrial respiration were enriched in the upregulated list of genes under the TAP-N15 condition in *Chlamydomonas* sp. MACC-216. Furthermore, enriched GO terms related to ACCase complex and acetyl-CoA oxidase activity were observed in the upregulated gene list. ACCase is an important enzyme that plays a role in the lipid synthesis pathway by catalyzing the formation of malonyl-CoA from acetyl-CoA in the plastid (chloroplast) and the cytosol of plants and algae [54]. Fatty acid synthase (FAS) then uses plastidial-derived malonyl-CoA for the de novo synthesis of fatty acids, while cytosol-derived malonyl-CoA is used for elongation of fatty acids in the endoplasmic reticulum [54]. However, no differential transcriptional regulation of the ACCase complex was observed in *Poryphyridium cruentum* between nitrogen-replete and -deplete conditions which indicates species-specific transcriptional regulation of this complex [27]. Furthermore, Huerlimann et al. [55], showed that changes in transcripts relating to acetyl-CoA were species-specific and depended on growth phase and nutrient conditions, and did

Table 3
List of selected genes relevant in nitrate metabolism. These 28 genes were used for heat map generation.

Gene description	Gene identifier	Gene symbol
Nitrate transporter	Cre04.g224700	*NRT1.1*
Nitrate transporter	Cre09.g410850	*NRT2.1*
High-affinity nitrate transporter	Cre09.g410800	*NRT2.2*
Nitrate/nitrite transporter	Cre09.g396000	*NRT2.3*
Nitrate/nitrite transporter	Cre03.g150101	*NRT2.4*
Nitrate/nitrite transporter	Cre03.g150151	*NRT2.5*
Nitrate/nitrite transporter	Cre02.g110800	*NRT2.6*
Nitrite transporter accessory protein	Cre09.g410900	*NAR2*
Nitrate reductase	Cre09.g410950	*NIT1*
Inorganic carbon channel localized at chloroplast membrane	Cre06.g309000	*LCIA, NAR1.2*
Formate/nitrite transporter	Cre04.g217915	*NAR1.3*
Formate/nitrite transporter	Cre07.g335600	*NAR1.4*
Nitrite transporter	Cre12.g541250	*NAR1.5*
Formate/nitrite transporter	Cre01.g012050	*NAR1.6*
Nitrite reductase	Cre09.g410750	*NII1*
Glutamine synthetase	Cre02.g113200	*GLN1*
Glutamine synthetase	Cre12.g530650	*GLN2*
Glutamine synthetase	Cre12.g530600	*GLN3*
Glutamine synthetase	Cre03.g207250	*GLN4*
Glutamate synthase, NADH-dependent	Cre13.g592200	*GSN1*
Glutamate synthase, ferredoxin-dependent	Cre12.g514050	*GSF1*
Transcription factor regulating nitrogen metabolism	Cre03.g177700	*NIT2*
Nitrogen regulatory protein PII	Cre07.g357350	*GLB1*
Urea carboxylase/allophanate hydrolase	Cre08.g360050	*DUR1*
Allophanate hydrolase	Cre08.g360100	*DUR2*
Urea active transporter	Cre08.g360200	*DUR3A*
Urea active transporter	Cre08.g360250	*DUR3B*
Urea active transporter	Cre17.g703800	*DUR3C*

not necessarily lead to fatty acid accumulation. GO terms related to amino acids biosynthesis, tetrapyrrole biosynthesis, chlorophyll biosynthesis and carotenoid biosynthesis were enriched GO terms in the upregulated gene list. With increasing photosynthesis and respiration, there is an increasing demand for proteins, chlorophyll, and carotenoids, and that is why corresponding terms were observed in the upregulated list. Furthermore, as tetrapyrrole compounds such as chlorophyll-a, chlorophyll-b, and cobalamin consist of nitrogen in their structure; these compounds may help accumulate nitrogen. Enzymes involved in the tetrapyrrole pathway were observed to be reduced under nitrogen starvation [24]. In the downregulated gene list of *Chlamydomonas* sp. MACC-216 under TAP-N15 condition, GO terms such as ion transport, and ammonium transmembrane transporter activity were enriched. These were all previously associated with nitrogen starvation responses. Furthermore, GO term zinc ion binding was also enriched in the downregulated gene list. This is a novel finding that has not been previously reported. Zinc has been known as an important component of various enzymes, a low concentration of zinc has been shown to help the growth of certain algae, whereas a high concentration of zinc has been shown to cause slow growth and reduced cell division [56]. As for ammonium transmembrane transporter activity, downregulation of *AMT2*, *AMT4*, *AMT5*, *AMT7*, and *AMT8*, respectively, was observed except *AMT6* which was found to be upregulated under TAP-N15 condition in *Chlamydomonas* sp. MACC-216. AMT6 is the largest ammonium transporter made up of 778 amino acids and may assist in reducing nitrogen quota. This transporter was the only downregulated transporter among ammonium transporters in *C. reinhardtii* under nitrogen-depleted condition probably to save nitrogen consumption [24]. Furthermore, González-Ballester et al. [57] showed higher expression of *AMT6* in microalgae grown under nitrate-replete condition compared to that under nitrate-deplete condition. A stress-related response, such as the response to freezing was observed to be downregulated. This suggests that microalgae grown under TAPN-15 conditions were not stressed, and that is why the downregulation of stress-related genes was observed. Moreover, downregulation of ion transport, transmembrane transport, and transporter activity GO terms was observed. Through our data, it was noticed that gene identifiers associated with the transport of ions were all downregulated for *Chlamydomonas* sp. MACC-216 under nitrate-replete condition. Interestingly, Schmollinger et al. [24] showed high induction of ion transport-related gene identifiers under nitrogen-depleted condition.

Previous studies have demonstrated that the expression of the *CrGLB1* gene is upregulated in the presence of nitrate [58]. However, no such upregulation was observed through this study either in *C. reinhardtii* cc124 or *Chlamydomonas* sp. MACC-216. The expression of *CrNZF1* was very similar in *C. reinhardtii* cc124 (~5 TPM) and *Chlamydomonas* sp. MACC-216 (~8 TPM) (Table S3). In fact, no differential expression of this gene was observed between TAP-N0 and TAP-N15 growth conditions in either microalga. *CrGLB1* encoded PII protein is responsible for coordinating the central C/N anabolic metabolism [59]. Furthermore, PII proteins have been shown to play a role in the regulation of triacylglycerol accumulation [59].

Visualization of expression of important nitrate metabolism-related genes was done through heatmap and the observed changes were further validated by RT-qPCR performed for selected genes. In *Chlamydomonas* sp. MACC-216, the expression of *NRT1.1* was higher under the TAP-N0 condition compared to the TAP-N15 condition. The NRT1 transporter is not widely studied in microalgae, although its functions are well-explored in plants. One of the best-studied NRT1 transporters from *Arabidopsis* is CHL1 or AtNRT1.1 which is a dual-affinity nitrate transporter that changes its affinity according to the nitrate conditions [60]. AtNRT1.1 is phosphorylated at low nitrate conditions and thereby functions as a high-affinity transporter, whereas, at high nitrate conditions, this transporter is dephosphorylated and becomes a low-affinity transporter. This explains why a higher expression of this transporter was observed under nitrate-deplete condition as it probably became a high-affinity nitrate transporter. Furthermore, an upregulation of *NRT2.1*, *NRT2.2* and *NRT2.4* was observed under TAP-N15 condition in *Chlamydomonas* sp. MACC-216. The NRT2 transporters are capable of transporting nitrate or nitrite, and some of them are two-component systems that necessitate the presence of the NAR2 (Nitrate assimilation-related component 2) protein to operate properly. Hence, NRT2.1 together with NAR2 constitutes a bispecific high-affinity nitrate/nitrite transporter and NRT2.2 together with NAR2 is a high-affinity nitrate transporter. The RT-qPCR analysis revealed a significant upregulation of the *NRT2.2* compared to the *NRT2.1*, suggesting that the transporter encoded by *NRT2.2* likely plays a crucial role in facilitating the transport of nitrate within microalgae (Fig. 7b). NRT2.4 transporter is associated with high-affinity transport of nitrite.

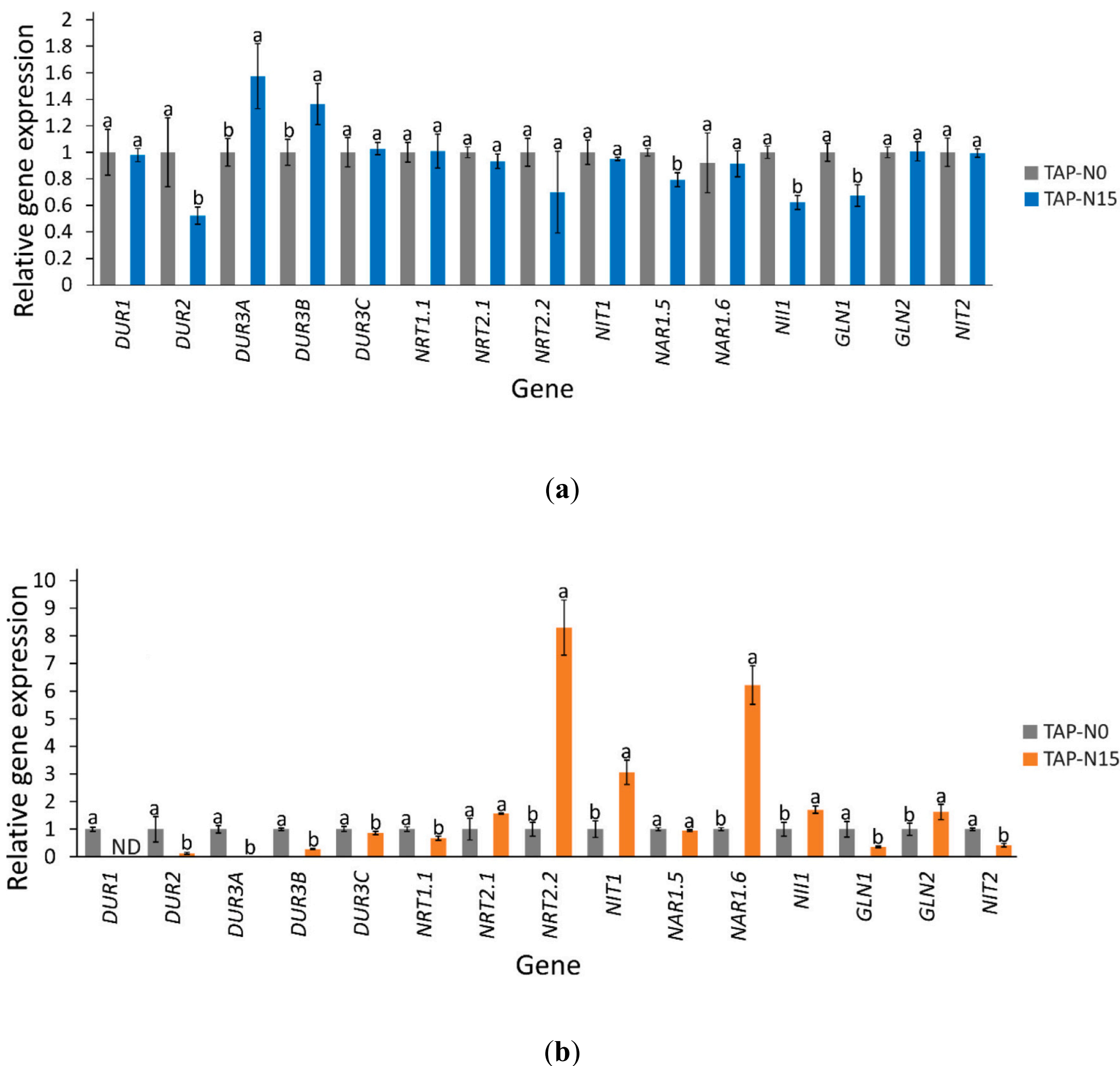


Fig. 7. RT-qPCR analysis. Relative gene expression of selected genes in *C. reinhardtii* cc124 (a) and *Chlamydomonas* sp. MACC-216 (b) for the validation of transcriptome data. For the normalization of relative gene expression, TAP-N0 condition was selected as the control. A relative gene expression value of <1 signifies downregulation, whereas a value of >1 signifies upregulation. X-axis represents the name of the gene. Y-axis represents the relative gene expression of a particular gene in either the TAP-N0 or TAP-N15 condition. Error bars represent standard deviations. Tukey's-test was performed for each gene separately. Lowercase letters signify statistical differences (p-value ≤ 0.05) as determined by Tukey's-test. qPCR amplification of the *DUR1* gene was not detected (ND) in *Chlamydomonas* sp. MACC-216 grown under TAP-N15 condition.

Furthermore, an upregulation of nitrate reductase (*NIT1*) and nitrite reductase (*NII1*) was observed. Nitrate reductase reduces nitrate to nitrite after that nitrite reductase catalyzes the reduction of nitrite to ammonium. Interestingly all the upregulated genes (*NRT2.1*, *NRT2.2*, *NRT2.4*, *NAR2*, *NIT1*, and *NII1*) observed in *Chlamydomonas* sp. MACC-216 under the TAP-N15 condition were downregulated in *C. reinhardtii* cc124 under the same condition. *C. reinhardtii* cc124 displayed similar expression of these genes under TAP-N0 and TAP-N15 conditions. Furthermore, upregulation of *NAR1.5*, and *NAR1.6* and downregulation of *NAR1.2*, and *NAR1.4* was observed under the TAP-N15 condition in *Chlamydomonas* sp. MACC-216. NAR1 transporters are known to play a role in the transport of nitrite and bicarbonate. Under the TAP-N15 condition, there was a significant upregulation of *NAR1.6* compared to *NAR1.5* in *Chlamydomonas* sp. MACC-216 (Fig. 7b). This points to the likelihood that the transporter encoded by *NAR1.6* plays a key role in the transport of nitrite. Apart from genes directly related to nitrogen assimilation and metabolism, downregulation of *GLN1* and *GLN4* genes coding for cytosolic GS1 (Glutamine synthetase) was also observed. Between *GLN2* and *GLN3* genes coding for chloroplastic GS2, *GLN2* was upregulated under the TAP-N15 condition in *Chlamydomonas* sp. MACC-216 in comparison to TAP-N0 condition.

Additionally, NIT2, a regulatory protein for the nitrate assimilation pathway showed no differential expression under TAP-N0 and TAP-N15 conditions in *C. reinhardtii* cc124. However, elevated expression of *NIT2* gene was observed under TAP-N0 condition in *Chlamydomonas* sp. MACC-216. Finally, genes responsible for urea assimilation (*DUR1* and *DUR2*) were expressed in a similar manner under both TAP-N0 and TAP-N15 conditions in *C. reinhardtii* cc124, indicating that these proteins might perform important functions for cells going through nitrogen starvation. However, genes responsible for urea transport (*DUR3A*, *DUR3B*, and *DUR3C*) showed higher expression under the TAP-N15 condition in *C. reinhardtii* cc124. Additionally, *DUR1*, *DUR2*, *DUR3A*, and *DUR3B* were also upregulated in *Chlamydomonas* sp. MACC-216 but only under TAP-N0 condition. Despite no urea being provided to either species of *Chlamydomonas* during cultivation, the expression of these genes related to urea metabolism lends evidence to the idea that these genes might have a role in nitrogen scavenging during nitrogen-starved condition. Previously, Park et al. [13] have also shown the upregulation of *DUR1*, *DUR2*, *DUR3B*, and *DUR3C* under nitrogen starvation. Furthermore, they also detected the presence of intracellular urea in

C. reinhardtii even when the growth medium was not supplemented with any urea [13]. Taken together, these results indicate that the upregulation of genes related to urea might indicate the activation of a survival mechanism in the absence of utilizable or any nitrogen source. Overall, the results from the heat map suggest that the expression of genes in *C. reinhardtii* cc124 grown under nitrate-deplete and -replete condition showed a highly similar pattern. No major difference was observed between the two conditions in this microalga which points towards the incapability of *C. reinhardtii* cc124 to sense and utilize nitrate as a nitrogen source. On the other hand, in *Chlamydomonas* sp. MACC-216, a clear difference in the gene expression patterns was observed between the two conditions.

The vast difference between the transcriptome of these two *Chlamydomonas* could be explained through the study of Pröschold et al. [61], where they showed that *C. reinhardtii* is divided into three sublines: Subline I ("Sager"-line), Subline II ("Cambridge"-line), and Subline III ("E/L"-line). In their study, they mentioned that *C. reinhardtii* belonging to Subline III are incapable of thriving solely on nitrate as their nitrogen source. This limitation can be attributed to the presence of mutations in *NIT1* and *NIT2* genes, where the existence of even a single mutation can impede microalgal growth on nitrate. Conversely, variants stemming from Subline I and Subline II can efficiently utilize nitrate for their growth. *C. reinhardtii* cc124 used in our study belongs to Subline III and that is why it cannot grow properly in the presence of nitrate. Furthermore, sequences of *NIT1* and *NIT2* genes were compared between *C. reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216 against a reference proteome which revealed larger differences in the *NIT2* sequence belonging to *Chlamydomonas* sp. MACC-216 (Method S1 & Fig. S2). Without additional mutants, it is difficult to confirm if the huge disparity in expression profiles between the two species is solely due to the variation in *NIT1* and *NIT2* sequences, or if additional regulatory elements are playing a role. This is because, we see similar expression of the *NIT2* gene in *C. reinhardtii* cc124 grown under both nitrate-deplete and -replete conditions, indicating that *C. reinhardtii* cc124 is somewhat unable to sense nitrate properly in the surroundings (Table S3). Since the sensing itself is an issue, variation in regulatory elements involved in nitrate sensing might play a role.

The knowledge about the diversity of nitrate metabolism within the *Chlamydomonas* genus can be important for ecological studies, as it contributes to our understanding of the adaptive strategies microalgae employ in response to varying environmental conditions. In-depth knowledge about the expression of important genes playing a role in nitrate metabolism was gained through the research carried out in the present study. Genes encoding nitrate transporter NRT2.2 and nitrite transporter NAR1.6 were shown to be highly expressed in the presence of nitrate in comparison to nitrate reductase and nitrite reductase encoding genes in *Chlamydomonas* sp. MACC-216, but similar expression of these genes was not observed in *C. reinhardtii* cc124 (Fig. 7). This kind of information can be crucial for selecting or engineering microalgae strains with specific gene expression traits for various biotechnological applications such as wastewater treatment.

5. Conclusions

Transcriptome analysis revealed only 45 genes to be differentially regulated in *C. reinhardtii* cc124 in the presence of nitrate compared to nitrate-deplete conditions. In contrast, 3143 genes showed different expression levels between nitrate-replete and nitrate-deplete conditions in *Chlamydomonas* sp. MACC-216. Genes playing a role in nitrate transport and reduction were differentially expressed in *Chlamydomonas* sp. MACC-216 only. The expression of genes involved in urea metabolism in *C. reinhardtii* cc124 was either similar in nitrate-deplete and -replete conditions or increased in nitrate-replete condition, which is intriguing given that the growth medium provided to this microalga did not contain urea as a nitrogen source. However, the same set of urea metabolism genes were only upregulated under nitrate-deplete condition in *Chlamydomonas* sp. MACC-216, indicating that scavenging and transport of urea might be a compensatory mechanism under nitrogen-limited condition. The absence of upregulation of established nitrogen sensing genes like *NIT2* and *CrNZF1* in *C. reinhardtii* cc124 hints at the potential existence of unidentified transcripts involved in nitrogen sensing in green microalgae. Finally, this study provides an example of the interesting phenomenon that taxonomically and morphologically strongly related green microalgae species from the same genus apply different strategies to utilize the essential macronutrient nitrogen.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103458.

Funding information

This research was funded by the following international and domestic funds: NKFI-2020-1.1.2-PIACI-KFI-2020-00020, NKFI-PD-146794 (SP) and the Lendület-Programme (GM) of the Hungarian Academy of Sciences [LP2020-5/2020] and by the Széchenyi Plan Plus National Laboratory Programme (National Laboratory for Water Science and Water Security, RRF-2.3.1-21- 2022-00008).

CRediT authorship contribution statement

Vaishali Rani: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Prateek Shetty:** Writing – review & editing, Visualization, Validation, Software, Formal analysis, Data curation, Conceptualization. **Gergely Maróti:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Acknowledgements

The authors would like to thank Seqomics Kft. (Mórahalom, Hungary) for the sequencing of the transcriptomes.

References

[1] E.W. Becker, Microalgae: Biotechnology and Microbiology, Cambridge University Press, Cambridge, 1994.

[2] A.J. Bloom, S.S. Sukrapanna, R.L. Warner, Root respiration associated with ammonium and nitrate absorption and assimilation by barley, Plant Physiol. 99 (1992) 1294–1301.

[3] M. Chen, H. Tang, H. Ma, T.C. Holland, K.Y. Ng, S.O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, Bioresour. Technol. 102 (2011) 1649–1655.

[4] G. Kim, G. Mujtaba, K. Lee, Effects of nitrogen sources on cell growth and biochemical composition of marine chlorophyte *Tetraselmis* sp. for lipid production, Algae 31 (2016) 257–266.

[5] T. Li, Y. Zheng, L. Yu, S. Chen, High productivity cultivation of a heat-resistant microalga *Chlorella sorokiniana* for biofuel production, Bioresour. Technol. 131 (2013) 60–67.

[6] M. Muthuraj, V. Kumar, B. Palabhanvi, D. Das, Evaluation of indigenous microalgal isolate *Chlorella* sp. FC2 IITG as a cell factory for biodiesel production and scale up in outdoor conditions, J. Ind. Microbiol. Biotechnol. 41 (2014) 499–511.

[7] S. Ruangsomboon, Effects of different media and nitrogen sources and levels on growth and lipid of green microalga *Botryococcus braunii* KMITL and its biodiesel properties based on fatty acid composition, Bioresour. Technol. 191 (2015) 377–384.

[8] M.C. de Graaf, R. Bobbink, J.G. Roelofs, P.J. Verbeek, Differential effects of ammonium and nitrate on three heathland species, Plant Ecol. 135 (1998) 185–196.
[9] J. Gerendás, Z. Zhu, R. Bendixen, R.G. Ratcliffe, B. Sattelmacher, Physiological and biochemical processes related to ammonium toxicity in higher plants, Z. Pflanzenernähr. Bodenkd. 160 (1997) 239–251.
[10] F.Q. Guo, J. Young, N.M. Crawford, The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in *Arabidopsis*, Plant Cell 15 (2003) 107–117.
[11] W.R. Scheible, M. Lauerer, E.D. Schulze, M. Caboche, M. Stitt, Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco, Plant J. 11 (1997) 671–691.
[12] H. Zhang, B.G. Forde, An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture, Science 279 (1998) 407–409.
[13] J.J. Park, H. Wang, M. Gargouri, R.R. Deshpande, J.N. Skepper, F.O. Holguin, D. R. Gang, The response of *Chlamydomonas reinhardtii* to nitrogen deprivation: a systems biology analysis, Plant J. 81 (2015) 611–624.
[14] S.S. Merchant, S.E. Prochnik, O. Vallon, E.H. Harris, S.J. Karpowicz, G.B. Witman, A. Terry, A. Salamov, L.K. Fritz-Laylin, L. Maréchal-Drouard, W.F. Marshall, The *Chlamydomonas* genome reveals the evolution of key animal and plant functions, Science 318 (2007) 245–250.
[15] N.M. Crawford, Nitrate: nutrient and signal for plant growth, Plant Cell 7 (1995) 859–868.
[16] E. Fernandez, A. Galvan, Inorganic nitrogen assimilation in *Chlamydomonas*, J. Exp. Bot. 58 (2007) 2279–2287.
[17] P. Fischer, U. Klein, Localization of nitrogen-assimilating enzymes in the chloroplast of *Chlamydomonas reinhardtii*, Plant Physiol. 88 (1988) 947–952.
[18] E. Sanz-Luque, A. Chamizo-Ampudia, A. Llamas, A. Galvan, E. Fernandez, Understanding nitrate assimilation and its regulation in microalgae, Front. Plant Sci. 6 (2015) 899.
[19] A. Camargo, Á. Llamas, R.A. Schnell, J.J. Higuera, D. González-Ballester, P. A. Lefebvre, E. Fernández, A. Galván, Nitrate signaling by the regulatory gene *NIT2* in *Chlamydomonas*, Plant Cell 19 (2007) 3491–3503.
[20] R.A. Schnell, P.A. Lefebvre, Isolation of the *Chlamydomonas* regulatory gene *NIT2* by transposon tagging, Genetics 134 (1993) 737–747.
[21] A. Quesada, A. Galván, R.A. Schnell, P.A. Lefebvre, E. Fernández, Five nitrate assimilation-related *loci* are clustered in *Chlamydomonas reinhardtii*, Mol. Gen. Genet. MGG 240 (1993) 387–394.
[22] J.J. Higuera, E. Fernandez, A. Galvan, *Chlamydomonas* NZF1, a tandem-repeated zinc finger factor involved in nitrate signalling by controlling the regulatory gene *NIT2*, Plant Cell Environ. 37 (2014) 2139–2150.
[23] H. Rismani-Yazdi, B.Z. Haznedaroglu, C. Hsin, J. Peccia, Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation, Biotechnol. Biofuels 5 (2012) 1–6.
[24] S. Schmollinger, T. Mühlhaus, N.R. Boyle, I.K. Blaby, D. Casero, T. Mettler, J. L. Moseley, J. Kropat, F. Sommer, D. Strenkert, D. Hemme, M. Pellegrini, A. R. Grossman, M. Stitt, M. Schroda, S.S. Merchant, Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism, Plant Cell 26 (2014) 1410–1435.
[25] L. Li, L. Zhang, F. Gong, J. Liu, Transcriptomic analysis of hydrogen photoproduction in *Chlorella pyrenoidosa* under nitrogen deprivation, Algal Res. 47 (2020) 101827.
[26] López García, A. de Lomana, S. Schäuble, J. Valenzuela, S. Imam, W. Carter, D. D. Bilgin, C.B. Yohn, S. Turkarslan, D.J. Reiss, M.V. Orellana, N.D. Price, N. S. Baliga, Transcriptional program for nitrogen starvation-induced lipid accumulation in *Chlamydomonas reinhardtii*, Biotechnol. Biofuels 8 (2015) 207.
[27] L. Wei, W. You, Z. Xu, W. Zhang, Transcriptomic survey reveals multiple adaptation mechanisms in response to nitrogen deprivation in marine *Porphyridium cruentum*, PLoS One 16 (2021) 1–21.
[28] R. Miller, G. Wu, R.R. Deshpande, A. Vieler, K. Gärtner, X. Li, E.R. Moellering, S. Zäuner, A.J. Cornish, B. Liu, B. Bullard, B.B. Sears, M.H. Kuo, E.L. Hegg, Y. Shachar-Hill, S.H. Shiu, C. Benning, Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism, Plant Physiol. 154 (2010) 1737–1752.
[29] V. Rani, G. Maróti, Assessment of nitrate removal capacity of two selected eukaryotic green microalgae, Cells 10 (2021) 2490.
[30] D.A. Cataldo, M. Haroon, L.E. Schrader, V.L. Youngs, Rapid colorimetric determination of nitrate in plant tissue by nitration in salicylic acid, Commun. Soil Sci. Plant Anal. 6 (1975) 71–80.
[31] L. Song, L. Florea, Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads, GigaScience 4 (2015) s13742-015.
[32] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114–2120.
[33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2_T^{-\Delta\Delta C}$ method, Methods 25 (2001) 402–408.
[34] T. Saito, M. Inoue, M. Yamada, Y. Matsuda, Control of gametic differentiation and activity by light in *Chlamydomonas reinhardtii*, Plant Cell Physiol. 39 (1998) 8–15.
[35] T. Kubo, J. Abe, T. Saito, Y. Matsuda, Genealogical relationships among laboratory strains of *Chlamydomonas reinhardtii* as inferred from matrix metalloprotease genes, Curr. Genet. 41 (2002) 115–122.
[36] M.L. Casais-Molina, S. Peraza-Echeverria, I. Echevarría-Machado, V.A. Herrera-Valencia, Expression of *Chlamydomonas reinhardtii* CrGPDH2 and CrGPDH3 cDNAs in yeast reveals that they encode functional glycerol-3-phosphate dehydrogenases involved in glycerol production and osmotic stress tolerance, J. Appl. Phycol. 28 (2015) 219–226.
[37] T. Driver, D.K. Trivedi, O.A. McIntosh, A.P. Dean, R. Goodacre, J.K. Pittman, Two glycerol-3-phosphate dehydrogenases from *Chlamydomonas* have distinct roles in lipid metabolism, Plant Physiol. 174 (2017) 2083–2097.
[38] M. Cai, L.H. He, T.Y. Yu, Molecular clone and expression of a NAD^+-dependent glycerol-3-phosphate dehydrogenase isozyme gene from the halotolerant alga *Dunaliella salina*, PLoS One 8 (2013) e62287.
[39] L. André, A. Hemming, L. Adler, Osmoregulation in *Saccharomyces cerevisiae*: studies on the osmotic induction of glycerol production and glycerol-3-phosphate dehydrogenase (NAD^+), FEBS Lett. 286 (1991) 13–17.
[40] A. Ben-Amotz, I. Sussman, M. Avron, Glycerol production by *Dunaliella*, Experientia 38 (1982) 49–52.
[41] H.D. Husic, N.E. Tolbert, Effect of osmotic stress on carbon metabolism in *Chlamydomonas reinhardtii*, Plant Physiol. 82 (1986) 594–596.
[42] R. León, F. Galván, Halotolerance studies on *Chlamydomonas reinhardtii*: glycerol excretion by free and immobilized cells, J. Appl. Phycol. 6 (1994) 13–20.
[43] A. Baich, Proline synthesis in *Escherichia coli*. A proline-inhibitable glutamic acid kinase, Biochimica et Biophysica Acta (BBA)-General Subjects. 192 (1969) 462–467.
[44] P.P. Saradhi, P. Mohanty, Involvement of proline in protecting thylakoid membranes against free radical-induced photodamage, J. Photochem. Photobiol. B Biol. 38 (1997) 253–257.
[45] K. Shah, R.S. Dubey, Effect of cadmium on proline accumulation and RNase activity in rice seedings: role of proline as a possible enzyme protectant, Biol. Plant. 40 (1997) 121–130.
[46] S. Binder, Branched-chain amino acid metabolism in *Arabidopsis thaliana*, Arabidopsis Book/American Society of Plant Biologists. 8 (2010) e0137.
[47] S.R. Kimball, L.S. Jefferson, Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis, J. Nutr. 136 (2006) 227S–231S.
[48] M.B. Price, J. Jelesko, S. Okumoto, Glutamate receptor homologs in plants: functions and evolutionary origins, Front. Plant Sci. 3 (2012) 235.
[49] J. Kang, S. Mehta, F.J. Turano, The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss, Plant Cell Physiol. 45 (2004) 1380–1389.
[50] S. Kang, H.B. Kim, H. Lee, J.Y. Choi, S. Heu, C.J. Oh, S.I. Kwon, C.S. An, Overexpression in *Arabidopsis* of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca^{2+} influx and delays fungal infection, Molecules & Cells (Springer Science & Business Media BV). 21 (2006) 418–427.
[51] H. Manzoor, J. Kelloniemi, A. Chiltz, D. Wendehenne, A. Pugin, B. Poinssot, A. Garcia-Brugger, Involvement of the glutamate receptor AtGLR3.3 in plant defense signaling and resistance to *Hyaloperonospora arabidopsidis*, Plant J. 76 (2013) 466–480.
[52] J. Kang, F.J. Turano, The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. 100 (2003) 6872–6877.
[53] F. Xu, J. Pan, Potassium channel KCN11 is required for maintaining cellular osmolarity during nitrogen starvation to control proper cell physiology and TAG accumulation in *Chlamydomonas reinhardtii*, Biotechnol. Biofuels 13 (2020) 129.
[54] R. Huerlimann, K. Heimann, Comprehensive guide to acetyl-carboxylases in algae, Crit. Rev. Biotechnol. 33 (2013) 49–65.
[55] R. Huerlimann, E.J. Steinig, H. Loxton, K.R. Zenger, D.R. Jerry, K. Heimann, The effect of nitrogen limitation on acetyl-CoA carboxylase expression and fatty acid content in *Chromera velia* and *Isochrysis* aff. *galbana* (TISO), Gene 543 (2014) 204–211.
[56] N.I. El-Agawany, M.I.A. Kaamoush, Role of zinc as an essential microelement for algal growth and concerns about its potential environmental risks, Environ. Sci. Pollut. Res. 30 (2023) 71900–71911.
[57] D. González-Ballester, A. Camargo, E. Fernández, Ammonium transporter genes in *Chlamydomonas*: the nitrate-specific regulatory gene *Nit2* is involved in *Amt1.1* expression, Plant Mol. Biol. 56 (2004) 863–878.
[58] Z. Zalutskaya, L. Kochemasova, E. Ermilova, Dual positive and negative control of Chlamydomonas PII signal transduction protein expression by nitrate/nitrite and NO via the components of nitric oxide cycle, BMC Plant Biol. 18 (2018) 305.
[59] Z. Zalutskaya, N. Kharatyan, K. Forchhammer, E. Ermilova, Reduction of PII signaling protein enhances lipid body production in *Chlamydomonas reinhardtii*, Plant Sci. 240 (2015) 1–9.
[60] C.H. Ho, S.H. Lin, H.C. Hu, Y.F. Tsay, CHL1 functions as a nitrate sensor in plants, Cell 138 (2009) 1184–1194.
[61] T. Pröschold, E.H. Harris, A.W. Coleman, Portrait of a species: *Chlamydomonas reinhardtii*, Genetics 170 (2005) 1601–1610.