

# Successful reversal of transgene silencing in *Chlamydomonas reinhardtii*

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## Abstract

*Chlamydomonas reinhardtii* has been successfully engineered to produce compounds of interest following transgene integration and heterologous protein expression. The advantages of this model include the availability of validated tools for bioengineering, its photosynthetic ability and its potential use as biofuel. Despite this, breakthroughs have been hindered by its ability to silence transgene expression through epigenetic changes. Histone deacetylases (HDAC) are main players in gene expression. We hypothesized that transgene silencing can be reverted with chemical treatments using HDAC inhibitors. To analyze this, we transformed *C. reinhardtii*, integrating into its genome the mVenus reporter gene under the HSP70-rbcs2 promoter. From 384 transformed clones, 88 (22.9 %) displayed mVenus positive (mVenus+) cells upon flow-cytometry analysis. Five clones with different fluorescence intensities were selected. The number of integrated copies was measured by qPCR. Transgene expression levels were followed over the growth cycle and upon SAHA treatment, using a microplate reader, flow cytometry, RT-qPCR, and western blot analysis. First, we observed that expression varies with the cell cycle, reaching a maximum level just before the stationary phase in all clones. Second, we uncovered that supplementation with HDAC inhibitors of the hydroxamate family, such as vorinostat (suberoylanilide-hydroxamic-acid, SAHA) at the initiation of culture increases the frequency (% of mVenus+ cells) and the level of transgene expression per cell over the whole growth cycle, through histone deacetylase inhibition. Thus, we propose a new tool to successfully trigger the expression of heterologous proteins in the green algae *C. reinhardtii*, overcoming its main handicap as an expression platform.

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## Summary

*Chlamydomonas reinhardtii* has been successfully engineered to produce compounds of interest following transgene integration and heterologous protein expression. The advantages of this model include the availability of validated tools for bioengineering, its photosynthetic ability and its potential use as biofuel. Despite this, breakthroughs have been hindered by its ability to silence transgene expression through epigenetic changes. Histone deacetylases (HDAC) are main players in gene expression. We hypothesized that transgene silencing can be reverted with chemical treatments using HDAC inhibitors. To analyze this, we transformed *C. reinhardtii*, integrating into its genome the mVenus reporter gene under the *HSP70-rbcs2* promoter. From 384 transformed clones, 88 (22.9 %) displayed mVenus positive (mVenus<sup>+</sup>) cells upon flow-cytometry analysis. Five clones with different fluorescence intensities were selected. The number of integrated copies was measured by qPCR. Transgene expression levels were followed over the growth cycle and upon SAHA treatment, using a microplate reader, flow cytometry, RT-qPCR, and western blot analysis. First, we observed that expression varies with the cell cycle, reaching a maximum level just before the stationary phase in all clones. Second, we uncovered that supplementation with HDAC inhibitors of the hydroxamate family, such as vorinostat (suberoylanilide-hydroxamic-acid, SAHA) at the initiation of culture increases the frequency (% of mVenus<sup>+</sup> cells) and the level of transgene expression per cell over the whole growth cycle, through histone deacetylase inhibition. Thus, we propose a new tool to successfully trigger the expression of heterologous proteins in the green algae *C. reinhardtii*, overcoming its main handicap as an expression platform.

Keywords: *Chlamydomonas*, transgene, epigenetic silencing, hydroxamate-type histone deacetylase inhibitor, vorinostat, mVenus, flow cytometry

## Introduction

*Chlamydomonas reinhardtii* is a haploid unicellular ciliated chlorophyte from the Viridiplantae lineage. As a model organism, it is used to study of and produce recombinant proteins, metabolites, and biofuels. One of its greatest advantages over yeast or bacteria is the presence of a photosynthetic system and its ability to fix CO<sub>2</sub>. *C. reinhardtii* can also be used to clean wastewater and require low space of land for its growth. Several tools have been developed to efficiently transform this microorganism (Jinkerson and Jonikas, 2015; Schroda, 2019). Despite these advances, studies exposed that nuclear transgene expression and metabolite production remain a challenge, as the microalgae had the ability to shut down transgene expression (Cerutti et al., 1997). This led to a very low yield of protein expressing colonies, a necessity to screen a huge number of transformants (Wang et al., 2022; Nouemssi et al., 2020), and a progressive loss of expression, costing time and money.

Epigenetic silencing is believed to be a main contributor of inefficient expression (Cerutti et al., 1997; Yamasaki and Ohama, 2011) hindering the exploitation of *C. reinhardtii* as a model system for synthetic biology or other applications. Interestingly, epigenetic modulation has been proposed as a possible defense mechanism to prevent expression of foreign DNA (Neupert et al., 2020; Shaver et al., 2010). In eukaryotic cells, this mechanism can be modulated by the level of chromatin condensation, through histone and DNA methylation and histone deacetylation leading to gene expression regulation (Casas-Mollano et al., 2007). These post-translational modifications drive a repressive-condensed chromatin state that prevents transcription factors access to promoters (Shaver et al., 2010). Studies have reported that nearly all detectable histone 3 (H3) included lysine methylation in *C. reinhardtii*, *i.e.* very little unmethylated H3 were found (Khan et al., 2018; Potdar et al., 2018; Waterborg et al., 1995). The majority of lysine residues in the position 4 were monomethylated (H3K4me1), while only a minority were trimethylated (H3K4me3). A strong positive correlation was found between H3K4me3 and a high level of H3 acetylation, while H3K4me1 was associated with a low level of H3 acetylation (Rommelfanger et al., 2021). Consistently, H3K4me1 was associated with inactive euchromatin at transgenic loci, whereas H3K4me3 was enriched in highly transcribed genes promoters (Strenkert et al., 2013; Strenkert et al., 2022). In addition to nucleosomal mechanisms, RNA-mediated

interference has also been demonstrated to modulate gene expression in *C. reinhardtii* (Rohr et al., 2004).

To overcome the gene silencing, several strategies have been developed like: hybrid promoters optimization (Schroda et al., 2000; Specht et al., 2015), addition of endogenous introns into coding sequences (Baier et al., 2018) and transgenes codon optimization. Moreover, mutant strains with enhanced protein expression have been generated by UV mutagenesis (Kurniasih et al., 2016; Neupert et al., 2009). These strains harbor mutations in histone deacetylase (*hdac*) and *DNA methyltransferase* genes. Although these breakthroughs contributed to an increase in expression, 50% of the transformant clones still do not express transgenes following transformation (Neupert et al., 2009). Surprisingly, Neupert et al showed that treated strains with HDAC inhibitors (sirtinol, trichostatin A and OSS\_128167) did not lead to an upregulation of transgene expression (Neupert et al., 2020). By contrast, Kaginkar et al. showed that treatment with some metal ions, light, curcumin, cinnamic acid, quercetin, sodium butyrate and 5-aza-2'-deoxycytidine could impact on stress-induced gene silencing phenotypes, using antibiotic resistance as a readout for transgene expression/silencing (Kaginkar et al., 2021).

In this study, we focused on deacetylation-dependent silencing mechanism in *C. reinhardtii* transformants. We hypothesized that chemical treatment with HDAC inhibitors could be used to restore high transgene expression. For that, we used nuclear transformants containing the *mVenus* reporter gene under the hybrid *HSP70/RBCS2* promoter and showed different levels of mVenus expression over time and in between clones by flow cytometry, a process that was unrelated to the number of transgene integration events, as measured by qPCR. A progressive loss of transgene expression was noted in most clones and in parallel mVenus expression fluctuated over the growth cycle reaching a maximum level just before stationary phase in all clones. Moreover, we showed that the addition of HDAC inhibitors (HDACi) of the hydroxamate family, such as vorinostat (suberoylanilide hydroxamic acid, SAHA) at initiation of *C. reinhardtii* culture, increased the frequency of transgene-expressing cells and the level of transgene expression per cell over the whole growth cycle. Thus, this study proposes a new tool to successfully trigger the expression of heterologous proteins, a simplified method to overcome nuclear gene silencing in the microalgae

*C. reinhardtii*.

## Materials and Methods

### *C. reinhardtii* cultivation and transformation

*C. reinhardtii* strain CC-1690 (wild-type strain; WT) was purchased from the *Chlamydomonas* Resource Center (The University of Minnesota, USA). Growth of *C. reinhardtii* was performed under mixotrophic conditions with Tris-acetate-phosphate (TAP) medium (Gorman et Levine, 1965) on agar plates or liquid in 6 well plates, 96 well plates or 125 mL shake flasks under  $50\pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  light intensity and a photoperiod of 16h light:8h dark cycles at  $21\pm 0.5^\circ\text{C}$ , and 130 rpm agitation for liquid cultures.

Nuclear transformation was carried out by electroporation following GeneArt™ *Chlamydomonas* Protein Expression Vector protocol (Invitrogen, Life technologies, Thermo Fisher Scientifics) using 0.5  $\mu\text{g}$  of linearized pOpt\_mVenus\_Paro vector (Laursen et al., 2015) by restriction endonucleases *Xba* I and *Kpn* I. Transformants were selected on TAP agar plates supplemented with paromomycin (10 mg.  $\text{L}^{-1}$ ) for 5-7 days.

### Treatment with histone deacetylase inhibitors

For all assays, *C. reinhardtii* WT and transformed colonies were initially transferred from agar plates to a liquid preculture in 6 well plates for 4-6 days, under culture conditions mentioned in section 2.1. Inoculums of  $10^5$  cells.  $\text{mL}^{-1}$  in late exponential phase of growth were used in this study.

For initial experiments, 200  $\mu\text{L}$  of *C. reinhardtii* cells grown to exponential phase were transferred into a 96-well plate (flat bottom; Corning) and incubated for 24 hours with individual HDACi, 50  $\mu\text{M}$  sirtinol,

2.5  $\mu\text{M}$  SAHA, 100  $\mu\text{M}$  OSS-128167, 1 mM nicotinamide (all from MedChem Express) and 5 mM sodium pyruvate (Fishers Bioreagents). A mix of all inhibitors was also tested. As a negative control, samples were incubated with DMSO.

SAHA analogs and other HDACi such as belinostat, dacinostat, panobinostat, mocetinostat, entinostat, and romidepsin (all purchased from MedChem Express) were also tested. Culture inoculums were transferred in 200  $\mu\text{L}$  of TAP medium in 96 well plates, with 5  $\mu\text{M}$  of each HDACi followed over a 6 days growth period. Other experiments were done to follow the impact of SAHA on growth and fluorescence levels, inoculum was transferred into 50 mL of TAP media in 125 mL shaking flasks and treated with SAHA at final concentrations of 2.5, 5 and 10  $\mu\text{M}$  for 6 to 12 days.

## Growth curve, chlorophyll and mVenus fluorescence detection using plate reader

Growth curves were obtained using a Synergy H1 plate reader (Biotek, Agilent), from three biological replicates for each *C.reinhardtii* strain studied as in (Molino et al., 2022). For SAHA treatments, 250  $\mu\text{L}$  of *C. reinhardtii* cells from 50 mL cultures were transferred, in triplicate, into 96 well flat bottom microplates (Corning Costar 96-Well, Cell Culture-Treated, Fisher Scientifics). Microalgae growth was done by tracking the optical density at 750 nm ( $\text{OD}_{750}$ , 750/8 nm). Area scan mode was used to measure the mean fluorescence intensity of mVenus (excitation 500/18 nm, emission 541/18 nm) and chlorophyll (excitation 475/18 nm, emission 650/18 nm). *C. reinhardtii* WT was used as negative control for mVenus fluorescence. Fluorescence intensity of mVenus was normalized to the  $\text{OD}_{750}$ .

## Flow cytometry

A Cytomics FC500 cytometer equipped with Argon (488 nm) and HeNe (633 nm) lasers were used to measure mVenus emission on the FL1 channel (525/15 nm), and chloroplast fluorescence on the FL4 channel (675/15 nm) (Beckman Coulter Life Sciences). At least 10,000 events were acquired. A homogeneous cloud of intact cells was first gated based on the size (forward scatter, FSC) and the granularity (side scatter, SSC). Then a daughter gate was selected on the cloud of cells with homogenous chlorophyll autofluorescence. The percentage (%) of mVenus<sup>+</sup> cells was measured on this daughter population, with a gate in the FL1 channel that (filter at 525/10 nm) excluded cells with autofluorescence levels similar to the negative controls. Cells were considered mVenus<sup>+</sup> when both the % of gated mVenus<sup>+</sup> cells (and >0.1%) and mVenus mean fluorescence intensity (MFI) were higher than the values of the wildtype (WT) cells used as a control. Propidium iodide (Thermo Fisher, 7  $\mu\text{M}$ ) was used to verify viability (Cheloni et al., 2014) and acquired on the FL3 channel (620/20 nm). To select palmelloid cells, a second gate that included events with a minimum of 2-fold increase in FSC mean intensity was drawn, similarly to (Cheloni and Slaveykova, 2021). In that case, cells were acquired on a Beckman Cytotflex S equipped with violet (405nm), blue (488nm), yellow-green (561 nm) and red (638 nm) lasers. Chlorophyll autofluorescence was detected in the PerCP channel (690/50 nm), mVenus on the FITC (525/40 BP). Statistics were obtained using BD FlowJo version 10 software (BD Biosciences, La Jolla, CA, USA, 2020).

## DNA extraction and qPCR for transgene relative quantity

Genomic DNA (gDNA) was extracted using the protocol from the *Chlamydomonas* Resource Center (The University of Minnesota, USA) with minor modifications. Two mL of a 6 days *C. reinhardtii* cell cultures were harvested and centrifuged in 1.7 tubes for 5 min at 4000 g. The cell pellet was resuspended in 500  $\mu\text{L}$  of CTAB-buffer (2% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 1,4 M NaCl, 20 mM EDTA pH 8.0 and 2% (v/v) 2-mercaptoethanol) and incubated at 65°C for 1h. The DNA was extracted with 500  $\mu\text{L}$  of chloroform/isoamyl alcohol (24:1). The upper phase was transferred and 0.7 volumes of isopropanol was added for 15 min at 4°C. The DNA was spin down at 4°C for 20 min at 12000 rpm. The pellet was washed twice with 1 mL of cold 70% ethanol and centrifuged at 4°C for 5 min at 12000 rpm. The supernatant was discarded and the pellet was air dry under the hood until the pellet was completely dried. The gDNA was dissolved in 50  $\mu\text{L}$

of TE-buffer (1 mM Tris-HCl pH 8.0 and 0,1 mM EDTA pH 8.0). gDNA yield and quality were determined by measuring the 260/280 ratios using a Nanodrop instrument (Thermo Fisher Scientific).

gDNA samples were subjected to qPCR amplification using Luna® Universal qPCR Master Mix (New England Biolabs). Briefly, 250 ng of gDNA were used as template in 20  $\mu$ L reactions according to manufacturer instructions. Initial denaturation was 2 min at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C and extension for 30 seconds at 60°C. Primers used to detect *mVenus* and *histone 3(h3)* (reference gene) were designed using PrimerQuest™ Tool (Integrated DNA Technologies, IDT) (primer sequences are listed in Table S1). The oligonucleotides were validated by performing a standard curve and through dissociation curves analysis (60-95°C for the melt curves). SYBR Green fluorescence was recorded in the FAM channel of a CFX connect real time system (Bio-Rad). PCR runs were analyzed with CFX Manager software (Bio-Rad). pOpt\_mVenus\_Paro was used to determine the copy numbers. Dilutions ranging from 5 to 1000 pg of vector were used to perform the standard curve. Plasmid copy number was estimated following this formula:

$$(\text{pOpt\_mVenus (ng)} * 6.0221.10^{23}) / (\text{pOpt\_mVenus (bp)} * 660 * 1.10^9)$$

(Integrated DNA Technologies, <https://www.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number>).

Relative mVenus gene copy numbers for each strain were determined using the equation of the standard curve  $\log(y) = ax + b$ ; where  $y$  = plasmid copy number and  $x$  = Ct. Each sample copy number was then determined with the formula  $10^{((Ct_{\text{sample}} - b)/a)}$ , as in (Masroori et al., 2016), normalized to the gDNA weight (ng). Relative mVenus gene detection was also calculated, using *h3* as an endogenous control (Veillette et al., 2013). Experiments were performed with three biological replicates.

## RNA extraction and RT-qPCR for mRNA relative quantification

Four mL of a 6 days *C. reinhardtii* grown culture were harvested and centrifuged in 1.7 mL tube for 5 min at 4000 g. Cells were lysed by immersion in liquid nitrogen for 1 min. Total RNA was extracted using Invitrogen™ TRIzol™ reagent (Life technologies, Thermo Fisher Scientific) according to the manufacturer protocol with minor modifications, including the addition of NaCl (final concentration of 100 mM) in isopropanol to improve nucleic acids precipitation. Samples were further treated with Turbo DNase (Invitrogen TURBO DNA-free Kit, Thermo Fisher Scientific) according to the manufacturer's instructions. RNA yield and quality were determined by measuring the 260/280 ratios using a Nanodrop instrument.

Samples of 100 ng total RNA were subjected to reverse transcription and qPCR amplification in a single reaction using the Luna<sup>(r)</sup> Universal One-Step RT-qPCR Kit Protocol (New England Biolabs). Briefly, 2  $\mu$ L of RNA were subjected to reverse transcription performed at 55°C for 10 minutes. Initial denaturation was 1 min at 95°C followed by 45 cycles of denaturation for 10 seconds at 95°C and extension for 30 seconds at 60°C. A melt curve analysis was performed from 60-95°C with an increment of 0.5°C each 5 seconds. Primers used for mVenus transcript and *h3* as a housekeeping gene were designed using PrimerQuest™ Tool (IDT) (primer sequences are listed in Table S1). SYBR Green fluorescence was recorded in the FAM channel of a CFX connect real time system. PCR runs were analyzed with Bio-Rad CFX Manager version 3.1 software. Relative mRNA expression levels were determined according to the  $2^{(-\Delta\Delta^{\tau})}$  method (Livak et Schmittegen, 2001). Experiments were performed using three technical replicates.

## Protein extraction and western-blot

Twenty-five mL of a 6 days *C. reinhardtii* cell cultures were harvested and centrifuged in 50 mL tube at 4000 g for 10 min at 4°C. The pellets were washed once with ice-cold PBS 1X supplemented with 5 mM sodium butyrate, to retain levels of histone acetylation. Then, pellets were weighed and resuspended with a ratio 0.5 g FW mL<sup>-1</sup> in Triton Extraction Buffer (TEB: PBS 1X containing 0.5% Triton X 100 (v/v), 0.02% (w/v) NaN<sub>3</sub>). PMSF, final concentration 2 mM, and protease inhibitor (32  $\mu$ L g FW<sup>-1</sup>) were subsequently added

(both from Thermo Fisher Scientific). Sonication was performed 6 times at 35% amplitude, 30 sec on, 30 sec off for 3 min total using Fisherbrand™ Model 505 Sonic Dismembrator (Thermo Fisher Scientific). Protein extracts were centrifuged at 14000 g for 30 min at 4°C. Supernatants containing the total soluble protein fractions were kept at -80°C to be used for western blot. Proteins were quantified with the RC DC Protein Assay Kit I (Bio-Rad).

To detect mVenus and Histone H3 Lysine 9 acetylated (H3K9ac), 50 µg and 25 µg of total proteins were loaded respectively, in 15% SDS-PAGEs. Purified mVenus protein from colony 21 using GFP trap agarose (Chromotek, Germany) was loaded as a positive control. Proteins were then transferred to the 0.2 µm PVDF membrane (settings: 1 mA constant and 25 V for 30 min). Primary antibodies for mVenus (27 kDa) and H3K9ac (15.4 kDa) detection were purchased from Cedarlane and from Agrisera, respectively. Both antibodies were diluted at 1:1000 in 3% BSA and were incubated overnight at 4°C. Actin (40 kDa) and histone 3 (H3, 15.4 kDa) detection was performed as internal standards of cytosolic and nuclear proteins, respectively, respectively. Blots were incubated with anti-actin-HRP solution, 1:10,000 in BSA 3%, from Abcam (Cambridge) and anti-H3 (1:1,000 in BSA 3%, Agrisera). After three washes with Tris-buffered saline, 0.1% Tween 20 (TBST) solution, blots were incubated for 1 hour in a 1:20,000 dilution, in 5% milk, of Immun-Star Goat Anti-Mouse (GAM)-HRP Conjugate from Bio-Rad to detect mVenus, and Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate from Bio-Rad to detect H3K9ac. After three washes of the membrane with TBST solution, protein detection was revealed using Clarity Max Western ECL Substrate-Luminol solution from Bio-Rad. Chemiluminescence detection and Ponceau S stained (Glacial Acetic Acid 5% v/v, Ponceau Red dye 0.1% m/v) of blots were visualized using ChemiDoc Imaging System with Image Lab Touch Software (Bio-Rad) and Image Lab Software (Bio-Rad). The molecular weight of the protein corresponding to the detected band was confirmed with the protein marker (Precision Plus Protein Dual Color Standards #1610374).

## Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 9.4.1, GraphPad Software, US). Data are expressed as means  $\pm$  SD of three biological replicated performed at least twice in independent experiments. Parametric tests (ANOVA and Student's *t* test) were used when population followed normal distribution, and non-parametric (ANOVA and Mann-Whitney test) were used when the population could not be assumed to be normally distributed. A *p* value  $< 0.05$  was considered to be significant.

## Results

### Screening of mVenus<sup>+</sup> transformants by flow cytometry

Electroporated cells with pOpt\_mVenus\_Paro linearized vector (Figure S1) were allowed to recover in liquid media for 24 hours and plated on Petri dishes containing TAP agar medium supplemented with paromomycin, as antibiotic selection, for 5-7 days. Cells were transferred twice in fresh media before fluorescence assessment in 6-day old liquid culture. Flow cytometry was used for the initial detection and screening of mVenus<sup>+</sup> positive colonies transferred to liquid medium. The initial gating strategy to screen mVenus<sup>+</sup> clones was as follow:  $>1\%$  mVenus<sup>+</sup> total cells and MFI  $> 1.5$ -fold compared to the WT (Figure 1 B). A total of 88 (22.9%) colonies over the 384 antibiotic-resistant were mVenus<sup>+</sup> at day 6 of culture (Figures 1A and S2). Among these positive colonies, 23 were selected and tested again to assess fluorescence stability 6 weeks later; 10 colonies (43.5%) were still mVenus<sup>+</sup>, while the others lost detectable levels of mVenus fluorescence signal (Figure 1C). We hypothesized that the loss in mVenus expression, despite antibiotic resistance, was due to silencing events.

## mVenus expression levels is modulated by hydroxamate family of HDAC inhibitors

Five transformants (numbered here as 16, 18, 20, 21, 22) were selected with different levels of fluorescence in order to assess if chemical treatment with silencing inhibitors could increase mVenus expression. *C. reinhardtii* WT was used as negative control. We used HDACi targeting HDACs classes I to IV. Sirtinol, nicotinamide target HDAC class III/sirtuin, and OSS-128167 is specific to sirtuin 6. SAHA inhibits HDAC class I, II & IV; and sodium butyrate is known as a HDAC class I & IIA inhibitor (Wang & al., 2019). HDACi were added at initiation of culture.

For most of the tested clones, treatment with SAHA (2.5  $\mu$ M) for 24 hours increased the frequency of cells expressing mVenus and its level of expression per cell (Figures 2A and 2B).

The % of mVenus<sup>+</sup> cells increased by 5.1-fold in clone 16 (mean: 13.3 to 68.1%) and there was a 4.6-fold increase in protein fluorescence intensity (MFI), when cells were treated with SAHA (Figure 2A). The rise was less marked in other clones (1.1- to 1.8-fold in frequency) with higher basal fluorescence, consistent with a baseline decreased silencing. OSS-128167 and sodium butyrate had a slight effect on the frequency and the mean fluorescence intensity of mVenus<sup>+</sup> cells, while none of the other inhibitors consistently improved expression levels of mVenus.

We further evaluated a 6-day treatment of SAHA and a new generation HDAC inhibitors, *i.e.* belinostat, dacinostat and panobinostat of hydroxamate-class like SAHA, mocetinostat and entinostat, two benzamide derivatives (HDAC class I inhibitor), and romidepsin, a bacterial-derived cyclic tetrapeptide (HDAC class I inhibitor) (Karagiannis et Rampias, 2021). All treatments were done with 5  $\mu$ M of inhibitors at initiation of culture (Figures 3A and 3B).

Clone 22 was replaced by clone 17 that exhibited less fluorescence at basal levels, *i.e.* mVenus expression was suspected to be more silenced in clone 17 compared to 22 (Figure 1C). Relative copy number of *mVenus* was assessed to verify if the differences between the clones were not due to the number of mVenus copies integrated into the genome. There were no differences in the amount of integrated mVenus in between the tested clones, as measured in copy number or relatively to endogenous gene (Figure S3).

Results showed that the hydroxamate class of HDACs inhibitors was the most potent in boosting both the frequency of mVenus cells in silenced clones (8.6-fold in clone 16 for SAHA (8,5 to 73,1%), 27-fold in clone 17 for SAHA (2,2 to 59,9%)) and its level inside each cell (MFI, 3-4-fold in clone 16 for SAHA, 2-5-fold in clone 17) at day 3 post-treatment (Figures 3A, 3B and S4A). Comparable results were obtained with the other inhibitors from this class, but SAHA was usually less toxic than dacinostat and panobinostat (growth curves and chlorophyll levels, although not to statistically significant levels) and was generally more potent than belinostat (Figure S4B and S4C).

Thus, we continued investigating the efficiency of this molecule (SAHA) to reverse silencing in *C. reinhardtii*.

## mVenus levels are modulated over time and treatment

Expression of mVenus was followed using both a microplate reader and a flow cytometer for 12 days after treatment with SAHA. In untreated, or solvent-treated cells, overall fluorescence peaked around day 6 in all samples (day 5-7), corresponding to the end of the exponential phase and the start of the stationary phase (Figures 4 A-C, S5A and B). Fluorescence levels of mVenus progressively decreased over the following 6 days of culture. When cells were treated with SAHA, the maximum detection of mVenus was also measured at the end of the exponential phase (day 4-7), reaching more than 74% of producing cells in all the clones (Figure 4C), and a maximum of fluorescence intensity overall and per cell (Figure 4A and 4B). Consistently with the low number of cells impacting on the flow cytometer sensitivity in the first 3 days, the analysis of the % of mVenus<sup>+</sup> cells varied at early time point (Figure 4C).

Nonetheless, SAHA treatment at initiation of culture steadily increased fluorescence around day 4-5 in all clones, but did not prevent the progressive decline in production when transformants advanced into stationary phase around day 7-8. At day 12, most clones declined to <10% of mVenus<sup>+</sup> cells (except 16 and 21) but had still more frequent population of mVenus expressing cells and more mVenus fluorescence signal per cells compared to untreated or DMSO treated cells (Figure 4A, 4B and 4C). SAHA did not seem to impact on overall growth of microalgae (Figure S5A) but a significant decline in chlorophyll fluorescence was noted (Figure S5B, One-way Anova, Dunett's multiple comparison test), suggesting that some metabolic pathways related to photosynthesis are altered. This prompted more investigation on SAHA's impact on cell fitness.

## SAHA's effect on viability, cell growth, palmelloid and chlorophyll levels

We treated cells at initiation of culture with increasing concentrations of the inhibitors and incubated them for 6 days. Viability was analyzed through membrane integrity confirmation using propidium iodide (PI) staining at day 6 on a flow cytometer. SAHA's addition at concentration of 2.5, 5 and 10  $\mu$ M seemed to slightly increase the % of PI<sup>+</sup> cells, but ~90% of the transformant cells remained intact and there was no significant increase compared to background levels in WT cells (Figure 5A). Different stress conditions have been reported to transiently induce palmelloid colonies in *C. reinhardtii* without impacting viability (de Carpentier et al., 2019). We observed that 6 days of treatment with 5 and 10  $\mu$ M of SAHA lead to the accumulation of small palmelloid colonies in *C. reinhardtii* (Figure 5C and D compared to Figure 5B), while motility was also progressively lost above 2.5  $\mu$ M. We quantified palmelloid cells formation by flow cytometry when transformants were treated with 5  $\mu$ M of SAHA and showed that in most cases palmelloid colonies increased at day 3 and 6 post-treatment (Figure 5E and S6A). These results suggest that SAHA modifies chlorophyll content and colony morphology but does not alter cell growth kinetic nor viability at concentrations of 10  $\mu$ M and lower.

SAHA's addition at higher concentration of 20, 40 and 80  $\mu$ M was also tested to see at which degree it could affect the growth kinetic and viability. Larger palmelloid colonies (or aggregates) were observed (Figure S6B) with increasing concentrations, while growth kinetic was inhibited partially with 20 and 40  $\mu$ M SAHA, and totally with 80  $\mu$ M SAHA (data not shown). Chlorophyll content was also strongly modified, as observed by the progressive loss of the usual dark green color of *C. reinhardtii* healthy culture in favor of a more yellow tint (20 and 40  $\mu$ M SAHA) or even red tint (80  $\mu$ M SAHA).

## SAHA upregulates mRNA transgene levels by inhibiting histone deacetylation.

Then, we measured the impact of SAHA treatment on transgene mRNA levels. SAHA being a HDAC inhibitor, its addition should increase the availability of the chromatin to transcription factors, and hence the mRNA levels. The relative mVenus mRNA expression over the housekeeping gene *h3* transcript expression increased 5.7- and 6.6-fold in clone 16 and 17 respectively at day 6 of cultures, when cells were treated with SAHA compared to the DMSO control (Figure 6A); the rise was also observed in other clones, although less marked (1.2- to 2.8-fold).

Finally, we verified by western-blot that histones acetylation was restored upon SAHA treatment (Figure 6B), leading to brighter band using the H3K9Ac antibody in all transformants, while the amount of histone 3 remained consistent. In addition, we could detect an increase in mVenus protein accumulation in clones 16, 17 and 18 at day 6 of cultures, but not in 20 and 21 the two transformants with the highest level of mVenus basal expression. Without treatment, mVenus was barely detectable in clones 16, 17, and 18, consistently with the low fluorescence signal detected by plate reader and FACS.

## Discussion

In this study, we propose a new tool to successfully trigger the expression of silenced heterologous proteins in the green microalgae *C. reinhardtii*. Integration of exogenous DNA into the microalgae nuclear genome predominantly occurs randomly, via non-homologous-end-joining (NHEJ), leading to a large and heterogeneous population of transformed cells with varied expression levels (Zhang et al., 2014; Nouemssi et al., 2020). Despite antibiotic selection, not all transformed cells express the targeted transgene at a desired level. This loss of expression occurred even though the resistance marker was downstream of the reporter expression cassette in the transformation vector. Hence, screening of mVenus<sup>+</sup> transformants by plate reader or flow cytometry becomes useful to target the transformants with the highest expressing level of transgene. As usually observed with *C. reinhardtii* nuclear transformants, many (97.4%) early positive transformants expressing the transgene of interest, which was not essential for survival, underwent transcriptional and/or post-transcriptional gene silencing mechanisms (Tran et Kaldenhoff, 2020). Thus, the number of positive transformants falls overtime after rounds of subcultures despite antibiotic selection. Our report demonstrated for the first time, to our knowledge, the use of HDAC inhibitors of hydroxamate-class like SAHA in *C. reinhardtii* to trigger protein expression.

Upon SAHA treatment, the expression level of the mVenus reporter protein can be recovered at doses that do not affect the cell growth. Treatment at initiation of cultures lead to a maximal production of mVenus at the end of the exponential phase (around day 6). However, SAHA did not prevent the progressive decline in mVenus production when transformants advanced into stationary phase, although cultures treated with SAHA still expressed more transgene.

The overall growth pattern of microalgae was not impacted upon treatment with 5  $\mu$ M SAHA, but a significant decline in chlorophyll fluorescence was observed. In addition, cell motility was progressively lost [?]2.5  $\mu$ M SAHA, while palmelloid formation was induced.

Some studies have shown that abiotic stress could transiently induce palmelloid in *C. reinhardtii* without impacting viability (Cheloni et Slaveykova, 2021; de Carpentier et al., 2022).

Cheloni et Slaveykova examined palmelloid colony formation upon micropollutants (MPs) exposure. The number of palmelloid and their size were dependent on MP concentration and exposure duration. Cells kept growing and dividing within the palmelloid and reverted to their unicellular lifestyle when colonies were harvested and inoculated in fresh medium, indicating that palmelloid formation is a common (and not specific) plastic response to different micropollutants. In mixed populations cultures, the unicellular population exhibited chlorophyll bleaching, membrane damage and oxidative stress, whereas palmelloids were unaffected. In a different study, Carpentier *et al.* reported on the characterization of an abiotic stress response that the algae can trigger, forming massive multicellular structures called aggregates, which are different from palmelloids. Aggregates are formed by a few tens, to several thousand cells, held together in an extracellular matrix, whereas palmelloids are composed of 4 to 16 cells surrounded by a cell wall. These aggregates constitute an effective bulwark within which the cells are efficiently protected from the toxic environment. Aggregation is not the result of passive agglutination, but rather of genetic reprogramming and substantial modification of the algae's secretome. Hence, the induction of aggregates and/or palmelloid following SAHA treatment, could help concentrate proteins (and even metabolites) within fewer cells linked together in these multicellular structures, which would not hinder significantly usual cellular metabolism.

High doses of SAHA (20, 40 and 80  $\mu$ M) strongly increased the number and promoted the larger size of the palmelloids and/or aggregations formation and inhibited the cell growth. There have been some studies using HDAC inhibitors of hydroxamate-class like SAHA in plant cell cultures (*Medicago truncatula* and *Bambusa multiplex*) to boost protein expression (Santos et al., 2017; Nomura et al., 2021), which reported cell toxicity induced by the tested HDACi. Toxicity was lower when the cell culture was treated at day 3, at the onset of exponential phase. In a different study, Nomura *et al.* boosted the production of two endogenous specialized metabolic compounds (3-*O*-*p*-coumaroylquinic acid and 3-*O*-feruloylquinic acid in a cell line culture of *B. multiplex* treated with Suberoyl bis-hydroxamic acid (SBHA), an analog of SAHA. Production

of both compounds was induced by SBHA at concentrations between 2  $\mu\text{M}$  and 100  $\mu\text{M}$ , but production decreased at concentrations [?] 50  $\mu\text{M}$ , mainly because of the cytotoxicity of SBHA. Interestingly, in their study, smaller initial cell density of 5% SCV (sedimented cell volume) lead to the strongest induction of both metabolites. This suggests that inoculum size might be an important contributing factor to the success of silencing reversal.

Upon SAHA-treatment, the relative mVenus mRNA expression and protein levels, together with fluorescence intensity increased in most clones. These results are consistent with SAHA *modus operandi* at the DNA levels, inducing gene expression. Transcriptional gene silencing is believed to be the main cause for transgene expression inhibition in *C. reinhardtii*, which is largely mediated by protein factors that place specific histone modifications onto nucleosomes at the transgene loci to trigger the formation of a repressive chromatin structure, a mechanism that may have evolved to protect the genome from invading DNA (Schroda, 2019). H3K4 and K9 monomethylated are some of the histone marks known to occur on nucleosomes in promoter regions of silent genes in *C. reinhardtii*, while H3K4 trimethylated and H3K9 acetylated appear in promoter regions of active genes (Yamasaki et al., 2008; Shaver et al., 2010; Strenker et al., 2013; Barahimipour et al., 2015; Schroda, 2019). SAHA triggered an increase in histone acetylation level of H3K9 (determined by western-blot) in all clones, while the level of H3 remained consistent. This further confirms that SAHA treatment restored expression by increasing histone acetylation levels, or by preventing histone deacetylation. In addition, Kaginkar *et al.*, using antibiotic resistance as readout for transgene expression in *C. reinhardtii* suggested that the use of some metal ions, light, curcumin, cinnamic acid, quercetin sodium butyrate, decitabine (5-aza-2'-deoxycytidine) could reverse stress-induced silencing, through inhibition of DNA methylation or histone deacetylation (Kaginkar et al., 2021). By contrast Neupert *et al.* could not induce expression using sirtuin inhibitors and other HDACs inhibitors, despite increasing histone acetylation levels (Neupert et al., 2020). In our hands, inhibition of methylation did not yield to an increase in mVenus expression, while hydroxamate-family HDAC inhibitors were very efficient. Multiple and distinct mechanisms responsible for silencing could occur in different clones. It might depend on the promoter, implying that triggering expression with some inhibitors might work for some clones and not others.

In this study, *mVenus* gene expression was driven under the hybrid *HSP70A-RBCS2* fusion promoter. Strenkert *et al.* (2011, 2013) demonstrated that the transgenic *HSP70A* promoters harbor lower levels of active chromatin marks than the native *HSP70A* but more than transgenic *RBCS2* promoters (Strenkert et al., 2013; Strenkert et al., 2011). The authors found that, first, heat shock transcription factor 1 (HSF1) binds to the promoter, second histone acetylation occurred, then nucleosomes were remodeled, and transcript accumulated. This suggested that the HSF1 recruits histone acetyltransferase (and other histone-modifying enzyme activities) to target promoters. HSF1 could constitutively form a scaffold at the transgenic *HSP70A* promoter, presumably containing mediator and TFIID, from which local chromatin remodeling and polymerase II recruitment to downstream promoters is realized. However, the authors also observed HSF1-independent histone H3/4 deacetylation at the *RBCS2* promoter after heat shock, suggesting interplay of specific and presumably more generally acting factors to adapt gene expression to the new requirements of a changing environment. Interestingly, in the case of the *HSP70A-RBCS2* fusion promoter, the chromatin state at the *HSP70A* promoter was dominantly transferred to *RBCS2* by HSF1, to recruit all the machinery necessary for transcription. Here, we show that HDAC inhibitors of hydroxamate-class like SAHA can further help maintain active chromatin marks at the *HSP70A-RBCS2* fusion promoters.

In summary, we uncovered a new tool to successfully trigger the expression of heterologous proteins in *C. reinhardtii*. This method could also be useful and applicable for recombinant production in other microalgae species and open the field to new studies.

## Conflict of Interest

The authors declare no conflict of interest. R.B., N.M., F.M-M., and I.D-P. are inventors of the patent application PCT/ US Application No. 63/453,541 based on this work.

## Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

## Author Contributions

R.B. and N.M. contributed equally to this work. N.M., F.M-M., and I.D-P conceived and supervised the project. R.B., N.M., E.F., S.B.N., and P.L. performed the experiments. R.B., N.M., and E.F. analysed the data. R.B., N.M., E.F., S.B.N., P.L., F.M-M., and I.D-P. wrote the paper with input from all the authors. I.D-P secured funding.

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## Figure legends

### Figure 1. Selection of mVenus<sup>+</sup> transformants by flow cytometry.

A. Heatmap representation of the % mVenus<sup>+</sup> cells in each transformant, as assessed by flow cytometry. mVenus fluorescence intensity was acquired on the FL1 channel (filter at 525/10 nm) on 384 transformed antibiotic resistant microalgae transferred to liquid TAP medium in four 96 well plates for 6 days. Negative controls (WT) are represented in wells 95 and 96 of each plate. Crossed wells are transformant that did not grow in liquid medium. B. Gating strategy and representative dot plots of a negative (left panel) and a positive (right panel) sample. C. Correlation between mean fluorescence intensity and % of mVenus<sup>+</sup> cells in 23 mVenus<sup>+</sup> transformants and a wild type strain re-assessed for mVenus production 6 weeks following the first screening.

### Figure 2. mVenus expression following treatment with silencing inhibitors.

A. Heatmap representations of the % of mVenus expressing cells (top) and of the mVenus delta mean fluorescence intensity (bottom) in clones 16, 18, 20, 21, 22 and WT. Liquid culture in 96 wells were treated with DMSO (0.1%), Sirtinol (SIRT, 50  $\mu$ M), vorinostat (SAHA, 2.5  $\mu$ M), OSS.128167 (OSS, 100  $\mu$ M), nicotinamide (NAD, 1mM), Sodium butyrate (ButNa, 5 mM), or a mix of all HDACi (Mix) for 24 hours. B. Representative pseudocolor dot plots of mVenus levels in transformants treated with HDACi or DMSO. \*:  $p > 0.05$ ; \*\*:  $p > 0.01$ , using one-way ANOVA Friedman's test with repeated measures.

### Figure 3. Hydroxamate-family of HDACs inhibitors are potent inducers of transgenic expression.

A. Bar graph showing the mean % of mVenus<sup>+</sup> cells acquired by flow cytometry at day 6 of culture in clones 16, 17, 18, 20, 21 and WT treated with DMSO or inhibitors in 96 wells liquid culture. B. mVenus mean fluorescence intensity curve plot over 5 days following inhibitors treatment in clones 16, 17, 18, 20, 21 and WT as measured using a microplate reader. mVenus fluorescence was acquired in area scan mode and normalized to the OD<sub>750</sub> acquired in the same reading mode. Finally, the levels of mVenus fluorescence intensity in the WT control (calculated similarly) was subtracted of each sample. Error bars represent standard deviation of biological triplicates.

**Figure 4. SAHA steadily increases transgene expression over culture period.**

Flask liquid cultures of clones 16, 17, 18, 20, 21 and WT were treated with 5 μM of SAHA and grown for 12 days. The mean fluorescence intensity (MFI) of mVenus was assessed every day. A. The MFI was measured on the overall culture using a microplate reader. B and C. represent the MFI and the % of mVenus<sup>+</sup> cells, respectively, measured across growth period using a flow cytometer

**Figure 5. SAHA induces a palmelloid phenotype in *C. reinhardtii*.**

A. SAHA's effect on cell viability (membrane integrity) was verified using propidium iodide (PI) staining and analyzed on a flow cytometer (FL3 channel). B-D. Representative microscopy pictures of liquid culture in 96 wells of transformants treated with DMSO, 5 and 10 μM of SAHA (respectively) for 6 days. E. Bar graph with means % of palmelloids in transformants following 5 μM SAHA treatment at day 3 and 6, as measured on a flow cytometer. FSC: forward scatter, SSC Side scatter. Error bars represent standard deviation of biological triplicates. Two-way ANOVA repeated value statistical test was used, \*\*\**p* < 0.001, \*\**p* < 0.01 (effect of SAHA on palmelloid formation).

**Figure 6. SAHA upregulates mRNA transgene level by inhibiting histone deacetylation.**

SAHA's effect on mRNA transgene levels and histone acetylation levels were verified by RT-qPCR and western-blot respectively. Flask liquid cultures of clones 16, 17, 18, 20, 21 and WT treated with 5 μM of SAHA and grown for 12 days were all tested at day 6. A. RT-qPCR bar graph result of relative mVenus mRNA expression normalized on histone*h3* in clones 16, 17, 18, 20, 21 and WT treated with SAHA and DMSO (negative control). B. Immunoblot analyses to determine the levels of mVenus (27 kDa upper panel), histone H3 Lysine 9 acetylated (H3K9ac, 15.4 kDa, below), histone 3 (H3, middle panel and actin (40 kDa, lower panel) in clones 16, 17, 18, 20, 21 and WT treated with SAHA and DMSO (negative control). Total soluble protein was separated by denaturing 12% SDS-PAGE, blotted and probed with specific antibodies as indicated. For mVenus and H3K9ac detection, 50 μg and 25 μg of total soluble proteins were loaded respectively. Purified mVenus was used as control. The blot, from the upper and lower panels, was detected with anti-mVenus, following by anti-actin detection. Middle panels blot was first incubated with anti-H3K9ac and then anti-H3. Molecular weights were deduced from co-migrating protein markers.

## Supplementary material legend

**Supplementary Table 1. Primer sequences used in this study.**

**Supplementary Figure 1. Vector used for nuclear transformation.**

pOpt\_mVenus\_Paro vector map from Laursen et al., 2015 (with minor modifications).

**Supplementary Figure 2. mVenus fluorescence intensity in transformants.**

Heatmap representation of the mVenus delta mean fluorescence intensity in each transformant, as assessed by flow cytometry. mVenus was acquired on the FL1 channel (filter at 525/10nm) on 384 transformed antibiotic resistant microalgae transferred to liquid TAP medium in four 96 well plates for 6 days. Negative controls (WT) are wells 95 and 96 of each plate. Crossed wells are transformant that did not grow in liquid medium (or that did not provide quantifiable data).

### Supplementary Figure 3. Transgene relative quantification by qPCR method

A. qPCR bar graph result of mVenus gene copy relative expression in clones 16, 17, 18, 20 and 21 are normalized to gDNA mass (ng). Means  $\pm$  SD (n=3) are plotted. B. mVenus gene detection in clones 16, 17, 18, 20, 21 was normalized to the endogenous gene Histone 3 and compare to the WT strain. Means  $\pm$  SD (n=3) are plotted.

### Supplementary Figure 4. Hydroxamate-type HDACs inhibitors effects on growth curve.

A. Bar graph showing the mean fluorescence intensity (MFI) of mVenus<sup>+</sup> cells  $\pm$  SD acquired by flow cytometry at day 3 of culture in clones 16, 17, 18, 20, 21 and WT treated with DMSO or inhibitors in 96 wells liquid culture. B. OD<sub>750</sub> curve plot over 5 days following inhibitors treatment in clones 16, 17, 18, 20, 21 and WT as measured using a microplate reader. OD<sub>750</sub> was acquired in scan mode. C. Chlorophyll fluorescence (RFU) curve plot over 5 days following inhibitors treatment in clones 16, 17, 18, 20, 21 and WT as measured using a microplate reader. Chlorophyll fluorescence was acquired in scan mode.

### Supplementary Figure 5. SAHA steadily increases transgenic expression over culture period.

Flask liquid cultures of clones 16, 17, 18, 20, 21 and WT were treated with 5 mM of SAHA and grown for 12 days. The OD<sub>750</sub> and chlorophyll fluorescence (RFU) were assessed every day. A. The OD<sub>750</sub> was measured on the overall culture using a microplate reader. B. The chlorophyll fluorescence (RFU) was measured on the overall culture using a microplate reader. Statistical analysis: Dunnett's multiple comparisons test, analyzing OD<sub>750</sub>, there was a statistically significance difference for clone 18: DMSO vs. SAHA \*\*  $p= 0.0088$ . Upon analyzing chlorophyll fluorescence, there were statistically significant differences comparing DMSO with SAHA treatment for WT ( $p= 0,0054$ ), for clone 16 ( $p= 0,0046$ ), clone 17 ( $p= 0,0080$ ), clone 18 ( $p= 0,0057$ ), clone 20 ( $p= 0,0151$ ), clone 21 ( $p= 0,0018$ ).

### Supplementary Figure 6. Single vs Palmelloid events gating strategy on a Cytoflex flow cytometer and Palmelloid microscopy.

A. Gating strategy and representative dot plots of a positive palmelloid event (upper panels) and a positive single event (lower panels). B. Representative microscopy picture of liquid culture in 96 wells of transformants treated with 20, 40 and 80  $\mu$ M SAHA for 6 days.

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**Supplementary Table 1. Primer sequences used in this study.**

Name	Sequence (5' to 3')
qPCR for transgene relative quantity	qPCR for transgene relative quantity
mVenus_F	AACCACTACCTGAGCTACCA
mVenus_R	ATATCCCTGCCCTCGATCTT
Histone H3_F	GAGATCCGCAAGTACCAGAAG
Histone H3_R	TCTTGAAGTCCTGGGCAATC

RT-qPCR for mRNA relative quantity	RT-qPCR for mRNA relative quantity
mVenus_F	GAAGCAGCACGACTTCTTCAAGAG
mVenus_R	TTCAGCTCGATGCGGTTTAC
Histone H3_F	GAGATCCGCAAGTACCAGAAG
Histone H3_R	TCTTGAAGTCCTGGGCAATC

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