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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 obstacle as an expression platform.

KEYWORDS
Chlamydomonas, epigenetic silencing, flow cytometry, hydroxamate-type histone deacetylase inhibitor, mVenus, transgene, vorinostat

Rémy Beauchemin and Natacha Merindol contributed equally to this work and shared as first authorship.

1 | 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_2. *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.[1,2] 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.[3] This led to a very low yield of protein expressing colonies, a necessity to screen a huge number of transformants,[4,5] and a progressive loss of expression, costing time, and money.

Epigenetic silencing is believed to be a main contributor of inefficient expression[3,6] 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.[7,8] 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.[9] These post-translational modifications drive a repressive-condensed chromatin state that prevents transcription factors access to promoters.[8] *C. reinhardtii* was shown to contain 17 genes annotated as *hdac*, including three Sirtuin2-type (HDAC class III, sirtuin class II, III, and IV), and HDAC homologous to *Arabiodopsis thaliana* type I, but no type II HDACs.[7] Studies have reported that nearly all detectable histone 3 (H3) included lysine methylation in *C. reinhardtii*, that is, very little unmethylated H3 were found.[10–12] The majority of lysine residues in the position four 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.[13] Consistently, H3K4me1 was associated with inactive euchromatin at transgenic loci, whereas H3K4me3 was enriched in highly transcribed genes promoters.[14,15] In addition to nucleosomal mechanisms, RNA-mediated interference has also been demonstrated to modulate gene expression in *C. reinhardtii*.[16]

To overcome the gene silencing, several strategies have been developed like hybrid promoters optimization,[17,18] addition of endogenous introns into coding sequences[19,20] and transgenes codon optimization. Moreover, mutant strains with enhanced protein expression have been generated by UV mutagenesis.[21,22] 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.[22] Surprisingly, Neupert et al. showed that treated strains with HDAC inhibitors (sirtinol targeting HDAC class III/sirtuin, trichostatin A inhibiting class I and II HDACs and OSS_128167 specific to sirtuin 6) did not lead to an upregulation of transgene expression.[7] By contrast, Kaginkar et al. showed that treatment with some metal ions, light, curcumin, cinnamic acid, quercetin, sodium butyrate (HDAC class I and IIA inhibitor), and 5-aza-2'-deoxycytidine (DNA methylation inhibitor) could impact on stress-induced gene silencing phenotypes, using antibiotic resistance as a readout for transgene expression/silencing.[23] The effect of other inhibitors such as hydroxamate-type vorinostat, belinostat, dacinostat, panabinostat that targets HDAC class I, II, and IV, and of mocetinostat and entinostat, benzamide derivatives specific to HDAC class I inhibitor have not been evaluated yet in *C. reinhardtii*.

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*.

2 | MATERIALS AND METHODS

2.1 | *C. reinhardtii* cultivation and transformation

Chlamydomonas 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[24] on agar plates or liquid in 6 well plates, 96 well plates or 125 mL shake flasks under 50 ± 10 µmol photons m^{-2} s^{-1} light intensity and a photoperiod of 16 h light:8 h dark cycles at 21 ± 0.5°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) as described in[4] using 0.5 µg of linearized pOpt_mVenus_Paro[25] or pOpt_Clover_Paro vector by both restriction endonucleases *Xba*I and *Kpn*I, electroporated with the Bio-Rad Genepulser Xcell in 4 mm cuvette at 0.5 kV, 50 µF, 800 Ω. Transformants were selected on TAP agar plates supplemented with paromomycin (10 mg L^{-1}) for 5–7 days. Colonies were replated once a week in fresh TAP solid medium. After two rounds of selection, 384 colonies were seeded in TAP liquid medium, including 4 WT and 4 empty vectors (EV, an antibiotic-resistant colony transformed

with a modified pOpt_mVenus_Paro in which mVenus sequence was removed). Five days later, mVenus fluorescence was measured on a flow cytometer. From this initial screen, 6 mVenus$^+$ clones from plate 1, 6 from plate 2, 7 from plate 3 and 3 from plate 4, which displayed the highest % of mVenus$^+$ cells, were selected. These 22 positive clones were replated 6 times (once a week for 6 weeks) on TAP solid medium. Colonies were reseeded in liquid medium and screened again 3 days later, when the cultures were in exponential phase. From here, the 5 clones with the highest mVenus$^+$ % were selected.

2.2 | Treatment with histone deacetylase inhibitors and ROS levels assessment

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 3.1. Inoculums of 10^5 cells mL^{-1} in late exponential phase of growth were used in this study.

For initial experiments, 200 µL of *C. reinhardtii* cells grown to exponential phase were transferred into a 96-well plate (flat bottom; Corning) and incubated for 24 h with individual HDACi, 50 µM sirtinol, 2.5 µM SAHA, 100 µM 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 µL of TAP medium in 96 well plates, with 5 µM 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 µM for 6 to 12 days.

CellROX orange (CellRox Orange Flow assay kit, Ex:545/Em:565, Molecular probes, Invitrogen) was used to measure reactive oxygen species following SAHA treatment following the manufacturer's instruction.

2.3 | 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.[26] For SAHA treatments, 250 µL 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 (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 OD_{750}.

2.4 | Flow cytometry

A Cytomics FC500 cytometer equipped with Argon (488 nm) and HeNe (633 nm) lasers were used to measure mVenus or Clover 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 granulosity (side scatter, SSC). Then a daughter gate was selected on the cloud of cells with homogenous chlorophyll autofluorescence. The percentage (%) of mVenus$^+$ (or Clover$^+$) 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$^+$ when both the % of gated mVenus$^+$ 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 µM) was used to verify viability[27] 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.[28] In that case, cells were acquired on a Beckman Cytoflex S equipped with violet (405 nm), blue (488 nm), 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).

2.5 | 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 milliliters 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 µL 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 1 h. The DNA was extracted with 500 µL 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 12,000 rpm. The pellet was washed twice with 1 mL of cold 70% ethanol and centrifuged at 4°C for 5 min at 12,000 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 µL 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 µL reactions according to manufacturer instructions. Initial denaturation was 2 min at 95°C followed by 45 cycles of denaturation for 15 s at 95°C and extension for 30 s 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:

(pOpt_mVenus (ng) * $6.022.10^{23}$) / (pOpt_mVenus (MW) * 1.10^{9}) according to Integrated DNA Technologies (https://www.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number).

mVenus gene copy numbers for each strain were determined from 250 ng of gDNA using the equation of the standard curve y = a*log(x) + b; where y = Ct and x = plasmid copy number. Each sample copy number was then determined with the formula $10^{((Ctsample-b)/a)}$, as in,[29] normalized to the gDNA weight (ng). Normalized relative mVenus gene detection was also calculated, using *histone3* and *pgk* as endogenous controls.[30] Experiments were performed with three biological replicates.

2.6 | RNA extraction and RT-qPCR for mRNA relative quantification

Four milliliters 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 Scientifics) 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 Scientifics) 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 Universal One-Step RT-qPCR Kit Protocol (New England Biolabs). Briefly, 2 µL of RNA were subjected to reverse transcription performed at 55°C for 10 min. Initial denaturation was 1 min at 95°C followed by 45 cycles of denaturation for 10 s at 95°C and extension for 30 s at 60°C. A melt curve analysis was performed from 60–95°C with an increment of 0.5°C every 5 s. Primers used for mVenus transcript and *h3* as a housekeeping gene were designed using PrimerQuestTM 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 $2^{(-\Delta\Delta Ct)}$ method.[31] Experiments were performed using three technical replicates.

2.7 | Protein extraction and western-blot

Twenty-five milliliters 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^{-1} in Triton Extraction Buffer (TEB: PBS 1X containing 0.5% Triton X 100 (v/v), 0.02% (w/v) NaN_3). PMSF, final concentration 2 mM, and protease inhibitor (32 µL g FW^{-1}) were subsequently added (both from Thermo Fisher Scientific). Sonication was performed 6 times at 35% amplitude, 30 s on, 30 s off for 3 min total using Fisherbrand Model 505 Sonic Dismembrator (Thermo Fisher Scientific). Protein extracts were centrifuged at 14,000 × *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. mVenus protein was purified from colony 21, and 50 ng was loaded as a positive control. The purification was performed from a 6-day-old culture, 1.10^{7} cells were resuspended in 200 µL of lysis buffer containing 10 mM Tris-HCl ph 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholate. The immunoprecipitation protocol was conducted using GFP-Trap Agarose and following the manufacturer's instructions (Chromotek, Germany). 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 were performed as internal standards of cytosolic and nuclear proteins, 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 h 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 and Image Lab Software (Bio-Rad). The bands' intensity

(adjust volume) was measured using the same software. The molecular weight of the protein corresponding to the detected band was confirmed with the protein marker (Precision Plus Protein Dual Color Standards #1610374).

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 9.4.1, GraphPad Software, US). Data are expressed as means ± 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.

3 | RESULTS

3.1 | Screening of mVenus$^+$ transformants by flow cytometry

Electroporated cells with pOpt_mVenus_Paro linearized vector (Figure S1) were allowed to recover in liquid media for 24 h and plated on Petri dishes containing TAP agar medium supplemented with paromomycin, as antibiotic selection, for 5–7 days. Colonies were transferred twice in solid fresh media before fluorescence assessment in 6-day old liquid culture. Flow cytometry was used for the initial detection and screening of mVenus$^+$ positive colonies transferred to liquid medium. The initial gating strategy to screen mVenus$^+$ clones was as follows: > 1% mVenus$^+$ total gated cells or an MFI > 1.5-fold compared to the WT and EV (empty vector) and > 0.1% mVenus$^+$ total gated cells (Figure 1B). A total of 89 (23,7%) colonies over the 376 antibiotic-resistant were mVenus$^+$ at day 6 of culture (Figures 1A and S2A). Among these positive colonies, 22 with the highest fluorescence, were selected and tested again to assess fluorescence stability 6 weeks later; two negative controls (an antibiotic-resistant colony transformed with a modified pOpt_mVenus_Paro in which mVenus sequence was removed: EV, and a WT colony not transformed) were also included. A sharp decrease in the % of mVenus$^+$ cells was observed for all colonies, except 21, with a mean difference of 24.26%; only 6 colonies had still over 1% mVenus$^+$ cells (Figure 1C, $p < 0.0001$, One-tailed paired t-test). We hypothesized that the loss in mVenus expression, despite antibiotic resistance, was due to silencing events.

3.2 | mVenus expression levels is modulated by hydroxamate family of HDAC inhibitors

The five transformants (numbered here as 16, 18, 20, 21, 22) with the highest % of mVenus$^+$ cells were initially selected to assess if chemical treatment with histone deacetylase inhibitors (HDACi) 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 which is specific to sirtuin 6. SAHA inhibits HDAC class I, II, and IV; and sodium butyrate is known as a HDAC class I and IIA inhibitor.[32] HDACi were added at initiation of liquid culture directly with cells. Concentrations of inhibitors were chosen based on published observations from algae, plants, fungus or animal cells. For most of the tested clones, treatment with SAHA (2.5 µM) for 24 h increased the frequency of cells expressing mVenus, as well as its level of expression per cell (Figure 2A,B).

In clone 16, the % of mVenus$^+$ cells and the value of mVenus fluorescence intensity (MFI) increased 5.1-fold (mean of 13, 3% to 68,1%, $n = 3$), and 4.6-fold (mean of 0,88 to 4,07, $n = 3$), respectively, when treated with 2.5 µM of SAHA. The rise in fluorescence was lower in other clones (1.1- to 1.8-fold for % of mVenus$^+$ cells, and 1.2- to 1.6-fold for MFI), with higher basal fluorescence, consistent with a lower baseline silencing. OSS-128167 and sodium butyrate had a smaller effect on the frequency and the mean fluorescence intensity of mVenus$^+$ cells compared to SAHA, while none of the other inhibitors consistently improved expression levels of mVenus.

We then tested the response of all initially positive 22 transformants to SAHA treatment (5 µM), 18 months post-transformation (Figure S2B). Transformants 16 and 17 could not produce detectable level of mVenus without SAHA, while clones 16, 17, 18, 20, 21, and 22 had their mVenus production increased upon SAHA treatment (Figure S2B). Overall, SAHA treatment significantly increased the frequency of mVenus$^+$ cells ($p = 0.001$, Wilcoxon's paired test). Still, SAHA could not improve mVenus levels over > 1% in the 16 remaining clones. It is possible that for some of these clones tested 18 months post-transformation, (1) autofluorescence early post-transformation led to the selection of false positive clones, or (2) that they lost transgene in their genome, or (3) that the silencing mechanism is not SAHA-sensitive.

In following experiments, clone 22 was replaced by clone 17 that exhibited much less fluorescence at basal levels, that is, mVenus expression was suspected to be more silenced in clone 17 compared to 22 (Figure 1C). We tested other inhibitors such as cinnamic acid (CAci, 1 mM),[33] lycorine (Lyco, 2,5 uM),[34] azacitidine (AZA, 1 µM)[35] together with sodium butyrate (SBut, 5 mM), OSS_128167 (OSS, 100 µM), sirtinol (Sirt, 50 µM), and vorinostat (SAHA, 5 µM) for 72 h, 18 months post-transformation (Figure S2C). The results confirmed that sodium butyrate had a small inducing effect, while SAHA was the most potent inducer of transgene expression in all selected transformants, while other known silencing inhibitors such as 5-aza-2'-deoxycytidine DNA methylation inhibitor had no detectable effect at the tested concentrations. Relative copy number of *mVenus* was assessed to verify if the differences between the clones were due to the number of mVenus copies integrated into the genome or not. 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).

As SAHA was the strongest inducer, we evaluated a 6-day treatment of SAHA and new generation HDAC inhibitors, that is, belinostat, dacinostat and panobinostat of hydroxamate-class like SAHA, mocetinos-

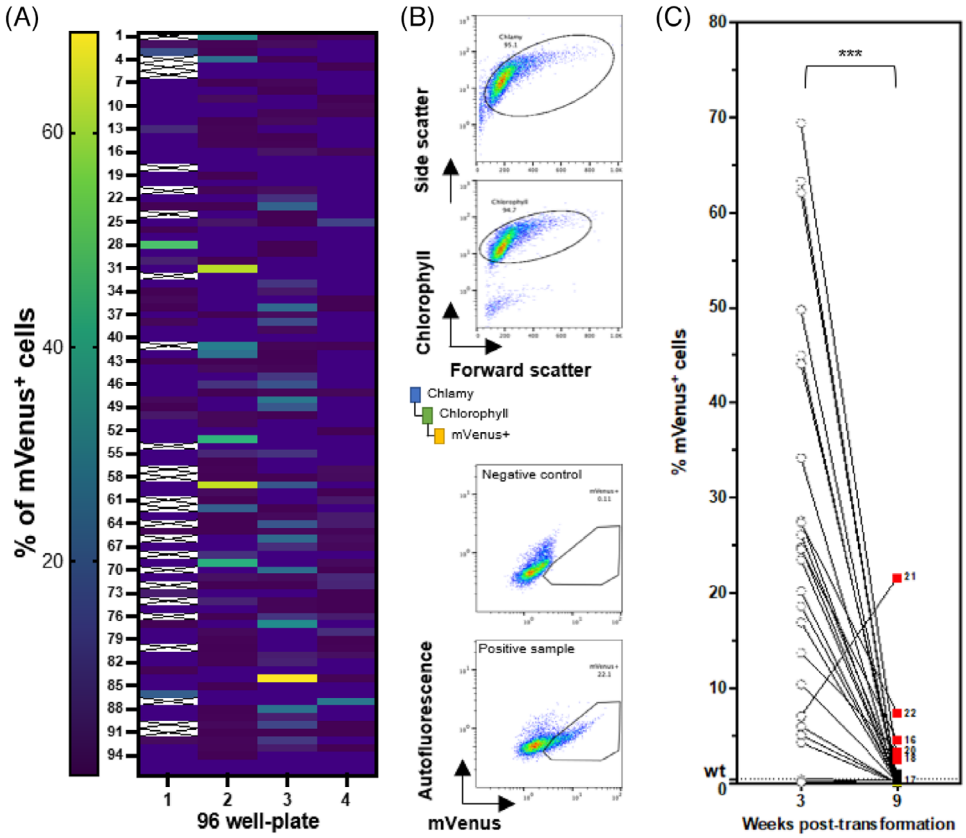

FIGURE 1 **Selection of mVenus$^+$ transformants by flow cytometry.** (A) Heatmap representation of the % mVenus$^+$ cells in each transformant, assessed by flow cytometry 3 weeks post-transformation. mVenus fluorescence was acquired on the FL1 channel (filter at 525/10 nm) from 376 transformed antibiotic resistant microalgae transferred to liquid TAP medium in four 96 well plates for 6 days. Negative controls (WT and EV (empty vector)) 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 (top panel) and a positive (bottom panel) sample. (C) Sharp loss of mVenus$^+$ cells in most of the 22 mVenus$^+$ clones between 3 and 9 weeks post-transformation (one tailed paired t-test, $p < 0.0001$). WT % of mVenus$^+$ cells was used to set background levels.

tat and entinostat, two benzamide derivatives (HDAC class I inhibitor), and romidepsin, a bacterial-derived cyclic tetrapeptide (HDAC class I inhibitor).[36] Treatments consisted of the addition of 5 µM of inhibitors at initiation of culture (Figure 3A,B).

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,B and S4A). Comparable results were obtained with the other inhibitors from this class, that is, SAHA, belinostat, dacinostat and panobinostat triggered transgene fluorescence following the same kinetic and at comparable levels, but SAHA was usually less toxic than dacinostat and panobinostat (as observed in growth curves and chlorophyll levels, although not 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*.

To confirm that SAHA treatment could induce the expression of other transgenes, we transformed *C. reinhardtii* with pOpt_Clover_Paro (Figure S5A). Flow cytometry was used for the initial detection and screening of Clover$^+$ positive colonies transferred to liquid medium, following the same method as described for mVenus$^+$ positive colonies. Nineteen positive transformants were detected over the 192 antibiotic-resistant (9,9%) and selected to be treated with SAHA (5 µM) in liquid culture for 6 days, 3 months post-transformation (positive clones were replated a total of 5 times within those 3 months) (Figure S5B and S5C). The mean % of Clover$^+$ cells was significantly increased by 18.45% in cultures treated with SAHA versus DMSO, confirming transgene induction in most of the positive clones selected.

3.3 | SAHA treatment at initiation of culture maximizes transgene expression in exponential phase

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 (Figure 4A-C, S6A and S6B).

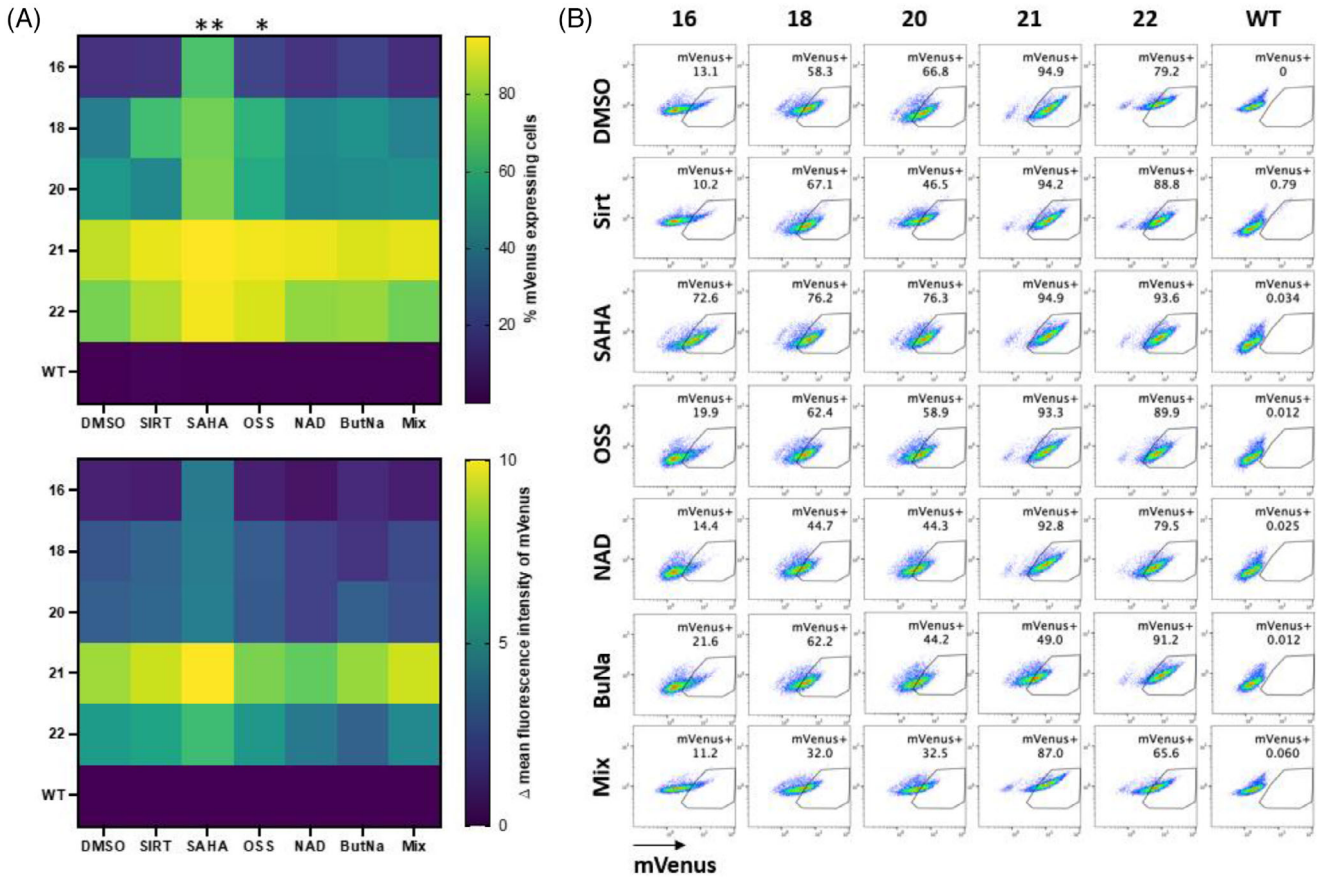

FIGURE 2 **mVenus expression following treatment with silencing inhibitors.** (A) Heatmap representations of the % of mVenus$^+$ cells (top) and the mVenus delta mean fluorescence intensity (bottom) in clones 16, 18, 20, 21, 22, and WT following silencing inhibitors treatment. Ninety-six well-liquid cultures were treated with DMSO (0.1%), Sirtinol (SIRT, 50 μM), vorinostat (SAHA, 2.5 μM), OSS_128167 (OSS, 100 μM), nicotinamide (NAD, 1 mM), Sodium butyrate (ButNa, 5 mM), or a mix of all HDACi (Mix) for 24 h. (B) Representative pseudocolor dot plots (of triplicates) 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.

Fluorescence levels of mVenus progressively decreased over the following 6 days of culture. When cells were treated with SAHA (5 μM), 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,B). Consistently with the low number of cells impacting on the flow cytometer sensitivity in the first 3 days, the analysis of the % of mVenus$^+$ cells varied at early time point (Figure 4C). In general, MFI values (mean abundance of transgene per cell) response correlated well with the frequency of mVenus$^+$ cells, which shows that SAHA induced expression of a higher quantity of transgene (MFI) in most of the population (%) of each clone, reaching the highest levels at day 5 and 6 (Figure 4B,C).

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$^+$ 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–C). SAHA did not seem to impact on overall growth of microalgae (Figure S6A) but a significant decline in chlorophyll fluorescence was noted (Figure S6B, One-way Anova, Dunett's multiple comparison test), suggesting that some metabolic pathways related to photosynthesis are altered.

The transient increase in transgene levels in exponential prompted us to study the optimal timing of SAHA treatment to obtain maximal expression. First, we compared the effect of SAHA treatment at initiation of culture, at day 0, versus in stationary phase, at day 7 (Figure 5A left and right panel, respectively). Colonies were transferred to a liquid pre-culture for 5 days and then diluted 1:25 to liquid media with SAHA (2.5 μM) or DMSO (cultures were in exponential phase). mVenus levels were measured every day for 10 days. We observed that SAHA treatment at initiation of culture (day 0) maximized transgene expression at days 4–5 in higher expressing transformants (18, 20, 21) as in lower producing ones (16 and 17) (Figure 5A, left panel), as seen in previous experiments. When cells progressed to the stationary phase, mVenus expression was lost. However, when treatment was applied to cells that had reached stationary phase (day 7), mVenus expression slightly increased in 18, 20, and 21, but could not be restored to

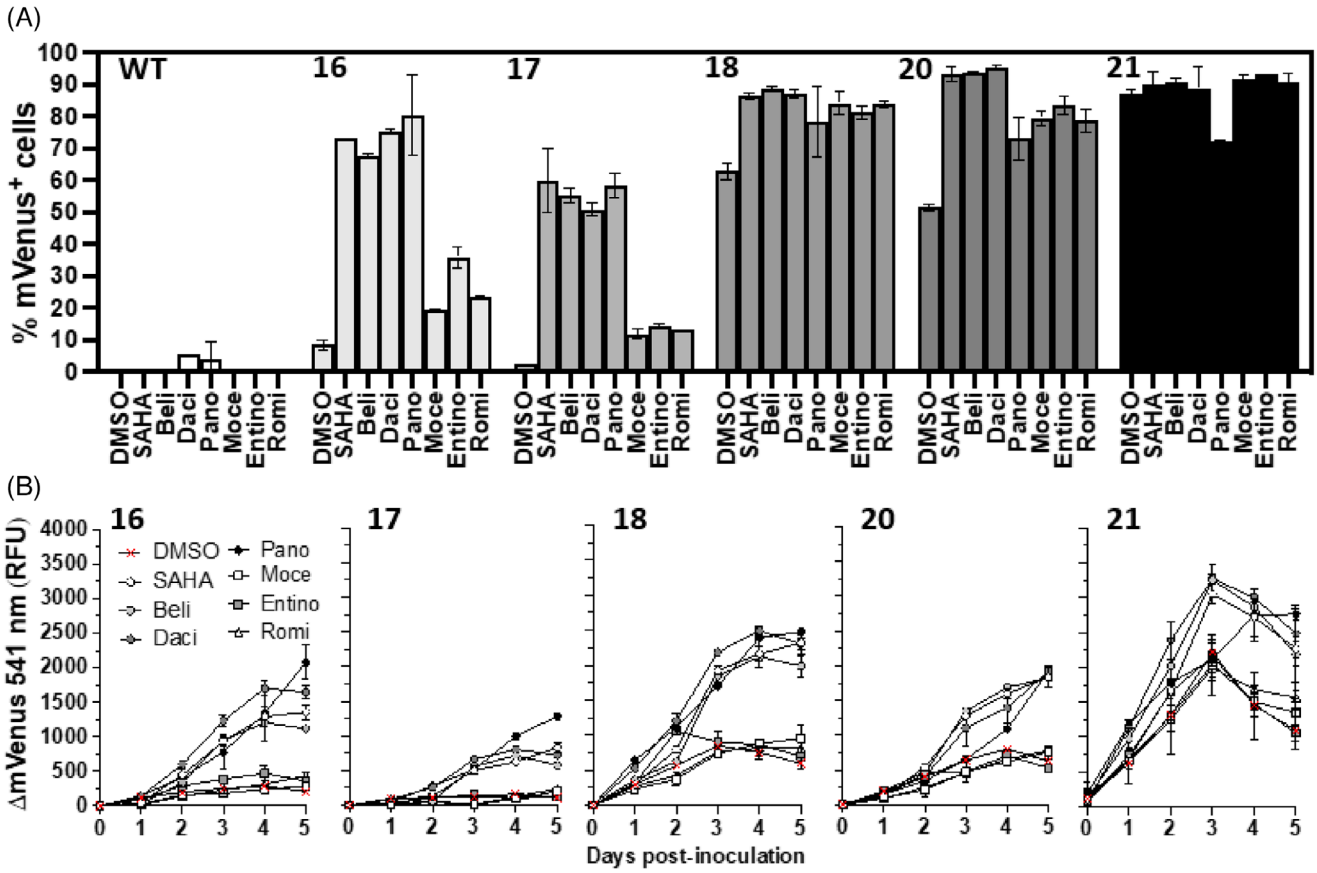

FIGURE 3 **Hydroxamate inhibitors are potent inducers of transgenic expression.** (A) Bar graph showing the mean % of mVenus$^+$ cells acquired by flow cytometry at day 6 of culture in clones 16, 17, 18, 20, 21, and WT treated with DMSO or inhibitors (5 μM) in 96 wells liquid cultures. (B) mVenus mean fluorescence intensity curve plot following inhibitors treatment in clones 16, 17, 18, 20, 21, and WT as measured using a microplate reader over 5 days. mVenus fluorescence was acquired in area scan mode and normalized to the OD_{750} 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. DMSO: dimetthylsulfoxide, SAHA: Vorinostat, Beli: Belinostat, Daci: Dacinostat, Pana: Panabinostat, Moce: Mocetinostat, Entino: Entinostat, Romi: Romidepsin.

the level observed at day 4 (Figure 5A, right panel). Thus, transgene expression activation by SAHA does not occur in stationary phase.

Second, as exponential phase is required for high transgene expression, we tested if we could keep mVenus expression high by maintaining cells in exponential phase and repeatedly treating with SAHA. To some extent, this would mimic a high throughput system of transgene production, where cells are kept in continuous exponential phase to maximize expression and production.

Transformants 16 and 21, and WT were transferred to a liquid preculture for 5 days and then diluted 1:50 to liquid media with SAHA (2.5 and 5 μM) or DMSO (cultures were in exponential phase) (Figure S7). At day 5, when cells reached the exponential phase again in liquid culture, half media was removed and replaced with fresh media containing SAHA or DMSO, and this 2-fold dilution was repeated at days 6, 7, 8, and 9. OD, mVenus and chlorophyll fluorescence were measured every day.

We observed that transgene expression was more induced in clone 16 at day 5 with 5 μM of SAHA (80.3-fold compared to DMSO (0.23% to 18.87%), compared to 2.5 μM (11.1-fold compared to DMSO (0.56% to 6.21%) (Figure 5B), and that transgene induction was transient (18.87% at day 5 and 3.83% at day 10 with 5 μM of SAHA), despite repeated daily SAHA treatment. Even though the level of transgene expression dropped, it was still higher than in DMSO-treated cells at day 10. Transgene expression also dropped in clone 21 when comparing day 5 to day 10, but interestingly, in this highly fluorescent transformant, mVenus expression was successfully kept high by the daily 2-fold dilution and SAHA had no significant impact (Figure 5B).

Finally, we tested if transgene expression could be recovered in cells that lost transgene expression following repeated SAHA-treatment. Following Figure 5B treatment, we rested transformant 16 by using a 1:25 dilution in fresh media in the absence of SAHA for 5 days. Then, cells were once again diluted 1:25 in fresh media containing 5 μM SAHA or DMSO and grown for 5 days (Figures S7A and S7B). Transgene expression was indeed induced after the rest period (by a 2.1-fold, with 8.20% at day 20 comparted to 3.83% at day 10, and by 80-fold compared to DMSO treated cells on day 20). However, the % of mVenus$^+$ cells did not reach the level that was initially observed (8.20% at day 20 vs. 18.87% at day 5) (Figure S7B).

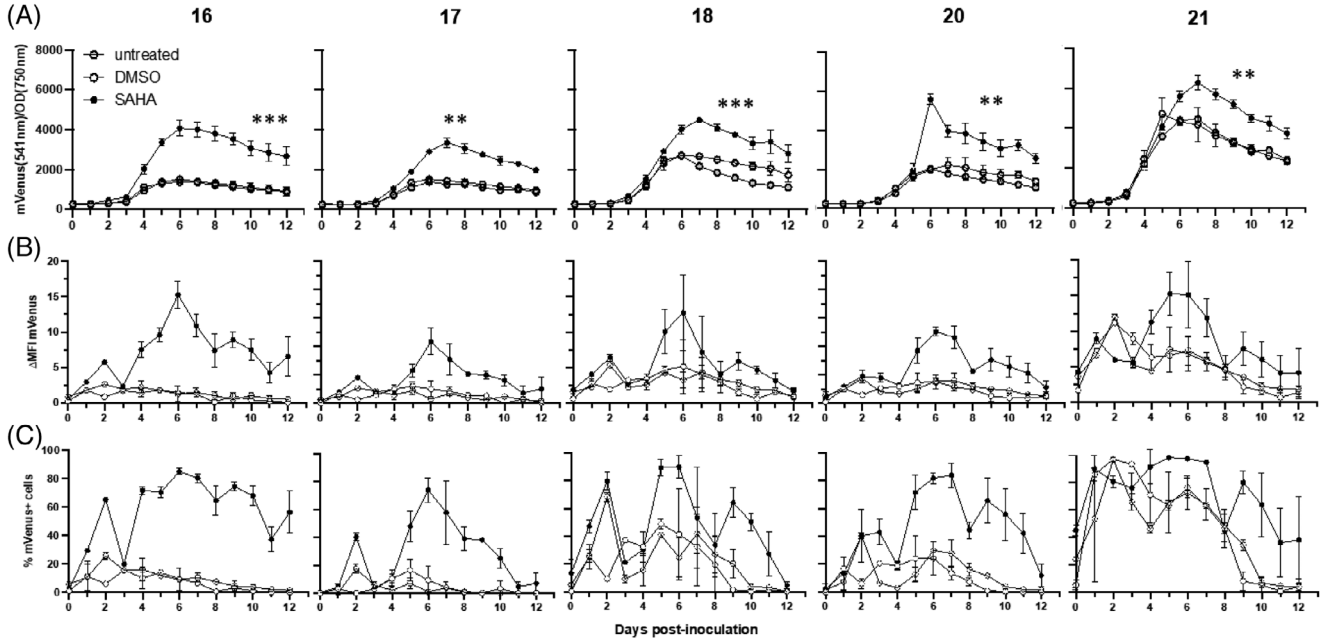

FIGURE 4 **SAHA consistently increases transgene expression over culture period**. Flask liquid cultures of clones 16, 17, 18, 20, 21, and WT were treated with 5 μM of SAHA and grown for 12 days. The mean fluorescence intensity (MFI) of mVenus was assessed every day. (A) The MFI was measured on the overall culture using a microplate reader. (B) and (C) represent the MFI and the % of mVenus$^+$ cells, respectively, measured across growth period using a flow cytometer. Multiple comparisons Dunnet's test with matching values was used to compare between treatment.

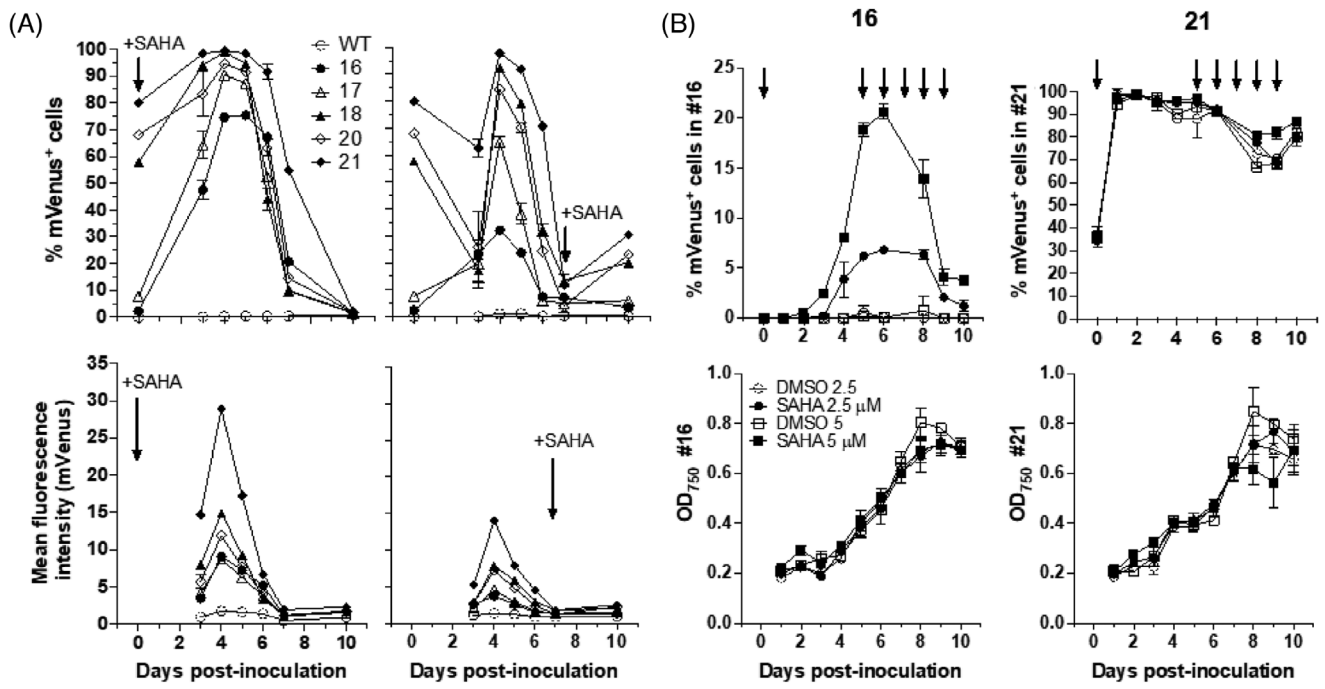

FIGURE 5 **SAHA triggers a robust but transient accumulation of transgene in exponential phase.** (A) Kinetic of mVenus fluorescence in culture treated with SAHA (2.5 μM) at day 0 (left panel) or at day 7 (right panel). Cells were grown in 24 well plates and fluorescence (% of mVenus$^+$ cells [top] and mean fluorescence intensity [bottom]) were measured by flow cytometry. The experiment was performed 4 months post-transformation in triplicates. Mean and standard deviations are shown, but only visible when deviating from the mean. (B) Kinetic of mVenus fluorescence (top) and growth (bottom) following repeated treatment of SAHA (2.5 or 5 μM) in cells artificially maintained in log phase. 16, 21, and WT were grown in six well plates and maintained in log phase by two-fold dilution in fresh media once a day from day 5 to 9. Arrows points at day of SAHA addition. Matching concentrations of DMSO were used as negative controls. This experiment was performed in triplicates 18 months post-transformation.

Overall, to produce transgenes, cells could be maintained in exponential phase in liquid culture for several cycles while adding SAHA once per harvest a few days before, but our results suggest that the most efficient way to use SAHA in a *C. reinhardtii* cell culture is to treat a fresh culture with SAHA at initiation, harvest at the end of exponential phase, and repeat the whole process with a fresh culture.

3.4 | SAHA's effect on viability, cell growth, palmelloid, chlorophyll levels, and ROS

Previous experiments suggested that SAHA addition impacted chlorophyll fluorescence. We performed several experiments to evaluate the level of stress caused by SAHA. 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. The % of PI^+ cells at day 6 shows a higher amount of non-viable cells for clone 16 treated with 2.5, 5, and 10 μM SAHA compared to DMSO (2.2-fold at 2.5 μM (5.7% to 12.6%), 1.8-fold at 5 μM (5.7% to 10.1%), and 1.7-fold at 10 μM (5.7% to 9.6%)) (Figure 6A). Similar results were obtained with other clones. Nevertheless, around 90% of the transformants cells remained intact with SAHA treatment, without significant increase when compared to background levels in WT cells. Different stress conditions have been reported to transiently induce palmelloid colonies in *C. reinhardtii* without impacting viability.[37] We observed that 6 days of treatment with 5 and 10 μM of SAHA lead to the accumulation of small palmelloid colonies in *C. reinhardtii* (Figure 6C,D compared to Figure 6B), while motility was also progressively lost above 2.5 μM. We quantified palmelloid cells formation by flow cytometry when transformants were treated with 5 μM of SAHA and showed that in most cases palmelloid colonies increased at day 3 and 6 post-treatment (Figures 6E and S8A). These results suggest that SAHA modifies chlorophyll content and colony morphology but does not alter cell growth kinetic nor viability at concentrations of 10 μM and lower.

SAHA's addition at higher concentration of 20, 40, and 80 μM was also tested to see at which degree it could affect the growth kinetic and viability. Larger palmelloid colonies (or aggregates) were observed (Figure S8B) with increasing concentrations, while growth kinetic was inhibited partially with 20 and 40 μM SAHA, and totally with 80 μM 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 μM SAHA) or even red tint (80 μM SAHA). Thus, increasing concentrations of SAHA cause increasing stress to *C. reinhardtii* cells, although they continue to proliferate and produce transgene up to 10 μM.

To further investigate stress induction, we measured reactive oxygen species (ROS) levels in cells grown in media containing SAHA (2.5 or 5 μM) or DMSO for 5 days (Figure 6F), and then upon repeated SAHA treatment following 2-fold dilution in fresh media aimed to maintain cells longer in exponential phase (Figures S7C and S7D). At day 5 post-treatment, corresponding to the peak of transgene production, ROS levels were slightly induced (< 5% of the cells were ROS^+) when 2.5 μM of SAHA was used at initiation of culture. No specific increase in ROS was observed when using 5 μM of SAHA in either WT, transformant 16 or 21, despite maximal transgene induction. However, at this stage, a 2-fold dilution in media containing 5 μM of SAHA resulted in an increase in ROS production 24 h later in all tested clones, up to 20%, while transgene expression remained stable, (6,5-fold compared to DMSO in clone 16 (2.37% to 15.31%) compared to 2.5 μM (5.5-fold compared to DMSO in clone 16 (0.93% to 5.16%) (Figure S7C and S7D), while most cells stayed ROS^-. These results indicate that SAHA-induced transgene production does not correlate with cellular stress levels. In addition, active concentrations of SAHA (2.5 and 5 μM) at initiation of culture might induce morphological changes, palmelloid cells and a small amount of ROS, but no severe adverse effects on algal cells is observed, similarly to the observed effect of low concentrations of micropollutants.[27]

3.5 | SAHA upregulates transgene mRNA and protein levels, and is associated with higher histone acetylation levels

SAHA is defined as a histone deacetylase inhibitor in several organisms (humans,[38] fungus,[39] plants,[40,41] and viruses[29]). As such, it indirectly triggers histone acetylation (in a non-sequence specific way), which leads to an increase in mRNA levels of genes that were initially silenced by deacetylation, including silenced transgenes. At protein levels, SAHA treatment leads to an increase in transgene production and histone acetylation which can be measured by a simple western blot (Figure 7A). We aimed at detecting these SAHA-induced effects in *C. reinhardtii* transformants. First, 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 7B); the rise was also observed in other clones, although less marked (1.2- to 2.8-fold). These results demonstrate that transgene production is induced at transcriptional levels, coherently with SAHA activity as a HDACi.

Lastly, we measured mVenus and acetylated histone accumulation upon SAHA treatment by western blot (Figures 7C,D and S9). A brighter band was observed using the H3K9Ac antibody in WT and in all transformants but 18, while the amount of histone 3 remained consistent. In addition, we could detect an increase in mVenus protein accumulation in all transformants at day 6 of cultures. Without treatment, mVenus was barely detectable in clones 16, and 17, consistently with the low fluorescence signal detected by the plate reader and FACS. Overall, SAHA-treatment was associated with an increased levels of acetylated histone 3 and of mVenus transgene (Figure 7D, $p < 0.01$, paired *t*-test), consistently with a mechanism of histone deacetylation inhibition.

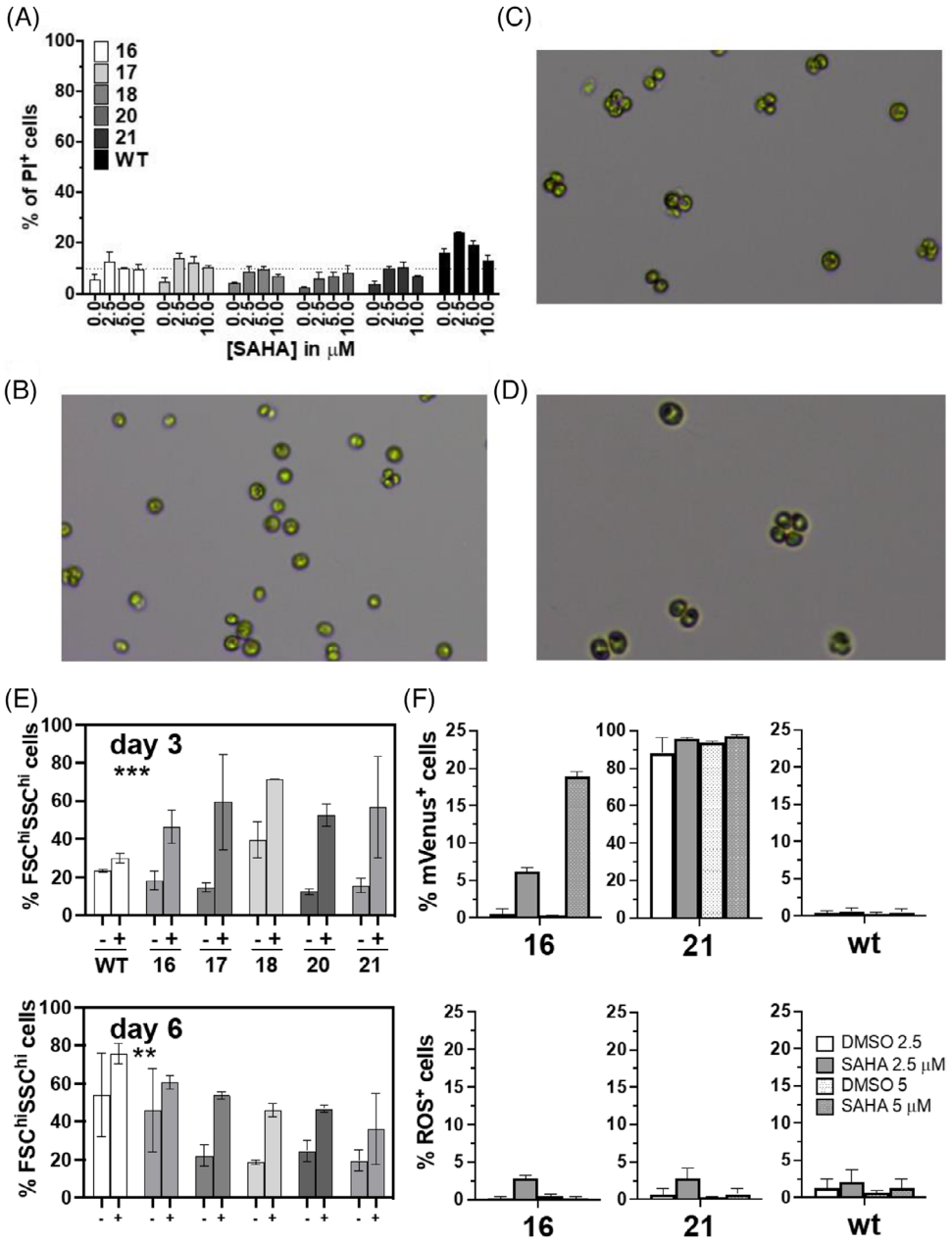

FIGURE 6 **SAHA induces a palmelloid phenotype in *C. reinhardtii* with low cytotoxicity.** (A) SAHA's effect on cell viability (membrane integrity) was verified using propidium iodide (PI) staining and analyzed on a flow cytometer (FL3 channel). (B-D) Representative microscopy pictures of liquid culture 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. Two-way ANOVA repeated value statistical test was used, $^{***}p < 0.001$, $^{**}p < 0.01$ (effect of SAHA on palmelloid formation). (F) mVenus and ROS levels were measured 5 days post-SAHA (2.5 or 5 µM) treatment by flow cytometry in transformant 16, 21 and in WT. Matching concentrations of DMSO were used as negative control. Error bars represent standard deviation of biological triplicates.

4 | 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.[4,42] Despite antibiotic selection, not all transformed cells express the targeted transgene at a desired level. This loss of expression occurs even though the resistance marker is downstream of the reporter expression cassette in the transformation vector. Hence, screening of mVenus$^+$ 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,

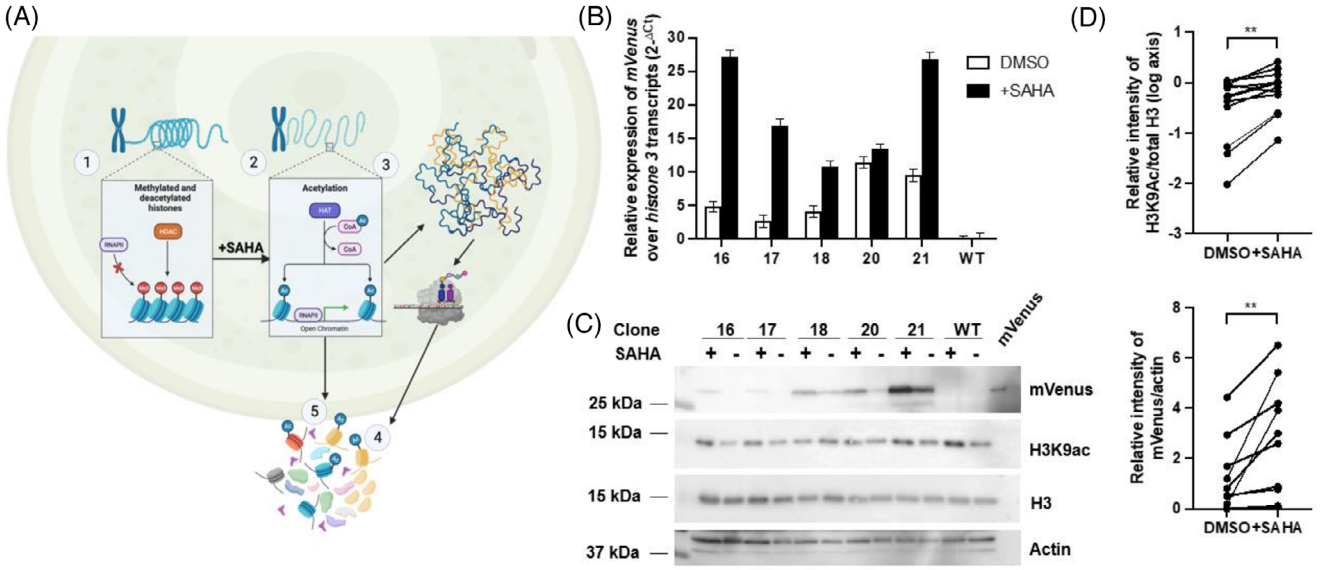

FIGURE 7 **SAHA upregulates transgene mRNA and protein levels, and is associated with higher histone acetylation levels.** (A) Summary of SAHA characterized mechanisms as a histone deacetylase inhibitor. (1) Chromatin in its compact form is associated with high methylation and low acetylation levels, a phenotype maintained by the activity of histone deacetylase among other proteins. (2) Upon SAHA treatment, histones are acetylated, associated with chromatin in open conformation enabling the RNA polymerase to bind to previously inaccessible, silenced promoters, and (3) to increase transcripts levels, including transgene's which levels can be measured by RT-qPCR. (4) Induced transcripts are translated into proteins that are detectable by western blot or fluorescence detector. (5) Chemical inhibition of histone deacetylase leads to an increase in overall acetylation levels of histone which can be revealed by western blot. (B) Relative expression of *mVenus* as measured by RT-qPCR and normalized on *histone 3* levels in five transformants grown in flasks and treated with SAHA (5 μM) or DMSO (solvant control) for 6 days. (C) Immunoblot analyses of mVenus (27 kDa), acetylated histone 3 (H3K9ac, 15.4 kDa), histone 3 (H3) and actin (40 kDa) in clones 16, 17, 18, 20, 21, and WT treated with SAHA (5 μM) or DMSO (solvant control) for 5 days. Total soluble proteins were separated by denaturing SDS-PAGE, blotted and probed with specific antibodies as indicated. For mVenus and H3K9ac detection, 50 μg and 25 μg total soluble proteins of protein samples were loaded respectively. Molecular weights were deduced from co-migrating protein markers. Full blots are shown in Figure S9. (D) Relative quantification of the adjusted band intensity of mVenus (normalized on actin) and of H3K9Ac (normalized on histone 3), in five transformants and WT assessed in two independent experiments. Values were extracted using the Image Lab software.

which was not essential for survival, underwent transcriptional and/or post-transcriptional gene silencing mechanisms.[43] Thus, the number of positive transformants falls overtime after rounds of subcultures despite antibiotic selection. Our report demonstrates for the first time to our knowledge, the use of HDAC inhibitors of hydroxamate-class like SAHA in *C. reinhardtii* to trigger transgene expression.

SAHA treatment at doses that do not affect the cell growth, enables the recovery of mVenus reporter protein expression. 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 and ROS levels were not impacted upon treatment with 5 μM SAHA, but a significant decline in chlorophyll fluorescence was observed. In addition, cell motility was progressively lost ≥2.5 μM SAHA, while palmelloid formation was induced. Some studies have shown that abiotic stress could transiently induce palmelloid in *C. reinhardtii* without impacting viability.[28,44]

Cheloni and 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 μM) strongly increased the number and promoted the larger size of the palmelloids and/or aggre-

gations 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,[41,45] 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 bishydroxamic acid (SBHA), an analog of SAHA. Production of both compounds was induced by SBHA at concentrations between 2 and 100 μM, but production decreased at concentrations ≥ 50 μM, 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.[2] 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.[2,8,14,46,47] Overall, SAHA triggered an increase in histone acetylation level of H3K9. Hence, SAHA treatment was associated with an increase in genomic histone acetylation and transgene production. 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.[23] By contrast Neupert et al. could not induce expression using sirtuin inhibitors and other HDACs inhibitors, despite increasing histone acetylation levels.[7] In our hands, inhibition of methylation using 5-aza-2'-deoxycytidine 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 also 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.[14,48] demonstrated that the transgenic *HSP70A* promoters harbor lower levels of active chromatin marks than the native *HSP70A* but more than transgenic *RBCS2* promoters.[14,48] 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.

There is a possibility that HDAC inhibitors of hydroxamate-class like SAHA could help maintain active chromatin marks at the *HSP70A-RBCS2* fusion promoters. Importantly, histone deacetylases also target non-histone proteins such as high-mobility group proteins 1 and 2 and α-tubulin.[49] In that sense, histone deacetylase inhibitors such as SAHA may affect other critical cellular processes. It will be interesting to assess its impact on the microalgae transcriptome and metabolome.

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.

AUTHOR CONTRIBUTIONS

Pamela Lavoie: Investigation; Methodology; Validation; Writing – review & editing. Serge Basile Nouemssi: Investigation; Methodology; Validation; Writing – review & editing. Fatma Meddeb-Mouelhi: Methodology; Project administration; Resources; Supervision; Writing – review & editing. Isabel Desgagné-Penix: Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing

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CONFLICT OF INTEREST STATEMENT

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.

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