

Article

Rapid Transcriptional Reprogramming Triggered by Alteration of the Carbon/Nitrogen Balance Has an Impact on Energy Metabolism in *Nostoc* sp. PCC 7120

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Abstract: *Nostoc* (*Anabaena*) sp. PCC 7120 is a filamentous cyanobacterial species that fixes N₂ to nitrogenous compounds using specialised heterocyst cells. Changes in the intracellular ratio of carbon to nitrogen (C/N balance) is known to trigger major transcriptional reprogramming of the cell, including initiating the differentiation of vegetative cells to heterocysts. Substantial transcriptional analysis has been performed on *Nostoc* sp. PCC 7120 during N stepdown (low to high C/N), but not during C stepdown (high to low C/N). In the current study, we shifted the metabolic balance of *Nostoc* sp. PCC 7120 cultures grown at 3% CO₂ by introducing them to atmospheric conditions containing 0.04% CO₂ for 1 h, after which the changes in gene expression were measured using RNAseq transcriptomics. This analysis revealed strong upregulation of carbon uptake, while nitrogen uptake and metabolism and early stages of heterocyst development were downregulated in response to the shift to low CO₂. Furthermore, gene expression changes revealed a decrease in photosynthetic electron transport and increased photoprotection and reactive oxygen metabolism, as well a decrease in iron uptake and metabolism. Differential gene expression was largely attributed to change in the abundances of the metabolites 2-phosphoglycolate and 2-oxoglutarate, which signal a rapid shift from fluent photoassimilation to glycolytic metabolism of carbon after transition to low CO₂. This work shows that the C/N balance in *Nostoc* sp. PCC 7120 rapidly adjusts the metabolic strategy through transcriptional reprogramming, enabling survival in the fluctuating environment.

Keywords: cyanobacteria; *Nostoc* sp. PCC 7120; transcriptomics; photosynthesis; carbon/nitrogen

1. Introduction

Cyanobacteria use light energy to fix inorganic carbon (C_i) and nitrogen (N), harvested from their aquatic environment, into the metabolic components required for growth and propagation. Environmental sources of C_i include dissolved CO₂ and bicarbonate (HCO₃⁻), while N can be supplied by nitrate (NO₃⁻), nitrite (NO₂⁻), ammonium (NH₄⁺), urea or N₂ (in diazotrophic cyanobacteria; reviewed in [1]). The tight coupling of the concentrations of C_i and N taken up from the environment prevents metabolic imbalance within the cell, which allows cyanobacteria to thrive amidst varying nutritional conditions. This is achieved in large part by transcriptional modifications that are triggered by fluctuations in the cellular homeostasis of organic carbon (C) and N, which are represented by changes in the relative abundances of key metabolite signals (reviewed in [2–4]). One such metabolite is 2-oxoglutarate (2OG), also known as α-ketoglutarate (αKG), which is a product of the tricarboxylic acid (TCA) cycle. The metabolite 2OG provides the inorganic carbohydrate skeleton for glutamate synthesis that occurs by the incorporation of NH₄⁺ in the glutamine synthetase/glutamine-oxoglutarate aminotransferase (GS/GOGAT) cycle. Cellular 2OG levels therefore represent the abundances of both

C and N (reviewed in [5,6]), making 2OG a central signalling metabolite that triggers transcriptional adjustments to restore C/N balance [7–9]. 2-phosphoglycolate (2PG) is another metabolite that controls transcriptional reprogramming in response to C/N balance [10], and 2PG is formed when Rubisco catalyses the oxygenation of RuBP (photorespiration), instead of the favoured carboxylation reaction between RuBP and CO₂ (reviewed in [4,11]). An increase in 2PG concentration therefore represents CO₂ deficiency, triggering transcriptional reprogramming designed to upregulate C_i import into the cell [12–15].

Nostoc (*Anabaena*) sp. PCC 7120 is a filamentous, diazotrophic cyanobacterium wherein C/N balance controls the formation of heterocyst cells specialised for fixing N₂ into NH₄⁺, while photosynthetic CO₂ fixation is restricted to vegetative cells (reviewed in [16,17]). In *Nostoc* sp. PCC 7120 and other heterocystous cyanobacteria, differentiation in cell structure and function is triggered by changes in C/N homeostasis and enacted by massive transcriptional reprogramming [1]. Emphasis on the impact of N concentration on heterocyst differentiation has revealed the central roles of 2OG and several regulatory proteins in instigating transcriptional and physiological responses to N deficit [6]. However, the transcriptional response of heterocystous cyanobacteria to C_i availability has drawn only little attention [18], in sharp contrast to that in non-diazotrophic species [12,13,15,19–22]. In the current study of the global transcriptome of *Nostoc* sp. PCC 7120, we found that a shift from 3% CO₂ to 0.04% CO₂ for 1 h can be largely attributed to changes in the levels of the metabolites 2PG and 2OG, which has a strong effect on genes involved in the import and metabolism of C_i and N. This study also identified that genes encoding factors involved in photosynthetic electron transport, glycolysis and iron homeostasis are regulated by C/N homeostasis, which is suggested to trigger a transition from efficient photoautotrophic growth and energy storage to photoinhibition and glycolysis.

2. Materials and Methods

2.1. Growth and CO₂ Stepdown

Nostoc sp. PCC 7120 cultures were grown in BG11 medium [23] buffered with 10 mM TES-KOH (pH 8.0) at 30 °C under constant illumination of 50 μmol photons m⁻² s⁻¹ with 120 rpm agitation, in air enriched with 3% (*v/v*) CO₂. During the exponential growth phase (OD₇₅₀ = 1.0), 2 mL samples were taken from three individual replicate cultures and frozen for RNA isolation. For CO₂ stepdown, the cultures were pelleted and the pellets washed once with fresh BG11, before resuspension in fresh BG11 and growth in air containing 0.04% (*v/v*) CO₂. After 1 h, 2 mL samples were collected from three replicates and frozen for RNA isolation.

2.2. RNA Isolation and Transcriptomics

Total RNA was isolated as described in [24]. Total RNA samples were submitted to the Beijing Genomics Institute (China) for library construction and RNA sequencing using Illumina HiSeq2500. RNA reads were aligned using Strand NGS 2.7 software (Avadis) using the *Nostoc* sp. PCC 7120 reference genome and annotations downloaded from Ensembl (EBI). Aligned reads were normalised and quantified using the DESeq package (R). Significantly differentially expressed genes were identified using a 2-way ANOVA. *p*-Values were adjusted for false discovery rate (FDR) using the Benjamini–Hochberg procedure.

3. Results

The transcriptome of *Nostoc* sp. PCC 7120 grown in BG11 under 3% CO₂-enriched air was compared with that of the same strain shifted from 3% CO₂ to 0.04% CO₂ for 1 h, revealing 230 genes to be upregulated >2-fold and 211 genes to be downregulated >2-fold, in the low CO₂ condition. The RNAseq data are available at the NCBI Sequence Read Archive (submission SUB8244772). As expected, given the short period under a new metabolic condition, no differences in the growth rates, lengths of filaments or frequencies of heterocysts (approximately 4% of all cells under 3% CO₂)

were observed between the cultures exposed to 0.04% CO₂ conditions, compared to those grown at 3% CO₂. Therefore, statistical tests of these parameters in the different cultures were not performed.

3.1. Uptake and Metabolism of Carbon and Nitrogen Are Inversely Responsive to Low CO₂ Conditions

The operons encoding three plasma membrane-localised HCO₃⁻ uptake systems were among the most strongly upregulated entities in *Nostoc* sp. PCC 7120 following CO₂ stepdown (Table 1). In cyanobacteria, the Cmp (BCT1) system is powered by ATP hydrolysis [25], while the SbtA and BicA systems depend on Na⁺ ions for HCO₃⁻ symport [21,26]. The upregulation of the operon encoding the Mrp Na⁺:H⁺ antiporter upon CO₂ stepdown may also be linked to HCO₃⁻ uptake through the extrusion of Na⁺ to support SbtA and BicA activity [27,28]. HCO₃⁻ uptake in cyanobacteria forms a major part of the carbon concentration mechanism (CCM), which also involves the concentration of cellular CO₂ into HCO₃⁻ by a customised NAD(P)H dehydrogenase (NDH-1) complex [29]. Genes *ndhF3*, *ndhD3* and *cupA*, which encode subunits that specialise the NDH1–MS complex for inducible CO₂ uptake, were upregulated after CO₂ stepdown (Table 1), as were several other genes encoding the core NDH-1M complex (see below). Notably, a putative *cupS* orthologue (*alr1320*), which is encoded separately from the *ndhF3/ndhD3/cupA* cluster in the *Nostoc* sp. PCC 7120 genome [30], was not differentially expressed (DE) in the current work. Genes encoding Rubisco and some carboxysome subunits were mildly upregulated in low CO₂ (1.3 to 1.7 fold change (FC); not shown), while other CCM components were not DE, suggesting that these components were already in sufficient abundance before CO₂ stepdown, whereas C_i uptake from the environment, especially HCO₃⁻ uptake, was apparently a primary concern for survival after 1 h under low CO₂.

The decrease in CO₂ was found to cause downregulation of the *nir* operon, which encodes subunits of an ATP-dependent nitrate (NO₃⁻) transporter, as well as NO₃⁻ and nitrite (NO₂⁻) reductases [31,32]. The *nir* operon is responsible for NO₃⁻ uptake and reduction to ammonia (NH₃), and is broadly conserved across cyanobacteria (reviewed in [5]). Unlike the *nir* operon, the majority of *nif* genes that encode subunits for the assembly and function of nitrogenase, which reduces atmospheric N₂ to NH₃, were not DE in the current work. Exceptions were *nifB* and *nifH2*, which were downregulated (Table 1). A gene that encodes a protein similar to the C-terminus of Mo-like nitrogenase (*alr1713*) was strongly downregulated, along with its neighbour (*asr1714*); however, the function of the encoded proteins is not known. Since heterocyst development is upregulated by a high C/N ratio (reviewed in [33]), it was not surprising to see many genes involved in the structural development of heterocysts repressed by the shift to low CO₂. In particular, the *hpd*, *hgl* and *dev* gene clusters that encode many components for the synthesis and export of glycolipids, which form the oxygen-impermeable heterocyst envelope (reviewed in [34,35]), and were downregulated in the current data. Notably, the *hep* genes encoding heterocyst outer layer polysaccharides were only moderately downregulated by the shift to low CO₂ (average FC −1.5, data not shown).

3.2. Expression of Genes Encoding Photosynthetic and Respiratory Components Responds to Low CO₂ Conditions

Expression of genes encoding photosystem II (PSII) core proteins D1 and D2 was upregulated after the shift to low CO₂ (Table 2), indicating an increase in the damage and turnover of PSII reaction centres [36,37]. In keeping with this, the genes encoding the two FtsH proteases involved in the degradation and turnover of damaged D1 protein were also upregulated [38,39]. We also found strongly induced expression of the *flv2–flv4* operon, as well as several genes encoding orange carotenoid proteins (OCPs) and early light-inducible proteins (ELIPs), all of which are associated with PSII photoprotection [40–44]. These expression data suggest that the shift to low CO₂ led to the over-reduction, damage and repair of PSII. Given this evidence for PSII over-reduction, it was surprising to find the gene encoding the “plastid” terminal oxidase (PTOX) as one of the most strongly downregulated in the current study (Table 2), being highly expressed under 3% CO₂ and strongly repressed in 0.04% CO₂. PTOX is part of a water–water cycle that moves electrons from reduced

plastoquinone (PQ) to O₂, and is thought to be an electron valve for balancing the photosynthetic redox state [45], which would presumably be important under low CO₂ (discussed below).

Virtually all genes encoding subunits of photosystem I (PSI) were substantially downregulated in the current study, which is in contrast to their increased expression in *Synechocystis* sp. PCC 6803 and unchanged expression *Synechococcus elongatus* PCC 7942 in low CO₂ [13,20]. PSI downregulation in *Nostoc* sp. PCC 7120 points towards a decrease in PSI electron transport that may be related to the diminution of the terminal electron acceptor CO₂. These conditions would also be expected to upregulate O₂ reduction and the subsequent formation of toxic superoxide radicals (O₂^{•-}); indeed, genes encoding a superoxide dismutase (SodB; *alr2938*) and peroxiredoxin (*all2375*), involved in reactive oxygen species (ROS) scavenging, were upregulated 3.2-fold and 2.3-fold, respectively (not shown). The expression of *isiB* (*alr2405*), which encodes a flavodoxin (Fld) that accepts electrons from PSI via a flavin mononucleotide cofactor [46], was also upregulated after low CO₂ treatment (Table 2), suggesting a shortage of oxidised ferredoxin (Fd) acceptors [47]. The upregulated expression of genes encoding F₀-F₁ ATP synthase points to an increased demand for energy in low CO₂, required for HCO₃⁻ import and CO₂ hydration (reviewed in [2]). Notably, the gene *alr1004* encoding an enzyme that converts glyoxylate to glycine for the detoxification of 2PG [18] was found to be downregulated after CO₂ stepdown, while other enzymes in the glycolate metabolism pathway were not DE.

Table 1. Differentially Expressed (DE) Genes Involved in Carbon and Nitrogen Metabolism.

Name ¹	Gene ID ¹	Description	Process	Fold Change ²	p-Value ³
<i>sbt</i> operon	<i>all2133–all2134</i>	Na ⁺ -dependent bicarbonate permease, P _{II} -like regulatory protein	Bicarbonate import	78.8	<0.001
<i>cmp</i> operon	<i>alr2877–alr2880</i>	ATP-dependent bicarbonate uptake subunits		36.4	<0.001
<i>bicA</i> operon	<i>all1303–all1304</i>	Na ⁺ -dependent bicarbonate permease, Na ⁺ :H ⁺ antiporter		8.7	<0.001
<i>mrp</i> operon	<i>all1837–all1843</i>	Na ⁺ :H ⁺ antiporter subunits	Na ⁺ extrusion, pH regulation	5.4	<0.001
<i>ndhF3</i>	<i>alr4156</i>	NDH-1MS subunit 5	CO ₂ uptake	2.5	0.002
<i>ndhD3</i>	<i>alr4157</i>	NDH-1MS subunit 4		2.0	<0.001
<i>cupA</i>	<i>alr4158</i>	NDH-1MS CO ₂ uptake subunit		5.3	<0.001
<i>nir</i> operon	<i>alr0607–alr0612</i>	Nitrate/nitrite reductase, ATP-dependent nitrate permease	Nitrate/nitrite import and metabolism	−3.5	0.017
<i>nirB</i>	<i>all0605</i>	Nitrate-dependent expression of <i>nir</i> cluster		−2.9	<0.001
<i>nifB</i>	<i>all1517</i>	Fe–Mo cofactor biosynthesis subunit	N ₂ fixation, heterocyst development and function	−2.0	0.002
<i>nifH2</i>	<i>alr0874</i>	Fe–S cluster-binding nitrogenase reductase		−3.2	0.024
<i>hgd, hgl</i> clusters	<i>all5341–alr5359</i>	Heterocyst glycolipid layer biosynthesis		−2.6	0.004
<i>dev</i> operon	<i>alr3710–alr3712</i>	ATP-binding subunit, membrane transport subunits		−2.1	0.004
<i>alr1713</i>	<i>alr1713</i>	Similar to Mo-dependent nitrogenase, C-terminus	Unknown	−5.6	0.002
<i>asr1714</i>	<i>asr1714</i>	Uncharacterised protein		−5.8	<0.001

¹ Shaded cells represent operons or clusters of neighbouring genes; ² Fold changes of genes upregulated or downregulated in low CO₂, compared to high CO₂, are coloured orange or green, respectively. In cases of multiple genes, average fold changes are shown; ³ p-values determined by moderated *t*-test. In cases of multiple genes, largest p-value is shown.

Table 2. Differentially Expressed (DE) Genes Involved in Photosynthesis and Respiration.

Name ¹	Gene ID ¹	Description	Process	Fold Change ²	p Value ³	
<i>psbAII</i>	<i>alr3727</i>	Photosystem II D1 protein	PSII electron transport	6.9	<0.001	
<i>psbAIII</i>	<i>alr4592</i>	Photosystem II D1 protein		1.9	0.003	
<i>psbAIV</i>	<i>all3572</i>	Photosystem II D1 protein		3.7	<0.001	
<i>psbD</i>	<i>alr4548</i>	Photosystem II D2 protein		3.4	<0.001	
<i>psaA</i>	<i>alr5154</i>	Photosystem I core protein A1	PSI electron transport	−1.9	0.035	
<i>psaB1</i>	<i>alr5155</i>	Photosystem I core protein A2		−1.9	0.038	
<i>psaB2</i>	<i>alr5314</i>	Photosystem I core protein A2		−2.2	0.031	
<i>psaC</i>	<i>asr3463</i>	Photosystem I Fe-S subunit		−2.2	<0.001	
<i>psaD</i>	<i>all0329</i>	Photosystem I reaction centre subunit 2		−2.7	<0.001	
<i>psaE</i>	<i>asr4319</i>	Photosystem I subunit E		−2.2	0.001	
<i>psaI</i>	<i>asl3849</i>	Photosystem I subunit I		−2.5	0.012	
<i>psaK</i>	<i>asr4775</i>	Photosystem I subunit K		−3.1	0.004	
<i>psaM</i>	<i>asr4657</i>	Photosystem I subunit M		−2.3	0.005	
<i>flv2</i>	<i>all4444</i>	Flavodiiron protein		Other photosynthetic/respiratory electron transport	23.7	<0.001
<i>all4445</i>	<i>all4445</i>	Unknown protein	31.8		<0.001	
<i>flv4</i>	<i>all4446</i>	Flavodiiron protein	16.7		<0.001	
<i>isiB</i>	<i>alr2405</i>	Flavodoxin	2.9		0.003	
<i>cytA</i>	<i>alr4251</i>	Cytochrome c ₆	−2.2		0.011	
<i>flv1B-flv3B</i>	<i>all0177–all0178</i>	Flavodiiron protein (heterocyst-specific)	−2.0		0.004	
<i>ptox</i>	<i>all2096</i>	Alternative plastoquinone oxidase	−65.0		<0.001	
<i>ftsH</i>	<i>alr1261</i>	FtsH protease	2.3		<0.001	
<i>ftsH2</i>	<i>all3642</i>	FtsH protease	2.4		<0.001	
<i>pec operon</i>	<i>alr0523–alr0527</i>	Phycocerythrocyanin synthesis	Metabolism/binding of light-harvesting pigments		−3.1	0.015
<i>chlL, chlN operon</i>	<i>all5076–all5078</i>	Protochlorophyllide reductase, ATP-binding protein		−3.3	<0.001	
<i>chlG</i>	<i>all4480</i>	Chlorophyll synthase 33 kDa subunit		2.2	0.003	
<i>hemH</i>	<i>alr4616</i>	Ferrochelatase		8.6	<0.001	
<i>ocp</i>	<i>all3149</i>	Orange carotenoid-binding protein		23.9	<0.001	
<i>ocp-like</i>	<i>all4941</i>	Orange carotenoid protein-like		3.1	0.010	
<i>asl3726</i>	<i>asl3726</i>	CAB/ELIP/HLIP superfamily		9.8	<0.001	
<i>asr5262</i>	<i>asr5262</i>	CAB/ELIP/HLIP superfamily		8.6	0.005	
<i>atpase cluster</i>	<i>all0004–all0010</i>	ATP synthase subunits		ATP synthesis	2.7	<0.001
<i>ndh-1 operon</i>	<i>alr0223–alr0227</i>	NDH-1 complex subunits		Electron and proton transport	2.3	0.005
<i>ndh-1 operon</i>	<i>all3840–all3842</i>		2.1		0.001	
<i>ndhB</i>	<i>all4883</i>		2.5		0.003	
<i>ndhN</i>	<i>alr4216</i>		1.9		0.001	
<i>alr1004</i>	<i>alr1004</i>		Alanine-glyoxylate transaminase		Glycolate metabolim	−2.7
<i>ndbA</i>	<i>all1553</i>	NDH-2 NAD(P)H:PQ reductase	Respiration	−2.1	<0.001	
<i>hupS</i>	<i>all0688</i>	Uptake hydrogenase, small subunit	H ₂ uptake/evolution	−2.5	0.002	
<i>nif/PPFOR</i>	<i>alr1911</i>	Pyruvate-ferredoxin/flavodoxin oxidoreductase		−42.4	<0.001	
<i>hox clusters</i>	<i>alr0750–all0752, alr0760–alr0766</i>	Bidirectional hydrogenase subunits, assembly and regulation		−73.9	0.002	
<i>ppsA</i>	<i>all0635</i>	Phosphoenolpyruvate synthase	Glycolysis	−107.4	<0.001	

¹ Shaded cells represent operons or clusters of neighbouring genes; ² Fold changes of genes upregulated or downregulated in low CO₂, compared to high CO₂, are coloured orange or green, respectively. In cases of multiple genes, average fold changes are shown; ³ p-values determined by moderated *t*-test. In cases of multiple genes, largest *p*-value is shown.

The downregulation of PSI abundance in response to low CO₂ can partially clarify the apparent PSII over-reduction discussed above. Lower PSI levels may also be linked to a decrease in the number of heterocysts, which contain a higher PSI:PSII ratio than in vegetative cells [16]. Although such a decrease in heterocysts was not observed after 1 h in low CO₂, suppressed heterocyst development was evident in the downregulation of *hgl* and *dev* clusters (Table 1), and this can also explain the suppression of genes encoding cytochrome c₆, Flv1B and Flv3B (Table 2) that operate predominately [48] or exclusively [49] in heterocysts. The gene encoding the small subunit of the heterocyst-specific uptake hydrogenase (HupS) was also downregulated here, reflecting the downregulation of the heterocystous nitrogenase activity under low CO₂.

We observed downregulation of the *pec* cluster that encodes the phycocerythrocyanin (PEC) parts of the light-harvesting phycobilisome (PBS) complex [50,51], while genes encoding the other

components of the PBS were not DE, indicating that the light-harvesting cross-section of PBS in *Nostoc* sp. PCC 7120 is altered in response to low CO₂. An operon encoding a subunit of the light-independent protochlorophyllide reductase (DPOR) was also downregulated after 1 h in low CO₂, while the expression of *chlG* and *hemH* genes, involved in later stages of chlorophyll and haem synthesis, respectively, were upregulated (Table 2).

Most genes encoding subunits of the NDH-1 complex were upregulated under low CO₂ (Table 2), probably to fulfil their role in CO₂ uptake as part of the NDH-1MS complex described above. In contrast, *ndbA* encoding NDH-2 was downregulated in the current study, suggesting a decrease in NDH-2-mediated respiration after the shift to low CO₂. In addition, the gene encoding phosphoenolpyruvate (PEP) synthase, which converts pyruvate to PEP that is consumed in the TCA cycle, was strongly downregulated (Table 2). Many genes encoding subunits of the bidirectional hydrogenase (Hox) were among the most strongly downregulated in response to low CO₂ conditions (Table 2), being both highly expressed in 3% CO₂ and strongly repressed in low CO₂. Hox reversibly catalyses the reduction of H⁺ to form H₂, powered by reduced Fd/Fld with electrons derived from either PSI or pyruvate, the latter route by way of pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR), which converts pyruvate to acetyl-CoA (reviewed in [52]). The *nifH* gene encoding PFOR followed a similar expression profile to Hox subunits, being another of the most strongly downregulated genes in the current study. The physiological role of the Hox enzyme is not known, but has been described as an electron valve that can maintain redox balance and store reducing power as H₂ during excess photosynthesis or fermentation [52–55]. The expression profile of Hox and PFOR genes suggests that pyruvate-powered hydrogen production is active under 3% CO₂ and inactivated by the shift to low CO₂.

3.3. Expression of Transcription Regulators Responds to Changes in CO₂ Conditions

The current study revealed substantial changes in the expression of several genes encoding transcription regulators, providing evidence of an ongoing cascade of transcriptional reprogramming after 1 h under low CO₂ (Table 3). Upregulated expression of the LysR-type regulator (LTTR) *cmpR* corresponds to the strong upregulation of its target, the *cmp* cluster encoding the BCT1 HCO₃[−] transporter (Table 1), as previously shown in *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 [56] and *Nostoc* sp. PCC 7120 [57]. Two sigB-type group 2 sigma factors, which have roles in the transcriptional response to low CO₂ and C/N balance [58–60], were upregulated after the shift to low CO₂ (Table 3). SigB has also been implicated in response to environmental stress and resistance to photoinhibition in *Synechocystis* sp. PCC 6803 [61,62], which is in line with the upregulation in this study of *groES* and *groEL* genes (4.3 to 5.5 FC; not shown) and photoprotective factors such as OCPs and the *flv2–flv4* operon (Table 2). Two homologous two-component response regulator clusters, which each comprise a histidine kinase and a DNA-binding regulator, were found to be upregulated by low CO₂. Of the two, the chromosomal *hik31* (*C-hik31*) operon was more highly upregulated, and has been found to be involved in the responses to oxygen concentration, light and metabolism [63,64]. A TetR-family transcription regulator with unknown function was also upregulated by low CO₂ (Table 3).

Another LTTR gene that was highly expressed under high CO₂ and was strongly downregulated after low CO₂ transition (Table 3) shared substantial sequence homology with the *ndhR* transcription repressor (also called *ccmR*) of *Synechocystis* sp. PCC 6803 [12,27]. In other cyanobacteria, *NdhR* represses the expression of CCM genes, including the *sbt* and *bicA* HCO₃[−] importers, the *mrrp* cluster and the *ndhF3/ndhD3/cupA* cluster [12,15,20,27,65]. The rapid downregulation of a putative *ndhR* orthologue in *Nostoc* sp. PCC 7120 may reveal the mechanism behind the strong upregulation of CCM genes after CO₂ stepdown in the current study (Table 1). Downregulation of the transcription enhancer *ntcB*, which increases N metabolism through upregulation of the *nir* operon [66], also correlates with downregulation of other N-related genes in the current work (Table 1). Expression of *ntcB* is controlled by *NtcA* [1]. As *ntcA* was not DE in the current study, downregulation of *ntcB* and other *NtcA* regulons may be due to the low CO₂-induced inactivation of *NtcA* (discussed below). Similarly, downregulated

transcriptional regulator genes *patB* (also called *cnfR*), *devH* and *nrrA* can also be attributed to inhibited NtcA activity [67–71]. These genes are expressed in heterocysts, where PatB upregulates the expression of *nifB* [72], DevH regulates heterocyst glycolipids [48,68,73] and NrrA induces expression of both the heterocyst regulator *hetR* and *fraF* encoding a filament integrity protein [70,74]. Both *nifB* and the *hgl* cluster were downregulated in this study (Table 1), while *hetR* and *fraF* were not DE (not shown).

Table 3. Differentially Expressed (DE) Genes Encoding Transcription Regulators.

Name	Gene ID	Description	Process	Fold Change ¹	p Value ²
<i>cmpR</i>	<i>all0862</i>	LysR-type transcriptional regulator	Regulates <i>cmp</i> cluster	3.1	<0.001
<i>sigB</i>	<i>all7615</i>	Group 2 sigma factor	Response to stress	4.6	<0.001
<i>sigB3</i>	<i>all7608</i>	Group 2 sigma factor		3.8	<0.001
<i>C-hik31 operon</i>	<i>all7583–all7584</i>	Two-component sensor His kinase, response regulator	Regulation of central metabolism in response to glucose, low O ₂	2.8	<0.001
<i>P-hik31 operon</i>	<i>alr1170–alr1171</i>	kinase, response regulator		1.6	0.001
	<i>all7523</i>	TetR family regulator	Unknown	2.4	0.046
putative <i>ndhR</i> orthologue	<i>all4986</i>	LysR-type transcriptional regulator	Repression of CCM expression	−146.2	<0.001
<i>ntcB</i>	<i>all0602</i>	LysR-type transcriptional regulator	Co-activation of <i>nir</i> operon	−2.4	0.002
<i>devH</i>	<i>alr3952</i>	CRP family transcriptional regulator	Het glycolipid biosynthesis	−2.2	0.006
<i>patB/cnfR</i>	<i>all2512</i>	Heterocyst patterning	Heterocyst development	−2.4	0.001
<i>nrrA</i>	<i>all4312</i>	OmpR family regulator		−2.1	<0.001

¹ Fold changes of genes upregulated or downregulated in low CO₂, compared to high CO₂, are coloured orange or green, respectively. In cases of multiple genes, average fold changes are shown; ² p-values determined by moderated *t*-test. In cases of multiple genes, largest *p*-value is shown.

3.4. Low CO₂ Conditions Influence the Expression of Metal Homeostasis Genes

A number of genes and gene clusters related to cellular homeostasis of iron (Fe) and other metals were found to be DE after CO₂ stepdown (Table 4). A gene cluster encoding subunits of a periplasmic ferrous Fe (Fe(II)) transporter [75,76] was strongly downregulated in the current study (Table 4), indicating a lower uptake of Fe from the environment under low CO₂. The *suf* cluster, encoding proteins involved in Fe mobilization and Fe–S cluster assembly, was also downregulated. The expression of both the Fe(II) transporter and the *suf* cluster is upregulated by Fe deprivation [77–79], suggesting a surplus of cellular Fe after low CO₂ treatment. Several neighbouring clusters of genes encoding subunits of metal cation efflux systems such as copper, nickel, zinc, cadmium and cobalt, were upregulated after low CO₂ treatment (Table 4). These genes have been implicated in heavy metal resistance [80,81], although the link to the current conditions is not clear. Taken together, low CO₂ appears to induce an active decrease in cellular metal content, which may be a strategy to avoid oxidative stress during the reducing conditions induced by an insufficient availability of photosynthetic electron acceptors.

Table 4. Differentially Expressed (DE) Genes Involved in the Transport and Metabolism of Metals.

Name	Gene ID	Description	Process	Fold Change ¹	p Value ²
Fe(II) transport operon	<i>alr2118–asr2120</i>	Ferrous iron transporter subunits	Periplasmic iron import	−42.6	<0.001
<i>suf</i> operon	<i>alr2492–alr2496</i>	ATPase, iron and sulphur transfer	Fe–S cluster assembly, transfer	−3.8	<0.001
Metal efflux cluster	<i>all7606–all7611</i>	Proton extrusion, cation efflux	Metal cation efflux, cellular metal homeostasis	3.1	<0.001
Metal efflux cluster	<i>all7616–all7619</i>	Cadmium/nickel/zinc/cobalt efflux system		3.9	<0.001
Metal efflux cluster	<i>all7629–all7633</i>	Cadmium/nickel/zinc/cobalt efflux system		5.0	<0.001
Cu ²⁺ efflux cluster	<i>alr7634–alr7636</i>	Putative copper efflux		5.1	0.002

¹ Fold changes of genes upregulated or downregulated in low CO₂, compared to high CO₂, are coloured orange or green, respectively. In cases of multiple genes, average fold changes are shown; ² p-values determined by moderated *t*-test. In cases of multiple genes, largest *p*-value is shown.

4. Discussion

4.1. Transcriptional Regulation in Response to CO₂ Stepdown is Triggered by Metabolites

Induction of the most strongly upregulated genes, the HCO₃[−] transporters (Table 1), after CO₂ stepdown, suggests a rapid transcriptional response that is highly sensitive to cellular C_i levels. In some unicellular cyanobacteria, repression of the CCM genes by NdhR (also called CcmR) can be modulated by both 2OG and 2PG [12,15,65,82]. Increased cellular 2PG concentration caused by increased photorespiration in low CO₂ leads to increased abundance of the NdhR–2PG complex that is unable to bind DNA to repress expression [4]. Additionally, declining 2OG levels due to lower CO₂ fixation and potentially lower TCA cycle activity decrease the abundance of the NdhR–2OG repressor complex, although it is unclear whether 2OG levels would actually decrease after only 1 h in low CO₂ due to the mobilisation of stored glycogen into the TCA cycle [2,15,82,83]. NADP⁺, another co-repressor of NdhR [82], is also theoretically far less abundant after CO₂ stepdown, due to decreased CO₂ fixation and lower NADPH consumption despite constant light conditions. Although a putative NdhR in *Nostoc* sp. PCC 7120 has not been studied, it appears that the LTTR encoded by *all4986* represents such an orthologue, and that the strong downregulation of *all4986* after CO₂ stepdown led to de-repression of the NdhR regulon, which includes the *ndhR* gene itself [84]. Previous transcriptomics studies have shown *ndhR* expression to be upregulated in *Synechocystis* sp. PCC 6803 after >3 h in low C_i conditions [12,13], which is in contrast to the strong downregulation of *all4986* seen here after 1 h (Table 3). This suggests that NdhR de-repression in response to CO₂ stepdown may be transient, and/or that expression of the NdhR regulon is also controlled by other transcription factors [18]. In the current study, de-repression by the putative NdhR is proposed to have caused a rapid and strong increase in HCO₃[−] and CO₂ uptake under C limitation, with the upregulated *cmp* operon (Table 1) inducing further increase in HCO₃[−] uptake. The *cmp* inducer CmpR is activated by 2PG or RuBP [82,85], both of which are in higher concentrations after CO₂ stepdown due to decreased CO₂ fixation. Furthermore, *cmpR* expression is also auto-upregulated (Table 3) [57]. In *Synechococcus* sp. PCC 7942 CmpR additionally upregulates the expression of PSII core subunits [86,87], found upregulated also in the current study alongside factors for PSII photoprotection and turnover, and downregulation of most PSI genes (Table 2). The overlap between cellular responses to either low CO₂ or high light stress is well documented [12,20,86,88], highlighting insufficient electron sinks similarly created by both high light and low CO₂, and resulting in the over-reduction of photosynthetic electron carriers [2]. Notably, several PSII photoprotection factors encoded by genes that were DE in the current study, including *psbAIII*, *flv4* and *sodB*, were shown to be regulated together with Rubisco and other CCM genes by another LTTR in *Nostoc* sp. PCC 7120 called PacR [18], suggesting the likely involvement of PacR in the transcriptional reprogramming seen here.

In addition to the LTTR transcription factors, the transcriptional response to low CO₂ is also regulated by LexA and the cyAbrB paralogues [89–92]. The vast change in expression of the *hox* operon after CO₂ stepdown (Table 2) may be related to the activity of LexA [93] and/or cyAbrB [90,94–96]. In *Synechocystis* sp. PCC 6803, cyAbrB2 controls the expression of many CCM components that were likewise found to be upregulated in this study (Table 1) [90], while the cyAbrB2 orthologue in *Nostoc* sp. PCC 7120 regulates the expression of FeSOD [96], also upregulated here. *Nostoc* sp. PCC 7120 cyAbrB1 has been recently implicated in transcriptional regulation of heterocyst differentiation [97], demonstrating a role close to the interface of C_i and N availability that suggests cyAbrB1/2 were likely active in the transcription regulation observed in the current study.

Given the transcriptional activation of NtcA, the master regulator of N metabolism, by high levels of 2OG upon a shift to low N (high C/N ratio) [7,98,99], it is widely assumed that a decrease in CO₂ leads to a decline in NtcA activity by decreasing the abundance of the 2OG–NtcA–PipX activator complex [100]. In the current study, lower NtcA activity was indeed evident in the downregulation of *nir* genes encoding NO₃[−] uptake and metabolism (Table 1), as well as downregulation of the *nir* co-activator *ntcB* (Table 3; reviewed in [101]). This transcriptional regulation would effectively bring N

metabolism into alignment with decreased C metabolism after CO₂ stepdown, despite the presence of N sources in the BG11 media. Overall, nearly 50% of genes downregulated in the current study have NtcA-binding promoters [71], although many NtcA-regulated genes, such as those involved in the uptake of NH₃ and urea, regulation of GS-GOGAT enzymes, as well as NtcA itself [71,102–104], were not DE after 1 h in low CO₂. The current work may therefore include only the early NtcA regulon. The NtcA-activated differentiation of vegetative cells to heterocysts occurs over approximately 24 h, via a cascade of transcriptional regulation that includes early upregulation of the co-activator *nrrA* [33]. The rapid downregulation in the current work of some heterocyst regulators, including *nrrA*, may block the commencement of heterocyst differentiation in response to relative N excess over C after CO₂ stepdown, and may signal an eventual decrease in the small number of heterocysts that are known to occur under high C/N [105]. Downregulation of NtcA activity in the current study appears to indicate a decline in 2OG levels after only 1 h in low CO₂, although an increased concentration of NH₃ derived from 2PG metabolism can also explain suppression the NtcA regulon under low CO₂ (low C/N ratio) [83]. N excess under low CO₂ is also evident in the –2.2 FC downregulated expression of cyanophycinase *chb2* (*all0571*; not shown), which is regulated by NrrA, suggesting a decrease in the mobilisation of stored N under low CO₂ (reviewed in [106]). It can also be argued that an increase in the ADP/ATP ratio under CO₂ stepdown, caused by decreased photosynthetic electron transport and rapid changes in metabolism, increases the abundance of both the ADP–PII–PipX complex and the inactive form of NtcA [6].

The strong downregulation of operons involved in ferrous Fe import and Fe–S cluster assembly in the current work (Table 4) suggests a connection between cellular Fe homeostasis and C/N balance in *Nostoc* sp. PCC 7120, which has been explored [107]. The current results indicate a CO₂ stepdown-induced cellular excess of Fe and other transition metals, which may be due to downregulation of Fe-rich PSI complexes (Table 2) [108] and/or an excess of reductant caused by insufficient electron sinks. Both conditions pose the danger of ROS formation, evidenced by upregulated expression of SOD, peroxiredoxin and protein chaperones.

4.2. Altered C/N Balance Modulates the Energetic Strategy of *Nostoc* sp. PCC 7120

The current study shows that a stepdown from 3% CO₂ in enriched air to 0.04% CO₂ (atmospheric) for just 1 h led to substantial reprogramming of global gene expression in *Nostoc* sp. PCC 7120 cultures. As discussed above, most of the transcriptional changes observed here can be directly attributed to metabolite signalling instigated by the alteration of the cellular C/N balance (Figure 1), initiated by the decrease in CO₂ concentration. Furthermore, these results highlight rapid transcriptional reprogramming of photosynthesis and energy metabolism in *Nostoc* sp. PCC 7120 in response to CO₂ levels (Figure 2). High CO₂ in light promotes a high rate of photosynthesis and the storage of photosynthate in the form of glycogen [13,109–111]. Strong expression of PEP synthase and PFOR under these conditions indicate glycolysis/gluconeogenesis through the metabolism of pyruvate, which is consumed in the TCA cycle to facilitate respiratory electron transport and to provide 2OG for amino acid synthesis (reviewed in [112,113]). Therefore, growth under 3% CO₂ somewhat resembles photomixotrophy, with photosynthetic and glycolytic metabolisms occurring concomitantly, even though glucose was not externally provided to cultures. Under these conditions, the cells experience a high C/N, which is evident in the relatively high expression of N metabolic genes, reflecting a cellular excess of 2OG [7,98,99]. In high CO₂, strong expression of Hox, which is important under mixotrophy and N deprivation [55], and PTOX, may provide a system to maintain redox poise [45], and in the case of Hox, also store surplus energy as H₂ [53]. The transfer of *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 cultures from high to low CO₂ showed that the toxic effects of 2PG transiently block Calvin–Benson–Bassham (CBB) activity [15,20,83] and this is also evident here in the upregulated expression of PSII repair, photoprotection and ROS scavenging enzymes in *Nostoc* sp. PCC 7120, which indicate over-reduction of the photosynthetic electron transport chain. Interestingly, detoxification of 2PG appeared to be downregulated through repression of *alr1004*

during CO₂ stepdown (Table 2), perhaps highlighting the importance of the metabolite for signalling during the early stages of C_i deprivation. We also observed downregulation of the terminal proteins in the phycobilisomes, suggesting modified harvesting of light energy to alleviate excitation pressure on the photosynthetic system. Under these conditions, inhibition of photoassimilation is compensated by glycolysis of stored glucose and CBB intermediates, providing an important supply of substrates for anaplerosis of the CBB and TCA cycles during acclimation to the transition [83,114,115]. In the current work, enhanced glycolytic activity is indicated by the upregulation of NDH-1, suggesting an increased reliance on respiratory electron transport for ATP generation, while strong downregulation of PEP synthase and PFOR after CO₂ stepdown may prevent diversion of pyruvate away from the TCA cycle. Notably, PEP abundance increased substantially in *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 after a shift from high to low CO₂ [20,83], while genes encoding both pyruvate kinase and PFOR were highly expressed in *Synechococcus elongatus* PCC 7942 after long-term acclimation to low CO₂, but not directly after the transition [20]. These findings support the results of this study and suggest that the metabolism of PEP and pyruvate are tightly regulated after the transition to low CO₂. This may be linked to the role of PEP and pyruvate as substrates to anaplerotic carbon fixation to produce TCA cycle intermediates oxaloacetate and malate (reviewed in [116]). During the adjustment to a low C/N ratio, TCA cycle activity generates 2OG for amino acid synthesis, utilising excess NH₄⁺ generated through 2PG detoxification [11,83].

This work has revealed rapid transcriptional reprogramming in *Nostoc* sp. PCC 7120 in response to a decrease in C_i availability, namely strong upregulation of CCM components and photoprotection, and downregulation of N uptake and early stages of heterocyst differentiation. Despite the vast increase in HCO₃⁻ uptake, glycolysis of stored C apparently plays an important role in energy metabolism at low CO₂, likely due to 2PG-induced inhibition of the CBB cycle. A majority of the transcriptional effects induced by low CO₂ in *Nostoc* sp. PCC 7120 can be attributed to 2PG modulation of CmpR and a putative NdhR homologue; however, the effects of changing abundance of 2OG and NtcA activity after 1 h in 0.04% CO₂, as well as the roles of other transcriptional regulators cannot be discounted. Finally, this work highlights the sensitivity of *Nostoc* sp. PCC 7120 to factors that influence the cellular C/N balance and demonstrates the speed at which genetic and metabolic reprogramming can take place, allowing rapid acclimation for surviving and thriving in the fluctuating environment.

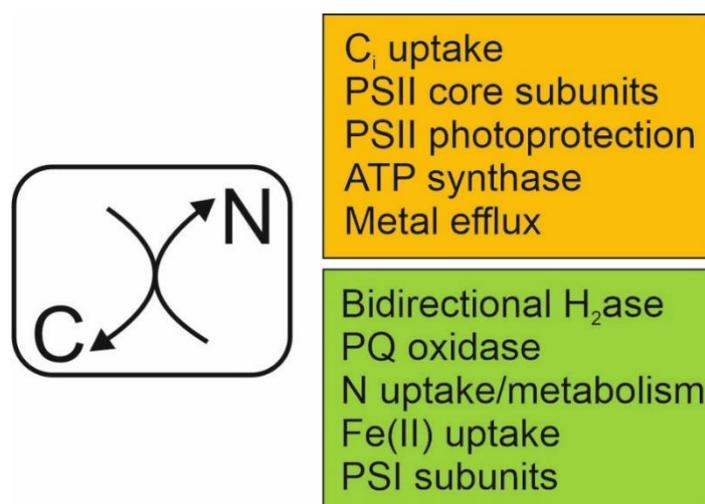


Figure 1. The transcriptional response of *Nostoc* sp. PCC 7120 cells to a change in the cellular concentrations of carbon and nitrogen (C/N balance). Decrease in the external CO₂ concentration of cultures causes a decline in the cellular C/N balance signalled by an increased production of 2PG and decreased 2OG levels relative to N. This metabolic change leads to upregulation of genes encoding processes depicted in orange, and downregulation of genes encoding processes depicted in green.

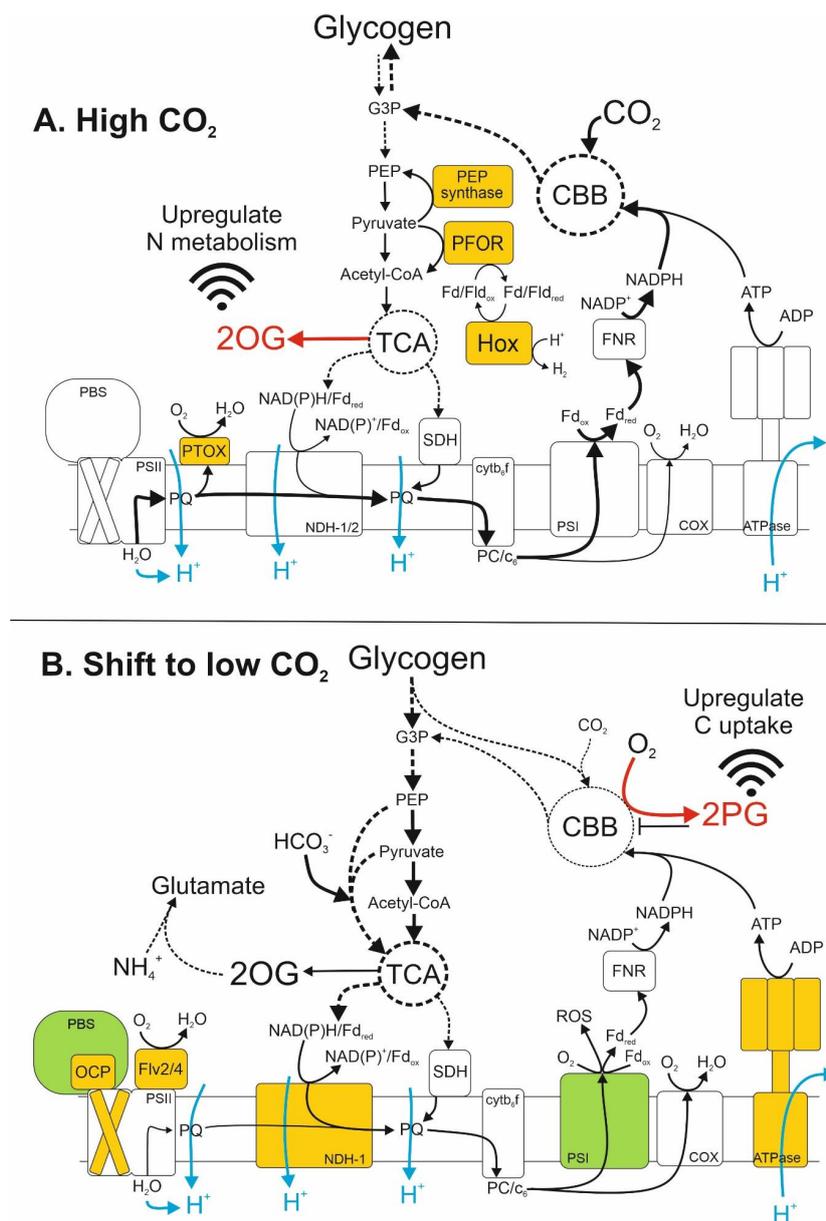


Figure 2. Schematic representation of the interactions between photosynthetic/respiratory electron transport and accumulation of 2OG and 2PG for signalling the C/N balance in *Nostoc* sp. PCC 7120 cells. Scheme is based on global transcriptomic profiling of *Nostoc* sp. PCC 7120 cultures under high CO₂ conditions and after CO₂ stepdown. (A) Under 3% CO₂, efficient photosynthetic electron transport and Calvin–Benson–Bassham (CBB) cycle activity enables gluconeogenesis of glyceraldehyde-3-phosphate (G3P), leading to accumulation of carbohydrate storage in the form of glycogen. Glycolysis of glycogen stores and/or photosynthate supplies pyruvate to the incomplete tricarboxylic acid (TCA) cycle, which produces reductant, ATP and succinate to drive respiratory electron transport through NAD(P)H-dehydrogenase (NDH) and succinate dehydrogenase (SDH), as well as other cellular processes. The TCA cycle also generates 2-oxoglutarate (2OG), which accumulates under high CO₂ due to a relative shortage of NH₄⁺ and glutamine. 2OG operates as a signal for upregulating genes involved in N uptake and metabolism. Strong expression of phosphoenolpyruvate (PEP) synthase converts pyruvate to PEP. Pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR) converts pyruvate to acetyl-CoA, reducing oxidised ferredoxin/flavodoxin (Fd/Flv_{ox}) that is consumed by bidirectional hydrogenase (Hox) for storage of excess energy as H₂. Strong expression of plastoquinone terminal oxidase (PTOX) maintains redox homeostasis of the plastoquinone (PQ) pool during high photosynthetic electron transport, while cytochrome c6 oxidase (COX) maintains the redox poise of lumenal electron

carriers cytochrome c_6 (c_6) and plastocyanin (PC). Genes encoding factors coloured orange are highly expressed under 3% CO_2 and strongly downregulated by the shift to 0.04% CO_2 . **(B)** After 1 h in 0.04% CO_2 , a deficiency of CO_2 electron acceptors leads to oxygenation of Rubisco, causing photorespiration that produces 2-phosphoglycolate (2PG). The CBB cycle and other metabolic pathways are inhibited by 2PG, which also signals upregulation of the transcriptomic response to low CO_2 . Low CBB activity causes over-reduction of the photosynthetic electron transport chain, leading to the production of reactive oxygen species (ROS) at photosystem I (PSI) and downregulation of PSI subunits. Increased reducing pressure on photosystem II (PSII) also causes upregulation of the PSII repair cycle and upregulation of PSII photoprotection by flavoproteins (Flv2/4) and orange carotenoid proteins (OCPs), as well as downregulation of phycoerythrocyanin in the phycobilisome (PBS). Glycolysis triggered by decreased CO_2 assimilation provides substrates for anaplerotic supplementation of the CBB and TCA cycles, including the production of oxaloacetate from PEP and bicarbonate (HCO_3^-). TCA cycle activity also produces 2OG for glutamate production from excess ammonium (NH_4^+) resulting from the shift from 3% to 0.04% CO_2 . Genes encoding factors coloured orange or green are upregulated or downregulated, respectively, 1 h after the shift from 3% to 0.04% CO_2 . Black arrows indicate the movement of electrons or ATP, dashed arrows summarise multiple enzymatic reactions in carbohydrate metabolism, blue arrows indicate the movement of protons. The red arrow in **(A)** shows the production of 2OG by TCA cycle activity, the red arrow in **(B)** shows the production of 2PG by Rubisco oxygenation in the CBB cycle and the black bar in **(B)** indicates CBB inhibition by 2PG.

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