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Effect of biphasic temperature regime on therapeutic recombinant protein production in the green alga *Chlamydomonas reinhardtii*

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Abstract

Microalgae are increasingly being considered for recombinant protein production because of low cultivation costs, absence of endotoxins and insusceptibility to human infectious agents. Despite these advantages, the yield of recombinant protein produced in microalgae is still low compared to more established expression systems and optimization at the genetic and cultivation levels is required for this new system to be economically viable. This study investigates the effect of biphasic temperature regimes on the yield of recombinant human interferon alpha 2a (IFN-α2a), a therapeutic protein known for its anti-cancer and anti-viral properties, produced by the model green alga *Chlamydomonas reinhardtii* (Cr.IFN-α2a). Biphasic growth is commonly employed to increase recombinant protein production in mammalian cell lines used for commercial production of therapeutic proteins, with a lowering of the temperature resulting in higher yields. In this study, lowering the temperature from 25°C to 15°C in mid-exponential growth phase increased the accumulation of Cr.IFN-α2a by 3.3-fold whilst it slowed down the growth of the three *C. reinhardtii* transgenic lines tested. In contrast, a rise of temperature from 25°C to 35°C accelerated cell growth, while negatively impacting the production of Cr.IFN-α2a. After a two-step chromatography purification, the Cr.IFN-α2a produced was estimated to be 53% pure with a yield of 90 µg/L of culture. The amino acid sequence of Cr.IFN-α2a was confirmed by mass spectrometry. However, the anti-viral activity of Cr.IFN-α2a was found to be 10 times lower than the human IFN-α2a standard produced using *E. coli* when challenged in a cytopathic effect (CPE) assay, likely due to the formation of aggregates. While the molecular mechanisms driving the accumulation of Cr.IFN-α2a at lower temperature remains unclear, our results support that reducing the temperature at the peak of expression is a valid strategy to increase the yield of recombinant Cr.IFN-α2a in *C. reinhardtii*.

Keywords: Biphasic growth; *Chlamydomonas reinhardtii*; nuclear transgenes; recombinant protein; interferon-alpha 2a.

1. Introduction

The model Chlorophyta (green algae) *Chlamydomonas reinhardtii* has recently attracted interest as a novel platform for the production of human therapeutic proteins [1]. Green algae offer advantages such as low production costs, scalability and the absence of endotoxins and human pathogens [2]. Endotoxins are lipopolysaccharides associated with the outer membrane of certain Gram-negative bacteria (including *Escherichia coli*), which cause an immunogenic response in humans and thus pose a problem in bacterial-derived therapeutic recombinant protein manufacturing. Endotoxins can trigger illness as serious as septic shock in patients and need to be carefully separated from the therapeutic, adding to the manufacturing time and cost [3]. In addition to being endotoxin-free, green algae have the ability to perform eukaryotic post-translational modifications such as glycosylation, which is often necessary for the production of biologically active and stable bio-pharmaceuticals [4]. While algal post-translational mechanisms have yet to be engineered to match that of mammalian glycosylation pattern for human therapeutics applications, the presence of post-translational machinery is a strong starting point. Despite the above-mentioned advantages, the production of recombinant proteins in *C. reinhardtii* is still in its infancy and the yield of recombinant protein has to be greatly improved for this system to be economically viable [5]. Large knowledge gaps surrounding the optimization of this new expression platform remain, and in particular the effect of culture conditions on the production of recombinant proteins.

The production of recombinant proteins in nucleus-transformed *C. reinhardtii* is generally associated with low transgene expression levels [6]. Possible reasons for poor transgene expression include (i) limited number of strong promoters; (ii) random integration leading to possible re-arrangements in the transgenic construct and regulatory sequences interruption in the host genome, insertion of multiple transgene copies and 'position effects', where the genomic region surrounding the transgene influences its level of expression [7, 8]; (iii) transgene silencing at both the transcriptional and post-transcriptional levels [9, 10]. While research is progressing to address these challenges, with for instance the implementation of introns and the foot-and-mouth-disease-virus 2a self-cleavage peptide [11, 12], knowledge on the manipulation of culture conditions to mitigate the aforementioned factors is limited. *C. reinhardtii* is tolerant to environmental variations (e.g., light, carbon source, temperature, etc.) [13], thus allowing for an array of cultivation modes to be explored. Few studies have compared the effects of phototrophic, heterotrophic and mixotrophic conditions on the production of recombinant proteins from nuclear transgenes in *C. reinhardtii*, with mixotrophy often yielding the highest recombinant protein quantity [5, 14]. To further inform bioprocessing strategies for recombinant production in *C. reinhardtii*, a biphasic temperature regime was developed in this study. In a biphasic temperature cultivation mode, cells are first grown under optimal temperature conditions for biomass generation and to initiate the production of the recombinant protein, this is then followed by a second phase whereby temperature is shifted to enhance and/or prolong the recombinant protein production phase. Given that *C. reinhardtii* cell division is arrested at temperatures above 39°C and below 14°C with temperature becoming lethal lethal below 0°C [15, 16], we selected the following temperature regimes: 15°C, 25°C (optimum) and 35°C. Schroda et al. [17] reported an increase in transgenes expression after heat-shock under the control of the *HSP70A/RBCS2* fusion promoter with *C. reinhardtii*. The same promoter was used in the current study, thus we hypothesized that an increase of

temperature could potentially enhance recombinant protein production. A decrease in temperature was also investigated, as it has proven to be effective in mammalian and bacterial systems [18-23]. Cultivation of mammalian cells at low temperatures is an established method used to increase recombinant protein yields, while maintaining or improving post-translational modifications [18-21]. Studies on Chinese hamster ovary (CHO) cell lines commonly used for commercial production of therapeutic proteins have shown that a temperature shift from 37°C to 31°C can increase recombinant protein yield by prolonging the protein production phase, maintaining cell viability, reducing apoptosis and protease activity [18, 20, 24], while also reducing growth rate and cell metabolism [18, 19, 24]. In bacterial systems, the induction of recombinant protein production is often performed at reduced temperatures following a shift from 37°C to temperatures ranging from 4 to 30°C [22]. Lowering the temperature after induction has shown to significantly enhance recombinant protein solubility, stability and folding [23].

The effect of temperature on recombinant proteins production has been previously investigated in chloroplast transgenes of *C. reinhardtii* [25]. Braun-Galleani et al. tested temperatures of 25°C, 30°C and 37°C in a monophasic mode (i.e. the temperature was kept constant during growth). Their results indicated that vivid Verde Fluorescent Protein (VFP) co-expressed with a gene encoding the *Escherichia coli* Spy chaperone was slightly more abundant at 30°C, while accumulation of a recombinant anti-microbial endolysin (Cpl-1) was more pronounced at 25°C under mixotrophic conditions. The authors concluded that culture optimisation of chloroplast transgenes is product-specific and needs to be optimized individually [25]. To our knowledge, the current study is the first to investigate the effect of biphasic growth regime on nuclear transgene of *C. reinhardtii*. Donnan et al. in 1985 reported that a biphasic temperature regime, where the temperature of wild-type *C. reinhardtii* 137c cultures was shifted from 30°C to 20°C,

resulted in a lower growth rate, while the opposite was observed for a temperature shift from 20°C to 30°C [26]. The authors noticed that the temperature change did not affect the commitment to divide once the daughter cells started growing. This impact of temperature on the rate of general metabolic processes is likely to affect the production of recombinant proteins in *C. reinhardtii* transgenic cell lines.

The human interferon alpha 2a (IFN-α2a) was chosen for use in this study as it is a relatively small soluble protein, consisting of 165 amino acids (~19 kDa), making it an appropriate model for the optimisation of a new recombinant expression system. IFN-α2a is recommended for the treatment of Chronic hepatitis B or C, hairy cell leukemia and chronic myelogenous leukemia [27, 28]. Since its initial approval by the Food and Drug Administration (FDA), IFN-α2a has established itself as a powerful anti-viral and anti-tumor treatment [27]. Effectiveness in treating the aforementioned conditions has made recombinant IFN-α2a a highly valuable therapeutic with a global market of 390 million US$ in 2018 [29]. IFN-α2a is currently produced recombinantly in *E. coli* (non-PEGylated ROFERON-A® and PEGylated Pegasys®, Roche) and the yeast, *Hansenula polymorpha* (PEGylated Reiferon Retard®, Rhein-Minapharm) [27]. Although the human IFN-α2a is naturally *O*-glycosylated in Thr106 [30, 31], the commercialized versions of IFN-α2a are not glycosylated. The absence of glycosylation does not affect the overall activity of the drug, but it leads to short plasmatic half-life due to sensitivity to proteases necessitating higher dosages and more frequent treatments [32, 33]. The ability to produce IFN-α2a in microalgae could prove useful in mitigating both the downstream refolding required for *E. coli* expressed proteins, as well as some of the cultivation costs and post-translational limitations associated with these expression systems. In this study, the glycosylation pattern of IFN-α2a was not investigated.

The *HSP70A/RBCS2* hybrid synthetic promoter used in this study is considered constitutive, although it is known to be most active during exponential growth phase with reduced expression observed once cultures enter the stationary phase [14, 34]. Therefore, we first investigated the production of IFN-α2a in transgenic cells of *C. reinhardtii* cultivated at optimal temperature (25°C) to identify the growth stage at which the accumulation of IFN-α2a was the highest. Once the production of IFN-α2a was reported to be maximal, the temperature was either dropped to 15°C or increased to 35°C to prolong or potentially increase the production of IFN-α2a. The results generated in this study will contribute to developing efficient cultivation strategies to maximize recombinant protein production in *C. reinhardtii* and to establish microalgae as a commercial platform for recombinant protein production.

2. Material and Methods

2.1. *C. reinhardtii* strain and culture conditions

The CC-125 wild type mt+ [137c] *C. reinhardtii* strain used in this study was obtained from the GeneArt® *Chlamydomonas* protein expression kit (Thermo Fisher Scientific). Strain 137c was axenically cultivated in Tris-acetate-phosphate (TAP) liquid medium [35] or TAP agar plates. The transformed cell lines were maintained on TAP plates supplemented with 5 - 15 µg mL^{-1} of Zeocin™ (Jomar Life Research, Australia) to keep a selective pressure. Liquid cultivation was performed in baffled flasks under continuous light (50 µmol photons m^{-2} s^{-1}) at 100 rpm. The temperature was set at 25°C, unless otherwise noted. The experiment described in this study was carried out on three independent cell lines (n = 3) grown at 25°C for 48 hours after inoculation (cell density of 3-4 x 10^4 cells mL^{-1}) before changing the temperature to either 15°C or 35°C, while keeping the controls at 25°C. Cells were sampled every 24 hours for cell

density (microscopic cell count), protein quantification (immunoblot) and gene expression analysis (qPCR).

2.2. Vector design

The pChlamy_4 backbone (GeneArt® *Chlamydomonas* protein expression kit, Thermo Fisher Scientific), designed to engineer *C. reinhardtii* nuclear genome, was used in this study. The transcript sequence of the human interferon-alpha 2a gene (https://www.drugbank.ca, Accession: DB00034) was optimised to match *C. reinhardtii* codon usage (Life Technologies Australia, Table S1), amplified by PCR (Phusion® High-Fidelity DNA Polymerase, New England Biolabs) using primers (forward: 5'-ATATAGAATTCATGTGCGACCTGCCCCAGACCCA-3' and reverse: 3'-ATATATTCTAGATTACTCCTTGCTGCGCAGGC-5'). The resulting amplicon was cloned into the pChlamy_4 vector between the EcoRI and XbaI restriction sites, resulting in *ifn-α2a* gene to be fused, via a foot and mouth disease virus (FMDV) 2a self-cleavage peptide, to the bleomycin resistance gene (*sh-ble)* from *Streptoalloteic hushindustanus* [12] (Figure S1) and confirmed by Sanger sequencing. Expression of the recombinant *ifn-α2a* gene was mediated by the chimeric constitutive *HSP70A/RBCS2* promoter with the 3'untranslated region (3'-UTR) from the ribulose bisphosphate carboxylate oxygenase small subunit 2 (*RBCS2*) [12]. The produced recombinant IFN-α2a protein was fused to a 6xHis tag at its C-terminus for immunoblot detection and purification purposes. The assembled plasmid was propagated in One Shot® TOP10 Chemically Competent *Escherichia coli* (Thermo Fisher Scientific) in Luria broth (LB) with 100 μg mL^{-1} of ampicillin, purified (Zyppy Plasmid Miniprep Kit, Zymo Research), and confirmed by Sanger sequencing, prior transformation of *C. reinhardtii*. An empty pChlamy_4 vector was also transformed into *C. reinhardtii* as control.

2.3. *C. reinhardtii* transformation and screening

C. reinhardtii strain 137c cells were transformed by electroporation (Gene Pulser Xcell™, Bio-Rad) following the instructions of the GeneArt® *Chlamydomonas* protein expression kit (Thermo Fisher Scientific). After electroporation, the cells were recovered for 16 hours in 10 mL of TAP medium supplemented with 40 mM sucrose, before being plated onto TAP agar plates containing 5 µg mL^{-1} of Zeocin™ and grown for 7-10 days (constant light, 50 µmol photons m^{-2} s^{-1} and 25°C). The transformants were then transferred onto TAP agar plates containing 15 µg mL^{-1} of Zeocin™ as a pre-selection step prior colony PCR. This step increased chances to select for transformants exhibiting high level of expression of the gene of interest. Indeed, the major benefit of the *she-ble FMDV2A* construct is that high-level of zeocin resistance correlates with higher fusion protein expression levels as zeocin is stoichiometrically sequestered [12, 36]. The correct insertion of the *ifn-α2a* transgene was then confirmed by screening 304 colonies by PCR (GoTaq® Flexi DNA Polymerase, Promega) with *ifn-α2a* specific primers (described in section 2.2.). Detection of the recombinant IFN-α2a protein in 118 positive colonies was performed by western blotting and immuno-detection as described in section 2.6. Three cell lines harbouring the full transgene (as confirmed by Sanger sequencing on *C. reinhardtii* genomic DNA) and producing interferon alpha 2a (Cr.IFN-α2a, "Cr" for *C. reinhardtii*) were selected for use in this study.

2.4. Automated cell counts

Algal suspension (500 µL) were sampled every 24 hours from each biological replicate. Cells were fixed with 0.22 µm filtered glutaraldehyde (final concentration of 2%) and stored at 4°C. The automated microscopic cell count was performed as described by Tran et al. [37].

2.5. Gene expression analysis (RT-qPCR)

Algal suspension (40 mL) were collected before temperature shift (48 h) and after temperature shift (72 h and 96 h). *C. reinhardtii* cells were pelleted at 3,000 x g for 3 min at 4°C, rinsed once with phosphate-buffered saline (PBS) and the pellets were snap frozen in liquid nitrogen. These were stored at -80°C until further analysis. RNA extraction and data acquisition were performed as previously described [38] with 1:10 dilutions of cDNA from each sample. To ensure that the cDNA samples were devoid of genomic DNA contamination a "no template" control, as well as a "no reverse transcriptase" control were included. The primers were designed using the software Primer3 version 4.1.0 (http://bioinfo.ut.ee/primer3/; Table 1) [39]. The *ifn-α2a* primers targeted the 3'-end of the transgene, including the His-tag, to correlate with immunoblotting results. Expression levels of the target gene (*ifn-α2a*) were normalized against two reference genes (*cblp* and *rpl13*; Table 1) to obtain normalized relative quantities (NRQ).

Table 1. Sequence-specific primers used in this study for RT-qPCR analysis. Gene IDs, primers sequences, amplicons length, melting temperatures, and RT-qPCR efficiencies are indicated. *ifn-α2a*: codon optimized human interferon alpha 2a gene (including 6x His-tag); *cblp*: *Chlamydomonas* β subunit-like polypeptide gene; *rpl13*: ribosomal protein L13 gene. The RT-qPCR efficiency for each primer set was determined from a cDNA dilution gradient of 27, 9, 3 and 1 ng and a linear regression model [40-42].

ID	Reference	Primer forward sequence	Primer reverse sequence	Amplicon length (bp)	Tm (°C)	Efficiency (%)
ifn-α2a	This study	AGGAGGGTCTAGAGGGCAAG	GTGGTGGTGGTGGTGGTG	83	60	93
cblp	Zhao et al., [43]	TGCTGTGGGACCTGGCTGA	GCCTTCTTGCTGGTGATGTTG	193	61.5	98
Rpl13	Whitney et al., [44]	AGCACGGCTAGAGACAGATG	TAGTGCGTGGCTGTTTGTTG	115	57.0	92

2.6. Digital droplet PCR

The QX200 Digital Droplet PCR (ddPCR) system (Bio-Rad) was used to quantify the absolute copy numbers of the transgene *ifn-α2a* in three cell lines (1, 2, 3) following the method described in [45]. In brief, 11.5 µl of EvaGreen Supermix (Bio-Rad), 0.2 µM of forward and reverse primers (same as qPCR, see Table 1), 5 µl of diluted (1:10000) DNA template (extracted from 30 mL cultures using a DNeasy Plant Mini kit, QIAGEN) and nuclease free water up to 23 µl were thoroughly mixed and 20 µl was used in the droplet generation step. The PCR reaction was performed on a C1000 Touch Thermal Cycler with 96-Deep Well reaction module (Bio-Rad) following the manufacturer's recommendations. The droplets were read with a QX200 Droplet Reader and QuantaSoft software ver. 1.7.4 (Bio-Rad). All samples displayed above 16,600 accepted droplets. A single threshold was applied on all samples and negative controls and the absolute number of transgene copies was used to estimate the number of transgene copies per cell in each line.

2.7. Cell lysing and western blot analysis

Prior to western blot, 20 mL of algal suspension were collected and centrifuged immediately at 3,000 x *g* for 3 min. The cell pellets were re-suspended in 200 µL of lysis buffer (50 mM Tris-HCl, 400 mM NaCl, 0.5% Tween 20, and protease inhibitor cocktail 1x (Sigma-Aldrich), pH 8.0), snap frozen in liquid nitrogen, and stored at -80°C for further analysis. The cells were lysed (TissueLyser LT, Qiagen) at 50 Hz for 5 minutes, by adding 250 µL of glass beads (0.5 mm) to the frozen cell pellet. Cell debris were removed by centrifugation (10,000 x *g*, 10 min at 4°C) and the extracted soluble proteins were then denatured by the addition of SDS-PAGE sample loading buffer (Laemmli) containing 2-Mercaptoethanol (1:10) for 10 minutes at 95°C. Proteins were separated on 4-20% SDS-PAGE (Mini-PROTEAN® TGX Stain-Free™ pre-cast gels, Bio-Rad) at 300 V and transferred on to a PVDF membrane using a Trans-Blot® Turbo™

Transfer system (Bio-Rad). After blocking with 5% skim milk, membranes were incubated with a mouse anti-6X His tag® antibody (Abcam, 1: 1,000 dilution in blocking solution) for 1 hour followed by another hour incubation with an anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Abcam, 1:3,000 dilution in blocking solution). The final blot was imaged using ChemiDoc MP System. Relative quantitation western blot data was obtained by normalising the intensity of the band corresponding to the Cr.IFN-α2a to the total amount of protein on the stain-free blot image and the Image Lab Software version 6.0.1 (Bio-Rad) as describe by Taylor et al. [46].

2.8. Cr.IFN-α2a purification and yield estimation

The cell line 1 was selected to determine the activity of the Cr.IFN-α2a produced by *C. reinhardtii* transformants as it showed higher Cr.IFN-α2a production and better FMDV 2A peptide cleavage efficiency than the two other cell lines based on immunoblot (Figure 1B). One litre of culture was grown in a shake flask in a biphasic temperature mode with a shift in temperature from 25°C to 15°C after 48 hours. The cells were harvested by centrifugation (2,500 x *g* for 5 min) at 96 h to maximise both biomass production and Cr.IFN-α2a yield. The pellet was re-suspended in lysis buffer containing imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5% Tween20, pH 7.4) to a 5:1 ratio buffer-sample, sonicated (QSonica, Fisher Scientific) (30% intensity, 5 min, 30 sec ON and 30 sec OFF) and subsequently centrifuged at 4˚C, 10,000 x g for 10 min to remove cell debris. The Cr.IFN-α2a was separated from the whole proteome by a two-step chromatography (ÄKTA™ Pure, GE Healthcare), with a first step of Immobilised Metal Affinity Chromatography (IMAC) (1 mL HisTrap™ FF Crude column, GE Healthcare) followed by an ion-exchange chromatography step performed using a 1 mL HiTrap™ Capto S (GE Healthcare). After purification, the Cr.IFN-α2a was estimated to be 53% pure by 4-20% SDS-PAGE gel separation (Bio-Rad) (Figure S7). The concentration

of recombinant Cr.IFN-α2a was calculated based on the total protein concentration in the pooled fraction (Pierce™ BCA Protein Assay Kit) and the previously determined purity.

2.9. Mass spectrometry on purified Cr.IFN-α2a

Prior to mass spectrometry analysis, the purified Cr.IFN-α2a protein contained in the pooled fractions was concentrated using the single-pot, solid-phase-enhanced sample-preparation (SP3) technology as described by Hughes et al. [47]. Then, the purified Cr.IFN-α2a was analysed using an Acquity M-class nanoLC system (Waters, USA) as described by Roediger et al. [48].

2.10. Anti-viral-based cytopathic effect (CPE) assay

The anti-viral-based cytopathic effect (CPE) assay was conducted by the company PBL Assay Science (NJ, USA) as per the protocol described on their website (www.pblassaysci.com). In brief, the purified Cr.IFN-α2a was analysed in triplicate alongside a human IFN alpha 2a standard obtained from human leukocyte mRNA expressed in *E.coli* (provided by PBL) in a viral challenge assay using the encephalomyocarditis virus (EMCV) on the human lung carcinoma cell line A549. An equal volume of serial two-fold dilutions of Cr.IFN-α2a samples and standards were added to the A549 cells in 96-well plates. The plates were then incubated 18-24 hours at 37°C/5% CO_2. A dilution of EMCV previously determined to kill 100% of the A549 cells within 40 hours was added to the plates. Six wells were reserved for the cell control (no IFN, no virus) and the virus control (no IFN, virus added). After complete killing of the control cells, in approximately 40 to 56 hours, the media was removed from the wells and the live cells were fixed and stained with crystal violet solution. After washing the unbound stain with cold tap water and overnight drying, the dye was solubilized, followed by absorbance reading at 570 nm. The data were then

analysed in GraphPad Prism using a sigmoidal fit (variable slope) and the EC50 values of the standard and Cr.IFN-α2a were determined as the dilution required to give 50% protection against the EMC virus.

2.11. Statistical analysis

Statistical analyses were done in GraphPad Prism 8.0. Shapiro-wilk test and Levene's test were used first to confirm normality and homoscedasticity of the data, respectively. A one-way ANOVA was applied to test the effect of the transgene insertion on the growth of three transgenic lines expressing *ifnα-2a* and one *C. reinhardtii* cell line transformed with the empty vector control compared to the wild-type strain. Significant effects were then analysed using post-hoc Dunnett's multiple comparisons test. Two-way ANOVAs were applied to test the effect of temperature regimes on *ifnα-2a* transcript levels (RT-qPCR data) and protein production data. Significant effects were analysed using post-hoc Tukey's HSD and Fisher's LSD tests, respectively. Finally, a two-way ANOVA was used to test the effect of temperature (15°C) on the growth of cell line 1 compared to 25°C. Significant effects were analysed using post-hoc Sidak's multiple comparisons test. The analyses tested the null hypothesis that temperature had no effect on growth, transgene expression and recombinant Cr.IFN-α2a production. The results were considered significant at $P < 0.05$ (Table S2). Throughout the manuscript, values given are mean ± SEM (n = 3 biological replicates).

3. Results

Three *C. reinhardtii* cell lines successfully produced a fully translated recombinant Cr.IFN-α2a (C-terminus His-tag detected by immunoblotting) of correct size (~22 kDa) (Figure 1B). Unprocessed ble2A-IFN fusion protein (~39.5 kDa) were also detected. The efficiency of the ble2A system was especially low in cell line 2 (Figure 1B). Complete insertion of the transgene in each cell line was confirmed by amplicon sequencing from genomic DNA. The three cell lines were therefore selected to assess the effect of a biphasic temperature regime on growth, transgene expression and Cr.IFN-α2a yield.

3.1. Chromosomal integration of *ifn-α2a* does not affect *C. reinhardtii* growth

The random integration of the transgene (*ifn-α2a*) into the nuclear genome did not affect the growth of the three transgenic lines, as no significant difference was observed with the wild-type strain and the cell line transformed with the empty vector at 25°C (Figure 1A). Although the hybrid *HSP70/RBCS2i1* promoter is considered constitutive, the production of Cr.IFN-α2a varied depending on the growth phase in the three *C. reinhardtii* transformants (Figure 1B). Peak production of Cr.IFN-α2a was observed at 24 and 48 hours of cultivation, which corresponded to the early/mid-exponential phase of growth (Figure 1A). These results informed the design of the following experiment, in which the temperature was shifted at 48 hours of cultivation in order to maximize both biomass and Cr.IFN-α2a production.

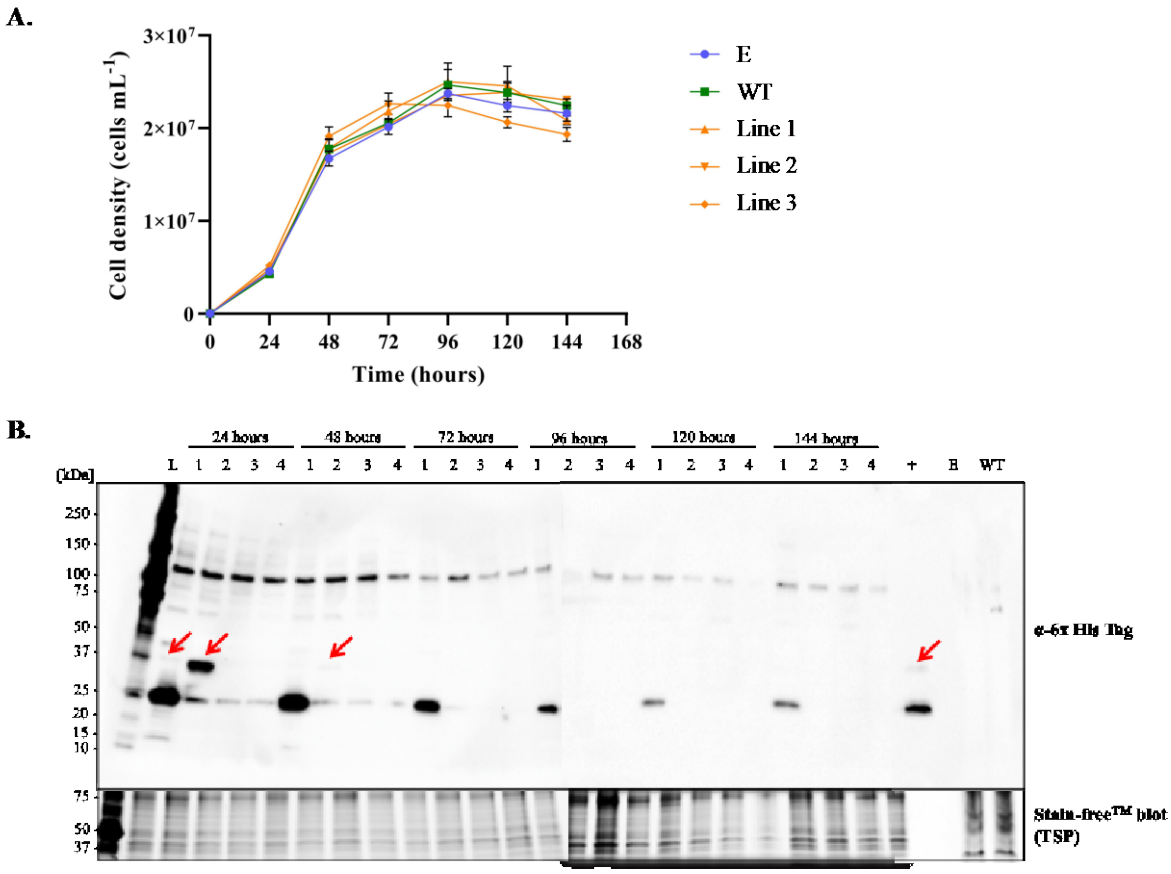


Figure 1- A. Cell density at 25°C of three independent *ifn-α2a* transformed *C. reinhardtii* cell lines (described as Line 1, Line 2 and Line 3) compared to the wild-type strain (WT) and one *C. reinhardtii* cell line transformed with the empty vector control (E). Error bars: Mean ± SEM (n = 3, for each independent cell line). No significant differences were found (Dunnett's multiple comparisons test, P>0.05) between the different cell lines. **B.** Western blot analysis of Cr.IFN-α2a production in the four *C. reinhardtii* transformants grown at 25°C. Cr.IFN-α2a production decreases in all cell lines after 48 hours. Immunodetection using raw cell extracts. WT and E samples were harvested at 72 hours. The sample "+" corresponds to Cr.IFN-α2a purified by affinity chromatography from Line 1 and confirmed by mass spectrometry, used as a positive control. The red arrows are indicating the unprocessed ble2A-IFN fusion protein (39.5 kDa). The stain-free™ blot shows the total soluble proteins (TSP) loaded in each lane. The figure was assembled from different images. The cell line 4 was not used in further experiments.

3.2. The biphasic temperature regime affected the growth of three *ifn-α2a* expressing lines

The growth profile of the three cell lines being identical, to assess the effect of the biphasic temperature regime on growth, transgene expression and Cr.IFN-α2a production, the data from the three independent cell lines were combined. This strategy allows to gauge the reproducibility of the results regardless of the transgene insertion site. The shift in temperature affected the growth of the three lines, with higher cell densities observed at higher temperature (35°C), and lower cell densities at lower temperature (15°C) (Figure 2A). Significant differences (Fisher's LSD, $P<0.05$) in cell densities were observed only 24 hours after the change in temperature. The cell density at 35°C dropped after 120 hours, while that at 15°C kept increasing. Reducing the temperature to 15°C seemed effective at slowing down cell growth and delaying death. The temperature shift did not result in higher cell mortality as the photosynthetic activities of the cells stayed high throughout the experiment (Figure S2). This is an important observation as lysed cells could release proteases and the recombinant protein into the medium, impacting on overall yield.

3.3. A lower temperature increases *ifn-α2a* gene expression and protein production

The effect of the temperature change on transgene expression and recombinant protein production was investigated. Lowering the temperature had a positive effect on the relative abundance of the *ifn-α2a* transcript, although not statistically significant (Figure 2B). The quantity of *ifn-α2a* mRNA kept increasing up to 96 hours at 15°C, while it was declining in the same time period at 25°C and 35°C. The quantity of recombinant Cr.IFN-α2a protein followed the same trend with a maximum of 3.3 and 3.5 times more Cr.IFN-α2a proteins being produced at 15°C compared to 25°C and 35°C, respectively, at 96 hours of growth (Fisher's LSD,

P<0.05) (Figure 2C,D). At 15°C, high levels of protein were observed up to 144 hours, whereas at 25°C and 35°C protein levels kept decreasing after 96 hours.

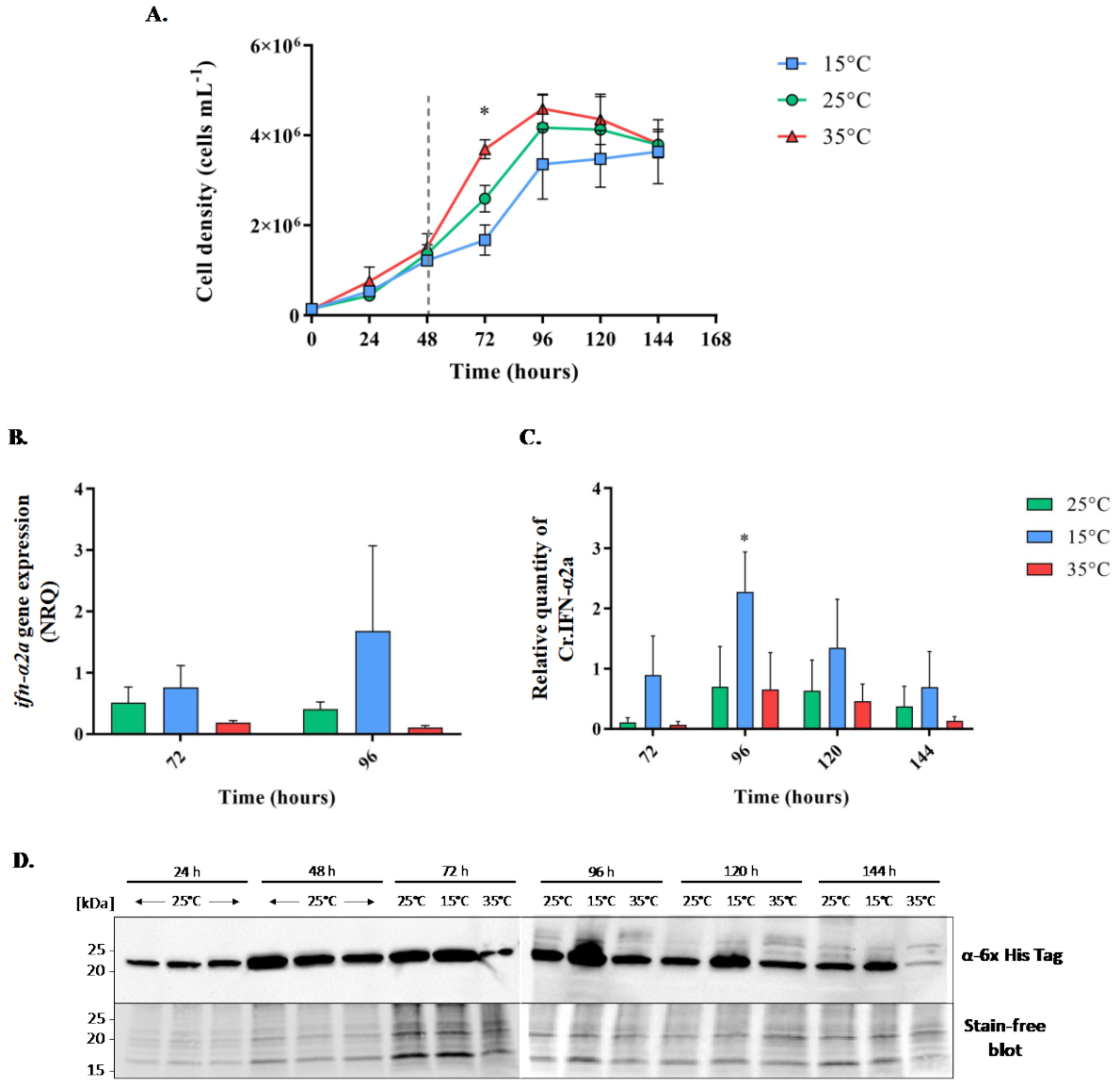


Figure 2- A. Combined cell densities of the three *ifn-α2a* transformed *C. reinhardtii* cell lines grown at different temperatures. The grey dashed line represents the time at which the temperature was shifted from 25°C to either 15°C or 35°C. **B.** Relative expression level (Normalized relative quantity, NRQ) of the *ifn-α2a* transcript and **C.** relative quantity of recombinant Cr.IFN-α2a protein after the temperature was changed from 25°C to 15°C and 35°C. The latter is based on relative band intensities from western blot images after

normalization to the total amount of protein loaded per lane (stain-free^{TM} blot) as shown in **D.** for cell line 1 (data for each cell line are shown in Figures S4, S5 and S6). Error bars: Mean ± SEM (n = 3, independent transgenic lines 1, 2 and 3). *Significant difference between 25°C, 15°C and 35°C (Fisher's LSD, P<0.05). The figure was assembled from different images.

3.4. IFN-α2a yield in crude extracts varies with estimated transgene copy numbers

The yield of Cr.IFN-α2a in the three cell lines were estimated from crude extracts of cells grown at 15°C and harvested at 96 hours (Table 2, Figure S3). The yield varied from 6.4 to 1.2 mg L^{-1}(0.1 – 0.02% TSP), which is in the range of what previous studies have reported for nucleus-coded recombinant proteins in *C. reinhardtii* [5, 12]. Whilst it is not possible to determine the actual number of copies of the intact and functional expression construct in the genome of *C. reinhardtii* without whole-genome sequencing, and we recently reported that random-chromosomal integration can cause massive rearrangements of the introduced DNA when integrated in the genome (ref George et al., 2020), we targeted a portion of the *Cr.ifn-α2a* coding sequence (ADD SOME DETAILS) with droplet digital PCR, to have an approximate estimate of the copies of the construct integrated. The ratio between these estimates and Cr.IFN-α2a yield varied between cell lines, with the highest amplicon copy number per cell yielding the highest quantity of Cr.IFN-α2a per litre. The cell line 1 was selected for scale-up and purification of Cr.IFN-α2a as it produced the highest level of fully processed Cr.IFN-α2a (~22 kDa).

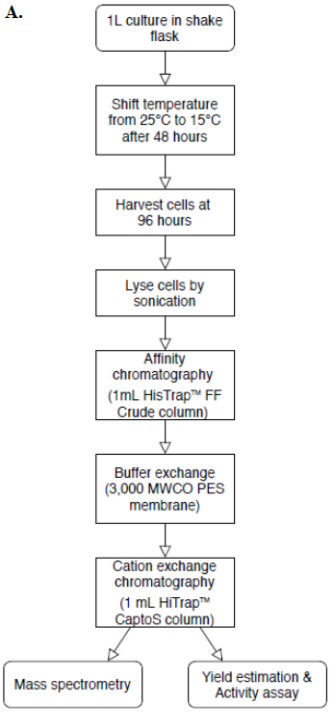
Table 2. Cr.IFN-α2a yield in crude extracts and transgene (T-DNA) copies per cell. Cr.IFN-α2a yield was determined by immunoblot against a commercial IFN-α2a of known concentration (Figure S3), while the number of T-DNA copies per cell was determined using digital droplet PCR.

Lines	Yield (mg L^{-1})	T-DNA copies $cell^{-1}$± SD
Line 1	6.4	3.36 ± 0.33
Line 2	5	2.34 ± 0.29
Line 3	1.2	0.73 ± 0.12

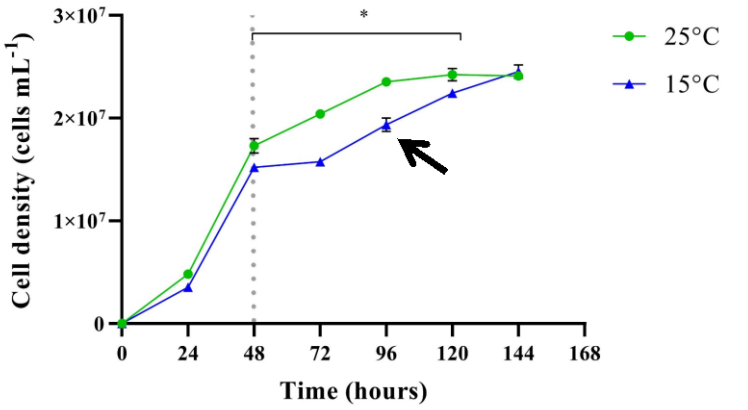
3.5. Low activity of the purified recombinant Cr.IFN-α2a

Figure 3A describes the purification process used in this study. The cell line 1 was up-scaled to 1 L in a shake flask, the temperature was shifted to 15°C and the cells were harvested at 96 hours as indicated by the arrow in Figure 3B. After a two-step chromatography purification, Cr.IFN-α2a was estimated to be 53% pure with a yield of 90 µg/L of culture or 4.5 fg per cell (Figure 3C, Figure S7). Only 1.5% of the Cr.IFN-α2a originally present in the raw cell extract were recovered. Protein loss during purification is a well-known issue [49], with more proteins lost for each step of chromatography added. In our case, ~50% of Cr.IFN-α2a were lost at each chromatography step. Further optimisation will be needed to increase the purity without compromising the yield.

A.



B.



C.

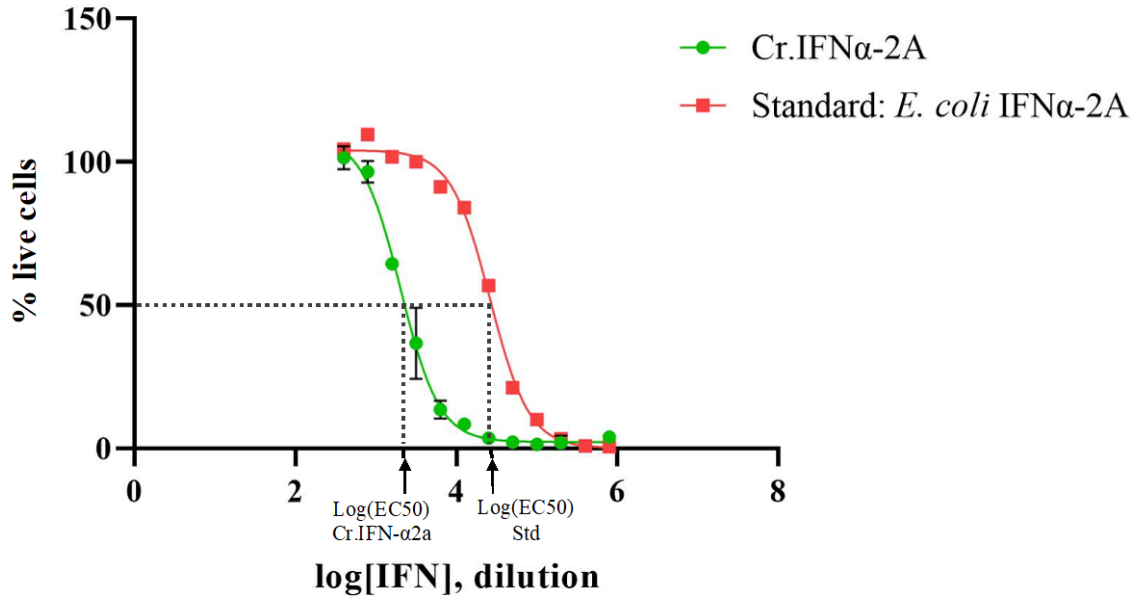
Total soluble proteins in the pooled fractions (µg)	Purity of Cr.IFN-α2a in the pooled fractions	Cr.IFN-α2a quantity in the pooled fractions (µg)	Cr.IFN-α2a yield (µg/L)	Cr.IFN-α2a yield (fg/cell)
170	53%	90	90	4.5

Figure 3- A. Flowchart of the Cr.IFN-α2a protein purification process. **B.** Cell density of cell line 1 grown at different temperatures prior to Cr.IFN-α2a harvesting and purification. The grey dashed line represents the time at which the temperature was shifted from 25°C to 15°C. Error bars: Mean ± SEM (n = 3). *Significant differences (Sidak's multiple comparisons test, P<0.001) between 25°C and 15°C. The arrow is indicating the time point at which the cells were harvested for Cr.IFN-α2a activity and yield determination. **C.** Calculation of the yield of Cr.IFN-α2a after a two-step purification of a 1 L culture harvested at 96 hours at 15°C and a cell density of 2 x 10^7 cells mL^{-1}.

Prior to the activity assay, the amino acid sequence of the purified protein was analysed by mass spectrometry to confirm the identity of the recombinant protein. The algal Cr.IFN-α2a showed a 79% coverage against the human IFN-α2a amino acid sequence (Figure S8), confirming that cell line 1 produced the recombinant human IFN-α2a. The activity of the Cr.IFN-α2a was assessed using a standard anti-viral interferon (IFN) bioassay, which consists in testing the ability of interferon alpha 2a to protect cells from cytopathic effect (CPE) of

certain viruses. The general preference for this bioassay is, in part historical, since this was the initial activity which defined these molecules. The other reason is that the CPE assay is amongst the most sensitive interferon bioassay, at least 100-times more sensitive than alternatives [50]. Cr.IFN-α2a was titrated in a CPE assay on A549 human lung carcinoma cells with encephalomyocarditis virus (EMCV) to determine the number of unit/ml of interferon needed to produce an inhibition of the cytopathic effect of 50% (EC50). The units of Cr.IFN-α2a activity were determined colorimetrically against the international reference standard for human IFN-α2a (produced in *E. coli*). We hypothesized that Cr.IFN-α2a would be as active as the standard. The Cr.IFN-α2a showed an anti-viral activity against the EMCV, although it was 10 times lower than the standard with 2.37 x 10^3 U/mL and 2.78 x 10^4 U/mL, respectively (Figure 4).

A.



B.

Sample	Cr.IFN-α2a	Standard	Std U/mL
EC50 Dilution	2.39 x 10^3	2.81 x 10^4	0.991
Activity (U/mL)	2.37 x 10^3	2.78 x 10^4	-

Figure 4- Results of the CPE assay. **A.** Data were fit to a sigmoidal dose response curve (variable slope) using the GraphPad Prism software package. The EC50 values were determined for each sample as the dilution from the stock required to give 50% protection against the EMC virus. **B.** The IFN-α2a concentration required to protect 50% of the cells was determined based on the Standard (Std). In this case 0.991 U/mL gives 50% protection. The anti-viral activity (U/mL) was then determined as: 0.991 x EC50 dilution.

4. Discussion

This study firstly demonstrated that temperature had an effect on *C. reinhardtii* growth, with a slower growth at 15°C compare to 25°C (Figures 2A and 3B). This effect was previously reported by Vitova et al. [51], who observed a longer cell cycle at lower temperature. They found that the average cell cycle duration under continuous illumination (150 µmol photons m^{-2} s^{-1}) at 15°C was about 34 h, compare to 21 h at 20°C and 15 h at 28°C. However, they reported a prolongation of the cell cycle to 25 h at 35°C, which contradicts the fast growth at 35°C observed in this study. In comparison, Lien et al. [52] estimated 35°C to be the optimal growth temperature for *C. reinhardtii*. The literature on the effect of the temperature on *C. reinhardtii* growth highly differs due to strain characteristics and cultivation mode (i.e., mixotrophy, phototrophy). However, there is concordance on the fact that temperatures below 14°C and higher than 39°C cause cell cycle arrest [15, 16]. In this study, the shift to 15°C delayed the growth by 24 hours but did not significantly affect the growth rate. The strain used here might be more resistant to cold temperatures. The cells also reached high density faster at 35°C than 25°C in mixotrophy, but the density dropped after 120 hours while it kept increasing at 15°C. At 35°C, the cell likely ran out of acetate faster than at 15°C, with a colder temperature likely slowing down the cellular metabolism, which is critical in a biphasic cultivation strategy. Maikova et al. [15] showed that temperatures ranging from 0°C to 14°C result in viable but not

dividing *C. reinhardtii* cells, with cells still growing at a slow rate (i.e. reported increase in diameter, chlorophyll and protein contents). Therefore, temperatures lower than 15°C could be further tested for recombinant proteins accumulation.

Lowering the temperature from 25°C to 15°C increased the abundance of Cr.IFN-α2a by a 3.3-fold factor at 96 hours in mixotrophy (Figure 2C), while prolonging Cr.IFN-α2a production up to 144 hours. In contrast, an increase in culture temperature to 35°C negatively impacted recombinant protein production (Figure 2C,D). These results correlate with studies on mammalian cells, which showed that lowering the culture temperature of CHO cells from 37°C to 33°C resulted in a 4-fold increase in the productivity of recombinant erythropoietin (EPO) [19], a 6-fold increase in recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) production [24] and a 1.4-fold increase in antibody (IgG) production [21]. Yoon et al. [19] explained this increase in productivity as being partially due to extended culture longevity resulting in the decreased release of proteolytic enzymes from dead cells. With a low culture temperature of 15°C inducing a prolonged exponential growth in *C. reinhardtii* and absence of cell death, it is legitimate to assume that similar mechanisms could take place in the microalgal cell. However, based on photosynthetic activity, cell mortality was low at 15°C and 35°C when higher abundance of Cr.IFN-α2a was only observed at 15°C. While reduced cell death probably contributes to Cr.IFN-α2a accumulation, it is not the main driver here. Further studies are needed at this stage to help determine if the higher Cr.IFN-α2a accumulation observed at lower temperature is due to a prolong expression period or overall reduced metabolism (reduced degradation) or a combination of both. Temperature also had an effect at the transcriptional level, as the transgene expression and Cr.IFN-α2a production followed the same trend, with the relative expression of *ifn-α2a* increasing when lowering the

culture temperature. The *HSP70A/RBCS2i1* promoter used in this study can be induced by both heat shock and light [17], but there is no evidence of it being responsive to low temperatures.

Wide discrepancy in the amount of recombinant protein produced between the three selected cell lines was observed, with cell line 1 producing more Cr.IFN-α2a than cell lines 2 and 3 (Figure 1B). As the growth curves of the three cell lines were similar, the discrepancy can be attributed to genomic "position effects", transgene copy number or post-transcriptional/post-translational silencing mechanisms due to the random insertion of the transgene into the nuclear genome [53, 54]. Copy number of transgenes and integration loci are key factors influencing transgene expression, but they cannot be controlled during random chromosomal integration events [10]. These factors can only be handled using targeted knock-in methods such as endonucleases-driven genome editing, which have recently shown to be successful in *C. reinhardtii* [55]. Although all three cell lines were transformed with the same method (i.e. electroporation), the number of transgenes inserted varied from ~1 to ~3. This difference in copy number correlated with the level of Cr.IFN-α2a produced, as the most productive cell line harboured the highest copy number. However, the variability in Cr.IFN-α2a production cannot be exclusively explained by the copy number, as the before-mentioned position effects also influenced the expression intensity of each transformant. The position effects were likely responsible for the low transformation efficiency of the *ifn-α2a* gene, which necessitated multiple transformations leading to a total number of 304 colonies screened by PCR. Over 118 transformants were found positive by colony PCR, but only 3 showed reasonable expression level on western blot and unaffected physiology. The screening of a transgene without a fluorescence, or enzymatic reporter was very laborious. Increasing the concentration of zeocin during the screening process helped to an extent, but a large number of zeocin resistant transformants were not producing Cr.IFN-α2a probably due to random recombination of the

fusion protein into the nuclear genome that lead to the *she-ble* gene being inserted without the *ifn-α2a* transgene. Recent work by George et al. [8] emphasized the unpredictability of random integration in microalgae, resulting in very large, highly concatenated insertion island with transgene and adjacent plasmid DNA being integrated in both sense and antisense orientations. Although George et al. used a different transformation method (i.e. biolistic bombardment), similar phenomenon could take place with electroporation. Therefore, finding the one positive outlier from a transformant population often result in much bigger effect on final product titer, as observed here with cell line 1. Cell line 2 also showed a relatively high titer, but the FMDV 2A efficiency was very low in this transformant. Similarly, Kong et al. [56] found that the ribosome skipping efficiency of the ble2A coding region was not uniform among their transformants. In contrast, Rasala et al. [12] did not detect unprocessed protein when using the FMDV 2A system for xylanase synthesis in the cell wall-deficient strain cc3395. Plucinak et al. [36] demonstrated that an extended version of the FMDV 2A peptide (39 amino acids) was more efficient than the ~20 amino acid used in this study, with an efficiency approaching 100% in cell wall deficient *C. reinhardtii* strain CC3491 producing mVenus. As noted by Kong et al., the efficiency of the ble2A system seems to be highly dependent on the following transgene sequence and/or the different strains characteristics [56], but use of the extended FMDV 2A peptide might help to alleviate these issues.

Interestingly, the production of Cr.IFN-α2a did not vary only based on the culture temperature, but the growth phase also seemed to have an influence. Indeed, the production of Cr.IFN-α2a peaked at 48 hours (early-log phase) at 25°C and then gradually decreased in the three cell lines (Figure 1). Lauersen et al. [14] also observed that the rates of protein production (relative to biomass) from an intracellular recombinant patchoulol synthase coded by a nuclear transgene in *C. reinhardtii* exhibited peaks of productivity between 24 and 48 h of cultivation

in the early logarithmic phase, after which the production rates steadily decreased [14]. The same observation was made for a recombinant bisabolene synthase, with the productivity of (E)-α-bisabolene peaking sharply between 48 and 72 h in mixotrophic conditions (under constant light) [34]. Unlike intracellular recombinant proteins, the productivity of secreted proteins seems dissociated from the growth phase as they accumulate in the medium [5]. When screening *C. reinhardtii* nuclear transformants for intracellular recombinant proteins, it is important to consider the growth phase effect as the "window of expression" might be missed. Indeed, the yield of intracellular recombinant proteins can be underestimated if measured at the wrong growth phase. Schroda et al. [17] reported that *HSP70A/RBCS2* promoter driven expression can be induced by exposure to low light intensity (40 µmol m^{-2} s^{-1}) after a period of darkness. The high expression observed in the early stage of growth (24 – 48 hours) in this study could be in part due to the cultures being inoculated from stationary (i.e. light limited) stock cultures. Once transferred into fresh medium, the higher amount of light (50 µmol m^{-2} s^{-1}) available per cell in the new low-cell-density culture might trigger higher expression of the transgene driven by the induced *HSP70A/RBCS2* fusion promoter.

In this study, we estimated the maximum yield at 15°C for intracellularly produced Cr.IFN-α2a to be around 6 mg L^{-1} (0.1% TSP) for crude extract or 90 µg L^{-1} after a two-step purification. Yields of nucleus-coded intracellular recombinant proteins are usually low in *C. reinhardtii* due to nuclear gene silencing mechanisms that are poorly understood [57, 58]. Barahimipour et al. [59] have reported yields as high as 1% TSP using codon-optimized transgenes in strains with impaired transgene silencing generated by UV mutagenesis. In contrast, in mammalian expression platforms, yields of recombinant proteins are typically above 1 g/L [60]. Generally, 10 mg L^{-1} is considered the minimum yield for commercial process development [61]. Yields of >10 mg L^{-1} have been reported in nuclear transgenes of

C. reinhardtii but for secreted proteins only [62, 63]. Ramos-Martinez et al. (2017) have produced a maximum of 15 mg L^{-1} of recombinant yellow fluorescent protein Venus, when Venus was C-terminally fused with synthetic glycomodules comprised of tandem serine (Ser) and proline (Pro) repeats of 10 and 20 units and secreted into the culture media. The yields reported in these studies are for un-purified recombinant proteins and are likely to be lower after purification. Indeed, secreted recombinant proteins are challenging to purify especially when produced by highly-expressing cell-wall deficient mutants of *C. reinhardtii* as aggregates of unassembled cell wall proteins form an insoluble glycoprotein framework, which impairs downstream processes such as filtration and chromatography [64]. Future studies should focus on optimizing the purification processes for recombinant proteins produced in *C. reinhardtii* as protein recovery is foreseen as one of the next challenges facing this expression platform.

The recombinant Cr.IFN-α2a produced in this study was functionally active, although its antiviral activity was 10 times lower than the standard human IFN-α2a produced in bacteria. The presence of contaminants (e.g. aggregates, unprocessed fusion proteins and co-purified cross-reacting material) could have impacted the activity of the purified extract. The high molecular weight bands observed on the SDS-PAGE gel and immunoblot (Figure S7) could be Cr.IFN-α2a aggregates strong enough to not dissociate under standard denaturing conditions. Aggregates can reduce Cr.IFN-α2a solubility and therefore potentially lower its activity. Interferons are proned to protein aggregation due to their hydrophobic nature [65]. IFN aggregation can be prevented by PEGylation, which consists in attaching a polyethylene glycol (PEG) polymer to a protein drug. PEGylation increases IFN activity and reduces the immunogenicity by masking the IFN protein hydrophobic sites [65, 66]. Performing gel filtration during the purification process also helps removing part of the large IFN aggregates and therefore increase the activity. El-Ayouty et al. [67] showed that three recombinant IFN-

α2a proteins partially purified from *C. reinhardtii* soluble extracts using anion exchange chromatography followed by gel filtration (sephadex-G50) were active in suppressing the growth and reproduction of vesicular stomatitis virus. In their study, Cr.IFN-α2a protein extracts had comparable anti-viral activity to the commercial bacterial PEGylated IFN (Pegasys®; Hoffmann-LaRoche, Basel, Switzerland) used as control [67]. Gel filtration would also be efficient at removing the unprocessed (ble2A-IFN) fusion proteins which co-purified during affinity and ion exchange chromatography (band at ~39 kDa on Figure S7) and might have contributed in lowering the anti-viral activity of the Cr.IFN-α2a protein. However, adding a third step of purification to the existent purification process would have likely reduced the yield of purified protein even further. Finding the right balance between purity and yield is critical and will need to be further studied and optimized.

5. Conclusion

This study demonstrated that the biphasic temperature regime (lowering temperature) is a valid strategy to increase the yield of recombinant protein IFN-α 2a from transgenic *C. reinhardtii* cultures. Cells were first grown under an optimal temperature (25°C) in order to increase cell biomass prior to lowering the temperature to 15°C to slow down recombinant protein degradation and lengthen the production phase. Decreasing temperature to 15°C was beneficial for Cr.IFN-α2a production with a 3.3 fold increase in protein production relative to control conditions (25°C). By demonstrating that temperature shift can significantly affect protein production from nuclear transgenes in *C. reinhardtii*, our results not only advance the field of recombinant protein production in microalgae, but also suggest that the dynamic modulation of temperature regimes should definitively be considered when assessing culture conditions and microalgae cell lines. Undoubtedly, within the last decade, *C. reinhardtii* has proved itself as an impending platform for recombinant protein production. However, in spite of the

extensive research already achieved, various optimization steps are still needed regarding the molecular machinery, the purification process, but also importantly, the culture conditions in order to reach its full potential as a commercial platform for recombinant protein production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR'S CONTRIBUTIONS

A.S.C. and M.F. conceived and designed the study; A.S.C. and N.K.W. performed the research; L.B. purified the recombinant Cr.IFN-α2a; N.S. performed the digital droplet PCR assay, J.A. generated preliminary data; M.P. performed statistical analysis; P.J.R. funded the research; A.S.C and N.K.W. wrote the manuscript with inputs from M.F., L.B., N.S., J.A., P.J.R and M.P.

SUPPLEMENTARY MATERIAL

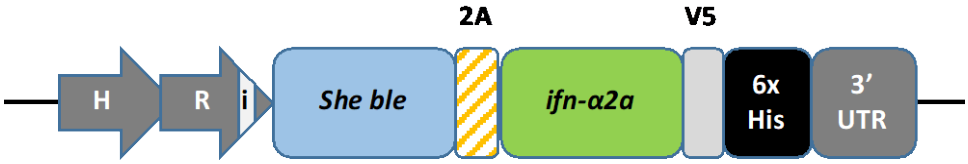

Fig. S1. Map of the construct used in this study. Vector pChlamy_4_Zeo_ifnα2a with the *HSP70A/RBCS2i1* promoter (H R i), zeocin resistance gene (*sh-ble*), *FMDV 2a* self-cleavage peptide (2a), human interferon-alpha 2a gene (*ifn-α2a*), V5 epitope, 6x His tag and 3' untranslated region (UTR) of the *rbcS2* gene.

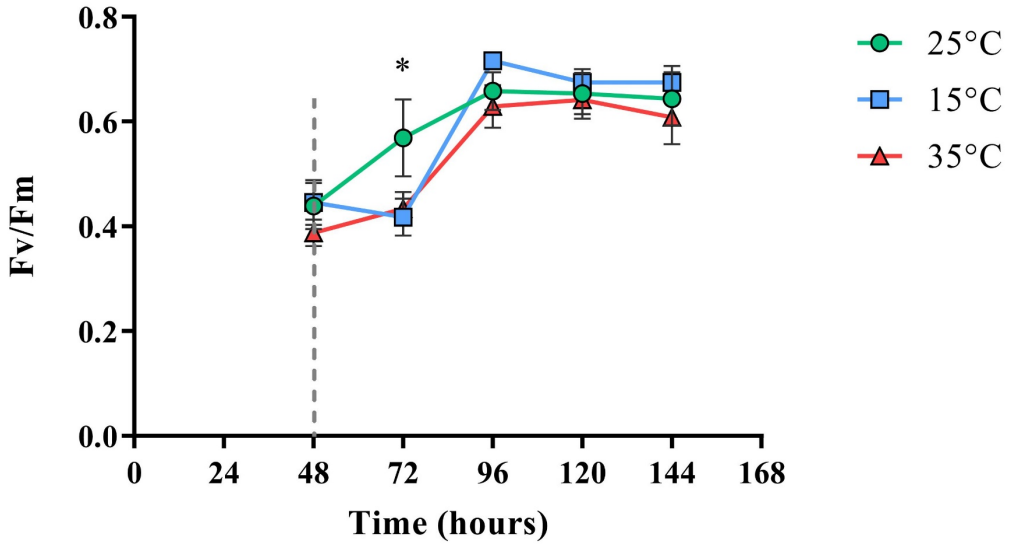

Fig. S2. Maximum quantum yield of photosystem II (Fv/Fm) after temperature shift. The Fv/Fm was measured by pulse-amplitude modulated fluorometry (PAM), as described in [38], for rapid assessment of the cell lines photosynthetic activity during the experiment, and to monitor for any photosystem stress caused by temperature shift. *Significant difference compared to 25°C (Fisher's LSD, $P<0.05$).

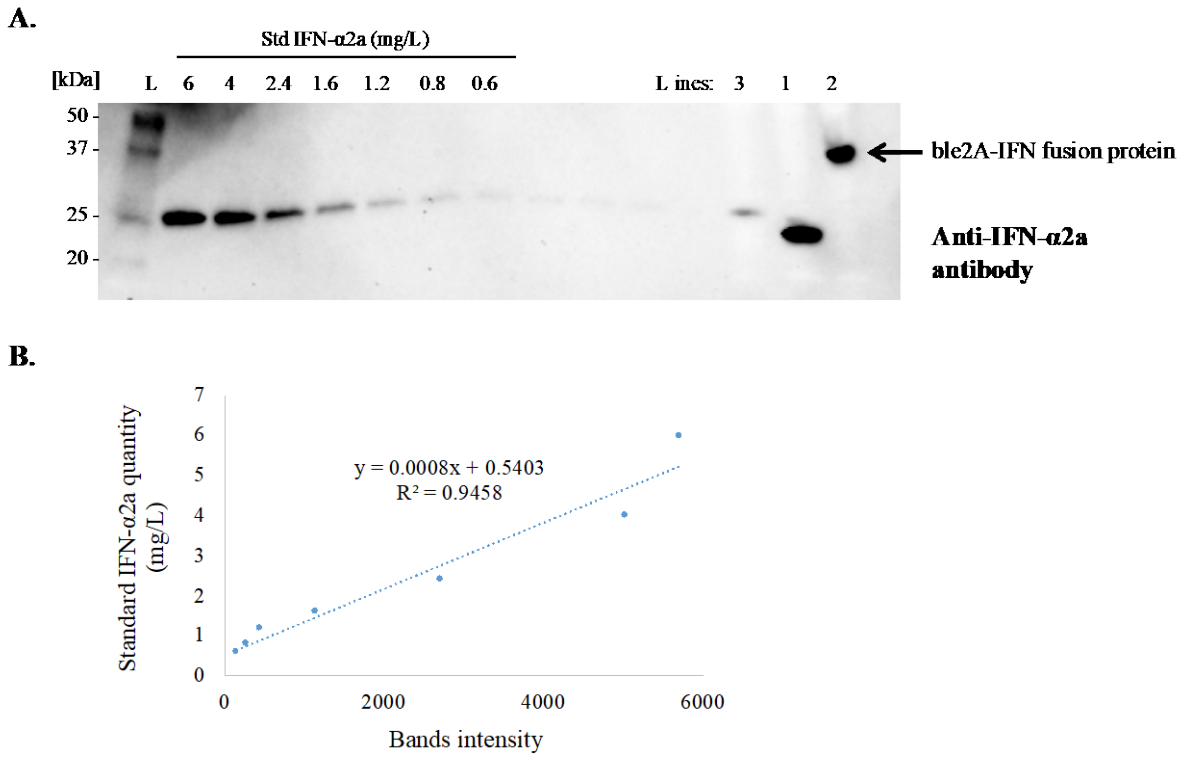

Fig. S3- Determination of Cr.IFN-α2a yield in crude extract. A. Western blot analysis of crude extracts from 30 mL of culture at 96 hours at 15°C. **B.** A commercial IFN-α2a produced in *E. coli* was used as standard (Sigma). A mouse anti-IFN-α2a primary antibody (1: 300 dilution in blocking solution) and an anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Abcam, 1:3,000 dilution in blocking solution) were used for immunoblotting. The concentrations of Cr.IFN-α2a in the crude extracts were determined using the Image Lab Software version 6.0.1 (Bio-Rad).

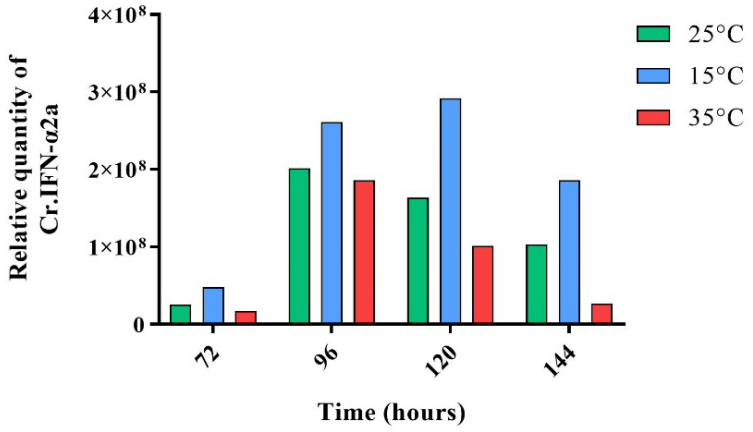

Fig. S4. Relative quantity of recombinant IFN-α2a protein after temperature shift from 25°C to 15°C and from 25°C to 35°C in Cell line 1. Quantification is based on band intensities from the western blot image presented in Figure 2D.

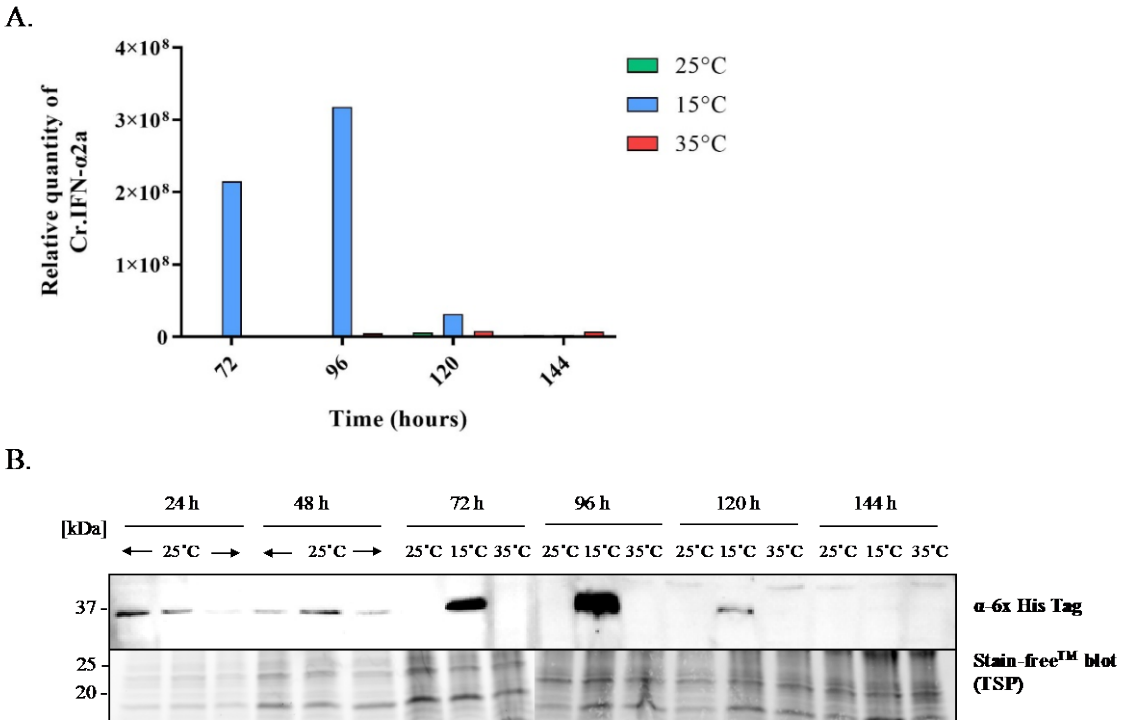

Fig. S5. (A) Relative quantity of recombinant IFN-α2a protein after temperature shift from 25°C to 15°C and from 25°C to 35°C in **Cell line 2**. Quantification is based on band intensities

from the western blot image (B) after normalization to the total amount of protein loaded per lane (stain-free™ blot). The figure was assembled from different images.

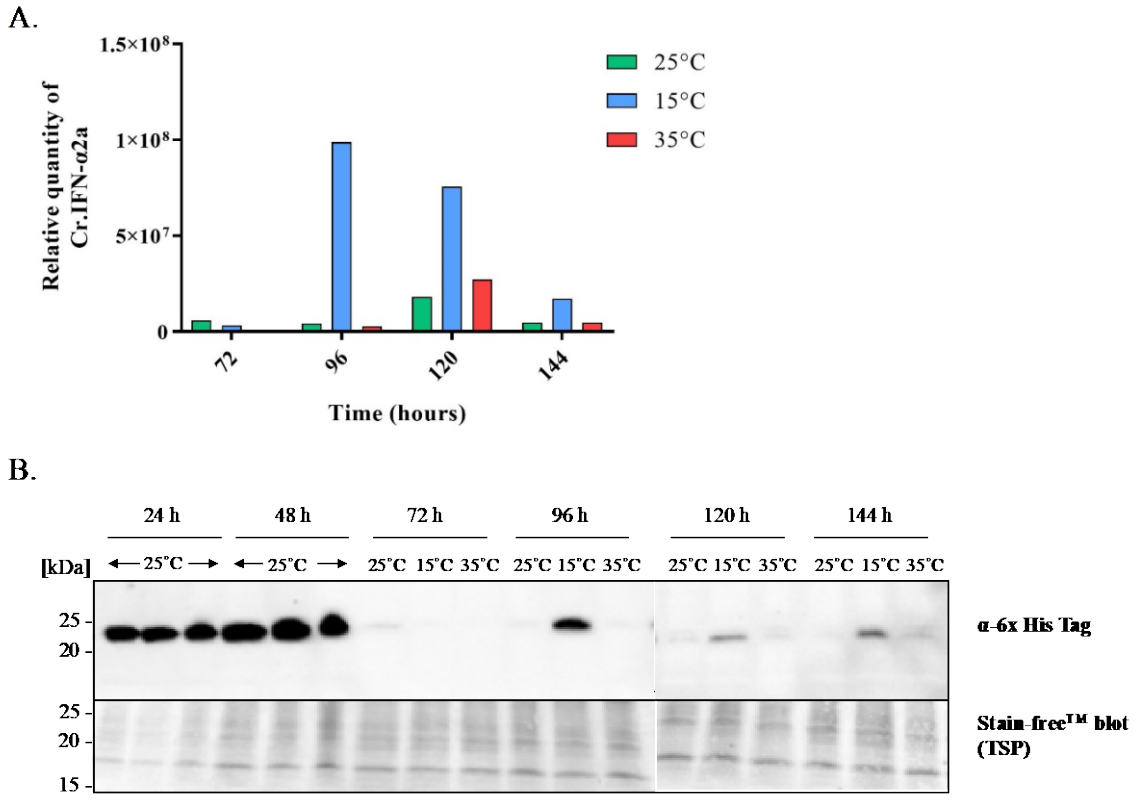

Fig. S6. (A) Relative quantity of recombinant IFN-α2a protein after temperature shift from 25°C to 15°C and from 25°C to 35°C in **Cell line 3**. Quantification is based on band intensities from the western blot image (B) after normalization to the total amount of protein loaded per lane (stain-free™ blot). The figure was assembled from different images.

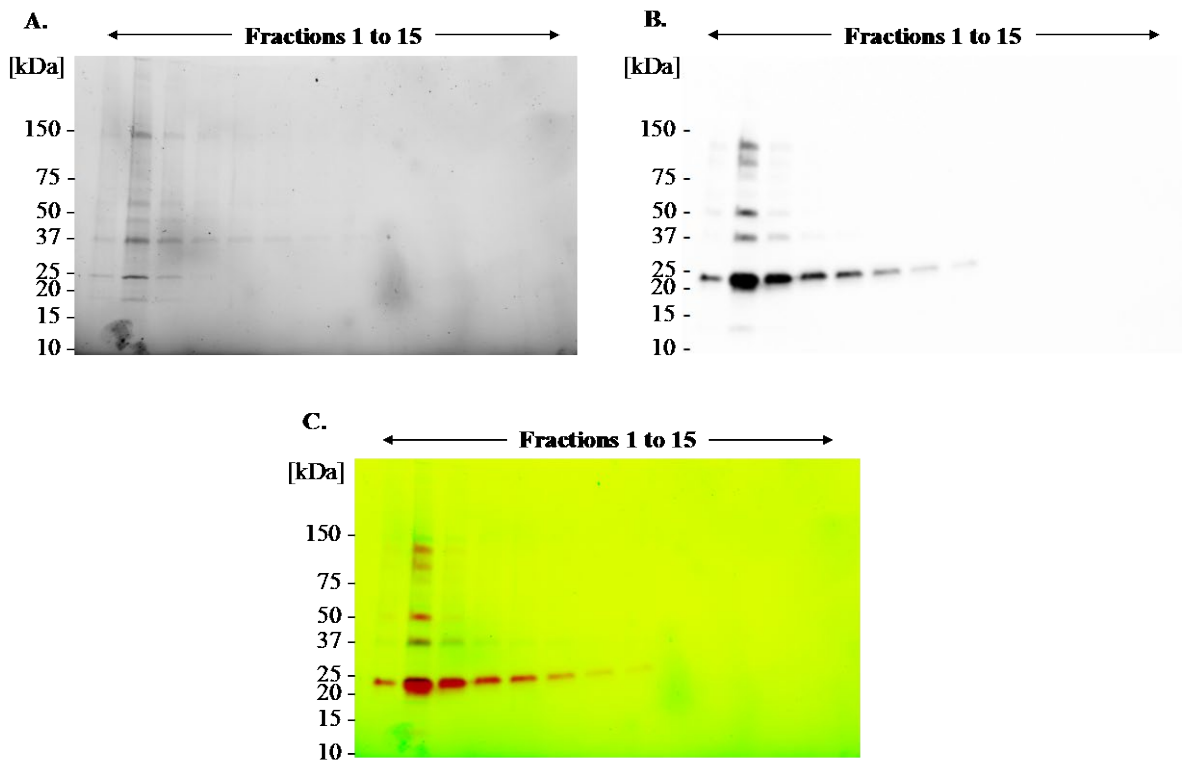

Fig. S7. (A) SDS-PAGE gel image of the fractions after cation exchange chromatography (HiTrap™ Capto S, GE Healthcare). (B) Immunoblot after incubation with mouse anti-6X His tag® antibody and anti-mouse HRP conjugated secondary antibody. (C) Overlay of the two images. The fully processed Cr.IFN-α2a is estimated to be ~ 22 kDa, the unprocessed fusion protein is visible at ~39 kDa, and the high molecular weight bands are likely to be aggregates as interferons are proned to protein aggregation due to their hydrophobic nature. The Cr.IFN-α2a was estimated to be 53% pure by superimposing the two images and calculating the abundance of Cr.IFN-α2a bands relative to the other bands on the SDS-PAGE gel image using the Image Lab Software version 6.0.1 (Bio-Rad).

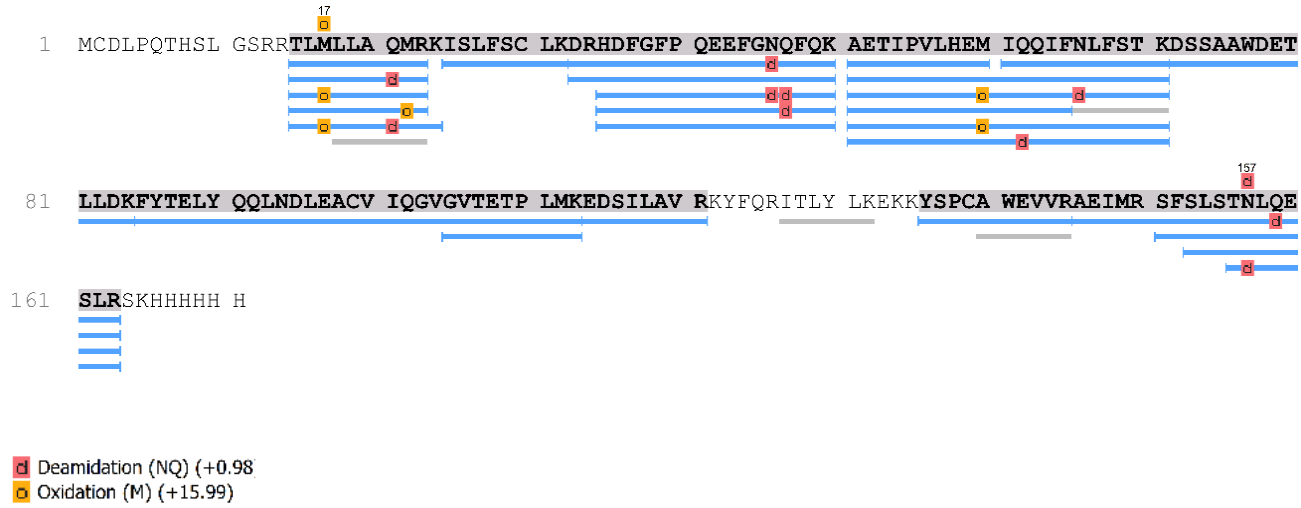

Fig. S8. Alignment of amino-acid sequence obtained by mass spectrometry analysis, showing a 79% coverage between the purified algal IFN-α2a and the reference human IFN-α2a amino acid sequence. The MS/MS data files were searched using Peaks Studio 10.0 against the human interferon alpha 2a amino acid sequence and a database of common contaminants with the following parameter settings. Fixed modifications: none. Variable modifications: propionamide, oxidised methionine, deamidated asparagine. Enzyme: semi-trypsin. Number of allowed missed cleavages: 3. Peptide mass tolerance: 10 ppm. MS/MS mass tolerance: 0.05 Da. The results of the search were then filtered to include peptides with a -log10P score that was determined by the False Discovery Rate (FDR) of <1%, the score being that where decoy database search matches were <1% of the total matches.

Table S1. Information about the human interferon-alpha 2a sequence used in this study.

Codon optimized nucleic acid sequence	Amino acid sequence	Protein molecular weight (Da)
>INF-alpha_2A_cre ATGTGCGACCTGCCCCAGACCCACAGCCTGGG CAGCCGCCGCACCCTGATGCTGCTGGCCCAGA TGCGCAAGATCAGCCTGTTCAGCTGCCTGAAG GACCGCCACGACTTCGGCTTCCCGCAGGAGGA GTTCGGCAACCAGTTCCAGAAGGCCGAGACCA TCCCCGTGCTGCACGAGATGATCCAGCAGATC TTCAACCTGTTCAGCACCAAGGACAGCAGCGC CGCCTGGGACGAGACCCTGCTGGACAAGTTCT ACACCGAGCTGTACCAGCAGCTGAACGACCTG GAGGCCTGCGTGATCCAGGGCGTGGGCGTGAC CGAGACCCCCCTGATGAAGGAGGACAGCATCC TGGCCGTGCGCAAGTACTTCCAGCGCATCACC CTGTACCTGAAGGAGAAGAAGTACAGCCCCTG CGCCTGGGAGGTGGTGCGCGCCGAGATCATGC GCAGCTTCAGCCTGAGCACCAACCTGCAGGAG AGCCTGCGCAGCAAGGAG	>DB00034 sequence CDLPQTHSLGSRRTLMLLAQ MRKISLFSCLKDRHDFGFPQE EFGNQFQKAETIPVLHEMI QQIFNLFSTKDSSAAWDETLL DKFYTELYQQLNDLEACVIQ GVGVTETPLMKEDSILAVR KYFQRITLYLKEKKYSPCAW EVVRAEIMRSFSLSTNLQESL RSKE	19,372

Table S2. Analyse of variance using GraphPad Prism 8.0 software. Df = degrees of freedom; MS = mean sum of squares. Bold face indicates statistical significance ($P < 0.05$).

A. One-way ANOVA and post-hoc Dunnett's multiple comparisons test results for the effect of the transgene insertion on growth.

Source of variation	DF	MS	F	P value
Cell lines	4	8.80e+11	0.7873	0.4535

Dunnett's multiple comparisons test (comparing the mean of each cell line with the mean of the wild-type strain)

Lines	Mean Diff.	Significant?	Summary	Adjusted P Value
WT vs. Empty vector	624710	No	ns	0.0902
WT vs. Line 1	-142857	No	ns	0.9753
WT vs. Line 2	88571	No	ns	0.9816
WT vs. Line 3	597619	No	ns	0.8781

B. Two-way ANOVA and post-hoc post-hoc Tukey's HSD test results for the effect of temperature regimes on *ifnα-2a* transcript levels (RT-qPCR data).

Source of Variation	Df	MS	F	P value
Interaction	2	0.5203	0.4866	0.6263
Time	1	0.2721	0.2545	0.6230
Temperature	2	1.825	0.2226	0.2226

Tukey's multiple comparisons test

	Mean Diff.	Significant?	Summary	Adjusted P Value
72 hour				
25°C vs. 15°C	-0.2441	No	ns	0.9551
25°C vs. 35°C	0.3252	No	ns	0.9220
15°C vs. 35°C	0.5693	No	ns	0.7825
96 hours				
25°C vs. 15°C	-1.278	No	ns	0.3193
25°C vs. 35°C	0.2968	No	ns	0.9345
15°C vs. 35°C	1.575	No	ns	0.1910

C. Two-way ANOVA and post-hoc post-hoc Tukey's HSD test results for the effect of temperature regimes on Cr.IFN-α2a production (western blot data).

Source of Variation	Df	MS	F	P value
Interaction	6	0.2483	0.4924	0.8056
Time	3	1.445	2.865	0.0655
Temperature	2	3.380	2.045	0.2102

Uncorrected Fisher's LSD test

	Mean Diff.	Significant?	Summary	Adjusted P Value
72 hour				
25°C vs. 15°C	-0.2441	No	ns	0.2892
25°C vs. 35°C	0.3252	No	ns	0.9522
15°C vs. 35°C	0.5693	No	ns	0.2638
96 hours				
25°C vs. 15°C	**-1.278**	**Yes**	*	**0.0397**
25°C vs. 35°C	0.2968	No	ns	0.9496
15°C vs. 35°C	**1.575**	**Yes**	*	**0.0347**
120 hour				
25°C vs. 15°C	-0.7103	No	ns	0.3379
25°C vs. 35°C	0.1762	No	ns	0.8104
15°C vs. 35°C	0.8865	No	ns	0.2341
144 hour				
25°C vs. 15°C	-0.3191	No	ns	0.6644
25°C vs. 35°C	0.2426	No	ns	0.7413
15°C vs. 35°C	0.5616	No	ns	0.4469

D. Two-way ANOVA and post-hoc Sidak's multiple comparisons test results for the effect of temperature on the growth of cell line 1.

Source of Variation	Df	MS	F	P value
Interaction	6	5.53e+12	36.55	P<0.001
Time	6	5.50e+14	3637	P<0.001
Temperature	1	3.92e+13	259.6	P<0.001

Sidak's multiple comparisons test

	Mean Diff.	Significant?	Summary	Adjusted P Value
25°C vs. 15°C				
0 hour	0.000	No	ns	>0.9999
24 hours	1266667	Yes	**	0.0030
48 hours	**2100000**	**Yes**	****	**<0.0001**
72 hours	**4633333**	**Yes**	****	**<0.0001**
96 hours	**4166667**	**Yes**	****	**<0.0001**
120 hours	**1800000**	**Yes**	****	**<0.0001**
144 hours	-433333	No	ns	0.7573

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