Nitrogen metabolism in the picoalgae *Pelagomonas calceolata*: disentangling cyanate lyase function under different nutrient conditions.

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Abstract

Among nitrogen sources compounds in the environment, cyanate (OCN^-) is a potential important actor given the activity and prevalence of cyanate lyase genes in microalgae. However, the conditions in which this gene is activated and the actual capacities of microalgae to assimilate cyanate remain underexplored. Here, we studied the nitrogen metabolism of the abundant and cosmopolite picoalgae *Pelagomonas calceolata* (Ochrophyta/Pelagophyceae) with environmental metatranscriptomes and culture experiments under different nitrogen sources (nitrate, ammonium, urea and cyanate) and concentrations. We observed that in nitrate-poor oceanic regions, the cyanate lyase gene is one of the most differentially expressed gene, suggesting that cyanate is an important molecule for *P. calceolata* persistence in oligotrophic environments. In the lab, we confirmed that this gene is overexpressed in low-nitrate medium together with several genes involved in nitrate recycling from endogenous molecules (purines and amino acids). *P. calceolata* is capable of growth on various nitrogen sources including nitrate, urea and cyanate but not ammonium. RNA sequencing of these cultures revealed that the cyanate lyase gene is surprisingly underexpressed in cyanate conditions indicating that this gene in not involved in extracellular cyanate catabolism to ammonia. Taken together, environmental datasets and lab experiments show that if the cyanate lyase is important in nitrate-poor environments it's probably to reduce the toxicity of cyanate as a consequence of endogenous nitrogenous compound recycling rather than the use extracellular cyanate to produce ammonium.

Introduction

Nitrogen is essential to many biological processes, such as photosynthesis, amino acid and nucleic acid biosynthesis, thus its bioavailability in the oceans impacts the growth of primary producers (Moore et al., 2013). Following gaseous dinitrogen fixation by diazotroph bacteria, inorganic ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the primary sources of fixed nitrogen for phytoplankton (Hutchins & Capone, 2022). Nitrate is the most abundant nitrogenous compound, whereas nitrite and ammonium are typically less abundant (Collos & Berges, 2002). Nevertheless, ammonium is considered a preferred source of nitrogen for the phytoplankton due to its lower energy cost during assimilation (Kumar & Bera, 2020). Majority of oceanic surface waters are depleted in inorganic nitrogen compounds because of the uptake photosynthetic organisms in the photic zone (Hutchins & Capone, 2022). A consequence of ocean warming is a potential enhanced stratification reducing the supply of nutrients to the euphotic zone (Fu et al., 2016). At the end of the twenty-first century, a decrease of nitrate concentration of 1.06 ± 0.45 $mmol.m^{-3}$ on average of surface water is projected under the IPCC high-emission scenario SSP5-8.5 (Kwiatkowski et al., 2020).

Since inorganic nitrogen sources concentration and availability can vary, photosynthetic organisms developed a large set of strategies to face temporal and spatial changes in nitrate, nitrite and ammonium availability. It has been shown that the optimisation of inorganic nitrogen uptake can be achieve with the regulation of the expression of transporters in several phytoplankton groups, especially in prasinophytes and diatoms (Lampe et al., 2019). Storage and recycling of nitrogen-rich proteins is also an important strategy in diatoms (Alipanah et al., 2015; Scarsini et al., 2022). Various phytoplankton can metabolise dissolved organic N (DON) such as urea, purines or amino acid (Kumar & Bera, 2020). In the ocean, DON concentration is often higher than DINs and contributes to autotrophic production and growth of primary producers, especially in coastal and estuarine environments (Sipler & Bronk, 2015). All these compounds have been shown to support the growth of microalgae in low N-conditions. For example, urea can be uptake as a nitrogen source by many phytoplankton species, such as diatoms, dinoflagellates and the bloom forming algae *Aureococcus anophagefferens* (Berg et al., 1997; Cerón García et al., 2005; García-Portela et al., 2020). Diatoms such as *P. tricornutum* and *T. pseudonana* present urea transporters that are overexpressed in N-limited conditions. *Emiliania huxleiyi* can use a broader range of organic nitrogen source, including urea, hydroxyurea, hydroxanthine, purines and small amides such as acetamides and formamides (Palenik & Henson, 1997).

Cyanate ion (OCN^-) is the smallest nitrogenous organic compound originally described as a toxic molecule altering structural and functional properties of proteins through carbamylation (Jaisson et al., 2011). In the oceans, cyanate is produced by terrestrial inputs, spontaneous decomposition of carbamoyl-phosphate or urea released by zooplankton or senescent diatom cells and photoproduction of dissolved organic matter (Widner et al., 2016). Cyanate concentration is measured up to 45 nM in ocean subsurface (Widner et al., 2018). Organisms actually capable of cyanate uptake and metabolism in the environment as well as underlying molecular mechanisms remain unclear. Growth on cyanate as sole N source was first described in *E. coli* then in cyanobacteria, in the ammonia-oxidizing archaea *Nitrososphaera gargensis*, in several yeasts, and in the mixotrophic dinophyceae *Prorocentrum* (Anderson et al., 1990; Hu et al., 2012, 2014; Kamennaya & Post, 2011; Linder, 2018; Palatinszky et al., 2015). In bacteria, the cyanate lyase enzyme (cynS gene) catalyses the bicarbonate dependant reaction of cyanate to ammonia and carbon dioxide (Johnson & Anderson, 1987). Cyanate lyase homologs are present in the genome of many organisms

including the dominant eukaryotic phytoplankton lineages (Mao et al., 2022). In the environment, the expression of cyanate lyase in most prokaryotic and eukaryotic phytoplankton is increased in N-limited environments, suggesting that these organisms might use cyanate as alternative N source (Dong et al., 2014; Mao et al., 2022; Smith et al., 2019; Wurch et al., 2011). ^{15}N stable isotope probing revealed that cyanate uptake could account for up to 10% of total nitrogen uptake in natural communities from offshore oligotrophic Atlantic, particularly in surface waters (Widner et al., 2016). Since cyanate lyase expression is as important as urease expression in the environment, it has been suggested that cyanate has a crucial ecological role (Mao et al., 2022). However, with the exception of *Prorocentrum,* the uptake of cyanate has not been shown in eukaryotic phytoplankton. A recent study have shown that cyanate enrichment in natural phytoplankton populations induces *Synechococcus* growth but not eukaryotic phytoplankton (Sato et al., 2023). This leads us to question the role of cyanate lyase in organic nitrogen assimilation in photosynthetic eukaryotes.

In order to disentangle the endogenous role of cyanate lyase in microeukaryotes and more globally have a better understanding of eukaryotic phytoplankton acclimation to changing N availability in the environment, we used the cosmopolite photosynthetic pico-eukaryote *Pelagomonas calceolata* (Pelagophyceae) as a model for pelagic phytoplankton (Guérin et al., 2022; Worden et al., 2012). *P. calceolata* genome (RCC100 strain) contains a large set of genes involved in nitrogen metabolism (Guérin et al., 2022). Among them, genes coding for arginase, urease and cyanate lyase may suggest the capacity of organic nitrogen compounds metabolism. In the environment, several nitrogen ions transporters, nitrate and nitrite reductases, glutamine synthetase, nitrate/nitrite sensing proteins and the cyanate lyase are significantly expressed at higher levels in low-N environments (Dupont et al., 2015; Guérin et al., 2022). Here we cultivated two *P. calceolata* strains under high or limited nitrate conditions and tested the effect of several inorganic (nitrate, ammonia) and organic (urea, cyanate) sources of nitrogen on *P. calceolata* growth. For all these conditions, we performed RNA sequencing and identified differentially expressed genes. We compared our results with a differential analysis of *P. calceolata* gene expression levels in environmental metatranscriptomes of *Tara* Oceans expedition according to *in situ* measurements of nitrate concentrations.

Results

In situ gene expression levels of *P. calceolata* according to nitrate concentration

To study the nitrogen metabolism of *P. calceolata in situ,* we used all metatranscriptomes of *Tara* Oceans datasets (Alberti et al., 2017). We aligned metatranscriptomic reads on the predicted mRNAs of *P. calceolata* RCC100 genome and selected 124 *Tara* samples where at least 75% of the genes are detected (at least 1 read aligned with more than 95% of identity over 80% of its length). For 112 of these samples with available *in situ* measurements of nitrate, 69 have nitrate concentrations below 2 µM and are considered "low-nitrate", and 43 have nitrate concentrations above 2 µM and are considered "high-nitrate" (Figure 1A). Interestingly, there is no significant correlation between nitrate concentrations and the relative abundance of *P. calceolata* (Figure 1B ; person= 0.2, p.value=0.11).

Differential expression analysis between "high-nitrate" and "low-nitrate" environments reveals 375 genes significantly overexpressed in low-nitrate samples (p-value < 0.01) and 6 of them with a log2 fold change (log2FC) higher than 2 (Figure 1B). Two of these genes are involved in inorganic nitrogen transport across cell membranes, a formate or nitrite transporter (PF01226 domain) and an ammonium transporter (PF00909 domain) as previously described (Guérin et al., 2022). In addition, a

purine transporter (uric acid/xanthine permease, K23887 domain), a formyltetrahydrofolate deformylase (10-FDF; EC 3.5.1.10), enzyme acting on carbon-nitrogen bounds and potentially related to ammonium recycling from glycine and an unknown gene carrying a RNA-binding domain of the Pumilio family (K17943 domain) are overexpressed in low-nitrate environments. The cyanate lyase (PF02560 domain) is the second most overexpressed genes in low-nitrate environments in *P. calceolata* genome. We also note the slight overexpression of a nitrate/nitrite transporter (K02575 domain), a carbon nitrogen hydrolase (PF00795 domain) and a dipeptidase (K08659 domain) suggesting an active recycling of nitrogen-rich molecule. Only 20 genes are slightly underexpressed in low-nitrate environments with a log2FC between 1 and 1.5 and most of them (14 genes) have unknown functions. Three Light Harvesting Complex proteins (LHC; PF00504 domain) are downregulated in low-nitrate environments suggesting a lower chloroplast activity.

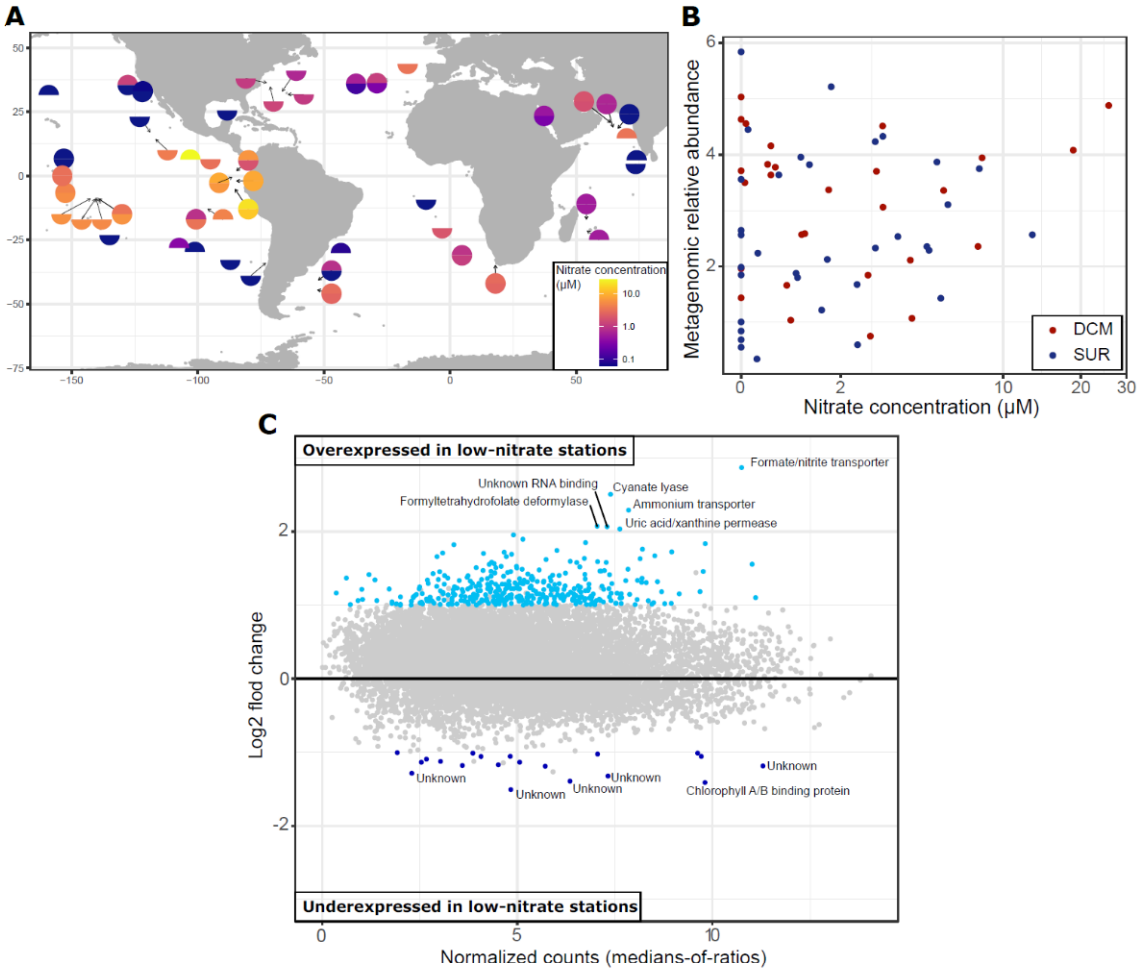

Figure 1. Abundance and transcriptomic response of *P. calceolata* to environmental nitrate concentrations. A) Nitrate concentrations measured during *Tara* Oceans expedition. The colour code indicates nitrate concentrations in µmol/l for surface and DCM samples in the upper and bottom part of each dot respectively. B) Relative abundance of *P. calceolata* in *Tara* samples estimated from metagenomics reads according to the concentration of nitrate (µM) C) *P. calceolata* gene expression levels between low-nitrate (NO_3^- < 2 µM, n=69) and high-nitrate samples (NO_3^- > 2 µM, n=43). Log2FC between low- and high-nitrate samples are given according to their mean expression level (normalized with DESeq2). Differentially expressed genes with p-value < 0.01 and log2FC >1 or < -1 are coloured in blue.

Growth and gene expression variations under low-N conditions

To estimate acclimation capacities of *P. calceolata* to low-nitrate conditions, we cultivated two strains representatives of the physiological diversity of this species complex. RCC100 (CCMP1214), isolated in the Pacific Ocean in 1973, is flagellated with a cell size of 1.5-2 µm. RCC697, isolated in the Indian Ocean in 2003, is significantly larger with cell diameter of 3 µm and non-flagellated. Since their isolation, both strains of *P. calceolata* are maintained in the Roscoff Culture Collection at 20°C in K media (Keller et al., 1987). These strains were cultivated in triplicates with different nitrate concentrations ranging from 50 µM to 882 µM for a minimum of 3 weeks with weekly dilutions with fresh media. The minimal nitrate concentration to observe cell growth was 220 µM for RCC100 and 50 µM for RCC697.

P. calceolata cells were harvested for RNA extraction in the following conditions: 220, 440 and 880 µM for RCC100 and 50, 220, and 880 µM for RCC697. Between 29.9 and 84.5 million paired-end RNA reads were sequenced for each sample then aligned on the predicted mRNAs of *P. calceolata* RCC100 genome. 86% of RCC100 genes were covered by at least one RCC697 read with on average 96.0% of identity showing the genetic proximity of these two strains. Correlations of gene expression levels within triplicates are higher (Pearson's correlation > 0.95) than correlations between conditions (Pearson < 0.81) with the exception of RCC697, 800 µM vs 200 µM of nitrate, that are highly correlated (Pearson > 0.92). These correlations indicate that all tested conditions induced a transcriptomic response of *P. calceolata* except the reduction to 200 µM of nitrate in RCC697.

Differential expression analyses were performed between the high-nitrate condition and each tested condition for each strain. For RCC100, compared to the high-nitrate condition, 1,282 are differentially expressed with 440 µM of nitrate and 799 are differentially expressed with 220 µM of nitrate (Figure 2 B,C). A large proportion these DEGs are in common between the two reduced nitrate conditions (604 DEG, 172 overexpressed and 434 underexpressed) indicating that the cells are already acclimated to low-nitrate in the 400µM experiment. For RCC697, only 4 genes are differentially expressed in the 220 µM condition showing that this nitrate reduction did not affect significantly this strain in contrast with RCC100. With only 50 µM of nitrate RCC697 shows an important transcriptomic response with 393 differentially expressed genes (251 underexpressed and 141 overexpressed). RCC100 and RCC697 have a similar response to the limitation of nitrate with a total of 95 common DEGs.

In RCC100 and RCC697, genes overexpressed under reduced nitrate concentration are enriched in amino acid catabolism and diverse type of transporters. Genes underexpressed in intermediate nitrate concentrations are enriched in translation initiation and elongation factors and in ribosomal proteins . DEGs involved in the nitrogen metabolism are described in the following paragraphs.

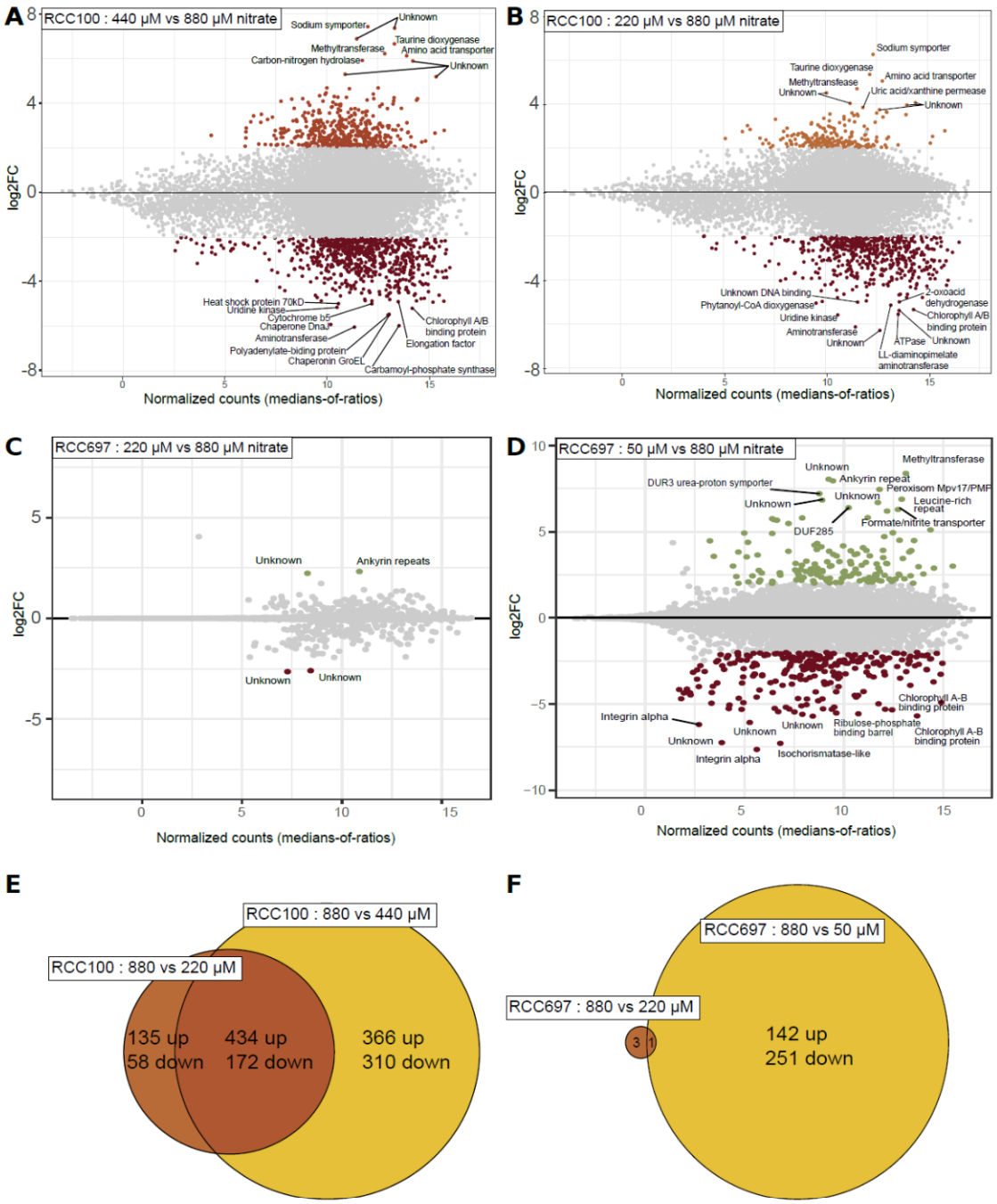

Figure 2: Transcriptomic response of *P. calceolata* to low-nitrate cultures. A-D) Differentially expressed genes of *P. calceolata* RCC100 in 441 µM (A) and 220 µM (B) of nitrate and RCC697 in 220µM (C) and 50 µM (D) compared to 880 µM of nitrate. Genes with p-value < 0.01 and log2FC > 2 are coloured. D, E) Euler diagram of DEGs in RCC100 (E) and RCC697 (F). The number of upregulated and downregulated genes is indicated.

Gene expression levels of *P. calceolata* RCC100 cultivated with different nitrogen compounds.

P. calceolata RCC100 was incubated with nitrate (882 µM, standard conditions), ammonium (882 µM), urea (441 µM) or cyanate (882 µM) for a minimum of 3 weeks with weekly transfer on fresh media. We observed similar cell density for nitrate, cyanate and urea in exponential growth phase

(Figure 3A). This result shows that *P. calceolata* is able to grow with cyanate or urea as sole source of nitrogen. The growth rate and maximal cell density is strongly limited on ammonium (Figure 3A). After polyA+ RNA extraction and sequencing, the 12 samples had between 45 million and 84 million paired-end reads that were used for the alignment on the predicted mRNAs of *P. calceolata* genome (RCC100). For all conditions, triplicates are highly correlated with Pearson correlations above 0.98. *P. calceolata* transcriptome under nitrate condition is more correlated with the cyanate and the ammonium conditions (pearson > 0.95) than with the urea conditions (pearson > 0.73 and < 0.82).

In the ammonium condition, despite a growth curve strongly impacted, only 71 genes are underexpressed and 45 are overexpressed compared to the nitrate condition. In contrast, *P. calceolata* RCC100 presents a good acclimation to cyanate and urea and many genes are differentially expressed (921 and 308 DEGs respectively). Taken together, cyanate, urea and ammonium experiments have 65 DEGs in common, 28 are overexpressed and 37 are underexpressed (Figure 3E, 3F). Among the 37 underexpressed genes, 8 are involved in nitrate transport and assimilation indicating a global downregulation of this pathway in the absence of nitrate in the media (see next paragraphs discussion). We also observed a downregulation of 6 LHC genes suggesting a decrease of the photosynthetic activity even if the alternative nitrogen source supports *P. calceolata* growth. Many overexpressed genes under alternative nitrogen source have unknown function (13 out of 28 genes). Yet, we note the presence of 3 genes involved in protein folding (2 Heat Shock Proteins and a Cyclophilin) and 3 genes involved in protein, fatty acid and carbohydrate catabolism (1 cysteine peptidase, the Phytanoyl-CoA deoxygenase and a Glycosyl hydrolase family 16) suggesting cellular stresses and activation of recycling in the absence of nitrate.

Only 7 genes are specifically overexpressed under cyanate (Figure 3E). Among them, one gene contains a domain of the transporter superfamily FepB (COG0614 ; ABC-type Fe3+-hydroxamate transport system) and carry one transmembrane domain. An enzyme carrying an alkyl-hydroperoxide reductase domain (AhpD-like, IPR029032) is also overexpressed under cyanate. This family of reductases is involved in the defense against reactive oxygen species (Hong et al., 2019). The 5 other genes are a Triosephosphate isomerase (K01803-EC:5.3.1.1), a Glycosyl transferase of family 90 (PF05686), a proline iminopeptidase (K01259-EC:3.4.11.5), and genes of unknown function. These genes are good candidates to be involved in cyanate uptake and metabolism.

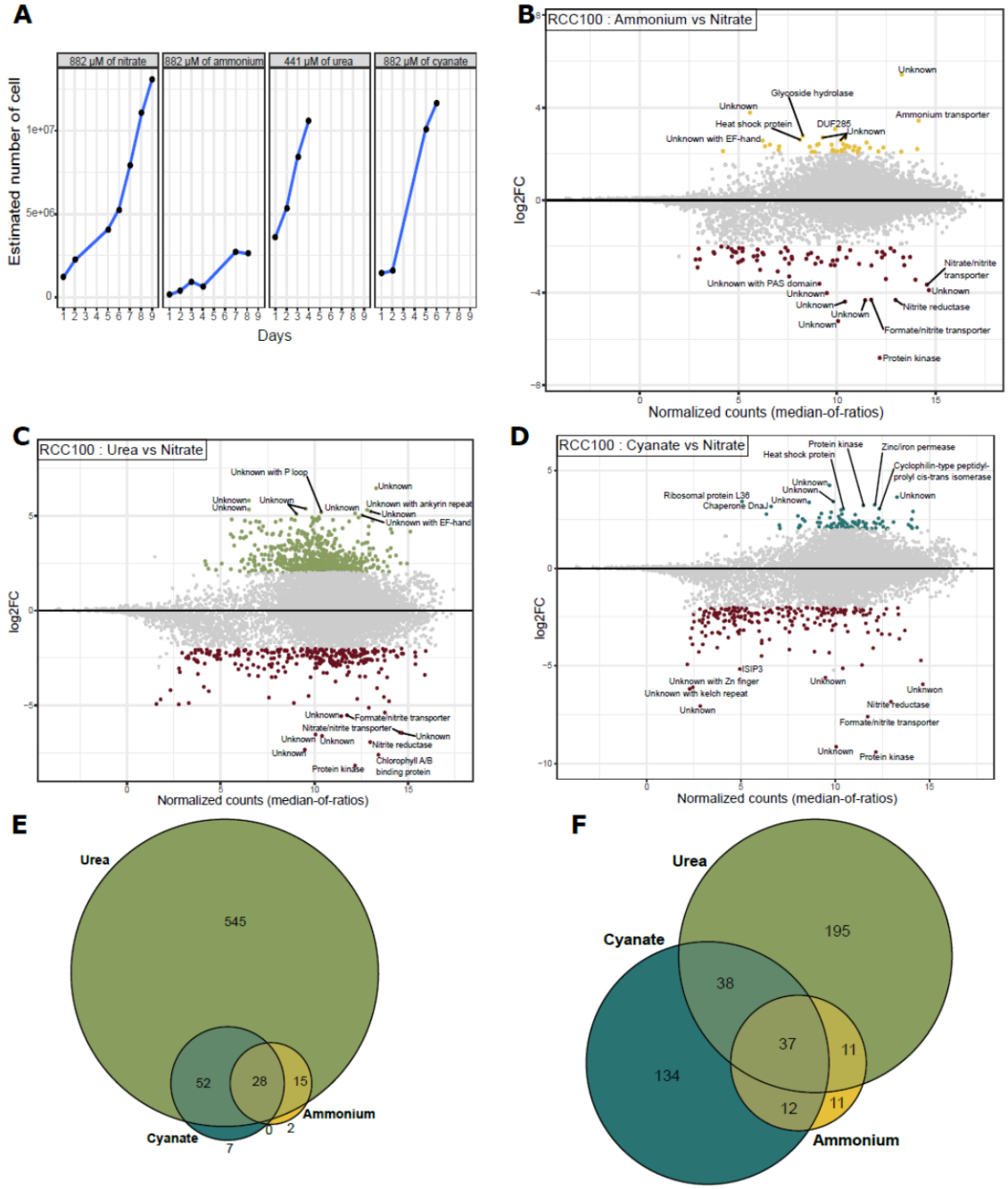

Figure 3. Growth and transcriptomic response of *P. calceolata* RCC100 cultivated with different nitrogen compounds. A) Growth of *P. calceolata* under various nitrogen sources (882 µM NO_3^-, 882 µM NH_4^+, 441 µM urea, 882 µM cyanate) measured by fluorescence converted into estimated number of cell. B, C, D) Differentially expressed genes in 882 µM of ammonium (B), 441 µM of urea (C) and 882 µM of cyanate (D) compared to 882 µM of nitrate. Genes with p-value < 0.01 and log2FC > 2 are coloured. E, F) Euler diagram of genes overexpressed (E) or under expressed (F) in at least one of the alternative nitrogen sources.

Nitrogenous compound transport

In each family of nitrogen compound transporters, we observed genes with important variations of expression when the nitrogen source or concentration is modified (Figure 4). The 2 high-affinity nitrate transporters (NRT2, K02575) are downregulated when nitrate is replaced by another nitrogen

source, but they are not regulated with the decrease of nitrate concentration. Two formate/nitrite transporter (FNT, PF01226) are underexpressed in all alternative N source. One FNT is a putative NAR1 transporter and potentially located on the chloroplast membrane. The other FNT is overexpressed in low-nitrate conditions *in situ* and in lab culture and putatively located on lysosome/vacuole membranes suggesting that this gene may be involved in the transportation of recycled nitrite products from intracellular vacuoles. Among the five ammonium transporters (Amt), one gene is overexpressed in low-nitrate conditions *in situ* and slightly overexpressed in the intermediate-NO_3 condition suggesting that this gene may be an high-affinity transporter. This gene is also downregulated when the nitrate is replaced by another nitrogen source including ammonium. Three other Amt genes are slightly underexpressed when nitrate is depleted and might be low-affinity transporters. Interestingly, the last low-affinity transporter is strongly overexpressed in urea, cyanate and ammonium conditions suggesting that this Amt is regulated according to the presence or absence of nitrate in the environment. One urea-proton symporter (K20989) present an opposite pattern of expression with an overexpression in low-nitrate in *P. calceolata* RCC697 and a downregulation in RCC100. The other urea transporter (PF03253) in *P. calceolata* genome is not differentially regulated in any of our tested conditions. Finally, the uric-acid/xanthine permease (K23887) overexpressed in low-nitrate environments *in situ* is also significantly overexpressed in low-nitrate cultures and is underexpressed when the nitrate is replaced by cyanate or urea. This expression pattern suggests that this transporter recycles purine degradation products under nitrate starvation but do not transport extracellular nitrogenous molecules.

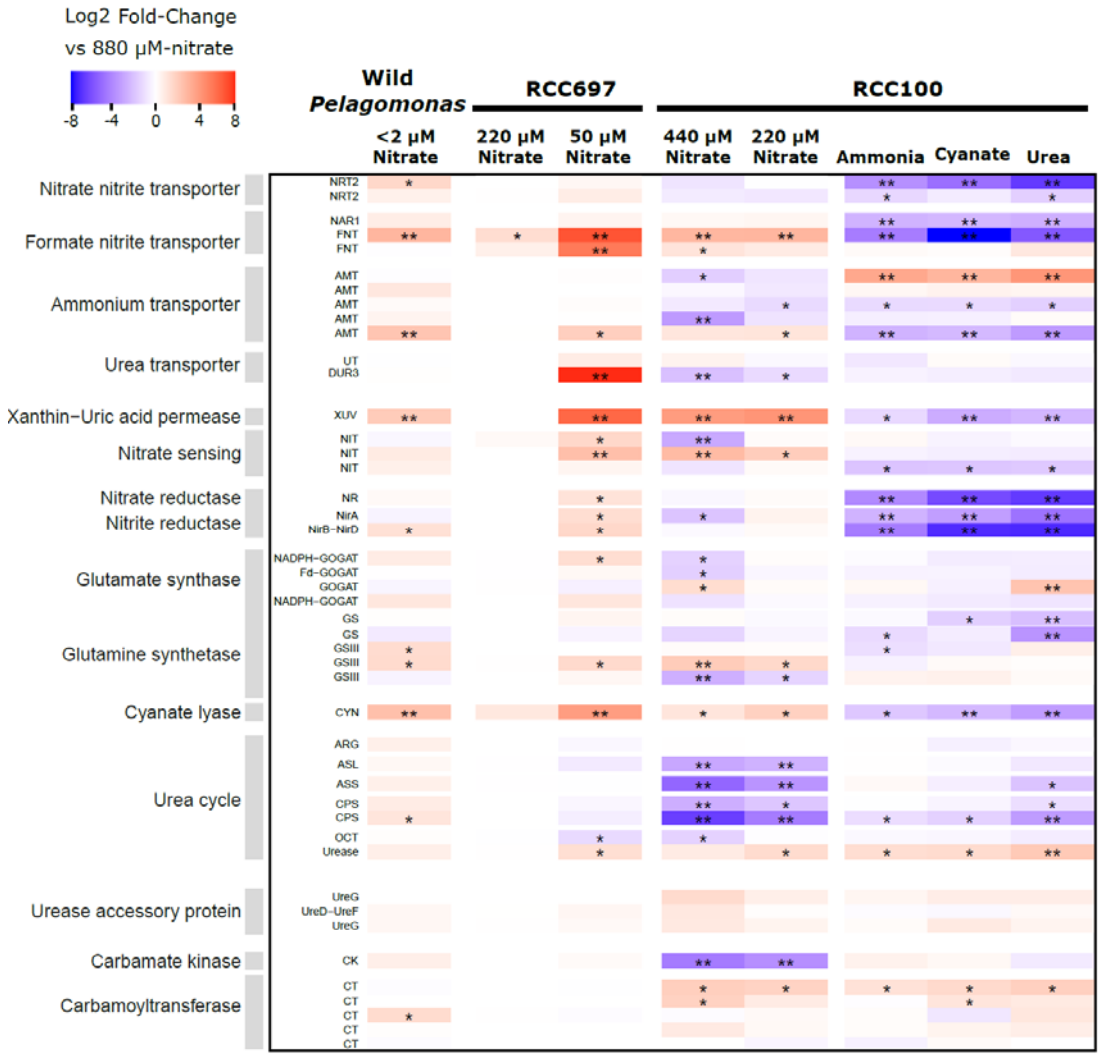

Figure 4. Differential expression of *P. calceolata* RCC100 genes involved in the nitrogen metabolism. Log2 fold changes are shown only when log2FC > 1 or < -1 and p-value < 0.01.

Nitrate sensing

Three genes carrying a nitrate/nitrite sensing domain (NIT) are present in *P. calceolata* genome. These genes have distinct expression patterns according to nitrate concentration or source (Figure 4, 5). The NIT-sensing gene carrying a transmembrane domain is overexpressed in low-nitrate samples but not differentially expressed when the nitrate is replaced by another nitrogen source. This gene has been shown to be overexpressed by *P. calceolata* in low-nitrate environments (Dupont et al., 2015; Guérin et al., 2022) but is not significant in our environmental DESeq2 analysis. The two other nitrate-sensing genes carry a serine–threonine/tyrosine kinase domain, and might play a role in phosphorylation-based signal transduction. One of these two genes is slightly underexpressed in all alternative nitrogen sources while the second present opposite pattern of expression in the two *P. calceolata* strains in reduced-nitrate conditions. This observation suggests that each gene has a specific role to respond to the intracellular or extracellular nitrate concentrations.

Nitrate reduction and storage

The NADH-dependant nitrate reductase (NR, K10534), the NADH-dependant nitrite reductase (NirB-NirD, PF01077) and the ferredoxin-dependant nitrite reductase (NirA, K00366) have the same expression pattern with higher gene expression levels when the nitrogen source is nitrate and a strong downregulation under urea, cyanate and ammonium (Figure 4). The downregulation of the nitrate reduction pathway in absence of nitrate is coherent with previous results on many algae including the diatom *P. tricornutum* and the Pelagophyte *A. anophagefferens* (Dong et al., 2014; Smith et al., 2019). Four of the 5 genes coding for glutamine synthetase (GS) in *P. calceolata* are differentially expressed in at least one experiment (Figure 4). Two GS are underexpressed when the nitrogen source is urea, suggesting that the urea uptake and metabolism do not require these genes. Two putatively mitochondrial GS are differentially expressed according to the nitrate concentration in the lab with opposite patterns. Four genes encode *P. calceolata* glutamate synthases (GOGAT). The putative Fd-GOGAT and one NADPH-GOGAT (GLT1, K00266) are predicted to be located in the chloroplast and are slightly underexpressed in low-nitrate samples. Reversely, the putatively mitochondrial GOGAT is slightly overexpressed in low-nitrate experiments and strongly when the nitrogen source is urea (Figure 4).

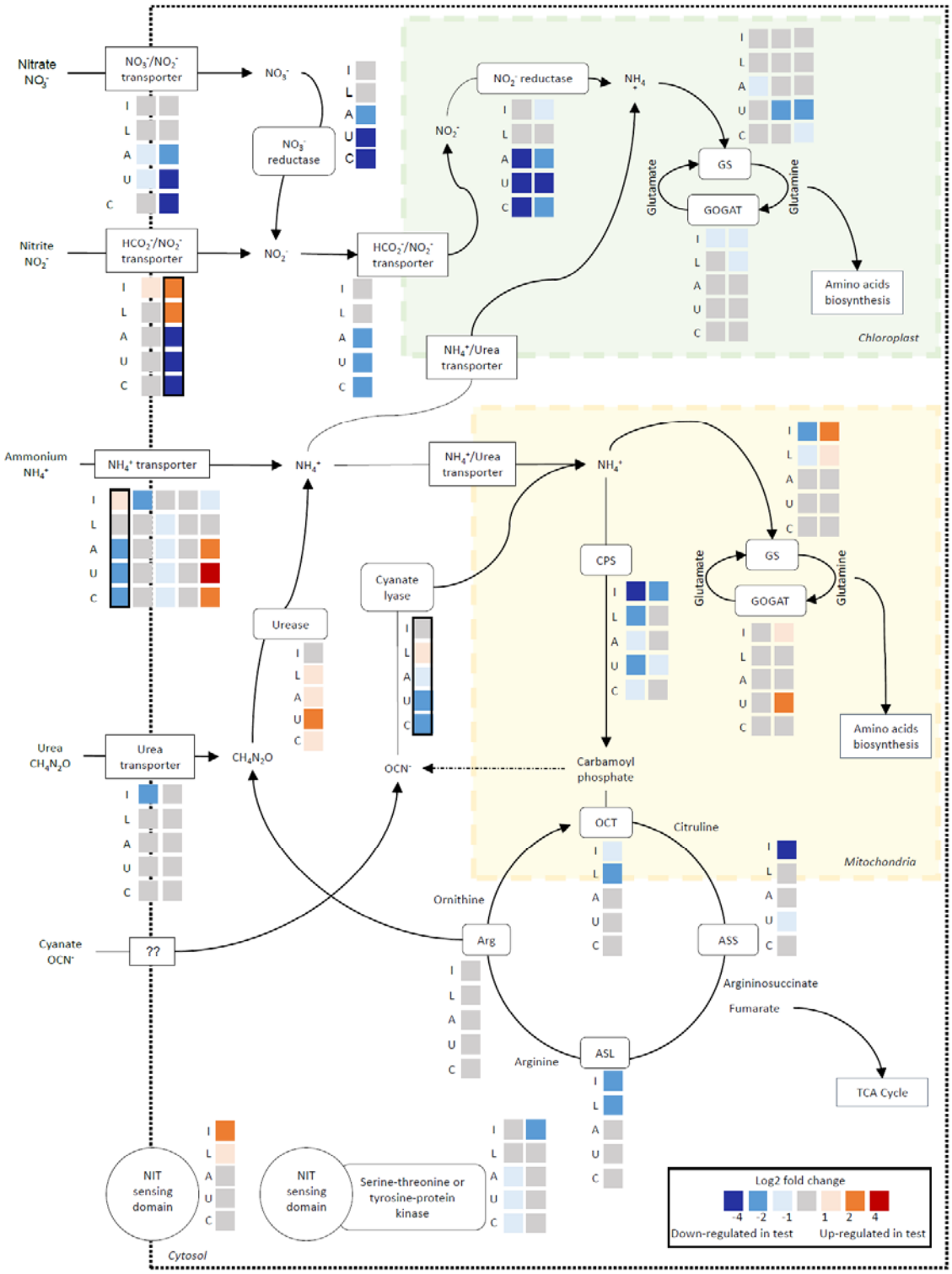

Figure 5. Metabolism of *P. calceolata* RCC100 under low-nitrate conditions or alternative nitrogen source. Each square represents the differential expression of one gene in one condition compared to 882 µM of nitrate. Each column is one gene and rows are the different culture conditions. I: intermediate nitrate (441 µM); L: low-nitrate (220 µM); A: ammonium; U: urea; C: cyanate. GS: glutamine synthetase; GOGAT: glutamate synthase; CPS: carbamoyl-phosphate synthetase; OCT: ornithine-carbamoyl transferase; ASS: arginosuccinate synthase; ASL: arginosuccinate lyase; Arg: arginase. The colour for each square indicates if the gene is underexpressed (red) or overexpressed (blue). Genes with black lines are overexpressed in environmental low-nitrate samples.

Cyanate lyase, urease and urea cycle.

Consistently with low-nitrate environments, *P. calceolata* cyanate lyase gene is slightly overexpressed in our low-nitrate experiment in both strains (Figure 4). Surprisingly, under alternative nitrogen source, including cyanate, the cyanate lyase is underexpressed. This result shows that the cyanate lyase is not involved in assimilation of extracellular cyanate and could instead be involved in nitrogen recycling from intracellular molecules (see discussion). *P. calceolata* encodes one urease (URE, K01427) and three urease accessory proteins: one UreD-UreF (K03190;K03188) and two UreG (PF02492). The urease is slightly overexpressed in low-nitrate samples and overexpressed in alternative nitrogen source, especially in urea. This result indicates that extracellular urea as well as intracellularly produced urea are hydrolysed into ammonia by the urease. The urea cycle in *P. calceolata* is composed of two carbamoyl phosphate synthetases (CPS), 1 ornithine carbamoyltransferase (OCT), 1 arginosuccinate synthase (ASS), 1 arginosuccinate lyase (ASL) and 1 arginase (Figure 4). Except for the arginase, all the genes involved in the urea cycle are overexpressed in high-nitrate conditions. This pattern is expected to remove excess of ammonia from the cell through the urea cycle.

Discussion

***P. calceolata* has common patterns of low-nitrate acclimation *in situ* and in lab cultures.**

In this study, we analysed the nitrogen metabolism of a pelagophyte *in situ* with *Tara* Oceans datasets and in the lab with culture experiments on two strains of *P. calceolata* under different nutrient conditions. Although we identified only 6 genes significantly differentially expressed in low-nitrate environments, 5 of them were also observed in our low-nitrate conditions in the lab. This result is remarkable for 2 reasons. Firstly, RCC100 strain is cultivated since 1973 in nitrate-rich conditions without environmental stress. Although important genomic modification (genome rearrangements, reduction, selection) may have appeared in the cultivated strain, the regulation of several genes in response to low-nitrate is conserved compared the wild *Pelagomonas*. Secondly, the minimal quantity of nitrate used in the lab to observe *P. calceolata* growth is one hundred times more important (50 µM) than the concentration measured in the environment where *P. calceolata* is relatively abundant (below 0.5 µM). Even with this important difference, the transcriptomic pattern in response to nitrate starvation is similar. One explanation is that the number of nitrate molecules accessible to each *P. calceolata* cell in the culture is strongly diluted by the high density of cells compared to cell concentration in the open ocean. The accessible nitrate in both situations may finally not be strongly different.

Despite the relative high abundance of *P. calceolata* in the environment, metatranscriptomes give only access to highly expressed genes. Therefore, lab experiments are needed to observe little variations and low expressed genes that are potentially critical and provide more in-depth results on the metabolism. In addition, considering the numerous environmental parameters affecting the gene expression levels in the environment, it is often difficult to isolate one parameter from the other to observe a specific response. In our analysis, we used the large diversity of environments sampled during *Tara* cruise to get within each group (low nitrate vs high nitrate) a large range of all other parameters. By doing this, we select only the genes that are directly affected by the nitrate concentration but we also strongly limit the number of significant DEGs. Finally, one limit of environmental metatranscriptomes across large geographical areas is to understand if observed gene expression variations are the results of acclimation (reversible short-term transcriptomic regulation) or adaptation (long-term selection). The complementation of *in situ* analysis with classical

transcriptomic allowed us to conclude that for the genes also differentially expressed in lab cultures, their expression variations in the environment probably result from short-term acclimation.

P. calceolata is genetically adapted to consume organic nitrogen compounds

We observed a similar growth of *P. calceolata* under nitrate, urea or cyanate as sole nitrogen source. This result shows that, as many other micro-algae, *P. calceolata* is adapted to consume organic nitrogen compounds. For example, *A. anophagefferens* has a greater growth when cultivated under urea compared to many others N sources such as nitrate or ammonium (Berg et al., 1997). The transcriptomic profile of *P. calceolata* is largely modified with organic nitrogen compounds but we did not observed overexpression of stress response genes. In our study, we disentangled the common transcriptomic response in the presence of urea, cyanate or ammonium from the specific response to each nitrogen source. The main common response in the absence of nitrate is the downregulation of the nitrate assimilation pathway, such as formate-nitrite and nitrate-nitrite transporters, nitrate and nitrite reductases. Interestingly, this pathway is not underexpressed when the nitrate concentration is reduced suggesting that the remaining nitrate in our low-nitrate conditions are enough to maintain this pathway. Because we were only able to divide by four the standard concentration, we cannot exclude that this pathway would have been downregulated at lower concentrations. Urea is the most studied alternative nitrogen source, and its presence negatively affects plastidic N assimilation in Pelagophyceae CCMP 2097 (Terrado et al., 2015). In *A. anophagefferens* it was observed that urea as N-sources triggered upregulation of genes involved in protein, amino acid, spermine and sterol synthesis (Dong et al., 2014), but these functions were not upregulated in *P. calceolata*.

Recycling of intracellular nitrogenous compounds is the dominant response under low-nitrate conditions.

Our results show that under low-nitrate, the main strategy is the recycling of intracellular nitrogenous compounds such as amino acids and nucleotides. A gene coding a xanthine/uracil/vitamin C permease (XUV), involved in the transport of purine degradation products, is overexpressed both in environmental and experimental low-nitrate conditions. Overexpression of XUV under low-nitrate condition has already been observed in several algae including the pelagophyte *A. anophagefferens* and the haptophyte *Prymnesium parvum* (Dong et al., 2014; Liu et al., 2015; Wurch et al., 2014). The expression of this permease in *P. calceolata* seems also linked to the catabolism of purines as a recycling mechanism in low-N environments and don't reflect the presence of purines in the environment. Enzymes necessary for the conversion of xanthine into urea and ammonia are present in *P. calceolata* genome but are not overexpressed in low-N conditions. This pathway is unlikely involved in case of purines recycling in contrast to *A. anophagefferens* when supplied with xanthine, (Gann et al., 2022). Recent research highlights that many micro-algae including ochrophytes are able to store nitrogen in purine crystals (Pilátová et al., 2022). Overexpression of the purine permease under low-nitrate could be a sign of crystals' nitrogen reallocation even if crystalline inclusions have not been reported in pelagophytes.

Role of the cyanate lyase in *P. calceolata*

In our study, we have shown that *P. calceolata* can grow under cyanate as sole nitrogen source. Among eukaryote algae, growth with cyanate as sole nitrogen source has only been shown in the dinophyceae *Prorocentrum donghaiense* in the expense of a lower growth rate (Hu et al., 2012).

Considering the large diversity of algae capable of growth under various organic nitrogen source, it is possible that cyanate would supports the growth of many taxa. It has been suggested that phytoplankton overexpressing the cyanate lyase in nitrate-low environments are therefore capable of cyanate uptake and metabolism (Dong et al., 2014; Mao et al., 2022). Although, *P. calceolata* cyanate lyase is overexpressed in low-nitrate environments (*in situ* and in the lab), we have shown that this gene is underexpressed when *P. calceolata* is cultivated with cyanate. In consequence, we believe that the cyanate lyase is not responsible for external cyanate metabolism and should not be used as a marker of cyanate uptake. In agreement with our results, cyanate supports the growth of several ascomycetes species despites the absence of cyanate lyase coding genes in their genome (Linder, 2018). Reversely, several yeasts with cyanate lyase genes were unable to grow under cyanate supporting the hypothesis that this gene is not involved in the assimilation of external cyanate in eukaryotes in contrary to prokaryotes. Thus, it is possible that another gene that remain to be identified supports this function. The alkyl-hydroperoxide reductase-like specifically overexpressed under cyanate in *P. calceolata* is a good candidate to participate in external cyanate metabolism. Cyanate transports into eukaryotic cells remain undescribed and the bacterial transporter of cyanate CynX is not conserved in eukaryotes (Widner & Mulholland, 2017). Among the 7 genes specifically overexpressed in cyanate condition, one gene carry homologies with the protein transporter domain ABC-type Fe3+-hydroxamate. This transporter, conserved across Ochrophytes is a candidate for the uptake of cyanate in *P. calceolata* cells and will require further studies.

The cyanate lyase function in *P. calceolata* is likely the detoxification of intracellularly produced cyanate. Cyanate can be generated through fast decomposition of carbamoyl-phosphate (Anderson et al., 1990; Ter-Ovanessian et al., 2021) or the slow decomposition of urea (Dirnhuber & Schütz, 1948). *P. calceolata* recycling of amino acids and protein in case of low nitrate supply. In this process, many enzymes are involved in the catabolism of metabolites, including large protein family carbamoyltransferase (K00612) that can produce carbamoyl-phosphate (CP). Two of the five genes coding for carbamoyltransferase are overexpressed in low-nitrate environment (Figure 4). We hypothesise that these enzymes increase intracellular concentration of CP, which is in principle taken up by the urea cycle. However, under low-nitrate conditions the urea cycle is downregulated, which could lead to an increase of intracellular cyanate, requiring the cyanate lyase activity to detoxify the cell.

Our results tends to show that the cyanate lyase of *P. calceolata* is involved in detoxification of intracellular cyanate, which is a by-product of carbamoyl-phosphate generated by amino-acid degradation and not taken up by the urea cycle under low-nitrate conditions. In complement to the work of Sato, Hashihama, and Takeda 2023 and the phylogeny of Mao et al. 2022, we hypothesise that the removal of intracellular cyanate is the main role of the cyanate lyase in eukaryotic microalgae instead of external cyanate metabolism as alternative N source.

Materials & Methods

Environmental metatranscriptomes and associated metadata

In order to detect gene expression of *P. calceolata* in the oceans, we used metatranscriptomic datasets from *Tara* Oceans and *Tara* Polar Circle expeditions (Alberti et al., 2017). We selected all available datasets from seawater samples of the photic zones (73 surface and 51 deep-chlorophyll maximum samples), and from 2 size-fractions ; 80 from the 0.8 - 5 µm and 44 from the 0.8-2000 µm. Metatranscriptomic reads were aligned on the 16,667 predicted mRNA sequences of *P. calceolata*

genome (ENA, PRJEB34158) with bwa-mem2 version 2.2.1 with default's parameters (Li et al., 2009). We selected reads aligned with more than 95% of identity over 80% of the read length. We kept nuclear genes covered by at a minimum of 10 reads in at least 10 samples. To eliminate putative cross-mapped genes (i.e. highly conserved genes which probably aggregate reads from other organisms), we removed genes detected in more than 90% of samples including those where *P. calceolata* is not present. Finally, only samples with more than 75% of *P. calceolata* genes were kept for the next steps. The environmental parameters measured during the expedition are available in the Pangaea database (https://www.pangaea.de/) (Pesant et al., 2015). Nitrate concentrations are calculated from *in situ* sensor (SATLANTIC) data, calibrated using water samples. Samples are considered "low-nitrate" if they contain less than 2 µM of nitrate. Differential expression analyses were conducted with DESeq2 package version 1.32.0 (Love et al., 2014) under R version 4.1.1 across the 15 617 genes and the 112 environmental samples with available *in situ* nitrate concentration measurements. Pairwise comparisons were performed across 43 "high-nitrate" samples (nitrate concentration > 2 µM) and 69 "low-nitrate" samples (nitrate concentration < 2µM) and with the function DESeq with default parameters and log2fold change values was calculated with lfcShrink function. We considered as differentially expressed genes with a p-value < 0.01 and a log2foldchange > 2 or < -2.

P. calceolata cultures in different nitrogen conditions

RCC100 and RCC697 strains of *Pelagomonas calceolata* were cultivated in artificial seawater (ASW) supplemented by L1 medium (as described in Guillard and Hargraves 1993). Briefly, ASW is prepared by the dissolution of 24.55g of sodium chloride (NaCl), 0.75g of potassium chloride (KCl), 4.07g of magnesium chloride hexahydrate ($MgCl_2$*6 H_2O), 1.11g of calcium chloride ($CaCl_2$), 2.95g of magnesium sulphate ($MgSO_4$) and 0.21g of sodium bicarbonate ($NaHCO_3$) in 1L of sterile water. 1 ml of Trace metals, vitamins and nutrients from the L1 medium Bigelow Kit (MKL150L) were added to reach following concentrations: 882 µM of sodium nitrate ($NaNO_3$), 36.2 µM of monosodium phosphate ($NaH_2PO_4^-$) and 106 µM of sodium silicate (Na_2SiO_3). Cultures were maintained at 20°C, under a 12:12h light-dark photoperiod and a blue light at the intensity of 20 µmol. $m^{-2}.s^{-1}$ of photosynthetic photons. RCC697 is maintained on an orbital shaker (Kühner) at 150 $r.min^{-1}$. For the nitrate depletion experiment, the nitrate concentration of the L1 medium was reduced to 441 µM, 220 µM, 110 µM or 50 µM. For the cyanate and the ammonium experiment, the nitrate was replaced by potassium cyanate (KOCN) or ammonium chloride (NH_4Cl) at the same concentration (882 µM). For the urea experiment, the nitrate was replaced by urea (CH_4N_2O) concentrated at 441 µM to keep the same concentration of nitrogen atoms in all conditions.

RCC100 and RCC967 were grown in each tested condition during an acclimation phase of minimum 8 days then diluted in fresh medium in triplicates during 5 to 9 days until they reached a minimum concentration of 10 million cells per ml. Growth and fluorescence was daily followed with a Qubit instrument (Invitrogen™ Qubit™ 3 Fluorometer Q33216, blue excitation at 470 nm, far red emission 665-720 nm), and cells were counted on a Thoma cell under microscope at the day of the harvesting (Marienfeld Thoma counting chamber, depth0.1 mm, 0640710). *P. calceolata* cells were harvested by filtration on a 1.2 µm mixed-cellulose esters membrane filter (MF-Millipore, rawp04700) with a peristaltic pump (SFP-100), then transferred in 15ml tubes, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

RNA extraction and sequencing

Flash-frozen filters with *Pelagomonas* cells were vortexed in QIAzol, then RNA were extracted following RNeasy Plus Universal Mini Kit procedure (Qiagen, Ref 73404). All extracted RNA were treated 6U of TURBO™ DNase (2 U/µl) (Thermo Fisher Scientific, Ref. AM2238) then purified with RNA Clean and Concentrator-5 kit (Zymo Research, Ref. ZR1016), keeping only large RNAs fractions (> 200 nt) for the RNAseq library preparation. 100 ng of treated RNA were used to create Illumina library (Illumina Stranded mRNA Prep, Ligation). Briefly, poly(A)+ RNAs were selected with oligo(dT) beads, chemically fragmented by divalent cations under high temperature, converted into single-stranded cDNA using random hexamer priming, followed by second strand synthesis and 3'-adenylated. A pre-index anchor was ligated and a PCR amplification step with 15 cycles was done to add a 10bp unique dual index adapter sequences (IDT® for Illumina® RNA UD Indexes, Ligation). All libraries were quantified by Qubit dsDNA HS Assay measurement. A size profile analysis was performed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced in 2x150 bp on Illumina NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA) in order to obtain 50 million paired-end reads. After Illumina sequencing, an in-house quality control process was applied to the reads that passed the Illumina quality filters (Alberti et al., 2017). Briefly, Illumina sequencing adaptors and primer sequences were remove, then low-quality nucleotides (Q < 20) were discarded from both ends of the reads. Sequences between the second unknown nucleotide (N) and the end of the read were also trimmed. We discarded reads shorter than 30 nucleotides after trimming with an adaptation of fastx_clean tool (https://www.genoscope.cns.fr/fastxtend/). In the last step, we discarded reads that were mapped to the Enterobacteria phage PhiX174 genome (GenBank: NC_001422.1), using bowtie2 v2.2.9 (-L 31–mp 4–rdg 6,6–local–no-unal) (Langmead & Salzberg, 2012). Remaining rRNA reads were removed using SortMeRNA v2.1 and SILVA databases (Kopylova et al., 2012; Quast et al., 2013).

Analysis of *P. calceolata* gene expression levels in different nitrogen conditions

Similarly, to the analysis of environmental metatranscriptomes, RNAseq reads were aligned with bwa-mem2 version 2.2.1 on the *P. calceolata* genome. Reads with a minimal size of 50 bp and aligned with more than 95% of identity over 80% of the read length were selected. 16 659 out of 16 667 genes were detected in at least one sample. We only kept nuclear genes, then normalised the gene expression levels in transcripts per kilobase per million mapped reads (TPM). Pearson's correlation matrices were computed with R using cor function between triplicates and across conditions, based on the gene expression normalised by TPM. Hierarchical clustering of Euclidian distance between samples was performed with dist and hclust functions of stats package on R version 4.1.1. Differential expression analysis on transcriptomic samples was carried out in the same way as for the metatranscriptomic DESeq2 analysis above. Identification of differentially expressed genes between control and test samples was performed by pairwise comparisons across standard condition (882 µM of NO3) and low-nitrate conditions (220 µM of NO3 or 441 µM of NO3) in one hand, and across standard condition and changing nitrogen sources conditions samples (882 µM of ammonium, 882 µM of cyanate and 441 µM of urea) in the other hand. We considered as differentially expressed genes those presenting a p-value < 0.01 and a log2foldchange > 2 or < -2. We specifically looked for log2foldchange of genes identified as involved in the nitrogen cycle in our previous study (Guérin et al., 2022). All figures were generated with ggplot2 version 3.4.1 except for the Euler diagrams that were made with eulerr version 6.1.1 and graphics version 4.0.3 under R version 4.0.3.

Author Contributions

N.G and Q.C conceived and planned this study with the support of P.W. A.T set up the culture chamber with important advices from P.G and I.P. N.G and C.S performed *P. calceolata* cultures with the strong support of C.O and L.B. B.V, E.B and G.M did RNA extractions, library preparations and sequencing coordinated by K.L. N.G and C.S carried out all bioinformatics analysis supervised by Q.C. N.G and Q.C wrote the paper. All authors contributed to the manuscript preparation and approved the final version of the paper.

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