1 **Overexpression of Calvin cycle enzyme fructose 1,6-bisphosphatase in**
2 ***Chlamydomonas reinhardtii* has a detrimental effect on growth**

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Abstract

Chlamydomonas reinhardtii and other green algae have potential for generating biofuels, pharmaceuticals, and other commercially valuable products. This potential could be improved by increasing rate of growth and biomass accumulation. Overexpression of cyanobacterial fructose-1,6-bisphosphatase (cyanoFBPase-II), an enzyme that catalyzes an irreversible step in the Calvin cycle, improves growth and biomass accumulation in higher plants, so we set out to determine if overexpression of *Chlamydomonas* FBPase (CrFBPase) has the same effect in *C. reinhardtii* by using a chloroplast expression system. To this end, we generated a chloroplast expression vector that includes the *C. reinhardtii* *FBP1* coding region synthesized with *C. reinhardtii* chloroplast codon-bias, flanked with *psbD* and *psbA* 5' and 3' regulatory sequences, respectively, and obtained transformants that integrated the vector properly into the chloroplast genome. *CpFBP1* transformants accumulated increased levels of CrFBPase protein and extracts contained ~1.4-fold-increased levels of FBPase enzyme activity compared to the wild type strain. Surprisingly, we found that under photoautotrophic growth conditions and/or with elevated CO₂ levels, this 40% elevation in FBPase activity had a negative effect overall on growth rate and biomass production. These results suggest that unlike the situation in higher plants, FBPase probably does not catalyze a rate-limiting step in the *C. reinhardtii* Calvin cycle.

Keywords: Calvin cycle, Growth and biomass, Fructose-1,6-bisphosphatase, FBPase, green microalgae, *Chlamydomonas*

1. Introduction

Microalgae hold great promise as a production platform for commercially useful products and as agents for wastewater bioremediation and carbon dioxide sequestration [1–5]. While substantial efforts are underway to engineer algae for specific biotechnology applications, such as production of nutraceuticals and biofuels, less attention has been focused on genetic modifications that promote better utilization of light and/or macronutrients, and therefore improved growth rates. Arguably, such advances would have a very positive impact on microalgal biotechnology.

Most reported investigations into genetic control of microalgal growth rates have utilized the model green alga *Chlamydomonas reinhardtii*, for which several powerful gene analysis and manipulation tools are available, including nuclear, chloroplast, and mitochondrial transformation systems, RNAi knockdown methods, hundreds of genetically characterized mutants, a sequenced genome, multiple transcriptomic analyses, and whole genome sequencing to identify phenotype-causative point mutations [3,4,6–9]. One potentially successful strategy for improving *C. reinhardtii* growth rates involves reduction of light stress through reduction of chlorophyll antennae size, as light-harvesting-complex mutants for the *LHCI* and *LHCII* genes that possess fewer or smaller chlorophyll antennae than wild type strains exhibit increased biomass productivity under controlled high light conditions [10–16], though it is not clear if these mutants grow faster under standard light conditions or in outdoor ponds [3,17]. Another promising

approach is the recently reported natural selection strategy, involving serial dilutions for >1,000 generations of *C. reinhardtii* populations growing under mixotrophic conditions, that produced a strain with a 35% increase in growth rate compared to the parent strain [18]. Interestingly, under photoautotrophic conditions, this fast-growing mutant grew at nearly the same rate as the starting strain, suggesting that it had become better adapted for acetate utilization than the parent strain but not for CO₂ utilization or photosynthesis.

For low-value biotechnology products such as fishery food, animal feed supplements and biofuels, microalgae are ideally cultured photoautotrophically so modifications that increase growth rate under these conditions will be especially useful. To this end, another potential strategy for improving algal growth and biomass accumulation is to modify the Calvin-Benson cycle (Calvin cycle), the light independent phase of photosynthesis during which CO₂ is fixed and glucose precursors are produced. The Calvin cycle can be divided into three stages—carbon fixation, reduction and regeneration—and includes twelve enzymatic reactions that consume nine molecules of ATP and six molecules of NADPH to produce one molecule of the glucose precursor G3P. Although several Calvin cycle enzymes are generally present at high levels, others are not. For instance, regeneration phase enzymes fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) have been reported to accumulate to relatively low levels in plants compared to other Calvin cycle enzymes [19,20] and mutants with reduced levels of either/both enzymes had reduced photosynthetic capacity [21–24]. Interestingly, overexpression of a bifunctional cyanobacterial FBPase/SBPase or cyanobacterial FBPase-II or *C. reinhardtii* SBPase in higher plants such as tobacco, lettuce and rice led to improved photosynthetic rate and rates of growth and biomass

production [20,25–27]. Transgenic tobacco with 2.3-fold higher FBPase activity and with 1.6- and 4.3-fold increased SBPase activity showed 1.3-, 1.5- and 1.5-fold increases of dry mass, respectively, compared with wild type controls. Similarly, overexpression of cyanobacterial FBPase/SBPase in transplastomic lettuce plants increased photosynthetic capacity and productivity by 1.3 fold and 1.6 fold, respectively [25,27]. Based on these reports, FBPase and SBPase seem to be promising candidates as targets for improving carbon flow, growth and biomass production in algae.

In this study we sought to first test the hypothesis that FBPase enzyme activity is limiting for *C. reinhardtii* growth, by expressing FBPase directly in the *C. reinhardtii* chloroplast. In *C. reinhardtii*, expression of transgenes from the chloroplast genome has several advantages compared to expression from the nuclear genome. One advantage is that genes integrate into the chloroplast genome by homologous recombination so it is possible to direct transgene integration into a single defined site, so that no host genes are disabled and all transformants can be genetically identical [28]. In addition, while nuclear transgenes are often subject to silencing effects, chloroplast transgenes are not and remain stably expressed indefinitely. Also the *C. reinhardtii* chloroplast genome is present in many (~75-100) copies per cell so it is often possible to obtain significantly higher protein expression from chloroplast transgenes [6,29,30]. In the case of *C. reinhardtii* FBPase (CrFBPase), which normally is synthesized in the cytoplasm and translocates and functions in the chloroplast, direct expression in the chloroplast should provide higher levels of stably-expressed, chloroplast-localized enzyme than expression from the nuclear genome.

Here we generated a new *C. reinhardtii* chloroplast expression vector that is based on but more versatile for cloning than chloroplast vectors previously reported by others [31]. We cloned a gene cassette with coding sequence for *C. reinhardtii* *FBP1* (the nuclear gene that encodes CrFBPase) into this vector and obtained transformants that contain the transgene properly integrated into the chloroplast genome. The CrFBPase transformants produced total CrFBPase protein at levels ~4-fold higher than the wild type strain (CC125) with total FBPase enzyme activity elevated ~1.4 fold. While we expected these transgenic lines to grow faster and to accumulate biomass more quickly than the wild type strain, they did not, suggesting that FBPase function is probably not rate limiting for growth in *C. reinhardtii*.

2. Materials and Methods

2.1. *Chlamydomonas* growth conditions for molecular and enzymatic activity analyses

Chlamydomonas reinhardtii wild type (WT) strain CC125 was purchased from the Chlamydomonas Resource Center, University of Minnesota. For DNA, RNA and protein analyses, *Chlamydomonas* cultures (WT and transformants) were grown under mixotrophic conditions with Tris Acetate Phosphate (TAP) medium, pH 7.0, at 24°C with continuous light (~126 microeinstein per second per square meter, $\mu\text{E m}^{-2} \text{s}^{-1}$) in a Percival (model# AR-60L, Scientific, Inc. Boone, Iowa) growth chamber.

2.2. Generation of chloroplast genome integration vectors *pWD1-CpFBP1* and *pWD2-CpFBP1*

136 To generate the final chloroplast expression vector pWD2-CpFBP1, we first
137 generated vector pWD1-CpFBP1, which was assembled as follows. A 2268-bp DNA
138 fragment was gene synthesized (Genscript) to encode a C-terminally FLAG-tagged
139 version of *C. reinhardtii* *FBP1* (with *C. reinhardtii* chloroplast gene codon bias), flanked
140 by 5' and 3' UTR sequences of the *C. reinhardtii* *psbD* (nt: 175858-176140) and *psbA*
141 (nt: 48481-48854) genes, respectively, with this chimeric *FBP1* gene itself flanked by
142 chloroplast genome sequences (250 bp each; corresponding to bp coordinates 46992-
143 47243 and 47349-47599, respectively, of the *C. reinhardtii* chloroplast genome) that
144 target integration of chimeric *FBP1* to an intergenic region downstream of the 3' UTR of
145 *psbA* and upstream of the neighboring 5S rRNA gene. The gene-synthesized *FBP1*
146 coding sequence excluded codons for aa 2-57, which the ChloroP 1.1 server [32] predicts
147 to be FBPase transit peptide sequence and are not necessary for protein expressed directly
148 in the chloroplast. The 2268-bp synthesized fragment was excised from its parent vector
149 (pUC57) with HindIII and SacI and subcloned into pBlueScript (KS⁺) at its HindIII and
150 SacI sites to generate plasmid pKS-CpFBP1. An *aadA* cassette that confers
151 spectinomycin resistance (Spec^R) was excised from plasmid p423 (Chlamydomonas
152 Resource Center) using restriction enzymes EcoRI and SpeI and subcloned into the
153 EcoRI and SpeI sites of plasmid pKS-CpFBP1 to generate vector pWD1-cpFBP1. The
154 final construct was sequenced to confirm that no unintended mutations had been
155 introduced during the synthesis or cloning steps, using primers indicated in Table 1.
156 After analysis of transformants revealed that pWD1-CpFBP1 did not permit homologous
157 recombination by double crossover into the chloroplast genome, we enlarged its 5' and 3'
158 chloroplast genome homology regions as follows. To enlarge the 3' homology region, we

used primers 3Hnew-for and 3Hnew-rev (Table 1) to PCR amplify an ~1.1-kb 3'-homology fragment using p322 (a vector containing the intergenic region between psbA-exon 5 and the 5S RNA gene, from the Chlamydomonas Resource Center) as template, and that also contains 20 bp at its proximal end that overlap SpeI/SacI digested pWD1-CpFBP1 at its SacI end. Then, 50-100 ng of digested vector and 250-500 ng of PCR amplified fragment were mixed with Gibson assembly master mix (Catalog # E2611S, New England BioLabs® Inc., USA), and the mixture was incubated at 50°C for 30 min as described in the Gibson assembly® protocol (E5510), New England BioLabs® Inc., and transformed into *E. coli*. A few clones determined by diagnostic restriction digests to have incorporated the larger 3' homology region were sequenced using primer 3Hnew-seq (Table 1) to confirm that no unintended mutations had been introduced during the cloning steps. One of these clones (pWD1-new3H-CpFBP1) was used to do another Gibson assembly to enlarge the 5' homology region. Primers 5Hnew-for and 5Hnew-rev (Table 1) were used to PCR amplify a region of vector to generate an ~1.3-kb 5'-homology region having 20-bp overlap with each end of SalI-digested pWD1-new3H-CpFBP1. Diagnostic tests of clones obtained from a bacterial transformation of the Gibson assembly mixture revealed that several had the correct-sized insert. The final pWD2-CpFBP1 vector was sequenced over the 5' homology region using primer 5Hnew-seq (Table 1) to confirm that no unintended mutations had been introduced during the cloning steps.

2.3. Chloroplast transformation

Cells of *C. reinhardtii* strain CC125 were prepared for chloroplast transformation as follows. Cells were inoculated from a plate into 50-mL TAP medium in a 125-mL flask and agitated at 150 RPM on a rotary platform shaker at 24°C with continuous light (as described above) to a density of $3\text{--}5 \times 10^6$ cells/mL. The culture was centrifuged at $3,000 \times g$ for 3 min in a Sigma 3K12 centrifuge and the pellet was resuspended in fresh TAP medium to a concentration of 30×10^6 cells/mL. 300 μL of this cell suspension was spread onto a 1.5% agar plate (TAP medium plus spectinomycin at 100 $\mu\text{g/mL}$) and air dried in a laminar flow hood for 10-15 min in preparation for bombardment.

Plasmid DNA to be transformed into CC125 cells was coated onto 0.5 μm diameter gold particle microcarriers (Seashell Technology, LLC., U.S.A.) according to the manufacture's protocol. Briefly, 10 μL of plasmid at 1 $\mu\text{g}/\mu\text{L}$ was mixed with 50 μL of gold particle solution, and then 60 μL of precipitation buffer was added. The suspension was centrifuged at $11,000 \times g$ for 10 sec in an eppendorf microfuge, then the DNA/gold particle pellet was washed with 500 μL 100% EtOH and resuspended with 60 μL of 100% EtOH and loaded in equal aliquots onto 6 macrocarriers (Bio-Rad Laboratories, Inc.) that were previously assembled into macrocarrier holders after washing in isopropanol then drying for 30-45 min in a desiccator.

Biolistic particle bombardment was performed using a helium-driven PDS-100/He system (Bio-Rad Laboratories, Inc.) with 1,350-psi rupture disk, as described in the Bio-Rad user's manual. The bombarded *C. reinhardtii* cells (on agar plates) were incubated in the dark at ambient temperature (26-28°C) for 24 hrs then transferred into the growth chamber (and grown with continuous light at 24°C as described above) then examined for appearance of colonies surviving selection after approximately 10-14 days.

204

205 **2.4. Genomic DNA extraction**

206 Genomic DNA (gDNA) was prepared as described in [33] and
207 <http://www.chlamy.org/methods.html>. Briefly, *C. reinhardtii* cells were inoculated from a
208 plate into 25 mL of TAP medium in a 125-mL flask and the culture was agitated at 150
209 RPM on a rotary platform shaker at 24°C with continuous light (as described above) to a
210 density of $3\text{--}5 \times 10^6$ cells/mL. Cells were pelleted in a 50-mL conical tube by
211 centrifugation at $3,000 \times g$ for 3 min at room temperature and the pellet was resuspended
212 in 0.5 mL of TEN buffer (10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl) by vortexing,
213 transferred into a 1.5-mL microcentrifuge tube, and centrifuged at $11,000 \times g$ for 1 min.
214 The supernatant was discarded and the pellet was resuspended in 150 μL of H_2O on ice.
215 300 μL of SDS-SB buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 100 mM Tris-HCl
216 pH 8.0) was added and the suspension vortexed to mix. The sample was first extracted
217 with 350 μL of saturated phenol: chloroform:isoamyl alcohol (25:24:1, v:v:v) by
218 vortexing for 5 min at room temperature then centrifuging at $11,000 \times g$ for 5 min before
219 transferring the top aqueous phase into a new 1.5-mL microcentrifuge tube. A second
220 extraction was performed by adding 300 μL chloroform:isoamyl alcohol (24:1, v:v) then
221 following the same steps as for the first extraction. gDNA was precipitated by adding 1
222 mL 95% cold EtOH at -20°C for 30 min and pelleted by centrifugation at $11,000 \times g$ for
223 15 min, washed with 70% cold EtOH, dried and resuspended and stored in 50 μL H_2O at
224 -20°C .

225

226 **2.5. Southern blot analysis to analyze integration of transgene DNA in transformants**

Southern blot analysis was performed as described [34]. Briefly, gDNA (5 µg) was digested with EcoRI for 4 hrs and electrophoresed on a 0.8% agarose gel in 250 mL of 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 50 mV for 2 hrs. The gel was then gently shaken with 0.25N HCl for 5 min, followed by 0.5M NaOH/1.5M NaCl for 30-60 min, then finally 0.5M Tris/1.5M NaCl for 30-60 min. The DNA fragments were transferred onto a nylon membrane (GE Water & Process Technologies, USA) overnight and crosslinked to the membrane by ultraviolet crosslinker (Company/brand; Model CL-1000, UVP Upland, CA, USA). The membrane was dried in a 65°C oven for 1 hr then stored with dessication.

The ³²P-radiolabeled probe used in this study was prepared by digesting pWD2-CpFBP1 with NheI and PstI, isolating the resulting ~ 3.0 kb-fragment, and using 50 ng in a DecaLabel DNA labeling kit (Thermo Scientific, USA) reaction according to the manufacturer's instructions. The hybridization was performed with rotation at 42°C for 2 hrs in a hybridization oven with 15 mL hybridization buffer (Rapid-hyb buffer; GE Healthcare Life Science, USA) and 100 µL of the radiolabeled probe mixture. The membrane was washed three times in solution A (15% 20X SSC and 1% SDS) at 65°C for 15 min each and four times in solution B (1.5% 20X SSC and 1% SDS) at 65°C for 15 min each. The blot was exposed to X-ray film (Thermo Fisher Scientific, Inc.) to determine the sizes of hybridizing fragments.

2.6. Western blot analysis

For analysis of exogenous (FLAG-tagged) FB Pase accumulation, cells from 25-mL cultures (~3-5 x 10⁶ cells per mL) were harvested by centrifugation at 3,000 x g for 3

min, then resuspended in 100 μ L SDS sample lysis buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 10% Glycerol, 100 mM DTT) and boiled for 3-5 min. Extracts were loaded onto 12% SDS-PAGE gels, and the proteins were electrophoresed then transferred onto a nitrocellulose membrane (Hybond-ECL, GE healthcare, UK) overnight using a Mini Trans-Blot® Electrophoretic Transfer Cell at 35 Milliamps. The following day, the membrane was blocked by incubating with blocking solution (10% Carnation non-fat milk in 1x PBST [0.01% Tween in PBS]) for 30 min. To detect FLAG-tagged FBPase, the blot was then reacted with primary anti-FLAG-mouse monoclonal antibody (Clontech Laboratories, Inc.) diluted at 1:1,000 in the primary blocking buffer (0.06 g BSA in 1 mL of 1x PBST [0.01% Tween in PBS]), washed 3 times in wash solution (80 g of NaCl, 14.4 g of Na₂HPO₄, 2 g KCl, 2.4 g KH₂PO₄, pH 7.4 resuspended in 1 L ddH₂O), then reacted with the secondary goat anti-mouse IgG peroxidase conjugate diluted 1:30,000 in the blocking solution. To detect the loading control protein (β -tubulin), primary mouse anti- β -tubulin monoclonal antibody (Active Motif) at 1:2,000 dilution was used with the same secondary antibody as was used for anti-FLAG antibody reactions, at 1:30,000 dilution. Blots were washed 6 times in 1x PBST buffer, then developed with Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.) chemiluminescence reagents (according to the manufacturer's directions) before exposing to X-ray film to detect antibody-positive bands.

For blots used to determine total FBPase accumulation in transgenic lines vs the wild type strain, western detection was performed as above except primary anti-*C.reinhardtii* FBPase antisera (raised against the peptide CGLGSTGQERVLDVN by

Genscript) was diluted 1:5,000, and goat anti-rabbit IgG peroxidase conjugate 2° antibody was diluted 1:50,000.

2.7. Colony genomic PCR to detect transgenes in transformants

Colony genomic PCR (gPCR) was performed as described in [35] using a T100™ Thermal Cycler (Bio-Rad Laboratories Inc.) and the following parameters: 98°C for 1 min, followed by 98°C for 10 sec, 30 cycles of 54°C for 20 sec, and 72°C for 10 sec as described in the Phusion High-Fidelity DNA Polymerase protocol (Thermo Fisher Scientific, Inc.). Briefly, first, for each reaction a *C. reinhardtii* colony growing on a TAP agar plate was resuspended in 50 µL of 5% chelex-100® in H₂O, and the mixture vortexed for 10 sec. The mixture was boiled for 10 min, immediately chilled on ice for 1 min, vortexed for 10 sec, and then centrifuged at 11,000 x g for 1 min. The supernatant was transferred into a new microcentrifuge tube and 1 µL of it was used in a 20 µL PCR with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Inc.).

2.8. FBPase Enzyme assay

FBPase activity in wild type and transformant *C. reinhardtii* lines was determined by a colorimetric assay that involves measuring increased absorbance of light (620 nm) by malachite green in the presence of phosphate released from fructose-1,6-bisphosphate by FBPase (described in [22] and Bioassay Systems® 2009). Briefly, cells were harvested by centrifugation at 3,000 x g from 25-mL cultures of CC125 and several WD2-CpFBP1 transgenic lines (for final assays, grown to density of 3-5x10⁶ cells/mL). The harvested cells were disrupted by a freeze-thaw cycle beginning at -80°C overnight or with flash

freezing in liquid nitrogen. Total protein extracts were prepared by resuspending the cell pellets in protein extraction buffer (50 mM Hepes, pH 8.2, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% TritonX-100, 2 mM benzamidine, 2 mM aminocaproic acid, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 mM dithiothreitol (DTT)) and the extracts were desalted by flowing through a NAP-10 column (GE Healthcare, UK) and eluting with desalting buffer (the extract buffer without Triton X-100). The protein extracts were aliquoted and stored at -80°C until use. Enzyme reactions were started by adding variable amounts of extract (5-20 µL but ultimately 15 µL for the final assays, as determined by initial tests) into 80 µL of the assay buffer (50 mM Tris, pH 8.2, 15 mM MgCl₂, 1.5 mM EDTA, 10 mM DTT, 2 mM FBP) and incubated at 25°C for 5-20 min; ultimately 5 min was determined to be the optimal time. Reactions were stopped by adding 50 µL of 1 M perchloric acid and centrifuging for 10 min at 11,000 x g. Each reaction sample (80 µL) was loaded into separate wells of a 96-well plate and 20 µL of malachite green reagent containing working reagents A and B (reagent A; 0.20% malachite green (CAS#: 123333-61-9), 4% ammonium molybdate (CAS#: 12054-85-2) and 12% sulfuric acid (CAS#: 7664-93-9) and reagent B; 15% polyoxyethylenesorbitan monolaurate (CAS#: 9005-64-5)) at a 100:1 ratio was added. After a 30-min incubation at room temperature, absorbance at 620 nm was measured by a plate reader (Infinite M200, Tecan Trading AG, Switzerland). Based on absorbance measured for the samples and for a standard [Pi] curve, enzyme activity was determined as described in the BioAssay Systems[®] manual (2009).

To establish optimal conditions for the phosphate (Pi)-release FB Pase enzymatic assay as described in [22], we set up two different experiments to determine at what stage

of growth cells should be harvested to prepare the extracts, how long the reaction should proceed (i.e., at what endpoint time should activity be measured), and how much enzyme extract should be used in each reaction. We used the WT strain for each of these tests. Cells were cultured then harvested at four different stages: early log ($\sim 1 \times 10^6$ cells/mL), mid log ($\sim 5 \times 10^6$ cells/ mL), late log ($\sim 8 \times 10^6$ cells/mL) and stationary phase ($>10 \times 10^6$ cells/mL) and then extracts were prepared then stored at -80°C until use. For testing optimal amount of enzyme extract, 5 μL , 10 μL , 15 μL or 20 μL of extract were used per reaction, along with a control (no extract). For testing optimal endpoint time for measuring phosphate release, reactions were allowed to proceed for 0, 5, 10 and 15 mins. Reactions were stopped by adding 50 μL of 1M Perchloric acid.

The experiment testing different extract amounts suggested that Pi release increase was linear as a function of extract volume, for all volumes tested, and that it was nearly equally robust for all growth stages other than stationary phase (data not shown). Based on this experiment we used 10-20 μL of extract made from cultures harvested at the mid-log phase, in all future experiments. For reactions using 15 μL enzyme extract, Pi release increased nearly linearly, for extracts made from all four growth stages, through approximately the first 10 min of the reaction, but then for most growth stages it declined significantly for later time points (data not shown). These results suggested that the Pi release reaction should be allowed to proceed for 5 min. In total, the results of these two sets of reactions suggested that mid-log phase is the optimal stage for harvesting cells, that reactions should be started with 15 μL extract, and that reactions should be stopped after 5 min.

2.9. Cell counts and biomass measurements

Algal cell counts and biomass were determined under 4 different culture conditions: mixotrophically in HSA (high salt medium supplemented with 14.6 mM NaOAc, pH 7.0) into which ambient air or air supplemented with 5% CO₂ was bubbled, or photoautotrophically in HSM (high salt medium, pH 7.0; Chlamydomonas Resource Center) into which ambient air or air supplemented with 5% CO₂ was bubbled. All cultures were grown in 500-mL flasks that were agitated on a rotary shaker, under continuous light (120 μ E) and at 24°C in a Percival growth chamber. Air was supplemented with 5% CO₂ by mixing room air with 100% CO₂ at a ratio of 20:1 in a Cole Parmer flow meter (air tube with glass float; model EW3217-29; CO₂ tube with stainless steel float; model EW03217-11) with air and CO₂ inlet pressures both set to 16 psi.

For each growth condition measurement, cultures were started in the following way. 5×10^5 cells/mL of CC125 or of FBPase-overexpressing transgenic line Cp2-TF1 were inoculated into 50 mL of HSA medium and grown for 4 days before transfer into 500 mL experimental medium to acclimate to the medium for 3-4 days. For cell count and biomass experiments, cells from cultures grown to $3\text{--}5 \times 10^6$ cells/mL prepared as described above were transferred into 500-mL flasks at a starting concentration of 5×10^5 cells/mL in a final culture volume of 200 mL. Cells were counted with a hemacytometer every 12 hrs to plot a growth curve, and cells were collected at 24, 36, 48 and 72 hrs after inoculation (early-, mid-, and late-exponential and stationary phase, respectively) for biomass measurements. Cells were collected by filtration onto pre-weighed glass

microfiber filters (GF/F Φ 47 nm, GE Healthcare Life Science) and the dry weight determined after drying overnight in an 80°C oven.

2.10. Statistical analysis and experimental replication

Biological/technical replicates and statistical analyses of data generated in this study were as follows. Growth curve analyses were performed on two biological replicate cultures for each growth condition with three technical replicates obtained and averaged for each data point of each biological replicate; the standard error of the mean was calculated for each biological replicate data point using Microsoft Excel and overall data is reported for both biological replicates (data for one biological replicate is displayed in Supplementary Materials). Enzymatic activity assays were performed on each of two biological replicates, with two technical replicates obtained for each biological replicate experiment; for each sample, the data for all four technical replicates (two for each of the two biological replicates) were pooled for ANOVA. Post-hoc testing with Dunnett's test determined which transformant samples were statistically different from the WT.

3. Results

3.1. Generation of a new *C. reinhardtii* chloroplast transformation vector (*pWD2Cp-FBP1*) to express *FBP1* (*CrFBPase*) from the chloroplast genome

Our initial efforts to express transgenic protein in *C. reinhardtii* using a nuclear transgene were hampered by apparent gene silencing effects (our unpublished results), similar to what others have reported for other *C. reinhardtii* transgenic strains [36,37]. In

contrast, chloroplast (Cp) transgenes are not silenced, and they often give rise to higher protein levels than nuclear transgenes [38–41]. Therefore we decided to express FBP1 protein directly in the Cp, using our newly generated Cp expression vector pWD2-CpFBP1. pWD2-CpFBP1 consists of a pBlueScript (pKS⁺) backbone into which was cloned a DNA fragment containing at its left and right ends ~1.3 and 1.1-kb regions, respectively, from the Cp genome (to target homologous recombination to the intergenic region between the *psbA* and *5S rRNA* genes). Internal to those targeting regions are a cassette containing the 1,125-bp *C. reinhardtii* *FBP1* coding sequence, synthesized with *C. reinhardtii* chloroplast codon bias and C-terminal FLAG epitope tag sequence, and an *aadA* gene cassette that confers spectinomycin resistance (Fig. 1a). pWD2-CpFBP1 was introduced into Cp transformation strain CC125 by biolistic particle bombardment. Spectinomycin resistant colonies appeared within 2 weeks of selection and 8 of these were maintained for further analysis.

3.2. Analysis of *FBP1* transgene integration into the Cp genome

To determine whether the *FBP1* and *aadA* gene cassettes had integrated properly in the 8 selected transformants, we prepared total genomic DNA (nuclear and organellar) from them then used PCR to amplify sequences between the *psbD* promoter/5' UTR region and the 5' end of the *FBP1* CDS, between the 3' end of the *FBP1* CDS and the *psbA* 3' UTR, and also an internal region of *aadA*, as shown in Fig. 1a (regions indicated by P1, P2, P3, respectively. For all transformants analyzed, fragments of the expected sizes were obtained (274 bp, 293 bp and 421 bp, respectively; data not shown) indicating that all transformants had incorporated at least parts of both *FBP1* and the *aadA* cassettes.

409

410 To determine whether the entire *FBP1* and *aadA* gene cassettes had integrated
411 properly via double homologous recombination, we used Southern analysis of EcoRI-
412 digested transformant DNAs probed with a radiolabelled DNA fragment that should
413 detect fragments of 5.6 kb and 8.9 kb if the integration had worked properly. The
414 expected bands were obtained for all transformants analyzed (Fig. 1b). This result
415 suggested that the ~1300-bp 5'- and 3' homology regions permitted successful targeted
416 integration of the *FBP1* and *aadA* genes into the Cp genome.

417

418 **Fig. 1 (1.5 columns)**

419

420 ***3.3. Western analysis of transgenic pWD2-CpFBP1 lines to determine accumulation of***
421 ***exogenous and endogenous CrFBPase***

422 To determine whether the pWD2-CpFBP1 transformants express FLAG-tagged
423 FBPase, we did western blot analysis of total protein extracted from eight transgenic lines
424 using anti-FLAG antibody. All eight transgenic lines tested accumulated a FLAG-
425 positive protein of ~45 kDa (exogenous CrFBPase), while no band was present in the
426 wild type control lane (Fig. 2a). This result and the Southern blot result described above
427 (Fig. 1b) indicate that Cp vector pWD2-CpFBP1 can successfully target genes to the Cp
428 genome and also permit expression of exogenous CrFBPase protein.

429

430 **Fig. 2 (1 column)**

431

To compare accumulation of FBPase-FLAG protein to endogenous FBPase in transgenic pWD2-CpFBP1 lines, we used anti-FBPase-peptide antisera to probe blots containing extracts from these strains and the CC125 control. The antibody detected a single band that migrated at ~45 kDa in all lines, suggesting that CrFBPase-FLAG runs in SDS-PAGE at the same size as endogenous CrFBPase, so it was not possible to directly compare the quantity of endogenous to transgenic CrFBPase protein in this way. However, by normalizing total CrFBPase band intensity from the 3 transgenic lines tested and from strain CC125 against the β -tubulin loading control band intensity for each strain, we determined that all transformants accumulate ~4-fold more FBPase-antisera reacting protein than the wild type strain (Fig. 2b), consistent with the fact that the FLAG-antibody western clearly indicates expression of the exogenous CrFBPase.

3.4. Analysis of FBPase enzyme activity in pWD2-CpFBP1 transformants

We next used a Pi release assay to measure FBPase activity in three of the pWD2-CpFBP1 lines to compare with the parental wild type strain CC125, to determine if the transformants had elevated levels of FBPase activity. As described in the Materials and Methods section, we first established optimal assay conditions for the experiment, namely cell culture density for extract preparation, amount of extract per reaction, and duration of the FBPase reaction, then used the assay to determine whether or not Cp2-TF transgenic lines expressing exogenous CrFBPase have increased levels of FBPase activity compared to the wild type. FBPase activity levels for all three Cp2-TF transgenic lines tested (Cp2-TF1, Cp2-TF2, and Cp2-TF7) after protein amount normalization were approximately 1.4 fold higher than for the parental strain (Fig. 3). This result suggests

that overexpressing CrFBPase protein in the *C. reinhardtii* chloroplast can enhance FBPase activity by up to 1.4 fold. Interestingly, the overexpression of FBPase activity was not proportional to the 4-fold increase of FBPase protein accumulation detected in these lines compared to the wild type (Fig. 2), but the increase in activity was clearly statistically significant.

Fig. 3 (1 column)

3.5. Effect of overexpression of CrFBPase on growth and biomass accumulation during mixotrophic and photoautotrophic growth, with and without supplementation with 5% CO₂

We next set out to determine whether a transgenic strain that overexpresses FBPase protein and activity can grow faster and/or accumulate more biomass than the wild type strain (CC125); a single line, Cp2-TF1, was chosen since extracts from all three tested lines contain approximately the same amount of FBPase activity, and this line possessed slightly more than the other lines that were tested. We tested growth under four different conditions: mixotrophic with 0.04% (atmospheric) CO₂ or 5% CO₂, and photoautotrophic with 0.04% CO₂ or 5% CO₂, expecting the transgenic line to have the largest advantage under photoautotrophic conditions, especially in the presence of 5% CO₂. Culture aliquots were harvested at regular intervals over a period of 3-7 days (depending on how long the cultures were growing) and were used to determine cell counts and to measure biomass. Under mixotrophic conditions, growth of Cp2-TF1 was very similar to that of parental strain CC125 when the strains were grown with

atmospheric CO₂ (Fig. 4a and Fig. S1a), but with 5% CO₂, the wild type strain reached a maximum cell density that was ~1.5 fold higher than for Cp2-TF1, and its maximum biomass accumulation was ~10-20% greater (Fig. 4b and Fig. S1b). So surprisingly, Cp2-TF1 exhibited slower growth than the wild type strain under mixotrophic conditions with 5% CO₂. Under photoautotrophic conditions with atmospheric CO₂, the wild type grew at a similar rate as Cp2-TF1 through the first 3 days of culture but remained in exponential phase longer and reached a maximum cell density that was ~1.4-fold higher (Fig. 4c and Fig. S1c), and with 5% CO₂, the wild type had a noticeably greater growth rate than Cp2-TF1 and reached a maximum cell density that was ~1.7 fold higher (Fig. 4d and Fig. S1d). By visual inspection, it was also clear that by 48 hours wild type cultures were obviously greener than Cp2-TF1 cultures (Fig. 5) despite the fact that all cultures had been started with ~the same number of cells themselves taken from cultures grown to the same stage of exponential growth. These results suggest that overexpressing FBPase activity up to 1.5-fold that of normal levels not only fails to improve growth and biomass accumulation, but instead, it hinders *C. reinhardtii* growth and biomass production during photoautotrophic and high CO₂ growth conditions.

Fig. 4 (2 columns), Fig. 5 (1.5 or 2 columns)

4. Discussion

In this study, we generated a new chloroplast transformation vector for *C. reinhardtii* and used it to overexpress *C. reinhardtii* FBPase in the chloroplast, with the goal of increasing FBPase activity, and cell growth and biomass accumulation. We did obtain transformants with elevated levels of FBPase protein and activity, but those transformants grew slower than the wild type strain under most conditions tested, and accumulated biomass at a slower rate. Our results suggest that contrary to the situation in plants such as tobacco, FBPase activity is not limiting for growth in *C. reinhardtii*, and possibly in other algae.

4.1. Accumulation of transgenic proteins in CpFBP1 transformants

Plasmid pWD2-CpFBP1 clearly is an effective Cp expression vector, as all Spec^R transformants obtained with this vector that were analyzed in this study accumulated substantial amounts of transgenic FBPase. All transformants tested by western blot had easily detectable levels of a protein that migrated at ~45 kDa (compared to the predicted 41 kDa for FLAG-tagged FBPase), and transformants had ~4-fold more total FBPase protein than the wild type strain, based on signals detected by anti-FBPase peptide antisera and the control antisera that detected β -tubulin; endogenous FBPase could not be distinguished from CrFBPase-FLAG in western blots. Plants possess two different FBPase genes, one that encodes a cytoplasmic FBPase that participates in gluconeogenesis, the other a chloroplast-targeted FBPase that functions in the Calvin cycle [43,44,49]. In contrast, *C. reinhardtii* FBPase protein appears to be encoded by a single nuclear gene, *FBP1*, and is believed to carry out both Calvin cycle and gluconeogenesis functions in the chloroplast [49]. Therefore, our peptide antibody

(generated against CrFBPase epitope CGLGSTGQERVLDVN, which ends 28 residues from the C-terminal end of the protein) should detect all CrFBPase, and our estimates of the degree of overexpression of FBPase in our transgenic lines should be accurate. However, it is in principle possible that *FBP1* encodes two different versions of FBPase, a cytoplasmic version and a chloroplast version, that migrate similarly in SDS-PAGE, and if this were the case, the degree of chloroplastic FBPase overexpression in our transgenic lines would be even greater than 4:1.

4.2. pWD2-CpFBP1 transformants have enhanced FBPase activity, but do not grow faster than the wild type

Based on Pi-release assay for FBPase activity, extracts prepared from pWD3-CpFBP1 transformants possess ~1.4-fold more FBPase activity per total cell protein than do extracts prepared from the wild type strain (Fig. 3). This finding was somewhat surprising, since these transformants accumulate ~4-fold more FBPase protein than the wild type strain. It is known that recombinant FBPase protein encoded by several plant and algal genomes is subject to redox regulation such that the enzyme is reversibly inactivated by oxidizing conditions, but our assays were performed using extracts that were prepared in buffer that contains 10 μ M DTT and using reaction buffer that also contained 10 μ M DTT, so none of the enzyme in our extracts should have been redox inactivated. It is possible that *C. reinhardtii* FBPase is regulated by some other, unknown posttranslational mechanism; it has been reported that substrate build up has a stabilizing effect on some FBPase proteins [42], and quite possibly the excess FBPase in our transformant lines could be less active than FBPase in the wild type line because there is

546 a much lower ratio of substrate to enzyme in the transformant lines. Transgenic FBPase-
547 FLAG might also not be as active as endogenous FBPase if the epitope tag interferes
548 somewhat with enzyme activity, or if the transgenic protein does not fold completely
549 normally in the Cp and so is less active than endogenous FBPase.

550 No doubt the most surprising finding of this study is that FBPase overexpression
551 not only failed to improve growth in the pWD2-CpFBP1 transformants, but also in most
552 cases led to an obvious decrease in growth and biomass production (Fig. 4). The failure
553 to improve growth is in stark contrast to what others have observed for overexpression of
554 cyanobacterial FBPase in tobacco [25]. In principle, a simple failure to improve growth
555 could be explained if the transgenic FBPase in our experiments were negatively regulated
556 in vivo by allosteric factors, and thus prevented from augmenting the activity of the
557 endogenous enzyme. Based on in vitro experiments, *C. reinhardtii* FBPase activity is
558 known to require a dithiol reductant (either dithiothreitol or reduced-thioredoxin) in the
559 presence of high Mg^{2+} concentration (8–16 mM) at a pH of 8.0 for optimal activity [43],
560 and it is possible that in our experiments, in vivo conditions were not conducive to high
561 FBPase activity [43]. However the fact that under some growth conditions
562 overexpression of FBPase had a measurable effect on growth, albeit a negative one,
563 suggests that the transgenic enzyme was active and increased the overall level of *in vivo*
564 FBPase activity.

565 Along these lines, the more puzzling outcome of our investigation was that
566 overexpression of FBPase in some cases inhibited growth. The most dramatic differences
567 were observed for cultures aerated with air supplemented with high levels (5%) of CO₂,
568 both under mixotrophic and photoautotrophic conditions. In fact under photoautotrophic

conditions with 5% CO₂, which we had expected to most favor the FBPase-overexpressing transformants, by stationary phase the cell count for the transformant strain was only ~60% that of the wild type, and biomass levels were also reduced. According to these results,,integration of a chloroplast vector that overexpresses FBPase (by ~1.4 fold in the Cp) somehow impedes growth and biomass accumulation under conditions most favorable for photosynthesis.

Two possible explanations for the negative effect by FBPase overexpression are that there is a significant energetic load of producing a 4-fold excess of FBPase protein that inhibits growth (possibly because of misfolding of the transgenic protein), or that somehow transgene integration impairs chloroplast function in our transformants. The first possibility seems unlikely since the growth disparities for transformant versus wild type strains were different for different growth conditions, while transgenic FBPase expression in the transformants should be approximately the same under each of the conditions. We expect that transgenic FBPase levels were likely constant under the different growth conditions because they were controlled by the 5' upstream region of the *C. reinhardtii psbA* gene, which is regulated at the translational level by the redox states of the ferredoxin–thioredoxin system and the plastoquinone pool [44,45]; there is no evidence to suggest that *psbA* expression is regulated by CO₂ or by acetate. The second possibility also seems unlikely because others have targeted chloroplast transgene integration to the same site used in our experiments (intergenic region between the *psbA* and 5S *rRNA* genes) without any reported effects on growth rate or biomass accumulation, suggesting that recombination into this region is not detrimental to growth [38].

A more plausible explanation for the fact that overexpression of FBPase impedes growth is that since FBPase functions at a branch point between the regenerative phase of the Calvin cycle and the biosynthesis of larger sugars [21], overexpression of FBPase could have a detrimental effect on synthesis of these sugars or other products. For instance, an increase in FBPase activity might deplete metabolites immediately upstream of FBP such as DHAP (dihydroxyacetone-phosphate) and G3P (glyceraldehyde-3-phosphate), as suggested in the model depicted in Fig. 6. These substrates could become limiting for the biosynthesis of sugars (or for tryptophan or thiamine, whose biosynthesis also depends on G3P), especially if any excess RuBP produced as a result of FBPase overexpression simply leads to a bottleneck somewhere else in the Calvin cycle prior to the production of DHAP and G3P. Under this scenario, the negative effect of FBPase overexpression would be worse for high CO₂ conditions than for normal CO₂ conditions, because Calvin cycle function is greater when CO₂ levels are higher, and elevated FBPase activity would create a more severe bottleneck. But where might the Calvin cycle bottleneck step be? One candidate is the step catalyzed by sedoheptulose 1,7-bisphosphatase (SBPase), which acts just downstream of FBPase. SBPase is a good candidate for executing a rate-limiting step because it appears to catalyze a rate-limiting step in the plant Calvin cycle [25]. If SBPase is rate limiting, our model predicts that overexpressing it, perhaps together with FBPase, should have the desired effect of increasing CO₂ assimilation, growth, and biomass production in algae.

Of course, there are still other possible reasons why increasing FBPase activity does not improve growth in *C. reinhardtii*. First, changes in levels of Calvin cycle (or other pathway) metabolites effected by elevated FBPase activity might be toxic, or might

lead to negative allosteric effects on enzymes whose activities have a positive influence on growth. Depletion of FBPase substrates that are positive allosteric factors for SBPase or other Calvin cycle enzymes would decrease the activities of those enzymes, and excess accumulation of FBPase products that are negative allosteric effectors would have the same effect. In this light, it would be informative to do a metabolomics analysis of the FBPase overexpressing transformant and the wild type to determine levels of key metabolites in the two strains.

Fig. 6 (1 column)

In any event, our results suggest that algae (or at least *C. reinhardtii*) may respond differently to perturbations of the Calvin cycle than do higher plants, whose growth and biomass are increased when either FBPase or SBPase are sufficiently overexpressed [25]. Probably both FBPase and SBPase are limiting in plants (whereas FBPase is likely not rate limiting in *C. reinhardtii*), so that overexpressing FBPase or SBPase can increase flux through the plant Calvin cycle, producing more G3P, and therefore improving plant growth and biomass. Interestingly, Tamoi and colleagues found that increasing FBPase by less than 1.7-fold and SBPase activity by less than 1.3-fold had little or no effect on growth [25]. Under photoautotrophic conditions with standard air, pWD2-CpFBP1 transformants grew and accumulated biomass at a similar rate compared with the wild type through the stationary phase (Fig 4c and Fig. S1c), or in other words for much of their growth period behaved like tobacco transformants that overexpressed FBPase to the same extent. We were unable to increase total FBPase to more than 1.4-fold the wild type

level in our pWD2-CpFBP1 transformants, and our efforts to overexpress SBPase using our chloroplast expression vector failed (data not shown), but we are currently attempting to overexpress a *Chlamydomonas SBP1* transgene from the nuclear genome and a cyanobacterial gene for an enzyme that catalyzes both FBPase and SBPase reactions [20,27]. As noted above, these experiments could provide a good test of the model we have proposed in Fig. 6. Furthermore, we are also generating new transformants that express FBPase from the chloroplast genome but at a lower level (due to lower transgenic genome copy number) to determine whether smaller increases in FBPase activity might impact growth differently. It will be interesting to see what effect these manipulations have on *C. reinhardtii* growth, as the results could provide insights into the steps that limit algal Calvin cycle output, and the sensitivity of the algal Calvin cycle to perturbations.

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Figure Legends

Figure 1. Integration of expression vector pWD2-CpFBP1 into the *C. reinhardtii* chloroplast genome. (a) Schematic depicting the insert region of chloroplast expression vector pWD2-CpFBP1 (top) and its integration into the region of the chloroplast (Cp) genome between the 5S rRNA gene and exon 5 of the *psbA* gene (middle and bottom). pWD2-CpFBP1 contains ~1.3-kb and 1.1-kb regions of homology to the Cp genome (nucleotides 45892-47242 and 47349-48407 for 5' and 3' regions, respectively; crosshatched boxes); a 1,125-bp *C. reinhardtii* *FBP1* coding fragment (white box)

flanked with 263 bp of *psbD* promoter/5'-UTR and 374 bp of *psbA* 3'-UTR (grey boxes); and a ~1.9 kb *aadA* cassette encoding Spec^R (white box) flanked by 652 bp of *atpA* promoter/5'-UTR and 436 bp of *rbcL* 3' UTR. The entire *FBP1* + *aadA* gene cassette is 3.7 kb. Numbers below EcoRI sites in the schematic of the Cp genome region indicate bp coordinates in the Cp genome. (b) Southern blot analysis of 9 pWD2-CpFBP1 transformants (Cp2-TF1-9). To determine whether the *FBP1* and *aadA* cassettes integrated properly into the Cp genome in Cp2-TF transformants, total genomic DNA was digested with EcoRI, electrophoresed, transferred onto a nylon membrane, and hybridized with a radiolabeled version of the ~3-kb NheI-PstI restriction fragment (line segment drawn above pWD2-CpFBP1 insert, top schematic). Fragments of the sizes expected for integration at the intended site (~8.9 and ~5.6 kb; indicated in panel a, bottom) were obtained, indicating proper integration. P1, P2, and P3 indicate PCR amplicons generated by genomic PCR, as an initial test for integration of the *FBP1* and *aadA* gene cassettes.

Figure 2. Western blot analysis of WD2-CpFBP1 transformants. (a) Detection of exogenous FBPase by FLAG antibody. Extracts were prepared from recipient strain CC125 and from 8 Cp2-TF transformants, subjected to 12% SDS-PAGE, and the proteins transferred to nitrocellulose and reacted with anti-FLAG antibody (top). All 8 Cp2-TF transformants analyzed showed accumulation of FBPase-FLAG protein, which was not detected in the CC125. The same blot was stripped and reprobed with an antibody against β -tubulin (β -TUB) protein as a protein loading control (bottom). (b) Detection of total FBPase by anti-FBPase antisera. Extracts were prepared from recipient strain CC125 and

from 4 WD2-CpFBP1 transformants (Cp2-TF1, 2, 7, and 8), subjected to SDS-PAGE, and the proteins transferred to nitrocellulose and reacted with anti-FBPase antisera (top). The same blot was stripped then reacted with anti- β -tubulin antibody as a loading control (bottom). All transformants appeared to accumulate at least 4-fold higher levels of FBPase than the recipient strain, as determined by Image J analysis of the band intensities and normalization of FBPase-antisera signal to β -tubulin signal.

Figure 3. FBPase activity in extracts of 3 pWD2-CpFBP1 transformants and recipient strain CC125. FBPase assays were performed in two biological replicates for three pWD2-CpFBP1 transgenic lines (Cp2-TF1, Cp2-TF2, Cp2-TF7) and for the recipient strain (CC125) by adding crude extract to reaction buffer and measuring Pi release (detected by malachite green absorbance at 620 nm, as described in Materials and Methods). Bars indicate fold change of FBPase activity for the transformants compared to CC125, combining the data for the two biological replicates (two technical replicates of each sample), following normalization for protein amount as determined by Bradford assay; * indicates significant difference compared with the control line, $p < 0.01$ (Cp2-TF1 and Cp2-TF2) or $p < 0.05$ (Cp2-TF7). FBPase activity in each sample was calculated as μ moles Pi released per μ g of protein per min. Error bars represent standard error for four combined replicates (two technical replicates for each of two biological replicates).

Figure 4. Growth and biomass analyses for Cp2-TF1 and CC125 cultured under mixotrophic and photoautotrophic conditions with atmospheric and 5% CO₂. Growth rate (cells/mL/day; a-d, left panels) and biomass (mg/mL; a-d, right panels) were

determined for pWD2-CpFBP1 transformant. Cp2-TF1 and for wild type strain CC125 grown mixotrophically with atmospheric (a) or 5% CO₂ (b) and for the same strains grown photoautotrophically with atmospheric (c) or 5% CO₂ (d). Growth rate and biomass were measured every 12 hrs by counting cell numbers and filtering, drying and weighing 5-10 mL of cell culture on Whatman paper, respectively. Under most conditions tested, transgenic line Cp2-TF1 (squares in growth curve plots, checkered boxes in biomass measurements) surprisingly exhibited slower growth and biomass accumulation than CC125 (circles in growth curve plots, stippled boxes in biomass measurements). One of two biological replicates is shown; a second biological replicate, which gave the same overall trend, is displayed in supplemental data Fig S1. Error bars in growth rate plots represent standard error of mean for triplicate technical replicates.

Figure 5. Visibly reduced growth under photoautotrophic conditions with 5% CO₂, as a result of FBPase overexpression. Flasks of CC125 and replicate Cp2-TF1 cultures grown photoautotrophically with 5% CO₂, are shown here at 24 hours (left), 48 hours (middle) and 72 hours (right; second and third flasks) post inoculation. Cp2-TF1 cultures were visibly less green than the CC125 cultures, especially at 48 hours. Cultures were started with same number of cells taken from exponential cultures grown to same culture density.

Figure 6. Model for the growth slowing effect of overexpressing FBPase in *C. reinhardtii*. Overexpression of FBPase slows *C. reinhardtii* growth under photoautotrophic and high CO₂ conditions. One possible explanation for this effect is

931 based on the assumption that FBPase is not a rate-limiting enzyme in the algal Calvin
932 cycle, and that one or more other enzymes acting downstream of FBPase are. Compared
933 to the situation in the wild type (top), overexpression of FBPase might decrease pools of
934 G3P available for synthesis of hexose sugars by converting fructose 1,6-bisphosphate
935 (FBP) into fructose 6-phosphate (F6P), which would accumulate to higher levels than
936 normal (bottom), or be converted to another Calvin cycle intermediate that builds up due
937 to a downstream bottle neck in the pathway. The end result of FBPase overexpression
938 would be reduced hexose production and slower growth.

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Table 1. List and sequences of oligonucleotide primers used in this study

Name	Sequences	Application
CpFBP1	5'-ATGATTAAAATTAAATAATAATAA-3'	pWD2-CpFBP1 sequencing
CpFBP2	5'-TGTATGTCAACCTGGTTCACGTTT-3'	
CpFBP3	5'-TTAGAAAGTTTCAC TAAAAAACAC-3'	
aadA1	5'-TGGCGGTACCACTGC CACTAAT-3'	
aadA2	5'-GAACAATACACTCCAGAAGTGA-3'	
aadA3	5'-GCGCGCAGATCAGTTGGAAGAATT-3'	
5HOMO1	5'-GGGAACAAAAGCTGGGTACCGG-3'	
5HOMO2	5'-CCTTCGGAGTATTAAAATAGGATG-3'	
3HOMO1	5'-GCCAGTGAGCGCGCGTAATACGAC-3'	
3HOMO2	5'-GGGAC GTGCTGTGGTCTTCCTAGT-3'	
5'UTR-region (P1)	For: 5'-CATTTTTATATCATAATAAT-3' Rev: 5'-GCGCTAGCCATTGCGTGTATCTCC-3'	Genomic PCR
3'UTR-region (P2)	For: 5'-ATTGCTGAACAAGCAGGTGG-3' Rev: 5'-CATAGGCAGTTGGCAGGACG-3'	
aadA-region (P3)	For: 5'-TATCGCCGAAGTATCAACTC-3' Rev: 5'-GAGAACATAGCGTTGCCTTG-3'	
5Hnew-for	5'-GTACCGGGCCCCCCTCGAGGTCGACATTTAAA TAATGCCTAGGAA-3'	pWD2-CpFBP1 Gibson assembly
5Hnew-rev	5'-GGCAGAAGCTTATCGATACCGTCGACGCAACTGC CACTGACGTCCC-3'	
3Hnew-for	5'-TGATTGTAAGAAAATGCGCTACTAGTCAATTATT CCAATTGAAACT-3'	
3Hnew-rev	5'-ACTCACTATAGGGCGAATTGGAGCTCATGCATCC ACCACTGCCTATTTTAAT-3'	
5Hnew-seq	5'-GGGTACCGGGCCCCCCTCG-3'	
3Hnew-seq	5'-CGACTCACTATAGGGCGAAT-3'	5', 3' homology region sequencing

Figure 1

a

b

1.5 columns

Figure 2

a

b

1 column

Figure 3

Figure 4

a

Mixotrophic growth, 0.04% CO₂

Mixotrophic biomass, 0.04% CO₂

b

Mixotrophic growth, 5% CO₂

Mixotrophic biomass, 5% CO₂

c

Photoautotrophic growth, 0.04% CO₂

Photoautotrophic biomass, 0.04% CO₂

d

Photoautotrophic growth, 5% CO₂

Photoautotrophic biomass, 5% CO₂

24 hrs

CC125

Cp2-TF1

Cp2-TF2

48 hrs

CC125

Cp2-TF1

Cp2-TF2

72 hrs Figure 5

CC125

Cp2-TF1

Cp2-TF2

Wild Type

Figure 6

FBPase OE

one column