Title: Impacts of genetically engineered alterations in carbon sink pathways on photosynthetic performance.

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Running head: Engineered sink metabolism has strong effects on photosynthetic electron flow

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32 homeostasis, metabolic sink, NADPH, photosystem, plastoquinone.

33

34 Abbreviations: C_i, inorganic carbon, primarily [HCO₃⁻+CO₂]; 2PG, initial product of photorespiration due
35 to the oxygenation reaction of RuBP by RubisCO; RuBP, ribulose biphosphate; RubisCO, ribulose
36 biphosphate carboxylase/oxygenase; 2OG, 2-oxoglutarate, α-ketoglutarate; CBB, Calvin-Benson-
37 Bassham; CEF, cyclic electron flow; LEF, linear electron flow; DCMU, diuron, 3-(3,4-dichlorophenyl)-1,1-
38 dimethylurea; PSI, photosystem I; PSII, photosystem II; DCBQ, 2,6-dichloro-1,4 -benzoquinone, artificial
39 electron acceptor of PSII; PQ/PQH₂, plastoquinone/plastoquinol; NADP⁺/NADPH, oxidized/reduced
40 nicotinamide adenine dinucleotide phosphate; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ oxidoreductase;
41 CCM, CO₂-concentrating mechanism;

42

Abstract

Genetic engineering of photosynthetic organisms typically redirects native metabolism towards desirable products, which thereby represent new metabolic sinks. There is limited information on how these modifications impact the evolved mechanisms of photosynthetic energy metabolism and cellular growth. Two engineered strains of *Synechocystis* sp. PCC 6803 with altered carbon sink capacity were assayed for their photosynthetic and CO₂ concentrating mechanism properties in conditions of high and low inorganic carbon (C_i) availability. In the Δ glgC mutant, glycogen cannot be synthesized and a carbon sink pathway has been effectively removed. The JU547 strain has been engineered by integration of the *Pseudomonas syringae* ethylene forming enzyme and provides a new sink. When cultured under high carbon conditions, Δ glgC displayed diminished photochemical efficiency, a more reduced NADPH pool, delayed initiation of the Calvin-Benson-Bassham cycle, and impairment of linear and cyclic electron flows. It also exhibited a large decrease in photochemical quenching indicative of the accumulation of Q_A⁻, normally associated with a reduced PQ pool, but appears instead to be the result of an undefined dissipative mechanism to spill excess energy. In the case of carbon sink integration, JU547 displayed slightly more oxidized PQ and NADPH pools and increased rates of cyclic electron flow and an enhanced demand for inorganic carbon as suggested by increase in the expression of the bicarbonate transporter, SbtA. Overall, the results highlight the importance of the native regulatory network of autotrophic metabolism in governing photosynthetic performance and provide cogent examples of both predictable and difficult to predict phenotypic consequences upon installation of new pathways in autotrophs.

1. Introduction

The cyanobacteria have long served as models for studying oxygenic photosynthesis and autotrophic carbon metabolism. This includes recent genetic engineering studies that investigate the feasibility of producing biofuels and economically valuable compounds that represent diversions of photosynthetic products to these alternative products (reviews, see references[1-3]). Presently there is limited information on how such engineered modifications will impact the evolved mechanism of photosynthetic energy metabolism and cellular growth. Conceivably, it is possible that the evolved regulatory mechanisms built into the native enzymes and cell regulatory circuits will be sufficient to accommodate the alterations in photosynthetic and metabolic flux imposed by the engineered pathways. That is, the introduction of genetic modifications leading to desired products or restricting flux to competing pathways may produce little in the way of deleterious or unexpected effects, apart from anticipated losses in cell growth and productivity due to partitioning of photosynthate away from growth substrate. Such introduced pathway alterations would be considered benign. On the other hand, it is also possible, in principle, that the introduced changes in metabolism will 'inappropriately' interact with the native regulatory mechanisms. In this case, native regulatory mechanisms and flux characteristics that normally give rise to evolutionary fitness and robustness in the natural system instead produce deleterious misregulation of gene and metabolic activity. For example, it was recently observed that carbon fixation capacity was severely diminished upon large diversion of photosynthate towards, 2,3-butanediol, an engineered sink [4]. However, the physiological basis for that observation remained unresolved. Indeed, an understanding of the 'interaction' between introduced pathways and photosynthetic function generally remains poorly understood. An important approach to this question is to examine photosynthetic performance in the face of contrasting limiting nutrient conditions because nutrient or light availabilities may have a strong influence on whether or not these hypothesized metabolic impacts become significant [2-4].

The availability of inorganic carbon (C_i) is very often the limiting factor in the growth of algae and cyanobacteria in natural and engineered settings [5-10]. For cyanobacterial growth, the two main substrate forms are CO_2 and bicarbonate (HCO_3^-). Cyanobacteria have evolved mechanisms to acquire and concentrate both forms in order to saturate the active site of RubisCO with CO_2 . The overall mechanism is termed the CO_2 -concentrating mechanism (CCM) [11-13]. The CCM consists of a set of HCO_3^- transporters and a set of redox powered CO_2 uptake (Cup) proteins, which together function to supply HCO_3^- to the carboxysome. The latter contains RubisCO, as well carbonic anhydrase [14]. The

94 action of the HCO_3^- transporters and Cup proteins results in high cytoplasmic concentrations of HCO_3^-
95 driving its transport across the carboxysome boundary through selectively permeable pores in the
96 surrounding proteinaceous shell. Carbonic anhydrase within the carboxysome converts incoming
97 bicarbonate into CO_2 thereby supplying RubisCO. The CCM also exists in two forms: a constitutive low
98 affinity, high flux uptake system and an inducible high affinity system that is controlled by C_i availability
99 via changes in NADP^+ and 2-oxoglutarate levels, which are metabolic signals that respond to changes in
100 C_i availability. An additional metabolic interface between the CCM and metabolism relates to the power
101 demands imposed by the operation of the CCM. The CCM requires both ATP and reductant (NADPH or
102 reduced ferredoxin, Fd_{red}), and therefore, the CCM is intimately connected to photosynthetic electron
103 transport. Accordingly, an important metabolic impact of the CCM involves its demands for ATP and
104 Fd_{red} /NADPH production. A stoichiometric accounting of the NADPH and ATP requirements of the Calvin-
105 Benson-Bassham (CBB) cycle shows a 2:3 ratio of NADPH to ATP required for carbon metabolism [15,
106 16], although there remains debate whether linear electron transport can fulfil this ratio (briefly
107 reviewed in [15]). Nevertheless, the demanded actual ratio, as tuned to metabolism, almost certainly
108 changes in response to changes in the environment: limited inorganic carbon availability and extremes
109 in pH and light intensity very likely modifies this ratio. Furthermore, the introduction of new metabolic
110 channels, and/or the closing of other channels, is predicted to impact these demands on the
111 photosynthetic mechanism. To cope with changes in the cellular demand ratio for NADPH/ATP,
112 oxygenic photosynthesizers have evolved mechanisms of returning electrons to the electron transport
113 chain, thereby increasing *pmf* (and therefore producing ATP) without the net production of Fd /NADPH.
114 Collectively, these mechanisms are referred to as cyclic electron flow (CEF; see Fig. 1). There are several
115 putative CEF pathways in cyanobacteria, involving SDH, NDH and FNR, but they remain poorly
116 understood. The availability of CEF pathways, combined with energy dissipation pathways provides the
117 photosynthetic electron transport system flexibility required to cope with fluctuations in light intensity
118 and nutrient supply.

Figure 1. Simplified model of electron flow and metabolism in *Synechocystis* sp. PCC 6803. The photosynthetic light reactions obtain electrons from water, producing gaseous oxygen as a result. Electrons are transferred through the photosynthetic electron transport chain ultimately reducing NADP^+ , forming NADPH. During these reactions, a proton motive force (pmf) is established and used to drive the formation of ATP. NADPH and ATP are used to energize the Calvin-Benson-Bassham (CBB) cycle in order to integrate inorganic carbon into cellular metabolism. Cyclic electron flow (CEF) returns electrons back to the electron transport chain and regulates the NADPH/ATP ratio as needed, although the regulatory mechanisms are not fully elucidated. In nutrient replete conditions, glycogen serves as a primary storage molecule for excess electrons and carbon skeletons. In the $\Delta glgC$ strain (red), an enzyme responsible for glycogen synthesis has been deleted, and glycogen synthesis is removed. In the JU547 strain (green), the ethylene forming enzyme (EFE) gene has been integrated into the genome. As a result, ethylene and succinate are formed as products. Succinate itself may serve as a form of CEF through the succinate dehydrogenase complex (SDH) and return electrons to the electron transport chain.

Here we perform a detailed analysis probing for possible changes in photosynthetic electron transport to address the question of how two types of engineered modification, one closing a metabolic sink, the other creating a new sink, are tolerated in the face of contrasting availability of the major photosynthetic macronutrient, inorganic carbon (Ci). Two engineered strains of *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) were chosen as models for analysis of differentially altered carbon metabolism.

The first strain, $\Delta glgC$, has a deletion of the gene encoding glucose-1-phosphate adenylyltransferase and cannot synthesize glycogen^[17]. Consequently, this mutant lacks a carbon sink available to the wild-type strain. The second strain, JU547, has been engineered to express the ethylene forming enzyme (EFE) from the organism *Pseudomonas syringae*^[18]. The ethylene-forming enzyme uses 2-oxoglutarate (2OG, aka α -ketoglutarate) as a cofactor for the synthesis of ethylene, a volatile compound, which escapes the medium as a gas. The JU547 strain therefore has an additional, engineered carbon and electron sink

available. Furthermore, the ethylene-forming reaction also produces succinate in addition to ethylene, which can serve as an electron donor to the plastoquinone pool through the succinate dehydrogenase complex, which may serve as a major donor of electrons during respiration [19]. Appropriately, lower 2OG and higher succinate concentrations have been reported in JU547 [20].

2. Methods

2.1 Strains and culturing conditions.

The glucose tolerant *Synechocystis* served as the background control strain and was maintained in standard BG-11 medium [21]. The $\Delta glgC$ and JU547 strains have been previously described [17, 18]. Experimental cultures were grown in BG-11 medium with the following modifications (here referred as LC7) to allow manipulation of the availability of inorganic carbon. The medium was buffered with 40 mM HEPES-KOH and adjusted to pH 7.0. Sodium carbonate was also omitted from the medium. Prior to inoculation into the modified media, the strains were grown for approximately 5 days in standard BG-11 in shaking flasks under an atmosphere of 3% CO₂ to ensure the cells were in a carbon replete physiological state. These starter cultures were then used to inoculate 700 mL of modified BG-11 in flattened 900 mL flasks, bubbling with 3% CO₂ at a flow rate of ~500 mL min⁻¹ and with illumination provided by Cool White fluorescent lamps with an incident intensity of ~ 80 $\mu\text{E m}^{-2} \text{s}^{-1}$. Physiological and biochemical assays were initiated using cultures that had reached an OD₇₅₀ between 0.6 and 1.0. These cultures were designated high carbon cultures (HC). To cause conditions of C_i limitation, the HC cultures were switched to bubbling with ambient air for 18-22 hours to produce low carbon cultures (LC). This mimics the culturing conditions previously used for analysis of transcriptional changes accompanying the downshift in CO₂ availability [22].

2.2 Culture harvesting and sample preparation.

Cells were harvested from culture by centrifugation (6000 x g, 10 min). Cells were resuspended in the modified BG-11 medium (above) that had been bubbled with CO₂-free air for at least 1 hour. The cells were washed in this manner two additional times with the final resuspension to a chlorophyll (Chl) concentration of 100-200 $\mu\text{g Chl} \cdot \text{mL}^{-1}$. These dense cell cultures were kept shaking under white light (approx. 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) and aliquots diluted to 5 $\mu\text{g Chl mL}^{-1}$ for assays. Cultures were assayed within 2 to 3 hours after harvesting. Values of Chl were estimated while cells were in suspension as described by Williams [23], and later normalized to actual concentrations determined in methanol extracts using an extinction coefficient of 12.95 [24]. Absorbance spectra and optical density due to scattering at 750 nm were measured with a UV-Vis Scanning Spectrophotometer (UV-2101PC, Shimadzu, Japan). Cell density

was estimated using an automatic cell counter (Cellometer Auto X4, Nexcelom Biosciences). Estimates of cell size were obtained using a Nikon Eclipse Ni series differential interference contrast microscope and a digital camera. The relative areas of cells were obtained by using area measurements of manually selected cells using ImageJ software (NIH, Maryland, USA).

2.3 Oxygen evolution assays.

Oxygen evolution was measured using a Clark-type electrode system maintained at 30°C. Bicarbonate-dependent oxygen evolution and consumption measurements were routinely performed in the presence of 5 mM sodium bicarbonate, unless otherwise stated. PSII capacity was determined using an oxygen electrode with the addition of 1 mM DCBQ and 1 mM potassium ferricyanide. Oxygen evolution was measured for 90 seconds under saturating illumination ($\sim 10,000 \mu\text{E m}^{-2} \text{s}^{-1}$), and rates of oxygen evolution were obtained from 40 to 80 seconds using a linear regression fit of the computer-acquired datapoints. Respiration was assayed immediately after rates of oxygen evolution and respiration rates were obtained by cessation of illumination and fitting oxygen consumption to a linear fit model.

2.4 P700 spectroscopy.

P700 absorbance was measured with a JTS-10 spectrophotometer (Bio-Logic, France) using a 620 nm actinic light and 705 nm interference filters over the sample and reference photodetectors. Assays were performed on whole cells in LC7 medium supplemented with 5 mM NaHCO_3 at a density corresponding to $5 \mu\text{g Chl mL}^{-1}$ unless otherwise stated. Before illumination, samples were dark-adapted 5 min with stirring. Stirring was terminated one minute before illumination. Basal levels of P700 absorbance were determined with measuring pulses 10 seconds before and immediately before illumination. Samples were then illuminated for 5 seconds at a nominal intensity of $2050 \mu\text{E m}^{-2} \text{s}^{-1}$ actinic light. Re-reduction of P700 was followed for 14 seconds after illumination was terminated. Measuring pulses were applied throughout the illumination and darkness period. Rates of oxidation and reduction were obtained using Bio-Logic software to obtain half times, which were converted to apparent rate constants. Unless noted, transients are representative traces from at least three biological replicates (independent cyanobacterial cultures).

In order to assay P700 activity during illumination (see Fig. 8, and Supplemental Fig. 1), samples were placed in the JTS-10 cuvette holder and stirred while under actinic illumination ($620 \text{ nm}, 45 \mu\text{E m}^{-2} \text{s}^{-1}$). Stirring was terminated after 10 min. Detection pulses were applied 10 seconds apart to determine basal levels of P700 oxidation during illumination. Actinic illumination was terminated as a 200 ms saturating pulse ($2500 \mu\text{E m}^{-2} \text{s}^{-1}$) was delivered, fully oxidizing P700. Re-reduction of P700 during

darkness was followed for 8 seconds after termination of the saturating pulse. Total P700 was taken as the difference between maximum and minimum absorbance values. Reduced P700 was measured as the difference between the basal absorbance value and the value at maximum oxidation (during saturating pulse) divided by total P700. Oxidized P700 can be determined by the difference between basal oxidation values (during illumination) and maximally reduced values (during darkness).

2.5 PAM Fluorescence.

Experiments utilized a Walz DUAL-PAM 100 (Walz, Germany) with (DUAL-DR) and NADPH (DUAL-(E/D) NADPH) units. Cell samples were diluted in an optical glass cuvette to a density corresponding to 5 $\mu\text{g Chl mL}^{-1}$ in LC7 medium supplemented with 5 mM NaHCO_3 and stirred under actinic illumination (nominally $53 \mu\text{E m}^{-2} \text{s}^{-1}$) for 4 minutes. Actinic illumination was terminated and samples were allowed to dark-adapt for 5 minutes before measuring traces were started. Dark-adapted chlorophyll fluorescence was measured for 60 seconds, at which point actinic illumination ($53 \mu\text{E m}^{-2} \text{s}^{-1}$) was provided for 300 seconds and then terminated ($t = 360 \text{ s}$). Post-illumination fluorescence was measured for an additional 140 seconds ($t = 500 \text{ s}$) and terminated. Multiple turnover flashes (nominally $20,000 \mu\text{E m}^{-2} \text{s}^{-1}$) were provided at 15 and 310 seconds. Samples were subjected to either a five minute, or one minute measuring sequence. The fluorescence measuring frequency was high during periods of illumination (1000 Hz) but lowered (200 Hz) during periods of darkness in order to minimize actinic effects. Transients as presented have been averaged (500 pts) for clarity, but non-averaged traces were used for calculation of decay rates and physiological parameters (i.e. F_0 , F_m). Unless noted, transients presented are representative traces from at least three biological replicates.

2.6 Immunoblot assays.

Aliquots of cells harvested for physiological analyses were pelleted and stored frozen until protein analysis. Pellets were resuspended to a density corresponding to 1 mg Chl mL^{-1} in 25 mM Tris-HCl (pH 7.5). Cells were lysed in a Bullet Blender 24 microfuge tube agitator (Next Advance Inc, USA) using an equal volume of cell suspension and 0.1 mm glass beads. ϵ -aminocaproic acid and PMSF, 1 mM each, were added prior to breakage in order to minimize proteolysis. After cell lysis, glass beads were removed by low speed centrifugation ($600 \times g$) and samples were quickly frozen in liquid nitrogen for later use. Cell extracts were solubilized using 2% SDS, 5 mM dithiothreitol (65°C , 10 min), centrifuged ($10,000 \times g$, 5 min), and loaded at a concentration of 1 $\mu\text{g Chl}$ on a 12% SDS-PAGE gel. Proteins were transferred to a PVDF membrane using a Bio-Rad semi-dry electrophoretic transfer apparatus. Commercially available (Agrisera, Sweden) rabbit antibodies targeting SbtA were used as primary

antibodies, anti-rabbit HRP-conjugated goat antibody was used as secondary antibody according to manufacturer's specifications, and 5% BSA was used as a blocking agent. Visualization of secondary antibody was performed using the chromogenic substrate 4-chloro-1-naphthol (4CN, Bio-Rad) and H₂O₂ as developing agents.

3. Results

3.1 Engineered strains differ in basic cell characteristics.

In order to understand the effects of altered carbon metabolism on photosynthetic efficiency, we first analyzed Chl content and cell density in each strain under our experimental conditions. The aim was to establish a baseline to better normalize physiological results. Often, results are compared on the basis of Chl concentration or sample turbidity. However, since the latter is sensitive to cell state, especially cell size, collection of additional information may be critical in evaluating differences in phenotype.

Figure 2. Changes in cell culture characteristics as a function of strain and inorganic carbon availability. Grey: WT, Red: $\Delta glgC$, Green: JU547. Error bars are standard deviations of three biological replicates. Panel A: Number of cells per OD₇₅₀ = 1. Panel B: Number of molecules per cell, as calculated from Chl determination and cell density counts.

Although culture turbidity is a convenient experimental parameter, we noticed consistent patterns of altered Chl concentrations per unit turbidity amongst the different strains. We next counted the number of cells per unit culture volume and found that the number of cells per unit OD₇₅₀ was different in the genetically modified strains (Fig. 2A). Moreover, cell counts became higher per OD₇₅₀ for all strains as C_i became more limiting. All of these observations suggest increased scattering per cell, which could reflect differences in cell size. Using differential interference contrast microscopy, it was found that

both engineered strains had a radius approximately 80% that of wild-type, and cell size additionally decreased for all strains after switching to low C_i conditions (see Supplemental Fig. 2). From these data, the number of Chl molecules per cell was calculated showing that both genetically modified strains have fewer Chl molecules per cell (Fig. 2B). In addition, as C_i becomes more limiting, transformant and WT strains have fewer Chl molecules per cell, and again, the cells themselves appear smaller. Although a detailed study of growth was not performed, growth rates of both genetically modified strains were lower in our hands (Supplemental Fig. 3), and an apparent trend worth further study is that faster growing cells were generally larger. Subsequent physiological assays were performed at similar Chl concentrations to simply minimize potential optical differences affecting actinic and measuring lights and, hereafter, cell parameters are normalized and presented on a per microgram of Chl basis. However, these results generally emphasize that interpreting comparative analyses of metabolic activities, such as electron transport, needs to be tempered by findings such as altered Chl per cell in engineered strains.

3.2 Carbon sink availability affects oxygen evolution and respiration.

Measurements of bicarbonate-dependent oxygen evolution and respiratory oxygen uptake in the dark were used to probe whole chain electron transport coupled to metabolism. Fig. 3A shows that the WT and JU547 strains have comparable rates of oxygen evolution and consumption during high carbon growth, normalized to Chl concentration. The $\Delta glgC$ strain has a pronounced impairment in overall oxygen evolution, similar to that seen in a starch synthesis mutant of *Chlamydomonas reinhardtii* [25]. The $\Delta glgC$ strain also responded similarly: the artificial electron acceptors DCBQ and PNDA completely alleviated the impairment of electron transport and oxygen evolution (not shown). Moreover, the relative amount of PSII in $\Delta glgC$ based upon total variable fluorescence due to a single turnover actinic flash is similar to the WT and the decay of fluorescence corresponding to electron transfer from Q_A to Q_B in dark adapted samples was not discernibly affected (not shown). All this points to the conclusion that a normal complement of PSII accumulates in the $\Delta glgC$ strain and that the impairment of oxygen evolution is due to alterations in downstream sink processes. The rates of oxygen evolution in the JU547 were slightly depressed in high carbon conditions as well. The rates of oxygen evolution per Chl increased in both transformants after the transition to low C_i conditions, however, $\Delta glgC$ was still lower than the other strains. Rates of respiration, as measured by oxygen consumption in the dark, showed that under high carbon conditions, all strains behaved similarly. On the other hand, the JU547 transformant had increased rate of respiration under low C_i conditions, perhaps due to extra succinate availability [20, 26].

Figure 3. Steady-state oxygen evolution and respiration measured in a Clark-type electrode. Grey: WT, Red: $\Delta glgC$, Green: JU547. Error bars are standard deviations of three biological replicates. Panel A: Bicarbonate dependent oxygen evolution under saturating light conditions ($\sim 10000 \mu\text{E m}^{-2} \text{s}^{-1}$) measured for 90 seconds. Panel B: Rates of oxygen consumption in 90 seconds of darkness following light exposure.

It has been reported that another glycogen metabolism mutant, $\Delta glgP$, exhibits a delay in CBB cycle activation, due to a low internal concentration of CBB metabolites necessary to obtain peak rates of the metabolically autocatalytic CBB cycle activity [27]. In order to test the hypothesis that the $\Delta glgC$ strain also exhibits a delay in CBB activation, oxygen evolution in the $\Delta glgC$ strain was monitored over 10 minutes (Fig. 4). High carbon grown cultures, in the presence of 5 mM bicarbonate and $180 \mu\text{E m}^{-2} \text{s}^{-1}$ of light, had a slow initial rate of oxygen evolution. This rate remained low for just over 5 minutes when rates of oxygen evolution increased to over 3 times their initial levels. When performed in the presence of 5 mM glucose, it is seen that initial rates of oxygen evolution are comparable to the later and fully activated rates seen in the glucose absent samples. Interestingly, low C_i treated $\Delta glgC$ samples do not display this trend. Rates of oxygen evolution begin high and gradually decrease, possibly due to the sensitivity of $\Delta glgC$ to photoinhibition.

Addition of glucose to these samples resulted in an overall lowering of oxygen evolution rate, but not a change in trend, similar to WT (not shown). The lack of delay in low C_i acclimated cultures may explain the slight increase in respiration in $\Delta glgC$ under these conditions (Fig. 3B), as the CBB metabolites are accumulated more quickly than in HC cultures during light exposure and are available following cessation of illumination. Overall, this points to a different pattern of metabolite distribution under the two C_i regimes with the LC state tending to have a greater catabolic flux including pathways that communicate with the CBB pathway, thereby allowing it to re-fill more even in the absence of carbon skeletons that are normally derived from the catabolism of glycogen.

Figure 4. Delayed induction of the CBB cycle in ΔglgC as assayed by oxygen evolution. Dark adapted cells were exposed to $180\mu\text{E m}^{-2} \text{s}^{-1}$ of light (in contrast to saturating intensities used for experiments in Fig. 3) and oxygen evolution was tracked over 10 minutes. Black: bicarbonate only; Grey: addition of 5 mM glucose. Dashed lines represent mathematical curve fits to either sigmoidal or linear equations. Panel A: High carbon grown cultures. Panel B: Low C_i acclimated cultures. These trends were not observed in either the WT or JU547 (not shown).

Consistent with this interpretation, it was previously observed that the HC $\rightarrow$ LC transition in WT results in increased levels of glycolytic intermediates including 3-PGA and PEP that appears to occur at the expense of hexoses [28-30]. At the same time, even with this alleviation, the rates were still lower than the wild-type and not commensurate with the content of PSII, which is similar to the wild-type. Therefore, additional factors are limiting whole chain net oxygen evolution. This could include, for example increase O_2 consumption via the flavodiiron proteins [31, 32].

3.3 Chlorophyll fluorescence induction shows interactions with metabolism and responses to C_i availability is different among the strains.

In order to assay the reduction state at acceptor side of PSII and downstream electron transport chain, Chl fluorescence was measured (Fig. 5). Fluorescence transients of the WT strain closely resembled those seen in previous experiments [33]. Chl fluorescence maintains a stable steady-state minimum fluorescence in the dark (F_0). Upon activation of an actinic light source, Chl fluorescence quickly (~ 1 second) reaches a local peak fluorescence value (F_p), transitioning through standard OJIP phases (not clearly resolved in this study, but for review see [34, 35]). Following the peak in fluorescence at F_p , fluorescence briefly declines to a brief stationary phase (F_s), before rising to a local maximum F_M . The cause of the F_p to F_s decline is still ambiguous in cyanobacteria, but in higher plants it is linked to proton uptake and acidification of the thylakoid lumen [36]. The F_s to F_M rise has been linked to State 2 $\rightarrow$ State 1 transition in cyanobacteria whose regulation is attributed to the oxidation of the PQ pool upon the

shift from the dark to light regime [37]. The decline from F_M to a terminal, steady-state fluorescence level, F_T , is highly C_i dependent and is largely due to complete activation of the CBB cycle [33].

Figure 5. Chlorophyll fluorescence transients of HC and LC cultures. Black: WT, Red: $\Delta glgC$, Green: JU547. Panels A & B: Chl fluorescence during a five minute exposure to actinic light ($53 \mu E m^{-2} s^{-1}$). Actinic light is turned on (up arrow) at 60 seconds and off (down arrow) at 360 seconds. Multiple turnover flashes are given at 15 and 300 seconds. Panels C & D: Chl fluorescence following one minute of actinic light ($53 \mu E m^{-2} s^{-1}$). Actinic light is turned off at 0 seconds. Assays were performed in modified BG-11 medium (LC7) supplemented with 5 mM $NaHCO_3$. Calculation of saturation parameters (below) use the light saturated, maximal values. Equal concentrations of Chl ($5 \mu g mL^{-1}$) are present and the raw F_0 values in these samples were highly similar.

In $\Delta glgC$ it is observed that the F_S to F_M rise appears to be delayed, indicating that state transitions may occur more slowly in this strain. The most striking difference seen in the $\Delta glgC$ mutant compared to WT is that the decline in fluorescence from F_M (~ 85 s) toward steady state F_T (~ 360 s) is replaced by a continued rise in fluorescence, especially in HC cells (Fig. 5 A & B). This suggests that the blockage of downstream electron sinks in carbon skeletons prevents the oxidation of PSII, which is usually taken to

be diagnostic of a highly reduced PQ pool in the electron transport chain. According to this explanation, electrons, unable to be consumed in the CBB cycle instead have a greater residence time in the intersystem electron transport chain impairing the photooxidation of the PQ pool, which impairs the photooxidation of Q_A^- leading to the high fluorescence state of PSII [38]. Alternative explanations such as increased fluorescence due to state transitions seem unlikely since the drop in fluorescence yield after termination of illumination remains relatively rapid, and not the tens of seconds associated with state transitions. Moreover, the high fluorescent state under active illumination is not due to C_i^- limitation since bicarbonate is present in excess [39, 40]. Thus, it would appear that under steady state conditions in actinic illumination at growth light intensities, the PQ pool is abnormally reduced in $\Delta glgC$. Furthermore, the fact that an artificial electron acceptor, DCBQ, can completely alleviate the low rates of O_2 evolution (not shown), indicates that an acceptor-side limitation is the cause. However, as shown below, the characteristics of P700 reduction kinetics, suggest that a highly reduced PQ pool may actually not exist in $\Delta glgC$, and therefore another explanation must be considered.

3.4 Post-illumination interactions between metabolism and the PQ pool.

Post-illumination kinetics of the three strains were further analyzed after short-term illumination (1 minute, Fig. 5, Panels C & D). The rise in Chl fluorescence 3-7 seconds after actinic illumination, F_N , has been associated with a return of electrons to the PQ pool via NDH-1 complex activity [33, 41]. In addition, a secondary rise, after approximately 30 seconds, F_R , is putatively associated with oxidation of sugars and CBB metabolism [42]. Under the assay conditions here, these post-illumination transients were present, but not very prominent, in the WT and JU547 strains (Figs. 5 C & D), suggesting efficient transfer of reductant to the CBB cycle and towards anabolic metabolism. Nevertheless, these peaks were easily observable in the $\Delta glgC$ mutant, consistent with a higher amount of photosynthetic reductant accumulated, probably as NADPH and/or reduced Fd. This accumulated reductant rapidly re-reduces the PQ pool ($t_{1/2} \sim 5$ sec) after the actinic illumination is terminated probably through NDH-1. In both HC and LC cells, the early NDH-associated peak, F_N , is present. However, under high carbon conditions, the 30 sec F_R rise is absent. Following acclimation to low C_i conditions the 30 sec post-illumination rise returns. This is consistent with the CBB cycle not fully activated for HC cells under these short illumination periods (1 minute). Switching to low C_i culturing eliminates the CBB delay and the F_R rise is observed consistent with the earlier assignment of the 30 sec post-illumination rise as due to the return of reductant via reduced carbon skeletons formed during the actinic period by the CBB [33]. In addition, the intensity of the F_R rise is more pronounced under longer illuminations, and the F_N rise is

diminished (Fig. 5B), whereas F_N is still observed under shorter illumination (Fig. 5D). Although not fully explored here, it seems likely that excess electrons are shunted through NDH complexes (giving the F_N peak) before the CBB cycle becomes fully activated. In this scenario, the more active CBB cycle in the LC cells (Fig. 4B) enables the flow of electrons into the form of CBB intermediates and trioses that can later feed reductant back into the PQ pool giving rise to the later F_R post-illumination peak. This corresponds to indirect CEF [43]. The fact that F_R is so pronounced in LC $\Delta glgC$ suggests that the absence of glycogen synthesis leads to the accumulation of higher levels of sugar that are not readily assimilated into anabolic metabolism and thus provide increased reductant to the membrane system through respiratory pathways. It is also worth noting that induction of the LC physiological state results in shifts in metabolism towards a more 'catabolic configuration' that leads to higher triose levels due to enhanced oxidative pentose phosphate pathway reactions [28-30], as already noted above as an explanation for the better filling of the CBB cycle intermediates in the dark providing for a lag-free induction of oxygen evolution in dark-adapted $\Delta glgC$ cells, in contrast to the HC condition.

3.5 Inorganic carbon availability affects LEF and CEF around PSI differently in engineered strains.

Cyclic electron flow functions to balance the stoichiometric requirements of ATP and NADPH [15]. Since these processes return electrons to the PQ pool, changes in PSI activity in the presence and absence of LEF can be used to assay CEF. PSI activity was analysed by measuring absorbance changes at 705 nm (nominally, P700) using a JTS-10 spectrometer (Bio-Logic, France) in the presence of bicarbonate only (LEF + CEF) and with the addition of DCMU to block PSII electron flow (CEF). Again, cells prepared in the HC and LC culture acclimation states are tested, but since excess C_i is present, the differences in P700 redox kinetics reflect differences among the strains and differences in their physiological state (e.g due to gene transcription and allosteric regulation differences) following acclimation to high and low C_i culture conditions.

Figure 6. P700 absorbance kinetics during 5 seconds of actinic illumination shows different redox states and dynamics among the different engineered strains. Actinic illumination (2050 μE) is turned on at 0 seconds and terminated at 5 seconds. Black/Grey: WT, Red: ΔglgC , Green: JU547. Panels A & B: Transients obtained in the presence of 5 mM HCO_3^- . Panels C & D: Transients obtained in the presence of 5 mM HCO_3^- and 10 μM DCMU. The data are presented normalized to measured chlorophyll concentration at a Chl concentration of 5 $\mu\text{g mL}^{-1}$.

Upon exposure of the sample to actinic illumination, P700 becomes oxidized, as shown by a decrease in absorbance (Fig. 6). High intensity actinic light exposure causes the oxidation of P700 to reach the maximum oxidation level (compare to DCMU trace). The comparative values of oxidation represented by the total negative deflection upon illumination can give relative contents of PSI per Chl, as samples were adjusted to equal Chl concentrations. As can be seen in the presence and absence of DCMU, both ΔglgC and Ju547 appear to have comparable concentrations of PSI relative to WT on a per Chl basis. In both transformant strains, rates of oxidation occur faster than in the WT under HC conditions (Fig. 7, top). This may reflect some differences in concentration of reduced PSI donor (plastocyanin and/or cytochrome c_{553}) in the PET chain or altered acceptor availability for PSI (i.e. Fd^+ , NADP^+). It is also seen that switching to low inorganic carbon availability does not appreciably affect the rate of oxidation.

Figure 7. Kinetic analysis shows alterations in photosynthetic cyclic electron transport is different in the different strains. Rate constants for P700⁺ oxidation and re-reduction due to 5 second pulse of saturating illumination in the absence (top) and presence (bottom) of PSII inhibitor, DCMU. Analysis of the kinetic profiles shown is from several experiments similar to that shown in Figure 6. Rate constants of oxidation and re-reduction are plotted for engineered and control strains Black/Grey: WT, Red: $\Delta glgC$, Green: JU547. Error bars are standard deviations of three biological replicates. Panel A: Transients obtained in the presence of 5 mM HCO₃⁻. Panel B: Transients obtained in the presence of 5 mM HCO₃⁻ and 10 μ M DCMU.

Following oxidation of P700, a transient re-reduction of the resultant P700⁺ (Fig. 6, top) occurs. Before this re-reduction, a brief delay is observed, where P700 remains maximally oxidized. Under HC conditions, this delay lasts ~180-200 ms in the WT strain. The delay is shortest in the JU547 transformant (<100 ms), and in $\Delta glgC$ the re-reduction is diminished and greatly delayed (~500 ms) (Fig. 7A, Fig. 8). It appears that the pool of reductant responsible for the transient re-reduction is greatly diminished in $\Delta glgC$ under HC conditions (see below regarding acceptor side differences). This loss of the re-reduction transient is highly reminiscent of similar alterations in P700 kinetics upon deletion of succinate dehydrogenase [44]. Interestingly, the diminished re-reduction transient observed in HC $\Delta glgC$ cells is partially restored by acclimation to low C_i conditions (Fig. 7B, Fig. 8). Note that the differences are not due to the absence of C_i in the assay, since both HC and LC samples have excess bicarbonate added and therefore, the differences must reflect changes in metabolism/enzyme activity

induced by the acclimation to low C_i . While the measurements do not take into account removal of electrons from the intersystem chain through respiratory oxidases, those rates are quite similar among the strains based on oxygen uptake measurements (Fig. 3B) except for JU547, which had a somewhat higher rate of respiration in LC conditions. Therefore, the characteristics of the re-reduction transient suggest two alternative, but not mutually exclusive, possibilities: 1.) the transient re-reduction of $P700^+$ is due to the reductant accumulated in the dark and exhausted by actinic illumination on the time scales used, or 2.) the transient re-reduction feature is due to changes in availability of PSI acceptors, where an oxidized pool of Fd and/or $NADP^+$ rapidly fills with electrons. This creates a transient inability to oxidize $P700^+$ at rates that match the influx of electrons from PSII. The pool of acceptors is then re-oxidized as the 'absorptive flux capacity' of secondary and tertiary acceptors is activated, causing a second phase of oxidation of $P700^+$ following the transient. As shown below, the NADPH pool in $\Delta glgC$ is more reduced, which should enhance the re-reduction transient, not diminish it as observed. This combined with the fact that it is restored in the LC state associated with increased catabolism and the similarity to the succinate dehydrogenase phenotype support the transient as being strongly dependent upon the flow of electrons into the thylakoid electron transport chain from cytoplasmic catabolic activity and that $\Delta glgC$ has a diminished flow of reductant via this path into the PQ pool.

In order to analyse CEF in isolation from PSII-dependent linear electron flow, actinic illumination was provided in the presence of DCMU to block electron donation from PSII. The $\Delta glgC$ mutant exhibits slower re-reduction of $P700^+$ that we attributed to CEF, which again, appears in conflict with the presence of a more reduced PQ pool during actinic illumination (Fig. 5). In the JU547, the increased rates of CEF may be explained through the activity of EFE genetic modification that yields ethylene. In addition to ethylene, succinate is a by-product of EFE. With additional succinate being created, succinate dehydrogenase is able to reduce available PQ, leading to indirect CEF to the PQ pool. Upon transition into LC conditions, the rate of $P700^+$ re-reduction in the WT strain remains unchanged, (Fig. 6D, Fig. 7). For the transformants however, this rate is altered. The LC cells of the $\Delta glgC$ mutant exhibit a large increase in the rate of re-reduction, probably owing to induction of the high affinity carbon uptake mechanism (see below), the alleviation of CBB cycle activation, and the inferred increase in the flux of reductant towards catabolic pathways [28-30]. This low C_i -induced flux appears to direct metabolism towards inputs into the electron transport chain such as SDH [45] and FNR and thus may not be related to CEF per se. The LC cells of JU547 exhibit a slight impairment of $P700^+$ re-reduction however, indicating that CEF may be down-regulated in favor of linear electron flow in these conditions.

3.6 Glucose feeding confirms a kinetically efficient supply of reductant to the electron transport chain from the cytoplasm.

To further evaluate the conclusion that the slowed rate of P700⁺ reduction in *ΔglgC* is due to a diminished flow of catabolic reductant into the intersystem electron transport chain, glucose was added to samples of high carbon grown *ΔglgC* and WT and allowed to incubate 5 minutes in darkness before starting the measuring traces (Figure 8A). Glucose reduces the PQ pool and thus should hasten the re-reduction of P700⁺. The addition of glucose had two prominent effects. First, the delay in the re-reduction transient is eliminated. In fact, the initial deflection of P700 oxidation does not appear to fully oxidize P700 (compare Figure 8A at time 0 seconds versus 2 seconds), implying that glucose is continuously supplying electrons to the electron transport chain, thus slowing the rate of P700 oxidation. Since the main route of catabolic reductant via glucose addition into the intersystem electron transport chain is succinate dehydrogenase and this enzyme reduces PQ, then it appears that the PQ pool in *ΔglgC* is more oxidized, not more reduced as suggested by the results of Chl fluorescence measurements (Fig. 5).

Figure 8. P700 absorbance kinetics of WT and *ΔglgC* strains grown under high carbon availability. Samples were incubated 5 minutes in darkness in the absence (solid lines) and presence (dashed lines) of 5 mM glucose before applying a 5 second saturating ($2050 \mu\text{E m}^{-2} \text{s}^{-1}$) actinic illumination. Black: WT; Red: *ΔglgC*. Panel A: Initial oxidation and transient re-reduction of P700 in samples upon actinic illumination. Actinic illumination was turned on at 0 seconds. Panel B: Re-reduction of P700 in samples upon termination of actinic light, with the addition of 10 μM DCMU. Actinic illumination was terminated at 5 seconds. The data are presented in uncorrected form with all samples at a Chl concentration of $5 \mu\text{g mL}^{-1}$.

3.7 Electron flow during steady-state photosynthesis.

To assay the electron flow to PSI during active photosynthesis, cells were illuminated at near growth light intensities ($45 \mu\text{E m}^{-2} \text{s}^{-1}$) for 10 minutes after which a 200 ms saturating pulse was delivered to completely oxidize P700 and illumination was terminated (Supplemental Figure S1). Rates of re-reduction after termination of illumination also give insight into the reduction state of the intersystem electron transport chain during steady state photosynthesis. In the illuminated HC cell samples, P700⁺ re-reduction occurs much more slowly in the $\Delta glgC$ transformant, with a rate of approximately 40 turnovers per second compared to 70 and 80 in the WT and JU547 strains (Figure 9A). These results are similar to the patterns of re-reduction observed during the 5 second illumination, albeit faster due to the build-up of reductant under illumination. The faster rates in all strains under these conditions presumably reflect the higher concentrations of accumulated reductant in this steady state assay. As before, the re-reduction of P700⁺ in $\Delta glgC$ in the HC state is comparatively slower than the WT (Fig. 9A, red bars). Acclimation to ambient air gassing (LC cells) increases the re-reduction rate in $\Delta glgC$ almost to that of WT values. The JU547 transformant also shows a slight increase in re-reduction rate as well, indicating that electron flux through the electron transport chain may be increased. Thus, again it is observed that the physiological state elicited during the acclimation to low C_i conditions restores a more WT-like PSI re-reduction flux in the $\Delta glgC$.

Figure 9. Steady-state actinic illumination electron transfer characteristics of photosystems under different carbon availability regimes. Grey: WT, Red: $\Delta glgC$, Green: JU547. Error bars are standard deviations of three biological replicates. Panel A: Reaction rates of P700⁺ re-reduction after a saturating pulse given during continuous $50 \mu\text{E m}^{-2} \text{s}^{-1}$ actinic light. Panel B: Quantum yield of photosystem I, as measured by $\text{P700}_{\text{red}}/\text{P700}_{\text{tot}}$. Panel C: Effective quantum yield of photosystem II, as measured by $(F_{m'} - F)/F_{m'}$, obtained from Chl fluorescence (Figure 5).

The observed quantum yield of PSI ($P700_{red}/P700_{tot}$), and relative PSII quantum yield ($(F_m' - F_s)/F_m'$) during steady state actinic illumination were obtained using saturating pulse techniques tracking P700 absorbance and Chl fluorescence (Fig. 9B & 9C). Overall, the quantum yields for JU547 are similar to the wild-type, whereas they are markedly lower in $\Delta glgC$. Greater than 90% of PSI are in a reduced condition (open) during steady-state photosynthesis in the WT and JU547 strains, whereas $\Delta glgC$ has only approximately 80% of PSI centers reduced under these relatively low actinic light conditions ($\sim 50 \mu E m^{-2} s^{-1}$) consistent with a more oxidized PSI donor pool. The relative quantum yield of PSII is evaluated on the basis of the remaining fraction of PSII centers in the sample that are not in a high fluorescent state (Q_A^-), but can be converted to that state by giving a saturating, high intensity flash. On this basis, JU547 has relatively open reaction centers, whereas $\Delta glgC$ has a considerably higher fraction of closed PSII centers. The latter is typically interpreted to indicate that the PQ pool is more reduced and is not keeping pace with the rate of electron flow from water to Q_A . However, the PQ pool appears relatively oxidized, not more reduced in $\Delta glgC$ based on the $P700^+$ re-reduction characteristics. This apparent discrepancy is further underlined upon consideration of photochemical quenching, q_p , which is calculated as $([F_m' - F_s]/F_m' - F_0)$. When q_p is calculated using the corresponding values from the same experiment, HC cells of the wild-type and JU547 each display values of ~ 0.8 , whereas $\Delta glgC$ is ~ 0.5 , which is normally taken to indicate a more reduced PQ pool. It is important to note that the PSII quantum yields obtained using this formula underestimate the actual quantum yield because it assumes well-coupled light-harvesting antennae, which is not the case in cyanobacteria [46]. Therefore, rapid adjustments in antenna coupling cannot be excluded as the basis for relatively higher fluorescence yields during steady state fluorescence in $\Delta glgC$ that render an appearance of lower levels of photochemical quenching. However, the fact that PSII electron acceptors can quench this higher fluorescence supports the conclusion that acceptor side limitation is indeed the cause of higher PSII fluorescence, despite strong evidence that the PQ pool is oxidized.

3.8 NADPH fluorescence shows a highly reduced NADPH pool due to impaired glycogen synthesis.

Analysis of downstream PSI processes was performed by measuring NADPH fluorescence. Previous studies have shown this to be a reliable method for measuring NADPH levels *in vivo* despite potential complications arising from interfering NADH fluorescence [33, 47, 48]. Wild-type NADPH fluorescence displays characteristic peaks and phases analogous to Chl fluorescence (Fig. 10). Samples acclimated to darkness reach a steady-state minimum, N_0 , where a minimum level of NADPH may be generated from the oxidative pentose phosphate pathway and other processes. Upon exposure to actinic illumination,

fluorescence increases in a monophasic rise to a local peak, N_p . NADPH fluorescence also displays analogous peaks and phases at the $P \rightarrow S \rightarrow M$ transitions seen in Chl fluorescence. However, very little decline is seen during the $P \rightarrow S$ transition. After approximately 30 seconds after the start of actinic illumination, NADPH fluorescence reaches a local maximum, N_M , and begins to decline. This decline has been associated with activation of the CBB cycle [33].

Figure 10. NADPH fluorescence kinetics during actinic illumination. Actinic illumination ($53 \mu E m^{-2} s^{-1}$) was activated at 60 seconds and terminated at 360 seconds. Black: WT, Red: $\Delta glgC$, Green: JU547. Panels A & C: High carbon grown cultures. Panels B & D: Low carbon grown cultures. Panels C & D: Detail of post-illumination NADPH oxidation. Numerical values represent average reaction rate constants, k (turnovers s^{-1}), as determined from curve fits (blue dashed lines) of three biological replicates.

The amplitude and kinetic features of light induced NADPH fluorescence of the JU547 strain resembled the WT, although under HC conditions the amplitude in JU547 was routinely lower consistent with the enhanced flow of reductant towards the production of ethylene. This difference was mostly lost for LC

JU547 cells, for reasons that are not clear, although presumably related to previously observed increases in catabolic metabolic pathways that occur upon adaptation of low C_i conditions [28-30]. In contrast, $\Delta glgC$ consistently displayed fluorescence values over two times greater than that of WT. Fluorescence continued to rise at a slow, constant rate, until delayed activation of the CBB cycle. After this point, fluorescence fell, but only to initial N_p values, not towards WT or JU547 values. Therefore, under steady-state photosynthesis, $\Delta glgC$ likely contains a larger and/or highly reduced NADPH pool.

Following the termination of actinic illumination, NADPH fluorescence quickly declines. A large dark reduction event after approximately 30 seconds of darkness is seen and may be associated with oxidation of triose and pentose sugars [33] and approximately coincides with the 30 second post-illumination peak in Chl fluorescence assigned to the transfer of that reductant to the PQ pool [33]. For the first 7 seconds after actinic illumination, NADPH fluorescence declines monoexponentially, as seen in Fig.s 9C & 9D. After this time, the rise culminating in the 30 second N_R peak begins to be seen. Decay curves were fitted using exponential decay models, and decay constants and half times were found. In each strain, the decay in NADPH fluorescence occurs more quickly in cultures switched to low carbon gassing, likely due to induction of the CCM and its NADPH consuming complexes. The difference is most prominent in the $\Delta glgC$ transformant, where decay rates were over twice as fast when cultures were switched to air gassing.

3.9 Transition to low carbon availability induces the CCM of transformant strains.

To assess whether proteins of the high affinity CCM were present and inducible in the transformant strains under these experimental conditions, immunoblot assays were performed using commercially available anti-SbtA antibody (Agrisera, Sweden), which is diagnostic of the high affinity CCM response. Cells in the HC and LC physiological states were obtained from the same cultures used for the above spectroscopic analyses and carefully loaded to contain equal amounts of Chl. Because 2OG serves as a signaling cofactor for the repression of CCM-related genes [49], these transformants with altered metabolism around 2OG may display mis-regulated expression of CCM proteins. The Sbt operon is under transcriptional control of the DNA-binding CcmR protein, whose mechanism uses 2OG as a co-repressor [49]. In WT cells, no detectable level of SbtA is observed in high carbon grown cultures, however, after switched to low carbon gassing, SbtA can be detected (Figure 10A). Similarly, transformant strains also show no detectable levels of SbtA when cultured under high carbon conditions. Low carbon culturing shows expression of SbtA in both transformants, with the JU547 transformant having a higher concentration of SbtA relative to WT. Since CcmR also regulates expression

of the *ndhF3/D3/cupA* operon [50], it is likely that these genes follow similar patterns as well, but further analysis was not performed. Overall, it appears that the diversion of photosynthate in JU457 may result in increased demand for C_i , as evidenced by the enhanced expression in LC JU457, yet the $\Delta glgC$ shows no differences in expression relative to the WT as hypothesized. Instead, it appears that the $\Delta glgC$ transformant retains near WT-like patterns of CCM gene expression with respect to changes in C_i conditions. As discussed earlier (see section 3.1) these results are expressed on a per Chl basis. However, since it was observed that the different cultures exhibit differences in cell number per OD_{750} and chlorophyll concentrations per cell (Fig. 1), a more thorough protein analysis may be warranted in future work. In addition, experiments assaying the whole cell affinity characteristics of each of the strains in the HC and LC state were performed and indicated that wild-type and JU457 exhibited the expected increases in affinity (Supplemental Fig. 4), whereas the evaluation of $\Delta glgC$ was more problematic due to the low activities of oxygen evolution in this strain.

Figure 11. Immunoblots from samples of wild-type and transformant strains under different inorganic carbon regimes. Samples containing 1 μ g Chl were loaded on a 12% SDS-PAGE gel and transferred to a PVDF membrane. Panel A: Immunoblot probed with α -SbtA as a primary antibody showing induction of the CCM-related *sbtA* gene.

4. Discussion

The aim of this work was to analyze how biotechnologically significant alterations in cyanobacterial carbon metabolism affect photosynthetic performance. The photosynthetic characteristics and CCM properties were examined in the ethylene-producing strain (JU457), which partitions at least 10% of their fixed carbon toward the release of ethylene [20, 26]. It has been engineered to express the

ethylene forming enzyme (EFE) from the organism *Pseudomonas syringae* [18, 20]. The ethylene-forming enzyme uses 2OG as a substrate for the synthesis of ethylene, a volatile compound, which escapes the medium as a gas. The JU547 strain therefore has an additional, engineered carbon and electron sink available. As an experimental contrast, the corresponding analysis was also performed on the well-studied $\Delta glgC$, which is deficient in glycogen biosynthesis and thus no longer has a key sink for photosynthate [51]. The $\Delta glgC$ transformant was originally constructed to explore ways to enhance product yields by eliminating a potentially competing sink pathway. However, the $\Delta glgC$ strain also proved interesting since it excretes organic acids, including 2OG, upon nitrogen starvation [51, 52]. This latter observation is relevant to C_i nutrition since 2OG plays a role in the regulation of the genes of the high affinity CCM [53].

4.1 Predicted and unpredicted alterations in photosynthetic performance.

The motivating hypothesis in this study was that strains with increased sink capacity, exemplified by the JU547, will exhibit increased demand for C_i and correlated effects on the characteristics of the photosynthetic mechanism such as enhanced electron transport capacity. Conversely, strains such as $\Delta glgC$, will exhibit opposite effects due to decreased sink capacity. Some of the predictions were supported and consistent with the previously identified alterations in metabolism. For example, we observed increased rates of respiration and faster re-reduction of oxidized P700 in JU547 presumably due to the closure of the TCA cycle via provision of succinate during the synthesis of ethylene with its consequent ability to reduce the electron transport chain [20, 26]. Succinate serves as an electron donor to the plastoquinone pool through the succinate dehydrogenase complex, which may serve as a major donor of electrons during respiration [19] and accounts for the predictable increase in electron flow to P700⁺. Likewise, the enhanced expression of the high affinity bicarbonate transporter was observed in JU547 is consistent with predictions for increased demand for C_i . On the other hand, the $\Delta glgC$ mutant proved more difficult to predict, with phenotypic characteristics that were strongly dependent upon whether or not the cells were subjected to C_i -limitation. While the hypothesized changes in high affinity CCM genes proved not to occur, there were unexpected features of electron transport that are revealing of the mechanisms by which the cyanobacterial photosynthetic apparatus adjusts to cope with stress.

4.2 Impaired glycogen metabolism has far-reaching effects even in the light and is affected by C_i availability.

Perhaps one of the biggest puzzles is an apparent redox paradox concerning the states of the PQ pool *vis-a-vis* the rate of electron flow through PSI in in $\Delta glgC$. Simply put, the high Chl fluorescence

characteristics (Fig. 5) would normally be interpreted as reflecting a more reduced intersystem electron transport chain and yet all other evidence points to the opposite. The re-reduction of $P700^+$ due to CEF is very slow, but fast rates can be restored by the addition of glucose, which shows 1.) that catabolism of glucose efficiently provides reductant to the intersystem electron transport chain and 2.) the PQ pool in $\Delta glgC$ is actually in a comparatively oxidized condition (Figs. 6, 7 and 8). This is true even for samples under steady state actinic illumination so it is not simply due to the depletion of carbon skeletons in the dark in $\Delta glgC$ (Fig. 9A). Based upon the steady-state electron transport measurements of photosystem relative quantum yields, it appears that PSII is even more 'closed' than PSI and may actually limit electron flow through the electron transport chain, which would yield a more oxidized PQ pool. Furthermore, besides steady-state linear electron flow, cyclic flow to the $P700^+$ is also retarded in the $\Delta glgC$ mutant as evidenced by the very slow rate at which $P700^+$ is re-reduced in the absence of electron input from PSII, which is alleviated by the addition of glucose (Fig. 8B). Moreover, growth under low C_i availability conditions also tends to alleviate this and many of the other observations suggesting that a shift in metabolic regulation towards a more catabolic configuration consistent with previous metabolomics studies of C_i -limitation [28-30].

4.3 Evidence for induction of relatively uncharacterized energy dissipation mechanism in $\Delta glgC$.

All of the $P700^+$ re-reduction kinetic results point toward a more oxidized PQ pool in $\Delta glgC$. And yet, by each measure of Chl fluorescence (Φ_{II} , q_P) or by simple inspection of the fluorescence induction traces (Fig. 5A, B), the fluorescence is higher in a manner usually interpreted to indicate a significantly more reduced condition of the PQ pool. One possible explanation for this physiological paradox may be the operation of the proposed RISE (reduction-induced suppression of electron flow) phenomenon, whereby PSII activity creates an overly reduced PQ pool that is correspondingly deficient in oxidized plastoquinone ([54]). Here it is hypothesized that deficiency in oxidized PQ thwarts the operation of the Q-cycle of cytochrome b_6f activity and results in an oxidized cytochrome c pool, leaving PSI reaction centers more oxidized during steady state photosynthesis. Another possible explanation for high fluorescence, yet slow re-reduction of $P700^+$ is that the $\Delta glgC$ mutation elicits a physiological state where PSII is sequestered in membrane domains away from PSI in a manner that does not permit diffusional communication of electron carriers at some point along the inter-system electron transport chain.

A third possibility for the apparent accumulation of Q_A^- is the operation of an alternative electron dissipation path that is strongly induced in $\Delta glgC$. In this case, the alternative electron path would need

to have an alternative electron acceptor pool capacity (distinct from, but analogous to the PQ pool), that also provides kinetic resistance to impede the oxidation of Q_A^- as this pool fills and is progressively depleted of oxidant. This hypothetical pool acts as an alternative photochemical quencher, much like the PQ pool, since its state also governs the oxidation Q_A^- , however, this is a dissipative pathway and does not contribute to anabolic photosynthetic processes. This could involve the flavodiiron proteins, Flv2/4, that are induced under low C_i conditions and have been shown to protect PSII [55]. Lastly, we cannot yet exclude that the possibility of the high fluorescent state is due to a diversion of excitation energy away from reaction centers. Such a model could involve a rapidly reversible uncoupling of the phycobilisomes to accommodate the reduced electron flux capacity through the electron transport chain and with excitation energy dissipated as fluorescence to account for the higher level of fluorescence yield. Although more work will be required to resolve the nature of this high fluorescence, it is worth noting that the results are reminiscent of the fluorescence properties assigned to an unusual form of cyanobacterial q_N associated with C_i depletion [46].

Despite the uncertainty of the redox poise of the intersystem electron transport chain, the redox state of the NADPH pool *seemingly* behaves as expected for the two engineered strains: addition of a new sink for carbon and reductant in the form of ethylene production in JU547 produces a modest yet reproducible shift to a more oxidized condition of the NADPH pool under steady state illumination. In contrast, closing a sink in the form of glycogen synthesis in $\Delta glgC$ results in a much more highly reduced NADPH pool. This indicates, as expected, that glucose synthesis with its concomitant consumption of NADPH constitutes a major avenue of metabolic reductant consumption in the light. However, this appears to be an oversimplified interpretation of what is happening in $\Delta glgC$ considering the alterations in the PQ pool and CEF. Assuming that NADPH donates electrons to CEF, a highly reduced NADPH pool is expected to produce a more reduced PQ pool and a faster rate of $P700^+$ re-reduction. However, the opposite was observed. The primary donor to the NDH-1 complexes is now believed to be Fd [56], yet there appear to be mechanism(s) allowing the oxidation of NADPH by NDH-1 complexes based on earlier results [57]. Thus, the present results with $\Delta glgC$ indicate that there exists a mechanism down-regulating CEF activity, and together with decreased electron flow from PSII into the PQ pool, results preventing the PQ pool from being over-reduced. In the case of in $\Delta glgC$, this mechanism would appear to be responding to the changed metabolism of sugars that result in impaired start-up of the CBB pathways (Fig. 4), which was previously shown to be the result of depletion of CBB cycle intermediates when glycogen metabolism is impaired [27].

Another aspect of the phenotype of the engineered strains was the effect of C_i availability on the mutant phenotype. For JU547 and the wild-type, the effects were quite modest, yet with $\Delta glgC$, the effects were quite dramatic. For each feature that showed significant impairment ranging from the delay in the start-up of O_2 evolution to the redox state of the PQ pool, growth under low C_i actually produced more 'healthy' physiological phenotype. The lag in oxygen evolution was observed in HC $\Delta glgC$, which by analogy with another glycogen mutant [58], is due to a slow accumulation of CBB intermediates that need to build to sufficient levels to exhibit autocatalysis and strong rates of CBB cycle-dependent oxygen evolution. Strikingly, LC cells did not exhibit this phenomenon suggesting that the physiological state accompanying LC conditions promotes great catabolic flux towards the production of the 'missing' intermediates, which is consistent with metabolomic analyses [28, 59]. The delay in CBB activation in HC $\Delta glgC$ cells also results in NADPH pools as might be expected given that a major sink for photosynthetic electrons has been thwarted due to the sluggishness of the CBB cycle.

In summary, it is clear that the impact of mutations directed at altering metabolic flux can have both expected and unexpected consequences with respect to the operation of the overall photosynthetic mechanism, and particularly, the activities of the electron transport chain. Even basic cell parameters important for performance evaluation, such as cellular Chl content, are sensitive to engineered alterations, although the mechanistic basis for these global cellular responses remains poorly understood. The findings with the already well-studied $\Delta glgC$ mutant were particularly striking, especially regarding its Chl fluorescence characteristics. The results were best interpreted to indicate the operation of a photoprotective mechanism that dissipates excess reductant from PSII. Overall, some initial predictions were verified, such as more oxidized pools of the redox carriers PQ and NADP and an enhanced expression of the high affinity CCM in JU547. However, some observations, notably in the $\Delta glgC$ mutant, could not be easily rationalized in the framework of simple enhanced or thwarted sink model.

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