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Research Article

Improving recombinant protein production in the *Chlamydomonas reinhardtii* chloroplast using vivid Verde Fluorescent Protein as a reporter

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Microalgae have potential as platforms for the synthesis of high-value recombinant proteins due to their many beneficial attributes including ease of cultivation, lack of pathogenic agents, and low-cost downstream processing. However, current recombinant protein levels are low compared to other microbial platforms and stable insertion of transgenes is available in only a few microalgal species. We have explored different strategies aimed at increasing growth rate and recombinant protein production in the *Chlamydomonas reinhardtii* chloroplast. A novel fluorescent protein (vivid Verde Fluorescent Protein, VFP) was expressed under the control of the native *atpA* promoter/5'UTR element. VFP levels were detected by western blotting, with increased protein levels observed when co-expressed with a gene encoding the *Escherichia coli* Spy chaperone. We used these transformant lines to study the effect of temperature, light and media on recombinant protein production and cell growth. VFP levels and fluorescence, assessed by flow cytometry, allowed a determination of improved cultivation conditions as 30°C under mixotrophic mode. These conditions were tested for the accumulation of an antimicrobial endolysin (Cpl-1) of potential commercial interest, observing that the outcome obtained for VFP could not be easily replicated for Cpl-1. This study suggests that recombinant protein expression is product-specific and needs to be optimized individually.

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1 Introduction

Traditionally, the production of recombinant proteins has been carried out using bacterial and yeast fermentation systems [1], or mammalian cell cultures [2]. Fermentation-based systems are generally low-cost, robust and flexible, with well-established procedures for the expression of heterologous genes in order to produce mostly small non-glycosylated proteins. However, these microbial systems have some significant disadvantages, such as being unable to perform appropriate post-translational modifications, which are crucial for the biological functionality of many eukaryotic proteins, and with a tendency to produce the proteins as insoluble aggregates. Additionally, bacterial hosts such as *E. coli* naturally produce endotoxins, which can lead to purification obstacles and potential adverse effects for the final users. As a consequence, several restrictions are faced when considering these systems for the production of bio-products for human application. On the other hand, mammalian cell cultures, although effective in producing properly folded active proteins, are expensive and pose drawbacks such as the use of complex media, sensitivity to shear stress, accumulation of toxic metabolites, possible pathogenic contamination and relatively low productivity [3, 4]. Trans-

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Abbreviations: GOI, gene of interest; **HSM**, high salt minimal medium; **TAP**, tris-acetate-phosphate medium; **TP**, TAP medium lacking acetate; **VFP**, vivid Verde Fluorescent Protein

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genic plants have gained significant attention in the last few years as a potential host for recombinant proteins due to the high levels of protein achieved and the low cost of cultivation [5, 6]. Nevertheless, critical issues arise from the lack of transgene containment and possible allergic reactions to plant antigens.

Microalgae are attracting increasing interest as alternative photosynthetic platforms for the synthesis of high-value proteins [3, 7–12]. These unicellular eukaryotes have the same photosynthetic machinery as higher plants; however, the energy from photosynthesis is directed to cellular growth and reproduction rather than maintaining differentiated structures, and thus the level of total soluble protein can be 30–50% of dry weight biomass. Potentially, microalgae offer all the benefits of higher plants coupled with the high productivity associated with microbial fermentations [4, 12, 13]. The green microalga *Chlamydomonas reinhardtii* is the most established model species for molecular genetic studies, and is an attractive platform for recombinant protein synthesis [14, 15]. Its nuclear, chloroplast and mitochondrial genomes have been fully sequenced and annotated, and genetic tools have been developed for the creation of engineered strains [4, 16, 17]. Additionally, *C. reinhardtii* can be grown in enclosed systems under phototrophic, heterotrophic and mixotrophic conditions, keeping the operation parameters controlled and reducing concerns about transgene containment [12, 18]. Importantly, methods for transgene expression in the chloroplast of *C. reinhardtii* are well established and a number of different groups have reported successful generation of lines producing high levels of recombinant proteins [19–22]. Insertion of foreign genes into the chloroplast genome has proved to be a reliable strategy since genes can be precisely targeted to a specific locus and relatively high levels of expression can be obtained without gene silencing issues. Additionally, the chloroplast possesses a variety of chaperones and disulfide isomerases that can assist in the correct folding of complex multi-component proteins such that they accumulate in the organelle without the formation of inclusion bodies [4, 10, 23–26].

Fluorescent reporter proteins such as green fluorescent protein (GFP) and luciferases are attractive tools to study and optimize recombinant protein expression. However, attempts to-date to use such reporters in the algal chloroplast have met with limited success, with poor levels of expression reported for native genes encoding a coral luciferase [27] or GFP [28, 29]. Improvements were achieved by using codon-optimized versions of GFP [28] and a bacterial luciferase [27], and by using different endogenous 5′ untranslated regions (UTR) to drive expression of the reporter gene [29]. Alternatively, the translational fusion of the gene for bacterial luciferase to the endogenous gene encoding the large subunit of the abundant protein Rubisco resulted in significant improvements in reporter protein accumulation [30]. Recently, a newly discovered reporter termed vivid Verde Fluorescent Protein (VFP) was isolated from the coral *Cyphastrea microphthalma* found in the Australian Great Barrier Reef [31]. This 26 kDa protein exhibits a maximum excitation and emission at 491 and 503 nm, respectively, and was shown to have superior fluorescence properties in terms of brightness and time exposure when compared to GFP and its variants. When expressed in *E. coli* or microinjected into zebrafish embryos, VFP fluorescence could be readily detected [31]. We were therefore encouraged to investigate its expression and use as a reporter protein in the chloroplast of *C. reinhardtii*.

In this study, we report the successful expression of a codon-optimized version of the VFP gene under the control of the promoter/5′UTR from the endogenous *atpA* gene in the chloroplast of two different strains of *C. reinhardtii*. We observed a marked difference in the level of VFP and fluorescence between the two strains, as judged by western blotting and confocal microscopy. In an attempt to further improve VFP yields a gene for the bacterial 'stabilizer' chaperone, Spy [32], was co-expressed with the VFP gene. To further explore and maximize the yield of this fluorescent protein, different cultivation modes comprising different media and temperatures were studied. Growth rate, protein accumulation and fluorescence detected by flow cytometry were analyzed in each case, and it was possible to determine improved conditions for VFP accumulation. We finally explored if these conditions were replicable for the accumulation of a recombinant protein of commercial interest, such as the antimicrobial endolysin Cpl-1. However, conditions that were optimal for VFP were found not to be the best conditions for the accumulation of the endolysin, indicating that the specificity of each protein dictates accumulation under different cultivation conditions.

2 Materials and methods

2.1 Strains and growth conditions

Two different *Chlamydomonas reinhardtii* strains were used for transformation: strain TN72 (strain T) originates from the cell-wall deficient strain *cw15* [19], and the Bst-same strain (strain B) originates from the wild-type strain CC-1021 [33]. Both strains have the *psbH* gene disrupted by the *aadA* cassette and hence are unable to grow under phototrophic conditions [33]. Standard growth conditions [19] were mixotrophic mode in tris-acetate-phosphate (TAP) medium with a light intensity of 30 µmol photons m^{-2} s^{-1} at 25°C. For cultivation condition studies, the modes tested were TAP in the dark (heterotrophic mode), TAP lacking acetate (TP) and light (phototrophic in TP), and high salt minimal medium (HSM) and light (phototrophic in HSM). Temperature values tested were 30 and 37°C. Cell growth was monitored by measuring optical density at 750 nm (OD_{750}).

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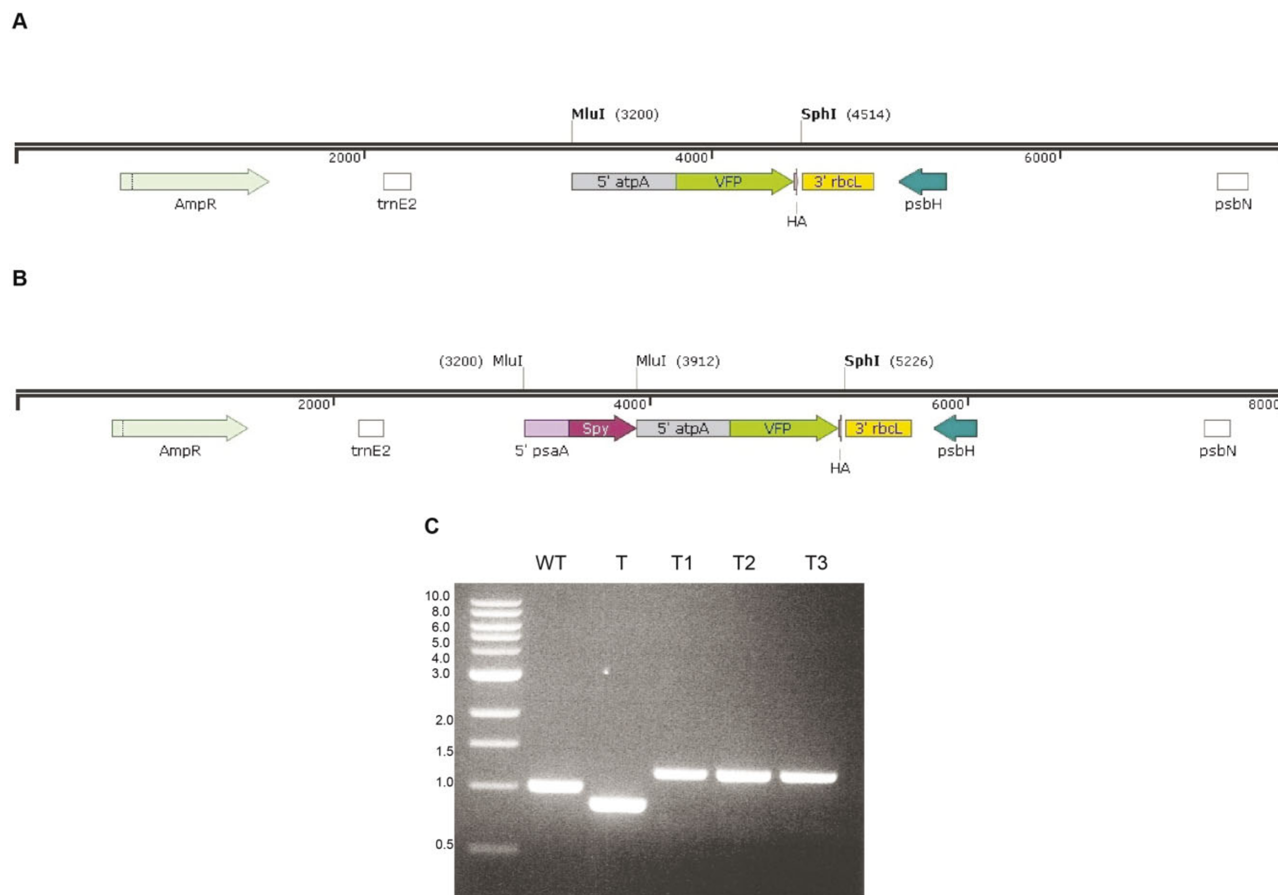

Figure 1. Schematic diagram of transformation plasmids (**A**) pASapI.VFP and (**B**) pASapI.VFP.Spy used to introduce *vfp* into the chloroplast genome of *Chlamydomonas reinhardtii*. For both plasmids *vfp* is fused to the *atpA* promoter/5' UTR and the 3' UTR from *rbcL*, and both carry a copy of the native *psbH* as the selectable marker, using phototrophic growth for selection of transformants. The presence of flanking regions from the endogenous *trnE2* and *psbN* regions ensures homologous recombination in that particular locus. (**C**) PCR analysis of transformants arising from strain TN72 transformed with pASap1.VFP in order to establish the presence of *vfp* and homoplasmy of the chloroplast genome. WT: the wild type strain CC-1021 as a control, T: the untransformed TN72 recipient, T1–T3: selected transformant lines.

2.2 Plasmid construction

Synthetic genes encoding VFP and Spy were designed for optimal translation in the *C. reinhardtii* chloroplast using the Codon Usage Optimizer program (codonusageoptimizer.org). Additionally, a sequence encoding an hemagglutinin (HA) epitope tag (YPYDVPDYA) was added to the 3' end of *vfp*, and restriction sites for *Sap*I and *Sph*I were added to the 5' and 3' ends of both genes to allow cloning into the pASapI chloroplast expression vector [19]. The genes were synthesized commercially by Geneart (Invitrogen) and cloned as *Sap*I-*Sph*I fragments into either pASapI (for *vfp*) or the related vector pSRSapI (for *spy*) that uses the *psaA* promoter/5'UTR instead of that from *atpA* [34]. This resulted in plasmids pASapI.VFP and pSRSapI.Spy. An additional plasmid, pASapI.VFP.Spy was created by excising the *psaA*-*spy* region from pSRSapI.Spy as a *Mlu*I fragment and cloning it into the *Mlu*I site of pASapI.VFP as shown in Fig. 1.

2.3 Chloroplast transformation

Chloroplast transformation was carried out using the glass bead agitation method as described in [19]. Transformants were selected based on restoration of *psbH* and recovery of phototrophy. Genomic DNA was extracted from transformant lines, and PCR analysis as detailed in [19] was carried out to confirm the homoplasmy of the plastome. (see Supporting information, Fig. S1, for the PCR strategy and primer sequences.)

2.4 Whole cell extract and western blot analysis

Whole cell extracts were prepared by resuspending samples in 0.8 M Tris-HCl pH 8.3, 0.2 M sorbitol and 1% β-mercaptoethanol v/v, and then boiling for 5 min in the presence of 10% SDS. Samples were centrifuged for 2 min at 21 000 x*g*, and the supernatant was loaded onto a 15% SDS-PAGE gel, all samples were normalized to the same

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cell density. The gel was blotted onto a Hybond ECL membrane (GE Healthcare) as described by Towbin et al. [35] using an electro-blotter (Trans-blot SD semi-dry transfer cell, Bio-rad). Western blot analysis was carried out using a rabbit anti-HA primary antibody at 1:2000 dilution and either an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody at 1:15 000 dilution when enhanced chemiluminescence (ECL) was used, or an anti-rabbit infrared dye 800 nm fluorophore linked (Li-Cor Biosciences, UK) at same dilution when infrared detection was used. For ECL detection, the membrane was incubated in the presence of SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, UK) according to the manufacturer's instructions and exposed to X-ray film. For infrared detection, the membrane was scanned using a quantitative fluorescence detection system (Odyssey Infrared Imaging System, Li-Cor Biosciences). To quantify the detected VFP, the 44 kDa commercial HA-tagged protein CARHSP1 (AbCam, UK) was included on western blots at known amounts of 50, 100 and 150 ng per well.

2.5 Flow cytometry

Flow cytometry was carried out using a Cyan ADP system (Beckman Coulter) fitted with a standard FITC filter and fluorescence was detected in the range 510–550 nm. A volume of 1 mL of each sample at an OD_{750} = 0.3 was used for each test. Control transformant lines with restored photosynthesis but lacking the VFP gene were used for every measurement to account for the auto-fluorescence of the algal cultures.

The median value obtained for the top 50% of the population was used as the parameter for fluorescence. Values above auto-fluorescence were attributed to VFP. The median value of fluorescence obtained for the VFP-expressing strain was compared to the value obtained from its negative control, and this shift was expressed as a percentage of fluorescence. Standard error was calculated for each condition measured (n = 4).

3 Results

3.1 Insertion of synthetic *vfp* into the chloroplast genome of *Chlamydomonas reinhardtii*

In order to examine whether the fluorescent protein, VFP [31] would function as a reporter in the *C. reinhardtii* chloroplast, a codon-optimized version of the *vfp* gene was designed and cloned into the chloroplast expression vector pASapI [19]. As shown in Fig. 1A, *vfp* is under the control of the promoter/5' UTR element from the endogenous *atpA*, together with the 3' UTR from the *rbcL*. As antibodies to VFP were unavailable, the *vfp* coding sequence was extended to include a single copy of the 9-residue hemagglutinin (HA) epitope to allow detection with anti-HA antibodies. The plasmid pASapI.VFP was further modified by introduction of a second transgene encoding a codon-optimized version of the *E. coli* Spy chaperone under the control of the *psaA* promoter/5' UTR (Fig. 1B). This small chaperone has been shown to aid protein folding [32] so it was of interest to see if VFP levels could be improved by Spy.

Chloroplast transformants were obtained by rescue of two different photosynthetic mutants that are derived from different lab strains, with Bst-same derived from a wild-type line and TN72 from a *cw15* mutant that is defective in cell-wall synthesis. Each strain was previously engineered to contain a disrupted copy of the chloroplast gene *psbH* that encodes an essential subunit of photosystem II. Selection for transformants is therefore based on restoration of phototrophy using the *psbH* carried on the plasmid and integration of the transgene(s) into a neutral locus between *trnE2* and *psbH* [19].

Transformant lines were recovered after three weeks and were subjected to three rounds of selection under phototrophic conditions to achieve homoplasmy of the polyploidy chloroplast genome [20]. Confirmation of this was obtained using a PCR-based assay described in detail in [19], as illustrated in Fig. 1C for pASapI.VFP transformants of strain TN72. In the PCR assay, a product of 1.0 kb is seen for a WT strain with an intact *psbH*, whereas the TN72 recipient generates a 0.85 kb band. Successful transformation of TN72 with the plasmid carrying the gene-of-interest (GOI) gives rise to a 1.2 kb band as seen in Fig. 1C. The absence of any detectable 0.85 kb PCR product from the three transformed lines (T1-T3) strongly indicates that the lines are homoplasmic for the engineered change. Homoplasmic transformant lines were similarly obtained for the other combinations of plasmid and recipient strain (Supporting information, Fig. S2).

3.2 VFP detection in the different transformant lines

VFP accumulation was assayed by western blotting using anti-HA antibodies, detecting a polypeptide of approximately 26 kDa. Loading was normalized to cell density in all analyses. Different transformants obtained for the same plasmid and strain showed an equivalent level of VFP, as observed in Fig. 2A for three lines obtained from the cell wall-deficient strain TN72 transformed with pASapI.VFP. A similar profile was observed for the transformants obtained from strain Bst-same, with equal levels of protein detected for each cell line (Supporting information, Fig. S3). In order to compare VFP accumulation in the two different genetic backgrounds, one representative transformant line from TN72 and Bst-same transformed with pASapI.VFP were directly compared at different time-points during growth (Fig. 2B). Surprisingly, a markedly lower VFP level is observed in samples from Bst-same in comparison to TN72 at all stages of cultivation, despite

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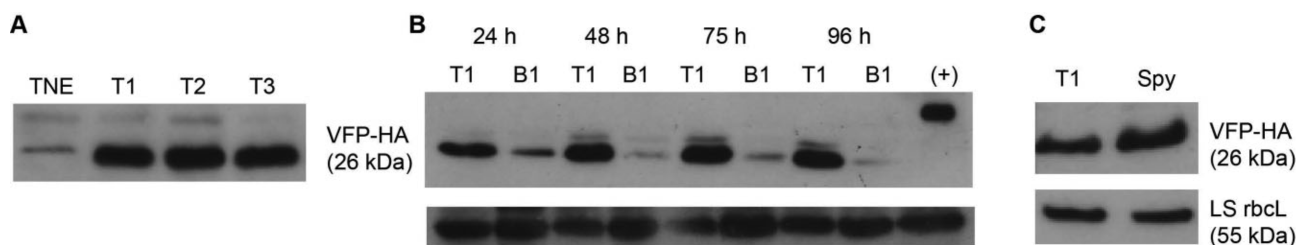

Figure 2. Western blot analysis of cell extracts using anti-HA antibodies to determine VFP accumulation in the different lines. All samples were normalized to cell density. (**A**) Three TN72 transformants obtained using pASapI.VFP (T1, T2, T3) or the empty pASapI vector (TNE), asa negative control (note: the band at ~27 kDa is an underlying non-specific band). (**B**) VFP accumulation at different stages of cultivation (numbers indicate hours) in a transformant obtained using pASapI.VFP using either TN72 as recipient (T1) or Bst-same (B1). The positive control (+) is from another TN72 transformant expressing a 40 kDa HA-tagged protein. (**C**) VFP accumulation in TN72 transformed with pASapI.VFP (T1) or pASapI.VFP.Spy (Spy), in which an approximately two-fold increase in VFP accumulation is observed in the presence of the Spy chaperone. In (**B**) and (**C**), the lower band shows binding with antibody against the large subunit of Rubisco, as a loading control.

the fact that both strains contain the same *atpA-vfp-rbcL* transgene construct introduced into the same chloroplast locus, and both strains were restored to phototrophic growth using the same selection strategy. We attribute this variation to genetic differences in the nuclear genome of one or other strain that affect chloroplast gene expression or protein stability, although genetic studies to demonstrate Mendelian inheritance of the low-expression phenotype [36] are required to establish this. Nevertheless, this finding underscores the importance of strain choice when engineering the chloroplast genome. In the light of these results, along with a lower fluorescence observed by confocal microscopy (Supporting information, Fig. S4) Bst-same was not used further and VFP expression was analysed exclusively in TN72.

Fig. 2C and Fig. S5 (Supporting information) present an initial examination of whether the *E. coli* Spy chaperone [32] could be synthesized in the chloroplast and whether its presence could improve VFP levels by aiding the folding of VFP into a stable form. As shown in Fig. S5, TN72 transformants expressing *spy* alone do accumulate detectable levels of the bacterial chaperone in the chloroplast. Therefore, a representative TN72 transformant generated using the *spy+vfp* construct shown in Fig. 1B was compared to a transformant expressing *vfp* alone (Fig. 2C). A comparison of levels using the large subunit of Rubisco as a loading standard showed an increase of approximately two-fold, indicating that the presence of the chaperone does aid in the accumulation of the recombinant protein.

To verify the functionality of VFP, flow cytometry was used to assess relative fluorescence. Cultures of the two strains shown in Fig. 2C were compared against a control strain TNE (TN72 transformed with the empty pASapI plasmid) and the shift of the median value of fluorescence was recorded as the fluorescence intensity of the *vfp*-expressing strain. The values of fluorescence shift obtained were 19.17% (SEM ± 0.83; $n = 4$) for the *vfp* only transformant (T1) and 22.30% (SEM ± 2.62; $n = 4$) for the *spy+vfp* transformant (Spy). The Spy strain therefore has a small increase in fluorescence emission, which correlates with the increase in protein level observed in the western blot.

3.3 Cultivation conditions and their effect on cell growth and VFP accumulation

Four different cultivation modes were evaluated in order to assess their influence on cell growth and protein accumulation. These were (i) mixotrophic growth in acetate-containing medium (TAP); (ii) heterotrophic growth in TAP medium (dTAP); (iii) phototrophic growth in TAP medium lacking acetate (TP); and (iv) phototrophic growth in an alternative medium – high salt minimal medium (HSM). Growth rates for strain T1 are given in Table 1, and show that mixotrophy yields the highest specific growth rate. There is also a pronounced difference in maximum cell density achieved providing a large advantage of mixotrophic growth in terms of biomass accumulation. *C. reinhardtii* is typically cultured at 25°C [15]. However, as shown in Table 1, an increase in temperature to 30°C has no detrimental effect on specific growth rate and maximum cell density. Indeed there is an increase of ~20% in growth rate under phototrophic conditions. On the other hand, a culture temperature of 37°C negatively affected both specific growth rate and final cell density. The most pronounced effect of the higher temperature was observed in mixotrophic mode, where maximum cell density determined at 96 h of cultivation dropped by ~40%. Importantly, we observed the same growth rates and maximum cell densities under all conditions studied for the control strain TNE, which demonstrated that there is no detrimental effect on growth attributable to VFP expression.

The effect of the different cultivation conditions was also evaluated for protein accumulation, and for this purpose strain T1 was grown under the four conditions at 25°C. A set of western blots carried out with samples harvested at different time points indicated that the protein level is consistently highest when cells are grown in het-

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Table 1. Specific growth rate and maximum cell density obtained for the *vfp*-expressing strain T1 grown at three different temperatures (25, 30 and 37°C) in different cultivation modes: tris-acetate-phosphate medium and light (TAP); tris-acetate-phosphate medium in the dark (dTAP); TAP medium lacking acetate and light (TP); and high salt minimal medium and light (HSM). Cells were grown in 25 mL flasks at 120 rpm of agitation and 30 μmol photons m^{-2} s^{-1}. Specific growth rates at stationary phase are an average from three independent experiments (SEM < 0.003 for all conditions).

Cultivation mode	25°C		30°C		37°C	
	Specific growth rate [h^{-1}]	Maximum cell density [OD_{750}]	Specific growth rate [h^{-1}]	Maximum cell density [OD_{750}]	Specific growth rate [h^{-1}]	Maximum cell density [OD_{750}]
Mixotrophic (TAP)	0.044	4.06	0.044	4.03	0.038	2.48
Heterotrophic (dTAP)	0.036	1.84	0.034	1.55	0.035	1.57
Phototrophic in TP (TP)	0.023	1.59	0.027	2.12	0.020	1.14
Phototrophic in HSM (HSM)	0.023	1.48	0.026	1.79	0.020	1.12

erotrophic mode (Fig. 3A). In addition, the VFP level appears to remain approximately constant at all time points measured. Considering this observation, samples for subsequent studies were harvested after 72 h of cultivation, when cultures were reaching stationary phase.

When cells were grown at 30 and 37°C, it was possible to observe an increase in protein accumulation over that obtained at 25°C for the mixotrophic condition only, with the remaining three cultivation conditions exhibiting similar protein levels at all temperatures analysed (Fig. 3B). Fluorescence was measured at 72 h of growth in all these cultivation conditions by flow cytometry and the shift in fluorescence detected for each strain and condition is presented in Fig. 3C. As observed, there is no major difference in the fluorescence emitted by both the T1 and Spy strains under the different cultivation conditions, which is in agreement with the western blot results. There appears to be a further increase in fluorescence for Spy at 30°C, which might indicate a stronger influence of the chaperone at this temperature; however, further work is needed to verify this finding.

In order to estimate the concentration of VFP produced, the commercial HA-tagged protein CARHSP1 was used in western blot analysis. This 44 kDa protein was loaded at three different concentrations and the intensity of the bands compared to the intensity of the bands obtained for T1 cultures grown in different conditions (Fig. 3D). As a result, we estimated the VFP level to be 1.65 mg L^{-1} culture in mixotrophic mode; 0.66 mg L^{-1} culture in heterotrophic mode; 0.63 mg L^{-1} culture in phototrophic (TP) mode, and 0.56 mg L^{-1} culture in phototrophic (HSM) mode. This data confirmed that the mixotrophic mode produces the highest protein level per volume of culture, which is related to the higher cell density achieved in this condition. When protein concentration is defined per unit of biomass, then the heterotrophic mode produces the highest VFP level (3.3 mg g^{-1} dry biomass).

With the data gathered for both growth rate and maximum cell density, together with VFP accumulation, we concluded that the highest protein level is obtained in mixotrophic cultivation at 30°C. We replicated these cultivation conditions using the chloroplast transformant TN72-Cpl in which the GOI encodes the antimicrobial endolysin Cpl-1 rather than VFP (H. Taunt and S. Purton, unpublished). TN72-Cpl was grown in the four different cultivation modes at 25 and 30°C and samples were analysed by western blot analysis. As seen in Fig. 3E, the accumulation of Cpl-1 at 25°C followed the trend observed for VFP, with higher protein level in mixotrophic mode. However, this difference was less pronounced at 30°C. Furthermore, less Cpl-1 protein accumulated at the higher temperature. This finding probably reflects differences in the efficiency of synthesis, folding or stability of VFP and Cpl-1 at the two different temperatures, and highlights the need to optimize temperature for the production of different recombinant proteins in the *C. reinhardtii* chloroplast.

4 Discussion

4.1 Recombinant protein accumulation

The chloroplast of *C. reinhardtii* has significant potential as a platform for the synthesis of valuable therapeutic proteins [1] although there is still a need to develop improved molecular tools for the high-level and regulated expression of transgenes [18]. Furthermore, a better understanding of parameters that influence recombinant protein levels is required to maximize productivity. Here we have developed a codon-optimized reporter gene and shown that *vfp* can be integrated and successfully expressed in the chloroplast genome. Additionally, the co-expression of a codon-optimized gene for the *E. coli* Spy chaperone appears to enhance VFP levels and suggests that such heterologous chaperones could aid in the stable accumulation of recombinant proteins in the chloroplast. Using VFP we have revealed a previously unreported issue with chloroplast transformation; namely the issue of strain variation on recombinant protein levels. All transformants generated using Bst-same as the recipient showed very low levels of VFP, whereas TN72 transformants showed levels at least ten-fold higher, despite

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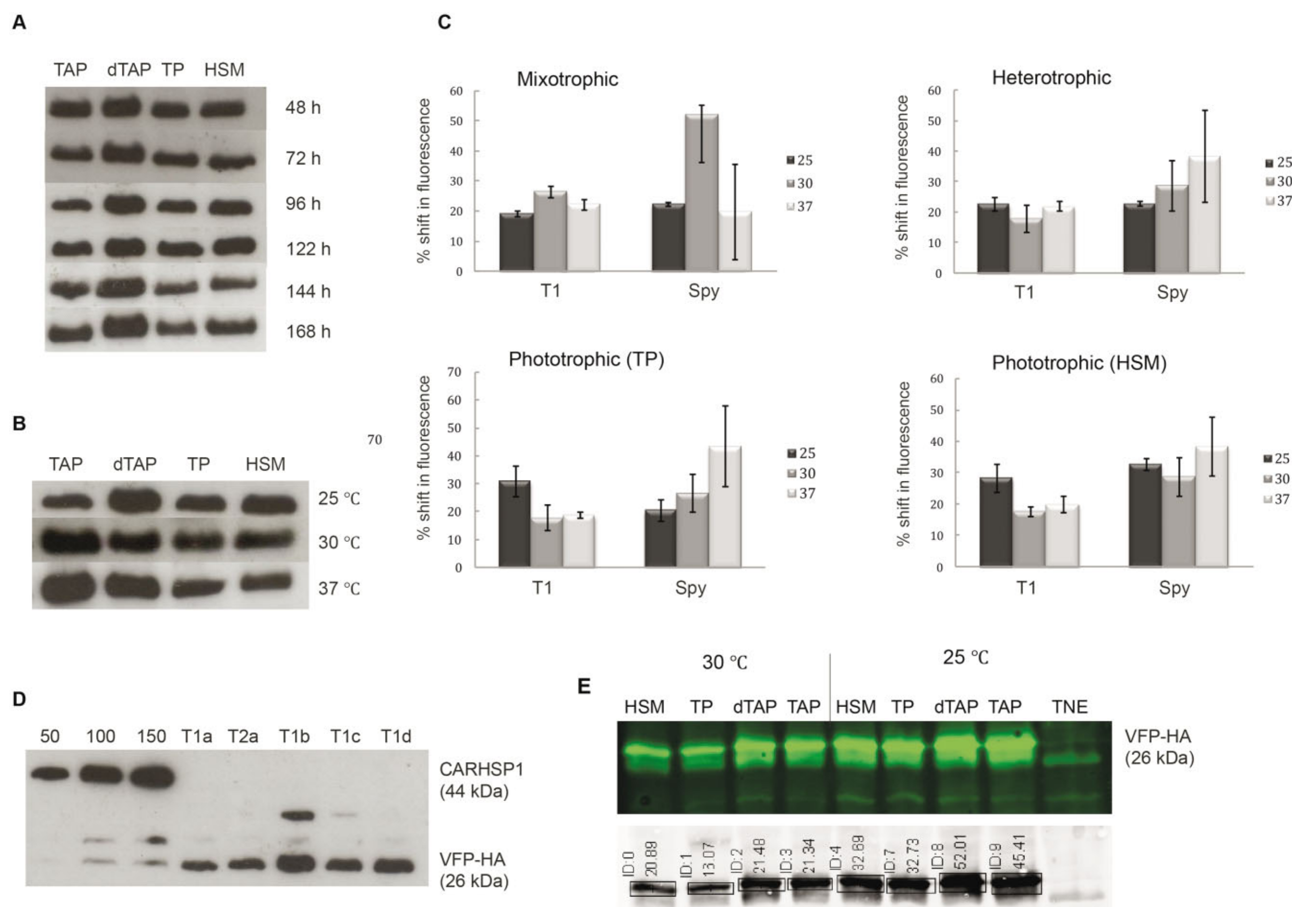

Figure 3. (**A**) VFP accumulation in TN72 transformed with pASapI.VFP (T1) at different stages of cultivation, indicated on the right side in hours. Cultures were grown in 25 mL shake flasks at 25°C, 120 rpm and 30 μmol m^{-2} s^{-1} in the four cultivation modes tested: mixotrophic (TAP), heterotrophic (dTAP), phototrophic with TP medium (TP), and phototrophic with HSM medium (HSM). Lanes were loaded with the same concentration of cell lysate, and equal loading was confirmed by blotting with antibodies to the Rubisco large subunit (not shown). (**B**) Comparison of VFP accumulation in T1 grown at different temperatures (25, 30 and 37°C). Cultures were grown as indicated in (**A**) and wells were loaded with the same concentration of cell lysate. (**C**) Fluorescence emission detected in the two strains developed for expression of VFP (T1 and the transformant 'Spy' co-expressing the chaperone) grown under the four different cultivation modes at the three different temperatures studied. Samples were harvested at late exponential phase (72 h) and fluorescence gathered is normalized to cell count (1×10^6 events). Values represent the shift in fluorescence in comparison to the respective control strain grown under the same condition. Error bars represent the standard error ($n = 2$). (**D**) Estimation of protein concentration in T1 using the HA-tagged standard protein CARHSP1 (44 kDa). Cultures were grown as described in (**A**). Samples, from left to right, are: CARHSP1 50/100/150 ng per well; transformants T1 and T2 grown in mixotrophic mode (T1a and T2a); T1 grown in heterotrophic mode (T1b); T1 grown in phototrophic mode in TP (T1c); and T1 grown in phototrophic mode in HSM (T1d). Samples were harvested in late exponential phase and lanes loaded with the same concentration of cell lysate. (**E**) Accumulation of endolysin Cpl-1 in a TN72 transformant line generated using a pSAapI.Cpl plasmid. The cell line was grown under the four different cultivation modes. Cultures were grown as detailed in (**A**) and protein was measured by infrared detection. The unspecific lower band serves as a loading control. The bottom gel shows the quantification values according to band intensity.

using an identical transformation strategy. The reason for this marked difference is unclear, although it is unlikely to be related to the presence/absence of a cell wall in the two strains and more likely reflects acquired mutations in one or other strain that influence gene expression or protein turnover in the chloroplast.

4.2 Fluorescence in microalgal whole cells

Whilst VFP was readily detectable by western blotting in the TN72 transformants, and fluorescence from the reporter protein was observed, the level of fluorescence was disappointingly low. Furthermore, the two-fold increase in protein level in the presence of Spy is not reflected in a similar increase in fluorescence. The reasons for this are unclear, but might reflect problems in the post-translational formation of the chromophore, or auto-fluorescence interference and/or signal quenching by the chlorophylls, flavonoids and carotenoids present in the algal cell. A recent report has highlighted the poor signal-to-noise ratio in *C. reinhardtii* of green fluorescent proteins and shown that proteins with fluorescence emission

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at longer wavelengths show greatly increased signal-to-noise ratios [37]. This study expressed the transgenes from the nucleus, but the findings suggest that longer wavelength variants of the GFP family, or a red fluorescent reporter such as ds-Red would also be more effective than VFP as visual reporters of chloroplast gene expression.

4.3 Effect of cultivation conditions on recombinant protein production

Our study of different cultivation conditions using VFP as a reporter did allow us to assess the impact of different parameters such as cultivation mode and temperature on cell growth, cell density and protein accumulation. We observed that a significantly higher cell density at stationary phase can be achieved using mixotrophy, in comparison to the other cultivation modes, a phenomenon that has been previously estimated by flux balance analysis [38] and observed experimentally for biomass yield [39]. On the other hand, protein accumulation appears to be favored under heterotrophic growth and provided the highest protein concentration, of 3.3 mg VFP g^{-1} dry biomass, which is twice as much protein as produced in the mixotrophic condition (1.62 mg VFP g^{-1} dry biomass). This difference could result from a decreased activity of light-induced proteases under heterotrophic cultivation, which would reduce protein degradation and hence increase accumulation. Additionally, it could be argued that the slower growth rate and lower cell density achieved in heterotrophic condition leads to a higher degree of protein synthesis, since the carbon source is still being utilized. Furthermore, mixotrophic cultivation produced slightly less protein (1.62 mg VFP g^{-1} dry biomass) than phototrophic growth (2 mg VFP g^{-1} dry biomass), and this is in agreement with other published results [40].

Three different temperatures were studied, and an increase in temperature to 30°C resulted in a similar specific growth rate and cell density as at 25°C, and slightly higher protein accumulation under mixotrophic cultivation. A further increase in temperature to 37°C showed a similar protein level, although at this temperature the specific growth rate and cell density were severely affected, and the increase in protein accumulation does not compensate for this biomass decrease. A similar negative effect on cell growth has been reported elsewhere [41] when *C. reinhardtii* cells were grown at 38°C; however, this research also reported an increase in biomass accumulation at 32°C, which we did not observe at 30°C. Importantly, our study of a second recombinant protein, Cpl-1, demonstrates that conditions need to be optimized for different recombinant proteins, since the optimal conditions for VFP accumulation (30°C and mixotrophic growth) were not mirrored by Cpl-1.

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