Title: Optimising light conditions increases recombinant protein production in *Chlamydomonas reinhardtii* chloroplasts.

Saskya E. Carrera Pacheco[1], Ben Hankamer[1*], Melanie Oey[1*]

[1]The University of Queensland, Institute for Molecular Bioscience, 306 Carmody Road, St Lucia, Australia

*corresponding authors: Dr Melanie Oey and Ben Hankamer, Institute for Molecular Bioscience, 306

Carmody Road, St Lucia, Australia; m.oey@uq.edu.au, phone +61 33462010

Saskya E. Carrera Pacheco, s.carrerapacheco@uq.edu.au,

Prof Ben Hankamer, b.hankamer@imb.uq.edu.au

Running title: Optimum light for recombinant proteins in microalgae

Keywords: Light, Recombinant Protein, Microalga, *Chlamydomonas reinhardtii*, Growth, Protein yield.

Word count: 6191

Summary

The green alga *Chlamydomonas reinhardtii* provides a platform for cheap, scalable and safe production of complex proteins. Despite the fact that chloroplast gene expression in photosynthetic organisms is tightly regulated by light, most expression studies have analysed chloroplast recombinant protein production under constant light. Here, the influence of light period and intensity on expression of green fluorescent protein (GFP) and a GFP- bacterial-lysin (PlyGBS) fusion protein was analysed. Protein yields were strongly influenced by the light period (6-24 h d^{-1}), the light intensity (0-450 $\mu E\ m^{-2}\ s^{-1}$) and trophic condition. Heterotrophic conditions showed low yields of both recombinant proteins due to low growth rates, despite high protein accumulation per cell. Mixotrophic conditions exhibited the highest yields for GFP (4 $mg.L^{-1}.d^{-1}$) under constant light at 35 $\mu E\ m^{-2}\ s^{-1}$ and GFP-PlyGBS (0.4 $mg.L^{-1}.d^{-1}$) under a light period of 15 h d^{-1} and 35 $\mu E\ m^{-2}\ s^{-1}$. This is due to the high growth rates and cellular protein content. For GFP-PlyGBS the maximum increase in cellular protein accumulation was ~24-fold, and in total protein yield ~10-fold, in comparison to constant light conditions (~200 $\mu E\ m^{-2}\ s^{-1}$). The highest yields under photoautrophic conditions were obtained under a 9 h d^{-1} light period. GFP yielded 1.2 $mg.L^{-1}.d^{-1}$ and GFP-PlyGBS 0.42 $mg.L^{-1}.d^{-1}$. This represented a ~5-fold increase in cellular protein accumulation for GFP-PlyGBS in comparison to constant light conditions (~200 $\mu E\ m^{-2}\ s^{-1}$). Optimising light conditions to balance growth and protein expression can significantly enhance overall recombinant protein production in *C. reinhardtii* cultures.

Introduction

Photosynthetic single-cell green algae (microalgae) provide a platform for the production of a wide range of complex proteins. They are increasingly recognised as being cheap, scalable and safe and able to complement bacterial, yeast, mammalian, insect, viral as well as higher plant systems in a number of ways. Bacterial and yeast systems offer well established low cost protein expression platforms but are limited in their ability to conduct sophisticated post-translational modifications essential for many complex proteins (Cereghino and Cregg, 1999, Swartz, 2001). Mammalian and insect cell cultures are capable of correctly folding and post-translationally modifying many proteins, but typically have lower expression yields and are generally significantly more costly and difficult to handle and scale. In addition, mammalian systems are also subject to contamination by human pathogens. Plant expression systems have advanced significantly, but the production of transformants can still require 6-12 months and transgene containment remains an issue (Mayfield et al., 2007).

In contrast, microalgae offer significant advantages. Transgenic expression cell lines can typically be generated in ~2–4 weeks (Mayfield et al., 2007) and support high rates of biomass production (~350 t algae biomass fresh weight ha^{-1} yr^{-1} vs. ~1.75 t ha^{-1} yr^{-1} for tobacco (Food and Agriculture Organisation, United Nations) (Stephens et al., 2013). These speed and yield factors offer significant cost advantages for scale up (Dove, 2002). Microalgae show high growth rates (similar to yeast), can be grown with simple inexpensive growth media consisting mainly of inorganic salts without any mammalian derived compounds (e.g. BSA), and require only simple and low-cost scalable bioreactors to enable controlled and contained cultivation suitable for Good Manufacturing Processes (GMP). A range of algal products have also been granted FDA approval on the basis that the production strains are classified as '*Generally Recognised As Safe*' (GRAS). This GRAS classification was simplified by the fact that microalgae are generally free of human, bacterial or viral pathogens (Hempel et al., 2011), bacterial endotoxins (Lu and Oyler, 2009) and prions (Specht et al., 2010). Purification is simplified by the absence of pyrogenic contaminants (e.g. bacterial lipopolysaccharide) and the use of CO_2 rather than organic carbon sources under photoautrophic conditions, which supports the maintenance of axenic cultures (inhibits yeast, bacterial and fungal contamination). Finally secretion or cell rupture release recombinant proteins from the cell (Rasala et al., 2012). The optimisation of expressed protein release is dependent on the strain and expression location and remains an active area of research (Spiden et al., 2013, Ramos-Martinez et al., 2017).

Chlamydomonas reinhardtii is one of the best established microalgal model systems, for both nuclear and chloroplast expression, with a wide variety of molecular tools already developed. The *C. reinhardtii* chloroplast makes up ~70% of the cell volume and is of particular interest for recombinant protein expression as it can effectively fold and disulfide-link proteins. A range of complex proteins have already been produced in the chloroplast. Examples include full-length monoclonal antibodies with 16 disulfide bonds (Tran et al., 2009), chimeric anti-cancer immunotoxins that could not be produced in *E. coli* or eukaryotic systems (Tran et al., 2013), and a wide variety of other therapeutic proteins such as erythropoietin, human fibronectin, interferon, pro-insulin, human vascular endothelial growth factor, wound healing high mobility group protein B1

(Chávez et al., 2016, Rasala et al., 2010), a White Spot Syndrome Virus vaccine-like protein for tiger prawns (Surzycki et al., 2009), and an orally applicable cholera vaccine (Gregory et al., 2013). Microalgal chloroplast expression therefore opens up the potential for new protein therapeutic development and low cost production. Currently, in the chloroplast, expression yields are typically in the 0.02-5% of total soluble protein (TSP) range, except for a few notable exceptions (e.g. 42% of TSP of VP28 (Surzycki et al., 2009)). Therefore production efficiencies can still be significantly improved.

Chloroplast gene expression is highly regulated by light, both in terms of quality and quantity (Goldschmidt-Clermont, 1997, Idoine et al., 2014). Given the obvious importance of light for photoautotrophic growth, it is surprising that most microalgal protein expression trials have been conducted under continuous illumination rather than natural diurnal, or otherwise altered light-dark cycles (He et al., 2007, Surzycki et al., 2009, Tran et al., 2013). While continuous light is expected to support the highest rates of growth, recent literature has also identified effects of incident light on protein expression (Goldschmidt-Clermont, 1997, Braun-Galleani et al., 2015, Gimpel et al., 2015).

Due to the lack of exogenous expression signals functional in *C. reinhardtii* chloroplasts, most recombinant protein expression is performed using endogenous regulatory elements. Consequently, it is likely that recombinant protein expression and accumulation is subjected to similar light regulation as the endogenous genes, at transcriptional, translational and protein degradation levels. The use of often unnaturally combined expression signals (e.g. promoter/UTRs from different genes), however, makes predictions of regulation effects difficult.

The focus of this study was to examine the effect of continuous light versus light/dark cycles, as well as light intensity, on the expression of two recombinant proteins; the Green Fluorescent Protein (GFP) reporter, and a bacterial lysin-GFP fusion protein (GFP-PlyGBS). Optimised protein production conditions were determined both on a per cell and per culture volume (mg L^{-1}) basis for photoautotrophic, mixotrophic and heterotrophic conditions.

Results

GFP and GFP-PlyGBS expression: To confirm the ability to produce GFP and the fusion protein GFP-PlyGBS in *C. reinhardtii*, expression constructs for each were transformed into wild-type CC125 and CC124 cells. PCR analysis confirmed that all putative positive transformants were homoplasmic. Successful production of GFP and GFP-PlyGBS under standard mixotrophic production conditions (TAP medium, 180-200 $\mu E\ m^{-2}\ s^{-1}$ constant light) was confirmed using native polyacrylamide gel electrophoresis (PAGE, Fig. 1a) and mass spectrometric protein identification. Each sample loading was normalised to the same optical density at 750 nm (OD_{750}), which was used as a proxy for cell number (pre-harvest and protein extraction); consequently the band intensity (Fig. 1a) corresponds to cellular accumulation levels of these recombinant proteins.

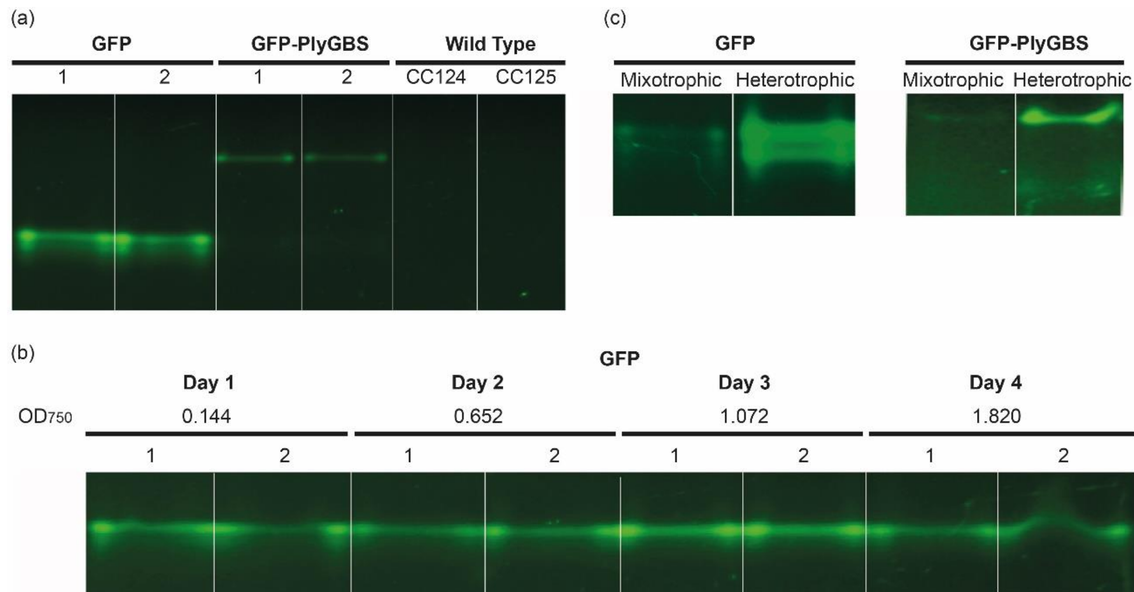

Figure 1. *C. reinhardtii* chloroplast recombinant protein accumulation levels visualised using native-PAGE. Sample loading was normalised on the basis of optical density (OD_{750}) as proxy for cell number. (a) GFP and GFP-PlyGBS fusion protein bands of duplicate samples. Chloroplast mutants of each expression cassette were grown in TAP medium under continuous light (180-200 µE m^{-2} s^{-1}). CC124 and CC125 wild type strains provide the negative controls. (b) Growth cycle independent GFP accumulation of duplicate strep-GFP mutants harvested at different growth stages during the 4-day time course. Synchronised microalgae cells were grown in TAP medium under continuous light (180-200 µE m^{-2} s^{-1}). (c) Mixotrophic versus heterotrophic expression of GFP and GFP-PlyGBS. Microalgae cells were grown in TAP medium under continuous light (mixotrophic, 180-200 µE m^{-2} s^{-1}) or continuous dark (heterotrophic).

Growth cycle does not influence recombinant protein accumulation: To test whether protein yields could be increased by harvesting at specific time points in the growth cycle, synchronised GFP mutants were freshly inoculated into TAP medium and grown at 180-200 µE m^{-2} s^{-1} under constant light for 4 days. To see if cellular protein content varied at different stages, protein levels were analysed by native-PAGE in duplicate at daily time points during the four day experiment. Cell samples were normalised based on OD_{750}. Figure 1b shows no noticeable change in fluorescence, suggesting a constant recombinant protein accumulation in the chloroplast under the conditions tested. Consequently, in subsequent analyses, samples were harvested at the end of mid-log phase; 4 days for mixotrophic cultivation and 5 days for photoautotrophic cultivation conditions.

Effect of light period and trophic condition at saturating light on cellular protein expression and growth: Microalgae can utilise inorganic CO_2 when grown photoautotrophically and mixotrophically, or organic carbon (e.g. acetate in TAP), when grown mixotrophically and heterotrophically, to support cellular metabolism and growth. This, in turn, can affect recombinant protein production. Figure 1c, in agreement with previous reports (Braun-Galleani et al., 2015), suggests that heterotrophic growth can accumulate higher amounts of protein per cell than mixotrophically grown cells exposed to constant light at saturating intensities (180-200 µE m^{-2} s^{-1}).

Little information is available on the influence of light/ dark cycles on recombinant protein expression. Consequently, the effect of varying the light period (0 h d^{-1} = dark (D), and 6, 9, 12 and 15 h d^{-1}) against a constant light control (CL, 24 h d^{-1}), was analysed on the expression of GFP and GFP-PlyGBS under photoautotrophic and mixotrophic

conditions at saturating light intensity (180-200 µE m^{-2} s^{-1}) and heterotrophic conditions. Recombinant protein production was quantified using the relative change in fluorescence signal to that under constant light and normalised on a cellular basis via OD_{750}.

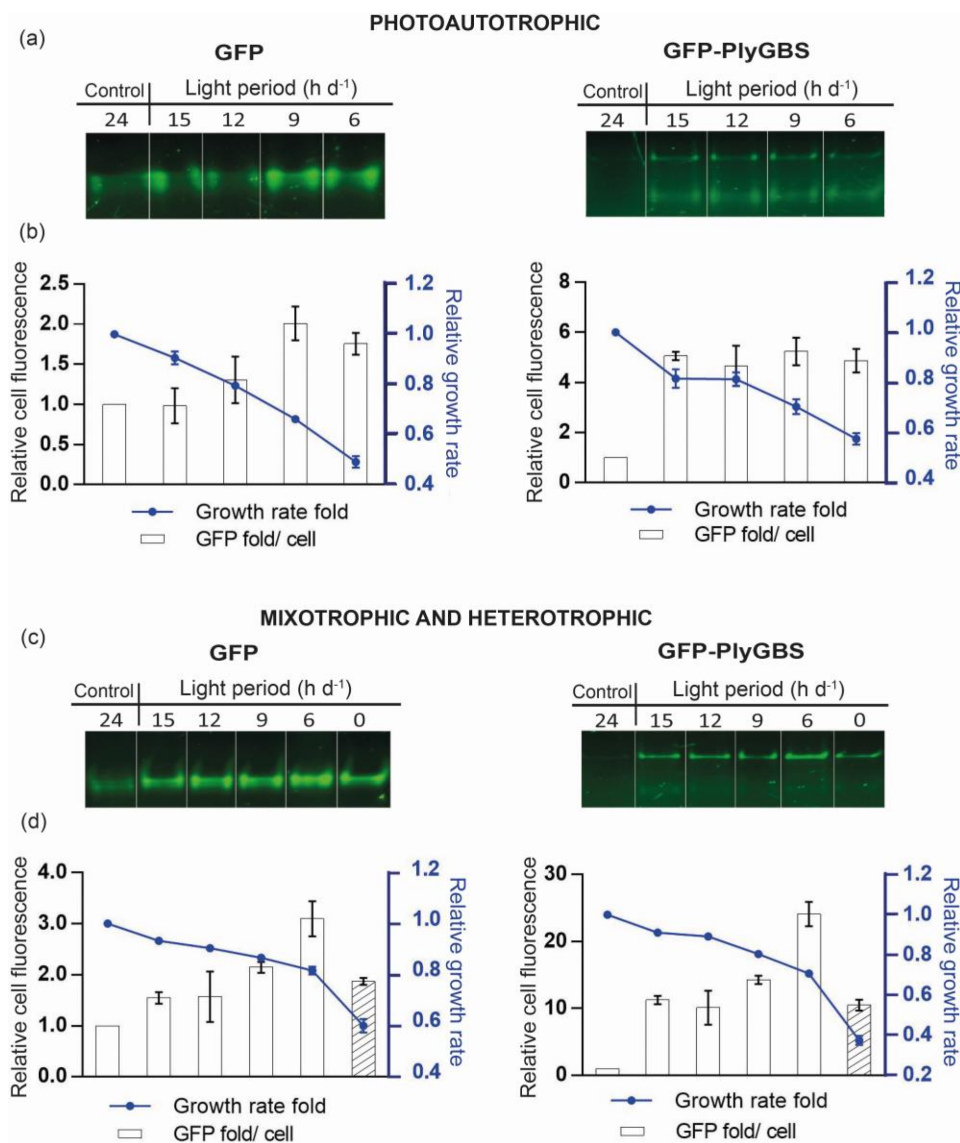

Figure 2. Quantitative analysis of the effect of light period at 200 µE m^{-2} s^{-1} on recombinant protein accumulation and cell growth under photoautotrophic, mixotrophic and heterotrophic conditions. Transgenic *C. reinhardtii* cultures were adjusted to the same cell density (OD_{750}) for native-PAGE. Intrinsic GFP fluorescence was used to measure recombinant protein production. (a) Native-PAGE gels showing fluorescent GFP and GFP-PlyGBS bands under photoautotrophic conditions. (b) GFP fluorescence levels of bands in (a) were quantified using Image Lab 5.2 software in the background subtraction mode.(c) Native-PAGE gels showing fluorescent GFP and GFP-PlyGBS bands under mixotrophic and heterotrophohic (Light period = 0 h d^{-1}; hatched pattern) conditions. (d) GFP fluorescence levels of bands in (c) were quantified using Image Lab 5.2 software in the background subtraction mode. Relative average growth rates were calculated for the duration of the experimental period (5 days photoautotrophic and 4 days mixotrophic). GFP fluorescence and growth rates are reported as the fold change relative to constant light (Control, 24). Error bars represent the standard deviation between biological replicates (n=2) of representative experiments.

Relative cellular protein production under photoautotrophic conditions: Native-PAGE analysis shows the effect of light period under photoautotrophic conditions (180-200 µE

m^{-2} s^{-1} light intensity) on recombinant GFP and GFP-PlyGBS accumulation, normalised to the same cell density (OD_{750}) (Fig. 2a). Figure 2b quantifies the expression levels relative to constant light (control) on a cellular basis. For both proteins, cellular yields peaked at a light period of 9 h d^{-1} (Fig. 2a & b). Here, GFP yields were ~2-fold those obtained under constant light, while GFP-PlyGBS accumulation showed an increase of ~5-fold over those exposed to constant light. This highlights the importance of light period in terms of recombinant protein production.

Interestingly, cellular GFP-PlyGBS levels (Fig. 2b right) are nearly constant for all light periods except under constant light, which were extremely low. The GFP-PlyGBS native-PAGE gels (Fig. 2a right) also showed a second lower molecular weight band similar in size to GFP alone, thought to be a degradation product. This suggests that GFP-PlyGBS accumulation could be increased further by preventing protein degradation.

The effect of light on cell growth rate (Fig. 2b blue line) was also determined for each algal strain and light condition. As expected, the constant 24 h d^{-1}light period yielded the highest growth rates for both strains. Overall, growth rates were reduced with decreasing illumination time (Fig. 2b blue line). Under a 9 h d^{-1} light period, the growth rate of the GFP and GFP-PlyGBS mutants dropped by about 30% compared to that of the 24 h light period control. Consequently a clear opposing effect of light period on protein accumulation and cell growth was observed (Fig. 2b).

Relative cellular protein production under mixotrophic and heterotrophic conditions: A similar pattern was observed under mixotrophic conditions (Fig. 2c & d), except that the highest cellular recombinant protein levels were obtained at an even lower light period of 6 h d^{-1}. One explanation for this is that the provision of acetate as an organic carbon source reduces the reliance of the cell on photosynthesis and enables protein production to persist longer under low light conditions (Fig. 2c & d). Under this light period (6 h d^{-1}) a ~3 times higher level of GFP and ~24 times higher level of GFP-PlyGBS expression was observed, compared to constant light (Fig. 2d). The fold increase of cellular recombinant protein accumulation by introducing dark periods compared to constant light was remarkably high in comparison to photoautotrophic conditions. In contrast, the growth rates of the culture were highest under constant light and, as expected, dropped with decreasing light period (Fig. 2d). Importantly, the drop in growth rate (~20-30%) at 6 h d^{-1} light period was significantly less than the associated increase in cellular protein accumulation (300-2,400%), explaining increased total protein yield under shorter illumination periods.

Interestingly, under the best conditions identified (light period of 6 h d^{-1}, mixotrophic) the cellular levels of both recombinant proteins, as well as the culture growth rates were higher than those of heterotrophic conditions (Fig. 2c & d). This demonstrates the importance of optimising light conditions.

Recombinant protein content under optimal light periods for photoautotrophic and mixotrophic conditions: Next we quantified the GFP fluorescence signal to determine actual cellular protein productivities (g recombinant protein Kg^{-1} biomass dry weight (BDW)) for the best light periods under photoautotrophic and mixotrophic conditions (Fig. 2b: 9 h d^{-1} and Fig. 2d: 6 h d^{-1} respectively) using known bacterial GFP standards. Recombinant protein yields (Fig. 3a, Table 1) were calculated to be 5.5 g Kg^{-1} BDW GFP and 1.4 g Kg^{-1} BDW GFP-PlyGBS under photoautotrophic conditions and 6.2 g Kg^{-1} BDW GFP and 1.9 g Kg^{-1} BDW GFP-PlyGBS under mixotrophic conditions (Table 1).

Importantly, despite the lack of an organic carbon source, photoautotrophic conditions yielded only ~11-26% less recombinant protein on a Kg^{-1} BDW basis than the best mixotrophic culture analysed.

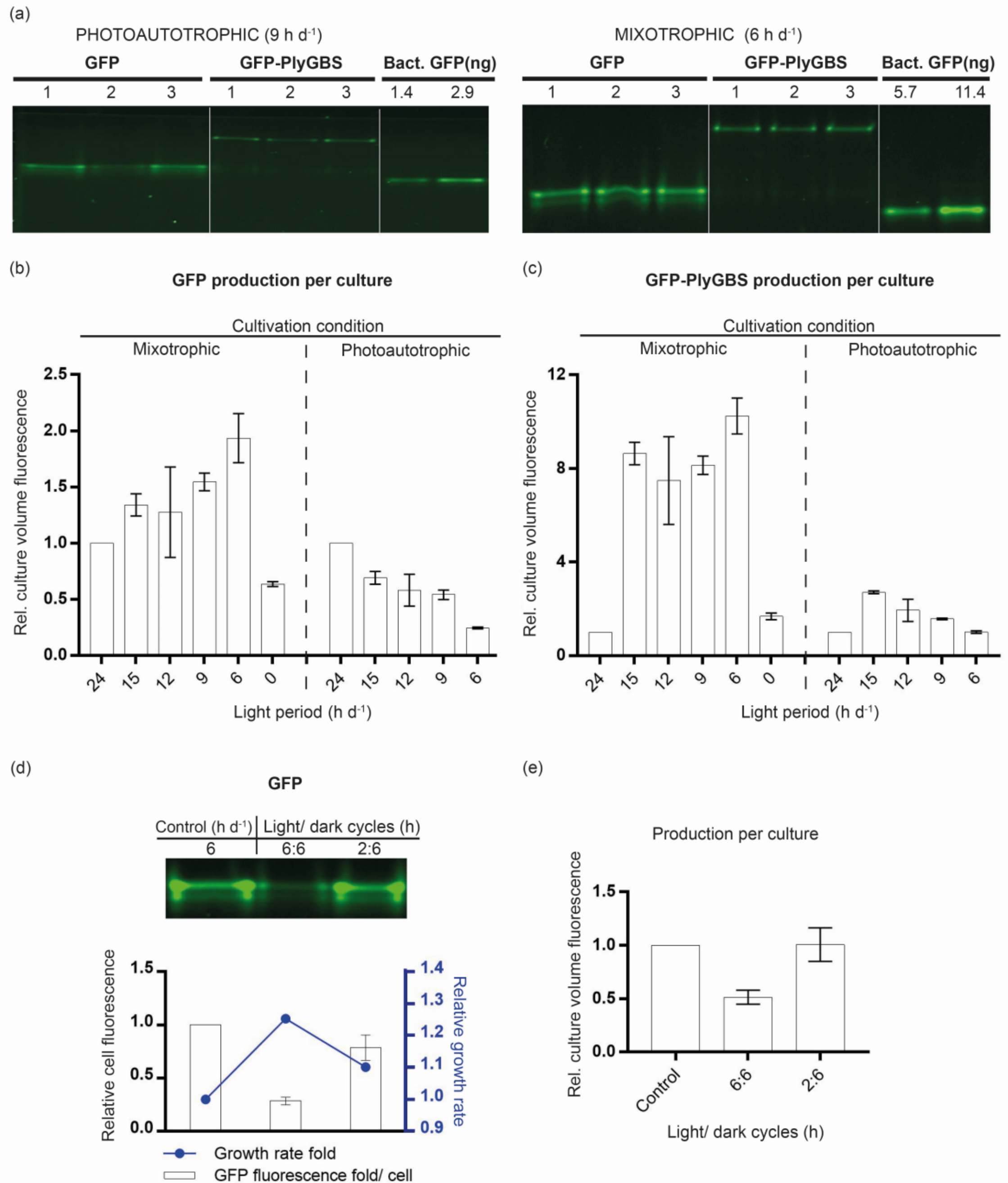

Figure 3. Recombinant protein production of mutants grown under different light conditions. Transgenic *C. reinhardtii* cultures were adjusted to the same cell density (OD_{750}) before loading a native-PAGE. GFP fluorescence was used to measure recombinant protein production. (a) GFP and GFP-PlyGBS quantification under the best identified light period. Mutants were grown under a light period of 9 h d^{-1} in photoautotrophic conditions and 6 h d^{-1} in mixotrophic conditions. Triplicate samples for each recombinant protein were loaded and their intrinsic GFP fluorescence was compared with known bacterial recombinant GFP standards. (b) Relative culture volume GFP fluorescence and (c) Relative culture volume GFP-PlyGBS fluorescence. Total recombinant protein production is reported as fold fluorescence per mL relative to constant light (24 h d^{-1}). Error bars represent the standard deviation (n=2) of representative experiments. In both cases the highest levels of production were obtained under mixotrophic conditions at 6 h d^{-1} light period. (d) Native-

PAGE gels showing GFP fluorescence of mutants grown under mixotrophic conditions at three different light/ dark cycles. Control experiment (6 h d^{-1} of constant i light), 6:6 (a total of 12 h d^{-1} light) and 2:6 (a total of 6 h d^{-1} light). GFP fluorescence of bands in the native-PAGE gel were quantified using the Image Lab 5.2 software in the background subtraction mode. Growth rates per day were measured over the duration of the experiment (4 days). GFP fluorescence and growth rates (blue line) are reported as fold change relative to the control. (e) Relative culture volume GFP fluorecence for the control, 6:6 and 2:6. Total recombinant protein production is reported as fold fluorescence per mL relative to the control. Error bars represent the standard deviation between the biological replicates (n=3). The production per culture in all cases was estimated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture under the respective cultivation condition.

Comparison of relative total protein yield per culture volume: Since maximum total protein yield is the desired outcome, the best overall production conditions were determined (Fig. 2b & d, Table S1) based on the maximum obtained cell density (OD_{750}) of a culture and the cellular fluorescent data and compared to that under constant light (Fig. 2b & d). The overall growth rates of heterotrophically grown cultures (OD_{750}) were slower than under mixotrophic and most photoautotrophic conditions and yielded lower biomass production (Table S1).

Mixotrophic: The optimal light period of 6 h d^{-1} under mixotrophic production gave a final GFP and GFP-PlyGBS concentration of 12.9 mg L^{-1} and 1.3 mg L^{-1} protein at the end point of 4 days respectively. This corresponds to average yields of 3.2 mg GFP L^{-1} d^{-1} and 0.32 mg GFP-PlyGBS L^{-1} d^{-1}. This corresponds to double the overall GFP protein yield per culture volume and a ~10-fold fold higher yield for GFP-PlyGBS, compared to constant light conditions (Fig. 3a, b & c, Table S1). Compared to heterotrophic conditions (0 h d^{-1} light period), the optimal 6 h d^{-1} light period showed ~3-fold GFP and 6-fold GFP-PlyGBS total protein yield per culture volume (Fig. 3b & c, Table S1). Collectively, this demonstrates that neither constant light nor total darkness is beneficial for protein production. Thus, optimising light conditions to balance protein production and cell growth can result in significant improvements in overall recombinant protein productivity.

Photoautotrophic: The optimal light period for cellular protein yield under photoautotrophic conditions was found to be 9 h d^{-1} (Fig. 2a & b). This resulted in final GFP and GFP-PlyGBS concentrations estimated at 6.0 mg L^{-1} and 2.1 mg L^{-1} protein at the end point of 5 days respectively (Fig. 3a). This corresponds to average productivities of 1.2 mg GFP L^{-1} d^{-1} and 0.42 mg GFP-PlyGBS L^{-1} d^{-1}(Table 1). However, for GFP, the total yields per culture were directly proportional to the accumulated biomass (OD_{750}; Table S1) and thus the highest yields (fluorescence fold mL^{-1}) were detected under constant light (Fig. 3b & c). For GFP-PlyGBS, the highest levels were detected under a light period of 15 h d^{-1} (a ~2.7-fold increase compared to constant light (Fig. 3c, Table S1)). This again demonstrates the importance of the light period on cell growth, and shows that GFP-PlyGBS is more sensitive to light than GFP.

Additionally, results from total soluble protein (TSP) quantification for the wild type and mutants of the GFP and GFP-PlyGBS showed that the observed increase in recombinant protein levels was a specific effect of the light period (Supplementary Fig. S1).

Recombinant protein accumulation is independent of light period in mixotrophic conditions, but affected by total light dose: To establish whether the optimal protein yields were because of light period or light dose, we next compared the best cellular protein accumulation under mixotrophic conditions (6 h d^{-1}, control) with two light/ dark cycles of 6:6 h L/D (giving same light period but 12 h total light d^{-1}) and 2:6 h L/D (giving the same 6 h light period d^{-1} as the control but at alternating cycle frequency).

Interestingly, the control light period of 6 h d^{-1} (Fig. 3d & e) and the 2:6 h L/D regime yielded similar total protein, regardless of the duration of the individual light period. This was more than that of the 6:6 L/D regime with 12 h light d^{-1}, even though the latter was divided into two 6 h light periods d^{-1}. These results suggest that the total hours of light (or the dose of mole of photons) provided during a 24 h d^{-1} cycle is more important than the length of each individual light period, both in terms of recombinant protein per cell (Fig. 3d) and total productivity per culture (Fig. 3e).

Light intensity effects on cellular protein accumulation under mixotrophic conditions: As the overall received light in a 24 h period was found to be more important than the duration of each light period, the effect of different light intensities (35, 100, 200 and 450 µE m^{-2} s^{-1}) on recombinant protein production was analysed next. Mixotrophic conditions had previously shown the highest changes in recombinant protein production when exposed to different light regimes, thus the optimal light period at both, cellular (6 h d^{-1}) and culture level (6 h d^{-1} and 15 h d^{-1}) was compared against the control (constant light) for each of the above light intensities (Fig. 4a). Quantification of the native-PAGE fluorescence normalised to OD_{750} showed that cellular GFP levels were highest at 35 µE m^{-2} s^{-1} both under 6 h d^{-1} and constant light and decreased with increasing light intensity (Fig. 4a). Under the 6 h d^{-1} light period this drop was more gradual than under constant light. As expected, under the 6 h d^{-1} light period, the highest growth rates were observed at a light intensity of 450 µE m^{-2} s^{-1} (Fig. 4a). In contrast, under constant (24 h d^{-1}) light the best growth rates were observed in the 100-200 µE m^{-2} s^{-1} range, suggesting light inhibition under constant light at higher intensities.

Overall, total protein yield on a culture level is shown in Figure 4b. This demonstrates that the highest yield of GFP was obtained under constant light at 35 µE m^{-2} s^{-1}, but that similar rates were also obtained under higher light intensities exposed over a shorter light period (i.e. 6 h d^{-1}, 35-100 µE m^{-2} s^{-1}), showing a higher light to protein conversion efficiency for the latter.

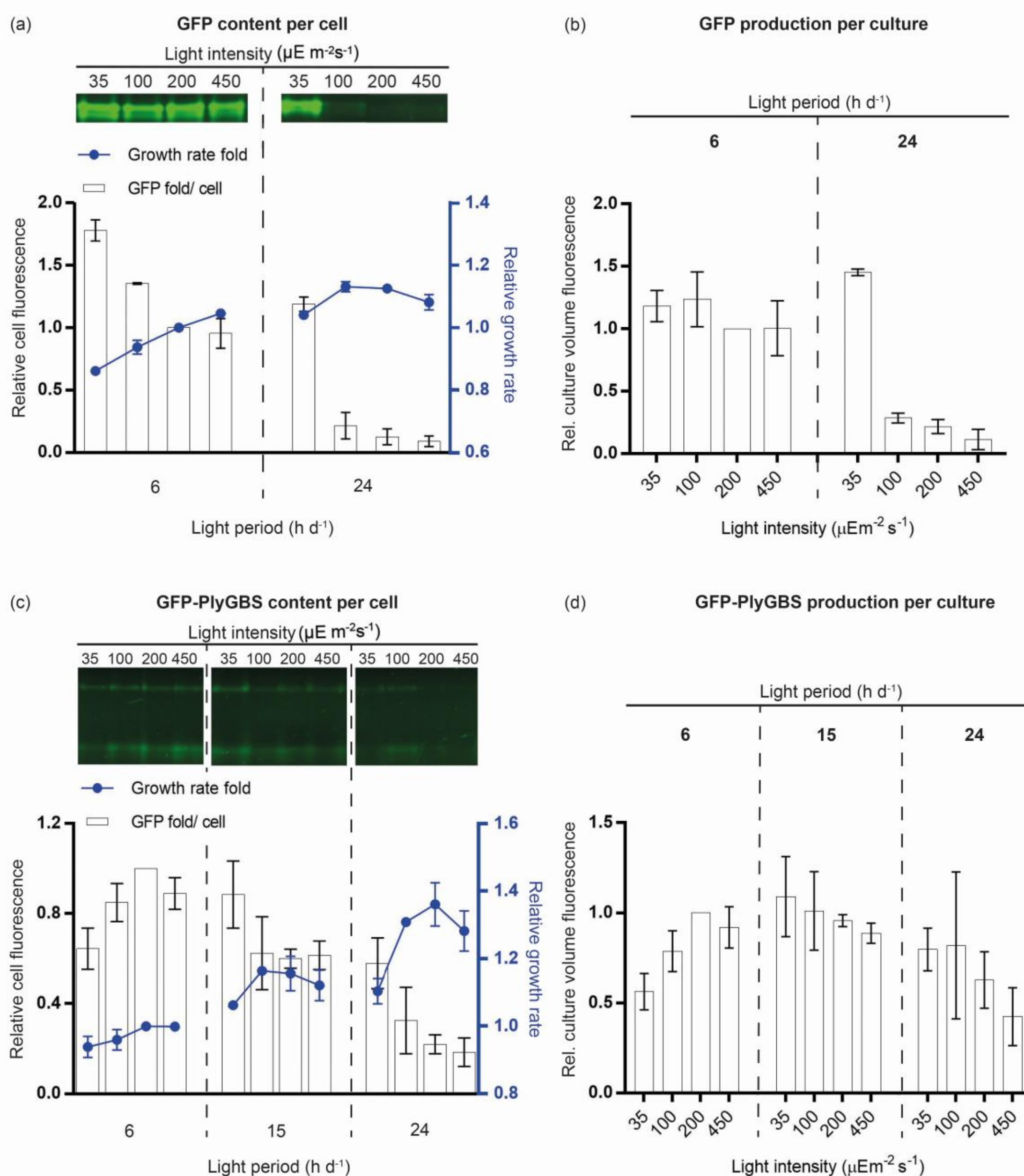

Figure 4. Effect of light intensity on GFP and GFP-PlyGBS production under mixotrophic conditions. Quantitative analyses of the effect of four different light intensities on recombinant protein accumulation and cell growth in transgenic *C. reinhardtii* cells. Microalgae samples were adjusted to the same cell density (OD_{750}) prior to protein extraction for native-PAGE gel and intrinsic GFP fluorescence analysis. GFP fluorescence of bands in the native gel were estimated by using the Image Lab 5.2 software in background subtraction mode. (a) Relative cell fluorescence was determined based on native-PAGE analysis of GFP mutants grown under mixotrophic conditions at two different light periods (6 h d^{-1} and constant light) and four light intensities (35, 100, 200 and 450 µE m^{-2} s^{-1}). Relative cell fluorescence is represented by the histogram and relative growth rates represented by the blue lines. (b) Relative culture volume GFP fluorescence was calculated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture**.** (c) Relative cell fluorescence detection determined based on native-PAGE analysis of GFP-PlyGBS mutants grown under mixotrophic conditions at three different light periods (6, 15 and 24 h d^{-1}) and four light intensities (35, 100, 200 and 450 µE m^{-2} s^{-1}). Relative cell fluorescence is represented by the histogram and relative growth rates are represented by the blue line. (d) Relative culture volume GFP fluorescence was calculated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture under mixotrophic cultivation conditions. Error bars represent the standard deviation between biological replicates of representative experiments (n=2).

A similar but less pronounced trend was observed for cellular levels of GFP-PlyGBS analysing light periods of 6 h d^{-1}, 15 h d^{-1} and constant 24 h d^{-1} light (Fig. 4c); the highest cellular protein accumulation (via relative cell fluorescence) were obtained at 200 µE m^{-2} s^{-1} light intensity at 6 h d^{-1} (Fig. 4c). However, the best relative growth rate was observed under constant light at 200 µE m^{-2} s^{-1} (Fig. 4c). The highest GFP-PlyGBS productivity per culture (Fig. 4d) was obtained at a light period of 15 h d^{-1} at 35 µE m^{-2} s^{-1}. This again supports the finding that there is a fine balance between growth and protein production to achieve maximum overall productivity.

Protein yields at best mixotrophic culture conditions: To compare the two highest overall protein yields per culture volume under mixotrophic conditions and optimised light period and intensity, first the protein yields per cell as g recombinant protein Kg^{-1} biomass dry weight (BDW) were estimated from correlations with known concentrations of bacterial GFP standards (Fig. 5a & b, Table 1). Maximum total protein yields per culture were then estimated from the final cell densities obtained after 4 days (Table 1, Table S2).

Table 1 shows that for GFP, the highest total protein yields under constant light at 35 µE m^{-2} s^{-1} was estimated to be 16.0 mg L^{-1}, giving an average of 4 mg L^{-1} d^{-1}. For GFP-PlyGBS the best total protein yields under a light period of 15 h d^{-1} at 35 µE m^{-2} s^{-1} was estimated at 1.6 mg L^{-1}, giving an average of 0.4 mg L^{-1} d^{-1}. The 10-fold change in yields between GFP and GFP-PlyGBS shows that expression levels are highly dependent on the protein and some may be more sensitive to light.

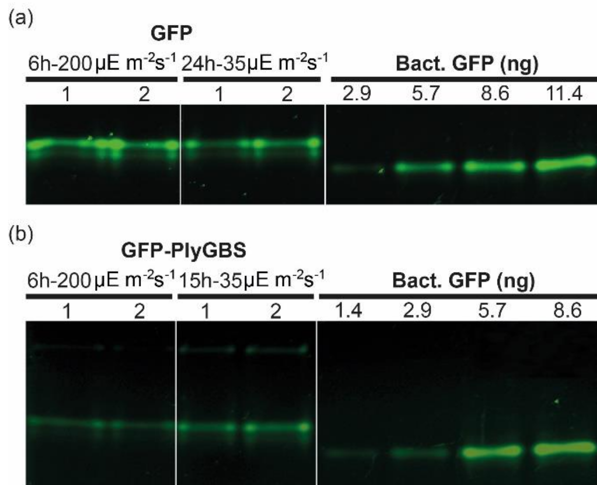

Figure 5. Quantification of GFP and GFP-PlyGBS produced under the best mixotrophic light length and intensity conditions. (a) GFP mutants grown under 6 h d^{-1} at 200 µE m^{-2} s^{-1} and constant light at 35 µE m^{-2} s^{-1}. (b) GFP-PlyGBS mutants grown under 6 h d^{-1} at 200 µE m^{-2} s^{-1} and 15 h d^{-1} light period at 35 µE m^{-2} s^{-1}. Duplicate samples for each recombinant protein and treatment were loaded and their intrinsic GFP fluorescence was compared with bacterial recombinant GFP standards.

Interestingly the yields under 6 h d^{-1} at 200 µE m^{-2} s^{-1} for both proteins (GFP max yield 13.5 mg L^{-1}; GFP-PlyGBS max yield 1.2 mg L^{-1}, Table 1) are not much less than the amounts obtained at the optimal light intensity of 35 µE m^{-2} s^{-1} under constant light and 15 h d^{-1} respectively (cf. 16 and 1.6 mg L^{-1}). This again suggests that light dose, rather than regime, is more important for optimal protein yield.

Discussion

Chlamydomonas reinhardtii can be grown under photoauto-, mixo- or heterotrophic conditions and is a well-established microalgae system for recombinant protein production. Interestingly, to date most protein yields are reported as percentages of total soluble protein and therefore only take cellular protein yields into account. While useful, this does not provide the necessary information to establish the suitability of these strains for scale up, as total protein yields depend both on the expressed protein yield per cell and the growth rate of these cells in the culture medium. Furthermore most of these yields were determined under constant light (He et al., 2007, Surzycki et al., 2009, Tran et al., 2013), typically 100-200 µE m^{-2} s^{-1}. While this light level is suitable for high biomass yields (Moon et al., 2013), it does not take into consideration light-mediated gene regulation effects. The expression of many of the genes in the chloroplast of *C. reinhardtii* is naturally regulated by light (Idoine et al., 2014). It is therefore likely that recombinant protein expression controlled by endogenous elements is subject to similar regulation. Thus, light conditions optimised for growth will not necessarily maximise recombinant protein production on a per cell basis, or in terms of total recombinant protein yields. Furthermore, when photosynthesis occurs, there are complex redox reactions taking place in the chloroplast, along with the production of reactive oxygen species and pH changes, all of which may affect protein stability.

Through a series of experiments (Figures 1-5), the best overall culture production conditions for GFP and GFP-PlyGBS for the tested photoautotrophic and mixotrophic conditions were identified as follows:

Photoautotrophic:

- **GFP:** 9 h d^{-1} (200 µE m^{-2} s^{-1}). Max Yield: 6.0 mg L^{-1} (~1.2 mg L^{-1} d^{-1}).
- **GFP-PlyGBS:** 9 h d^{-1} (200 µE m^{-2} s^{-1}): Max Yield: 2.1 mg L^{-1} (~0.42 mg L^{-1} d^{-1}).

Mixotrophic:

- **GFP:** constant light (35 µE m^{-2} s^{-1}). Max Yield: 16.0 mg L^{-1} (~4 mg L^{-1} d^{-1}).
- **GFP-PlyGBS:** 15 h d^{-1} (35 µE m^{-2} s^{-1}): Max Yield: 1.6 mg L^{-1} (~0.4 mg L^{-1} d^{-1}).

A central theme that emerged from these studies was that light had an important impact on cellular recombinant protein production but also played an important role in cell growth; the combination of both influenced the overall culture productivity. Under photoautotrophic conditions, while short light periods had a positive impact on cellular protein accumulation, the severe impact on growth rates (Fig. 2a & b) shifted the overall culture production to be the best at the longest light periods tested to support cellular growth (Fig. 3b & c). For GFP, a very stable protein, this was constant light, whereas for the optimum overall production for GFP-PlyGBS a minimum dark period was required, leaving 15 h light d^{-1}. This boosted the cellular protein accumulation ~5-fold to outcompete the 20% decrease in growth caused by the introduced dark period (Fig. 2b).

Similarly, when examining different light periods under the same light intensity, short light periods under mixotrophic conditions resulted in much higher cellular protein levels but decreased growth rates (Fig. 2c & d). However, the resulting low growth rates were by far outcompeted by the protein increase and thus shifted the overall productivity to the shortest light period tested (6 h d^{-1}) (Fig. 3b & c; Table S1). Interestingly, despite the ability of *C. reinhardtii* to grow heterotrophically, these conditions yielded lower total

protein productivity compared to mixotrophic conditions, as increased cellular protein accumulation could not make up for the severe growth impairment. Surprisingly, the introduction of different light period cycles (Fig. 3d & e) and light intensities (Fig. 4) revealed that potentially the overall mole of photons per day received by the cell, play an important role in balancing protein production and cellular growth.

Figure 6 summarises the mixotrophic results from Figure 4 above and shows the inverse relationship between the mole of photons m^{-2} d^{-1} provided over a 24 hour period and the relative cellular recombinant protein accumulation, relative cell growth as well as overall relative culture productivity. Figure 6a highlights that the best recombinant protein yield improvements on a per cell basis were obtained below 5 mol photons m^{-2} d^{-1}. The fact that cellular recombinant protein accumulation was in the majority of cases high under 35 μE m^{-2} s^{-1} for both proteins, regardless of the total mole of photons provided per day (i.e. ~0.76 during 6 h d^{-1} light period and ~3 under constant light) but dropped with increasing light intensity (Fig. 4a-d), suggests that there may also be a light intensity threshold above which photosynthesis is favoured over recombinant protein production.

Figure 6b shows that the best biomass growth was observed in the 5- 20 mol photons m^{-2} d^{-1} range, while Figure 6c highlights the fact that the best overall culture production improvements were typically obtained at the intersect between the optimal relative cellular productivities and optimal biomass growth.

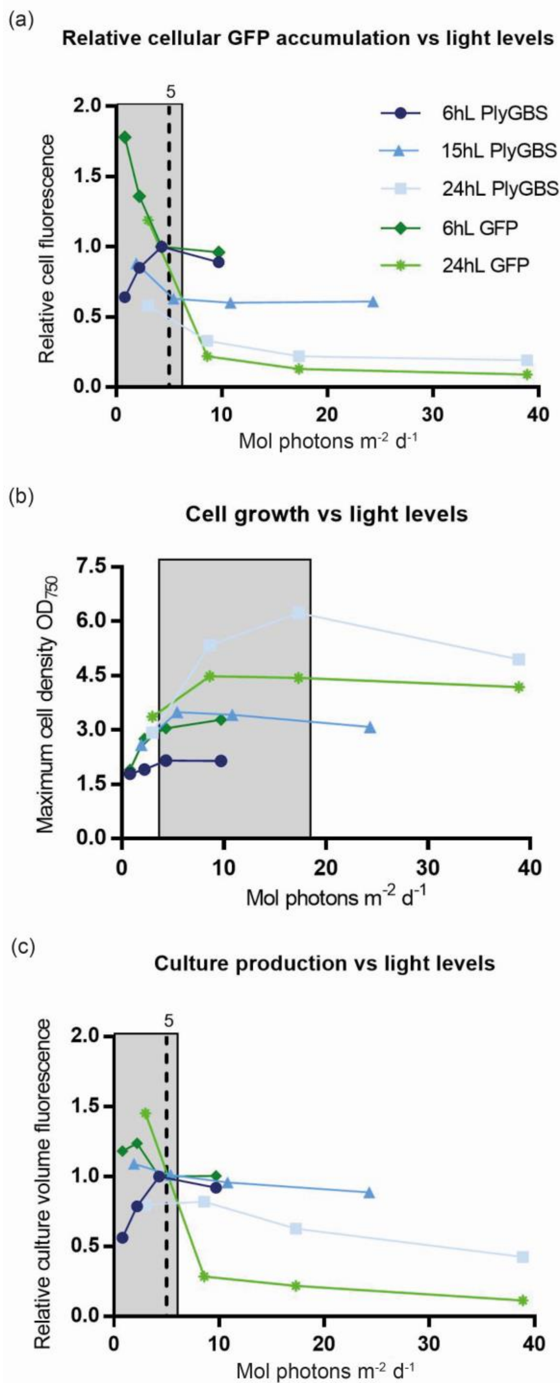

Figure 6. Relative GFP and GFP-PlyGBS fluorescence as a function of light levels. GFP levels shown in green. PlyGBS shown in blue. GFP mutants were grown under light periods of 6 h d^{-1} and constant light, GFP-PlyGBS mutants were grown under light periods of 6 h d^{-1}, 15 h d^{-1} L and constant light, both proteins under 35, 100, 200 and 450 µE m^{-2} s^{-1} light intensity (four data points) based on which the mole of photons per day values were calculated. Relative GFP fluorescence and maximum cell density (OD_{750}) were taken from the average values of representative experiments (n= 2) normalised to 6 h d^{-1} light period at 200 µE m^{-2} s^{-1}. (a) Relative GFP cell fluorescence vs. mol photons m^{-2} d^{-1}. (b) Maximum cell density (OD_{750}) at 4 days of the experiment vs. mol photons m^{-2} d^{-1}. (c) Relative GFP culture volume fluorescence vs. mol photons m^{-2} d^{-1}. In all conditions tested the best recombinant protein production levels were found to be below 5 mol photons m^{-2} d^{-1}. The grey box represents the area where the relative fluorescence or cell density were the highest.

Light effect on transcription, translation and protein degradation:

The specific reasons why dark periods were beneficial for protein accumulation are unclear. Since both recombinant proteins are expressed using the 16S rRNA promoter and the transcript levels of the endogenous gene and other chloroplast genes during the diurnal cycle are relatively stable (Boschetti et al., 1990, Idoine et al., 2014), it is not expected that transcription is the primary reason for the observed light dependent changes in recombinant protein yields per cell.

Translation: Chloroplast gene expression is primarily regulated during translation (Marín-Navarro et al., 2007) and previous studies have shown translation to be the main limiting step in recombinant protein production (Coragliotti et al., 2011). Specifically since *atpA* underlies light-activated translation (Coragliotti et al., 2011) it is possible that chloroplast translation capacity is a limiting factor for atpA 5'UTR driven protein production under high light conditions. The chloroplast being central to photosynthesis, much of its protein synthesis machinery may also be required to repair photodamaged photosynthetic proteins during high light illumination (i.e. 200 µE m^{-2} s^{-1} constant illumination or above) (Grebanier et al., 1978, Satoh et al., 1983, Ohad et al., 1984). This would be expected to adversely affect recombinant protein production. This is supported by the fact that the highest cellular recombinant protein yields were obtained at low light levels during mixotrophic conditions, during which photosystem repair is minimal.

Proteases: An important component of functional photosynthesis is also the protein degradation process. The chloroplast contains over twenty proteases, with ATP-dependent proteases reportedly responsible for most of the proteolysis within it (Adam et al., 2001, Preiss et al., 2001, Surzycki et al., 2009). Clp in the stroma and FtsH on thylakoid membranes are the major conserved ATP-dependent multimeric protease complexes that catalyse processive protein degradation in their respective sub-organellar compartments (Nishimura et al., 2016). The transcript level of ClpP, which encodes the proteolytic subunit of Clp, is down-regulated under dark conditions (Idoine et al., 2014). This is consistent with the findings that reduced protease activity was observed in mixotrophic low light conditions (Fig. 2c) compared to photoautotrophic conditions (Fig. 2a). It is therefore possible that inhibition of protease activity under heterotrophic conditions, as is reported to be the case (Preiss et al., 2001, Braun-Galleani et al., 2015), could explain the increased cellular recombinant protein yields obtained under these conditions.

A potential explanation for the increase in protein levels observed in mixotrophic and photoautotrophic conditions involving dark periods compared to the purely heterotrophic conditions or continuous light, is thus a combination of gene activation during the light period (Idoine et al., 2014), and increased protein accumulation due to reduced protein degradation during dark periods.

Conclusion

This study found that optimising recombinant protein yields in the chloroplast requires the balance of cellular protein expression levels and growth rates, which appear to be in conflict; when cellular expression was high, grow rates were low and *vice versa*.

A similar trend for mixo- and photoautotrophic conditions was observed in terms of light limitation; shorter light periods at saturating light intensities or constant low light exposure, both benefitted recombinant protein accumulation. The fact that low constant light can produce high yields, suggests that protein expression is possible under low levels of photosynthetic activity. However, light limiting conditions had a negative impact on biomass growth. Thus, photoautotrophic growth required longer light periods compared to mixotrophic conditions. Not surprisingly, mixtrophic growth produced more than 3-fold higher yields than photoautotrophic conditions for production of GFP. Remarkably, however, GFP-PlyGBS had similarly high yields for both trophic conditions under optimised light. It should be noted that the latter protein had low relative yields, ~10-fold less than GFP. This may due to its much larger size, being a fusion protein, some other unexplained translational inhibition or accessibility to proteases. Furthermore, heterotrophic growth produced low total yields, despite high cellular protein accumulation due to extremely poor growth, showing that some light is vital for the health of algae cells.

This knowledge provides valuable information to assist with the design of next generation high-efficiency systems. Typically, microalgae photobioreactors have large surface area to volume (SA: V) ratios to maximise light capture. The discovery that low light levels deliver the best recombinant protein productivities is important, as it enables the design of photobioreactors with lower SA: V ratios which significantly reduce capital costs. Moreover, this study revealed that the total proportion of time in the dark, rather than the specific dark period, was important for cellular and total protein production (Fig. 3d). This showed that recombinant protein production can be optimised for normal day night cycles which have light periods between those tested (6 – 24 h). This could enable the development of industrial biotechnologies that are optimised for available ambient light.

Finally, this study showed that while there were clear trends on light effects for both GFP and GFP-PlyGBS), conditions for recombinant protein production are best optimised on a case by case basis for individual proteins.

Experimental procedures

Plasmids: Transformation vectors pMO146 (GFP) and pMO169 (GFP-PlyGBS fusion) were built using a gateway-assisted vector construction approach (Oey et al., 2014). Vector pMO146 carried an expression cassette containing a codon-optimised DNA sequence encoding a Strep-tagged GFP, whereas vector pMO169 contained a codon-optimised sequence encoding a Strep-tagged GFP-TEV-PlyGBS fusion protein. Codon optimisation was performed *in-house*. Both expression cassettes were driven by the 16S rRNA promoter and were under the control of *atpA* 5' and 3'-UTRs. A selectable marker gene, *aadA,* was included in the plasmids to confer resistance to spectinomycin and streptomycin.

Algal strains, culture conditions and transformation: *Chlamydomonas reinhardtii* strains CC124 and CC125 were purchased from the Chlamydomonas Resource Center (St. Paul, Minnesota, USA) and were transformed by biolistic bombardment as described by (Oey et al., 2014). Transformants were selected and cultivated on Tris-acetate-phosphate (TAP) (Gorman and Levine, 1965) 1.2% agar plates supplemented with 150 mg L^{-1} spectinomycin until colonies appeared. Standard liquid culture conditions were in TAP under constant incident light (~180 to 200 µE m^{-2} s^{-1}), a culture temperature of

25±2°C with 250 rpm agitation speed. For light and nutrient optimisation experiments, strain CC124 mutant pMO146 and strain CC125 mutant pMO169 were cultivated under mixotrophic (TAP) and photoautotrophic (Photoautotrophic *Chlamydomonas* Medium, PCM, (Oey et al., 2013)) conditions. Both media were supplemented with antibiotic (300 mg L^{-1} spectinomycin). Photoautotrophic growth was maintained under 1.0±0.3% of CO_2 and 2 L min^{-1} of air.

Homoplasmy screening: Genomic DNA was isolated using the phenol-chloroform-isoamylalcohol method (Thomson and Henry, 1995). PCR amplification of sequences present in the wild-type phenotype with primers Cr-Seq-fwd (5'ACTTAAAGCGACAGGTACTTCCG3') and Cr-Seq-rev (5'CGTTTATATTATGGCTGGATTAGGTC3') were used to confirm positive clones and plastome homoplasmy.

Light experiment: To determine the conditions yielding the best algae growth and protein expression, six different light periods over a 24 hour day were tested using TAP and PCM media. The total experiment duration was 4 days for mixotrophic conditions and 5 days for photoautotrophic conditions. The experiments were carried out using a robotic growth chamber (Tecan Freedom Evo, Tecan Austria GmbH, Grödig, Austria) (Radzun et al., 2015, Wolf et al., 2015) which was programmed for continuous 24, 15, 12, 9, 6 and 0 hours light and respective dark period per day at saturating light (180 to 200 µE m^{-2} s^{-1}) conditions by an Arduino® integrated circuit controller and software (Yarnold et al., 2016). Microwell plates were fitted in shakers adapted with light-emitting diodes (LEDs) which irradiated the plates from the bottom. Additionally, alternating light cycles were assessed for the best combination of hours of light and dark determined from the light period assay. Three sets of 6 h light and 18 h dark periods per day were alternated. Initial algae culture was grown in 6 well microwell plates at an inoculation density OD_{750} of ~0.1. Cells for inoculation were grown at ~180 - 200 µE m^{-2} s^{-1} light intensity, 25±2°C and a 250 rpm agitation speed. Experiments were performed in replicates. Specific number of experiments are shown in the figure legends. For light intensity experiments algae cells were grown at four light intensities: 35, 100, 200 and 450 µE m^{-2} s^{-1} at 6 and 24 h d^{-1} for GFP and 6, 15 and 24 h d^{-1} light period for GFP-PlyGBS mutants. Mixotrophic conditions at 25±2°C and 250 rpm agitation speed were used. The total experiment duration was 4 days.

Growth rate (µ) determination: Cell growth was monitored daily by using a SmartSpec^{TM} 3000 spectrophotometer (Bio-Rad Laboratories Pty Ltd, Hercules CA, USA) using (OD_{750}) as a proxy to estimate growth rates. Growth curves were generated after 4 or 5 days from the daily data and growth rate was calculated based on equation $\mu = ln(\Delta OD_{750})/\Delta t$ (Oey et al., 2013). Averages and standard deviations were calculated from the replicates.

Protein extraction: To determine protein yields, all samples were adjusted to the same OD_{750} before harvesting. 5 mL of algae culture was spun down (10 min, 500×g, RT), the cell pellet was resuspended in 200 µL of protein extraction buffer (HEPES-KOH, pH 7.5 [50 mM], potassium acetate [10 mM], magnesium acetate [5 mM], EDTA [1 mM], 2x Complete^{TM} Protease Inhibitor- EDTA free (Roche Australia, Brisbane, Queensland, Australia) with DTT [1 mM, final concentration]. Cells were lysed using the Navy beads lysis kit and the Bullet Blender® (Next Advance, Inc. USA) (3 min, 12 speed, 4 °C). Samples were centrifuged (10 min, 12000×g, 4 °C) and recombinant GFP carrying a

Strep-tag was isolated from the total soluble protein using 10 µl *Strep*-Tactin® Sepharose® 50% suspension (IBA, Göttingen, Germany) per sample according to manufacturer's specifications.

GFP fluorescence intensity determination and gel quantification: Recombinant proteins were analysed on a 15 % native-Polyacrylamide Gel Electrophoresis (PAGE). GFP fluorescence was detected by the ChemiDoc™ MP Imaging system (Bio-Rad Laboratories Pty Ltd, Gladesville, New South Wales, Australia) and the intensity was determined using the Image Lab 5.2 software in background subtraction mode (Bio-Rad Laboratories). Averages and standard deviations of the replicates (number of replicates is indicated in the figure legends) were calculated for all treatments. A calibration series of bacterial recombinant GFP was used to estimate the amount of algal recombinant protein.

Biomass dry weight quantification (BDW) and protein yield: Algae cells grown in TAP and PCM media were first adjusted to the same OD_{750} as used for protein extraction. Empty and dry glass-fibre filters (Whatman GF/F) were pre-weighed twice (average weight used). A volume of algae culture was filtrated onto the filter then dried in an oven at 85 °C for 3 days. Filters were placed in a desiccator to cool before weighing on a precision scale. Weight change was registered and used for BDW calculation. Protein yield was calculated based on the BDW and protein concentration estimated from bacterial recombinant GFP.

Bradford assay for TSP protein quantification: Wild-type algae CC125, GFP and GFP-PlyGBS mutants were grown in TAP media under continuous light, complete dark and 6 h d^{-1} light period. OD_{750} was adjusted in all the cultures and algae cells were lysed as described in the protein extraction section. The supernatant was used to determine the total soluble protein using a Bradford protein assay (Bio-Rad protein assay). A standard curve was generated by using bovine gamma globulin as a control. Standards and algal protein samples were quantified in triplicates at 595 nm in a PowerWave XS microwell plate reader (Bio-Tek instruments, Inc., Highland park, USA).

Acknowledgements

Dr Gordon King who kindly provided the bacterial GFP plasmid used in this study. Dr Ian Ross for his thoughtful comments on the manuscript. Dr Jennifer Yarnold for her helpful input and for proof-reading the manuscript. We also thank the NHMRC for their support (grant No. APP1074296).

Author Contributions

Conceived and designed the experiments: M. Oey, S. Carrera Pacheco.
Performed the experiments: S. Carrera Pacheco.
Analysed the data: S. Carrera Pacheco, M. Oey, B. Hankamer.
Drafting the article and revising it critically: S. Carrera Pacheco, M. Oey, B. Hankamer.
Final approval of the article: B. Hankamer.
B. Hankamer (b.hankamer@imb.uq.edu.au) and M. Oey (m.oey@uq.edu.au) as corresponding authors take responsibility for the integrity of the work as a whole, from inception to finished article.

Conflict of interest, informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

All authors agree to authorship and submission of the manuscript for peer review.

References

ADAM, Z., ADAMSKA, I., NAKABAYASHI, K., OSTERSETZER, O., HAUSSUHL, K., MANUELL, A., ZHENG, B., VALLON, O., RODERMEL, S. R., SHINOZAKI, K. & CLARKE, A. K. 2001. Chloroplast and Mitochondrial Proteases in Arabidopsis. A Proposed Nomenclature. *Plant Physiology,* 125**,** 1912-1918.

BOSCHETTI, A., BREIDENBACH, E. & BLÄTTLER, R. 1990. Control of protein formation in chloroplasts. *Plant Science,* 68**,** 131-149.

BRAUN-GALLEANI, S., BAGANZ, F. & PURTON, S. 2015. Improving recombinant protein production in the Chlamydomonas reinhardtii chloroplast using vivid Verde Fluorescent Protein as a reporter. *Biotechnology Journal,* 10**,** 1289-1297.

CEREGHINO, G. P. L. & CREGG, J. M. 1999. Applications of yeast in biotechnology: protein production and genetic analysis. *Current Opinion in Biotechnology,* 10**,** 422-427.

CHÁVEZ, M. N., SCHENCK, T. L., HOPFNER, U., CENTENO-CERDAS, C., SOMLAI-SCHWEIGER, I., SCHWARZ, C., MACHENS, H.-G., HEIKENWALDER, M., BONO, M. R., ALLENDE, M. L., NICKELSEN, J. & EGAÑA, J. T. 2016. Towards autotrophic tissue engineering: Photosynthetic gene therapy for regeneration. *Biomaterials,* 75**,** 25-36.

CORAGLIOTTI, A. T., BELIGNI, M. V., FRANKLIN, S. E. & MAYFIELD, S. P. 2011. Molecular Factors Affecting the Accumulation of Recombinant Proteins in the Chlamydomonas reinhardtii Chloroplast. *Molecular Biotechnology,* 48**,** 60-75.

DOVE, A. 2002. Uncorking the biomanufacturing bottleneck. *Nat Biotechnol,* 20**,** 777-9.

GIMPEL, J. A., HYUN, J. S., SCHOEPP, N. G. & MAYFIELD, S. P. 2015. Production of recombinant proteins in microalgae at pilot greenhouse scale. *Biotechnology and Bioengineering,* 112**,** 339-345.

GOLDSCHMIDT-CLERMONT, M. 1997. Coordination of Nuclear and Chloroplast Gene Expression in Plant Cells. *International Review of Cytology,* 177**,** 115-180.

GORMAN, D. S. & LEVINE, R. P. 1965. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. *Proceedings of the National Academy of Sciences of the United States of America,* 54**,** 1665-1669.

GREBANIER, A., COEN, D., RICH, A. & BOGORAD, L. 1978. Membrane proteins synthesized but not processed by isolated maize chloroplasts. *The Journal of Cell Biology,* 78**,** 734-746.

GREGORY, J. A., TOPOL, A. B., DOERNER, D. Z. & MAYFIELD, S. 2013. Alga-Produced Cholera Toxin-Pfs25 Fusion Proteins as Oral Vaccines. *Applied and Environmental Microbiology,* 79**,** 3917-3925.

HE, D.-M., QIAN, K.-X., SHEN, G.-F., ZHANG, Z.-F., LI, Y.-N., SU, Z.-L. & SHAO, H.-B. 2007. Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in Chlamydomonas reinhardtii chroloplasts. *Colloids and Surfaces B: Biointerfaces,* 55**,** 26-30.

HEMPEL, F., LAU, J., KLING, L. A. & MAIER, U. 2011. Algae as protein factories: expression of a human antibody and the respective antigen in the diatom *Phaeodactylum tricornutum*. *Plos One,* 6**,** 1-7.

IDOINE, A. D., BOULOUIS, A., RUPPRECHT, J. & BOCK, R. 2014. The Diurnal Logic of the Expression of the Chloroplast Genome in Chlamydomonas reinhardtii. *PLOS ONE,* 9**,** e108760.

LU, Y. & OYLER, G. 2009. Green algae as a platform to express therapeutic proteins. *Discovery Medicine,* 8**,** 28-30.

MARÍN-NAVARRO, J., MANUELL, A. L., WU, J. & P. MAYFIELD, S. 2007. Chloroplast translation regulation. *Photosynthesis Research,* 94**,** 359-374.

MAYFIELD, S. P., MANUELL, A. L., CHEN, S., WU, J., TRAN, M., SIEFKER, D., MUTO, M. & MARIN-NAVARRO, J. 2007. Chlamydomonas reinhardtii chloroplasts as protein factories. *Current Opinion in Biotechnology,* 18**,** 126-133.

MOON, M., KIM, C. W., PARK, W.-K., YOO, G., CHOI, Y.-E. & YANG, J.-W. 2013. Mixotrophic growth with acetate or volatile fatty acids maximizes growth and lipid production in Chlamydomonas reinhardtii. *Algal Research,* 2**,** 352-357.

NISHIMURA, K., KATO, Y. & SAKAMOTO, W. 2016. Chloroplast Proteases: Updates on Proteolysis within and across Suborganellar Compartments. *Plant Physiology,* 171**,** 2280-2293.

OEY, M., ROSS, I. L. & HANKAMER, B. 2014. Gateway-Assisted Vector Construction to Facilitate Expression of Foreign Proteins in the Chloroplast of Single Celled Algae. *PLOS ONE,* 9**,** e86841.

OEY, M., ROSS, I. L., STEPHENS, E., STEINBECK, J., WOLF, J., RADZUN, K. A., KÜGLER, J., RINGSMUTH, A. K., KRUSE, O. & HANKAMER, B. 2013. RNAi Knock-Down of LHCBM1, 2 and 3 Increases Photosynthetic H2 Production Efficiency of the Green Alga Chlamydomonas reinhardtii. *PLOS ONE,* 8**,** e61375.

OHAD, I., KYLE, D. J. & ARNTZEN, C. J. 1984. Membrane protein damage and repair: removal and replacement of inactivated 32-kilodalton polypeptides in chloroplast membranes. *The Journal of Cell Biology,* 99**,** 481-485.

PREISS, S., SCHRADER, S. & JOHANNINGMEIER, U. 2001. Rapid, ATP-dependent degradation of a truncated D1 protein in the chloroplast. *European Journal of Biochemistry,* 268**,** 4562-4569.

RADZUN, K. A., WOLF, J., JAKOB, G., ZHANG, E., STEPHENS, E., ROSS, I. & HANKAMER, B. 2015. Automated nutrient screening system enables high-throughput optimisation of microalgae production conditions. *Biotechnology for Biofuels,* 8**,** 65.

RAMOS-MARTINEZ, E. M., FIMOGNARI, L., & SAKURAGI, Y. 2017. High-yield secretion of recombinant proteins from the microalga Chlamydomonas reinhardtii. *Plant Biotechnology Journal, 15*(9), 1214-1224.

RASALA, B. A., LEE, P. A., SHEN, Z., BRIGGS, S. P., MENDEZ, M. & MAYFIELD, S. P. 2012. Robust Expression and Secretion of Xylanase1 in Chlamydomonas reinhardtii by Fusion to a Selection Gene and Processing with the FMDV 2A Peptide. *PLOS ONE,* 7**,** e43349.

RASALA, B. A., MUTO, M., LEE, P. A., JAGER, M., CARDOSO, R. M. F., BEHNKE, C. A., KIRK, P., HOKANSON, C. A., CREA, R., MENDEZ, M. & MAYFIELD, S. P. 2010. Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii. *Plant Biotechnology Journal,* 8**,** 719-733.

SATOH, K., NAKATANI, H. Y., STEINBACK, K. E., WATSON, J. & ARNTZEN, C. J. 1983. Polypeptide composition of a Photosystem II core complex. Presence of a herbicide-binding protein. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 724**,** 142-150.

SPECHT, E., MIYAKE-STONER, S. & MAYFIELD, S. 2010. Micro-algae come of age as a platform for recombinant protein production. *Biotechnology Letters,* 32**,** 1373-1383.

SPIDEN E. M., YAP, B. H. J., HILL, D. R. A., KENTISH, S. E., SCALES, P. J., & MARTIN, G. J. O. 2013. Quantitative evaluation of the ease of rupture of industrially promising microalgae by high pressure homogenization. *Bioresource Technology*, 140, 165-171.

STEPHENS, E., ROSS, I. & HANKAMER, B. 2013. Expanding the microalgal industry – continuing controversy or compelling case? *Current Opinion in Chemical Biology,* 17**,** 444-452.

SURZYCKI, R., GREENHAM, K., KITAYAMA, K., DIBAL, F., WAGNER, R., ROCHAIX, J.-D., AJAM, T. & SURZYCKI, S. 2009. Factors effecting expression of vaccines in microalgae. *Biologicals,* 37**,** 133-138.

SWARTZ, J. R. 2001. Advances in Escherichia coli production of therapeutic proteins. *Current Opinion in Biotechnology,* 12**,** 195-201.

THOMSON, D. & HENRY, R. 1995. Single-step protocol for preparation of plant tissue for analysis by PCR. *Biotechniques,* 19**,** 394-397.

TRAN, M., VAN, C., BARRERA, D. J., PETTERSSON, P. L., PEINADO, C. D., BUI, J. & MAYFIELD, S. P. 2013. Production of unique immunotoxin cancer therapeutics in algal chloroplasts. *Proceedings of the National Academy of Sciences,* 110**,** E15–E22.

TRAN, M., ZHOU, B., PETTERSSON, P. L., GONZALEZ, M. J. & MAYFIELD, S. P. 2009. Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. *Biotechnology and Bioengineering,* 104**,** 663-673.

WOLF, J., ROSS, I. L., RADZUN, K. A., JAKOB, G., STEPHENS, E. & HANKAMER, B. 2015. High-throughput screen for high performance microalgae strain selection and integrated media design. *Algal Research,* 11**,** 313-325.

YARNOLD, J., ROSS, I. L. & HANKAMER, B. 2016. Photoacclimation and productivity of Chlamydomonas reinhardtii grown in fluctuating light regimes which simulate outdoor algal culture conditions. *Algal Research,* 13**,** 182-194.

Tables

Table 1. Summary of GFP and GFP-PlyGBS expression data collected from cultures grown under the best light regime and intensity for mixotrophic and photoautotrophic conditions.

Culture Conditions	Light regime (light/dark hours)	Light intensity ($\mu E\ m^{-2}\ s^{-1}$)	Protein	Mutant	Growth rate (d^{-1})[a]	Maximum cell density (OD_{750})[b]	Protein Yield (g Kg^{-1} BDW)	Dry Biomass per culture (g L^{-1})[c]	*Volumetric yield (mg L^{-1})*	*Volumetric productivity (mg L^{-1} d^{-1})*
Mixotrophic[d]	6/ 18	180-200	GFP	pMO146#3	0.60±0.01	1.66±0.07	6.2	2.1	12.9	3.2
	6/ 18	180-200	GFP-PlyGBS	pMO169#5	0.53±0.01	1.25±0.02	1.9	0.7	1.3	0.3
Photoautotrophic	9/ 15	180-200	GFP	pMO146#3	0.53±0.01	1.37±0.05	5.5	1.1	6.0	1.2
	9/ 15	180-200	GFP-PlyGBS	pMO169#5	0.63±0.01	2.08±0.11	1.4	1.4	2.1	0.4
Mixotrophic	24/0	~35	GFP	pMO146#3	0.83±0.01	3.31±0.01	11.5	1.4	16.0	4.0
+ intensities[e]	6/18	~200	GFP	pMO146#3	0.79±0.01	2.78±0.01	13.5	1.0	13.5	3.4
	15/9	~35	GFP-PlyGBS	pMO169#5	0.75±0.01	2.59±0.01	1.6	1.0	1.6	0.4
	6/18	~200	GFP-PlyGBS	pMO169#5	0.72±0.01	2.31±0.01	1.8	0.7	1.2	0.3

[a]Based on 4 and 5 days (d) of growth for mixotrophic and photoautotrophic conditions, respectively.
[b]At the end of the experimental period, 4 and 5 days (d) of growth for mixotrophic and photoautotrophic conditions, respectively.
[c]Based on the maximum cell density (OD_{750}) at the end of the experimental period.
[d]Growth conditions under 6 h light/ 18 h dark regime without light intensity adjustment.
[e] Growth conditions under different light regimes with light intensity adjustment.

Figure legends

Figure 1. *C. reinhardtii* chloroplast recombinant protein accumulation levels visualised using native-PAGE. Sample loading was normalised on the basis of optical density (OD_{750}) as proxy for cell number. (a) GFP and GFP-PlyGBS fusion protein bands of duplicate samples. Chloroplast mutants of each expression cassette were grown in TAP medium under continuous light (180-200 µE $m^{-2} s^{-1}$). CC124 and CC125 wild type strains provide the negative controls. (b) Growth cycle independent GFP accumulation of duplicate strep-GFP mutants harvested at different growth stages during the 4-day time course. Synchronised microalgae cells were grown in TAP medium under continuous light (180-200 µE $m^{-2} s^{-1}$). (c) Mixotrophic versus heterotrophic expression of GFP and GFP-PlyGBS. Microalgae cells were grown in TAP medium under continuous light (mixotrophic, 180-200 µE $m^{-2} s^{-1}$) or continuous dark (heterotrophic).

Figure 2. Quantitative analysis of the effect of light period at 200 µE $m^{-2} s^{-1}$ on recombinant protein accumulation and cell growth under photoautotrophic, mixotrophic and heterotrophic conditions. Transgenic *C. reinhardtii* cultures were adjusted to the same cell density (OD_{750}) for native-PAGE. Intrinsic GFP fluorescence was used to measure recombinant protein production. (a) Native-PAGE gels showing fluorescent GFP and GFP-PlyGBS bands under photoautotrophic conditions. (b) GFP fluorescence levels of bands in (a) were quantified using Image Lab 5.2 software in the background subtraction mode.(c) Native-PAGE gels showing fluorescent GFP and GFP-PlyGBS bands under mixotrophic and heterotrophohic (Light period = 0 h d^{-1}; hatched pattern) conditions. (d) GFP fluorescence levels of bands in (c) were quantified using Image Lab 5.2 software in the background subtraction mode. Relative average growth rates were calculated for the duration of the experimental period (5 days photoautotrophic and 4 days mixotrophic). GFP fluorescence and growth rates are reported as the fold change relative to constant light (Control, 24). Error bars represent the standard deviation between biological replicates (n=2) of representative experiments.

Figure 3. Recombinant protein production of mutants grown under different light conditions. Transgenic *C. reinhardtii* cultures were adjusted to the same cell density (OD_{750}) before loading a native-PAGE. GFP fluorescence was used to measure recombinant protein production. (a) GFP and GFP-PlyGBS quantification under the best identified light period. Mutants were grown under a light period of 9 h d^{-1} in photoautotrophic conditions and 6 h d^{-1} in mixotrophic conditions. Triplicate samples for each recombinant protein were loaded and their intrinsic GFP fluorescence was compared with known bacterial recombinant GFP standards. (b) Relative culture volume GFP fluorescence and (c) Relative culture volume GFP-PlyGBS fluorescence. Total recombinant protein production is reported as fold fluorescence per mL relative to constant light (24 h d^{-1}). Error bars represent the standard deviation (n=2) of representative experiments. In both cases the highest levels of production were obtained under mixotrophic conditions at 6 h d^{-1} light period. (d) Native-PAGE gels showing GFP fluorescence of mutants grown under mixotrophic conditions at three different light/ dark cycles. Control experiment (6 h d^{-1} of constant i light), 6:6 (a total of 12 h d^{-1} light) and 2:6 (a total of 6 h d^{-1} light). GFP fluorescence of bands in the native-PAGE gel were quantified using the Image Lab 5.2 software in the background subtraction mode. Growth rates per day were measured over the duration of the experiment (4 days). GFP fluorescence and growth rates (blue line) are reported as fold change relative to the control. (e) Relative culture volume GFP fluorecence for the control, 6:6 and 2:6. Total recombinant protein production is reported as fold fluorescence per mL relative to the control. Error bars represent the standard deviation between the biological replicates (n=3). The production per culture in all cases was estimated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture under the respective cultivation condition.

Figure 4. Effect of light intensity on GFP and GFP-PlyGBS production under mixotrophic conditions. Quantitative analyses of the effect of four different light intensities on recombinant protein accumulation and cell growth in transgenic *C. reinhardtii* cells. Microalgae samples were adjusted to the same cell density (OD_{750}) prior to protein extraction for native-PAGE gel and intrinsic GFP fluorescence analysis. GFP fluorescence of bands in the native gel were estimated by using the Image Lab 5.2 software in background subtraction mode. (a) Relative cell fluorescence was determined based on native-PAGE analysis of GFP mutants grown under mixotrophic conditions at two different light periods (6 h d^{-1} and constant light) and four light intensities (35, 100, 200 and 450 µE $m^{-2} s^{-1}$). Relative cell fluorescence is represented by the histogram and relative growth rates represented by the blue lines. (b) Relative culture volume GFP fluorescence was calculated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture**.** (c) Relative cell fluorescence detection determined based on native-PAGE analysis of GFP-PlyGBS mutants grown under mixotrophic conditions at three different light periods (6, 15 and 24 h d^{-1}) and four light intensities (35, 100, 200 and 450 µE $m^{-2} s^{-1}$). Relative cell fluorescence is represented by the histogram and relative growth rates are represented by the blue line. (d) Relative culture volume GFP fluorescence was calculated based on the maximum cell density (OD_{750}) and the per-cell-fluorescence measurements of the respective culture under mixotrophic cultivation conditions. Error bars represent the standard deviation between biological replicates of representative experiments (n=2).

Figure 5. Quantification of GFP and GFP-PlyGBS produced under the best mixotrophic light length and intensity conditions. (a) GFP mutants grown under 6 h d^{-1} at 200 µE $m^{-2} s^{-1}$ and constant light at 35 µE $m^{-2} s^{-1}$. (b) GFP-PlyGBS mutants grown under 6 h d^{-1} at 200 µE $m^{-2} s^{-1}$ and 15 h d^{-1} light period at 35 µE $m^{-2} s^{-1}$. Duplicate samples for each recombinant protein and treatment were loaded and their intrinsic GFP fluorescence was compared with bacterial recombinant GFP standards.

Figure 6. Relative GFP and GFP-PlyGBS fluorescence as a function of light levels. GFP levels shown in green. PlyGBS shown in blue. GFP mutants were grown under light periods of 6 h d^{-1} and constant light, GFP-PlyGBS mutants were grown under light periods of 6 h d^{-1}, 15 h d^{-1} L and constant light, both proteins under 35, 100, 200 and 450 µE $m^{-2} s^{-1}$ light intensity (four data points) based on which the mole of photons per day values were calculated. Relative GFP fluorescence and maximum cell density (OD_{750}) were taken from the average values of representative experiments (n= 2) normalised to 6 h d^{-1} light period at 200 µE $m^{-2} s^{-1}$. (a) Relative GFP cell fluorescence vs. mol photons $m^{-2} d^{-1}$. (b) Maximum

cell density (OD_{750}) at 4 days of the experiment vs. mol photons $m^{-2} d^{-1}$. (c) Relative GFP culture volume fluorescence vs. mol photons $m^{-2} d^{-1}$. In all conditions tested the best recombinant protein production levels were found to be below 5 mol photons $m^{-2} d^{-1}$. The grey box represents the area where the relative fluorescence or cell density were the highest.

Supplementary material

Total soluble protein: To ensure that the observed increase in recombinant protein yields was not due to an overall cellular protein increase, total soluble protein (TSP, Bradford assay) was determined for the wild-type CC125, as well as for GFP and GFP-PlyGBS expressing cell lines, under three light periods (0, 6 and 24 h of light d^{-1}). Supplementary Figure S1 shows that under dark conditions (0 h d^{-1}), TSP levels were relatively constant; upon illumination (6 and 24 h d^{-1} light periods), the wild-type accumulated more TSP than the expression cell lines. These findings confirm that the observed increase in recombinant protein accumulation is not due to an overall increase in TSP but due to a specific effect of the altered light regimes on the recombinant protein production.

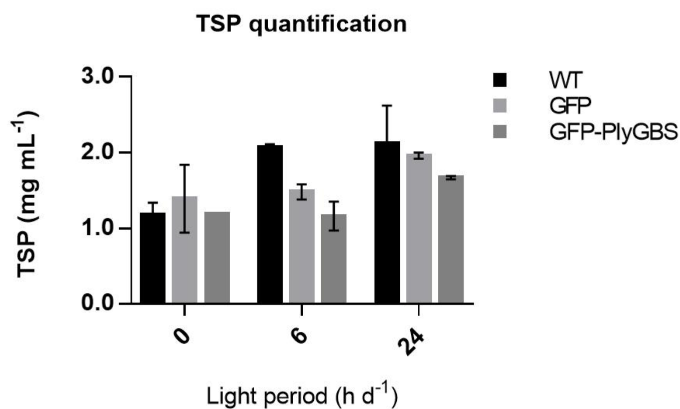

Figure S1. Total Soluble Protein (TSP) quantification. Comparative analysis of wild type and transgenic *Chlamydomonas reinhardtii* total soluble protein. Soluble proteins were extracted from wild type CC125, GFP and GFP-PlyGBS mutant cells grown under three different light periods (0, 6 and 24 h d^{-1}). TSP concentration (mg mL^{-1}) was calculated by a Bradford protein assay. Error bars represent the standard deviation (n=3).

Table S1. Maximum cell density and GFP fluorescence fold per mL calculated for GFP and GFP-PlyGBS mutants grown under different light periods and two trophic conditions.

Culture Conditions		GFP		GFP-PlyGBS	
	Light regime (light/dark hours)	**Maximum cell density (OD_{750})[a]**	**GFP fluorescence fold / mL[b]**	**Maximum cell density (OD_{750})[a]**	**GFP fluorescence fold / mL[b]**
Mixotrophic (TAP)[c]	24/0	**2.99±0.03**	1.00±0.00	**2.92±0.04**	1.00±0.00
	15/9	2.39±0.08	1.34±0.10	2.26±0.05	8.64±0.49
	12/12	2.18±0.05	1.27±0.40	2.13±0.02	7.49±1.87
	9/15	1.95±0.05	1.55±0.08	1.65±0.04	8.14±0.39
	6/18	1.66±0.07	**1.93±0.22**	1.25±0.02	**10.25±0.77**
	0/24	0.82±0.08	0.64±0.02	0.47±0.03	1.68±0.14
Photoautotrophic (PCM)[c]	24/0	**5.44±0.25**	**1.00±0.00**	**7.86±1.16**	1.00±0.00
	15/9	3.73±0.30	0.69±0.06	3.45±0.20	**2.71±0.06**
	12/12	2.38±0.11	0.58±0.14	3.42±0.18	1.94±0.47
	9/15	1.37±0.05	0.54±0.04	2.08±0.11	1.57±0.03
	6/18	0.65±0.09	0.25±0.00	1.15±0.04	1.00±0.05

[a] Average values for maximum cell density from three replicates at the end of the experimental period
[b] Average values from duplicates of cellular fluorescence per mL of culture normalised to constant light
[c] Cultures grown at approximately 180-200 µE m^{-2} s^{-1}

Table S2. Maximum cell density and GFP fluorescence fold per mL calculated for GFP and GFP-PlyGBS mutants grown under different light periods at four light intensities.

Culture Conditions			GFP		GFP-PlyGBS	
	Light regime (light/dark hours)	**Light intensity ($\mu E\ m^{-2}\ s^{-1}$)**	**Maximum cell density (OD_{750})[a]**	**GFP fluorescence fold / mL [b]**	**Maximum cell density (OD_{750})[a]**	**GFP fluorescence fold / mL [b]**
Mixotrophic (TAP)	24/0	35	3.36±0.30	**1.45±0.03**	2.93±0.02	0.80±0.12
		100	**4.49±0.36**	0.29±0.04	5.34±0.01	0.82±0.41
		200	4.43±0.42	0.22±0.06	**6.23±0.08**	0.63±0.16
		450	4.19±0.09	0.12±0.08	4.95±0.06	0.42±0.16
	15/9	35	-	-	2.59±0.01	**1.09±0.22**
		100	-	-	3.50±0.01	1.01±0.22
		200	-	-	3.42±0.03	0.96±0.03
		450	-	-	3.09±0.03	0.89±0.06
	6/18	35	1.90±0.30	1.18±0.12	1.79±0.01	0.56±0.10
		100	2.75±0.23	1.24±0.22	1.91±0.01	0.79±0.11
		200	3.04±0.52	1.00±0.00	2.15±0.01	1.00±0.00
		450	3.28±0.10	1.01±0.22	2.14±0.01	0.92±0.11

[a] Average values for maximum cell density from three replicates at the end of the experimental period
[b] Average values from duplicates of cellular fluorescence per mL of culture normalised to 200 $\mu E\ m^{-2}\ s^{-1}$ light intensity at 6 hd^{-1} light period tested