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ORIGINAL ARTICLE

Transcriptomic analysis illuminates genes involved in chlorophyll synthesis after nitrogen starvation in *Acaryochloris* sp. CCMEE 5410

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Abstract *Acaryochloris* species are a genus of cyanobacteria that utilize chlorophyll (chl) *d* as their primary chlorophyll molecule during oxygenic photosynthesis. Chl *d* allows *Acaryochloris* to harvest red-shifted light, which gives them the ability to live in filtered light environments that are depleted in visible light. Although genomes of multiple *Acaryochloris* species have been sequenced, their analysis has not revealed how chl *d* is synthesized. Here, we demonstrate that *Acaryochloris* sp. CCMEE 5410 cells undergo chlorosis by nitrogen depletion and exhibit robust regeneration of chl *d* by nitrogen repletion. We performed a time course RNA-Seq experiment to quantify global transcriptomic changes during chlorophyll recovery. We observed upregulation of numerous known chl biosynthesis genes and also identified an oxygenase gene with a similar transcriptional profile as these chl biosynthesis genes, suggesting its possible involvement in chl *d* biosynthesis. Moreover, our data suggest that multiple prochlorophyte chlorophyll-binding homologs are important during chlorophyll recovery, and light-independent chl synthesis genes are more dominant than the light-dependent gene at the transcription level. Transcriptomic characterization of this organism provides crucial clues toward mechanistic elucidation of chl *d* biosynthesis.

Keywords Acaryochloris · Chlorophyll *d* · Nitrogen starvation · Nitrogen recovery · RNA-Seq · Transcriptomics

Abbreviations

chl	Chlorophyll
PAR	Photosynthetically active radiation

Bruce J. Wittmann and Jeremy D. King have contributed equally to this work.

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Introduction

Oxygenic photosynthesis occurs in plants, algae, and cyanobacteria. For most oxygenic phototrophs, usable light occurs between 400 and 700 nm. This spectral region is referred to as photosynthetically active radiation (PAR). Some algae and cyanobacteria are able to extend photosynthetic growth to 750 nm (Chen and Blankenship 2011). The extension of photosynthetic growth into the far-red is significant as it allows these organisms to survive below other phototrophs (Larkum and Kühl 2005). Growth in far-red light is successful for two reasons. First, normal oxygenic photosynthesis only depletes PAR, transmitting nearly all light above 700 nm (Larkum and Kühl 2005). Secondly, photon flux is relatively high from 700 to 750 nm, providing ample energy for growth (Chen and Blankenship 2011). In fact, the photon flux from 700 to 750 nm is 19 % that of PAR (Chen and Blankenship 2011). The great potential for far-red photosynthesis has made it a major target for improving crop yields (Ort et al. 2015).

Some cyanobacteria employ red-shifted chlorophylls (chl) *d* and *f* for growth on far-red light (Miyashita et al. 1996; Chen et al. 2010). Chls *d* and *f* are identical to chl *a* except for the addition of a formyl group at the C3 position or C2 position, respectively (Scheer 1991; Willows et al. 2013; Chen 2014). The formyl substitution in these positions red shift the Qy band in methanol from 665 nm in chl *a* to 696 nm in chl *d* and 706 nm in chl *f*, allowing for absorption of longer wavelength photons (Chen and Blankenship 2011). *Acaryochloris marina* MBIC11017 was the first organism discovered to contain chl *d* (Miyashita et al. 1996). Chl *d* is the dominant chlorophyll in *A. marina*, with only trace amounts of chl *a* (Miyashita et al. 1996). *Acaryochloris* species have been found in diverse locations, occurring as an epiphyte of red algae (Murakami et al. 2004), on the underside of ascidian colonies (Miyashita et al. 1996), and the interior of microbial mats (Miller et al. 2005). In these environments, *Acaryochloris* species are shaded by other oxygenic phototrophs, receiving only far-red light (Larkum and Kühl 2005). Several species containing chlorophyll *f* are now known (Chen et al. 2012; Airs et al. 2014; Gan et al. 2014a; Miyashita et al. 2014; Behrendt et al. 2015). All chl *f* organisms contain a gene cluster for the biosynthesis of unique reaction centers and antennas (Gan et al. 2014a, b). The chl *f* gene cluster occurs in taxonomically distant cyanobacteria, suggesting it is horizontally transferred between cyanobacteria (Gan et al. 2014a). The gene cluster is induced by growth in far-red light. When chl *f* organisms are grown in white light, no chl *f* is made (Gan et al. 2014b). Unlike chl *d* in *Acaryochloris* species, chl *f* is only a minor pigment which accounts for less than 10 % of all chlorophyll under far-red growth (Gan et al. 2014b). Some chl *f* organisms also contain very low levels of chl *d* (Airs et al. 2014; Gan et al. 2014a). Despite the rapid growth in discovery of organisms using chl *d* and *f*, little progress has been made in determining their biosynthesis.

The sequencing of the *A. marina* MBIC11017 and *Acaryochloris* sp. CCMEE 5410 genomes revealed conserved biosynthetic machinery for the production of chl *a* starting from 5-aminolevulinate (Swingley et al. 2008; Miller et al. 2011). The production of protoporphyrin IX proceeds through the cyanobacterial protoporphyrinogen oxidase hemJ (Kato et al. 2010). The genes required to convert protoporphyrin IX to chl *a* are well conserved and homologous to those of other cyanobacteria (Swingley et al. 2008). Genes for both the light-independent protochlorophyllide reductase and light-dependent protochlorophyllide reductase are present (Yamazaki et al. 2006). Both sequenced *Acaryochloris* genomes contain bciA- and bciB-type divinyl reductases, while all other sequenced cyanobacteria contain only a single type of divinyl reductase. The biosynthesis of chl *d* proceeds through chlorophyllide *a* or chl *a* (Schliep et al. 2010). The oxygen in the formyl group of chl *d* is derived from molecular oxygen and not water (Schliep et al. 2010). Interestingly, transformation of *A. marina* with chlorophyllide *a* oxygenase (CAO) produced a novel chlorophyll with formyl substitutions at both the C3 and C7 positions (Tsuchiya et al. 2012). Examination of *Acaryochloris* genomes revealed a few candidates as likely chl *d* synthases (Swingley et al. 2008), although it is still unclear what genes are involved in chl *d* synthesis.

An increased understanding of chlorophyll biosynthesis in *Acaryochloris* species would benefit prioritizing candidate chl *d* synthases. The physiology of chlorophyll regulation is underexplored in *Acaryochloris* species. The ratio of chl *d* to chl *a* has been examined by growth under different light regimes. Cells grown under far-red light contain less chl *a* than cells grown under white light (Duxbury et al. 2009). Generally, low light intensity is correlated with increased amounts of chl *d*. In all cases, chl *a* levels change very little ranging from 3 to 5 % (Gloag et al. 2007). In non-diazotrophic cyanobacteria, chlorophyll biosynthesis is regulated by nitrogen availability. When starved for nitrogen, non-diazotrophic cyanobacteria degrade their chlorophylls, possibly as a mechanism to recover nitrogen (Sauer et al. 2001). If returned to a nitrogen-replete condition, the non-diazotrophic cyanobacteria will resume chlorophyll production. It is unknown if, in general, non-diazotrophic, chl *d*-containing organisms degrade their chlorophylls in response to nitrogen starvation, and if they will resume chlorophyll production when returned to nitrogen.

In this study, we tested the effect of nitrogen starvation and repletion on chlorophyll degradation and biosynthesis in the chl *d*-containing *Acaryochloris* sp. CCMEE 5410. This strain was isolated from the Salton Sea, where high concentrations of dissolved organic carbon and nitrogen can be found (Miller et al. 2005; 2011). For this reason, the nitrogen starvation experiment was better suited for strain CCMEE 5410 than *A. marina* MBIC11017. We found that *Acaryochloris* sp. CCMEE 5410 degrades its chl *d* similar to chl *a*-containing cyanobacteria, and that nitrogen repletion results in a rapid increase in chl *d* production. We tracked global gene expression during the chl *d* burst to understand the nitrogen response, pigment biosynthesis, and oxygenases possibly involved in chl *d* biosynthesis.

Materials and methods

Growth conditions and nitrogen starvation

Acaryochloris sp. CCMEE5410 was grown in BG-11 supplemented with 35 g/L NaCl (hereafter sBG-11) (Swingley et al. 2005). We substituted 35 g/L NaCl for the Instant Ocean normally used in marine BG-11, as Instant Ocean contains trace amounts of nitrogen. This substitution did not appear to alter growth in any significant manner. Growth was performed at 24 °C under 18 µE illumination. For nitrogen starvation experiments, a 10L master culture of *Acaryochloris* sp. CCMEE5410 was grown in a 13L carboy with bubbling and stirring for aeration. 9 L of the master culture was split into 1 L aliquots and concentrated by centrifugation. The cells of six of the bottles were each sterilely resuspended in 1.0 L of nitrogen-free sBG-11, and the cells of the remaining three bottles were resuspended in standard sBG-11. Cultures were transferred to nine different 2.8 L Fernbach flasks for nitrogen starvation experiments. Nitrogen starvation was allowed to proceed for 144 h. Cells were again harvested by centrifugation. The three Fernbachs grown in normal sBG-11 were resuspended in normal sBG-11 again. Three of six nitrogen-free cultures were resuspended in normal sBG-11, and the remaining three cultures were resuspended in nitrogen-free sBG-11.

Chl *d* monitoring

Chlorophyll *d* levels in all nine Fernbachs were monitored by methanol extraction. 1 mL of culture was sterilely removed and harvested by centrifugation. To avoid disrupting the pellet, 950 microliters of media was removed. Each pellet was resuspended in 0.79 g of methanol (1 mL) (Li et al. 2012). Pellets were resuspended by vortexing and allowed to sit for 5 min. Debris was removed by centrifugation. Spectra of the extracted samples were recorded on a Shimadzu UV 1800 UV–Vis spectrophotometer.

RNA isolation

50 ml of *Acaryochloris* sp. CCMEE5410 liquid cultures were harvested by centrifugation and stored in 3 ml of RNAShield (Zymo Research) for 30 min at room temperature, and then stored at 4 °C until all samples were harvested and stored. RNA-stabilized cells were divided into two tubes and centrifuged again to remove RNAShield. The cells from one set containing all 15 samples (5 time points, biological triplicates) were used to isolate total RNA, while the rest were stored at −80 °C as backup samples.

Total RNA was isolated using ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research), following the manufacture's protocol for removing most small RNAs. The total RNA was treated with TURBO DNase I (Ambion) for 30 min at 37 °C (6 U enzyme to up to 6 µg of total RNA). After DNase I treatment, total RNA samples were cleaned using RNA Clean & Concentrator kit (Zymo Research), and eluted in 20 µl of nuclease-free water. Total RNA was quantified using Qubit RNA-HS after appropriate dilutions.

rRNA depletion from *Acaryochloris* total RNA

5 µg of DNase I-treated, cleaned total RNA of each sample was used to deplete ribosomal RNA using RiboZero rRNA Removal Kit Bacteria (Epicentre, currently Illumina). Ribosomal RNA was depleted according to the manufacturer's protocol, except for a minor modification: during rRNA probe-RNA sample hybridization step at room temperature, samples were vigorously mixed by vortexing every 2 min for 10 min. After rRNA depletion, samples were cleaned up using RNAClean XP beads (Agencourt) following manufacturer's protocol. Aside from quality and quantity checking, all of rRNA-depleted RNA samples were used to generate cDNA. First-strand cDNA was synthesized using 1 µl SuperScript II (Invitrogen), 1 µl 10 mM dNTP mix, 0.5 µl Random Primers (3 µg/ml, Invitrogen), 4 µl 5× buffer, 1 µl RNase inhibitor (included in RiboZero kits), and 2 µl 0.1 M DTT at 24 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. Second strand synthesis mix [61.75 µl nuclease-free water, 10 µl NEBuffer 2, 3 µl 10 mM dNTP mix, 5 µl *E. coli* DNA polymerase I, 0.25 µl RNase H per sample; all enzymes were from New England Biolabs (NEB)] was added to the first-strand cDNA and incubated for 2 h at 16 °C. 1 µl *E. coli* DNA ligase was added to each reaction, and the samples were incubated at 16 °C for another 15 min, before cleanup using Qiagen PCR purification kit. Double-

stranded cDNA were quantified using Qubit DNA HS kit, and 50 µl of the eluate containing all cDNA was used to generate an Illumina sequencing library.

Illumina library preparation for RNA-Seq

A protocol adapted from Fisher et al. (2011) and Bowman et al. (2013) was used to generate an Illumina library. Double-stranded cDNA in 50 µl EB buffer (Qiagen) was fragmented to ~200 bp using Covaris E-220 and micro-TUBEs at a recommended setting by Covaris. ~50 µl of fragmented DNA was recovered from Covaris tubes and transferred to a semi-skirted 96-well PCR plate. 20 µl of end repair mix (7 µl 10× T4 ligase buffer, 2.8 µl 10 mM dNTPs, 3.5 µl T4 polymerase, 3.5 µl T4 polynucleotide kinase, 0.7 µl *E. coli* DNA polymerase I, 2.5 µl nuclease-free water; all enzymes were purchased from NEB) was added to the fragmented DNA to blunt and phosphorylate the ends at room temperature for 30 min. 130 µl of AMPure XP beads (Agencourt) was added to each well to remove reagents from previous step. The 96-well plate was placed on a magnetic plate after pipetting thoroughly to mix, and supernatant discarded when it became clear. The beads + DNA on the walls of 96-wells were washed with 150 µl 80 % ethanol once, before eluting the DNA with 32 µl of water. 18 µl of dA-tailing mix [5 µl 10× NEBuffer 2, 1 mM dATP, 3 µl Klenow fragment (3′-5′ exo-)] was added to the beads + eluted DNA to add dA-tail at 37 °C for 30 min. After dA-tailing, 110 µl of 20 % polyethylene glycol 8000 2.5 M NaCl was added to precipitate DNA onto the magnetic beads. The plate was placed on a magnet again to collect DNA and washed with 80 % ethanol. Barcoded adapters were added to the eluted DNA (20.5 µl water added to DNA with beads) to 10:1 molar ratio, and 6.5 µl of ligation mix was added to ligate adapters at room temperature for 20 min. 21 µl of 20 % PEG 2.5 M NaCl was added to precipitate DNA on beads, and two barcoded samples were pooled after washing for elution. Adapter-ligated DNA was then PCR amplified using KAPA HiFi Hot start Readymix (KAPA Biosystems) and gel purified using Zymoclean Gel DNA Recovery Kit (Zymo Research). Resulting purified DNA samples were quantified using Qubit DNA HS kit and combined to yield a 10 nM pool in EB buffer. 20 µl of the pool was submitted to a HiSeq-2500 single read, 50-bp sequencing run at Genome Technology Access Center at Washington University.

RNA-Seq data analysis

Raw Illumina reads were demultiplexed by barcodes and trimmed using an in-house Python script and mapped to an indexed supercontigs of *Acaryochloris* sp. CCMEE 5410 (Joint Genome Institute, taxon ID 2513237397, IMG submission ID 8895, NCBI taxon ID 310037) using Bowtie2 (Langmead and Salzberg 2012). The mapped SAM files were converted to sorted BAM files with BAM index files using SAMtools (Li et al. 2009; Li 2011), featureCounts (Liao et al. 2014) was used to count the number of reads per coding sequence, and the raw result was used for differential expression analysis using DESeq 2 (Love et al. 2014) in R Programing Language.

Quantitative PCR (qPCR)

Total RNA samples isolated as described above were used as templates to generate first-strand DNA. 10 ng of template first-strand DNA was mixed with 0.6 µl each of 10 µM primers (16S rRNA gene forward primer AYKO228: CACACTGGGACTGAGACACG; reverse primer AYKO229: CTGCTGGCACGGAGTTAGC; 25147 37811 (a CAO homolog) forward primer AYKO462: AT CGAGCCAACCCACGATCT; reverse primer AYKO463: TCCGGGATAATCCGAGGAGA) and 10 µl 2× SYBR Select Master Mix (Life Technologies) in triplicates. Real-time reaction was run following a SYBER Select Master Mix protocol in a Bio-Rad CFX96 qPCR system, and data were analyzed using Bio-Rad CFX Manager software.

Results

Nitrogen repletion restores chlorophyll *d* levels in *Acaryochloris* cells after nitrogen starvation

Nitrogen starvation in non-diazotrophic cyanobacteria results in chlorosis (Sauer et al. 2001) but has never been tested in an organism where chl *d* is the major chlorophyll. To test if nitrogen starvation induces a reversible chlorosis in *Acaryochloris* sp. CCMEE 5410, we harvested and transferred log-phase cells into sBG-11 with and without nitrogen. Cells transferred to nitrogen-free media ceased chlorophyll production immediately, and began chlorophyll degradation after 72 h of starvation (Fig. 1). When re-introduced into media with nitrogen, the nitrogen-starved cells began to produce chl *d* after a 24-h lag phase (Fig. 1). The production of chl *d* was extremely high in the first 72 h of nitrogen repletion, nearly matching the total chlorophyll production of control cells grown continuously with nitrogen (Fig. 1). Thus, strain CCMEE 5410 responds to nitrogen depletion and repletion similar to chl *a*-containing non-diazotrophic cyanobacteria. Cells that remained nitrogen starved continued to degrade chlorophyll, eventually turning yellow (Online Resource 1, Fig S1). We used log-phase cultures for experiments on transcript induction during nitrogen repletion, because nitrogen

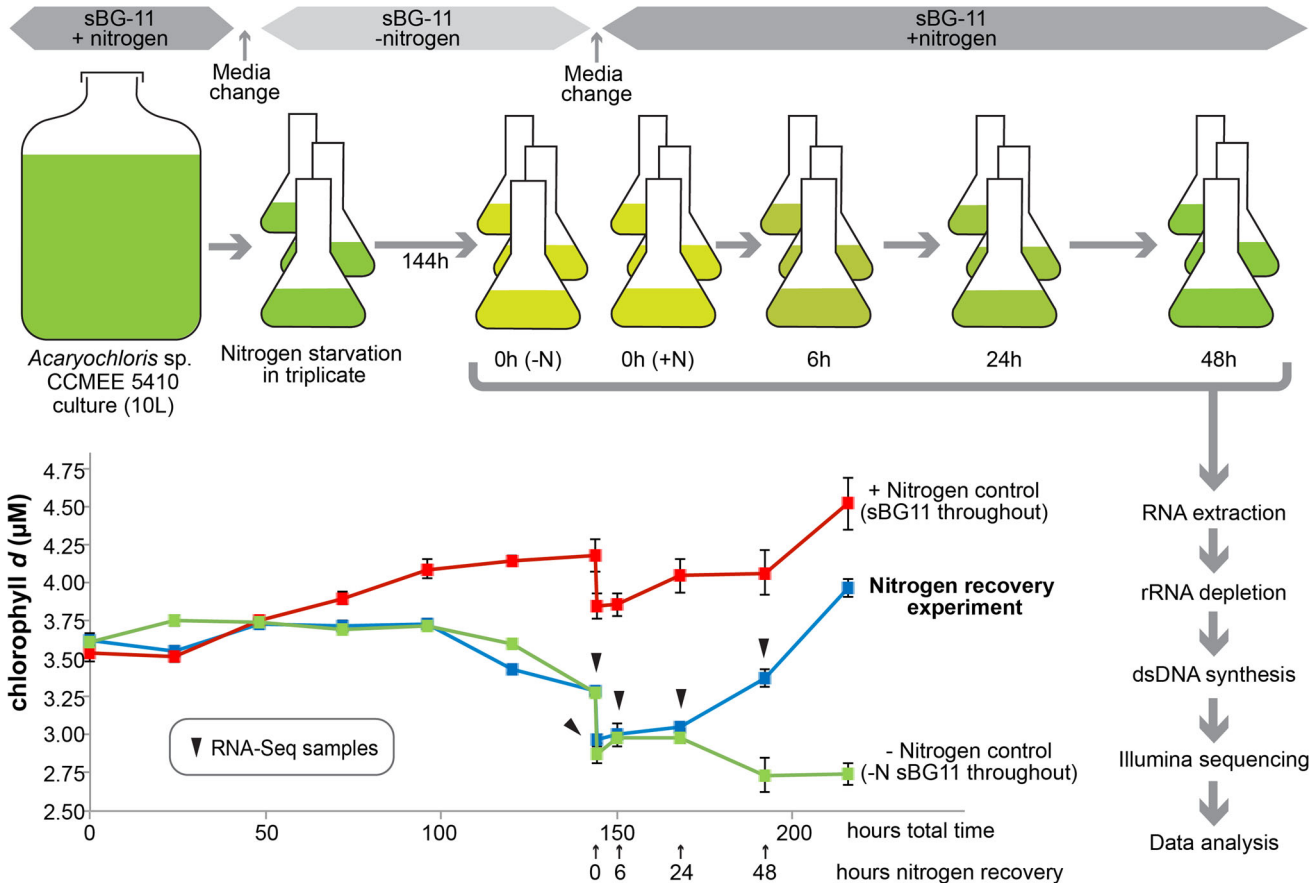

Fig. 1 Experimental setting (nitrogen starvation, nitrogen recovery time course). *Acaryochloris* sp. CCMEE 5410 was inoculated in sBG-11 in a 1L culture. The starter culture was then washed to remove nitrogen and resuspended in 1L nitrogen-free sBG-11 cultures in triplicate. As control experiments, two sets of triplicates were prepared for −N sBG-11 throughout and +N sBG-11 throughout, and Chl *d* levels were measured during the entire time period (graph). After 144 h of nitrogen depletion, the cultures were washed and resuspended in +N sBG-11 to start nitrogen recovery. Arrowheads in the graph indicate where in the time course RNA-Seq samples were harvested

starvation had no obvious effect on stationary cells after 2 weeks of starvation.

Rapid change of gene expression upon nitrogen repletion/media change

We observed a rapid transcriptional change in genes related to nitrogen utilization upon media change from sBG-11 without nitrogen to sBG-11 with nitrogen, which occurred within 30 min (Fig. 1, Online Resource 1, Fig. S2). The most upregulated gene upon media change was 2514736195 (gene ID)/ACCM5_010100011169 (locus tag), which is an *Acaryochloris*-specific hypothetical protein with a domain of unknown function (DUF) 4278 (see Online Resource 2). 2514739787/ACCM5_010100029183 (hypothetical protein with DUF 4278) has homology to glutamine synthetase inactivating factor IF7 and was one of the most upregulated genes after media change. This gene was upregulated immediately after media change, but the expression levels decreased in the subsequent time points. Branched-chain amino acid/urea ABC transporter operon (2514734055-63) was actively transcribed during nitrogen starvation, but it was significantly down-regulated in response to media change, and an alternate urea transport system became active in sBG-11 with nitrogen (Figs. 2, 3). Transcriptional data suggest that cyanate and nitrile were utilized during nitrogen starvation. Glutamine synthetase type III (GlnN) appears to be the major enzyme during nitrogen starvation, but type I (GlnA) seems to become dominant after the media change (Fig. 3). Differential usage of glutamine synthases was also reported in other cyanobacteria including *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 (Reyes et al. 1997; Sauer et al. 2000). Global nitrogen regulatory protein NtcA homolog, 2514734623/ACCM5_010100003288, was only mildly upregulated (2–2.4 fold) during nitrogen repletion.

Lead, cadmium, zinc, mercury transporting ATPase/Copper-translocating ATPase genes showed strong

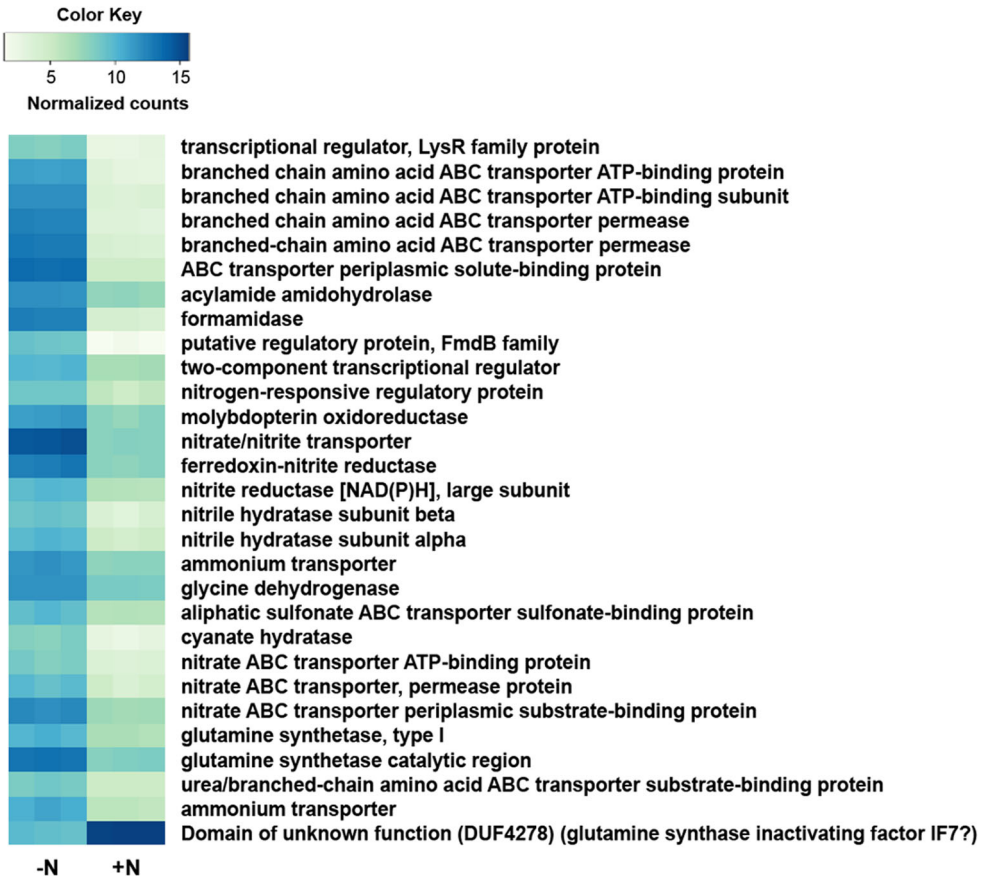

Fig. 2 A heat map of genes that showed rapid response to media change. Each *square* of the heatmap represents gene expression (normalized counts) per one of biological triplicates. −N, sBG-11 without nitrogen, +N, sBG-11 with nitrogen

upregulation after media change, so did multicopper oxidase/copper resistant protein genes. On the other hand, iron uptake genes were unchanged (e.g., ABC transporter ATP-binding protein 2514734558/ACCM5_010100002963; Fe(II)-dependent oxygenase superfamily protein 251 4737075/ACCM5_010100015618; iron-sulfur cluster assembly accessory protein, 2514734605/ACCM5_0101 00003198) or slightly upregulated (e.g., iron ABC transporter permease 2514736016/ACCM5_010100010268; iron-regulated protein A precursor (similar to Imelysin, implicated in iron uptake), 2514736372/ACCM5_010 100012084; iron deficiency induced protein A, 2514739 865/ACCM5_010100029590). There were also multiple transcriptional regulators and signaling-related genes that were upregulated after media change.

Significantly regulated genes during nitrogen recovery

Ribosomal protein genes were primarily upregulated earlier during nitrogen recovery (~6–24 h post-media change), but other genes displayed various timing of up- or down-regulation (Fig. 3). CO_2 fixation and carbon metabolism genes showed gradual upregulation, peaking at 24 or 48 h (Fig. 3). Photosynthesis-related genes were gradually upregulated and remained high at 48 h. Some PS gene transcripts remained abundant during nitrogen starvation, such as *psbA* and *psbD*. *psaA* and *psaB* genes were the most expressed genes overall, followed by *psbC*, *psbD, psbB*, "carbohydrate porin" (2514737530/ACCM5_010 100017868), *psbA, psbD*, and another copy of *psbA*. Several prochlorophyte chlorophyll–binding (pcb) proteins are highly upregulated throughout the recovery (Fig. 3; Online Resource 1, Fig. S3). Surprisingly, allophycocyanin beta subunit is also highly upregulated during recovery despite no evidence of phycobilin protein absorption in strain CCMEE 5410. Lipid-related genes such as KASII were upregulated during nitrogen recovery. Chl synthesis genes were actively transcribed and upregulated during nitrogen recovery, reaching a peak at 24 h time point and were slightly lower at 48 h (Fig. 4). Chlorophyll *d* synthase candidates are shown in Fig. 5. Some oxidase reactions are predicted to result in the production of formaldehyde. Genes involved in formaldehyde detoxification are upregulated during nitrogen recovery (Fig. 5).

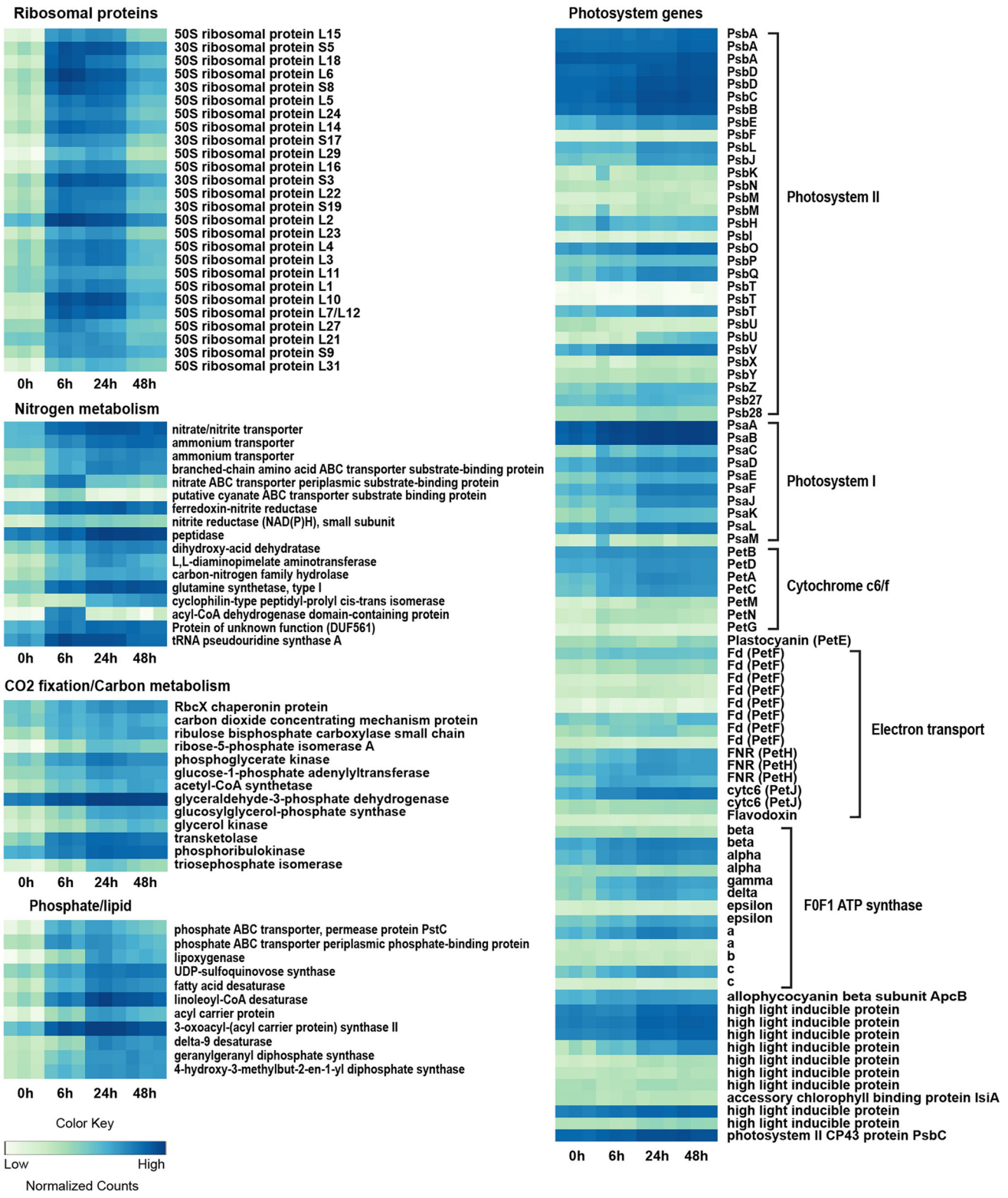

Fig. 3 Summary of significantly regulated genes during nitrogen recovery time course. The genes in the heatmaps were at least fourfold up-or down-regulated during nitrogen repletion, except genes encoding GlnN and NtcA were included as a comparison, and the map of photosystem genes includes comprehensive list of annotated genes. 0, 6, 24, 48 h indicate time points in hours after media change

Discussion

Nitrogen recovery

The time course of nitrogen-related genes showed that the CCMEE 5410 cells undergo rapid adaptation from nitrogen-deprived to nitrogen-replete environment, mediated by the glutamine synthetase inactivation factor IF7 and two differentially utilized glutamine synthetases, type I and type III (Fig. 2, 3). Thereafter, it appears that cells recover normal protein synthesis capacity by generating more ribosomes, sometime after 6 h post-nitrogen repletion (Fig. 3). Photosynthesis-related genes seem to recover after 24 h of nitrogen repletion, but some genes such as *psbA* maintained high levels of transcripts even when the cells are nitrogen starved (Fig. 3). An alternative explanation for these highly abundant transcripts is that their mRNAs are more stable than other mRNA in strain CCMEE 5410. Our current data cannot distinguish between higher transcript stability and higher levels of transcription.

The effect of nitrogen repletion on chlorophyll biosynthesis in strain CCMEE 5410

We found that nitrogen starvation induces chlorosis in *Acaryochloris* sp. CCMEE 5410 similar to other non-diazotrophic cyanobacteria (Sauer et al. 2001). The yellow phenotype is similar in appearance to mutants in nitrate reduction in *A. marina* (Watabe et al. 2015), indicating that nitrogen limitation in general lowers chlorophyll levels. Chlorophyll degradation begins after 96 h and continues until nitrogen repletion (Fig. 1). The degradation of chlorophyll *d* suggests that formylation of the C3 position does not prevent *Acaryochloris* species from degrading chlorophyll *d*. It is possible that chlorophyll *d* is converted back into chlorophyll *a* for degradation. In plants, the enzyme chlorophyll *b* reductase is responsible for converting chlorophyll(ide) *b* back into chlorophyll(ide) *a* for degradation (Folly and Engel 1999). Alternatively, the enzymes of *Acaryochloris* might be capable of degrading chlorophyll *d* directly. Chlorophyll levels recovered

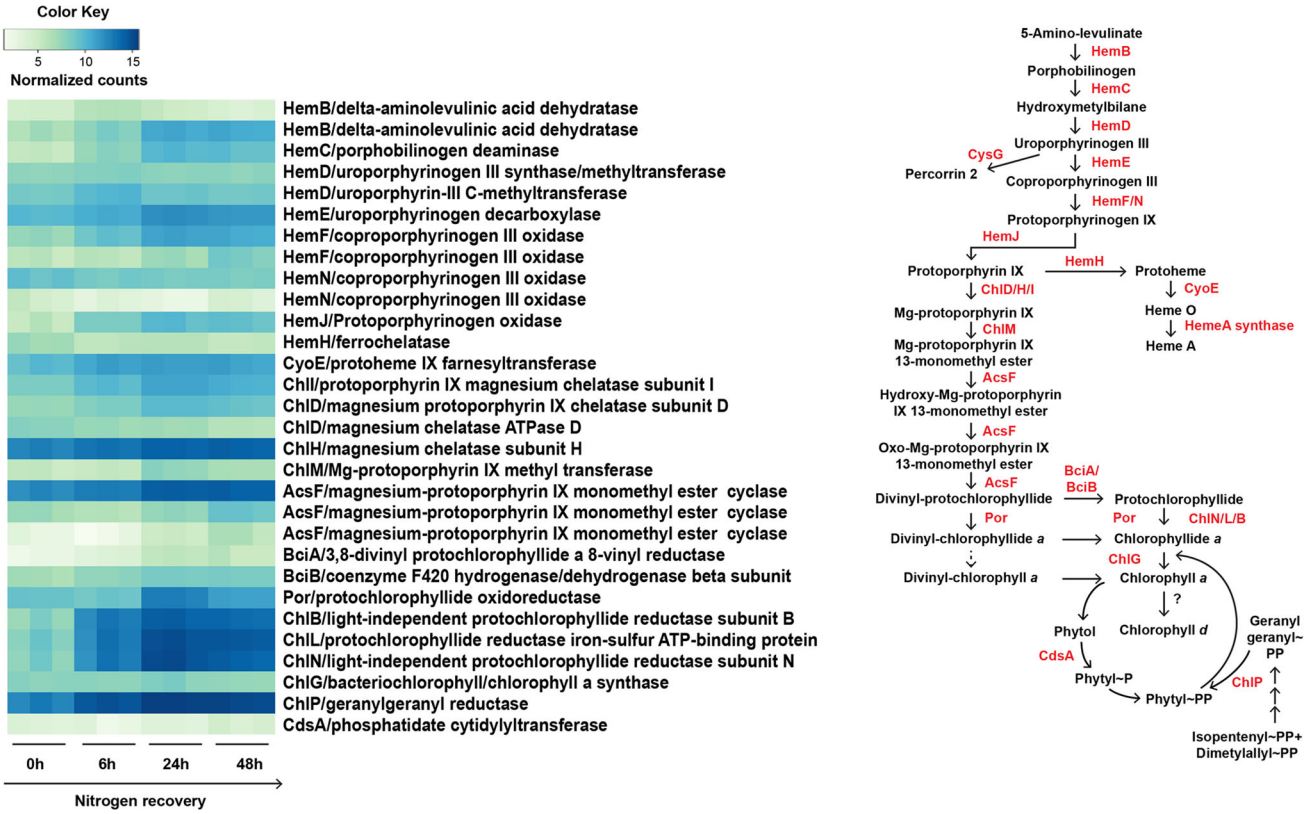

Fig. 4 Summary of heme/Chl synthesis gene expression profiles during nitrogen recovery. Most genes involved in heme and chlorophyll synthesis are upregulated during nitrogen recovery. Chlorophyll synthesis genes are transcribed most at 24 h and slightly less at 48 h than 24 h. Expression levels of the DPOR genes were higher than those of the LPOR gene

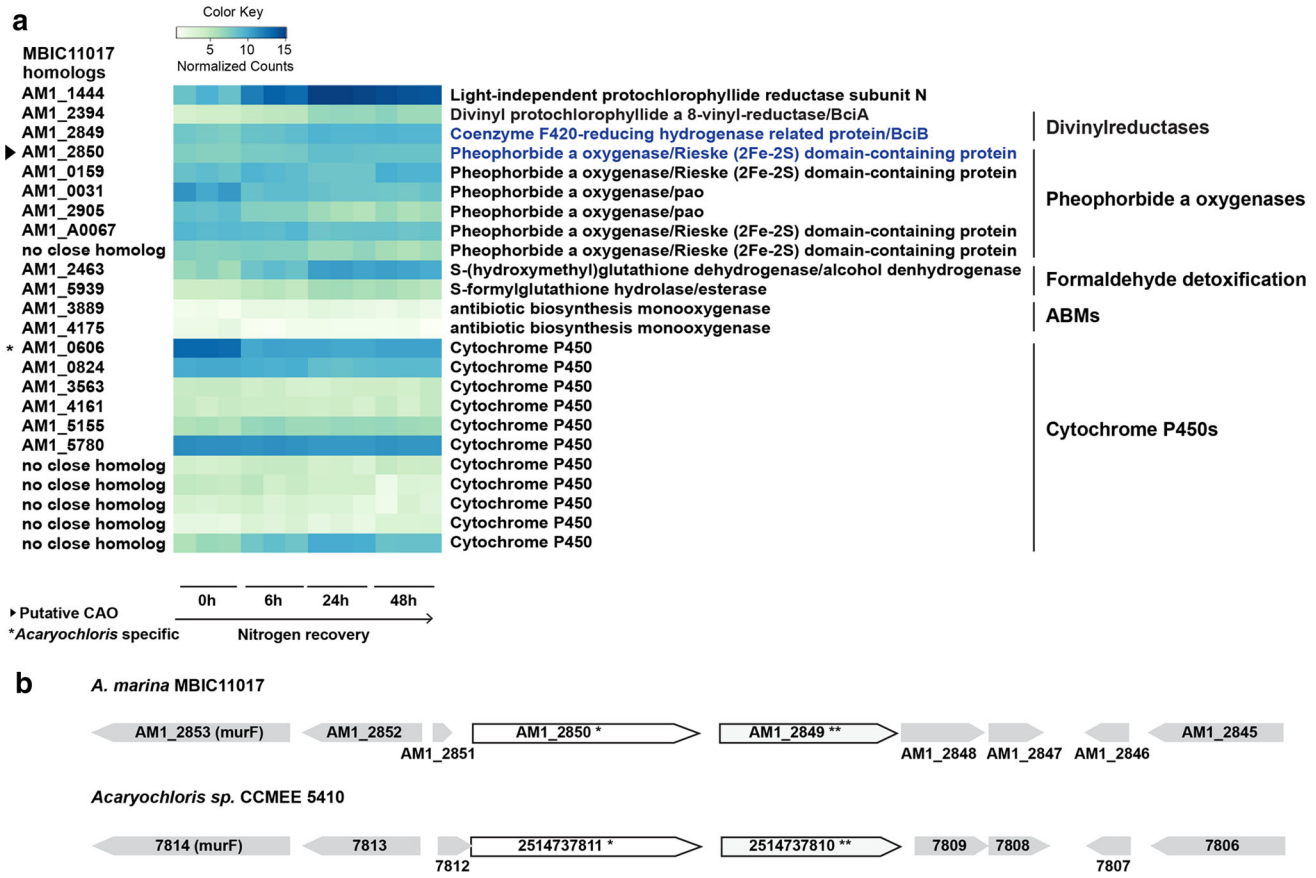

Fig. 5 Genes that could be involved in Chl *d* synthesis. **a** A heatmap of the candidate genes during nitrogen recovery. One of the DPOR genes, *chlN*, was included to compare expression levels. Genes encoding P450s are unchanged or down-regulated. **b** Genomic organization of the *bciB* homologs and Rieske domain-containing protein genes in *A. marina* MBIC11017 and *Acaryochloris* sp. CCMEE 5410. *BciB homologs; **Rieske domain-containing proteins

quickly after nitrogen repletion. After 6 h of recovery, chlorophyll degradation ceased. The chlorophyll production was obvious by 24 h after recovery (Fig. 1). Thus, nitrogen availability can be used to regulate chlorophyll levels in strain CCMEE 5410.

Chlorophyll biosynthesis genes in strain CCMEE 5410 are highly redundant. Strain CCMMEE 5410 encodes both the oxygen-dependent (*hemF*) and oxygen-independent (*hemN*) type coproporphyrinogen III oxidases (Heinemann et al. 2008), and encodes both light-dependent (*lpor*) and light-independent (*chlBLN*) protochlorophyllide reductases (Reinbothe et al. 2010). It is also the only organism with both *bciA*- and *bciB*-type divinyl reductases (Liu and Bryant 2011; Chen et al. 2016). Both divinyl reductases appear to act on the 8-vinyl and not the 3-vinyl(Chen et al. 2016). It is surprising that the analogous coproporphyrinogen III oxidases, protochlorophyllide reductases, and divinyl reductases are all upregulated during nitrogen recovery (Fig. 4). We expected the aerobic and light-dependent pathways to be favored under our growth conditions of continuous light and shaking. The light-independent protochlorophyllide reductase is particularly surprising as it is thought to be oxygen sensitive (Yamazaki et al. 2006), yet was one of the most upregulated sets of genes (Fig. 4). The low light levels (20 µE) of our experiment might explain the abundance of the light-independent protochlorophyllide reductase. In mutants of the cyanobacteria *Plectonema boryanum* with only the light-independent protochlorophyllide reductase active, cells grow well in low light (25 µE) but fail to grow in high light (170 µE) (Fujita et al. 1998). These *P. boryanum* mutant cells will grow under high light if oxygen is continuously removed by nitrogen purging (Yamazaki et al. 2006). If we had grown strain CCMEE 5410 under higher light, we might expect that the light-independent protochlorophyllide reductase would be less abundant. Alternatively, the high abundance of the *chlBLN* transcripts could be due to deactivation of the protein by oxygen. In addition to analogous pathways, there are also duplications in porphobilinogen synthase (*hemB*), uroporphyrinogen-III synthase (*hemD*), *hemN*, *hemF*, and Mg-protoporphyrin IX monomethyl ester cyclase (*acsF*). All duplications are

likewise upregulated during nitrogen recovery. It appears that the entire complement of chlorophyll biosynthesis genes respond to nitrogen recovery (Fig. 4), allowing for restoration of chlorophyll levels.

RNA-seq data implicate genes involved in chl *d* synthesis

Comparing the gene expression data and chl *d* recovery data, it is consistent that gene expression preceded actual recovery of chl *d* molecules. We wanted to correlate the expression level of various oxygenases in strain CCMEE 5410 with chlorophyll production levels. We focused on several families of oxygenases whose chemistry is compatible with converting chl(ide) *a* to chl(ide) *d*: 1) cytochrome P450 family (Denisov et al. 2005), 2) Rieske oxidase (RO) family (Barry and Challis 2013), and 3) antibiotic biosynthesis monooxygenase (ABM) family (Sciara et al. 2003) (Fig. 5a). Strain CCMEE 5410 encodes eleven different cytochrome P450s (pfam00067). The expression data for each of the cytochrome P450s do not correlate with chl *d* production. The Rieske oxidase family is known to be involved in the metabolism of chlorophylls. Chlorophyllide *a* oxygenase (CAO) is responsible for the production of chlorophyll *b* (Tanaka et al. 1998), and pheophorbide *a* oxygenase (PAO) is involved in the degradation of chlorophylls in higher plants (Pruzinská et al. 2003). Strain CCMEE 5410 has six Rieske oxidase family members. One Rieske oxidase (2514737811/ACCM5_010100019298) is slightly upregulated during chlorophyll biosynthesis, and the trend was confirmed by qPCR (Online Resource 1, Fig. S4). Interestingly, this Rieske oxidase is on an operon with divinyl reductase homolog (*bciB*, 2514737810/ACCM5_010100019293, Fig. 5b). The BciB/RO operon only occurs in *Acaryochloris* species. Finally, there are two ABM family members in strain CCMEE 5410 (2514736144/ACCM5_010100010914 and 2514736431/ACCM5_010100012379). ABM family members carry out ring opening oxidation reactions on heme in non-canonical heme oxygenases (Skaar et al. 2004; Chim et al. 2010), similar to PAO in chlorophyll degradation. ABM heme oxygenases are structurally unrelated to the canonical heme oxygenases characterized in humans and cyanobacteria (Skaar et al. 2004). 2514736144 is on an operon with *psbV* and cyt c_6 and has homologs in other species. On the other hand, 2514736431 is encoded as a monocistronic gene and occurs only in *Acaryochloris* species with no other homologs in Genbank or JGI databases. Both ABMs are not highly expressed and not upregulated during recovery (Fig. 5a). Formaldehyde is the expected byproduct in conversion of chl *a* to chl *d* by RO and ABM family members. Formaldehyde is the known byproduct of the alternative heme oxygenase IsdG from *Staphylococcus aureus* (Streit et al. 2015). Indeed, we found that the formaldehyde detoxification system is upregulated during chlorophyll biosynthesis (Fig. 5a).

Response to metal ions

Our finding of strong temporal upregulation of heavy metal transporting ATPase/copper-translocating ATPase genes and multicopper oxidase/copper resistant protein genes was unexpected. The transcriptional data implied that the strain CCMEE 5410 was under excess metal stress after growth medium was changed from sBG-11 without nitrogen to sBG-11 with nitrogen (Online Resource 2). It is possible that sBG-11 medium that we used for this study had higher levels of metal ions than the strain CCMEE 5410 naturally prefers, so that the cells were showing a detoxification response of excess metals. In fact, many cyanobacteria require very low metal levels for growth (Mann et al. 2002). Studies using IMK media, which contain significantly lower concentrations of transition metals, reported much better growth of *A. marina* (Tsuchiya et al. 2012). In *Synechocystis* sp. PCC 6803, it is reported that plastocyanin protein levels increase when cells were subjected to higher copper concentration (Giner-Lamia et al. 2012), but the expression of the gene encoding predicted plastocyanin was unchanged in *Acaryochloris* sp. CCMEE 5410 (Online Resource 2; 2514736351/ACCM5_010100011979).

We identified four MerR family transcriptional regulators, one LysR family transcriptional regulator, one two-component sensor kinase, and two two-component response regulators that were expressed at higher levels at media change (Online Resource 2). Since MerR family transcriptional regulators are usually involved in metal transport or resistance, they might be involved in reacting to excess metals in the fresh medium. The LysR family transcriptional regulator (2514734055/ACCM5_010100000430) appears to regulate the branched-chain amino acid transporter system (2514734056-60), as it is located in the same operon. Some of the most upregulated genes after media change were 2514738481/ACCM5_010100022667 (thioredoxin) and 2514738564/ACCM5_010100023082 (glutaredoxin), which are known to be essential for arsenic detoxification (Ji and Silver 1992; López-Maury et al. 2009; Pandey et al. 2013).

Conclusion

Nitrogen starvation is found to induce chlorosis in strain CCMEE 5410. Nitrogen repletion leads to a rapid burst in chlorophyll production. Transcriptomic data of known chlorophyll biosynthesis genes during nitrogen recovery

revealed an expression profile which matches chlorophyll *d* production. We examined the expression of the oxygenases present in strain CCMEE 5410 during recovery. Only a single oxygenase, 2514737811/ACCM5_010100019298, followed the expression pattern of known chlorophyll biosynthesis genes.

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