

Development and implementation of optogenetic tools for controlling cellular processes in *Chlamydomonas reinhardtii* and *Physcomitrium patens*

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Abbreviations

AboB12	Adenosylcobalamin
ACR	Anion channelrhodopsins
AD	Activation domain
AMPK	AMP-activated protein kinase
AOC5	Cationic amino acid transporter
AOI	Area of interest
AQUA	Advanced quick assembly
AR	Hsp70A/RbcS2 promoter
AsLOV2	LOV2 domain of <i>Avena sativa</i> Phototropin 1
AtLOV2	LOV2 domain of <i>Arabidopsis thaliana</i> Phototropin 1
ATP	Adenosine triphosphate
ATR	All-trans-retinal
AtUBQ10	<i>Arabidopsis thaliana</i> ubiquitin 10 promoter
BLINK	Blue-light-induced K(+) channel
BOff	Blue light gene repression switch
BlueOn	Blue light gene activation switch
C120	DNA cognate sequence of EL222
CaMV35S	Cauliflower mosaic virus 35S promoter
CarH	HTH-type transcriptional repressor
CarO	CarH-specific operator sequence
CCR	Cation channelrhodopsins
cDNA	Complementary DNA
cGMP	Guanosine 3',5'-cyclic monophosphate
ChR2	<i>C. reinhardtii</i> channelrhodopsin 2
CRISPR	Clustered regularly interspaced short palindromic
CRISPRa	CRISPR activation
CRY2	CRYPTOCHROME 2
DBD	DNA-binding domain
DBS	DNA-binding sequence
dCas9	CRISPR associated protein 9 nuclease deficient
E	Macrolide-responsive repressor protein
EGFP	Enhanced green fluorescence protein

EL222	Transcription factor 222 from <i>Erythrobacter litoralis</i>
ETC	Electron transport chain
etr	DNA cognate sequence of E
FAD	Flavin adenine dinucleotide
FCCP	carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone
FLuc	Firefly luciferase
FMN	Flavin mononucleotide
GAF	cGMP phosphodiesterase/adenylyl cyclase/FhlA domain
GLuc	Gaussia luciferase
GOI	Gene of interest
GPCRs	G protein-coupled receptors
GRAS	Generally Recognized as Safe
gRNA	guide RNA part containing the 20 bp target sequence
HSM	Sueoka's high salt media
HTH	Helix-turn-helix
HygR	Hygromycin B resistance
IME	Intron mediated enhancement
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
KanR	Kanamycin resistance
KRAB	Transcriptional repressor domain from human Krüppel Associated 8 Box
LED	Light-emitting diode
LOV	Light-Oxygen-Voltage
MLS	Mitochondrial leading sequence
MNE	Mean normalized expression
mRNA	Messenger RNA
MTS	Mitochondrial targeting sequences
NA	Not applicable
NHEJ	Non-homologous end joining
NIR	Near-infrared
NLS	Nuclear localization sequence
nptII	Neomycin phosphotransferase
NLuc	Nanoluciferase

NTS	N-terminal targeting signal
OMM	Outer mitochondrial membrane
P35S	Cauliflower mosaic virus 35S promoter
PA _t U6-26	<i>A. thaliana</i> U6-26 RNA polymerase III promoter
ParoR	Paromomycin resistance
PCA	Protoplast Culture Arabidopsis medium
PCB	Phycocyanobilin
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PFA	Paraformaldehyde
PhCMV _{min}	Minimal human cytomegalovirus immediate early promoter
PhyB	Phytochrome B
PIF	Phytochrome interacting factor
PM	Plasma membrane
PMF	Proton motive force
POI	Protein of interest
PPSAD	Promoter of photosystem I protein D
PSV40	Simian virus 40 early promoter
PULSE	Plant Usable Light Switch-Elements
RD	Repression domain
RedOn	Red light gene activation switch
RLU	Relative Luminescence Units
RLuc	Renilla luciferase
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription–quantitative PCR
SEM	Standard error of the mean
SRDX	EAR repression domain from <i>A. thaliana</i>
SSFO	Channelrhodopsin-2 variant with longer open states (mutations in D156 and C128)
TALEN	Transcription activator-like effector nucleases
TAP	Tris-acetate-phosphate medium
TMH	Transmembrane helices

TMRE	Tetramethylrhodamine ethyl ester
TMRM	Tetramethylrhodamine methyl ester perchlorate
TPSAD	Terminator of photosystem I protein D
Tnos	Nopaline synthase terminator from <i>A. tumefaciens</i>
TULIP	Tunable, light-controlled interacting protein tag
TV	Activation domain composed by 6x TAL and 2x VP64 and NLS sequence
UTR	Untranslated region
UV	Ultraviolet
UVM4	<i>C. reinhardtii</i> UV-mediated mutant 4
UVR8	UV RESISTANCE LOCUS 8
VEGF	Vascular Endothelial Growth Factor
VP16	Herpes simplex virus-derived transactivation domain
VPR	Activation domain composed by the fusion of three transcriptional activators (VP64, p65 and Rta)
wt	Wild type
βTUB2	β2-tubulin promoter
Δψ _m	Mitochondrial membrane potential

Summary

Fundamental research studies and biotechnological applications often require precise control over cellular processes. Synthetic biology, particularly optogenetics, offers the possibility of controlling diverse cellular processes, such as gene expression or ion fluxes across different membranes like the mitochondrial membrane, with high spatiotemporal and quantitative resolution by engineering tools that respond to light. Since the early 2000s, optogenetic tools have been applied in animal systems, but their implementation in plants only began a few years ago. This delay is primary due to the crucial role of light in the survival of photosynthetic organisms. Most implementations have been in the model plant *Arabidopsis thaliana*, with less focus on other model green organisms such as *Chlamydomonas reinhardtii* and *Physcomitrium patens*. These organisms are used in different research fields, including photosynthesis and development, and are also of significant interest for biotechnology.

In this thesis, we expand the palette of optogenetic tools available for these two organisms. Firstly, in *P. patens*, we established the PULSE system, a plant light-controlled gene expression system that is not affected by the normal growth conditions, namely cycles of white light and darkness. Additionally, we implemented for the first time in this moss the CRISPR-activation (CRISPRa) system, which allows control over endogenous promoters, and combined it with the PULSE system to enable light-controlled regulation of endogenous promoters in *P. patens*. Secondly, in *C. reinhardtii*, we developed an optogenetic platform with several characterized components for the standardized and rapid implementation of optogenetic tools to control gene expression. Using this opto-toolbox and all the knowledge derived from the quantitative characterization of various molecular components, such as synthetic promoters, optoswitches and other regulatory sequences, we created the first optogenetic repression system in *C. reinhardtii*, termed *CrBOff*. Furthermore, we proposed canavanine as a novel antibiotic selection marker for *C. reinhardtii* and combined it with *CrBOff* to control antibiotic resistance with light. Moreover, we made progress towards generating light-controlled mitochondria energetics in *C. reinhardtii* by targeting light-controlled channels and pumps (opsins) into the inner mitochondrial membrane, and we presented a method to monitor changes in mitochondrial membrane potential of this alga.

This work demonstrates the extensive potential that synthetic biology and optogenetic offer for controlling and studying various biological processes in different organisms. We believe that the tools developed in this work will accelerate and enable more advanced and sophisticated biotechnological and biological studies in *P. patens* and *C. reinhardtii*.

1 Introduction

1.1 Introduction to Synthetic Biology

Synthetic biology is an interdisciplinary field that combines engineering and biology principles, with the final purpose of creating new biological systems, or redesign existing ones, to achieve specific predictable functions or applications. It merges the biological approach of simplifying biological systems to understand nature, with the construction and standardization philosophy of engineering. Altogether, the synthetic biology approach can be summarized on the combination of biological components or “building blocks” to generate novel biological systems or devices following a rational systematic model based on the “Design-Build-Test” cycle (Hirschi et al., 2022; Kelwick et al., 2014; X. E. Zhang et al., 2023).

Its roots go back to 1961, when Jacob and Monod described the *lac* operon in *Escherichia coli*. Their research highlighted the existence of regulatory circuits that mediate the response of cells to the environment. From this moment, the idea of assembling novel regulatory circuits combining existing molecular components began to take shape (Jacob & Monod, 1961). In the next decades, researchers started to identify and characterize the cellular components that constitute the building blocks necessary to engineer gene circuits. Major advances that contributed to the fast development of molecular biology were the chemical synthesis of biological molecules (Kung et al., 1965), molecular cloning techniques such as the polymerase chain reaction (PCR; Cohen et al., 1973; Mullis et al., 1986), and the first methods for sequencing DNA molecules (Maxam & Gilbert, 1977; Sanger et al., 1977).

The progress in molecular biology, biochemistry and theoretical approaches enabled the beginning of synthetic biology in the first year of the 21st century, when Gardner reported the engineering of a genetic toggle switch that oscillates between two states in response to environmental signals (Gardner et al., 2000), Elowitz and Leibler a repressilator based on a triple negative feedback that results in oscillation of protein expression (Michael B. Elowitz & Stanislas Leibler, 2000), and Becksei and Serrano an autoregulatory gene network (Becksei Attila & Serrano Luis, 2000). These first circuits were developed for bacteria models, but in the following decades they were expanded to other organisms, like mammalian and plants (Andres et al., 2019; Lienert et al., 2014).

Synthetic circuits allow to control in a precise manner biological processes at the molecular level. This control offers many possibilities ranging from designing synthetic circuits to dissect and study biological networks, to the generation of biosensors for monitoring the environment

(Gregorio-Godoy & Míguez, 2013; Saltepe et al., 2018). Many synthetic circuits are based on chimeric transcription factors. These transcription factors combine a DNA-binding and a transactivation or repressor domain (Lienert et al., 2014). In mammalian cells, the first inducible synthetic gene switch was based on a chimeric transcription factor constituted by the binding domain of the tetracycline repressor (TetR) and the activator domain VP16 from *Herpes simplex* virus. The binding of TetR to its operator sequence tetO, brings the VP16 transactivation domain to the close proximity of a minimal promoter, inducing the downstream expression of a target gene. The binding of this synthetic transcription factor, and therefore the expression of the target gene, is controlled by the antibiotic tetracycline (Gossen & Bujard, 1992).

The synthetic regulatory circuits mentioned above fall within one of the key research areas of synthetic biology: the design of genetic circuits, whose aim is to make novel highly controlled and predictable regulatory pathways by combination of simplified modules. However, synthetic biology involves other key research areas as well. These include the engineering of synthetic chromosomes and genomes that could confer new functionalities to the host, and the design of customized metabolic pathways for *in vivo* synthesis of desired products. Additionally, another branch of synthetic biology focuses on the creation of proteocells with reduced genomes, which can serve as simplified model organism for biological studies or as highly efficient biotech production platforms. All these research areas benefit from computational techniques that focus on creating computational models to facilitate the design of synthetic biology technologies (Bin Sajid et al., 2023; Deplazes, 2009).

In this regard, synthetic biologists of all the different research areas have already made notorious advances in controlling and modifying cellular behaviour, and have fulfilled groundbreaking accomplishments (Jewett & Ellis, 2017). Notable examples include the full synthetic production of opioids or the antimalaria drug in yeasts (Galanie et al., 2015; Paddon et al., 2013; Ro et al., 2006), the generation of a cancer invading bacteria capable of invading mammalian cells upon sensing of tumour microenvironment conditions (Anderson et al., 2006), and the synthesis of the sixteen yeast chromosomes and bacterial genomes, showcasing the functionality of synthetic genomes with the creation of *Synthia*, the first living organism based on synthetic DNA (Dymond et al., 2011; Gibson et al., 2010; Ostrov et al., 2016; Venter et al., 2022; Zhao et al., 2023).

Another illustration of the diverse applications of the different synthetic biology approaches is the enhancement of cancer immunotherapy based on CAR-T cells. These cells, engineered in the laboratory by genetically modifying T-cells to target and eliminate cancer cells, have been

improved using synthetic biology techniques. For example, synthetic biology Boolean-logic gates have been employed to enable CAR-T cells to distinguish between normal and cancerous cells, thereby reducing off-target effects. Additionally, synthetic switches have been developed to enhance control over T-cell activity or even to drive customized therapeutic response programs (Dannenfelser et al., 2020; Giordano-Attianese et al., 2020; Jan et al., 2021; Roybal et al., 2016; Tousley et al., 2023; Wu et al., 2015; Zhu et al., 2024).

All of this reflects the potential of synthetic biology and how it is promoting advancements in various areas of biology by providing innovative solutions to a wide range of challenges.

1.2 Optogenetics

One of the primary goals of synthetic biology is the development of innovative methods for manipulating biological systems at the molecular level, aiming for higher predictability and control compared to traditional biological methods. The control of molecular processes is crucial in molecular biology for numerous purposes, including the study of cellular functions (such as genes or protein roles), unveiling disease mechanisms, developing treatments, as well as engineering metabolic pathways for bioactive compound production, among many others. In this context, optogenetics, a branch of synthetic biology utilizing light to regulate biological processes, has become an ideal tool for extremely precise biological process control with minimized side effects and high reversibility and spatiotemporal resolution. In essence, optogenetics is the research area that integrates optics and genetic engineering with the aim of transforming light-sensing proteins into switches capable of controlling cellular processes (Dugué et al., 2012).

The first optogenetic experiment was performed in 2002. Using yeast as experimental model, a gene expression control system was developed based on the red light-dependent interaction of a plant phytochrome and its interacting factor PIF3 (Shimizu-Sato et al., 2002). A year later, the opsin Channelrhodopsin-2 (ChR2), a light-gated ion transporter, from *Chlamydomonas reinhardtii* was heterologously expressed in *Xenopus oocytes* and mammalian cells, and its ability to depolarize the cell membrane in response to light was demonstrated (Nagel et al., 2003). In 2005, the field of optogenetics was founded with the introduction of Channelrhodopsin-2 into mammalian neurons to depolarize its plasma membrane potential in a light dependent manner (Boyden et al., 2005).

In the following years, the capacity of opsins to transport ions was exploited to modify the neuronal membrane potential of different model organisms, such as *Caenorhabditis elegans*

(Nagel et al., 2005), *Drosophila sp.* (Schroll et al., 2006) or non-human primates (Han et al., 2009). As early as 2006, they were used to restore vision in blind mice (Bi et al., 2006). Nowadays, clinical trials are in progress to use opsin-based optogenetics to restore vision in patients with retinitis pigmentosa (Sahel et al., 2021; Sakai et al., 2022).

These optogenetic approaches based on opsins, known as opsin-based optogenetics or optogenetics 1.0, not only revolutionized the field of neuroscience, but have also been used to manipulate cardiac, pancreatic or plant cells (Deisseroth, 2015; Entcheva & Kay, 2021; Ferenczi et al., 2019; Nussinovitch et al., 2014; Reinbothe et al., 2014; Rost et al., 2017; Zhou et al., 2021).

Apart from opsin, other photoreceptors have been discovered, as well as engineered, during the last decades, expanding the possibilities of optogenetics (Ziegler & Möglich, 2015). These photoreceptors can be combined with different synthetic biology blocks, like transactivation domains, promoters or targeting sequences, to control a broad range of different cellular processes, including gene expression, protein recruitment or organelle positioning (Duan et al., 2015; Hannanta-Anan et al., 2019; Konrad et al., 2023; Mansouri et al., 2019; Ohlendorf & Möglich, 2022; Pérez et al., 2022; Van Bergeijk et al., 2015; Yamada et al., 2020). This diverse optogenetic toolbox can be applied to a wide range of valuable processes such as apoptosis control or biofuel production (Hughes et al., 2015; Reshetnikov et al., 2022; Smart et al., 2017). The branch of optogenetics that makes use of photoreceptors not involved in ion transport is known as optogenetics 2.0 or non-opsin-based optogenetics.

Nowadays, there is huge range of opsins and non-opsin photoreceptors across the visible spectrum that one could use to control cellular signalling and behaviour with light.

The use of optogenetics has several advantages when comparing it with chemical inducible systems. First, optogenetics has highest spatial and temporal resolution, even at the single-cell level. The properties of light allow to induce (and stop inducing) the desired process in a fast way, due to the instantaneous appearance and disappearance of light, whereas chemicals need to be transported inside the cell and then removed or degraded for the signal to disappear. Also, different from optogenetics, chemical induced systems can diffuse, causing off-target effects. Second, optogenetic control is typically very sensitive and can be quantitatively regulated depending on the light intensity and the exposure time. Lastly, optogenetics is reversible and non-invasive, particularly for non-photoresponsive organisms, while chemical induction is often toxic for the cells (Omelina et al., 2022; Shemesh et al., 2017).

1.2.1 Optogenetics 1.0: opsins

Optogenetics 1.0 or opsin-based optogenetics focuses on the precise manipulation of cellular ion fluxes with light through the use of opsins.

Opsins are light-responsive transmembrane proteins that sense light via its chromophore retinal. The complex formed by the retinal and the opsin is known as rhodopsin. Most of the rhodopsins have 7 transmembrane alpha-helical domains, being the chromophore covalently bound to the seventh transmembrane domain (O. P. Ernst et al., 2014). Opsins can be classified in two groups, microbial or type I rhodopsins, and animal or type II rhodopsins. Animal rhodopsins are found in eukaryotes, they function as G protein-coupled receptors (GPCRs) and are involved in animal vision. Light triggers the isomerization of type II rhodopsin's cofactor from 11-cis-retinal to all-trans-retinal (ATR), resulting in phototransduction (Hofmann & Lamb, 2023).

In this section, type I rhodopsins, which are more relevant for this thesis, would be explained in more detail. Microbial opsins can be found in bacteria, fungi, algae and archaea. The chromophore of these opsins is all-trans-retinal, which isomerizes to 13-cis-retinal in response to light. The isomerization triggers changes in the chromophore environment that cause a conformational change in the opsin, resulting in ion transport. The reversion of 13-cis-retinal to ATR inactivates the opsin (Deisseroth, 2015; Kandori, 2021; F. Zhang et al., 2011).

Among microbial rhodopsins, two subgroups can be distinguished: light-gated ion channels and light-gated ion pumps. The main difference between channels and pumps is that pumps unidirectionally transport ions in an active way, even against gradient, and they only transport one ion per photon absorbed, while channels passively transport a given number of ions per cycle, dissipating the existent gradient. For this reason, optogenetic applications comprising pumps normally require higher light intensities and expression levels (Kandori, 2015; Rozenberg et al., 2021).

Pumps can be further subdivided in four categories: inward chloride pumps, outward sodium pumps, inward proton pumps, and outward proton pumps. The first discovered microbial rhodopsin was the outward proton pump *HsBR* (Oesterhelt & Stoeckenius, 1971), followed by other outward proton pumps like Archaelhodopsin-3 from *H. sodomense* (Mukohata et al., 1999) or MAC from *L. maculans* (Waschuk et al., 2005). It was not until 2016 that inward proton pumps, e.g. PoXeR, were discovered (Inoue et al., 2016). Lastly, chloride and sodium pumps, like *NpHR* or *KR2*, respectively, are non-proton transport pumps that might be used to modify membrane potential without altering the pH (Inoue et al., 2013; F. Zhang et al., 2007).

Light-gated ion channels can be classified, depending on the nature of the ions transported, in two subgroups: anion channelrhodopsins (ACR) and cation channelrhodopsins (CCR) (Kato, 2021). The most widely used opsin is channelrhodopsin 2 (ChR2) of *Chlamydomonas reinhardtii*, which is a non-selective cation channelrhodopsin that transports mainly protons, but is also permeable for other cations as Na^+ , K^+ and Ca^{2+} (Nagel et al., 2003). In 2005, Channelrhodopsin 2 was firstly used to depolarize hippocampal neurons, and since then it has been extensively used in neurobiology (Boyden et al., 2005; Rost et al., 2017).

Natural anion channelrhodopsins were discovered in *Guillardia theta* (GtACR1 and GtACR2). Before this finding, anion channelrhodopsins were being engineered from CCRs and optimized to try to minimize the transport of cations (Berndt et al., 2016; Govorunova et al., 2015; Kato et al., 2018; Wietek et al., 2015).

Recently, enzymorhodopsins were identified. This type of opsins have eight transmembrane domains and are coupled to an enzyme, that can be a guanylyl cyclase, a phosphodiesterase or a histidine kinase (Mukherjee et al., 2019; Tsunoda et al., 2021).

Since the discovery of the first opsin in the 70s, several rhodopsins have been found and engineered. The engineering efforts have been mainly focused on modifying the photocurrent, light sensitivity, kinetics, ion selectivity and activation wavelength properties, and improving its heterologous expression. Enhancing the photocurrent and the sensitivity minimize the invasiveness as lower irradiance is required. Most of the opsins respond to blue or green light, thus several efforts have been made to generate more red-shifted variants, like NeoR or ChrimsonR (Broser et al., 2020; Klapoetke et al., 2014). The latter was used to partially restore vision in a blind patient (Sahel et al., 2021). Red-shifted variants represent advantages due to the higher penetrance of red light and allow for dual-colour experiments in which blue and red shifted variants can be combined to even bidirectionally modulate the membrane potential (Klapoetke et al., 2014; Vierock et al., 2021).

1.2.1.1 Optogenetics to control mitochondrial energetics

Microbial rhodopsins have been mainly used to manipulate plasma membrane potential. However, since 2017, they have been used to modify the mitochondrial membrane potential ($\Delta\psi_m$) by its targeting to the inner mitochondrial membrane (IMM) (Amitrano et al., 2021; Berry, Baldzizhar, et al., 2020; Berry, Trewin, et al., 2020; Berry & Wojtovich, 2020; P. Ernst et al., 2019; Chen et al., 2022; Imai et al., 2019; Tkatch et al., 2017). Opsin-controlled mitochondrial membrane potential allows to study mitochondrial-related processes, such as

ATP production, reactive oxygen species (ROS) generation, mitochondrial protein transport, apoptosis or mitophagy, and mitochondrial related diseases such as Parkinson, with spatiotemporal resolution, which is not possible when using uncoupling agents as carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that irreversibly dissipate the mitochondrial membrane potential.

The targeting of the opsins to the IMM has been achieved by fusing opsins to mitochondrial targeting sequences (MTS) of IMM proteins as mitofilin, COX8 (subunit VIII of cytochrome C oxidase), ABCB10 (ATP-binding cassette transporter) or SDHC1 (succinate dehydrogenase complex subunit C). This has been achieved in *Drosophila sp.*, *C. elegans* and in different types of mammalian cells, including CD8⁺ T-cells, cardiomyocytes and pancreatic β -cells (Amitrano et al., 2021; Berry, Baldizhar, et al., 2020; Berry, Trewin, et al., 2020; P. Ernst et al., 2019; Imai et al., 2019; Tkatch et al., 2017).

Both light-controlled depolarization and hyperpolarization of mitochondrial membrane potential have been successfully accomplished. First, it was observed that opsin-mediated hyperpolarization increases ATP production, independently of oxygen consumption, and reduces ROS generation. Moreover, Imai and collaborators showed that activation of the opsin reduced the effects of Parkinson in a Parkinson's disease model (Amitrano et al., 2021; Berry, Trewin, et al., 2020; Imai et al., 2019). Second, depolarization causes a decrease in ATP production and increases oxygen consumption and Ca²⁺ uptake. Tkatch and coworkers demonstrated the spatial resolution capacity of this technique by manipulating single populations of mitochondria within the mitochondrial network of a cell (Berry, Baldizhar, et al., 2020; Chen et al., 2022; P. Ernst et al., 2019; Tkatch et al., 2017).

Mitochondria hyperpolarization or energization was achieved with the proton pumps deltarhodopsin dR from *Haloterrigena sp.* arg-4, and MAC, from *Leptosphaeria maculans*, which transport protons outside the matrix (Amitrano et al., 2021; Berry, Trewin, et al., 2020; Ihara et al., 1999; Imai et al., 2019; Waschuk et al., 2005). Pump ion transport direction depends on its orientation. MAC fused to a different mitochondrial targeting peptide, which confers a different topology, was also fruitfully used to depolarize mitochondria (Berry, Baldizhar, et al., 2020). Apart from the above-mentioned pumps, channelrhodopsin-2, and variants of this CCR with longer open states, were also used with de-energization purposes (P. Ernst et al., 2019; Nagel et al., 2003, 2005; Tkatch et al., 2017; Yizhar et al., 2011).

A more detailed description of the optogenetic tools available for mitochondrial research can be found in (Deen-Rozalen et al., 2024).

1.2.2 Optogenetics 2.0: non-opsin photoreceptors

Non-opsin-based optogenetics or optogenetics 2.0 employs non-opsins photoreceptors to control cellular processes like subcellular protein localization, gene expression and cell differentiation (Kolar & Weber, 2017).

These photoreceptors or light sensing proteins can be found in every kingdom, but mainly in bacteria, fungi and plants. There is a wide variety of photoreceptors among all the visible light spectrum that have been used in optogenetics (Fig. 1). In all of them, the light perception is mediated by a chromophore, that varies depending on the type of photoreceptor. The website OptoBase (<https://www.optobase.org/>) is an online photoreceptor database that serves as a guide for the selection of the ideal optogenetic tool for a certain purpose (Fan & Lin, 2015; Kolar et al., 2018; Ziegler & Möglich, 2015).

Blue-light sensing photoreceptors are the most commonly used for optogenetic purposes. Examples of these are LOV (light-oxygen-voltage), BLUF (blue light using FAD) and cryptochromes, which are responsible of circadian rhythm regulation in plants and animals (Chaves et al., 2011; Christie, Gawthorne, et al., 2012; Glantz et al., 2016; Masuda, 2013). One representative member of the latter is cryptochrome 2 (CRY2) from *A. thaliana* (Mas et al., 2000; Taslimi et al., 2016). LOV, BLUF and cryptochromes use FAD or FMN as a chromophore. This chromophore is reduced upon blue light illumination, resulting in a conformational change in the photoreceptor. This conformational change can result, for instance, in exposure of a domain or oligomerization. Optogenetic tools based on these photoreceptors do not require chromophore supplementation, since FMN and FAD are present in every organism (Christie, Gawthorne, et al., 2012), which eliminates the limitation that may arise from having to supplement the chromophore exogenously.

Covering the green range of the spectrum, there are cobalamin-dependent photoreceptors as CarH from *Thermus thermophilus* that forms tetramers, in the presence of adenosylcobalamin (AboB12), that dissociate upon green light illumination (Cheng et al., 2016).

In the area of UV-B, we find the tryptophan-dependent UVR8 photoreceptor from *A. thaliana*. UVR8 forms homodimers in the dark. Light absorption by its chromophore causes a conformational change that breaks this homodimer, allowing the formation of a heterodimer with constitutively photomorphogenic 1 (COP1; Christie, Arvai, et al., 2012; Favory et al., 2009).

UV-B light has a short wavelength, making it difficult to penetrate tissues. For this reason, for *in vivo* applications, red light responding systems are more suitable. Plant phytochromes and bacteriophytochromes respond to red and far-red light. In these cases, light absorption depends

on a tetrapyrrole chromophore that can be phytochromobilin (PΦB), phycocyanobilin (PCB) or biliverdin, depending on the origin of the phytochrome (Auldrige & Forest, 2011; Paik & Huq, 2019).

Another type of photoreceptors are cyanobacteriochromes, which absorb light in all the visible spectrum (violet, red, and green), and also use PCB as chromophore (Rockwell et al., 2013). Recently, an optogenetic system named BICYCL (bidirectional light inducible dimers), based on a cyanobacteria photoreceptor, was engineered to control protein-protein interactions (Jang et al., 2023). In the last decade, the PCB synthesis pathway was introduced in mammalian cells, opening the possibility to use red-light optogenetic systems in this biological chassis without the external addition of chromophores (Müller, Engesser, Timmer, et al., 2013; Uda et al., 2017).

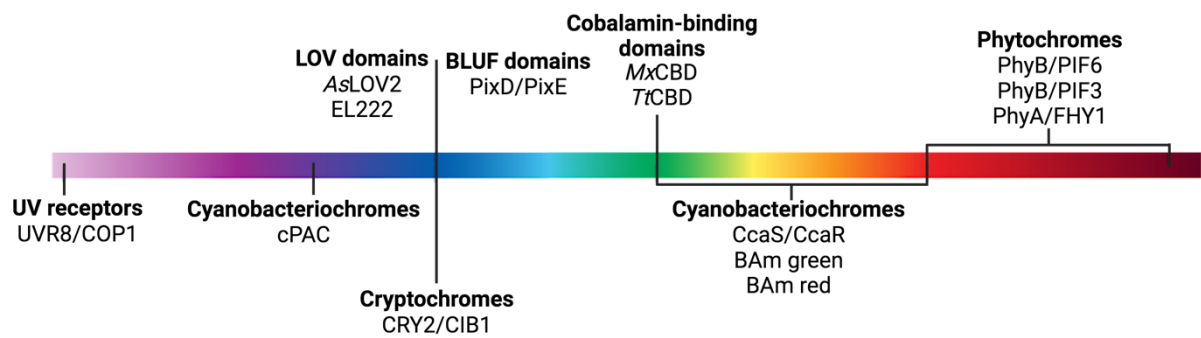


Figure 1. Overview of some non-opsin based optogenetic switches positioned according to the light wavelength they respond to. UVR8, *A. thaliana* UV resistance locus 8; COP1, *A. thaliana* constitutively photomorphogenic 1; cPAC, cyanobacteriochrome-based photoswitchable adenylyl cyclase; CRY2, *A. thaliana* cryptochrome 2; CIB1, *A. thaliana* cryptochrome-interacting basic helix-loop-helix 1; LOV, light-oxygen-voltage; AsLOV2, LOV2 domain of *Avena sativa* phototropin 1; EL222, *Erythrobacter litoralis* transcription factor 222; BLUF, blue light using FAD; PixD, ; PixE, ; MxCBD, *Myxococcus xanthus* cobalamin binding domain; TtCBD, *Thermus thermophilus* cobalamin binding domain; CcaS/CcaR, *Synechocystis sp.* cyanobacteriochrome two component system; BAm green, binder of Amg2-Green state; BAm red, binder of Amg2-Red state; PhyB, *A. thaliana* phytochrome B; PIF6, *A. thaliana* phytochrome-interacting factor 6; PIF3, *A. thaliana* phytochrome-interacting factor 3; PhyA, *A. thaliana* phytochrome A; FHY1, *A. thaliana* far-red elongated hypocotyl 1.

Based on these photoreceptors, many optogenetic tools have been engineered to control different molecular processes, like protein localization, gene expression or organelle positioning. In the next subchapter, the optogenetic tools used to control gene expression in the frame of this thesis are explained in more detail.

1.2.2.1 Optogenetic control of gene expression in the frame of this thesis

Optogenetic control of gene expression involves the development of systems that can be classified into two types based on their activation outcomes: those that activate gene expression and those that repress gene expression. Specifically, gene expression control with light has been achieved through the generation of chimeric transcription factors that combine photoreceptors, with transcription regulator and DNA-binding domain (DBD) modules from transcription factors. The transcription regulator can be an epigenetic modifier, a transactivation domain (AD), or a repression domain (RD). The DBD specifically recognizes an operator sequence, the DNA binding sequence (DBS), in the synthetic promoter. When engineering synthetic promoters for gene-expression-activation systems, the DNA binding sequence is placed in the close proximity of a minimal promoter. Contrary, in repressor systems, the minimal promoter is replaced by a constitutive promoter to yield expression of the gene of interest, that can be then downregulated on command with the gene-switch (Baaske et al., 2018; Christie & Zurbriggen, 2021; Konrad et al., 2023; Müller et al., 2013; Ohlendorf & Möglich, 2022).

In optogenetic systems employing photoreceptors that form light-controlled heterodimers, one subunit is fused to the transcription regulator while the other is fused to the DBD. The light-induced heterodimerization locates the AD or RD near the promoter, thereby inducing or repressing gene expression, respectively. In some cases, this heterodimerization is the direct consequence of a light-induced conformational change, as observed in plant phytochromes (Fig. 2). In other cases, light induces the dissociation of a homodimer, allowing the formation of a heterodimer, as seen in the UVR8 photoreceptor. In certain photoreceptors, instead of heterodimerization, light triggers the formation or dissociation of oligomers or homodimers, altering the binding of the photoreceptor to its operator sequence. In such cases, the photoreceptor, that contains the DBD, is bound to the AD/RD, and light regulates the binding or dissociation from the operator sequence (Fig. 2; Isomura, 2021; Müller et al., 2015; Ohlendorf & Möglich, 2022; Yamada et al., 2020).

An alternative approach to regulate the activity of endogenous promoters, rather than using synthetic promoters, involves employing a nuclease-deficient Cas9 (dCas9) instead of a DNA binding domain. dCas9 can be targeted to any endogenous promoter by designing guide RNAs (gRNAs) for the specific promoter (Nihongaki et al., 2015; Polstein & Gersbach, 2015).

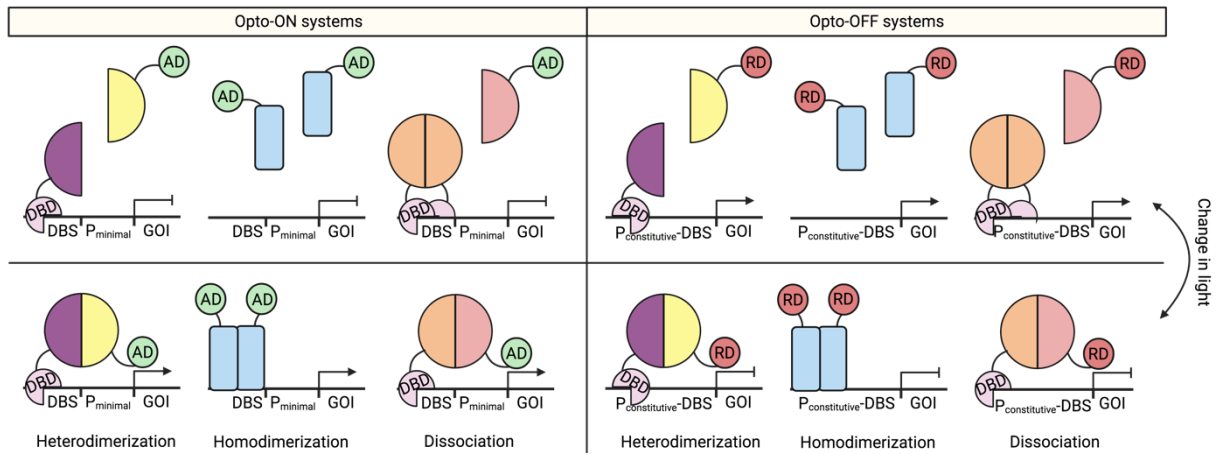


Figure 2. Illustration of the different strategies to engineer optogenetic switches for transcriptional activation (Opto-ON) or repression (Opto-OFF) depending on the photoreceptor used. Optogenetic synthetic promoters always have a cognate operator site recognized by a DNA-binding domain or a photoreceptor. In Opto-ON synthetic promoters this DNA binding sequence (DBS) is next to a minimal promoter (P_{min}), positioning of the transactivation domain (AD) close to P_{min} is sufficient to induce expression. Instead, Opto-OFF synthetic promoters have the DBS imbedded in a constitutive promoter, when the repression domain (RD) is in the proximity of the promoter region, gene expression ceases. The strategy to control the positioning of the AD and RD varies depending on the mechanism that the photoreceptor undergoes when exposed to light. If a photoreceptor forming heterodimers is used, one of the components is fused to a DNA binding domain and the other to the AD or RD. The same approach can be used when a homodimer-forming photoreceptor that dissociates upon light illumination, allowing heterodimer formation, is used. Instead, the AD or RD can be directly fused to photoreceptors that bind a cognate sequence upon light-dependent homodimerization or oligomerization.

In this work, one LOV-based and one phytochrome-based optogenetic systems were employed. LOV domains were identified in phototropins, which are plant blue-light receptor kinases. Phototropins have two LOV domains, from which the LOV2 domain holds the photoreceptor activity (Christie, Gawthorne, et al., 2012). Relevant to this work is the light-dependent DNA binding protein EL222, from the gram-negative bacteria *Erythrobacter litoralis*. EL222 contains a LOV2 domain that is connected through a α -helix to a DNA binding helix-turn-helix (HTH) domain. In darkness, the HTH is attached to the LOV core, preventing the binding to DNA. The conformational change, promoted by blue light illumination, breaks this interaction, resulting in homodimerization of EL222 and binding of the HTH to the C120 DNA motif (Nash et al., 2011).

Regarding plant phytochromes, these photoreceptors have two isoforms: a biologically inactive Pr state and a biologically active Pfr state. Red light illumination results in the transition from the red-light absorbing Pr state to the far-red light absorbing Pfr state. The structural changes in Pfr promote the interaction with phytochrome interacting factors (PIFs) and other

transcription factors, and its transport to the nucleus. The reversion of the Pfr to the Pr state occurs in darkness, but can also be prompted by applying far-red light, providing a more fine-tuned temporal control in phytochrome-based optogenetic systems (Möglich et al., 2010; Rockwell et al., 2006). In this thesis, optogenetic systems utilizing phytochrome B (PhyB) from *Arabidopsis thaliana* were employed. These PhyB-based optogenetic systems are based on the split-transcription factor principle, and thus comprise two engineered modules: one involves the fusion of PhyB with a transactivation domain, and the other consists of the combination of PhyB interacting factors, such as PIF3 or PIF6, with a DNA binding domain (Levskaya et al., 2009; Müller, Engesser, Metzger, et al., 2013).

1.2.3 Optogenetics in green cellular systems

Members of the viridiplantae clade (green plants), as the model organisms *Chlamydomonas reinhardtii*, *Physcomitrium patens*, *Nicotiana benthamiana*, or *Arabidopsis thaliana*, depend on light energy to perform photosynthesis and have light-signalling pathways that control different processes, such as phototaxis, phototropism, flowering, or germination (Galvão & Fankhauser, 2015; Witman, 1993). This dependency on light makes the implementation of optogenetic tools in plants more challenging than in organisms that can perform in dark conditions. Moreover, many of the photoreceptors used for engineering these light-switches have their origin in plants, making orthogonality difficult to achieve (Konrad et al., 2023). These circumstances explain why optogenetics in plants lagged behind compared to other disciplines. Nonetheless, in the last years, plant synthetic biology researchers have designed hi-tech and creative solutions to overcome some of the challenges that were hampering the implementation of optogenetics in plants. The first light-inducible gene expression system adapted to plants was the red system, based on the interaction of the plant components PhyB and PIF6. This system was used in *N. benthamiana* and *P. patens* protoplasts, and it can be potentially applied to transgenic plants, since the system is inactive upon far-red light illumination. However, this implies that supplementation of white light with a given additional intensity of far-red light would be necessary (Müller et al., 2014), and therefore, this approach would not be suitable for plants growing under sunlight. This problem was circumvented with the creation of PULSE (Plant Usable Light-Switch Elements), an optogenetic system that combines a red-ON module and a blue-OFF module, that recognize a synthetic promoter that controls the expression of the gene of interest (GOI; Ochoa-Fernandez et al., 2020). This combination allows the activation of the target gene only under monochromatic red light, but

not under dark, white, or blue light. The blue-OFF module is based on the fusion of EL222 protein of *Erythrobacter litoralis* with the EAR repression domain (SRDX). The red-ON module is formed by a truncated version of PhyB (PAS-GAF-PHY domains only) fused to the transactivation domain VP16, and a shorter version of PIF6 (amino acids 1-100) linked to the DBD of the macrolide repressor protein from *E. coli*, called E protein. White light is a mixture of all visible light wavelengths, including blue and red light. Under white light conditions, both switches bind the synthetic promoter, being the repressor activity dominant and thus making the system off. Under dark conditions, none of the light switches bind the promoter, and therefore the system is also inactive. Only under monochromatic red light the activation module binds its binding sequence while the repressor module remains unbound, therefore making the system on and allowing the synthetic promoter to drive the expression of the GOI. Consequently, PULSE can be used in transgenic green plants growing under normal photoperiods of white light and darkness. This optogenetic system was established in *A. thaliana* and *N. benthamiana*.

C. reinhardtii can grow heterotrophically, independent of light, making it compatible with optogenetic systems that are active under white light, when grown in heterotrophic conditions. In this sense, a cryptochrome-based optogenetic tool was implemented in the alga to control hydrogen production using blue light (Wang et al., 2017).

Plant photoreceptors and photosynthetic pigments (chlorophyll a and b, and carotenoids) mainly absorb light in the blue and red area of the spectrum (Paik & Huq, 2019; Rungrat et al., 2016), hence another strategy to generate plant optogenetic tools, interfering as little as possible with plant light signalling, is the implementation of systems responding to green light, as the CarH system. CarH is a bacterial protein, that under dark conditions, forms tetramers and binds to its recognition sequence CarO. Its fusion to a transactivation domain allows the induction of gene expression in dark, whereas upon green light illumination the tetramer disassemble and dissociates from the binding sequence. One of the disadvantages of this system is that requires the addition of the chromophore AboB12, which is not present in plants (Chatelle et al., 2018). In 2022, a green-light-based optogenetic system was engineered to control protein interaction and gene expression in chloroplasts. This system is based on a cyanobacterial photoreceptor that requires keto-carotenoid as a chromophore. The authors engineered the keto-carotenoid synthesis pathway, allowing the use of this opto-system without the need of chromophore supplementation in the generated transgenic plants (Piccinini et al., 2022).

Apart from these tools to control gene expression, in 2019 a switch to control potassium fluxes with blue light was engineered and applied into plants. This system, termed BLINK (Blue

Light-Inducible K⁺ channel), combines a potassium channel and a LOV2 domain responding to blue light that regulates the channel activity. Implementation of the opto-switch has been shown to increase biomass production in *Arabidopsis sp.* (Papanatsiou et al., 2019).

As described in section 1.2.1, the most common way to optogenetically control ion transport is by the use of opsins. Significant progresses have been made in the last years to make opsin-based optogenetics possible in plants. Most of the opsins available respond to blue or green light, thus opsin-expressing photosynthetic organism should be grown in red light to avoid activation of the light-dependent transporter. Opsins require its cofactor retinal, not only to function properly but also to maintain high expression levels and prevent its degradation (Nagel et al., 2013). To overcome the limitation that plants do not produce retinal, the Chr2 XXL variant, which requires low levels of retinal, was used in *N. benthamiana* and *A. thaliana*. Unfortunately, retinal supplementation was still required (Reyer et al., 2020). Very recently, Zhou *et al.*, introduced a bacterial dioxygenase into the chloroplast of *A. thaliana* to convert all the β -carotene available in the chloroplast into retinal, therefore expanding the possibilities of opsin-based optogenetics in plants. They demonstrated the potential of this engineered plants by expressing different anion-channelrhodopsins, like *GtACR1* and 2, to control stomatal closure and plant growth (Zhou, Ding, Duan, et al., 2021; Zhou, Ding, Gao, et al., 2021; Zhou, Ding, Nagel, et al., 2021).

In summary, optogenetic tools to precisely control ion fluxes and gene expression have been established in *A. thaliana*. These tools have been further engineered to function without the need of opsins cofactor supplementation or disturbing growth under normal conditions (white light and darkness), as seen with the PULSE system. Additionally, there is also a green-light induced optogenetic system available for *A. thaliana*, providing the opportunity to combine different optogenetic systems for simultaneous and independent control of various genes with different light wavelengths. These huge advancements lagged behind in other model organisms, such as *C. reinhardtii*, where only one system to control gene expression with light is available, and *P. patens*, where no systems are available that do not interfere with normal growth conditions. Expanding the palette of optogenetic tools in these model organisms will enhance the potential for high-resolution control of cellular processes, thereby expanding the possibilities in fundamental research studies and biotechnology applications.

1.3 Model organisms used: *Chlamydomonas reinhardtii* and *Physcomitrium patens*

1.3.1 The green alga *C. reinhardtii*

Green algae are a very heterogeneous group in which we can find the genus *Chlamydomonas*, from the phylum Chlorophyta. Among this genus, the most common species in scientific laboratories is *Chlamydomonas reinhardtii* (Pröschold et al., 2001). *C. reinhardtii* can be found in soil and pelagic zones of lakes (Sasso et al., 2018). It is a 10 micrometers single-celled alga with an ovate shape. Regarding its structure, it has a multilayered glycoprotein cell wall and two anterior flagella that are used for motility. Occupying almost half of the cell volume, a big chloroplast can be identified containing at least one pyrenoid. The chloroplast partially surrounds the nucleus, which is normally centrally located and contains a noticeable nucleolus. The nuclear membrane is continuous with the endoplasmic reticulum and the Golgi apparatus. *C. reinhardtii* also contains mitochondria distributed in the cytoplasm, a contractile vacuole and a bright orange eyespot, a carotenoid-rich organelle involved in light perception and phototaxis. This green flagellate is a haploid organism containing 17 nuclear chromosomes, its three genomes (nuclear, plastid and mitochondrial) can be genetically manipulated and were sequenced in the last decades (Boynton & Gillham, 1993; Gallaher et al., 2018; Kindle, 1990; Merchant et al., 2007; Remacle et al., 2006). Propagation of this microalga is typically asexual, but sexual mating of isogamous gametes can occur under certain stress conditions (Beck & Acker, 1992; Harris et al., 2009). Its growth conditions are well established since decades and its cultivation is very easy, inexpensive and fast, with a doubling time of 6-8 hours (Harris, 2001; Schroda & Remacle, 2022). It can be grown in three different conditions: autotrophically using light and CO₂ to perform photosynthesis, heterotrophically when cells are in darkness and use acetate as carbon source, and mixotrophically when light and acetate are present (Harris et al., 2009).

C. reinhardtii has different characteristics that makes it very interesting both for fundamental research and biotechnology. It is a model organism for fundamental research of several cellular and molecular biology processes including photosynthesis, since photosynthetic-related genes can be mutated without impeding survival, thanks to its heterotrophic metabolism (Eberhard et al., 2008), cilia and flagella biology (Wingfield & Lechtreck, 2018), biofuel production (Scranton et al., 2015), circadian clock (Matsuo & Ishiura, 2011), and heavy metal homeostasis (Hanikenne, 2003), among others (Salomé & Merchant, 2019). On the other hand, regarding biotechnology applications, there is an increasing need to abandon fossil fuel-based production,

and an interesting substitute would be the use of photosynthetic organisms like plants, algae and cyanobacteria, which can convert CO₂ into valuable products. In comparison with cyanobacteria, the use of plants and algae for recombinant protein production has some benefits, since they can make glycosylation modifications and fold proteins better than bacteria or yeast (Yan et al., 2016). Moreover, algae offer a significant advantage over plants as they can be cultivated on a large scale without competing for agriculture land, even in wastewater, thereby expanding the possibilities for circular economy approaches. In this regard, in Europe, the majority of the microalgae cultivation, accounting for 71 %, occurs in photobioreactors (Kuech, Breuer and Popescu, 2023). Additionally, this unicellular green alga is recognized as a GRAS (Generally Recognized as Safe) organism, opening the possibility to employ it as a green factory for biomedical purposes (Fernández et al., 2021; Masi et al., 2023; Rosales-Mendoza et al., 2020; Sathya et al., 2023; Ummalyma et al., 2021).

The first *C. reinhardtii* strain was isolated in 1945 in Massachusetts (Harris et al., 2009). Since then, hundreds of strains have been engineered from this common ancestor, including the mutant collection CliP (Li et al., 2015). All these strains can be found in a repository in the Chlamydomonas Resource Center (<https://www.chlamycollection.org/>). The *cw15* wall-less strains have a defective cell wall (Davies and Plaskitt, 1971), making nuclear transformation quite easy by the so-called glass bead method, that consist in vortexing a mixture of glass beads, linearized DNA and alga cells (Kindle, 1990). The DNA enters the cell and randomly integrates by non-homologous end joining (NHEJ) in double strand breaks (Shahar et al., 2020). Nuclear transformation, a requisite for genetic engineering, has been improved since the first reports in 1982 (Rochaix & Van Dillewijn, 1982). However, despite the easiness of the process, high levels of nuclear transgene expression are difficult to achieve due to certain limitations. First, the genome average GC content of *Chlamydomonas* is 64 %, quite high when compared to other organisms like humans, *E. coli* or *A. thaliana*, which decreases the success rate of common molecular cloning techniques. Second, it is codon biased, meaning that some codons are barely used, which makes codon optimization indispensable to achieve transgene expression (Barahimipour et al., 2015; Fuhrmann et al., 1999; Weiner et al., 2018). Third, silencing mechanisms are quite efficient in this green alga. The silencing of transgenes occur mainly at the transcriptional level, through histone modifications, but might also take place post-transcriptionally by small RNAs (Cerutti et al., 1997b; Schroda, 2019). To mitigate the effect of silencing and increase expression efficiency, certain measures should be adopted. In addition to codon optimization, expression can be boosted by integrating introns into the transgene, like *Rbcs2i1* and *Rbcs2i2* from the *Rbcs2* gene, that may contain enhancer

sequences or promote transcription elongation by intron mediated enhancement (IME) (Baier et al., 2018, 2020; Eichler-Stahlberg et al., 2009; Lumberras et al., 1998; Rose, 2008; Schroda, 2019; Schroda & Remacle, 2022). To facilitate this process, the Intronserter tool was developed. This tool helps with codon optimization and intron insertion design (Jaeger et al., 2019). Gene expression levels also depend on the promoter used. Some available promoters for nuclear expression in *C. reinhardtii* are HSP70A, RBCS2, β TUB2, and the ammonium-starvation-inducible NIT1 promoters (Davies & Grossman, 1994; Ohresser et al., 1997; Schroda et al., 2000; Stevens et al., 1996). However, the most frequently used promoters for strong gene expression are the PSAD promoter (Fischer & Rochaix, 2001) and the synthetic AR promoter (Schroda et al., 2000), which is a fusion of HSP70A and RBCS2 promoters.

However, it has been shown that promoter strength and gene expression levels also vary depending on the transgene, since the nucleosome positioning also plays a role in silencing. For this reason, it is important to test different promoters when generating synthetic constructs aiming to achieve high expression levels (Lodha & Schroda, 2005; Schroda, 2019; Schroda & Remacle, 2022). Moreover, since the exogenous DNA randomly integrates into the genome, position effects of the chromosomal integration site also need to be considered, and therefore several transformants should be tested seeking for high expressing candidates (Shahar et al., 2020). During integration, it has been shown that chromosomal rearrangements may occur and that *C. reinhardtii* DNA derived from lysed cells can be incorporated. These effects are more prominent when big plasmids are transformed. Considering all these obstacles, it has been described that extensive screening is needed to successfully generate new *Chlamydomonas* transgenic lines (R. Zhang et al., 2014).

In the last fifteen years, strains with increased transgene expression levels have been generated by UV mutagenesis, given rise to the UVM4 and UVM11 strains, that harbor a mutation in the Sir2-type histone deacetylase (SRTA) gene (Neupert et al., 2009, 2020). In 2016, a similar strain was developed following the same approach but starting from a methyltransferase (MET1) mutant (Kurniasih et al., 2016).

The most extensive molecular kit currently available for algae is the one developed for *C. reinhardtii*. In 2018, the MoClo kit was generated (Crozet et al., 2018), a collection of genetic parts that includes promoters, antibiotic resistance genes, secretion signals, targeting peptides, reporters, introns, purification tags, terminators, etc., all of them codon optimized for *Chlamydomonas*. Nowadays, many reporters that allow a sensitive and quantitative analysis of gene expression are available for this alga, like Gaussia or Renilla luciferase and different

fluorescence proteins (GFP, mCherry, mVenus...) (Fuhrmann et al., 1999, 2004; Rasala et al., 2013; Shao & Bock, 2008). Regarding antibiotic resistance genes, the most commonly used are the eubacterial *aadA* gene against spectinomycin and streptomycin, and *aphVII* and *aphVIII*, from *Streptomyces rimosus*, that confer resistance to hygromycin B and paromomycin, respectively (Berthold et al., 2002; Cerutti et al., 1997a; Sizova et al., 2001). All the genetic parts included in the MoClo kit (GoldenGate cloning method-based) can be easily assembled, reducing the time and effort needed to test different combinations of promoter-transgene or promoter-terminator, making it easier to find the best combination to achieve higher expression levels.

Additionally, Lauersen and colleagues created the Poptimized (pOpt) plasmids, that contain an antibiotic resistance cassette for *Chlamydomonas* and a multiple cloning site, to easily clone the gene of interest, surrounded by a strong promoter-terminator combination (Lauersen et al., 2015).

Despite the transgene expression issues that this model alga has, the mentioned advances in generating efficient transgene expressing strains, a MoClo kit for fast assembly and testing, and the improvements in transgene design with tools like *intronserter*, make *Chlamydomonas* a suitable and promising platform for synthetic biology approaches. However, there is a lack of tools for the control of biological processes with quantitative and high spatiotemporal resolution and with minimize invasiveness. So far there is only one optogenetic system available to control gene expression, or others based on the addition of compounds, like nitrate or salts (Beltran-Aguilar et al., 2019; Koblenz & Lechtreck, 2005; Wang et al., 2017), which offer less temporal resolution than light inducible systems.

The broad implementation of optogenetic tools in *C. reinhardtii* would enable the precise control of molecular components, allowing for the study of complex biological networks and cellular processes in ways previously unattainable.

1.3.2 The moss *P. patens*

The bryophyte *Physcomitrium patens* belongs to the family Funariaceae and can be found in North America, Europe and East Asia (Medina et al., 2019; Rensing et al., 2020). The most commonly used ecotype in the laboratory is Gransden, which was collected in UK in 1962 (Engel, 1968). *P. patens* belongs to the clade of Viridiplantae, and it plays an important role to decipher processes like terrestrialization, since it is evolutionary positioned between microalgae, like *C. reinhardtii*, and vascular plants, as *A. thaliana* (Prigge & Bezanilla, 2010).

Mosses are simpler than vascular plants, but they share many biological processes and conserved pathways, which makes them a very interesting model to study processes like biotic or abiotic stress responses, organelle-organelle communication or hormone signaling. Therefore, in the last decades, *P. patens* has been used as a model for cell biology and evolutionary developmental (evo-devo) studies (Falz & Müller-Schüssele, 2019; Rensing et al., 2020; Richardt et al., 2010).

One difference between this non-vascular land plant with *A. thaliana* is that it has a haploid gametophyte phase which is predominant. Its life cycle starts with the germination of the spore, giving rise to filaments that conform the protonemal tissue. Two different cell types are distinguished in the protonemata: the chloronemal and the caulonemal cells. The chloronemal cells are the first emerging cells from the spore, containing numerous chloroplasts and a perpendicular cross wall. Some days after the appearance of chloronemal cells, caulonemal cells can be distinguished. Caulonemal cells exhibit less chloroplasts, have an oblique wall and grow faster than chloronemal cells (Menand et al., 2007). The transition of young to adult gametophyte comes along with a bud formation in the caulonemal cells. This bud will generate a leafy gametophore in which, upon maturation, gametes are produced. *P. patens* is a monoecious plant that harbors, in a single gametophore, the male gametes in the antheridia, and the female gametes in the archegonia. After fertilization, the diploid sporophyte is formed, it contains a foot, a seta and a capsule where spores are produced by meiosis. Once mature, the spores are released from the capsule (Fig. 3; Cove et al., 2006; Rensing et al., 2020; D. G. Schaefer & Zrýd, 2001).

P. patens whole life cycle can take place in the laboratory and it is relatively short when compared to seed-plants like *A. thaliana*. Moreover, this moss can regenerate a full plant from a single cell or a piece of gametophyte, allowing easy vegetative propagation. Additionally, gamete formation can be induced in the laboratory by incubating the plants at fifteen degrees. Protoplasts of protonemata tissue can be easily isolated and transformed by transient permeabilization of the cell membrane with polyethylene glycol (PEG) (D. Schaefer et al., 1991; D. G. Schaefer & Zrýd, 2001). After transformation, protoplasts can regenerate an adult plant, making the generation of stable transgenic lines way easier and faster than in vascular plants. Regarding the implementation of optogenetic systems into plant model organisms, *P. patens* protoplasts represent an ideal platform to test synthetic biology molecular tools in a fast manner, thanks to the availability of transient transformation protocols and the high levels of cotransformation efficiency, which allow testing multiple components with low molecular cloning efforts (Ochoa-Fernandez et al., 2016). Furthermore, homologous recombination is

very efficient in this green organism, allowing to generate knock outs straightforwardly and making it an ideal organism to perform genetic studies.

Another advantage of working with *P. patens* is that the CRISPR/Cas9 genome editing technology is established (Collonnier et al., 2017). This technology allows targeting and disrupting multiple genes by introducing several guide RNAs, overcoming the common problems derived from gene functional redundancy in plants, especially in reverse genetic approaches (Lopez-Obando et al., 2016). The double-stranded breaks produced by the Cas9 enzyme can also be repaired by homologous recombination when a homologous template is supplied. This approach brings a lot of possibilities, such as substituting certain nucleotides in endogenous genes or modifying endogenous promoters (Rensing et al., 2020; Yi & Goshima, 2020).

The genome of *Physcomitrium patens*, organized in 27 chromosomes, was sequenced in 2008, being the fourth plant genome fully sequenced. Its plastid and mitochondrial genomes are also available (Lang et al., 2008, 2018; Reski et al., 1994; Sugiura et al., 2003; Terasawa et al., 2007). This fact, together with the high homologous recombination efficiency, and its high regeneration capacity, makes it a very interesting model for biology studies, and synthetic biology and biotechnology approaches. In this regard, this moss can be industrially grown in up to 500 L bioreactors, and has been used as a biological chassis to produce biopharmaceuticals and cosmetic products, some of them already in the market while others are in clinical trials (Decker & Reski, 2020; Michelfelder et al., 2017; Reski et al., 2018). Remarkably, Müller *et al.* demonstrated the possibility of controlling the production of a recombinant human growth factor (hVEGF) in *P. patens* using a red-light induced optogenetic system (Müller et al., 2014). The implementation of this optogenetic system in *P. patens* introduced the possibility to control transgene expression with high spatiotemporal resolution, addressing a previous limitation, since until then inducible systems in *P. patens* mainly relied on chemicals and hormones (Kubo et al., 2013; Zeidler et al., 1996). Nonetheless, the optogenetic tool implemented in our lab by Müller and colleagues is not suitable for plants cultivated under normal conditions (white light and darkness), as white light would trigger gene expression. Hence, there remains a necessity for an inducible system enabling high controlled manipulation of biological processes without interfering with normal growth. Such a system would expand the possibilities for biotechnological applications and fundamental research relying on precise spatiotemporal and quantitative control of gene expression.

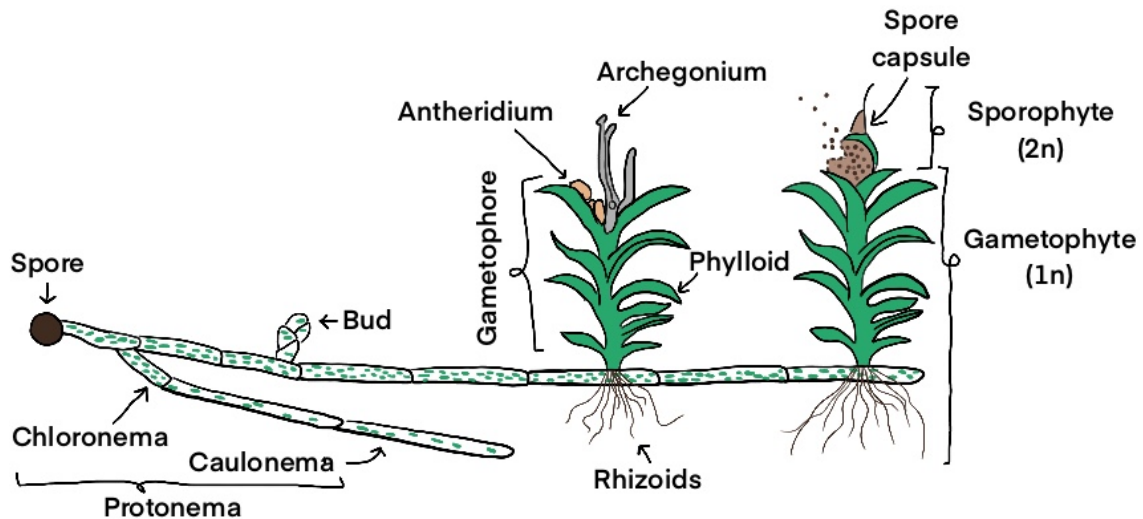


Figure 3. Diagram depicting the life cycle of *P. patens*, illustrating its two alternating generations: the gametophyte and the sporophyte. Spore germination initiates the development of protonemal tissue, comprising chloronema and caulonema cells. Buds, arising from caulonemal cells, give rise to the gametophore, characterized by leaf-shaped phylloids and rhizoids. In mature gametophores, gametes are produced within the antheridia and archegonia. Following fertilization, the sporophyte forms, where spores are formed. Adapted from figure 1 in Strotbek et al., 2013.

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2 Aims

This work focuses on engineering and implementing optogenetic tools in two green model organisms, *Physcomitrium patens* and *Chlamydomonas reinhardtii*, in which there is a general lack of tools to precisely control and study cellular processes. The primary aims of this thesis are:

- Implementing the PULSE optogenetic system, a light-controlled gene expression system suitable for plants growing under white light conditions, and CRISPRa technologies to control endogenous promoters in the moss *Physcomitrium patens*.
- Creating an optogenetic toolbox, and procedures to expedite and standardize the implementation of optogenetic systems for regulating gene expression in *C. reinhardtii*. This includes the quantitative characterization of different molecular components, such as promoters and reporters, and advancing towards the expression of large plasmids encoding more than two transcriptional units.
- Engineering and implementing optogenetic switches to control activation and repression of gene expression in *C. reinhardtii*.
- Establishing a methodology for monitoring changes in mitochondrial membrane potential in *C. reinhardtii* and progressing towards the development of optogenetically regulated mitochondria.

3 CHAPTER I: Implementing optogenetic tools to control gene expression in *P. patens* protoplasts

One of the primary objectives of this thesis is the establishment of the PULSE system, an optogenetic system compatible with standard growth conditions (white light and darkness), in *P. patens*. To expedite the implementation of this system, we aimed to implement it into protoplasts, an ideal platform for rapid testing of synthetic biology tools, since they can be transiently transformed avoiding the time-consuming generation of stable lines. Protoplasts experiments typically require around 24 h, enabling researchers to evaluate several constructs quickly and select the most effective one for subsequent generation of the stable line.

To achieve this, we initially established protocols for protoplast isolation and efficient cotransformation with multiple plasmids simultaneously, thereby eliminating the need for cloning large constructs and further reducing the time and effort required for testing this large optogenetic system.

The work conducted to establish the PULSE optogenetic system in *P. patens* protoplasts is outlined in the manuscript titled “PULSE-optogenetic control of gene expression in the moss *P. patens*” (manuscript 1), which is in its final stages of preparation for submission. Additionally, it shows the establishment of the CRISPR activation system for the first time, and its control with light by combining it with PULSE. It also includes the preliminary data analysis of a multiplexed RNA-seq of protoplasts transformed with PULSE controlling a gene involved in the production of cytokinins.

3.1 Manuscript 1: PULSE-optogenetic control of transient gene expression in the moss *P. patens*

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Author contribution:

O.D.-R. designed and performed the experiments, analysed the data and wrote the manuscript. M.M. and O.D.-R performed the RNA-seq experiment and data analysis, L.S.-M. and S.M.B contributed to the RNA-seq data interpretation and conceptualization, U.U.-G. contributed to the design and conceptualization of PULSE-controlled CRISPRa, Y.C. and T.V conceptualized the PULSE-controlled cytokinin production. M.D.Z. designed, conceptualized and supervised the project, analysed the data and wrote the manuscript.

Abstract

The moss *Physcomitrium patens* is employed as a host in biotechnology and as a model organism for fundamental research studies, mainly related to plant development, physiology, and evolution. These studies often require the control of specific genes. However, achieving precise and reversible control of gene expression without disrupting normal growth conditions remains challenging due to the limited inducible tools available for this moss.

In this study, we explored the implementation of the PULSE optogenetic system in *P. patens* protoplasts, a system that enables precise and reversible control of gene expression with red light and is compatible with plants growing under ambient light conditions. We characterized the functionality and properties of the PULSE system in *P. patens*, demonstrating significantly higher gene induction under red light compared to white light, as well as rapid activation and deactivation kinetics of the system.

Furthermore, we established the CRISPR activation (CRISPRa) technology and integrated it with PULSE. This innovative approach not only introduced the possibility of enhancing endogenous gene expression with CRISPRa but also the light-controlled modulation of endogenous promoter activity through its combination with PULSE. These newly implemented synthetic biology tools enhance the potential of *P. patens* as a model for fundamental research and biotechnological applications, opening the possibility to control biological processes with unprecedented precision.

Introduction

The moss *Physcomitrium patens* has emerged as a promising model organism for biology, including synthetic biology, and biotechnology (Decker & Reski, 2020; Reski et al., 2018). Evolutionary closer to higher plants than microalgae, *P. patens* offers insights into fundamental biological processes that are not easily studied in other model organisms, and serves as an evolutionary bridge that provides valuable information for understanding the genetic basis of plant development and adaptation (D. Cove et al., 2006; Falz & Müller-Schüssele, 2019; Lang et al., 2008; Prigge & Bezanilla, 2010; Rensing, 2017; Rensing et al., 2020). However, it shares some characteristics with single-celled algae, like a predominant haploid phase, which makes the generation of transgenic lines easier than in seed plants. Moreover, *P. patens* offers unique advantages that make it an attractive platform for genetic engineering and synthetic biology applications, like efficient homology recombination, ensuring high-rate of gene targeting, the capacity to regenerate a whole plant from a single cell, and the possibility of assembling DNA fragments *in vivo*, similar to yeast (Kamisugi et al., 2006; King et al., 2016). For all this, together with its simple growth requirements, rapid life cycle, and well-established transformation protocols, it is considered an ideal organism for genetic manipulation (D. J. Cove et al., 2009). This moss has served as a chassis for the production of recombinant proteins with biopharmaceutical potential, like the large and complex human complement factor H (FH) (Michelfelder et al., 2017; Reski et al., 2015). Additionally, it has been engineered to match the human glycosylation pattern, a pivotal consideration in the development therapeutic proteins, as glycosylation influences protein structural integrity and activity (Huether et al., 2005; Parsons et al., 2013). Noteworthy, for industrial applications, this moss can be grown in photobioreactors without the need of a carbon source (Hohe & Reski, 2002), making its production cheaper than in mammalian cell lines, one of the current main biopharmaceutical hosts, and avoiding the contamination with human pathogens that may occur when using mammalian cells as biotechnology chassis (Sahreen et al., 2023).

Many biotechnological applications and fundamental research studies rely on the expression of heterologous genes or the modulation of endogenous one. Dynamic modulation of specific gene activity can help in a broad set of scenarios ranging from deeper understanding of gene function to optimization of the synthesis of valuable compounds, while it also helps to mitigate potential negative effects associated with constitutive expression of some genes (Singh et al., 2022; Sosa-Carrillo et al., 2023). Therefore, the availability of inducible molecular switches to control gene expression is crucial to fully harnessing the potential of *P. patens* in fundamental

research, synthetic biology and biotechnology. In *P. patens* there are different inducible systems based on chemicals, such as tetracycline or β -estradiol, as well as heat shock (Kubo et al., 2013; Saidi et al., 2005; Zeidler et al., 1996). These switches provide precise control over gene expression; however, they typically offer low spatiotemporal resolution. In this sense, optogenetics offers a precise and spatiotemporal control that is not possible to achieve with chemically inducible systems. Light, as an inducer, compared to chemicals, has minimized toxicity and invasiveness and allows to reversibly control gene expression by simply switching the light source on or off (Omelina et al., 2022). However, since plants require life to survive, implementation of optogenetic tools that do not interfere with normal light-responses and growth of photosynthetic organisms is challenging.

In 2014, a red-light-inducible optogenetic system was implemented in *P. patens* protoplasts effectively inducing the production of the vascular endothelial growth factor, VEGF (Müller et al., 2014). However, since this system is active under white light, there remains a need for the establishment of an optogenetic system suitable for *P. patens* growing under ambient light. Recently, the PULSE system was engineered and implemented in both *A. thaliana* and *N. benthamiana* (Ochoa-Fernandez et al., 2020). This optogenetic system combines a red-light-inducible switch (Red_{on}) with a blue-light-regulated repressor (B_{off}). The integration of these components enables the induction of gene expression exclusively under red light conditions. Whereas in cycles of darkness/white light it is off. Here, we implemented the PULSE system in *P. patens* protoplasts. Protoplasts are an ideal platform to study in a fast and simplified manner signaling pathways, since they retain the identity of the tissue while being highly amenable to chemical treatments or microscopy (Jiang et al., 2013). We tested the functionality of the PULSE system and characterized its gene induction capacity and reversibility. Additionally, we employed the PULSE system to control the expression of an isopentenyl transferase gene, which is involved in the biosynthesis of cytokinins (CKs), using light. We analysed the effects of its expression through RNA-seq analysis.

To further expand the use of this organism as a synthetic biology host, a CRISPR-Cas9-based technology, previously absent in *P. patens*, was combined with the PULSE system to enable precisely controlled transcription of endogenous loci. Within this strategy, the expression of dead Cas9 (dCas9), fused to a transactivation domain, was induced with PULSE. This innovative approach not only addresses the prior absence of CRISPR-Cas9-derived gene activator in *P. patens* but also introduces a mechanism for regulating the expression of endogenous genes. This could potentially be utilized, for instance, to generate inducible and

reversible knockouts. The implementation of PULSE and its integration with CRISPR-Cas9 activation in *P. patens* improves the precision and flexibility in regulating gene expression in this moss species, advancing its potential as a synthetic biology host and expanding the possibilities in developmental and metabolism studies.

Materials and methods

Plasmid generation

A detailed description of plasmids and oligonucleotides designed, constructed and used in the frame of this thesis are listed in tables S1 and S2. DNA fragments of interest were amplified according to table S1 using polymerase chain reaction (PCR), with oligonucleotides provided by Sigma Aldrich or Integrated DNA Technologies, using Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB). 1 % agarose gel was used to separate DNA fragments. For gel extractions, NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used. Plasmids were assembled using Golden Braid, Gibson assembly or AQUA cloning (Beyer et al., 2015; Gibson et al., 2009; Sarrion-Perdigones et al., 2011) and transformed into chemically competent *E. coli* strain 10-beta (NEB). Plasmids were isolated from *E. coli* using Wizard Plus SV Miniprep DNA Purification Systems (Promega) and NucleoBond Xtra Midi kit (Macherey-Nagel). Newly constructed plasmids were verified by restriction enzyme (NEB) digestion and DNA sequencing (Microsynth).

E. coli transformation and growth conditions

E. coli strain 10-beta (NEB) was grown at 37°C in LB broth supplemented with agar (4 % m/V) and kanamycin (25 µg/mL), ampicillin (100 µg/mL), chloramphenicol (25 µg/mL) or spectinomycin (100 µg/mL) when required. Plasmid were transformed into chemically competent cells by heat shock as described elsewhere (Froger & Hall, 2007).

P. patens growth conditions, protoplast isolation and transformation

P. patens (ecotype Gransden) protonemata tissue was grown at 22 °C, in a 16:8-light:dark regime, on modified BCDAT media plates (5 mM (NH₄)₂C₄H₄O₆, 1mM MgSO₄·7H₂O, 1.84 mM KH₂PO₄, 10 mM KNO₃, 0.04 mM FeSO₄·7H₂O, 10 mM H₃BO₃, 0.22 mM CuSO₄·5H₂O, 0.24 mM KBr, 0.09 mM Al₂O₁₂S₃, 2 mM MnCl₂·4H₂O, 0.19 mM ZnSO₄·7H₂O, 0.66 mM LiCl, 0.23 mM CoCl₂·6H₂O, 0.17 mM KI, 0.12 mM SnCl₂·2H₂O, 1 mM CaCl₂·2H₂O, 0.8 % agar) covered with cellophane (Roth K423.1) (<http://moss.nibb.ac.jp>). Protonemata tissue was weekly propagated as described elsewhere (Bezanilla, 2008). For protoplast isolation, four 4/5-days-old protonemata plates were digested with 0.5 % driselase (Sigma Aldrich, cat. no. D8037-5G) in 0.5 M mannitol for 1.5 h in an orbital shaker at 80 rpm, protected from light. The suspension was passed through a 100 µm and 70 µm filter (Greiner Bio One), consecutively, and centrifuged during 10 min at 100 g (without breaks). Pelleted protoplasts were washed twice by centrifugation in 10 mL of 0.5 M mannitol. Polyethylene-glycol-

mediated transformation was carried out as described elsewhere (Liu & Vidal, 2011) with some modifications. Briefly, protoplasts were counted using a Rosenthal Chamber, prior to centrifugation and resuspension of the protoplasts in 3M solution (15 mM MgCl₂, 5 mM MES, 0.465 mannitol, pH 5.8) at a final concentration of 1.2×10^6 protoplasts/mL. 40 µg of single plasmids or mixtures of different plasmids were mixed with 500 µl protoplast suspension and 550 µl of PEG solution (4g PEG₄₀₀₀, 2.5 mL of 800 mM mannitol, 1 mL of 1 M CaCl₂ and 3 mL of H₂O). After 25 min incubation at room temperature, 2.2 mL of W5 (2 mM MES, 154 mM NaCl, 125 mM CaCl₂·2H₂O, 5 mM KCl, 5 mM glucose, pH 5.8) was added, followed by centrifugation. Transformed protoplasts were resuspended in 1.6 mL PCA (0.32% (wt/vol) Gamborg's B5 basal salt powder with vitamins (bioWorld), 58.4 mM sucrose, 3.4 mM CaCl₂·2H₂O, 5 mM MES, 2 mM MgSO₄·7H₂O, 0.342 mM l-glutamine, 444 mM glucose, 8.4 µM calcium pantothenate, 2% (vol/vol) biotin from a biotin solution 0.02% (wt/vol) 0.1% (vol/vol) in H₂O, pH 5.8, sterile filtered. Prior to use 0.1% (vol/vol) Gamborg's B5 Vitamin Mix and 64.52 µg µL⁻¹ ampicillin were added) or W5 buffer with 5 % fetal bovine serum (FBS, PAN, cat. no. P30-3602) and 64.52 µg µL⁻¹ ampicillin.

Light experiments in *P. patens*

Transformed protoplasts were divided in 24-well plates (400 µL/well) or 96-well plates (200 µL/well). As described elsewhere (Ochoa-Fernandez et al., 2016, 2020), samples were kept in the dark or illuminated with custom-made LED light-boxes, containing LEDs of different wavelengths (455 nm-blue, 660 nm-red, 740 nm-far red and 4000K-white). For white light condition, white light was supplemented with blue light and far-red light, to mimic the sunlight spectra equivalent ratio of blue, red and far-red light. To avoid unintended activation of the optogenetic systems, handling of the samples was carried out under green light conditions (510 nm).

Luminescence analysis of *P. patens* protoplasts

FLuc activity was determined as described elsewhere (Ochoa-Fernandez et al., 2020). Briefly, three or four 80 µl technical replicates of *P. patens* protoplast suspensions in PCA media were transferred into a 96-well white flat-bottom plate (Costar). 20 µl of FLuc substrate (0.47 mM d-luciferin (Biosynth AG), 20 mM tricine, 2.67 mM MgSO₄·7H₂O, 0.1 mM EDTA·2H₂O, 33.3 mM dithiothreitol, 0.52 mM adenosine 5'-triphosphate, 0.27 mM acetyl-coenzyme A, 5 mM NaOH, 264 µM MgCO₃·5H₂O, in H₂O, pH 8) were added immediately prior to measurement. Luminescence was measured for 20 min with an integration time of 0.1 s, using a Centro XS3 LB960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).

For protein kinetic experiments, transformed protoplasts were resuspended in W5 buffer with 5 % fetal bovine serum (FBS, PAN, cat. no. P30-3602), 64.52 $\mu\text{g } \mu\text{L}^{-1}$ ampicillin and 2.4 % FLuc substrate (50 mM D-Luciferin Firefly (Biosynth AG) in 0.1 M triphosphate buffer, pH 8). Three 200 μL technical triplicates were loaded into a 96-well, white, flat-bottom plate (Costar). Plate was sealed with TopSeal-A Plus (PerkinElmer). Luminescence was measured every 30 min with an integration time of 0.1 s, utilizing the Centro XS3 LB960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Protoplasts RNA isolation and quantitative reverse transcription PCR

Samples containing $1\text{--}3 \times 10^5$ protoplasts in W5 buffer with 5 % fetal bovine serum (FBS, PAN, cat. no. P30-3602) and 64.52 $\mu\text{g } \mu\text{L}^{-1}$ ampicillin, were centrifuged during 3 min at 100 g. Pellets were frozen in liquid N₂ for subsequent RNA isolation with the RNeasy Plant Mini Kit (Qiagen). To reduce DNA contamination, samples were treated with DNase I (Thermo Scientific). Conversion of RNA to cDNA was performed using the LunaScript RT SuperMix Kit (New England Biolabs, NEB). For quantitative PCR, 15 ng of cDNA were mixed with specific primers (listed in table S3) and GoTaq qPCR Master Mix (Promega), containing BRYT Green Dye. Cycling conditions, according to the manufacturer, were set in a qTOWER³ G Real-Time PCR Thermal Cycle (Analytik Jena). Expression levels of all the samples were measured in triplicates.

Multiplexed RNA-seq

Transformed protoplasts were resuspended in W5 buffer with 5 % fetal bovine serum (FBS, PAN, cat. no. P30-3602) and 64.52 $\mu\text{g } \mu\text{L}^{-1}$ ampicillin and incubated during 12h in darkness. Subsequently, protoplasts were either kept in dark conditions or exposed to 10 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ of red light (660 nm) for 3, 6, 9, 12, 18 or 24h. When indicated, 1 μM of N⁶-(2-Isopentenyl)adenine (2iP), from a 100 μM stock solution in H₂O, was added. H₂O was used as a mock treatment. After incubation, 1,250 protoplasts from each sample were used for RNA-seq. Multiplexed RNA-seq, including mRNA extraction and capture, reverse transcription and cDNA amplification, and sequencing library preparation, was performed using an adapted version of the AccuraCode® TCR kit according to the manufacturer's instructions (Singleron Biotechnologies GmbH). mRNA molecules from each sample were barcoded with specific oligos. The DNA library was sequenced using HiSeq PE150 (Illumina, Inc.).

For the data analysis, a modified version of the *P. patens* reference genome was used. Briefly, the *P. patens* reference genome V3.57 (Fasta and GTF files) was downloaded from Ensembl Plants (Harrison et al., 2014) and modified by adding the transformed heterologous genes and

the chloroplast and mitochondrial genomes (downloaded from <https://genomevolution.org/coge/OrganismView.pl?dsgid=33928>).

The processing of the Fastq raw data was performed using CeleScope® (v 1.17.0, <https://github.com/singleron-RD/CeleScope>, Singleron Biotechnologies GmbH). Out of 290,394,886 raw reads, 93.53 % passed the Q30 Phred score. Of those, 0.84 % were removed after trimming with Cutadapt (Martin, 2011) for being too short or for containing poly A or user-defined adapters. Reads were aligned to the modified *P. patens* reference genome using STAR (Dobin et al., 2013), and subsequently assigned to genes using featureCounts (Liao et al., 2014). Out of the 96 wells, 6 wells were discarded due to low quality. The average reads per well was 2,920,566 and the total number of genes mapped was 27,371.

Downstream analysis was done using R software for statistical computing version 4.3.3 (R Core Team, 2021). Differentially expressed genes were identified using DESeq2 with the default parameters (Love et al., 2014). Venn diagrams were obtained using Venny 2.1 (Oliveros, 2007-2015). Fisher's exact test was done using the function `fisher.test` from the R package `stats` version 4.3.3. Heatmaps and hierarchical clustering were performed using the R package `pheatmap` version 1.0.12 (<https://CRAN.R-project.org/package=pheatmap>). *P. patens* orthologs of *A. thaliana* genes were obtained using the `g:Orth` function of `g:Profiler` (Kolberg et al., 2023).

Software

Except for the multiplexed RNA-seq, data analysis was performed using GraphPad Prism 9 for MacOS version 9.0.0 and Microsoft Excel (version 16.16.27 for Mac). Benchling [Biology Software] was used for molecular cloning design. Inkscape 1.2.2 (b0a8486, 2022-12-01) and BioRender.com were employed for graphical design.

Statistics

Data shown are representative of at least three independent experiments, except for the multiplexed RNA-seq, where only one experiment was performed, including replicates. Plotting and statistical analysis were performed with GraphPad Prism 9 for MacOS version 9.0.0. Statistical outliers were determined and excluded as described elsewhere (Jacobs & Dinman, 2004).

Results

Implementation of PULSE in *P. patens* protoplasts

The PULSE gene expression system, developed by Ochoa-Fernandez and colleagues in 2020, combines a red-light-inducible (Red_{on}) system with a blue-light-repressible (B_{off}) optoswitch. The previously implemented Red_{on} module in *P. patens* (Müller et al., 2014) is a red/far-red bi-stable system, based on the interaction of Arabidopsis Phytochrome B (amino acids 1-650) with the phytochrome interacting factor PIF6 (amino acids 1-100). Under red light, Phytochrome B, that is coupled to the transactivation domain VP16, interacts with PIF6, which is fused to the macrolide repressor protein from *E. coli* (E protein). The E protein binds to its cognate sequence, *etr*, which is placed in the synthetic promoter, therefore PhyB-PIF6 interaction positions VP16 close to the synthetic promoter, resulting in gene expression activation (Fig. 1a). A major drawback of the system for use in plants is that this interaction also occurs upon white or blue light illumination. As a solution, the combination with the B_{off} module ensures that gene expression induction occurs only under red light. The blue repression system is based on the *Erythrobacter litoralis* photoreceptor EL222. Upon blue light exposure, EL222 fused to the *A. thaliana* trans-repressor domain SRDX forms a homodimer. The dimerization allows EL222-SRDX to bind to its specific target DNA sequence within the synthetic promoter, thereby repressing gene expression (Ochoa-Fernandez et al., 2020). The PULSE synthetic promoter consists of eight repetitions of the E protein (*etr*) and five repetitions of EL222 (C120) cognate sequences. These sequences are positioned upstream of the human cytomegalovirus minimal promoter (hCMVmin), which drives the expression of the gene of interest. Figure 1a,b details the comparison of the light- controlled activation patterns and molecular composition of the Red_{on} and PULSE systems. We tested the control of the expression of the reporter Firefly luciferase with the PULSE system in *P. patens* protoplasts and compared it with the Red_{on} system. To assess this, we transformed protoplasts with the different plasmids (Fig. 1b) and incubated them for 24-hours under red, blue, or white light, or darkness. As a light-independent control, we used the synthetic transcription factor constituted by the fusion of the E protein directly to the activation domain VP16. Notably, the PULSE system exhibited a higher induction rate under red light compared to the Red_{on} module alone, achieving a 26.9-fold induction above protoplasts in white light (Fig. 1c).

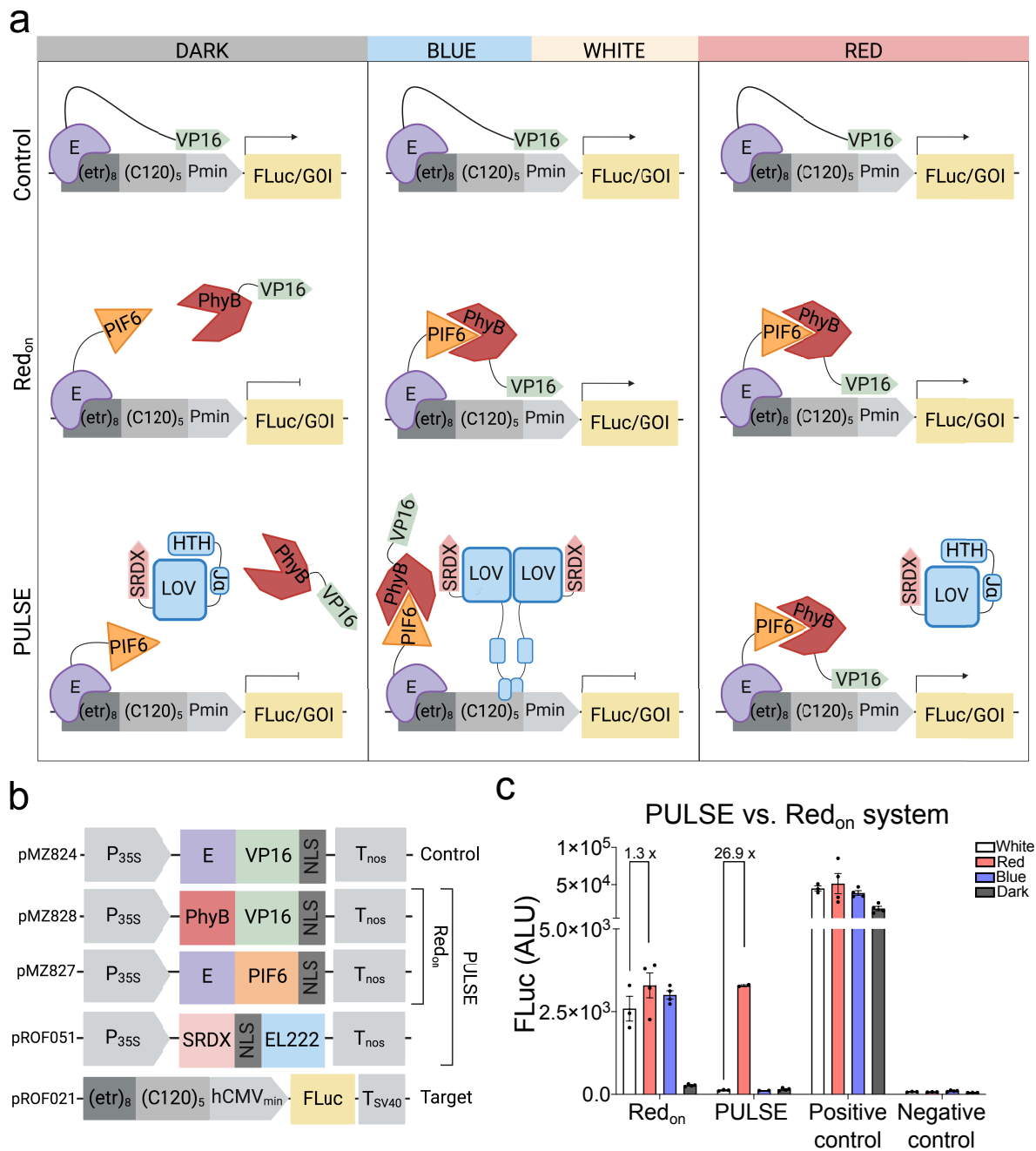


Figure 1. Comparison of PULSE and the Red_{on} system in *P. patens* protoplasts. (a, b) Positive control, Red_{on} and PULSE systems mechanism and their molecular configuration. The control, a synthetic transcription factor, consists of the DNA-binding protein E fused to the VP16 transactivation domain. The Red_{on} system is based on the reconstitution of the split synthetic transcription factor upon interaction of Phytochrome B with its interacting factor PIF6. PULSE combines the Red_{on} system with the BOff system, in which EL222 photoreceptor from *E. litoralis* is fused to the repression domain SRDX. Firefly expression is driven by the synthetic optogenetic promoter (Popto), that contains the DNA binding sequences of the E protein (etr) and EL222 (C120), and the human CMV minimal promoter. Control, Red_{on} and PULSE components are under the control of the 35S promoter. (c) Quantitative characterization of PULSE and Red_{on} systems. *P. patens* protoplasts were transformed with the target plasmid and the control, the Red_{on} or the PULSE plasmids. In the negative control, cells were transformed only with the target plasmid. FLuc activity was determined after 24 h incubation of the protoplasts in darkness, or under 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of white, red, or blue light. The mean of the

luciferase activity, expressed as absolute luminescence units (ALU), and standard error of the mean (SEM) are plotted (n=4). Data are representative of three independent experiments. FLuc, Firefly luciferase; GOI, gene of interest; P_{35S}, CaMV35S promoter; NLS, nuclear localization sequence; T, terminator; SRDX, SRDX repressor domain; HTH, helix-turn-helix; LOV, Light-Oxygen-Voltage; EL222, *E. litoralis* photoreceptor; PhyB, first 650 amino acids of phytochrome B; PIF6, first 100 amino acids of phytochrome interacting factor 6; hCMVmin, human cytomegalovirus minimal promoter; E, macrolide repressor DNA-binding protein E.

To evaluate the influence of the promoter used to drive the expression of the components constituting the PULSE system (*i.e.*, E-PIF6, PhyB-VP16, EL222-SRDX), two variants of the system, termed PULSE and Ubi-PULSE, were transformed into *P. patens* protoplasts. In the PULSE configuration, all components are placed under the control of the constitutive Cauliflower Mosaic Virus 35S promoter, while in the Ubi-PULSE version the *A. thaliana* Ubiquitin 10 promoter controls the expression of the three components. We observed more than 2 times higher induction rates when the CaMV35S promoter was utilized. However, studies have demonstrated that the AtUBQ10 promoter exhibits increased stability, persistence and results in higher expression levels compared to the CaMV35S promoter in stable transgenic lines of tobacco and Arabidopsis (Grefen et al., 2010). Additionally, it has comparable performance to other promoters in *P. patens* transgenic lines (Peramuna et al., 2018). Consequently, since generation of stable lines is easier when a single plasmid is used, we investigated the induction rates achieved when all components were driven by the AtUB10 promoter and encoded in a single plasmid, including the target of the PULSE system. A comparison of this version, named Full-PULSE, with Ubi-PULSE, where the components are encoded in co-transformed separate plasmids under the AtUBQ10 promoter, revealed remarkably lower leakiness and higher induction rates in the Full-PULSE system than when co-transforming the Ubi-PULSE plasmids (Fig. 2).

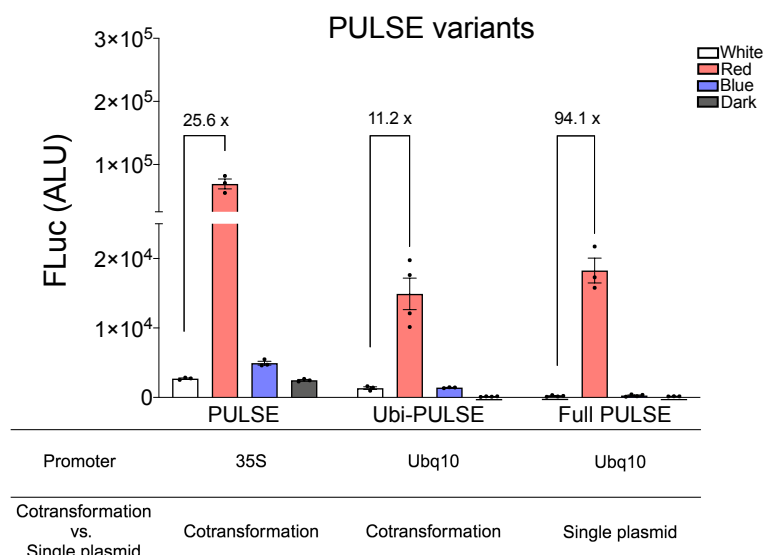


Figure 2. PULSE optimization and quantitative characterization of different variants in the promoter architecture and plasmid configuration (PULSE, Ubi-PULSE and Full-PULSE). In the PULSE and Ubi-PULSE configurations, the three different blocks of the PULSE system (this is E-PIF6, PhyB-VP16 and EL222-SRDX) and the target plasmid were encoded in different plasmids and mixed for co-transformation into protoplasts. In the Full-PULSE configuration, a single plasmid containing the target and the PULSE components was used. CaMV 35S or *A. thaliana* Ubiquitin 10 promoters were used to drive the expression of the PULSE components. The different configurations were transformed into *P. patens* protoplasts (PULSE plasmids: pMZ827, pMZ828, pROF051, pROF021; Ubi-PULSE plasmids: pROF104, pROF105, pROF117, pROF021; Full PULSE plasmid: pROF345). Protoplasts were exposed for 24 h to white, blue, or red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$), or kept in the dark, before quantification of firefly, expressed as ALU. The ALU mean and SEM are represented ($n=4$). Data are representative of three independent experiments. ALU, absolute luminescence units; FLuc, Firefly luciferase; 35S, CaMV35S promoter; Ubq10, *A. thaliana* ubiquitin 10 promoter.

Characterization of PULSE in *P. patens* protoplasts

To achieve a deeper understanding of the dynamics of the PULSE system and determine the ideal conditions for future experiments, we analysed the impact of the light intensity employed to modulate the activity of the system and the on-off kinetics of PULSE at the protein and mRNA levels (Fig. 3).

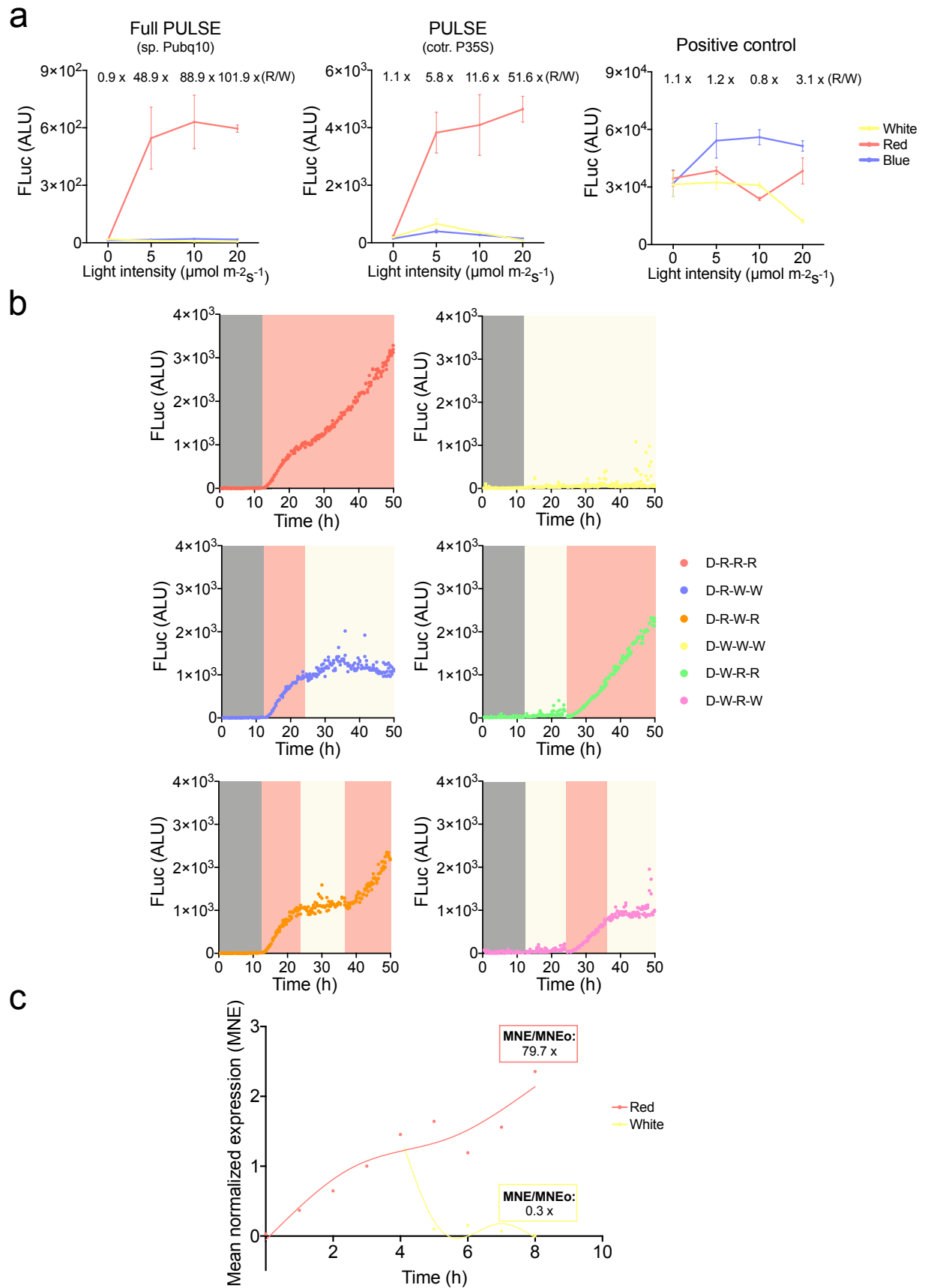


Figure 3. PULSE characterization in *P. patens* protoplasts. (a) Blue, red, and white light intensity dose-response curves for the light-independent control, PULSE and Full-PULSE configurations. Transformed protoplasts were exposed for 24 h to the indicated light intensities of white, blue, or red

light. Subsequently, firefly activity was quantified as absolute luminescence units. Mean ALU and SEM are plotted (n=4). **(b)** On-Off protein kinetics of the Full-PULSE version. Protoplasts were incubated 12 h in the dark prior to illumination with red or white light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). Every 12 h, samples were transferred to the indicated light condition or maintained with the same illumination. Luminescence was measured every 30 min. ALU for each replicate are plotted (n=3). **(c)** On-Off mRNA kinetics of PULSE. Protoplasts were incubated 20 h in the dark, before illumination with red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 4 h under red light, half of the samples were transferred to white light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). Samples were collected every hour. mRNA levels are shown as mean normalized expression (MNE). Firefly luciferase transcript was normalized against the reference gene ubiquitin; n=4 technical replicates for each transcript. Spline line is represented. **(a, b, c)** Data are representative of three independent experiments. FLuc, Firefly luciferase; P35S, CaMV35S promoter; Pubq10, *A. thaliana* ubiquitin 10 promoter.

To study the effect of the light intensity employed, *P. patens* protoplasts transformed with the Full-PULSE (in one plasmid) or PULSE versions, or the light-independent control, were incubated under 0, 5, 10 and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue, white or red light. The results showed that leakiness is reduced as light intensity increases. However, as shown in the light-independent control, high white light intensities may result in low levels of toxicity (Fig. 3a).

The on-off kinetics of the system were investigated at both protein and mRNA levels. For protein kinetics experiments, transformed protoplasts underwent a 12-hour incubation in darkness to allow the expression of PULSE components before illumination with $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of either white or red light for another 12 h. Subsequently, protoplasts were shifted to the alternate light wavelength or maintained under the same conditions for successive 12 h periods, aiming to analyse repression and induction dynamics. Firefly protein levels were continuously monitored during four 12-hour periods (Fig. 3b). Transcriptional analysis of Firefly was performed by incubating protoplasts for 4 h in red light and then splitting the sample in two: one to be transferred to white light for additional 4 h and the other maintained under red light. Protoplast samples were collected every hour and frozen in liquid N₂ for posterior messenger RNA quantification. Firefly mRNA levels showed rapid reversibility of the system, as only 1 hour of white light exposure was sufficient to revert to the initial state (Fig. 3c), indicating the high temporal control that can be achieved with this optogenetic system in *P. patens*.

CRISPR-Cas9-derived gene expression control with PULSE in *P. patens* protoplasts

CRISPR-Cas transcriptional activation (CRISPRa) offers the possibility of regulating endogenous gene expression (Dominguez et al., 2016; Pickar-Oliver & Gersbach, 2019). To achieve optogenetic control over gene expression driven by any endogenous promoter in *P.*

patens, we combined CRISPRa technology with PULSE, as previously demonstrated in *A. thaliana* protoplasts (Ochoa-Fernandez et al., 2020). In this approach, PULSE was utilized to regulate the expression a nuclease-deficient Cas9 protein (dCas9) fused to a transactivation domain. Specifically, the dCas9 employed in this study is the catalytic-inactivated form of the human codon-optimized coding sequence of the *Streptococcus pyogenes* Cas9 protein, with mutations D10A and H840A, and its corresponding level 0 Golden Braid plasmid is available in Addgene #75399 (Vazquez-Vilar et al., 2016). This dCas9 variant was fused with the strong transactivation domain TV (Li et al., 2017; Ochoa-Fernandez et al., 2020). We compared its activity in *P. patens* with that of a maize codon optimized and intronized dCas9 fused to TV (zdCas9io-TV, Grützner et al., 2021) and with that of the dCas9m4-VPR fusion, previously used in mammalian cells (Chavez et al., 2015; Fig. S1). The dCas9m4 variant carries additional mutations (A23D, D59A, D839A, and N863A) compared to dCas9 (Mali et al., 2013). dCas9-TV and zdCas9io-TV demonstrated higher induction rates compared to dCas9m4-VPR. Although zdCas9io-TV showed slightly better induction rates than dCas9-TV, the latter was chosen for further experiments due to its lower leakiness.

Subsequently, we used the dCas9-TV to regulate the expression of a synthetic promoter consisting of one or three repetitions of a protospacer sequence, characterized by Boyle et al., 2017, placed upstream of the human CMV minimal promoter. Firefly luciferase was incorporated downstream of the synthetic promoter to quantitatively characterized the system. To target the synthetic promoter, we used a guide RNA against the protospacer (Fig. 4a).

Under red-light illumination, PULSE-controlled dCas9-TV resulted in 3- or 8.7-times greater activation of the synthetic promoter compared to white light, when employing one or three repetitions of the protospacer in the synthetic promoter, respectively (Fig. 4b). Constitutive induction of dCas9-TV with the synthetic transcription factor E-VP16 served as a positive control and an indicator of the maximum activation capacity of dCas9-TV in *P. patens* when targeting the aforementioned synthetic promoter.

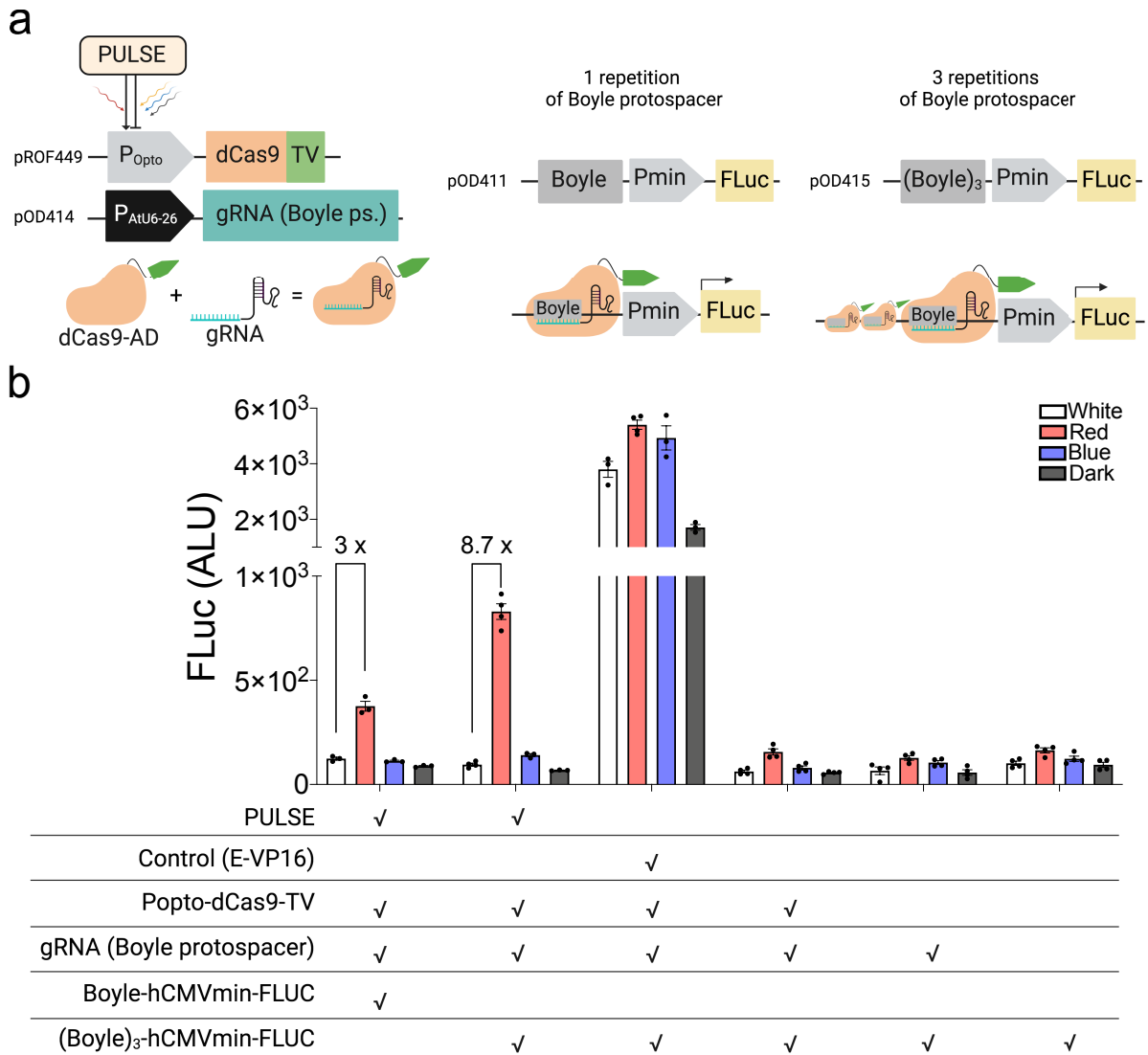


Figure 4. PULSE-controlled expression of CRISPRa in *P. patens* protoplasts. (a) Experimental depiction of the PULSE-controlled expression of dCas9-TV, which under red light is expressed and targets a synthetic promoter formed by the hCMV minimal promoter and one or three repetitions of the gRNA target, termed Boyle protospacer, encoded in pOD411 or pOD415, respectively. (b) The different indicated experimental setup combinations were transformed into *P. patens* protoplasts and incubated for 24 h under white, blue, or red light ($15 \mu\text{mol m}^{-2} \text{s}^{-1}$), or darkness. Firefly luminescence activity was quantified and expressed as ALU. Mean and SEM are plotted ($n=4$). Data are representative of three independent experiments. FLuc, Firefly luciferase; Popto, PULSE optogenetic promoter; dCas9, nuclease-deficient Cas9; TV, TV transactivation domain; $P_{\text{AtU6-26}}$, *A. thaliana* U6-26 promoter; gRNA, guide RNA; Boyle ps, Boyle protospacer; AD, activation domain.

PULSE-controlled cytokinin production in *P. patens* protoplasts

To explore the potential applications of PULSE in *P. patens*, such as controlling metabolism, we placed a gene involved in the cytokinin biosynthesis pathway, named isopentenyl transferase (IPT), under the control of the PULSE system. We used two different IPT genes: the *P. patens* *ISOPENTENYL TRANSFERASE-1* gene (*PpIPT1*), which has been shown to

upregulate cytokinin production in overexpression lines (Coudert et al., 2019), and the *A. tumefaciens* IPT gene (AgroIPT), which has been demonstrated to induce production of CK in *P. patens* (Schulz et al., 2000). The aim was to induce CK production only under red light. To achieve this, we transformed *P. patens* protoplasts with the PULSE system (pMZ827, pMZ828, pROF051) controlling either *PpIPT1* (PULSE-*PpIPT1*; pOD356) or AgroIPT (PULSE-AgroIPT; pOD357).

To determine whether we could control CK synthesis with PULSE, we performed RNA-seq analysis to identify changes in the expression of genes involved in CK synthesis and signalling pathways. We studied the impact of different illumination durations (3, 6, 9, 12, 18 and 24 h) with $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light and compared these with samples kept in darkness. Multiple time points were used because the time required for CK production and to observe subsequent changes in CK downstream gene expression is unknown. As a control, we placed the Firefly reporter gene under the control of the PULSE system (PULSE-FLuc; pROF021), which is not involved in CK pathways.

Additionally, we inoculated protoplast samples transformed with the PULSE-FLuc constructs with or without $1 \mu\text{M}$ of the adenine-based cytokinin N^6 -(2-Isopentenyl)adenine (2iP) at 3, 12, and 24h in darkness under red light. This was done to identify genes involved in the CK signalling pathway in *P. patens* protoplasts (Fig. 5).

Given the number of samples to be analysed, we performed multiplexed RNA-seq (Fig. 5; Fig. S2), which enables sequencing of up to 96 different samples simultaneously, all of which need to be collected at the same time. Therefore, the experimental setup was as follows: protoplasts were transiently transformed with different plasmid combinations (PULSE-*PpIPT1*, PULSE-AgroIPT or PULSE-FLuc). All samples were incubated in darkness for 12h to allow expression of all PULSE components. After the dark incubation, samples were either exposed to red light or kept in darkness as indicated in Fig. 5. Additionally, 2iP was added to some of the PULSE-FLuc samples at the specified time points. Samples were collected simultaneously before proceeding with the multiplexed RNA-seq workflow (Fig. S2).

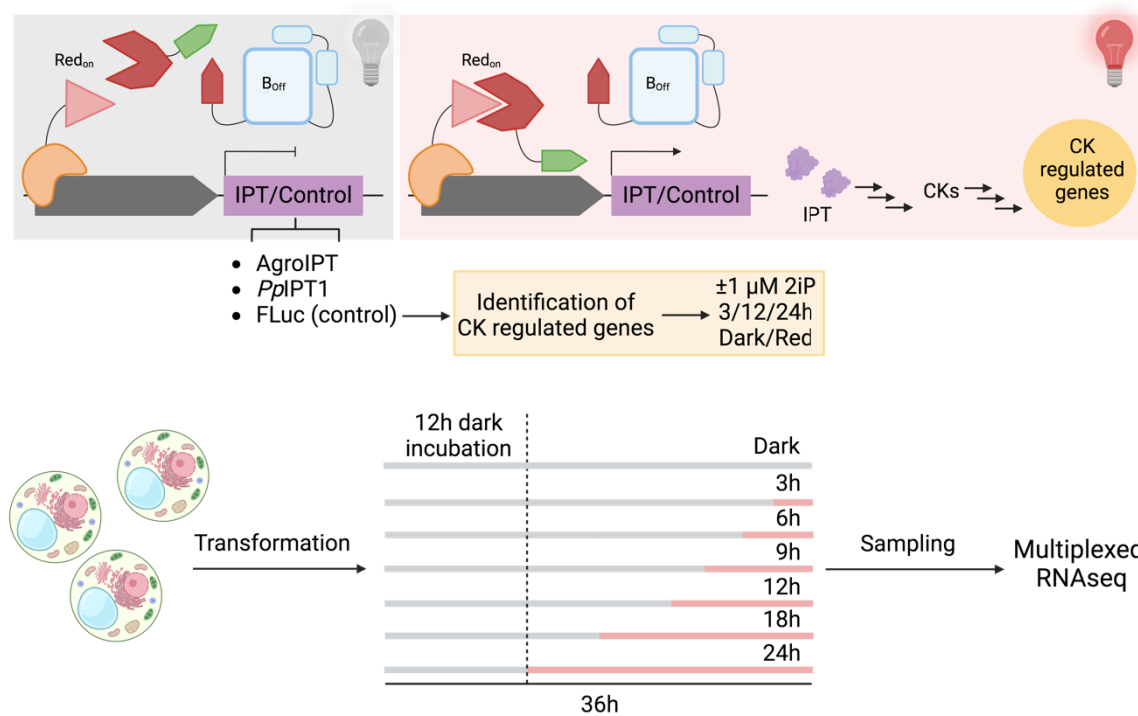


Figure 5. PULSE-controlled cytokinin production in *P. patens* protoplasts. Depiction of the experimental setup for PULSE-controlled expression of IPT genes involved in cytokinin production from *A. tumefaciens* (AgroIPT) or *P. patens* (*Pp* IPT1), as well as the Firefly reporter gene (used as control). Under red light, expression of the IPT genes should lead to CK production, resulting in changes in the expression of CK-regulated genes. Protoplasts transformed with the combinations PULSE-AgroIPT, PULSE-*Pp* IPT1, or PULSE-FLuc were exposed to varying durations of red light for gene induction. The effects of inducing AgroIPT, *Pp* IPT1, or FLuc expression were analysed using multiplexed RNA-seq. Highlighted in yellow are additional sample conditions used to identify CK-regulated genes: PULSE-FLuc treated with either 0 or 1 μM of the cytokinin 2iP. CK addition was carried out at 3, 12, and 24h in samples incubated in both darkness or under red light. IPT, Isopentenyl transferase; Agro, *A. tumefaciens*; *Pp*, *P. patens*; FLuc, Firefly luciferase; CK, cytokinin; 2iP, N⁶-(2-Isopentenyl)adenine.

To investigate the impact of adding cytokinins or inducing IPT genes in *P. patens* protoplasts, we analysed the differentially expressed genes (DEGs) among various sample conditions: (i) impact of CK addition (CK versus no CK), (ii) effects of inducing IPT genes (PULSE-AgroIPT or PULSE-*Pp* IPT1 versus PULSE-FLuc/Control). The amount of induced or repressed genes is shown (Fig. 6). As a summary, DEGs are presented together, regardless of the specific time point at which samples were exposed to red light or inoculated with CK.

Comparison of samples with or without external addition of 1 μM of 2 iP reveals that only a low number of genes (fewer than 40) show differential expression, regardless of whether samples were incubated under red or dark conditions (Fig. 6a). This suggests that the addition of cytokinin to protoplasts either did not effectively induce significant changes in gene expression or has minimal impact on protoplasts.

Regarding the DEGs between PULSE-AgroIPT and PULSE-FLuc (control) samples, a similar number of DEGs is observed under both dark and red conditions, suggesting potential leakiness in AgroIPT expression (Fig. 6b). However, in the comparison between PULSE-*Pp*IPT1 and the control, there is a distinct induction or repression of genes under red light compared to darkness (Fig. 6c, top). For this comparison, the amount of DEGs observed under the different durations of red light illumination is also shown (Fig. 6c, bottom).

Furthermore, DEGs within the two different light condition (red versus dark) was analysed for each sample (PULSE-AgroIPT, PULSE-*Pp*IPT1, and PULSE-FLuc; Fig. 6d). Similar numbers of DEGs were found between IPT samples and the FLuc sample, indicating that red light presence might be the primary driver of differential gene expression. Therefore, to identify genes specifically induced or repressed by red light, termed “red genes”, in the IPT samples, Venn diagrams comparing the DEGs in the IPT and control samples were generated (Fig. 6e). The commonly induced or repressed genes under red light between the IPT and control samples were excluded from the lists of DEGs for PULSE-AgroIPT and PULSE-*Pp*IPT1 under Red vs. Dark conditions. This allowed for the analysis of DEGs attributable solely to IPT gene expression, independent of red light effects (Fig. 6f).

To study whether *Pp*IPT1 and AgroIPT PULSE-induction results in CK production, differences in the expression of CK-responsive genes when these IPT genes are expressed were analysed. Orthologs of *A. thaliana* CK-responsive genes (Bhargava et al., 2013) in *P. patens* were used as CK-responsive genes. Heatmaps of the expression levels of these CK-responsive genes and clustering of different samples based on these levels were performed (Fig. 7). Red-light illumination resulted in higher expression levels of these genes compared to samples kept in darkness, regardless of whether the gene controlled by PULSE was one of the IPT genes or FLuc (control). However, the expression levels of most of these genes were higher when the IPT genes were expressed. In fact, these two samples clustered separately from the control sample. This suggests that the red-light induction of the IPT genes results in the differential expression of CK-responsive genes. Additionally, the external addition of cytokinins alters the expression of CK-responsive genes in a similar manner, regardless of whether this is done in the presence of red light or in darkness.

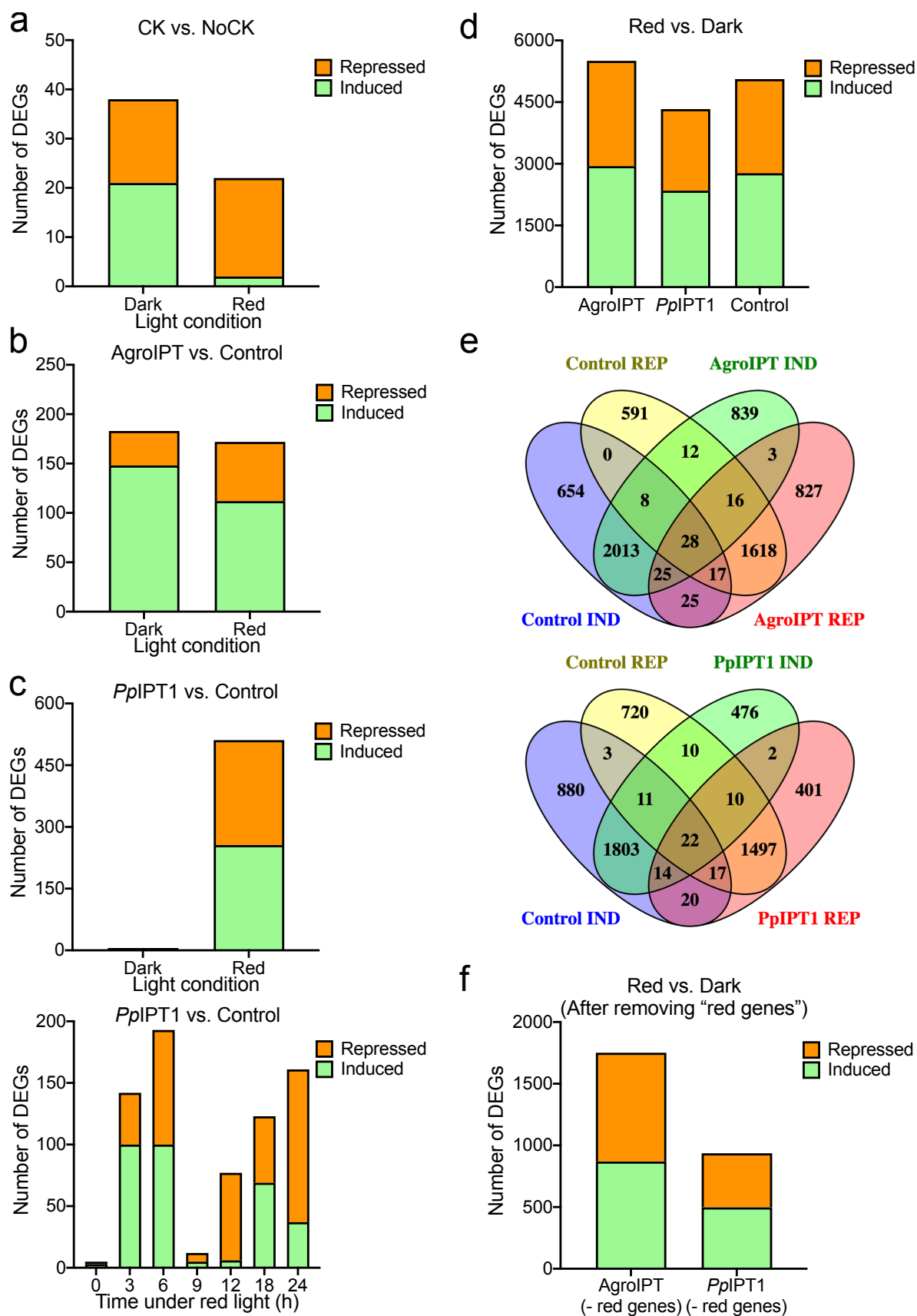


Figure 6. Differentially expressed genes in the analysed conditions. (a) Number of induced and repressed genes in control samples treated with cytokinin versus untreated samples under dark and red light conditions. (b) Number of induced and repressed genes in PULSE-AgrolIPT versus PULSE-FLuc (control) samples under dark or red light. (c) Number of induced and repressed genes in PULSE-PpIPT1

versus PULSE-FLuc (control) samples aggregated across all red light time points (top) or separated by time points (bottom). **(d)** Number of induced and repressed genes in red versus dark conditions for the three different samples: PULSE-AgroIPT, PULSE-*Pp*IPT1, and PULSE-FLuc (control). **(e)** Venn diagrams of DEGs in (d), showing the overlap of DEGs in the IPT and the control samples. Overlapping genes are considered as red-light affected genes, “red genes”. **(f)** Number of induced and repressed genes in red versus dark conditions for PULSE-IPT samples after removal of “red genes”. Filtering parameter for DEG: Log₂FC greater or lower than 0.5, adjusted p-value < 0.05. PULSE-FLuc samples, n=3; PULSE-AgroIPT and PULSE-*Pp*IPT1 samples n=4. Unless specified, the DEGs from different red light time points are plotted together. DEG, differentially expressed genes; IPT, Isopentenyl transferase; Agro, *A. tumefaciens*; *Pp*, *P. patens*; CK, cytokinin; REP, repressed; IND, induced.

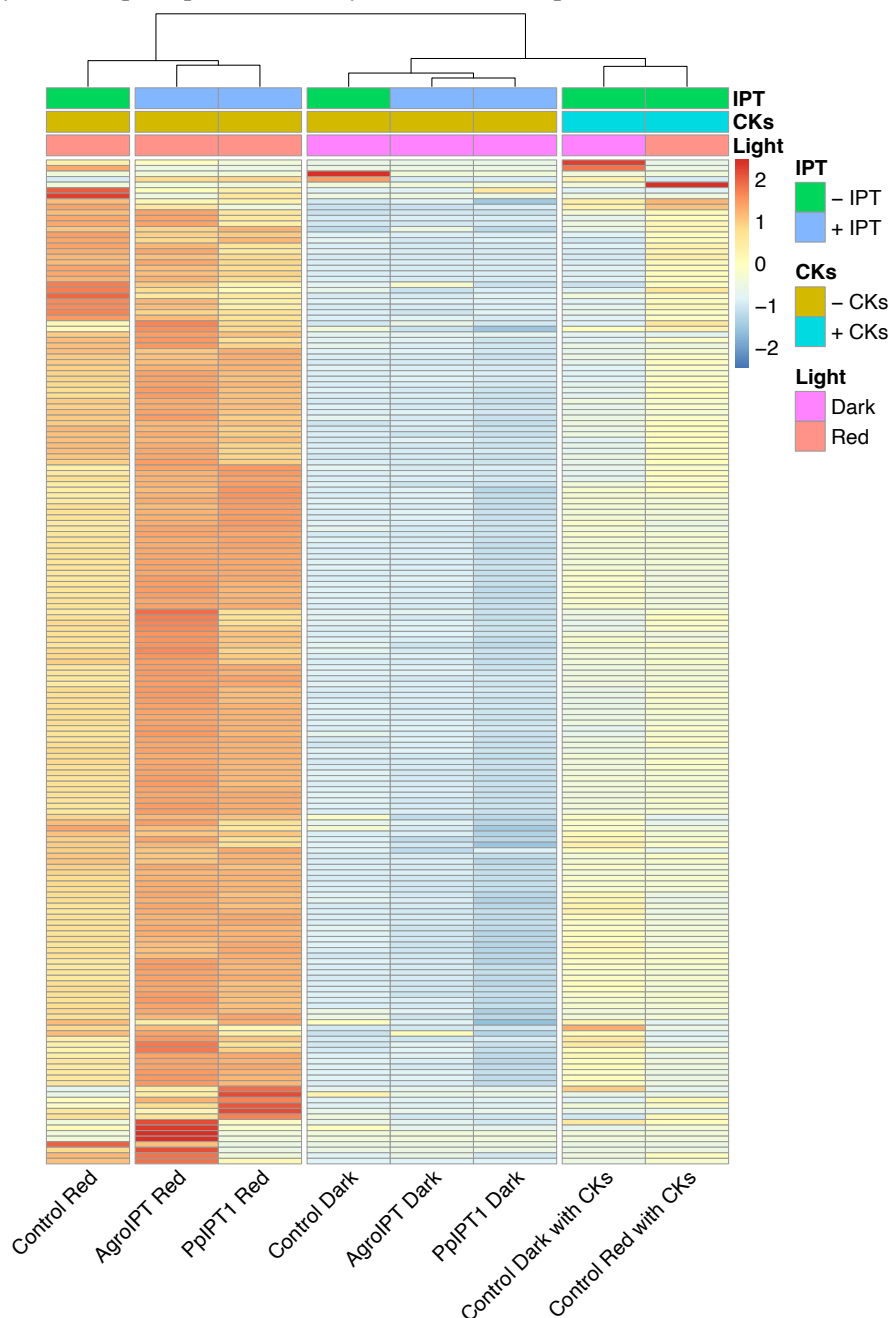


Figure 7. Expression of *P. patens* orthologs of *A. thaliana* CK-responsive genes. Heatmap and hierarchical clustering based on the gene expression of *P. patens* orthologs of CK-responsive genes in *A. thaliana* (Bhargava et al., 2013) under different analysed conditions: PULSE-FLuc (referred to as Control), PULSE-AgroIPT, and PULSE-*Pp*IPT1 in both red light and darkness, as well as Control

samples with and without the addition of 1 μ M 2iP in the presence of red light or in darkness. Red color indicates higher expression levels, while blue color indicates lower expression levels. IPT, Isopentenyl transferase; Agro, *A. tumefaciens*; *Pp*, *P. patens*; CK, cytokinin.

Additional tests were performed to analyse the enrichment of cytokinin-related genes differentially expressed under the different conditions. Genes differentially expressed in the sample treated with CK in darkness, compared to the untreated sample, were considered cytokinin-related genes. The enrichment on these cytokinin-related genes in the different conditions was analysed using Fisher's exact tests. These tests suggest that *Pp*IPT1 might be more efficient than AgroIPT in inducing CK-related responses in *P. patens* protoplasts, as there is a higher enrichment of CK-related genes in the *Pp*IPT1 sample under red light compared to the control sample, than in the AgroIPT sample (Fig. S3). However, further analyses are required to confirm these observations.

Discussion

The moss *P. patens* is a model organism for exploring different aspects of plant development, physiology, and evolution. The study of such biological processes often requires precise manipulation, in both space and time, of biochemical reactions or signaling pathways. While chemical inducible systems provide a degree of control, they are limited in spatiotemporal resolution and may induce toxic effects (Kubo et al., 2013; Saidi et al., 2005; Zeidler et al., 1996). Optogenetics, conversely, offer the possibility of controlling gene expression with light, thereby providing high spatiotemporal and quantitative control, while minimizing toxicity and invasiveness.

Until now, only the Red_{on} optogenetic system had been implemented in *P. patens*. However, this opto-tool is not suitable for plants under normal growth conditions (cycles of white light and darkness) because it responds to white light as well. In 2020, this issue was addressed in *A. thaliana* and *N. benthamiana* with the introduction of the PULSE system. PULSE combines the Red_{on} system with a B_{Off} system, allowing the induction of gene expression only under red light, while under dark and white light the system is in its OFF state. Therefore, our objective was to implement this advanced optogenetic tool in *P. patens* to bridge the gap for a system that allows plants to grow under normal light conditions while offering precise control over gene expression, thereby broadening the opportunities for researchers to conduct sophisticated and finely tuned experiments.

Here, we established and characterized the PULSE optogenetic system in *P. patens* protoplasts. We performed a comparative analysis of its functionality under different light conditions in parallel to the Red_{on} system. Notably, the induction of gene expression under red light compared to white light with PULSE was found to be 20.7 times higher than with the Red_{on} system. Moreover, we observed lower leakiness and higher induction rates when all PULSE components were encoded in a single plasmid instead of in different plasmids that were cotransformed. Additionally, our investigation into the light-dose response revealed that increasing the light intensity reduces the leakiness, likely due to more repression by the B_{Off} system. The On-Off kinetics analysis demonstrated the high reversibility of the PULSE system, which allows high temporal control.

Another powerful synthetic biology tool for fundamental research and biotechnological applications is the CRISPR activation (CRISPRa) technology. CRISPRa is used to enhance gene expression by employing a catalytically inactive Cas9, known as “dead” Cas9 (dCas9), fused to an activation domain. dCas9-AD can be targeted to any specific endogenous or synthetic promoter using specific guide RNAs. While CRISPR-Cas9 is increasingly utilized in

P. patens for generating knockouts and enhancing homologous recombination efficiency (Mallett et al., 2019), the use of dCas9 in *P. patens* has been reported only once for CRISPR interference (CRISPRi) applications, which involve inhibiting promoter activity by targeting dCas9 to it and thereby repressing gene expression. However, the observed dynamic range was low, indicating the need for further optimization in this species (Khan et al., 2024). Here, we demonstrated, for the first time, the application of CRISPRa technology in *P. patens*, thereby opening up possibilities for overexpressing endogenous genes. Furthermore, we combined the CRISPRa technology with the PULSE system, enabling light-controlled CRISPRa activity and thereby allowing precise modulation of endogenous promoter activity using light.

We also assessed the potential applications of the PULSE system in elucidating plant signaling pathways by controlling IPT genes involved in the cytokinin biosynthesis pathway. Our goal was to showcase the versatility of PULSE for a wide range of studies, including investigations into plant signaling and metabolism. In this case, as a proof of principle, we have focused on the regulation of cytokinins, but it could also be used and applied to other signaling pathways. Beyond its role as a model organism for fundamental research, *P. patens* has also served as a chassis for recombinant proteins production. We believe that the establishment of CRISPRa and the PULSE system in *P. patens* protoplasts will not only facilitate the study of signaling and cellular processes in *P. patens* with high spatiotemporal resolution, but could also enable the controlled production of biopharmaceuticals. In conclusion, the establishment of the PULSE system in *P. patens* protoplasts offers a valuable tool for investigating plant signaling pathways and cellular processes with unprecedented precision and control.

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Supplementary information

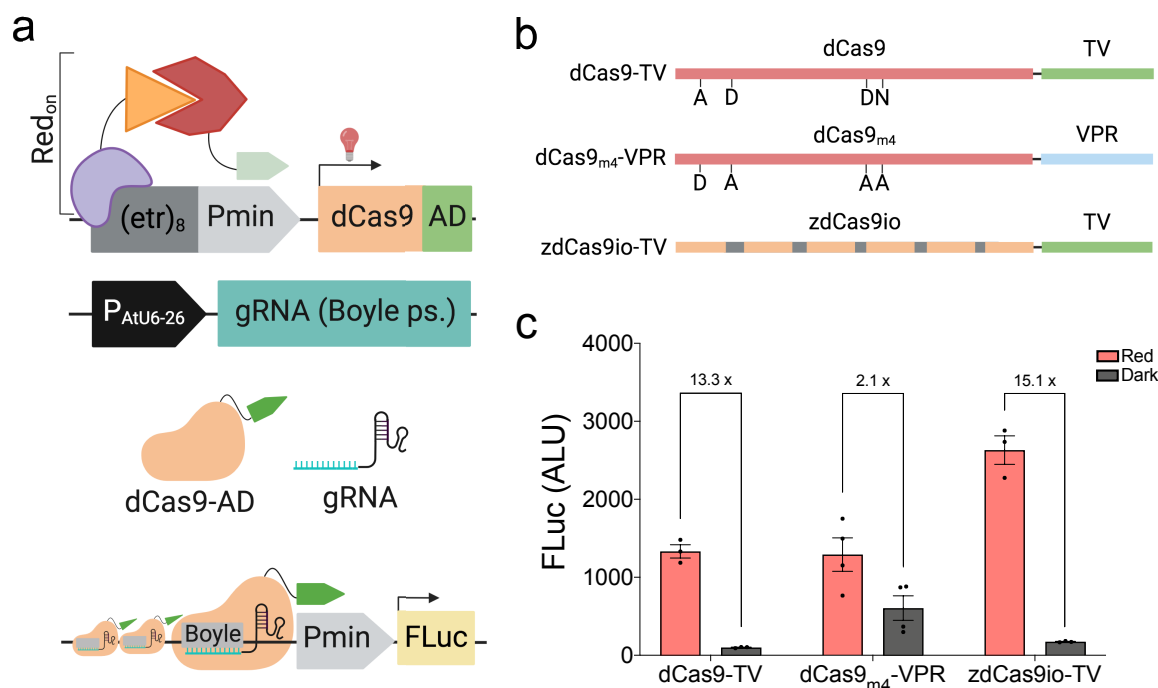


Figure S1. Comparison of different dCas9-AD variants activities in *P. patens* protoplasts. (a) Depiction of the mechanism of the Red_{on}-controlled expression of different dCas9 fused to a transactivation domain. Red light induces the expression of the different variants, which target a synthetic promoter consisting of three repetitions of the Boyle protospacer and the hCMV minimal promoter. (b) Schematic illustration of the dCas9-AD fusion variants sequence: dCas9-TV (pROF449), dCas9_{m4}-VPR (pKT634), and zdCas9io-TV (pUU617). Human or maize codon-optimized dCas9 sequences are represented in red or yellow, respectively. Introns are represented in grey. The TV and VPR transactivation domains are depicted in green or blue, respectively. (c) The plasmids of the different dCas9-AD variants were cotransformed with the Red_{on} system (pMZ827 and pMZ828), gRNA (pOD414), and target (pOD415) plasmids. *P. patens* protoplasts were incubated for 24 h under red light (15 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or darkness. The activity of dCas9-AD variants was compared by measuring Firefly luminescence activity, expressed as ALU. Mean ALU and SEM are plotted (n=4). Data are representative of three independent experiments. FLuc, Firefly luciferase; Pmin, minimal promoter; dCas9, nuclease-deficient Cas9; TV, TV transactivation domain; VPR, VPR transactivation domain; P_{AtU6-26}, *A. thaliana* U6-26 promoter; gRNA, guide RNA; Boyle ps, Boyle protospacer; AD, activation domain.

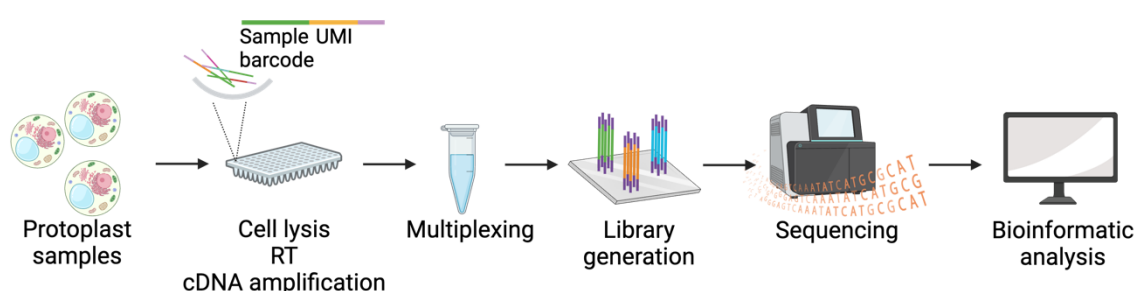


Figure S2. Multiplexed RNA-seq workflow. Following sample collection, individual cell suspensions are lysed in a 96-well plate. mRNA is labelled with specific barcoded oligos within the same plate,

followed by reverse transcription and PCR amplification of the cDNA. The amplified cDNA is pooled together and subjected to library preparation for next-generation sequencing (NGS). Bioinformatic analysis employs the barcoding system to assign each molecule to its respective well. RT, reverse transcription.

Comparison		CK vs. NoCK (Red)	Control Red vs. Dark	AgroIPT Red vs. Dark (- red genes)	Pp IPT1 Red vs. Dark (- red genes)	AgroIPT vs. Control (Red)	Pp IPT1 vs. Control (Red)
Induced genes in CK-dark vs. Induced genes	CK-Dark induced genes	21	21	11	10	21	21
	Induced genes	2	1937	629	346	112	253
	Expected common genes	0,003	3,169	0,712	0,352	0,183	0,414
	Identified common genes	0	10	1	0	8	9
	p-value	1	0	0,521	1	4,87E-12	9,35E-11
	Enrichment	0	3,155	1,405	0	43,616	21,735
Repressed genes in CK-dark vs. Repressed genes	CK-Dark repressed genes	17	17	8	11	17	17
	Repressed genes	20	2290	875	435	41	182
	Expected common genes	0,027	3,033	0,720	0,486	0,054	0,241
	Identified common genes	0	15	1	1	0	7
	p-value	1	5,38E-10	0,530	0,392	1	1,78E-09
	Enrichment	0	4,945	1,389	2,057	0	29,029
Induced genes in CK-dark vs. Repressed genes	CK-Dark induced genes	21	21	11	10	21	21
	Repressed genes	20	2290	875	435	41	182
	Expected common genes	0,033	3,747	0,990	0,442	0,067	0,298
	Identified common genes	2	13	2	3	0	2
	p-value	0	8,74E-06	0,260	0,008	1	0,035
	Enrichment	60,919	3,470	2,020	6,788	0	6,714
Repressed genes in CK-dark vs. Induced genes	CK-Dark repressed genes	17	17	8	11	17	17
	Induced genes	2	1937	629	346	112	253
	Expected common genes	0,003	2,566	0,518	0,387	0,148	0,335
	Identified common genes	0	1	1	2	0	2
	p-value	1	0,881	0,581	0,085	1	0,030
	Enrichment	0	0,390	1,932	5,172	0	5,967

Enrichment 0-1 1-2 2-5 >5

Figure S3. Enrichment in CK-related genes in the different samples. Analysis of enrichment in cytokinin-related genes across various conditions. It shows the overlap between genes induced or repressed by CK treatment in darkness and those induced or repressed in other comparisons. Specifically, the enrichment of genes induced or repressed in the CK vs. NoCK (dark) comparison was calculated for the different comparisons (CK vs. NoCK in red, Control in red vs. dark, AgroIPT or PpIPT1 in red vs. dark excluding the “red genes”, and AgroIPT or PpIPT1 vs. control upon red light illumination). Fisher’s Exact Test was conducted on the DEGs (Log₂FC greater or lower than 0.5, adjusted p-value < 0.05). The DEGs from the different CK treatment or red light time points were combined for these calculations. CK, cytokinin; IPT, Isopentenyl transferase; Agro, *A. tumefaciens*; Pp, *P. patens*.

Table S1. Plasmids design and used. pUPD2, pDGB and GB plasmids were acquired from the Golden Braid collection (Sarrion-Perdigones et al., 2013; <https://gbccloning.upv.es>).

ID	Description and cloning procedure	Insert	Backbone	Reference
pOD343	ppIPT1 (Pos. B3-B5) Insert was amplified from B05 with oligos oOD550 and oOD551. BsmBI restriction-ligation reaction (GoldenBraid).	<i>P. patens</i> IPT1	pUPD2	This work

pOD344	AgroIPT (Pos. B3-B5) Insert was amplified from A83 with oligos oOD552, oOD553, oOD554 and oOD555. BsmBI restriction-ligation reaction (GoldenBraid).	Agrobacterium IPT	pUPD2	This work
pOD356	(etr)₈-(C120)₅-PhCMVmin::ppIPT1::Tnos Insert was amplified from pOD357 with oligos oOD629 and oOD630. Backbone was digested with BsrGI and SacII. Both fragments were assembled using AQUA cloning.	(etr) ₈ -(C120) ₅ -PCMVmin	pOD358	This work
pOD357	(etr)₈-(C120)₅-PhCMVmin::AgroIPT::Tnos BsaI restriction-ligation reaction (GoldenBraid).	pROF053, pROF054, pROF059, pOD344, GB0037	pDGB 3alpha1	This work
pOD358	P35S::ppIPT1::Tnos BsaI restriction-ligation reaction (GoldenBraid).	GB0030, pOD343, GB0037	pDGB 3alpha1	This work
pOD411	Boyleprotospacer-PhCMVmin::FLUC::TSV40 Insert was amplified from pUU024 with oligos oOD648 and oOD649. Backbone was digested with BsmBI and SbfI. Both fragments were assembled using AQUA cloning.	Boyle protospacer	pROF021	This work
pOD414	PAtU6-26::gRNA(Boyleprotospacer)-sgRNA Backbone was amplified with the oligo pairs oOD654 and oOD655, and oOD656 and oOD657. Insert was added in the oligos. Both fragments were assembled using Gibson cloning.	gRNA (Boyle protospacer)	GB1221	This work
pOD415	(Boyleprotospacer)₃-PhCMVmin::FLUC::TSV40 Insert was amplified from pUU024 with oligos oOD662 and oOD663. Backbone was digested with AatII and SbfI. Both fragments were assembled using AQUA cloning.	(Boyle protospacer) ₃	pOD411	This work
pOD417	(etr)₈-(C120)₅-PhCMVmin::dCas9m4-VPR::T35S Insert was amplified from pKT634 with oligos oOD652 and oOD653. Backbone was amplified with oligos oOD664 and oOD665. Both fragments were assembled using Gibson cloning.	dCas9m4-VPR	pROF449	This work
pMZ824	P35S::E-VP16-NLS::TpA			Müller et al., 2014
pMZ827	P35S::E-PIF6(1-100)-NLS::TpA			Müller et al., 2014
pMZ828	P35S::PhyB(1-650)-VP16-NLS::TpA			Müller et al., 2014
pROF053	(etr)₈ (Pos. A1)			Ochoa-Fernandez et al., 2020
pROF054	(C120)₅ (Pos. A2)			Ochoa-Fernandez et al., 2020

pROF059	PhCMVmin (Pos. A3-B2)			Ochoa-Fernandez (unpublished)
pROF021	(etr)₈-(C120)₅-PhCMVmin::FLUC::TSV40			Ochoa-Fernandez et al., 2020
pROF051	P35S::SRDX-NLS-EL222::Tnos			Ochoa-Fernandez et al., 2020
pROF104	PAtUbq10::E-PIF-NLS::Tnos			Ochoa-Fernandez et al., 2020
pROF105	PAtUbq10::PhyB-VP16-NLS::Tnos			Ochoa-Fernandez et al., 2020
pROF117	PAtUbq10::EL222-SDRX::Tnos			Ochoa-Fernandez et al., 2020
pROF345	Tnos::KanR::Pnos Tnos::PIF6-E::PAtUbq10 Tnos::Cerulean-NLS::PAtUbq10(short) Tnos::VP16-PhyB::PAtUbq10 Tnos::EL222-SDRX::PAtUbq10 T35S::FLuc::PhCMVmin-(C120)₅-etr₈			Ochoa-Fernandez (unpublished)
pROF449	(etr)₈-(C120)₅-PhCMVmin::dCas9-TV::T35S			Ochoa-Fernandez et al., 2020
pKT634	(etr)₈-PhCMVmin::dCas9m4-VPR::TSV40			Tang (unpublished)
pUU024	Boyleprotospacer-PhCMVmin::SEAP::TpA PSV40::FLUC::TSV40			Urquiza (unpublished)
A83	pGK52-AgrobacteriumIPT			Yoan Coudert
B05	pDON-R 221-IPT1 stop			Yoan Coudert
GB0030	P35S (Pos. A1-B2)			Sarrion-Perdigones <i>et al.</i> , 2013
GB0037	Tnos (Pos. B6-C1)			Sarrion-Perdigones <i>et al.</i> , 2013
GB1221	PAtU6-26::gRNA(Psidfr)-sgRNA			Selma <i>et al.</i> , 2019
pRSET	PT7-driven bacterial expression vector			Novagen

Table S2. Oligonucleotides used for cloning.

Name	Sequence (5' → 3')
oOD550	GCGCCGTCTCGCTCGAATGTACATGTTGACGCACCG
oOD551	GCGCCGTCTCGCTCAAAGCTTACATAGAGGTCGTCAAGGA
oOD552	GCGCCGTCTCGCTCGAATGGATCTGCGTCTAATTTTCG
oOD553	GCGCCGTCTCGAAGTCTCCTCGTGTGCTAAC
oOD554	GCGCCGTCTCGACTTTCATGAACGTGGCCAAG

oOD555	GCGCCGTCTCGCTCAAAGCCTAATACATTCCGAATGGATGAC
oOD629	AACATAGTATCGACGGAGCCGATTTTGAACCGCGGTGATCACAGGCAGCAACG
oOD630	ACAGACAGGCCTGAGCACGGTGCCTCAACATGTACATTACCGGTAGGCTGGATCG
oOD648	AGTGCCACCTGACGTCGTCGACGATCGACCTGCAGGGACGCATAAAGATGAGACGC
oOD649	TATGGAGGTCAAAACAGCGTGGATGGCGTCTCCAGGCGATCTGACGGTTCACTAAAC G
oOD652	ATAGAAGACACCGGGACCGATCCAGCCTACCGGTAATGGACAAGAAGTACTCC
oOD653	GATCGGGATCGAAGATCCATTCCAAGCCTAGTTACACAAGTACAATGCAGTG
oOD654	CCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCC
oOD655	CCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCGTCTCATCTTTATGCGTCCACTAC TTCGACTCTAGCTG
oOD656	GTTTTAGAGCTAGAAATAGCAAG
oOD657	CAGGATCTTGCCATCCTATG
oOD662	CACATTTCCCCGAAAAGTGCCACCTGACGTCGTGACGCATAAAGATGAGACGCTGGC GACGAGACGCATAAAGATGAGAC
oOD663	GCGACCAGCGTCTCATCTTTATGCGTCCCTGCACCAGCGTCTCATCTTTATGCGTCGGT CGACCAGCGTCTCATCTTTAT
oOD664	TACCGGTAGGCTGGATCG
oOD665	TAACTAGGCTTGGAATGGATCTTCGATCCCGATCCGGCCATGCTAGAGTCCG

Table S3. Oligonucleotides used for RT-qPCR.

Name	Description	Sequence (5' → 3')	Reference
oOD590	<i>P. patens</i> ubiquitin Fwd	AGTGGATGACTCCGGTAAG	Thelander et al., 2022
oOD591	<i>P. patens</i> ubiquitin Rev	CAGTAGTGTCGGTCGAAATG	Thelander et al., 2022
oOD592	Fluc Fwd	GGGCGTTAATCAAAGAGG	This work
oOD593	Fluc Rev	GTCTTCGTCCCAGTAAGC	This work

4 CHAPTER II: Implementing optogenetic tools in *C. reinhardtii*

One of the main goals of this thesis is the implementation of optogenetic tools in *C. reinhardtii* to expand the experimental approaches for the precise control and study of cellular processes in this model alga.

Part of this work was done in the frame of the project A13 of the DFG collaborative research center SFB1208 “Optogenetic membrane manipulation of protein targeting to mitochondria and plastids” in collaboration with the group of Dr. Sven Gould from the Molecular Evolution Institute. The main aim of this project was to develop optogenetic tools to study the targeting of proteins to mitochondria and plastids. Mitochondria and plastids are two organelles of endosymbiotic origin present in plants and algae. Due to their reduced genome, they need to import thousands of nucleus-encoded proteins from the cytosol. Both organelles are surrounded by two membranes and have protein import machineries responsible of importing proteins with high specificity (Schleiff & Becker, 2011). However, since dual targeting has been evidenced in *Arabidopsis*, this has arisen questions on how dual targeted proteins can be recognized without compromising the high specificity of protein import into these eukaryotic cell compartments (Chris Carrie et al., 2009; Christopher Carrie & Small, 2013).

It is known that mitochondrial protein import depends on the electrochemical gradient present in the inner mitochondrial membrane (Geissler et al., 2000; Martin et al., 1991). Moreover, the charge of the N-terminal targeting signal (NTS) ensures specific import by interacting with proteins in the membranes of chloroplasts and mitochondria, and with the electrochemical gradient at the inner mitochondrial membrane and the thylakoid membrane (Neupert, 2015).

Chlamydomonas reinhardtii represents an ideal model to study the role that mitochondrial membrane potential plays in organelle protein import because it can switch between aerobic and anaerobic metabolism, where the mitochondrial membrane potential is lost. However, it is not possible to study exclusively the role of the mitochondrial membrane potential without modifying the metabolic status of the cell.

In this sense, optogenetic tools can be used to interrogate, with a temporal and quantitative resolution, the elements involved in organelle membrane identity, by controlling the mitochondrial membrane potential and the expression of proteins involved in this process with light, decoupled from the physiological state of the cell.

Therefore, in this work we aimed to develop:

(A) Optogenetic switches for gene expression control: the generated opto-switches would provide the means to precisely manipulate gene expression levels with high controllability,

including the expression of different components involved in protein targeting to organelles facilitating the analysis of their roles. The work related to the generation of “**Optogenetic switches for gene expression control in *C. reinhardtii***” is represented in the manuscript 2 and the subchapter “Optogenetic switches for transcriptional activation in *C. reinhardtii*”:

- Manuscript 2 – A toolbox for implementing optogenetic regulation of gene expression in *C. reinhardtii*. This article summarizes the work done to generate an optogenetic toolbox for *C. reinhardtii*, that contains all the components required for implementing optogenetic tools (promoters, reporters, transactivation domains, etc.). It also details the establishment of the first optogenetic gene-repression system in *C. reinhardtii*, the *CrBOff* system, which is controlled by blue light. The manuscript is in its final stages of preparation and will be submitted for publication in Q3 2024.
- Optogenetic switches for transcriptional activation in *C. reinhardtii*: *CrBlueOn* and *CrRedOn* systems. In this section the efforts to generate an optogenetic system for transcriptional activation are summarized. It explores the creation of optogenetic switches activated by red (*CrRedOn*) or blue light (*CrBlueOn*).

(B) Optogenetically controlled mitochondria: by integrating opsins into the inner mitochondrial membrane of *C. reinhardtii*, it would become feasible to precisely manipulate mitochondrial membrane potential. This tool would enable the investigation of the impact of mitochondrial membrane potential in protein targeting decoupled from the physiological state of the cell. The work focused on the design of “**Optogenetically controlled mitochondria**”

is summarized in the draft manuscript 3:

- Manuscript 3 – Towards optogenetic control of mitochondrial membrane potential ($\Delta\psi_m$) in *C. reinhardtii*. In this chapter, we describe the establishment of a method to monitor changes in $\Delta\psi_m$ in isolated mitochondria from *C. reinhardtii*. Additionally, the strategy to modify $\Delta\psi_m$ with light in *C. reinhardtii*, by the targeting of opsins into the inner mitochondrial membrane, is presented, and the mitochondrial targeting of two different opsins is shown. However, final experiments to confirm the activity of these targeted opsins and their ability to modify $\Delta\psi_m$ are still pending for the finalization of the manuscript.

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4.1 Manuscript 2: A toolbox for implementing optogenetic regulation of gene expression in *C. reinhardtii*

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Author contribution:

O.D.-R., designed, performed and supervised experiments, analysed the data, and wrote the manuscript. S.F. and L.-L.D. performed experiments related to the implementation and characterization of the *CrBOff* system, and its use for controlling canavanine resistance, respectively. K.T. contributed to the data analysis of the reporter characterization experiments. P.K.R. and S.B.G. contributed to the design, data analysis, and supervision of the bioreactor experiment. D.P. designed and supervised the canavanine-related experiments, M.D.Z. designed, conceptualized and supervised the project, analysed the data and wrote the manuscript.

Abstract

The microalga *Chlamydomonas reinhardtii* is a model organism for fundamental research and biotechnological innovation. Despite advancements in heterologous expression efficiency, there remains a lack of tools for precisely controlling molecular and cellular processes, including the control of gene expression. Conventional inducible systems based on chemicals or heat shock do not offer high temporal and quantitative resolution, and are highly invasive. To address this, we developed an optogenetic toolbox customized for *C. reinhardtii* and proposed a standardized workflow for testing and implementing light inducible molecular switches in this model alga. Furthermore, we quantitatively characterized various promoters and reporters, and delved into the importance of promoter selection and transcriptional unit orientation within plasmids for optimal optogenetic tool and transgene expression efficiency. Additionally, we engineered the first blue light gene-repression system for *C. reinhardtii*, *CrBOff*, based on the EL222 photoreceptor, enabling precise gene expression control without the addition of chemicals. Moreover, we introduced a novel resistance marker based on canavanine, and combined it with the *CrBOff* system for light-regulated conditional resistance to canavanine. Overall, our study lays the groundwork for the widespread implementation of optogenetics in *C. reinhardtii*, providing valuable insights and tools for future research and biotechnological applications.

Introduction

The microalga *Chlamydomonas reinhardtii* is a model organism that is widely used for fundamental research studies and biotechnology applications (Kuech, Breuer and Popescu, 2023; Salomé & Merchant, 2019). Despite the challenges associated with genetic manipulation and foreign gene expression in this single-celled alga, researchers have significantly improved the efficiency of heterologous gene expression. These advancements include sequencing its genome, establishing methods for nuclear, mitochondrial, and plastid transformation, creating lines with enhanced transgene expression efficiency, developing the Intronserter online tool for the design of optimized intron-containing transgenes, and even generating a standardised Modular Cloning Kit (MoClo) and a genome-wide mutant library (CLiP; Boynton & Gillham, 1993; Crozet et al., 2018; Jaeger et al., 2019; Kindle, 1990; Li et al., 2015; Merchant et al., 2007; Neupert et al., 2009; Remacle et al., 2006).

However, in molecular biology and biotechnology, there is often a need for inducible, controllable expression of foreign genes. This necessity arises, for example, when expressing genes whose continuous expression results in detrimental effects on the host, when knockouts are lethal, or when precise timing or levels of gene expression need to be tightly regulated (Corrado & Karali, 2009; Kallunki et al., 2019). Unfortunately, some of the inducible systems that traditionally have been used in *C. reinhardtii* to regulate transgene expression, mainly based on chemicals or heat shock, can also have negative effects on algae growth and metabolism (Beltran-Aguilar et al., 2019; Ferrante et al., 2008; Kim et al., 2020; Koblenz & Lechtreck, 2005; Lee et al., 2018; Ohresser et al., 1997). Furthermore, the medium replacement, sometimes required to stop the induction, can be costly and arduous when cultivating microalga in large ponds or photobioreactors. Optogenetics offers the possibility to control gene expression with light in a fast, cheap and reversible way, with spatiotemporal and quantitative resolution and minimizing the invasiveness and side effects of chemical-inducible systems (Omelina et al., 2022; Shemesh et al., 2017). Light-inducible gene expression systems have been widely applied in mammalian, bacteria, yeasts and plants allowing a highly regulated gene expression induction (Christie & Zurbriggen, 2021; Müller et al., 2015; Ohlendorf & Möglich, 2022; Pérez et al., 2022). However, to the date, only one optogenetic system, based on *Arabidopsis thaliana* cryptochrome 2, has been engineered for *C. reinhardtii* (Wang et al., 2017), to induce H₂ production in response to blue light.

Here, we implemented a blue light optogenetic system for the repression of gene expression in *C. reinhardtii* (CrBOff). The BOff system, based on the EL222 photoreceptor from

Erythrobacter litorialis, was previously engineered for its use in mammalian cells and *A. thaliana* (Baaske et al., 2018; Ochoa-Fernandez et al., 2020). The heterotrophic metabolism of the microalga *C. reinhardtii* allows its cultivation in the dark, where the system is inactive, whereas blue light illumination activates the light switch, repressing gene expression. The presence of blue light in white light would allow the use under normal growth conditions, i.e. white light as activation light, which is more cost-effective for industrial purposes.

We generated *C. reinhardtii* CrBOff stable transgenic lines, in which repression rates of up to 8.1-fold of a gene downstream of the strong constitutive promoter β 2-tubulin were achieved. Furthermore, we demonstrated that the cultivation of these lines can be upscaled in photobioreactors without compromising the functionality of the CrBOff system. Additionally, we implemented a new selection marker in *C. reinhardtii* utilizing the antibiotic canavanine and employed the CrBOff system to regulate resistance to canavanine with light, thereby showing the potential application of the CrBOff system.

Prior to the establishment of this optogenetic system, we generated an optogenetic toolbox with dozens of components codon-optimized for *C. reinhardtii*, which are needed for the implementation of a variety of optogenetic tools in this alga. This set includes photoreceptors, DNA binding domains, repressor and transactivation domains, and reporters, among others. Some of these components, such as reporters and promoters, were quantitatively characterized (performance, function, applicability). We found that certain *C. reinhardtii* constitutive promoters are light-sensitive. We identified the β 2-tubulin and Psad promoters as the less light-sensitive ones, therefore they represent the most adequate option for engineering optogenetic tools. Furthermore, we analysed the effect in gene expression of the relative orientation of two transcriptional units (promoter-gene-terminator) within a plasmid, concluding that convergent ($\rightarrow\leftarrow$) orientation increases the expression efficiency of both genes. Likewise, after comparing the effect of darkness, white, blue and red light on *C. reinhardtii* growth and photosynthesis, we selected the photosynthetically active blue and red lights for optogenetic experiments, since there were no major differential effects among them. The generation of this opto-toolbox opens up the implementation and design of customized optogenetic systems to control gene expression in a faster way. In this work, a blue-light repression system was engineered, enabling the control of gene expression with light in *C. reinhardtii*, as demonstrated through the light-mediated regulation of *AOC5*, a gene involved in conferring resistance to the novel selection marker in *C. reinhardtii*, canavanine (Ma, Kim et al., 2013).

Materials and methods

Plasmid generation

A detailed description of plasmids and oligonucleotides designed, constructed and used in the frame of this study are listed in tables S1 and S2. Heterologous genes were codon-optimized for *C. reinhardtii*: gene design was done with Intronserter (Jaeger et al., 2019), synthesis of the codon optimized genes was performed by GeneArt Thermo Fisher Scientific and introns were integrated using Golden Braid cloning (<https://gbcloning.upv.es>). DNA fragments of interest were amplified according to table S1 using polymerase chain reaction (PCR), with oligonucleotides provided by Sigma Aldrich or Integrated DNA Technologies, using Q5 High-Fidelity DNA Polymerase with GC enhancer solution (New England Biolabs, NEB). 1 % agarose gel was used to separate DNA fragments. For gel extractions, NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used. Plasmids were assembled using Golden Braid, Gibson assembly or AQUA cloning (Beyer et al., 2015; Gibson et al., 2009; Sarrion-Perdigones et al., 2011, 2013) and transformed into chemically competent *Escherichia coli* strain 10-beta (New England Biolabs, NEB). Plasmids were isolated from *E. coli* using Wizard Plus SV Miniprep DNA Purification Systems (Promega) and NucleoBond Xtra Midi kit (Macherey-Nagel). Newly constructed plasmids were verified by restriction enzyme (New England Biolabs, NEB) digestion and DNA sequencing (Microsynth).

E. coli and *C. reinhardtii* strains, transformation and growth conditions

E. coli strain 10-beta (New England Biolabs, NEB) was grown at 37°C in LB broth supplemented with agar (4 % m/V) and kanamycin (25 µg/mL), ampicillin (100 µg/mL), chloramphenicol (25 µg/mL) or spectinomycin (100 µg/mL) when required. Plasmid were transformed into chemically competent cells by heat shock as described elsewhere (Froger & Hall, 2007).

C. reinhardtii strains (UVM4, CC5385, CC4533-AOC5; <https://www.chlamycollection.org>; Li et al., 2015; Neupert et al., 2009) were routinely grown in Tris-acetate-phosphate (TAP) medium (Gorman & Levine, 1965) supplemented with agar (1.2 % m/v), ampicillin (0.05 mg/mL) and, when required, hygromycin B (10 µg/mL) or paromomycin (10 µg/mL). Liquid cultures were grown in TAP with ampicillin (0.05 mg/mL) at 160 rpm under 50 µmol photons m⁻² s⁻¹ long day 16:8 h light cycle, at 22 °C. For UVM4 growth and photosynthesis characterization experiments, cells were also grown in Sueoka's high salt (HS) media (Sueoka, 1960).

For *Chlamydomonas* transformation, the glass bead method was followed (Kindle, 1990; Lauersen et al., 2015). A TAP culture of $1-3 \times 10^6$ cells/mL was set to a final concentration of 3×10^8 cells/mL. 1×10^8 cells were mixed with 2 μ g of linearized plasmid and 300 mg of 0.5 mm glass beads, prior to 15 s vortexing at high speed. Transformations were spread on TAP-agar plates with appropriate antibiotics. 20 h incubation in darkness was followed by 5-7 days of constant white light illumination ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Luminescence analysis of *C. reinhardtii* experiments

C. reinhardtii clones were transferred into flasks or 6-, 12- or 96-well plates containing TAP medium and grown for 3-5 days under white light ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). For FLuc, GLuc, RLuc and NLuc routinely activities determination, 80 μ l of *Chlamydomonas* culture were transferred into a 96-well white flat-bottom plate (Costar). For FLuc quantification, 20 μ l of FLuc substrate were added (0.47 mM d-luciferin (Biosynth AG), 20 mM tricine, 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM $\text{EDTA} \cdot 2\text{H}_2\text{O}$, 33.3 mM dithiothreitol, 0.52 mM adenosine 5'-triphosphate, 0.27 mM acetyl-coenzyme A, 5 mM NaOH, 264 μM $\text{MgCO}_3 \cdot 5\text{H}_2\text{O}$, in H_2O , pH8). For GLuc, RLuc and NLuc analysis, 20 μ l coelenterazine were added (472 mM stock solution in methanol, diluted 1:15 in PBS prior to use; Carl Roth, Karlsruhe, Germany, no. 4094.4). Luminescence was measured for 20 min with an integration time of 0.1 s, using Centro XS3 LB960 Microplate Luminometer (Berthold Technologies).

For the reporter secretion characterization experiments, cells were centrifuged at 3,000 rpm for 5 min and supernatant was collected. To determine luminescence activities of cell lysates, cell pellets were resuspended in RIPA (150 mM NaCl, 20 mM Tris pH 7.5, 0.1 % NP40, 5 mM EDTA) or Lysis Buffer 1 (25 mM Tris/HCl pH 7.8, 1 % triton X-100, 15 mM MgSO_4 , 4 mM EGTA) supplemented with 1 tablet of Protease Inhibitor Cocktail (cOmplete, Mini EDTA-free Protease Inhibitor Cocktail, Roche) and, when indicated, with 1 mM DTT. The solution was stirred for 15 min at 700 rpm prior to measurement. Supernatants and cell lysates were measured in the same way as the whole culture.

Light experiments in *C. reinhardtii*

For high throughput light experiments in which more than 90 colonies were screened, *C. reinhardtii* plates containing colonies were kept in dark for 1-2 days, prior to transferring into a 96-well plate with 250 μ L/well of TAP. After resuspension, 100 μ L of each well were transferred to 2 independent 96-well plates containing 150 μ L of TAP.

For light experiments with less than 50 colonies, *C. reinhardtii* lines were grown in TAP medium under white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) and a long day 16:8 h light cycle for 4-7 days. 1 or 2 days before the light experiment, medium was exchanged and cultures were incubated in the dark. Next, liquid cultures were diluted to the same OD₇₅₀ or cell count and transferred to 12- or 6-well plates.

96-, 12- or 6-well plates were illuminated using custom-built LED light-boxes containing LEDs of different wavelengths (455 nm-blue, 660 nm-red) with usually $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red or blue light for 24 h (unless otherwise indicated), before luminescence analysis.

For light experiments conducted in the photobioreactor FMT 150 equipped with a red and blue bi-colour LED panel (Photon Systems Instrument), *C. reinhardtii* lines were initially transferred from plate to 50 mL TAP media and cultivated for 5 days. Subsequently, the culture was scaled up to 500 mL, with this growth phase occurring under a long day cycle of 16 hours light and 8 hours dark, with a white light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. 3 days prior to the light experiment, the medium was replaced, and cultures were incubated in darkness. For the light experiment itself, 2 L of culture containing 2×10^6 cells/mL was prepared and divided into two photobioreactors, one illuminated with blue light and the other with red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). To prevent accidental activation of the optogenetic systems, all samples handling was carried out under safe light conditions (510 nm-green).

For normalization, either cell counts acquired using a Cell counter CASY (OMNI Life Science) or OD₇₅₀ measured from 200-300 μL of culture in transparent 96-well flat-bottom plates (Costar) using a BMG Labtech CLARIOstar (BMG Labtech GmbH) were utilized.

Canavanine experiments in *C. reinhardtii*

For the spot tests, TAP agar plates containing different indicated concentrations of canavanine (0, 0.25, 0.5 and 1 mg/mL) were prepared. Cultures with 0.5, 1.5 or 3×10^6 cells/mL were prepared, and a 4 μL drop was spotted onto each plate. Plates were then incubated in constant white light ($50\text{-}100 \mu\text{mol m}^{-2} \text{s}^{-1}$) before picture acquisition.

For the light experiments, liquid cultures with 1×10^6 cells/mL were prepared and divided into two 12-well plates (2 mL/well), 20 μL of the corresponding canavanine dilution, for a final concentration per well of 0, 0.005, 0.1 and 0.5 mg/mL, were added. Plates were then incubated under red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 48 h of incubation 200 μL of the cultures were transferred to a 96-well flat-bottom plates (Costar), and pictures were taken. OD₇₅₀ was subsequently measured using a BMG Labtech CLARIOstar (BMG Labtech GmbH).

L-canavanine sulfate salt, purchased from Sigma-Aldrich, was diluted in miliQ water. Sterile miliQ water was used as mock.

Growth, chlorophyll content and maximal quantum yield of photosystem II quantification

Chlamydomonas reinhardtii cell suspension density was determined using a Cell counter CASY (OMNI Life Science).

Chlorophyll content was quantified as described elsewhere (Porra et al., 1989). Briefly, 1 mL of *C. reinhardtii* culture was centrifuged, and the pellet resuspended and vortexed in 1 mL methanol. After centrifugation, 100 µl of supernatant absorption was measured at 652, 665 and 750 nm using a BMG Labtech CLARIOstar (BMG Labtech GmbH) in transparent 96-well flat-bottom plates (Costar). Chlorophyll content was calculated as: Chlorophyll a (µg/mL) = $[16.29 \times (A_{665} - A_{750})] - [8.54 \times (A_{652} - A_{750})]$; chlorophyll b (µg/mL) = $[30.66 \times (A_{652} - A_{750})] - [13.58 \times (A_{665} - A_{750})]$; total chlorophyll (µg/mL) = (Chl a + Chl b) x dilution factor.

Photosynthetic parameters were performed using the fluorometer Imaging-Pam-Maxi (Heinz Walz GmbH) and the ImagingWin software. Prior to the measurement the cultures were incubated 15 min in the dark. F_o and F_m parameters were measured, for 3 areas of interest (AOI) within each sample, by applying a saturation pulse. The maximal quantum yield of photosystem II was calculated as $F_v/F_m = (F_m - F_o)/F_m$.

Software

Data was analysed using GraphPad Prism 9 for MacOS version 9.0.0 and Microsoft Excel (version 16.16.27 for Mac). Benchling [Biology Software] was used for molecular cloning design. Inkscape 1.2.2 (b0a8486, 2022-12-01) and BioRender.com were employed for graphical design.

Statistics

Multiple *t*-test and two-way ANOVAs for determination of statistical significance were performed with GraphPad Prism 9 for MacOS version 9.0.0. Statistical outliers were determined and excluded as described elsewhere (Jacobs & Dinman, 2004).

Results

Towards implementing light-control of gene expression in *C. reinhardtii*

Generating a molecular opto-toolbox: modules and elements required

Currently, only one optogenetic tool that regulates an artificial miRNA with blue light, resulting in induction of H₂ production, is available for *C. reinhardtii*. However, its capacity to control protein-coding gene expression was not shown. Additionally, the absence of any other optogenetic system, restricts the ability to independently control more than one gene or repress gene expression, which could be achieved through the broader implementation of optogenetic tools in this model alga. The development and implementation of optogenetic tools to control gene expression require the combination of many different synthetic biology building blocks, including light sensing proteins, DNA binding domains and the corresponding DNA binding sequence, transactivation and repressor domains (Müller et al., 2015; Ochoa-Fernandez et al., 2020). These building blocks can be combined in multiple ways to generate different optogenetic systems, that will have distinctive properties depending on the modules used (Fig. 1a). To facilitate a faster, less cloning-intensive, and more standardized process for generating novel optogenetic systems in *C. reinhardtii*, we developed a toolbox containing many of the necessary building blocks for assembling opto-switches. To achieve sufficient gene expression in *C. reinhardtii*, all the optogenetic blocks were codon-optimized and intron-integrated (Fig. 1b).

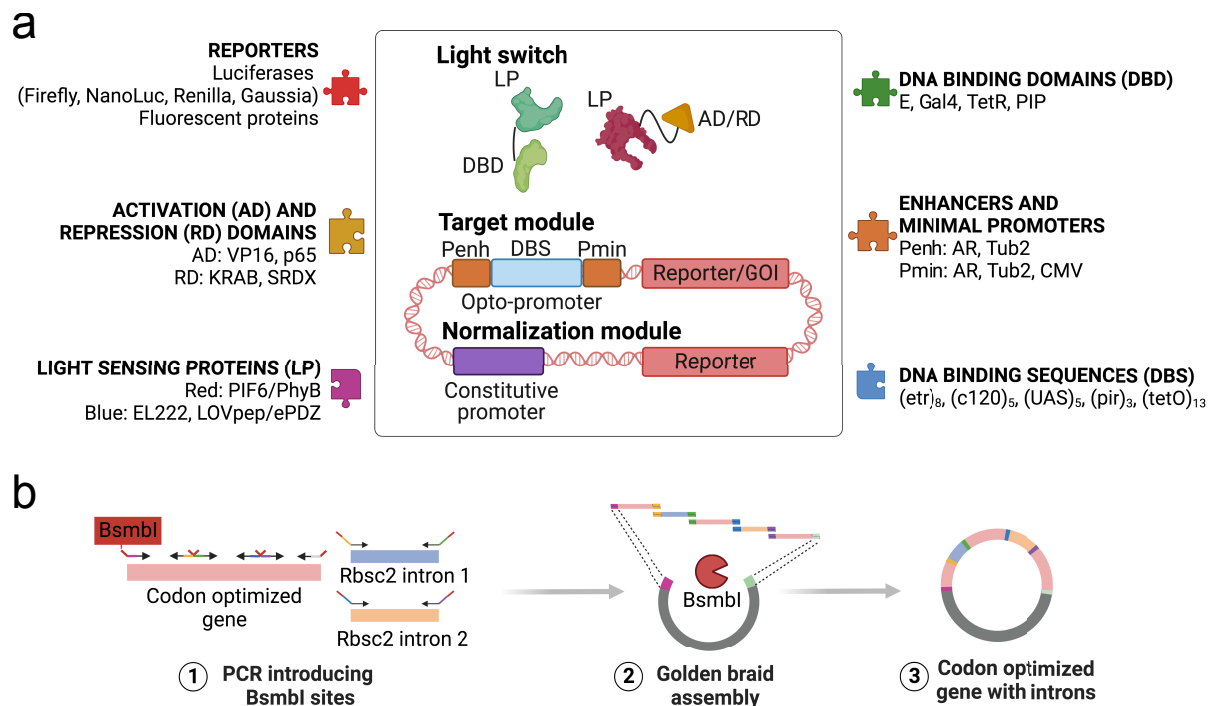


Figure 1. Genetic parts required to engineer light-regulated gene expression systems for *C. reinhardtii*. (a) Elements necessary to develop optogenetic systems were codon optimized for *C.*

reinhardtii, including different reporters, transactivation (AD) and repressor domains (RD), light sensing proteins (LP), enhancers (Penh), minimal promoters (Pmin), DNA binding domains (DBD) and their respective DNA binding sequences (DBS). **(b)** Schematic overview of the cloning stages to obtain the *Chlamydomonas* codon-optimized parts, including introns, in a Golden Braid compatible backbone.

Moreover, all the components present in the toolbox were designed to follow the MoClo syntax (Fig. 2), to facilitate cloning and ensure compatibility with the *C. reinhardtii* MoClo kit (Crozet et al., 2018).

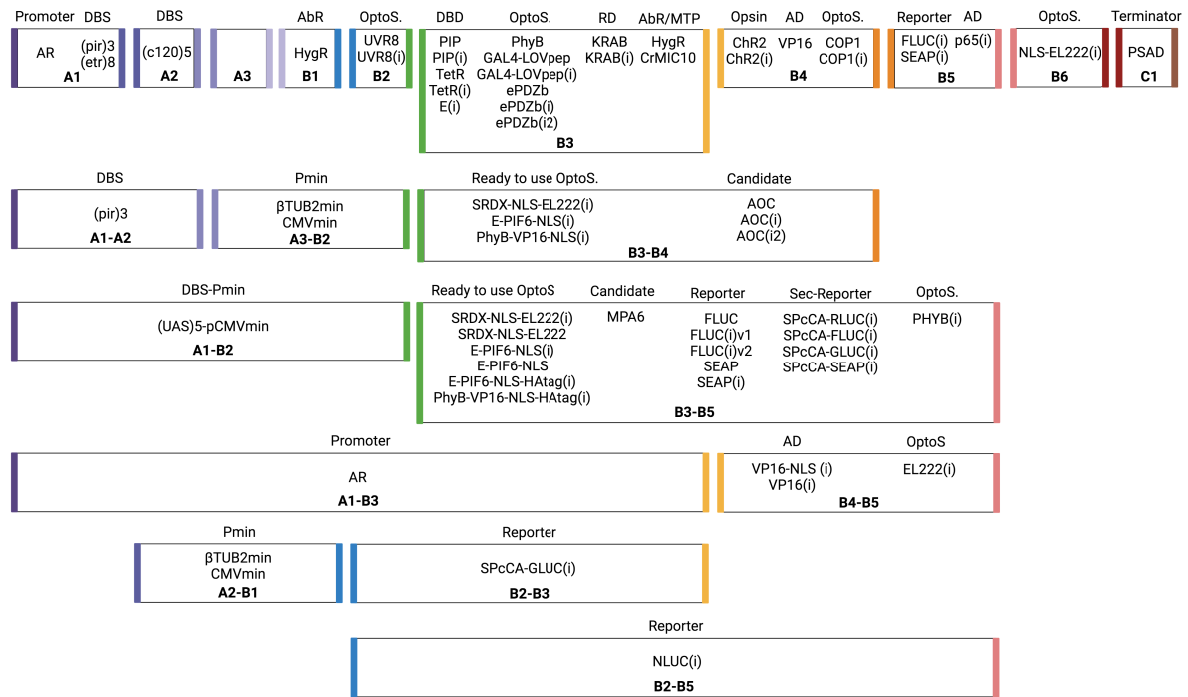


Figure 2. *C. reinhardtii* optogenetic MoClo-based toolkit. Optogenetic elements, codon-optimized for *C. reinhardtii* and following the *Chlamydomonas* MoClo kit rules, generated in the frame of this work. The color code indicates the upstream and downstream overhang pair, which follows the Plant MoClo syntax (Crozet et al., 2018). An (i) indicates the presence of an intron.

Quantitative characterization of different reporters

Luciferase reporter assays are widely used to quantitatively analyse gene expression, and allow the characterization of synthetic biology and optogenetic tools used to control gene expression, in a low invasive and fast way (Müller et al., 2015; Ochoa-Fernandez et al., 2020). Previous work has shown that Firefly (FLuc), Gaussia (GLuc), Renilla (RLuc) and Nano (NLuc) luciferases are active in *C. reinhardtii* (Crozet et al., 2018; Fuhrmann et al., 2004; Schroda, 2019; Shao & Bock, 2008). Here, we quantitatively compare the activity and sensitivity of these four bioluminescence proteins by measuring 96 clones resulting from transforming *C. reinhardtii* UVM4 with constructs encoding each of the luciferases under the control of the synthetic AR promoter. The luciferase activity was measured during 20 minutes. GLuc, NLuc

and RLuc showed the typical “flash” type kinetics, while FLuc has a glow-light behavior. For all of them the mean of the measurements in the linear range was calculated and plotted (Fig. 3).

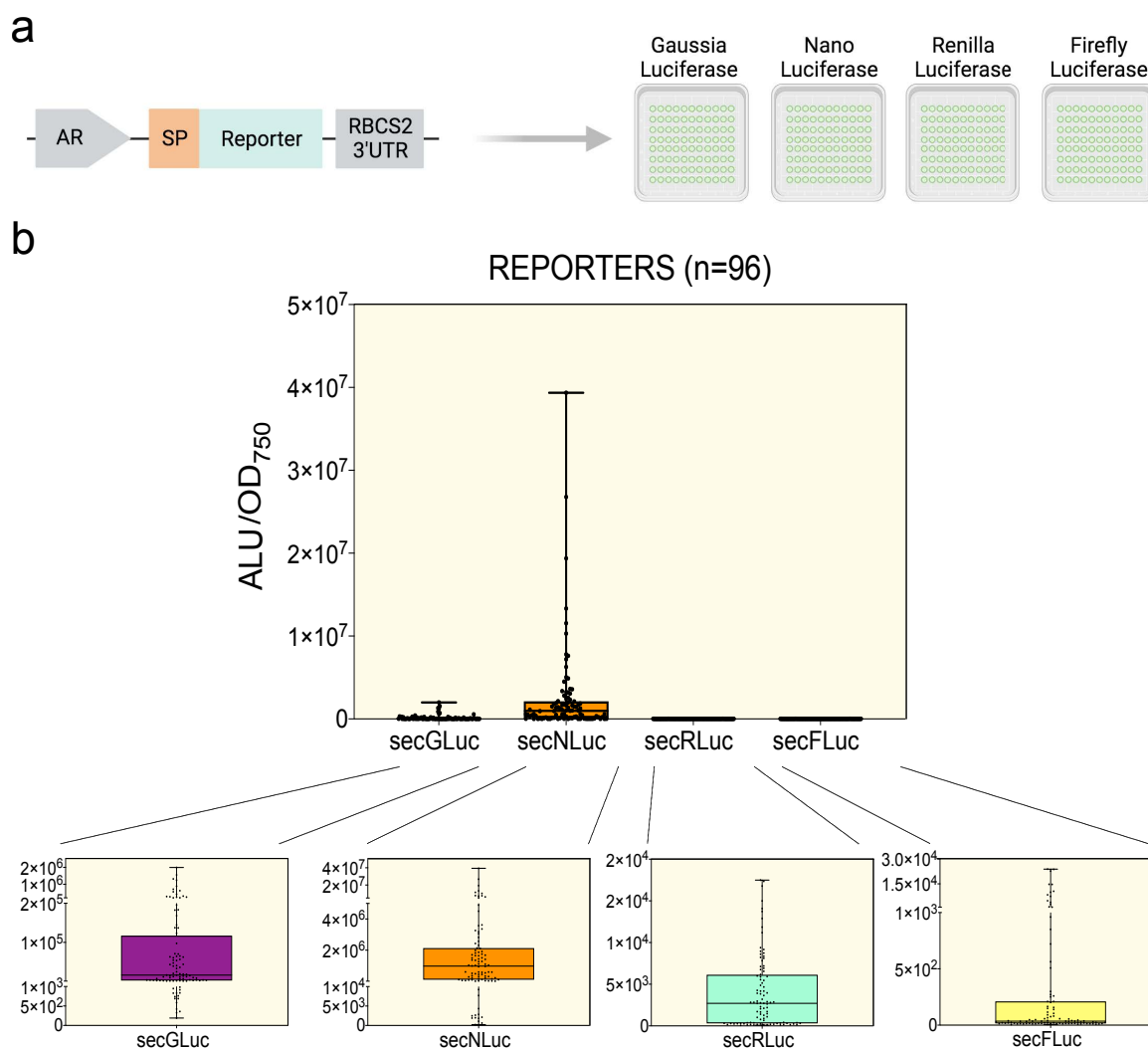


Figure 3. Quantitative characterization of different reporter genes activities (Gaussia, Nano, Renilla and Firefly Luciferases) in *C. reinhardtii*. (a) Scheme representation of the constructs transformed into UVM4 (plasmids: secGLuc-pOpt_gLuc_Hyg, secNLuc-pOD008, secRLuc-pOD043, and secFLuc-pOD179): reporters were fused to a secretion peptide and expressed under the control of the AR promoter. For each reporter, 96 independent transformants were screened. (b) Top, overview of the luciferase activity expressed as absolute luminescence units (ALU) normalized to OD₇₅₀ for each reporter. Bottom, detailed depiction of the distribution of 96 clones expressing each reporter (n=96). Data is representative of three independent measurements. AR, HSP70A-RBCS2 promoter; SP, secretion peptide; SecGLuc, secreted Gaussia luciferase; SecNLuc, secreted Nanoluciferase; SecRLuc, secreted Renilla luciferase; SecFLuc, secreted Firefly luciferase.

Dual luciferase assays allow to monitor the activity of two luciferases that use different substrates. These assays are routinely used when developing gene-expression inducible systems (e.g., optogenetic systems). One luciferase is employed to monitor the inducible

promoter activity, while the other one is used for normalization purposes. In this case, GLuc, RLuc or NLuc, that use coelenterazine as substrate, can be combined with FLuc, which oxidases D-luciferin.

Moreover, secretion of these luciferases allows monitoring their activity over time in a non-invasive way by determining their activities in medium aliquots. Secretion efficiency was compared by quantifying luminescence activity of *C. reinhardtii* strains stably transformed with one luciferase fused to a secretion signal and placed under the control of the synthetic constitutive promoter AR (secGLuc-pOpt_gLuc_Hyg; secNLuc-pOD008; secRLuc-pOD043; secFLuc-pOD179, FLuc-pOD381) (Fig. 4a,c). Untransformed wild type background was used as negative control. The ratio between the luminescence activity of the luciferase-expressing strains and the wild type background was calculated in the supernatant, whole cell culture and cell lysate. The whole cultures and supernatants showed comparable ratios for GLuc, NLuc and RLuc, whereas FLuc supernatant displayed almost 3 times lower ratio in comparison with the whole culture, possibly indicating an incomplete secretion. DTT was added to the lysis buffer to reduce the oxidative effect of the buffer on coelenterazine. RIPA buffer was used due to its lower signal in comparison to LB1 (Fig. 4b).

For subsequent dual luciferase assays in *C. reinhardtii*, Firefly and Gaussia luciferases were selected. Gaussia luciferase showed greater sensitivity than Renilla luciferase, but lower bioluminescent signal than Nanoluciferase. However, Nanoluciferase high signals could saturate the luminescence plate reader detector if transgenic clones have high expression levels.

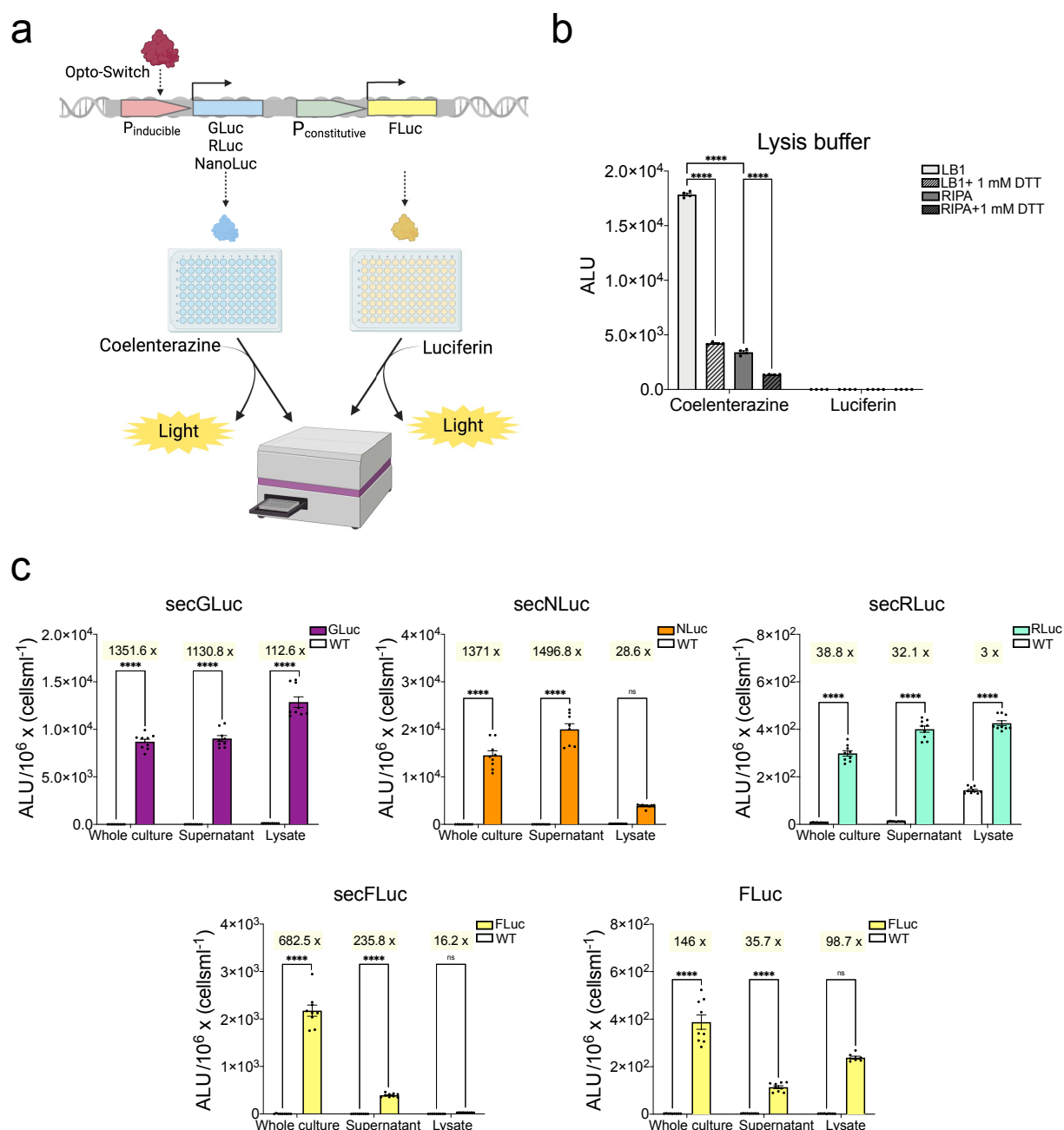


Figure 4. Establishment of the dual luciferase assay in *C. reinhardtii*. (a) Luciferase dual assay scheme. (b) Basal luminescence of luciferases substrates, luciferin and coelenterazine, in two lysis buffers: lysis buffer 1 (LB1) and RIPA buffer, in the presence and absence of the reducing agent DTT. Luminescence is given as absolute luminescence units (ALU). Error bars indicate standard error of the mean (SEM; n=4). (c) Quantitative luciferase activity in whole cell culture, supernatant and lysate, for the different assayed gene expression reporters. RIPA buffer was used for lysing the cells. The wild type UVM4 background was used as negative control. Absolute luminescence units were normalized to the cell count. Mean and SEM of three independent algae cultures measured in triplicates are represented (n=9). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (two-way ANOVA). SecGLuc, secreted Gaussia luciferase; SecNLuc, secreted Nanoluciferase; SecRLuc, secreted Renilla luciferase; SecFLuc, secreted Firefly luciferase; FLuc, Firefly luciferase.

Quantitative characterization and light sensitivity of different constitutive promoters

The activity of optogenetic systems rely on the adequate expression of its components. To examine in detail the strength of different constitutive promoters and their activity in relation to the downstream genes, the chimeric HSP70A-RBCS2 (AR) promoter, the heterologous cauliflower mosaic virus 35S (35S) promoter and the *C. reinhardtii* endogenous β 2-tubulin (β TUB2) and PsaD promoters were placed upstream of Firefly and Gaussia luciferase reporters. Measurement of the light signal of 96 transgenic lines, derived from the transformation of *C. reinhardtii* UVM4 with each different construct, showed that there is a relation between the promoter strength and the regulated gene. The PsaD promoter efficiently drives the expression of both luciferases, while the 35S promoter activity seems to be low in both cases. However, β TUB2 promoter results in higher expression levels of Firefly in comparison with AR promoter, but lower expression levels when the controlled gene is GLuc (Fig. 5). Conversely, clones with higher expression can be identified when a version of Firefly harboring less introns (FLuc-v1) is placed under the control of the AR promoter, instead of the β TUB2 promoter. This might reflect a dependency between the downstream gene structure and the activity of the promoter (Fig. 6).

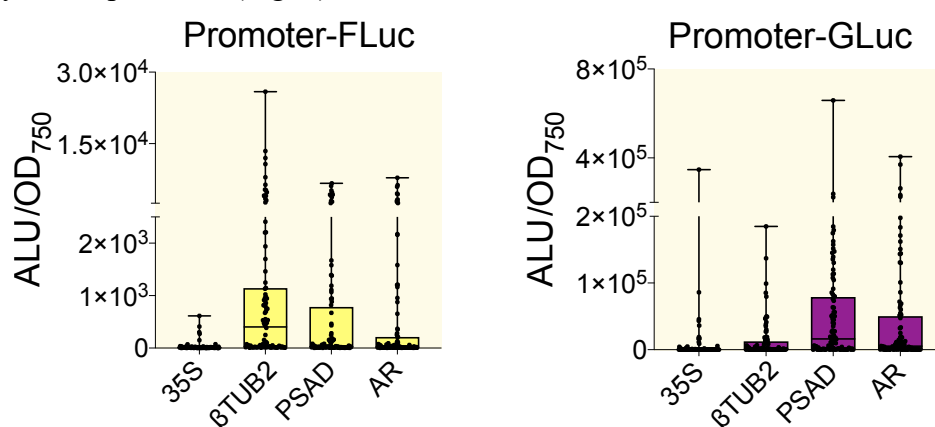


Figure 5. Quantitative comparison of the strength of four different constitutive promoters when combined with two different reporters, Firefly and Gaussia Luciferases. Strains were transformed with the different promoter-reporter combinations (pOD383, pOD253, pOD384, pOD380, pOD205, pOD206, pOD207, pOpt_gLuc_Hyg). For each promoter-reporter combination box, dots represent the luciferase activity given as absolute luminescence units (ALU) and normalized to OD₇₅₀ of 96 clones. Data is representative of three independent measurements. 35S, CaMV 35S promoter; β TUB2, β 2-tubulin promoter; PsaD, PsaD promoter; AR, HSP70A-RBCS2 promoter; FLuc, Firefly luciferase; GLuc, Gaussia Luciferase.

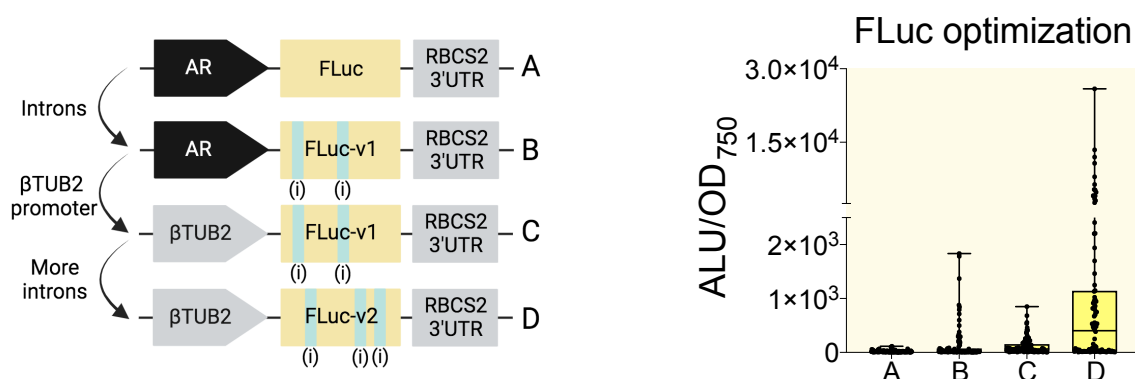


Figure 6. Optimization of Firefly Luciferase expression. Left, process to get a functional FLuc under the control of the βTUB2 promoter (plasmids encoding the different FLuc versions: A-pOD204; B-pOD179; C-pOD254; D-pOD253). Right, FLuc activity in the different stages of optimization. Absolute luminescence units (ALU) normalized to OD₇₅₀ of 96 clones is shown. βTUB2, β2-tubulin promoter; AR, HSP70A-RBCS2 promoter; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; FLuc, Firefly luciferase; (i), intron.

Furthermore, to understand if the 35S, βTUB2, PSAD and AR promoters have light-dependent regulation. We analysed the effect of different light wavelengths in the activity of these promoters. For each promoter, 12 transgenic lines, expressing GLuc, were illuminated with monochromatic blue or red light, followed by luciferase activity determination. The ratio between blue and red luciferase activity was calculated for each strain. In general, for the four different promoters, GLuc expression was higher in blue than in red light. However, the number of strains displaying a ratio close to 1 varies for the different promoters (35S>βTUB2>PsaD>AR) (Fig. 7).

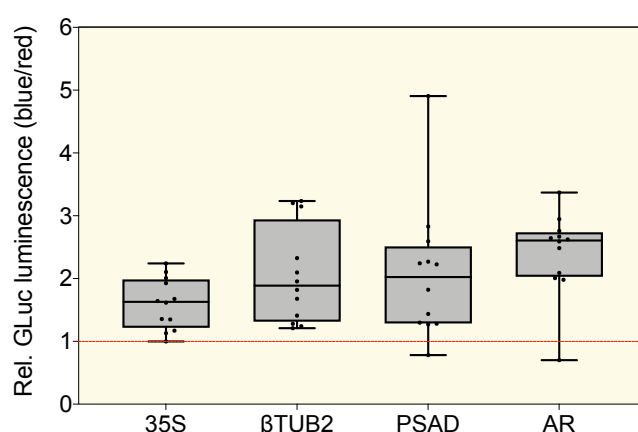


Figure 7. Blue/Red light relative GLuc expression under the control of four different constitutive promoters. For each promoter, twelve independent *Chlamydomonas* transformed clones (35S-pOD205; βTUB2-pOD206; PSAD-pOD207; AR-pOpt_gLuc_Hyg) were kept in liquid culture under red (20 μmol m⁻² s⁻¹) or blue light (20 μmol m⁻² s⁻¹). GLuc absolute luminescence units (ALU) normalized to OD₇₅₀ were determined after 24 h. Four samples from each light condition were measured

and averaged. The data shows the Blue/Red ratio in each clone (n=12). 35S, CaMV 35S promoter; β TUB2, β 2-tubulin promoter; PSAD, PsaD promoter; AR, HSP70A-RBCS2 promoter.

Based on the strength and the effect of light on the different promoters, β 2-tubulin and PsaD promoters were selected as the more adequate promoters for optogenetic purposes as they show less light-dependent variations and strong activity.

Effect of light on *C. reinhardtii* growth and photosynthetic parameters

To establish the conditions for optogenetic experiments, we grew *C. reinhardtii* cultures in four different light conditions (dark, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white, 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red or 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue light) in the presence or absence of a carbon source (acetate). Growth curves, maximal quantum yield of photosystem II (PSII) and the chlorophyll content were analysed for all the setups.

The growth curves show a faster growth under heterotrophic than in autotrophic conditions. As expected, *C. reinhardtii* is unable to grow in the absence of light and a carbon source. The addition of acetate allows its growth in the darkness; however, this is slower compared to cultures grown in the presence of white, red or blue light. Additionally, addition of acetate reduces the growth differences between cultures grown in blue or red light (Fig. 8a).

Independently of the addition of acetate, differences in the maximal quantum yield of PSII between 24-h blue and red cultures were not significant (Fig. 8b). The chlorophyll content tended to increase in blue light cultures even in the presence of acetate. This tendency could also be observed in cultures grown in the absence of white light and a carbon source (Fig. 8c). In general, after 24-h the differences in the measured parameters between all the light conditions tend to increase.

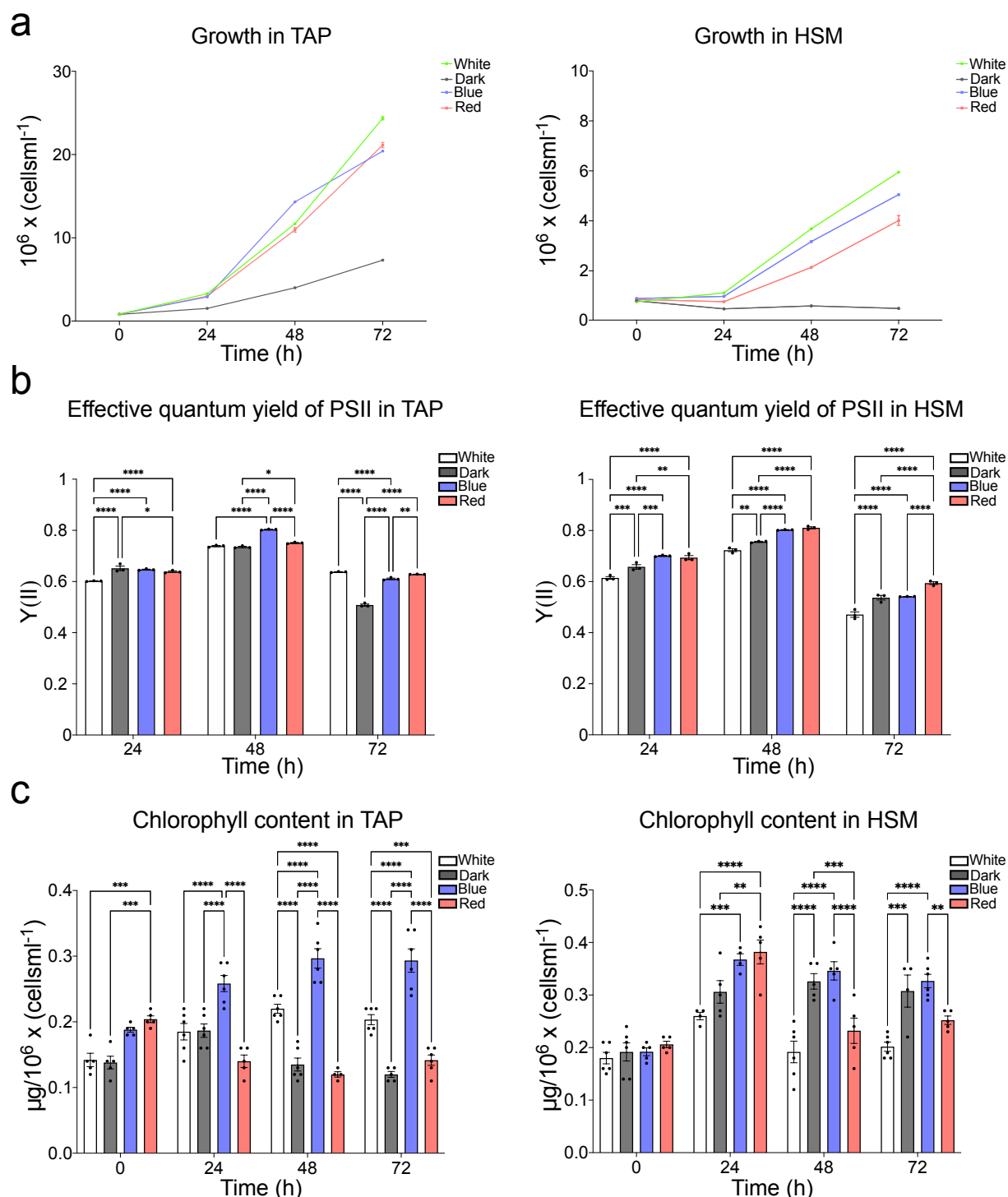


Figure 8. UVM4 characterization under different growth conditions. *Chlamydomonas* UVM4 cultures were grown in darkness, white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$), red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$), in the presence (TAP) or absence (HSM) of acetate. Samples were collected at 24, 48 and 72 h after incubation with the different light conditions, and different parameters were measured: **(a)** Growth curves. Cell density mean and SEM values are plotted for three independent biological samples measured in triplicates ($n=9$); **(b)** maximal quantum yield of photosystem II. Mean and SEM are plotted for three independent biological samples ($n=3$); **(c)** chlorophyll content. Mean and standard error of the mean are plotted for three independent biological samples measured in duplicates ($n=6$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (two-way ANOVA).

Effect on gene expression of the orientation of the normalization module in the construct

In the development of optogenetic systems to control gene expression, dual luciferase assays allow to use one luciferase as a reporter, to quantitatively assay the behavior of the optogenetic tool (reporter module), while the second luciferase can be employed for normalization or screening purposes (normalization module). We investigated the effect on gene expression of the architecture of the construct, i.e., relative orientation of the two modules. Two constructs, FF (pOD382) and FR (pOD341), were generated in which FLuc and GLuc were placed under the control of the β TUB2 promoter. In FF both modules are in the forward orientation, while in FR the FLuc module is in reverse orientation with respect to the GLuc module. GLuc and FLuc activities were measured for 96 transgenic lines transformed with either FF or FR. The number of clones that expressed one of the luciferases, both or neither, was quantified (Fig. 9). FR orientation resulted in more clones expressing both luciferases, therefore this configuration was used for subsequent experiments.

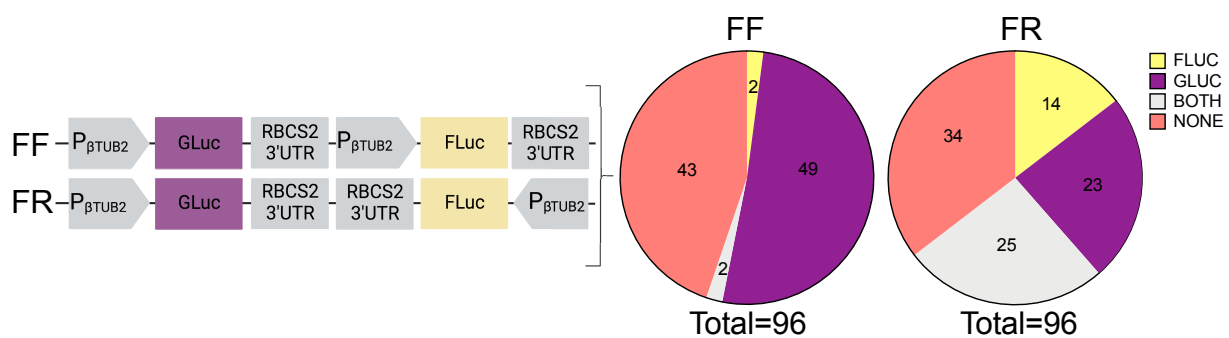


Figure 9. Effect of the orientation of two open reading frames in FLuc and GLuc expression levels.

Left, molecular configuration of the constructs, both luciferases are under the control of the β TUB2 constitutive promoter. Right, number of clones expressing GLuc, FLuc, both luciferases or none of them (n=96). Absolute luminescence units normalized to OD₇₅₀ higher than 100 are considered as a real gene expression. FF, forward-forward; FR, forward-reverse; P β TUB2, β 2-tubulin promoter; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; FLuc, Firefly luciferase; GLuc, Gaussia luciferase.

Minimal promoters and enhancers

Minimal or core promoters are essential for the formation of the transcription initiation complex, but upstream regulatory elements or enhancers are necessary to regulate and drive gene expression. We quantitatively compared the activity of two minimal promoters, the widely used cytomegalovirus minimal promoter (hCMVmin) and the β TUB2 minimal promoter from *C. reinhardtii*, defined in this work. Three constructs were generated in which GLuc was placed under the control of the minimal promoters or the β TUB2 full promoter (control; Fig. 10a). All the constructs have a FLuc normalization module that was used to check

whether the expression levels between the transformations with the different constructs were comparable. 96 independent transformants were screened to obtain a broad representation of the activity of these promoter variants when integrated into different loci, allowing us to rule out the effect caused by integration at different genome positions. As expected and in accordance with our data, expression levels are considerably reduced when minimal promoters, instead of the full promoter, are upstream of GLuc (Fig. 10b). To further characterize the β TUB2 promoter and determine whether a weaker promoter could be engineered out of this strong promoter, the proximal promoter region was divided in two enhancer parts, that were independently placed upstream of the β TUB2 minimal promoter controlling GLuc. Both segments of the proximal promoter were able to drive gene expression, but with a lower strength than the full proximal promoter (Fig. 11). These engineered weaker promoters could be used for applications in which lower transgene expression levels are required.

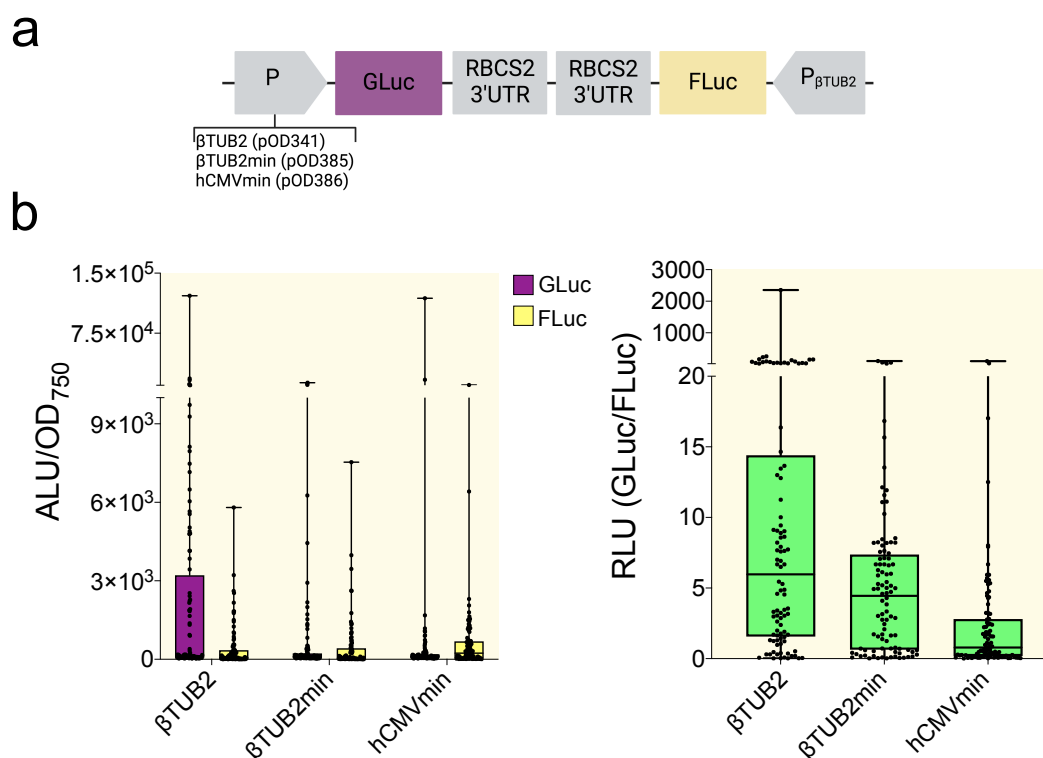


Figure 10. Quantitative characterization of the β TUB2 and hCMV minimal promoters. (a) Molecular structure of the plasmids transformed. FLuc under the control of the β TUB2 constitutive promoter is used as an indicator of the general gene expression levels of the clones. GLuc is placed under the control of the β TUB2min (pOD385), hCMVmin(pOD386) and β TUB2 constitutive promoter (pOD341). **(b)** GLuc and FLuc activity, expressed as absolute luminescence units (ALU) normalized to OD₇₅₀ (left), and GLuc/FLuc ratio, expressed as relative luminescence units (RLU; right), of 96 UVM4 clones transformed with each plasmid. β TUB2, β 2-tubulin promoter; β TUB2min, β TUB2 minimal promoter; hCMVmin, cytomegalovirus minimal promoter; FLuc, Firefly luciferase; GLuc, Gaussia luciferase.

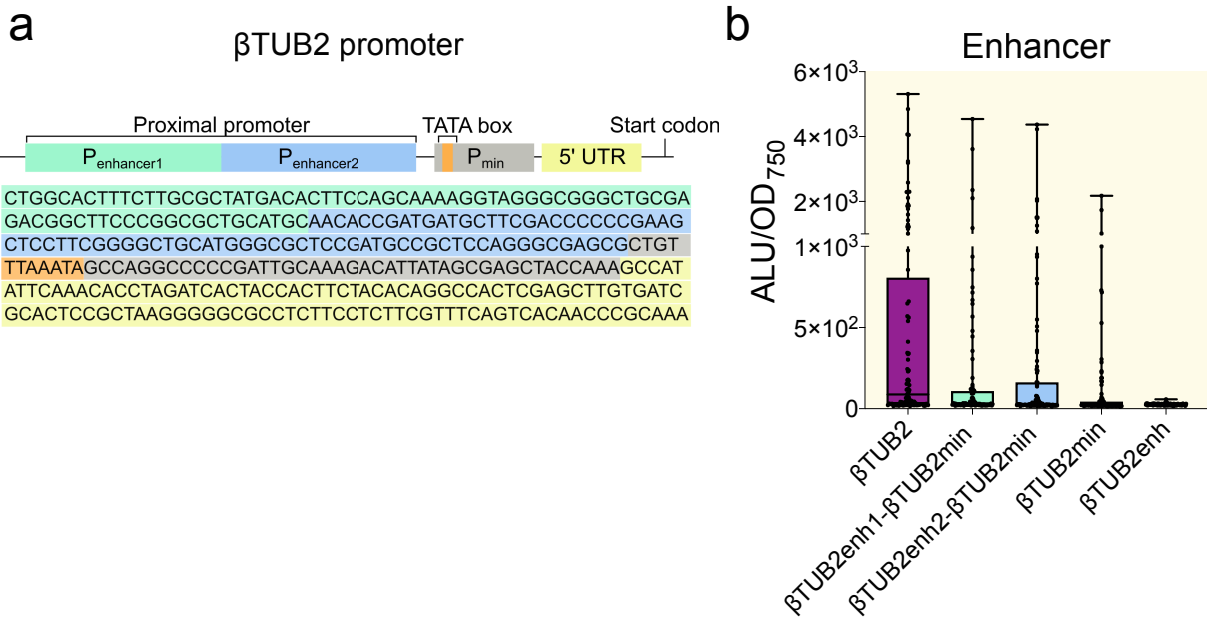


Figure 11. Characterization of the β TUB2 promoter enhancer region. (a) β TUB2 promoter structure and sequence. The proximal promoter was subdivided in two regions of equal size, termed enhancer 1 (enh1) and enhancer 2 (enh2). (b) Box and whiskers plot shows the GLuc activity distribution of 96 clones harbouring Gaussia under the control of the β TUB2 constitutive promoter (pOD341), the fusion of enhancer 1 (pOD397) or enhancer 2 (pOD396) with β TUB2min, the β TUB2min alone (pOD385), or the β TUB2 enhancer region (pOD387). Absolute luminescence units (ALU) were normalized to OD₇₅₀. β TUB2, β 2-tubulin promoter; β TUB2min, β TUB2 minimal promoter; β TUB2enh, β TUB2 enhancer region; UTR, untranslated region.

Blue-light repression of gene expression in *C. reinhardtii*

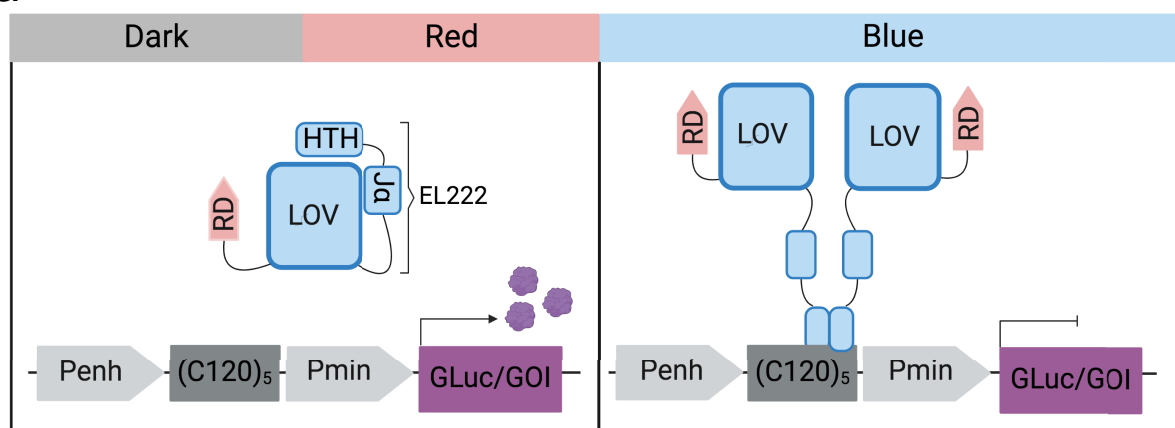
Design and construct generation of an optogenetic, blue light repression system

Due to the absence of an optogenetic repression system in *C. reinhardtii*, we engineered a blue-light-controlled gene repression system, named *CrBOff*, capable of repressing gene expression with light (Fig. 12). For this, we used the EL222 photoreceptor from *E. litoralis*, which is fused to a repression domain. EL222 has a N-terminal LOV domain and a DNA-binding C-terminal HTH domain. Upon blue light illumination, the photoreceptor undergoes a conformational change that results in EL222 dimerization. As a dimer, EL222 binds to the target sequence, C120, which is placed in the optogenetic promoter. The binding brings the repression domain into close proximity to the promoter region resulting in repression of the downstream gene of interest or reporter. In the absence of blue light, EL222 is unable to bind and repress gene expression (Fig. 12a).

We evaluated different constructs with different architectures and molecular components of the *CrBOff* system in order to obtain a final optimized version with the best properties (e.g., high dynamic range). The different construct versions tested are summarized in Fig. 12b and table

S3. In the optimized version, β TUB2 and PsaD promoters were selected for the engineering of the opto-tool, given its lower light-sensitivity in comparison with other promoters. The constitutive PsaD promoter drives the expression of EL222 fused to the repressor domain SRDX (light-switch). EL222 binding sequence is integrated into the β TUB2 constitutive promoter, constituting the optogenetic promoter. In the target module, GLuc is placed under the control of the optogenetic promoter, whereas FLuc is downstream of the β TUB2 constitutive promoter, in reverse orientation, and serves as a normalization module. Constructs containing both target and light switch modules (pOD302), or only one of them (pOD306-target, pOD235-light switch), were assembled and tested in *C. reinhardtii* to evaluate the CrBOff functionality.

a



b

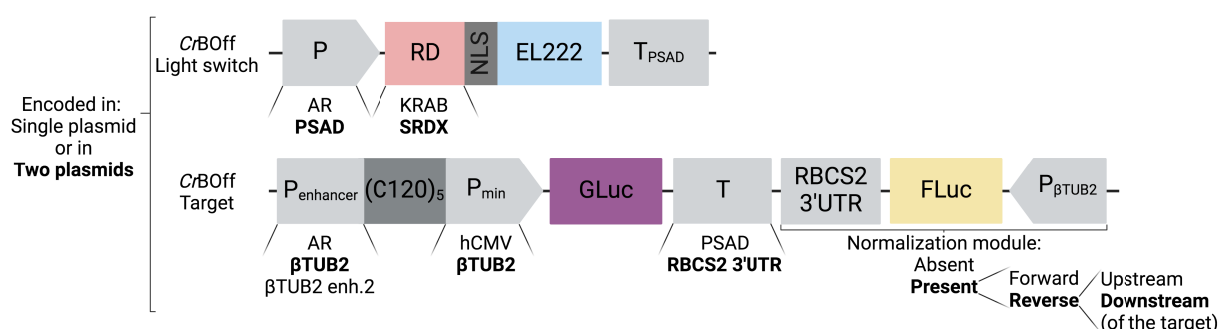


Figure 12. Mode of function and molecular design of the blue-light repression system (BOff). (a)

Mode of function. GLuc or a GOI is under the control of the light regulated promoter consisting on five repetitions of the binding sequence of EL222, C120, embedded in a constitutive promoter. Blue light illumination results in the dimerization and binding of EL222 to its binding sequence in the optogenetic promoter, which brings the repression domain into close proximity of the promoter region. **(b)** Molecular design and overview of the CrBOff plasmid variants generated and tested. Final CrBOff plasmid characteristics are highlighted in bold. Penh, promoter enhancer region; Pmin, minimal promoter; hCMV, cytomegalovirus promoter; β TUB2, β 2-tubulin promoter; β TUB2enh.2, β TUB2 enhancer region 2; PSAD, PsaD promoter or terminator; AR, HSP70A-RBCS2 promoter; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; GLuc, Gaussia luciferase; FLuc, Firefly

luciferase; GOI, gene of interest; EL222, *E. litoralis* photoreceptor; RD, repressor domain; HTH, helix-turn-helix domain; J α , J alpha helix; LOV, light-oxygen-voltage sensing domain; SRDX, SRDX repressor domain; KRAB, KRAB repressor domain.

Screening workflow for gene expression optogenetic tools in *C. reinhardtii*

Given the differences in growth between blue light and dark conditions, red light instead of darkness was selected as non-activating light condition. Cultures grown in blue and red light does not show significant differences in growth, especially after 24 h. However, to be sure that differences in gene expression are due to the light switch and not because of differences in the physiology of the cells or other consequences derived from genome positioning effects, a screening method in which the target and the light switch were transformed in two independent and subsequent phases was established. This approach allows to use the strain only harboring the target module as control. Since random integration of transformed DNA into *C. reinhardtii* results in high variability of gene expression among clones, the two-phases transformation workflow is accompanied by extensive screening in each step of the workflow. Light experiments are also conducted prior to transforming the light switch to ensure the selection of target-clones where gene expression is unaffected by light due to reasons other than the light switch activity. Figure 13 outlines the entire process, typically spanning around eight weeks (steps 1 and 3 each take about two weeks, while steps 2 and 4-6 take three days each, plus additional time for growing the cultures).

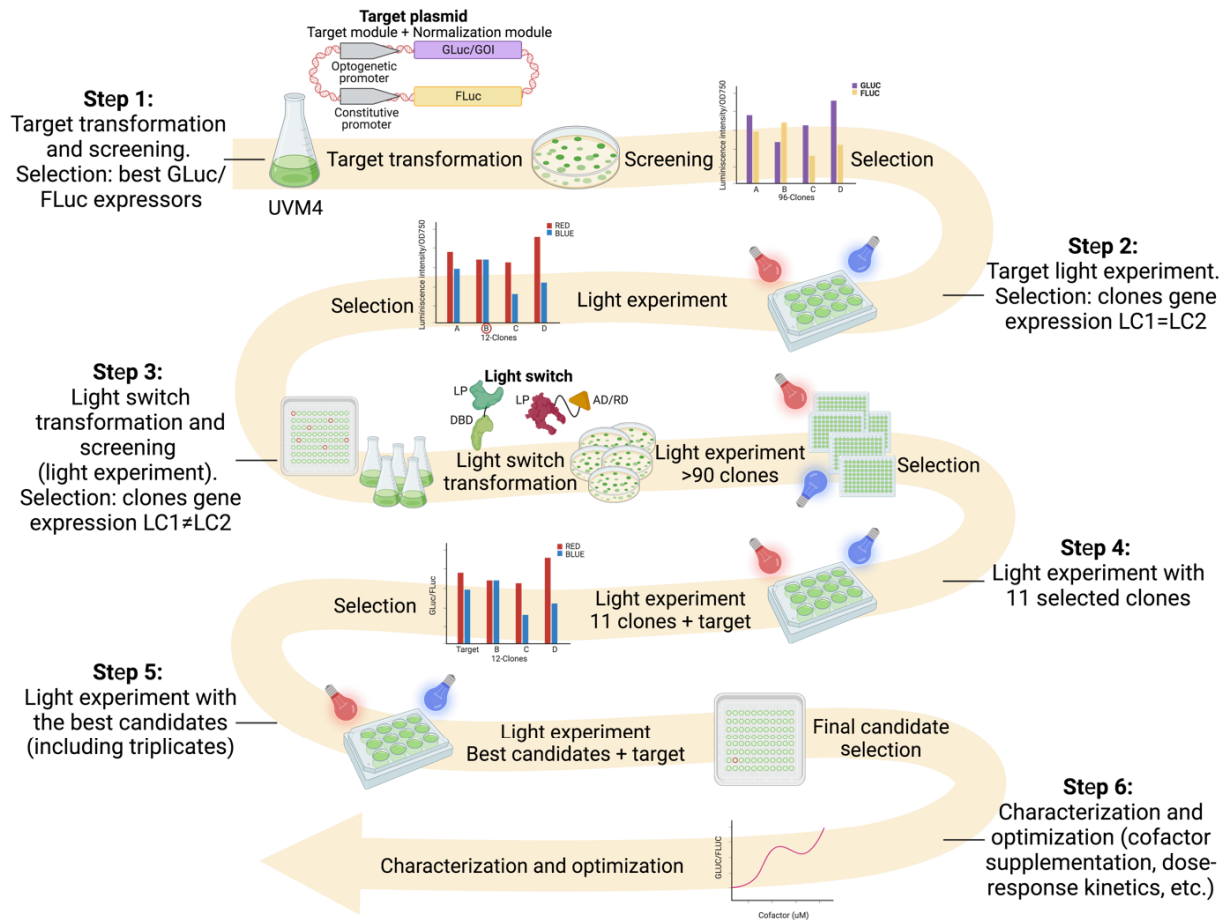


Figure 13. Overview of the different steps required to establish and screen optogenetic tools in *C. reinhardtii*. Step 1: *C. reinhardtii* UVM4 strain transformation with a plasmid encoding the target module (GLuc or a GOI under the control of a light-regulated promoter) and the normalization cassette (FLuc downstream of a constitutive promoter). 96-clones were screened based on GLuc and FLuc activities. If an inducible system is being engineered, clones with low or none GLuc values would be selected, whereas when engineering a repressible system medium-high GLuc values are preferred. Step 2: light experiment performed with selected clones from step 1 to identify clones in which expression is light-independent. Clones displaying luciferase activity independent of the light condition were selected (i.e., gene expression in light condition 1 equal to gene expression in light condition 2). Step 3: transformation of the optogenetic light responding element(s) in the clones from step 2. GLuc and FLuc was determined for 94 clones exposed to different light treatments, and those displaying differential GLuc activity were selected (i.e., gene expression in light condition 1 different from gene expression in light condition 2; for blue-inducible or red-repressible systems selection of clones with expression in Blue > Red; for blue-repressible or red-inducible systems, selection of clones with expression in Blue < Red). Step 4: additional light experiment with clones from step 3. Clone selection based on best optogenetic performance (e.g., high dynamic range). Step 5: light experiment with biological triplicates with selected clones from step 4. Step 6: characterization of the optogenetic system (e.g., dose-response kinetics, On-Off dynamics) and optimization (e.g., cofactor supplementation, components redesign). GLuc, gaussia luciferase; FLuc, firefly luciferase; GOI, gene of interest; LC, light condition.

The six steps depicted were followed to develop the *CrBOff* system and are described in detail in the subsequent sections.

Screening and selection of *CrBOff*-target transgenic lines (steps 1 and 2)

Following the two-phases transformation workflow, the *CrBOff* target module (pOD306) was transformed in UVM4. Gaussia and Firefly luciferases were measured in 96 independent transgenic lines, finding that 24 clones expressed both luciferases. Based on their GLuc values, out of this 24, 11 clones were selected for further testing (low GLuc, clone 94; medium GLuc, clones 21, 37, 44, 48, 56; medium-high GLuc, 5, 20, 45, 80; high GLuc, 12) (Fig. 14).

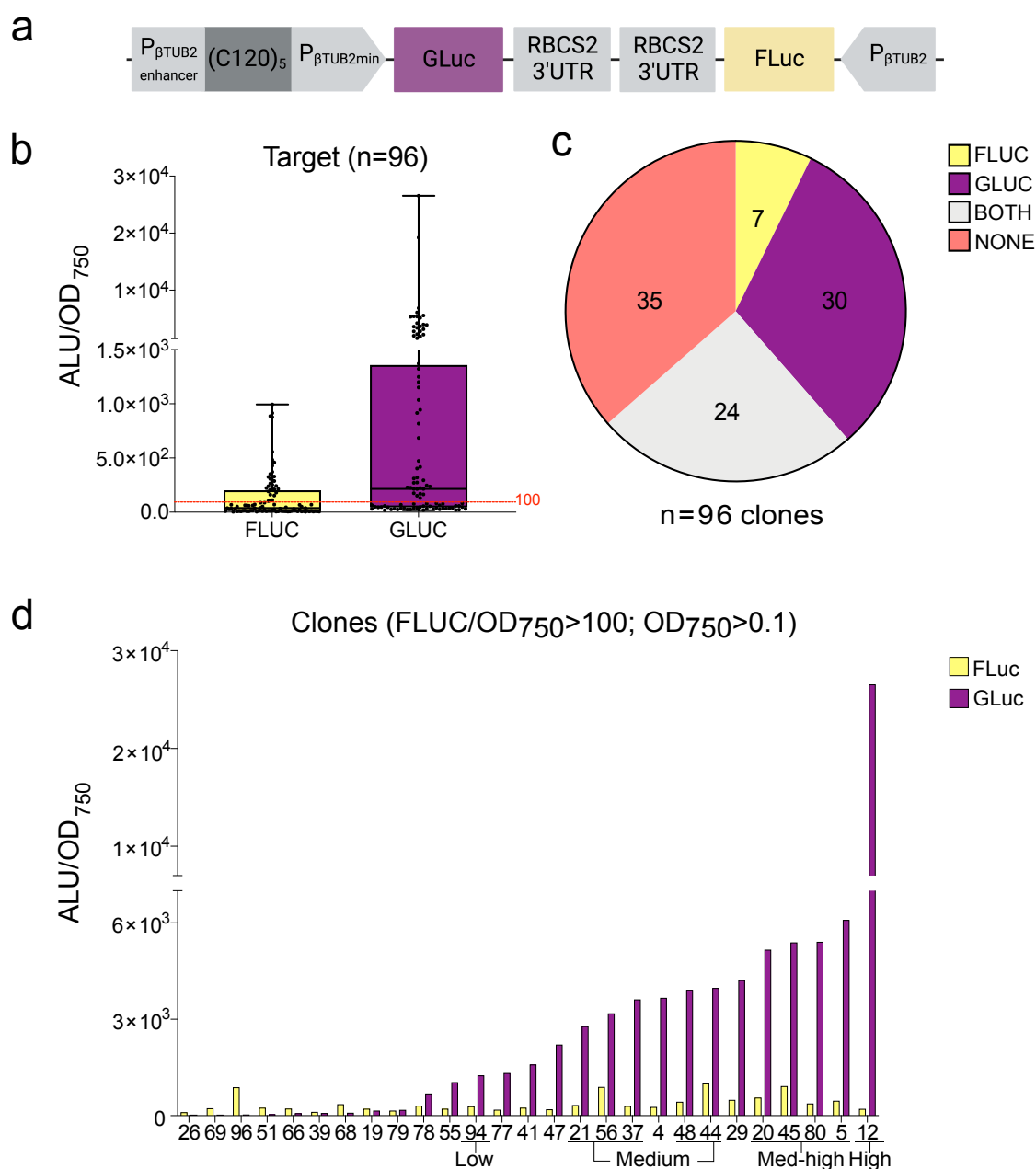


Figure 14. Screening of 96 UVM4 clones transformed with the target of the blue light-repression system (*CrBOff*). (a) Target construct design. The synthetic promoter is composed of the enhancer

region of the β TUB2, five repeats of the binding sequence of EL222, C120, and the minimal core promoter of β TUB2 that drives the expression of GLuc. In reverse orientation, β TUB2 promoter drives the constitutive expression of the normalization reporter FLuc. **(b)** Overview of GLuc and FLuc activities of the screened clones. Box and whiskers plot of the luciferase activity, expressed as absolute luminescence units (ALU) normalized to OD₇₅₀, for each independent clone (n=96), with the whiskers delineating the minimum and maximum values. **(c)** Number of clones expressing GLuc, FLuc, both luciferases or none of them (n=96). Absolute luminescence units normalized to OD₇₅₀ higher than 100 are considered as real gene expression. **(d)** Detailed depiction of GLuc and FLuc activity of clones with values of FLuc/OD₇₅₀ and OD₇₅₀ higher than 0.1. Clones selected for further experiments are underlined and classified in low, medium, medium-high or high, based on the ALU/OD₇₅₀ value. P β TUB2, β 2-tubulin promoter; P β TUB2min, β TUB2 minimal promoter; P β TUB2enhancer, β TUB2 enhancer region; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; FLuc, Firefly luciferase; GLuc, Gaussia luciferase.

A light experiment was performed with the 11 selected clones to ensure that there were no differences in gene expression under blue and red light (Fig. 15). Five clones, with different GLuc values and low differences in the two light conditions, were selected for transformation with the light switch (lowest GLuc values: 37 and 48; medium GLuc values: 94; medium-high GLuc values: 44 and 45). Selection of clones with different GLuc values enables us to later investigate the light switch's capacity to repress varying levels of gene expression. Additionally, it remains uncertain whether clones showing high GLuc expression levels are due to integration in genomic regions that are challenging to repress, such as downstream of a strong constitutive promoter. Similarly, choosing clones with low levels of gene expression may impede us to fully explore the repression potential of the optogenetic system. Including a wide range of gene expression levels will facilitate the study of all these aspects.

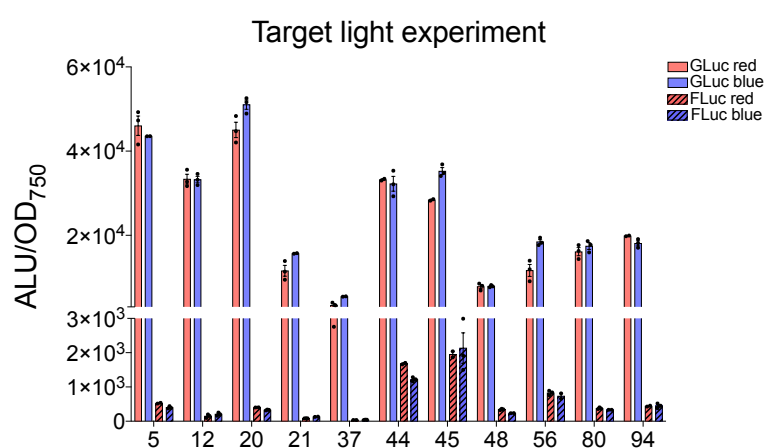


Figure 15. Light experiment with selected UVM4 clones transformed with the target of the blue light-repression system (CrBOff). Liquid cultures were maintained in darkness for two days before illumination with red or blue light (20 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After 24 h, GLuc and FLuc activities were determined. Mean and SEM are plotted for n=3 samples.

Characterization of two different repression domains (step 3)

We analysed two versions of the blue-light repression module by fusing EL222 to either the EAR repression domain (SRDX) from *A. thaliana* or the Krüppel-associated box domain (KRAB) from humans. These two constructs were independently transformed in the selected *CrBOff*-target transgenic lines (37, 44, 45, 48, 94; Fig. 16a). From each transformation event, 94 clones were screened by growing them in blue and red light. The luciferase ratio under the two light conditions was compared between the target-transgenic lines and the light-switch expressing lines. The target transgenic lines and the lines transformed with KRAB-EL222 had a ratio close to 1, which indicates no differences in gene expression between the two light conditions. Differently, some of the clones harbouring the SRDX-EL222 version had lower expression levels under blue than under red light, and therefore, SRDX repression domain was selected for subsequent experiments. Nevertheless, line 48 transformed with SRDX-EL222 showed a ratio close to 1, so it was discarded for the next steps of the workflow (Fig. 16b,c).

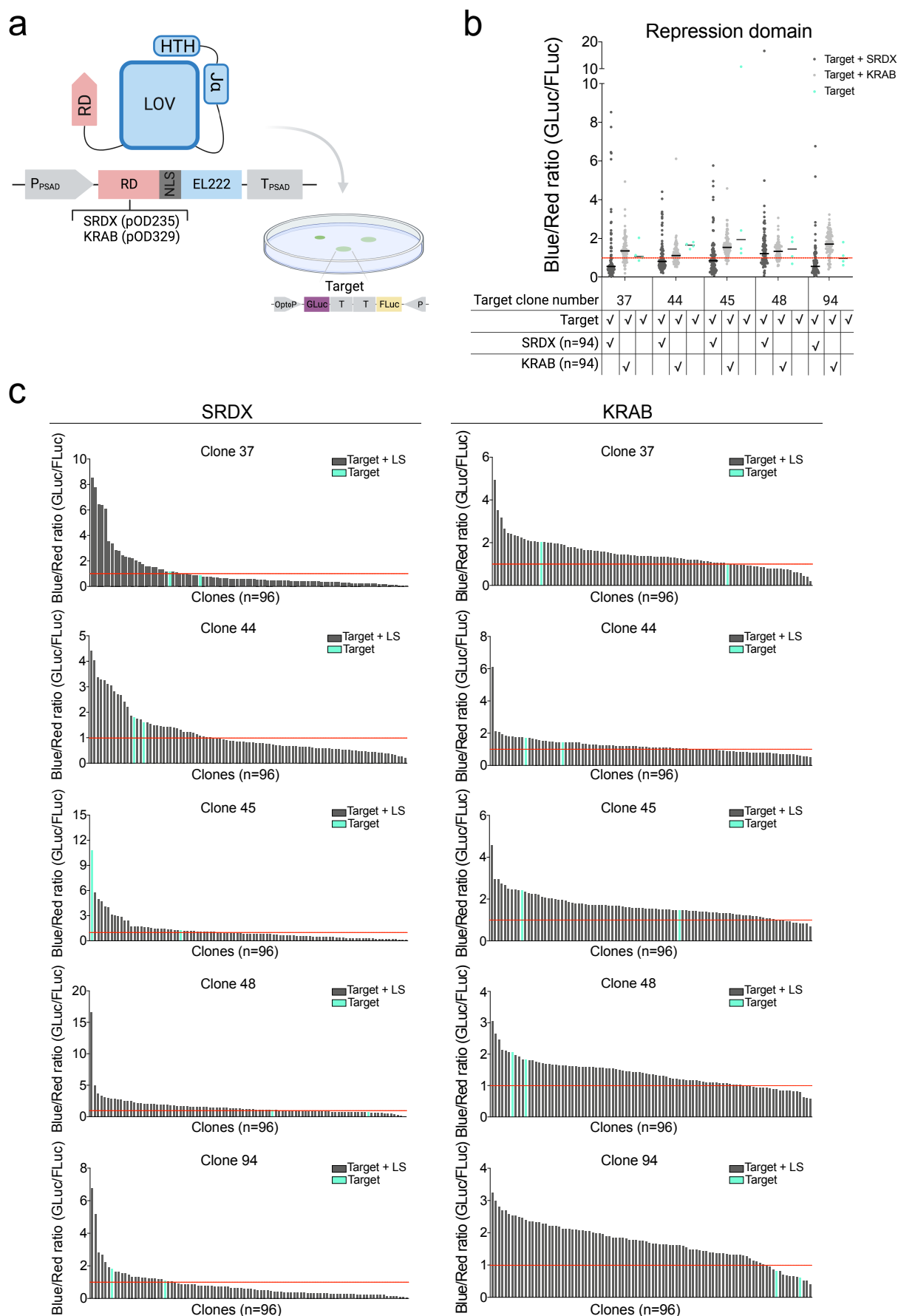


Figure 16. Characterization of two repression domains for the blue-light repression system in *C. reinhardtii* (CrBOff). (a) Selected UVM4 clones harbouring the target module of the CrBOff system

(target clones) were transformed with blue-light responsive element fused to either the SRDX (pOD235) or KRAB (pOD329) repressors, under the control of the PsaD promoter. **(b)** Quantitative comparison of SRDX and KRAB repressors. For each selected target clone, 94 clones transformed with pOD235 or pOD329 were illuminated with blue or red light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. GLuc and FLuc activities were measured and normalized to OD₇₅₀. The ratio between the normalized GLuc and FLuc was calculated. Comparison of the two repressors and the target clones is shown. The median of the blue/red ratio from all the clones is plotted (n=94). For the target clones four replicates were measured (n=4). **(c)** Depiction in descending order of the blue/red ratio of each clone (n=94) with SRDX or KRAB, target clones were measured twice (n=2). P_{PSAD}, PsaD promoter; NLS, nuclear localization sequence; T, terminator; RD, repressor domain; SRDX, SRDX repressor domain; KRAB, KRAB repressor domain; HTH, helix-turn-helix; LOV, Light-Oxygen-Voltage; EL222, *E. litoralis* photoreceptor; GLuc, Gaussia luciferase; FLuc, Firefly luciferase.

A functional blue light repression system in *C. reinhardtii* (steps 4 and 5)

In order to confirm the functionality of the blue-light repression system in *C. reinhardtii* and select clones with the best dynamic range, additional light experiments in 12 (Fig. 17a) or 6-well (Fig. 17b) plates were performed. Clone 58 from the target-line 37, clone 27 from the target-line 44, and clone 70 from the target-line 94 were selected as final candidates showing a repression rate of 7.5, 2.4 and 8.1, respectively. An overview of the screened and selected lines throughout the screening workflow is summarized in table 1.

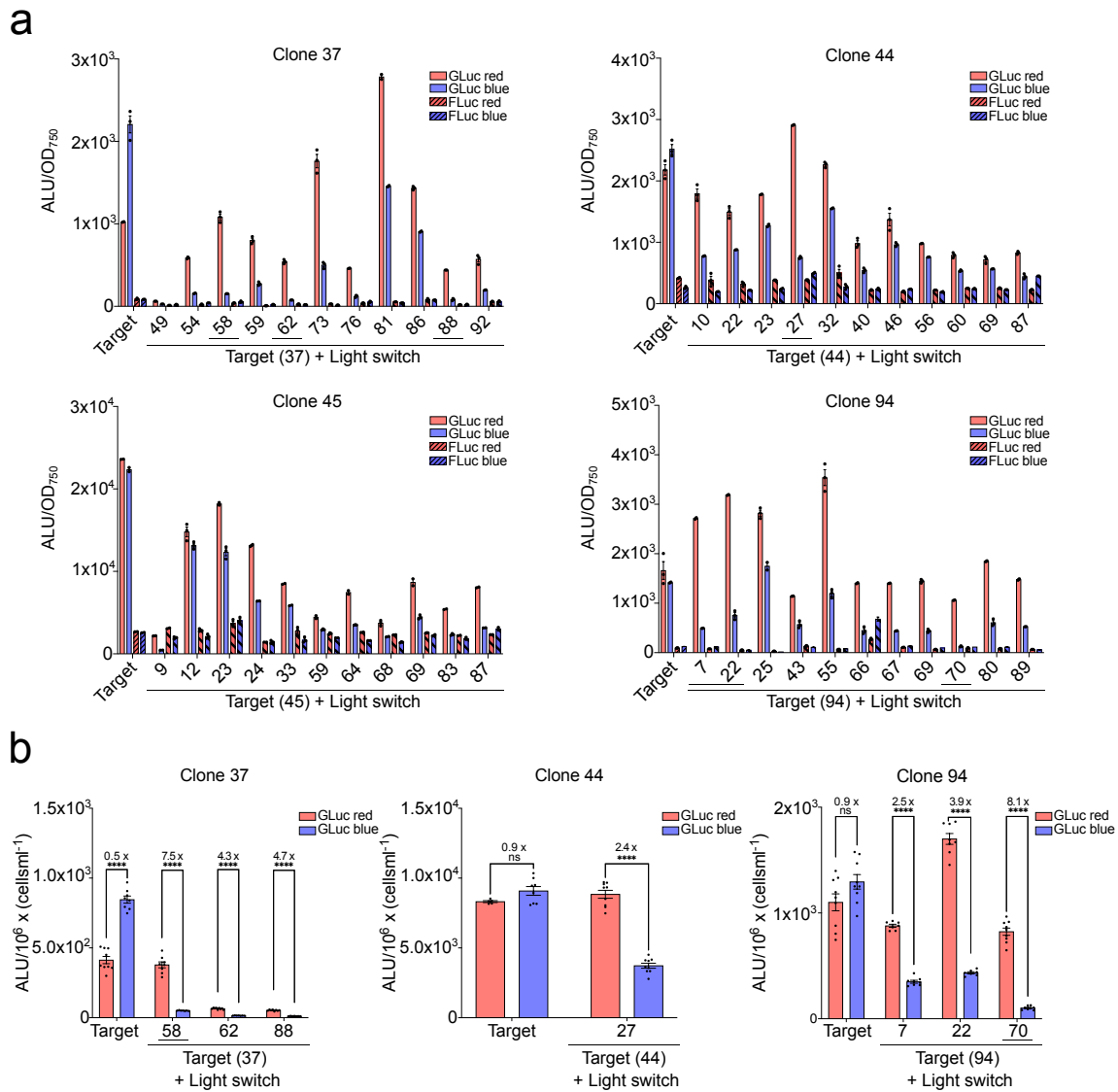


Figure 17. Light experiment with selected clones transformed with the blue light-repression switch (*CrBOff*). Cultures were grown in darkness for two days before illumination with red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 24 h GLuc and FLuc activities were determined. **(a)** For each target line (37, 44, 45, 94), eleven independent clones, transformed with the light sensing element of the *CrBOff* system, were screened in 12-well plates. Clones selected for further testing are underlined. GLuc and FLuc activity expressed as absolute luminescence units (ALU) normalized to OD_{750} . Mean and SEM are plotted for $n=3$ samples. **(b)** Light experiment of selected clones from (a), performed in 6-well plates. Transgenic lines selected are underlined. Mean, SEM and red/blue ratio of three independent algae cultures sampled in triplicates is represented ($n=9$) for each line. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (multiple t -test). GLuc, Gaussia luciferase; FLuc, Firefly luciferase.

Table 1. Overview of the *CrBOff*-UVM4 lines selected. From the 96-UVM4 clones, transformed with the target of the *CrBOff* system, five were selected and transformed with the light sensing element of the *CrBOff* switch, from which 96 clones were screened. Eleven clones selected for further testing, harbouring all the components of the *CrBOff* system, are indicated. The final candidates are highlighted in yellow.

Target transformation (n=96)	Light switch transformation (n=96)										
37	49	54	58	59	62	73	76	81	86	88	92
44	10	22	23	27	32	40	46	56	60	69	87
45	9	12	23	24	33	59	64	68	69	83	87
48	None of the 96 clones were selected										
94	7	22	25	43	55	66	67	69	70	80	89

Effect of the addition of the cofactor FMN on the *CrBOff* repression activity (step 6)

Conformational changes induced by light on EL222 are known to be triggered by the blue-light excitation of its cofactor flavin mononucleotide (FMN; Nash et al., 2011). We tested if the exogenous addition of FMN improved the repression capacity of the *CrBOff* opto-tool. 0, 5 and 10 μM FMN was added to liquid cultures of the three selected *CrBOff*-lines (target37-LS58 and target94-LS70). No significant increasing differences in GLuc expression were found under blue and red light after the addition of FMN (Fig. 18).

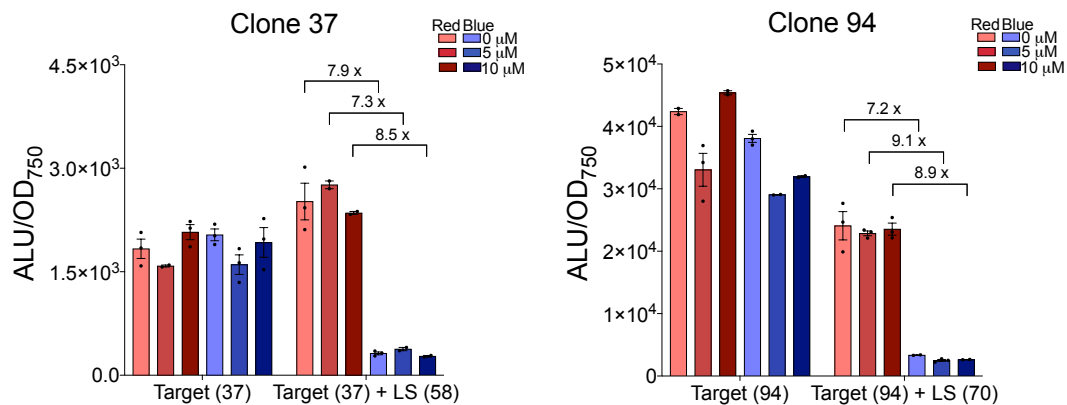


Figure 18. Impact of FMN cofactor in Gaussian luciferase activity in three independent *CrBOff* transgenic lines. Algae cultures were kept in the dark two days before the experiment. The indicated concentration of FMN were added to each line prior to 24 h cultivation in red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). Gaussian luciferase activity was measured and expressed as absolute luminescence units (ALU) normalized to OD_{750} . For each target line (37 and 94), one clone transformed with the light sensing element of the *CrBOff* system was screened (58 and 70, respectively). Mean and SEM are plotted for $n=3$ samples. LS, light switch.

Light dose-response of the *CrBOff* system (step 6)

To test whether the *CrBOff* system activation light intensity could be reduced without significantly affecting the repression capacity, light experiments with 5, 10 and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$

¹ blue light were performed (Fig. 19a). The lowest light intensity tested was sufficient to repress gene expression. Next, to confirm that repression is achieved under settings similar to normal growth conditions (i.e., white light), we simulated a white light-like condition by supplementing red light with two different blue light intensities (5 and 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was used and showed to allow repression of the system (Fig. 19b).

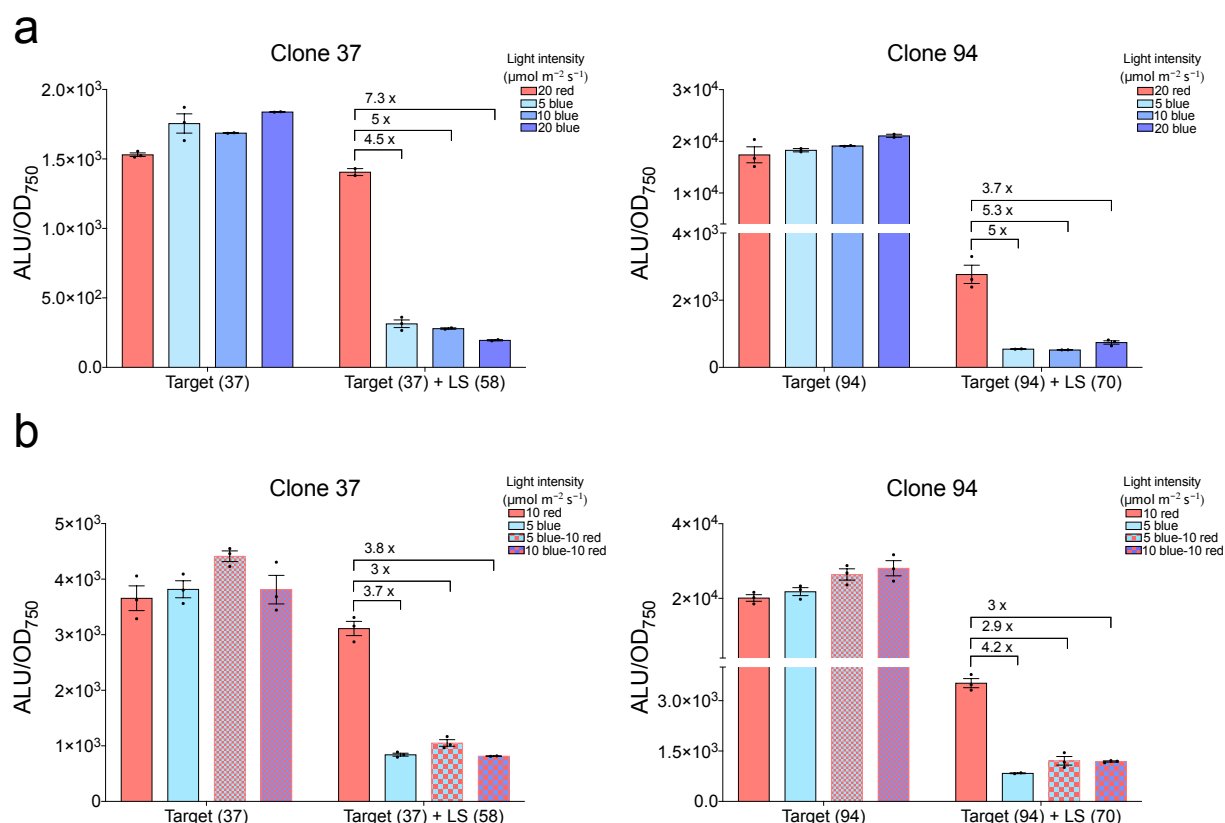


Figure 19. *CrBoff* repression capacity under different light intensities. Characterization under (a) monochromatic blue or red light, (b) blue, red and red light supplemented with increasing intensities of blue light. *C. reinhardtii* cultures were kept in darkness two days prior to 24 h illumination with the indicated light intensities. Gaussia luciferase activity was measured and expressed as absolute luminescence units (ALU) normalized to OD₇₅₀. For each target line (37 and 94), one clone transformed with the *CrBoff* light switch was screened (58 and 70, respectively). Mean and SEM are plotted for n=3 samples.

Performance of upscaled cultures of the *CrBoff*-line in photobioreactors

To assess scalability for biotechnological applications of *CrBoff*-line cultures while preserving *CrBoff* system activation and functionality, we conducted light-experiments in photobioreactors. Specifically, cultures of the *CrBoff*-line (target94-LS70) were scaled-up and incubated in two 1 L photobioreactors FMT 150 (Photon Systems Instrument) equipped with red and blue LED panels. In one of them, the culture was illuminated with 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light, while in the other, the culture was exposed to an equal intensity of red light (Fig. 20a). Samples were collected at various time points (between 0-48 hours) to measure GLuc

luciferase activity and OD₇₅₀ (Fig. 20b). The observed higher GLuc activity under red light compared to blue light suggests the feasibility of utilizing the *CrBOff* system in larger cultures in photobioreactors.

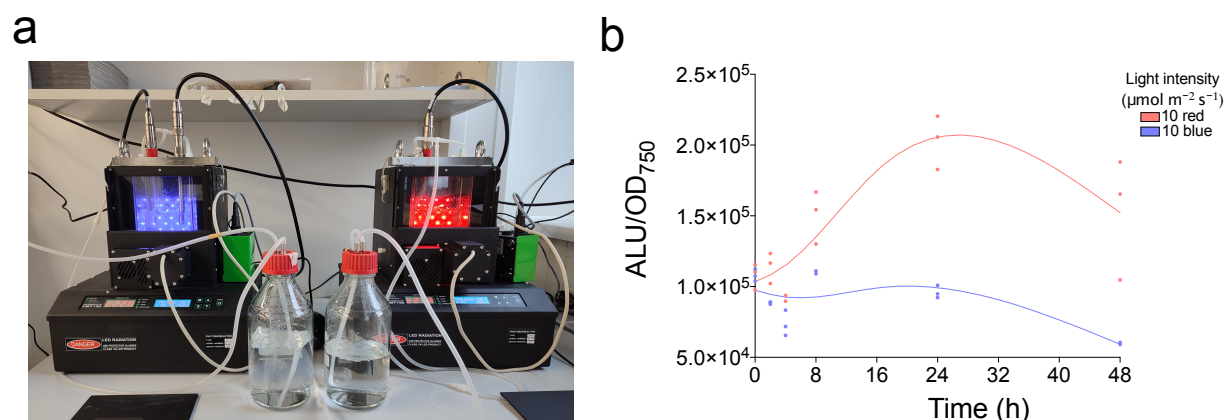


Figure 20. *CrBOff* system activity in cultures cultivated in photobioreactors. (a) The setup of the photobioreactors. Photobioreactors FMT 150 (Photon Systems Instrument) equipped with a red and blue bi-colour LED panel. **(b)** Photobioreactor light experiment. Cultures of the *CrBOff* line (target94-LS70) were grown in darkness for three days before being scaled up to 2 L, with an initial OD₇₅₀ of 0.15. Culture was split in two and incubated in the 1 L photobioreactors under either red or blue light (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$). GLuc luciferase activity was measured at 0, 2, 4, 8, 24, and 48 h, and is expressed as absolute luminescence units (ALU) normalized to OD₇₅₀. Spline curves and the values of n=3 technical replicates are shown.

Optimizing the *CrBOff* system for reduced expression levels

Following confirmation of the *CrBOff* system's activity, we explored the possibility of engineering a *CrBOff* system with basal lower expression levels, where little to no gene expression occurs under blue light repression conditions. To achieve this, we modified the *CrBOff* target promoter by reducing its strength. This was accomplished by removing one of the enhancer regions from the proximal promoter, previously characterized in this study (refer to Fig. 11). We then compared the activity of this newly generated optogenetic weak promoter with the previously characterized stronger *CrBOff* optogenetic promoter (Fig. 21a).

To assess its performance, we followed once again the two-phases transformation workflow. Transformation with the two different versions of the *CrBOff* target (strong and weak) plasmid resulted in clones exhibiting generally lower Gaussia luciferase expression levels with the weak promoter compared to the strong promoter. Specifically, the median expression levels across 96 clones were 64.9 for the weak promoter and 214.2 for the strong promoter (Fig. 21b).

As was done with the original *CrBOff* system, different target-clones transformed with the light switch were screened. For one of these clones (target7-LS81, being the target pOD399), the repression capacity of the system under blue light was examined and compared with one of

the clones previously characterized with the original system (target94-LS70, being the target pOD306). It was observed that Gaussia luciferase activity under blue light was lower than in red for both clones. However, with the weaker *CrBOff*-target, activation of the *CrBOff* system resulted in Gaussia luciferase activity levels that were indistinguishable from the background (Fig. 21c).

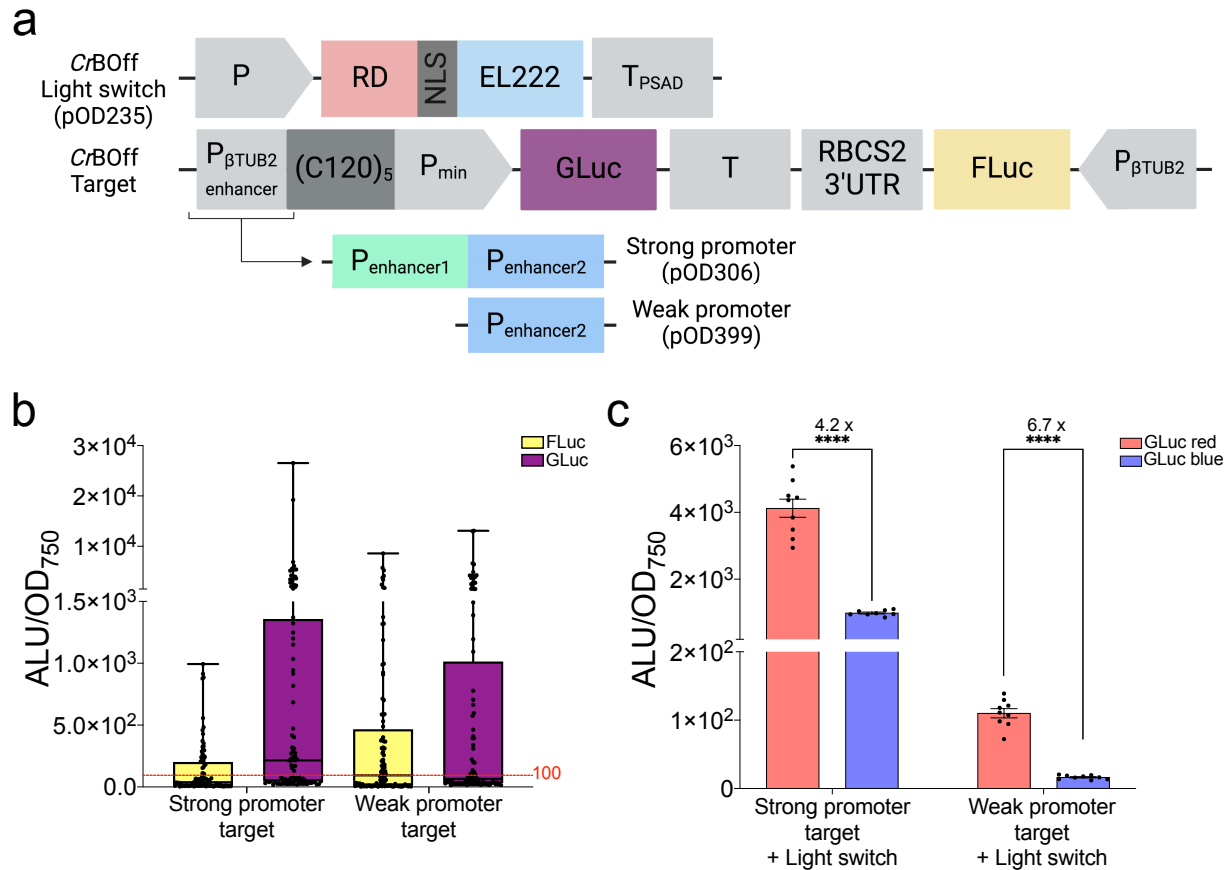


Figure 21. Characterization of a tighter *CrBOff* system with lower overall expression levels. (a) Design of the weaker *CrBOff* target promoter construct. The newly designed weak optogenetic promoter (pOD399) contains only the second enhancer region of the β TUB2, whereas the original *CrBOff* target promoter (pOD306) includes the full proximal promoter, upstream of the five repeats of the binding sequence of EL222, C120, and the minimal core promoter of β TUB2. **(b)** Overview of GLuc and FLuc activities of the 96 screened clones transformed with either the strong promoter version (pOD306) or the weak promoter version (pOD399). Box and whiskers plot illustrating luciferase activity, expressed as absolute luminescence units (ALU) normalized to OD₇₅₀, for each independent clone (n=96), with whiskers indicating the minimum and maximum values. **(c)** Light experiment comparing one selected clone transformed with the weak promoter version (target7-LS81) with the previously characterized clone (target94-LS70). Both clones transformed with the *CrBOff* light switch (LS; pOD235). Experiment conducted in 6-well plates. Gaussia luciferase activity was measured in cultures kept in darkness 2 days before cultivation during 24 h under red or blue light (20 μ mol m⁻² s⁻¹), was measured. Mean, SEM, and the red/blue ratio of three independent algae cultures sampled in triplicates are represented (n=9) for each clone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (multiple t -test). P, promoter; RD, repression domain; NLS, nuclear localization sequence; EL222, *E. litoralis* photoreceptor; T_{PsAD}, PsaD terminator; P β TUB2, β 2-tubulin

promoter; $P_{\beta TUB2}$ enhancer, $\beta TUB2$ enhancer region; P_{min} , minimal promoter; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; GLuc, Gaussia luciferase; FLuc, Firefly luciferase.

CrBOff system impact on general gene expression levels

In order to verify that the lower expression levels under blue light are due to specific binding of the CrBOff system to its target sequence, and not to undesired general repression effects on gene expression, the CrBOff light switch was transformed in 4 independent lines harbouring the target module of CrBOff lacking the binding sequence of EL222 (C120). The evaluation of the luciferase ratio under blue and red light for 94 clones of each transformation event confirms that the repression activity of the CrBOff system is due to the specific binding of EL222 to its cognate DNA sequence (Fig. 22).

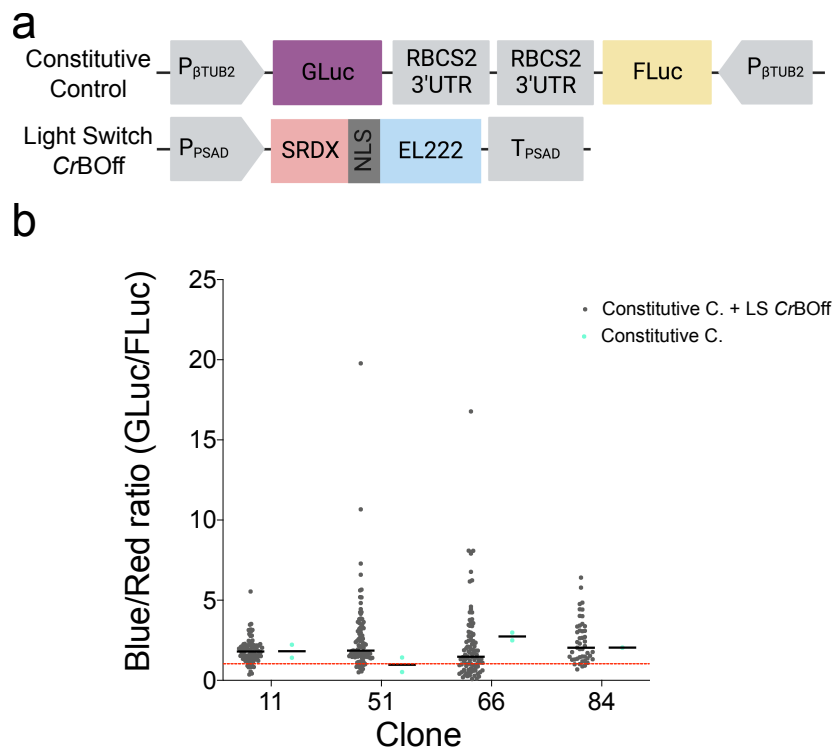


Figure 22. Specificity of the blue-light repression system in *C. reinhardtii* (CrBOff). (a) Transformed constructs scheme. Selected UVM4 clones harbouring the target module of the CrBOff system lacking the recognition sequence of EL222 (Constitutive control lines; pOD341) were transformed with the light-switch of CrBOff (pOD235). (b) For each constitutive control line (11, 51, 66, 84), 94 clones transformed with the CrBOff light-switch were illuminated with blue or red light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. Subsequently, GLuc and FLuc activities were measured and normalized to OD_{750} . The ratio between the normalized GLuc and FLuc was calculated. Comparison of the light-switch harbouring clones and the constitutive control lines is shown. The median of the blue/red ratio from all the clones is plotted ($n=94$). For the constitutive control clones, two replicates were measured ($n=2$). $P_{\beta TUB2}$, $\beta 2$ -tubulin promoter; P_{PSAD} , PsAD promoter; NLS, nuclear localization sequence; T_{PSAD} , PsAD terminator; LS, light switch; constitutive C., constitutive control; SRDX, SRDX repressor domain; EL222, *E. litoralis* photoreceptor; RBCS2 3'UTR, 3'-untranslated region of the Rubisco subunit 2 gene; GLuc, Gaussia luciferase; FLuc, Firefly luciferase.

CrBOff-controlled light-dependent resistance to canavanine, a novel selection marker in *C. reinhardtii*

It has been proposed that the gene *AOC5* (Cre07.g329050), which encodes a cationic amino acid transporter, may be involved in the import of both arginine and canavanine (Vallon & Spalding, 2009). Canavanine, although structurally similar to arginine, disrupts proper protein folding when introduced as an analog into proteins, resulting in cell death in wild type cells when exposed to it. In order to confirm the involvement of *AOC5* in canavanine transport, wild type and *AOC5* knockout mutant cells were fed with different concentrations of canavanine (Fig. 23a). Our findings indicate that the *aoc5* strain shows insensitivity to canavanine compared to its wild type genetic background, confirming the role of *AOC5* in canavanine transport and suggesting its potential use as a selection marker in *C. reinhardtii*.

Furthermore, we aimed to regulate canavanine resistance with light by placing the *AOC5* gene under the control of the *CrBOff* system (*CrBOff-AOC5*; Fig. 23b) to create a blue-light-induced conditional mutant capable of growing in the presence of canavanine solely under blue light conditions (Fig. 23c).

To accomplish this, the *aoc5* mutant was transformed with the *CrBOff-AOC5* construct (pOD364). For one of the transgenic clones resulting from the transformation and the *aoc5* mutant, growth was evaluated under both red and blue light conditions, across various concentrations of canavanine (Fig. 23d). It was observed that even low concentrations of canavanine (0.005 and 0.1 mg/mL) were sufficient to hinder growth under red light, with growth recovery occurring under blue light. This behaviour was not observed in the *aoc5* strain, which exhibited growth unaffected by light conditions in the presence of canavanine.

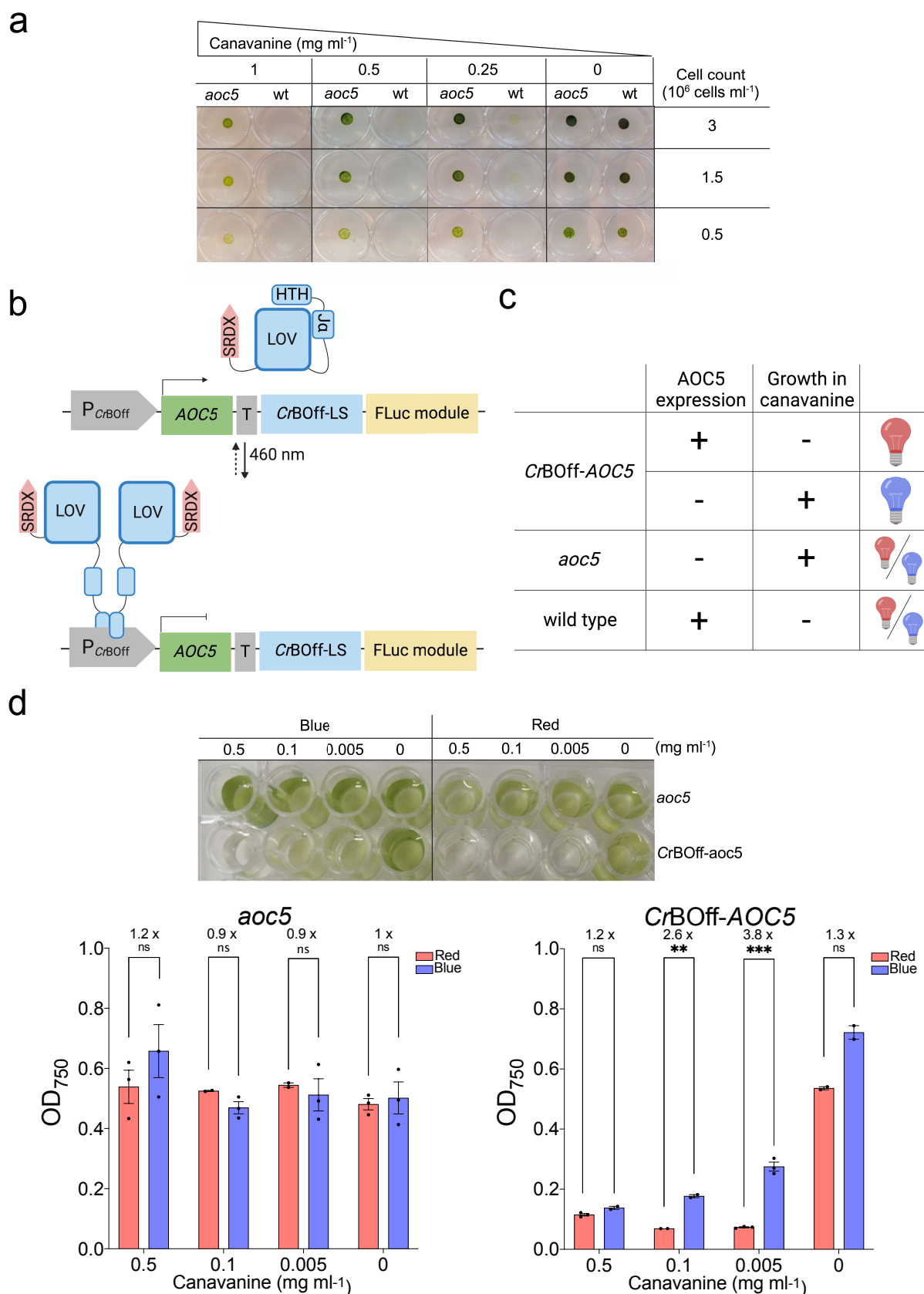


Figure 23. Establishing and light-controlling a novel selection marker in *C. reinhardtii*. (a) Growth of *C. reinhardtii* wildtype and an AOC5 knockout mutant spotted on agar medium with varying concentrations of canavanine (0, 0.25, 0.5, 1 mg/mL) after 72 h under constant white light. (b) *CrBOff*-controlled resistance against canavanine. The AOC5 gene, placed under the control of the *CrBOff*

system, is repressed upon blue light illumination. **(c)** Expected growth and AOC5 expression of *CrBOff-AOC5* (*aoc5* mutant transformed with pOD364; clone 9), *aoc5* mutant, and wild type strains under blue or red light conditions. **(d)** Light experiment comparing the growth of the *aoc5* mutant and a *CrBOff-AOC5* clone (*aoc5* mutant transformed with pOD364; clone 9) at different canavanine concentrations (0, 0.005, 0.1, 0.5 mg/mL). Cultures were maintained in darkness for 1 day before preparing new cultures with a final concentration of 1×10^6 cells/mL. These cultures were divided into two 12-well plates (2 mL/well), with 20 μ L of different canavanine stock solutions added to the wells. Cultures were then incubated under red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 48 h before picture acquisition and OD₇₅₀ measurement. Upper part, overview picture of the cultures. Lower part, mean and SEM of OD₇₅₀ from three independent algae cultures. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (multiple *t*-test). AOC5, cationic amino acid transporter; P_{CrBOff}, optogenetic promoter of the *CrBOff* system; *CrBOff*-LS, light switch module of the *CrBOff* system; FLuc module, firefly normalization module; T, terminator; SRDX, SRDX repressor domain; HTH, helix-turn-helix; LOV, Light-Oxygen-Voltage; wt, wild type.

Discussion

Chlamydomonas reinhardtii is a well-studied model organism with significant potential for biotechnological applications and fundamental research (Arias et al., 2023; Harris, 2001; Masi et al., 2023; Salomé & Merchant, 2019; Scranton et al., 2015). The sequencing of its three genomes, the development of the MoClo kit and the CLiP mutant library, and the availability of protocols for genomic manipulation, make it an ideal chassis for fundamental research, and biotechnological and synthetic biology applications (Boynton & Gillham, 1993; Crozet et al., 2018; Li et al., 2015; Kindle, 1990; Merchant et al., 2007; Remacle et al., 2006). Numerous studies require precise control over biological processes, such as gene expression. However, in *C. reinhardtii*, there are few tools available for controlling gene expression, primarily relying on chemicals or temperature changes, which provide limited spatiotemporal control and hinder the possibility of conducting more advanced studies (Beltran-Aguilar et al., 2019; Ferrante et al., 2008; Kim et al., 2020; Koblenz & Lechtreck, 2005; Lee et al., 2018; Ohresser et al., 1997). Optogenetic gene expression systems address these limitations by using light as an inducer, offering high quantitative and spatial resolution, as well as reversibility (Omelina et al., 2022). Optogenetic tools for controlling gene expression have been widely used in various model organisms, including mammalian cells, yeast and bacteria (Müller et al., 2015; Ohlendorf & Möglich, 2022; Pérez et al., 2022; Salinas et al., 2017; Yamada et al., 2020). However, challenges have been encountered when implementing light-inducible systems in photosynthetic organisms due to their reliance on light for survival (Christie & Zurbriggen, 2021). In 2020, the establishment of the PULSE system in *A. thaliana* marked a significant advancement in enabling light-controlled gene expression in plants growing under white light (Ochoa-Fernandez et al., 2020). Despite the advantage of *C. reinhardtii*'s ability to grow heterotrophically in dark conditions, facilitating the use of optogenetic systems, only one blue-light regulated optogenetic system has been developed in this microalga to date. This system controls the activation of an artificial miRNA with light; nevertheless, its potential to induce protein-coding gene expression in *C. reinhardtii* has not been demonstrated (Wang et al., 2017). Therefore, there is a need for optogenetic systems that demonstrate control over protein-coding gene expression as well as systems to repress gene expression.

This study aims to lay the groundwork for the efficient and standardized implementation and evaluation of optogenetic tools for regulating gene expression in *C. reinhardtii*. The key steps involved include the engineering of an Opto-toolkit compatible with the MoClo kit for *C. reinhardtii*, quantitative analysis of different reporters, activity and light-sensitivity

characterization of certain promoters, optimization and performance assessment of a dual luciferase assay, identification of optimal light conditions for testing the functionality of new blue-light regulated optogenetic system, and analysis of minimal promoters and enhancers required for constructing optogenetic promoters in *C. reinhardtii*.

Our findings revealed that NLuc exhibited the highest sensitivity among the reporters tested, followed by GLuc. RLuc and FLuc activities were generally lower, potentially complicating the distinction between clones expressing low values from background. Dual luciferase assays, in which two luciferases utilizing different substrates are simultaneously measured, are very convenient when examining gene expression regulation or implementing inducible systems, since one of the luciferases can serve as a control for normalizing the assay. Here, we optimized the dual luciferase assay specifically for *C. reinhardtii*. FLuc, the only luciferase capable of utilizing luciferin instead of coelenterazine, was combined with GLuc. NLuc could also have been used, however we recommend using a lower substrate concentration to prevent saturation of the luminescence plate reader.

For the characterization of optogenetic systems, we used and advocate for the implementation of FLuc for normalization due to its lower sensitivity, and GLuc for monitoring the activity of the inducible or repressible promoter. Secreted GLuc and non-secreted FLuc were employed, since our findings confirm the appropriate secretion of GLuc into the media when fused to a secretion signal, whereas incomplete secretion of FLuc was observed when fused to the same secretion signal.

Furthermore, we investigated the orientation of the normalization module (FLuc) relative to the GLuc module and discovered that reverse orientation resulted in a higher number of transformants expressing both luciferases. This empirical observation suggests that this orientation may be preferable when encoding more than one transcriptional unit in a single plasmid.

The choice of promoters is critical for the dynamic range of optogenetic systems, including both the optogenetic promoter and the promoter responsible for driving the expression of components forming the light-switch. We observed that the CaMV 35S promoter exhibited lower activity in *C. reinhardtii*, as shown before (Díaz-Santos et al., 2013; Ruecker et al., 2008; Schroda, 2019), compared to the endogenous promoters β 2-tubulin and PsuD, and the synthetic AR promoter. Additionally, we found that promoter activity may vary depending on the downstream gene; this observation aligns with what was described by Schroda in 2019, based on findings previously reported by other researchers (Schroda, 2019). On the other hand,

promoter activity was generally higher in blue light than in red light for most of the screened transformants, which might indicate a light-dependent activity of the promoters evaluated; this difference in activity within the two light conditions was less pronounced when the β TUB2 promoter was used. Based on these findings, the β 2-tubulin promoter was selected for engineering optogenetic promoters. To achieve this, its minimal and core promoter regions were defined and characterized. Our data revealed that regions defined as the minimal promoter, including the TATA box, and the enhancer region alone were insufficient to drive high levels of gene expression, which are achieved when both regions are combined. Furthermore, we generated two weaker synthetic promoters derived from the β TUB2 promoter by depleting certain parts of the enhancer region.

During the characterization of optogenetic systems in organisms like *C. reinhardtii*, that undergo divisions during the experiment and which growth is affected by light, it is crucial to incorporate normalization elements, robust controls, and minimize differences resulting from growing in different light conditions, in order to be able to distinguish between alterations in total expression levels attributable to the inducible system or to variations in cell number and energy status. Our findings demonstrate that $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red or blue light elicits similar effects on growth when a carbon source, acetate, is present. Additionally, our data also suggests that analysing the activity of the optogenetic systems after 24 h, and preferably no later than 48 hours, is advisable, as changes in PSII quantum yield and chlorophyll content accumulates with time.

Moreover, we developed a 2-step workflow for testing of optogenetic systems to control gene expression in *C. reinhardtii*, involving independent transformation of the target and light-switch components, and several screening steps under different light wavelengths. The independent transformation offers the possibility of using the strain containing only the target as a control.

As a proof of concept, we employed the generated optogenetic toolbox to construct a blue-light repression system for *C. reinhardtii* (*CrBOff*) and followed the 2-step workflow for its testing and establishment in the microalga. In *CrBOff*, the EL222 photoreceptor binds its target sequence, C120, in the presence of blue light, repressing gene expression due to its fusion to a repression domain. We evaluated two repression domains, SRDX from *A. thaliana* and KRAB from humans, finding only the first one to be active in *C. reinhardtii*. The engineering and optimization of the *CrBOff* system were conducted based on the design-build-test-learn cycle, involving the design, construction, testing, and iterative refinement of different plasmids

encoding different versions of the system until the final *CrBOff* construct was obtained (Table S3). The final *CrBOff* system achieved repression rates of up to 8.1-fold, comparable to those obtained with the corresponding *BOff* system in *A. thaliana*, and is also suitable for scaled-up cultures cultivated in photobioreactors, the main production method used in Europe for microalgae production (71 %, compared to 19 % in ponds and 10 % in fermenters; Kuech, Breuer and Popescu, 2023). Additionally, the *CrBOff* optogenetic promoter underwent additional engineering to facilitate its use when lower levels of transgene expression or complete gene expression shutdown are required.

On top of this, we established a novel selection marker in *C. reinhardtii* based on resistance to arginine analog canavanine in cells lacking the *AOC5* gene. Furthermore, we combined this marker with the *CrBOff* system to induce drug resistance, and therefore growth in the presence of canavanine, exclusively under the presence of blue light, when the expression of *AOC5* is reduced. The *CrBOff-AOC5* line could be employed when controlled growth is required or for investigating the early effects of misfolded protein accumulation on protein homeostasis in *C. reinhardtii* (Schroda et al., 2015; Work & Brandman, 2021).

In summary, this study establishes the foundations for the implementation of optogenetic tools to regulate gene expression in *C. reinhardtii*. It provides valuable insights into promoter activity, the importance of transcriptional unit orientation within plasmids, and presents a workflow and necessary tools for future research and development of optogenetics in *C. reinhardtii*.

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Supplementary information

Table S1. Plasmids design and used. All the pCM0 plasmids are from the *C. reinhardtii* MoClo-kit (Crozet et al., 2018). pUPD2, pDGB and GB plasmids were acquired from the Golden Braid collection (Sarrion-Perdigones et al., 2013; <https://gbcloning.upv.es>). ChRe, genes coming from *C. reinhardtii*; ChRe+, genes codon optimized for *C. reinhardtii*.

ID	Description and cloning procedure	Insert	Backbone	Reference
pOD004	(etr)₈-PhCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pROF053, pROF060, pCM0-051, pCM0-082, pCM0-114	pDGB 3alpha1	This work
pOD008	Par::SPcCA-NLUC(ChRe+)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pCM0-078 with oligos oOD031 and oOD032. Backbone was digested with AatII and EcoRV. Both fragments were assembled using AQUA cloning.	NLUC(ChRe+)	pOpt_gLuc_Hyg	This work
pOD017	Pβtub2min (Pos. A2-B1) Insert was amplified from pCM0-021 with oligos oOD007 and oOD008. BsmBI restriction-ligation reaction (GoldenBraid).	Pβtub2min	pUPD2	This work
pOD034	HygR(APHVII)#noSTOPcodon (Pos. B3) Insert was amplified from pOpt_gLuc_Hyg with oligos oOD096 and oOD050. BsmBI restriction-ligation reaction (GoldenBraid).	HygR(APHVII) without STOP codon	pUPD2	This work
pOD037	HygR(APHVII)#noSTOPcodon (Pos. B1) Insert was amplified from pOpt_gLuc_Hyg with oligos oOD097 and oOD052. BsmBI restriction-ligation reaction (GoldenBraid).	HygR(APHVII) without STOP codon	pUPD2	This work
pOD038	F2A(ChRe+) (Pos. B2) Insert was amplified from pCM0-087 with oligos oOD053 and oOD054. BsmBI restriction-ligation reaction (GoldenBraid).	F2A (ChRe+)	pUPD2	This work
pOD039	ChR2[p.1-315](ChRe) (Pos. B4) Insert was amplified from <i>C. reinhardtii</i> gDNA with oligos oOD055, oOD056, oOD057 and oOD058. BsmBI restriction-ligation reaction (GoldenBraid).	ChR2[p.1-315]	pUPD2	This work
pOD043	Par::RLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pAES14 with oligos oOD092 and oOD093. Backbone was digested with NdeI and EcoRV. Both fragments were assembled using AQUA cloning.	RLUC(ChRe+)	pOpt_gLuc_Hyg	This work
pOD044	F2A(ChRe+) (Pos. B2-B3) Insert was amplified from pCM0-087 with oligos oOD053 and oOD241. BsmBI restriction-ligation reaction (GoldenBraid).	F2A(ChRe+)	pUPD2	This work
pOD045	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+) (Pos. B3-B5) Insert was amplified from 20AD5RLP with oligos oOD177 and oOD187. BsmBI restriction-ligation reaction (GoldenBraid).	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)	pUPD2	This work
pOD046	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+) (Pos. B3-B5)	E(ChRe+)-PIF6(ChRe+)-	pUPD2	This work

	Insert was amplified from 20AD5RKP with oligos oOD107 and oOD119. BsmBI restriction-ligation reaction (GoldenBraid).	NLS(SV40)(ChRe+)		
pOD047	FLUC(ChRe+) (Pos. B3-B5) Insert was amplified from 20AD5RJP with oligos oOD091, oOD106, oOD077 and oOD090. BsmBI restriction-ligation reaction (GoldenBraid).	FLUC(ChRe+)	pUPD2	This work
pOD048	PIP(ChRe+) (Pos. B3) Insert was amplified from 20AD5RIP with oligos oOD188 and oOD195. BsmBI restriction-ligation reaction (GoldenBraid).	PIP(ChRe+)	pUPD2	This work
pOD049	SEAP(ChRe+) (Pos. B3-B5) Insert was amplified from 20AD5RHP with oligos oOD139, oOD140, oOD142, oOD138, oOD141 and oOD158. BsmBI restriction-ligation reaction (GoldenBraid).	SEAP(ChRe+)	pUPD2	This work
pOD050	PHYB(ChRe+) (Pos. B3) Insert was amplified from 20AD5RGP with oligos oOD122, oOD137, oOD120 and oOD121. BsmBI restriction-ligation reaction (GoldenBraid).	PHYB(ChRe+)	pUPD2	This work
pOD051	VP16(ChRe+) (Pos. B4) Insert was amplified from 20AD5RFP with oligos oOD160 and oOD166. BsmBI restriction-ligation reaction (GoldenBraid).	VP16(ChRe+)	pUPD2	This work
pOD052	KRAB(ChRe+) (Pos. B3) Insert was amplified from 20AD5REP with oligos oOD196 and oOD203. BsmBI restriction-ligation reaction (GoldenBraid).	KRAB(ChRe+)	pUPD2	This work
pOD053	TetR(ChRe+) (Pos. B3) Insert was amplified from 20AD5RDP with oligos oOD171, oOD167, oOD168, oOD169, oOD170 and oOD176. BsmBI restriction-ligation reaction (GoldenBraid).	TetR(ChRe+)	pUPD2	This work
pOD054	GAL4(ChRe+)-LOVpep(ChRe+) (Pos. B3) Insert was amplified from 20AD5SAP with oligos oOD204 and oOD216. BsmBI restriction-ligation reaction (GoldenBraid).	GAL4(ChRe+)-LOVpep(ChRe+)	pUPD2	This work
pOD055	ePDZb(ChRe+) (Pos. B3) Insert was amplified from 20AD5R7P with oligos oOD210 and oOD224. BsmBI restriction-ligation reaction (GoldenBraid).	ePDZb(ChRe+)	pUPD2	This work
pOD056	UVR8(ChRe+) (Pos. B2) Insert was amplified from 20AD7EEP with oligos oOD222 and oOD234. BsmBI restriction-ligation reaction (GoldenBraid).	UVR8(ChRe+)	pUPD2	This work
pOD058	COP1(ChRe+) (Pos. B4) Insert was amplified from 20AD7ECP with oligos oOD063 and oOD076. BsmBI restriction-ligation reaction (GoldenBraid).	COP1(ChRe+)	pUPD2	This work
pOD059	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from 20AD5RLP with oligos oOD177, oOD181, oOD183, oOD184, oOD186 and oOD187. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD069, oOD182, oOD185 and oOD156. BsmBI restriction-ligation reaction (GoldenBraid).	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work

pOD061	VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B4-B5) Exons were amplified from 20AD5RFP with oligos oOD242, oOD161, oOD162, oOD163, oOD165 and oOD245. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD124, oOD131, oOD164 and oOD135. BsmBI restriction-ligation reaction (GoldenBraid).	VP16(ChRe+)-NLS(SV40)(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD062	PHYB(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from pOD050 with oligos oOD120, oOD126, oOD129, oOD133, oOD136 and oOD137. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD127, oOD128, oOD134 and oOD135. BsmBI restriction-ligation reaction (GoldenBraid).	PHYB(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD065	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from 20AD5RKP with oligos oOD107, oOD108, oOD110, oOD115, oOD118 and oOD119. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD109, oOD100, oOD116 and oOD117. BsmBI restriction-ligation reaction (GoldenBraid).	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD066	FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from pOD047 with oligos oOD077, oOD082, oOD085, oOD102, oOD105 and oOD106. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD083, oOD084, oOD103 and oOD104. BsmBI restriction-ligation reaction (GoldenBraid).	FLUC(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD067	SEAP(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from pOD049 with oligos oOD142, oOD147, oOD150, oOD154, oOD157 and oOD158. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD148, oOD113, oOD155 and oOD156. BsmBI restriction-ligation reaction (GoldenBraid).	SEAP(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD068	SPcCA-RLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Insert was amplified from pAES14 with oligos oOD247, oOD248, oOD249 and oOD250. BsmBI restriction-ligation reaction (GoldenBraid).	SPcCA-RLUC(ChRe+)	pUPD2	This work
pOD071	E(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3) Insert was amplified from pOD065 with oligos oOD251 and oOD252. BsmBI restriction-ligation reaction (GoldenBraid).	E(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pUPD2	This work
pOD082	FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B5) Insert was amplified from pOD092 with oligos oOD253 and oOD106. BsmBI restriction-ligation reaction (GoldenBraid).	FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pDGB pUPD2	This work
pOD091	FLUC(ChRe+) (Pos. B3-B5) Insert was amplified from 20AD5RJP with oligos oOD254, oOD077, oOD106 and	FLUC(ChRe+)	pUPD2	This work

	oOD255. BsmBI restriction-ligation reaction (GoldenBraid).			
pOD092	FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Exons were amplified from pOD091 with oligos oOD077, oOD082, oOD085, oOD102, oOD105 and oOD106. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD083, oOD084, oOD103 and oOD104. BsmBI restriction-ligation reaction (GoldenBraid).	FLUC(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD106	SEAP(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B5) Insert was amplified from pOD067 with oligos oOD266 and oOD158. BsmBI restriction-ligation reaction (GoldenBraid).	SEAP(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pUPD2	This work
pOD108	SPcCA(ChRe+) (Pos. B3-B4) Insert was amplified from pCM0-089 with oligos oOD268 and oOD269. BsmBI restriction-ligation reaction (GoldenBraid).	SPcCA	pUPD2	This work
pOD112	HygR(APHVII)#noSTOPcodon (Pos. B3) Insert was amplified from pCM0-092 with oligos oOD043 and oOD050. BsmBI restriction-ligation reaction (GoldenBraid).	HygR(APHVII) without STOP codon	pUPD2	This work
pOD113	HygR(APHVII)#noSTOPcodon (Pos. B1) Insert was amplified from pCM0-092 with oligos oOD051 and oOD052. BsmBI restriction-ligation reaction (GoldenBraid).	HygR(APHVII) without STOP codon	pUPD2	This work
pOD114	MIC10(ChRe)#noSTOPcodon (Pos. B3) Insert was amplified from cDNA with oligos oOD270, oOD271, oOD272 and oOD273. BsmBI restriction-ligation reaction (GoldenBraid).	CrMIC10 without STOP codon	pUPD2	This work
pOD115	Par::HygR(APHVII)-F2A(ChRe+)-MIC10(ChRe)-ChR2[p.1-315](ChRe)-mVenus(ChRe)::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-008, pOD113, pOD038, pOD114, pOD039, pCM0-102, pCM0-114	pDGB 3alpha1	This work
pOD119	Par (Pos. A1) Insert was amplified from pCM0-008 with oligos oOD274 and oOD275. BsmBI restriction-ligation reaction (GoldenBraid).	AR promoter	pUPD2	This work
pOD120	Par::HygR(APHVII)-F2A(ChRe+)-SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-008, pOD113, pOD038, pOD059, pCM0-114	pDGB 3alpha2	This work
pOD123	Par-(C120)_s-PhCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pOD119, pROF054, pROF059, pOD108, pCM0-107, pCM0-114	pDGB 3alpha1	This work
pOD124	Par-(C120)_s-PhCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.2}::Tpsad Par::HygR(APHVII)-F2A(ChRe+)-SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad	pOD123, pOD120	pDGB 3omega1	This work

	BsmBI restriction-ligation reaction (GoldenBraid).			
pOD125	P35S::HygR(APHVII)-F2A(ChRe+)-GLUC(ChRe+)-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	GB0030, pOD112, pCM0-087, pCM0-107, pCM0-114	pDGB 3alpha1	This work
pOD127	Pβtub2::HygR(APHVII)-F2A(ChRe+)-GLUC(ChRe+)-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-021, pOD112, pCM0-087, pCM0-107, pCM0-114	pDGB 3alpha1	This work
pOD128	Ppsad::HygR(APHVII)-F2A(ChRe+)-GLUC(ChRe+)-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-016, pOD112, pCM0-087, pCM0-107, pCM0-114	pDGB 3alpha1	This work
pOD129	PIP(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3) Exons were amplified from 20AD5RIP with oligos oOD188, oOD189, oOD190, oOD191, oOD194 and oOD195. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD192, oOD113, oOD185 and oOD193. BsmBI restriction-ligation reaction (GoldenBraid).	PIP(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD130	SPcCA-FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Insert was amplified from pOD082 with oligos oOD278 and oOD106. BsmBI restriction-ligation reaction (GoldenBraid).	SPcCA- FLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pUPD2	This work
pOD131	SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Insert was amplified from pOpt_gLuc_Hyg with oligos oOD268 and oOD280. BsmBI restriction-ligation reaction (GoldenBraid).	SPcCA- GLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pUPD2	This work
pOD132	SPcCA-SEAP(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5) Insert was amplified from pOD106 with oligos oOD279 and oOD158. BsmBI restriction-ligation reaction (GoldenBraid).	SPcCA- SEAP(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pUPD2	This work
pOD136	Par::HygR(APHVII)-F2A(ChRe+)-MIC10(ChRe)-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-mVenus(ChRe+)::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-008, pOD113, pOD038, pOD114, MAC synthesized by Genearth Thermofisher in pos. B4, pCM0- 102, pCM0-114	pDGB 3alpha1	This work
pOD139	KRAB(ChRe+)-{Irbcs2.2} (Pos. B3) Exons were amplified from 20AD5REP with oligos oOD196, oOD200, oOD202 and oOD203. Intron was amplified from pOpt_gLuc_Hyg with oligos oOD134 and oOD201. BsmBI restriction-ligation reaction (GoldenBraid).	KRAB(ChRe+) with intron 2 from Rbcs2	pUPD2	This work
pOD140	TetR(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3) Exons were amplified from 20AD5RDP with oligos oOD171, oOD172, oOD173, oOD174, oOD175 and oOD176. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD124,	TetR(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work

	oOD100, oOD116 and oOD135. BsmBI restriction-ligation reaction (GoldenBraid).			
pOD141	GAL4(ChRe+)-LOVpep(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3) Exons were amplified from 20AD5SAP with oligos oOD204, oOD208, oOD211, oOD212, oOD215 and oOD216. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD209, oOD113, oOD213 and oOD214. BsmBI restriction-ligation reaction (GoldenBraid).	GAL4(ChRe+)-LOVpep(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD142	ePDZb(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3) Exons were amplified from 20AD5R7P with oligos oOD210, oOD217, oOD220, oOD221, oOD223 and oOD224. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD185, oOD156, oOD218 and oOD219. BsmBI restriction-ligation reaction (GoldenBraid).	ePDZb(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD143	UVR8(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B2) Exons were amplified from 20AD7EEP with oligos oOD222, oOD226, oOD229, oOD230, oOD233 and oOD234. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD227, oOD113, oOD231 and oOD232. BsmBI restriction-ligation reaction (GoldenBraid).	UVR8(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD144	P65(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B5) Exons were amplified from 20AD7EDP with oligos oOD228, oOD235, oOD236, oOD239, oOD243 and oOD244. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD083, oOD100, oOD240 and oOD135. BsmBI restriction-ligation reaction (GoldenBraid).	P65(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD145	COP1(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B4) Exons were amplified from 20AD7ECP with oligos oOD063, oOD064, oOD067, oOD072, oOD075 and oOD076. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD065, oOD066, oOD185 and oOD074. BsmBI restriction-ligation reaction (GoldenBraid).	COP1(ChRe+) with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD151	(UAS)₅-PhCMVmin (Pos. A1-B2) Insert was amplified from pKT136 with oligos oOD295 and oOD293. BsmBI restriction-ligation reaction (GoldenBraid).	(UAS) ₅ -PhCMVmin	pUPD2	This work
pOD152	(UAS)₅ (Pos. A1-A2) Insert was amplified from pKT136 with oligos oOD295 and oOD296. BsmBI restriction-ligation reaction (GoldenBraid).	(UAS) ₅	pUPD2	This work
pOD175	Ppsad::ChR2[p.1-315](ChRe)-GFP(ChRe+):Tpsad Par::ParoR(APHVIII):3'UTRrbc2 Insert was amplified from pOD115 with oligos oOD313 and oOD299. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.	ChR2[p.1-315]	pJR38	This work
pOD177	Ppsad::MIC10(ChRe)-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-	CrMIC10-MAC(ChRe+)	pJR38	This work

	GFP(ChRe+):Tpsad Par::ParoR(APHVIII):3'UTRrbc2 Insert was amplified from pOD136 with oligos oOD314 and oOD303. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.			
pOD179	Par::SPcCA-FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}:3'UTRrbc2 Par::HygR(APHVII):3'UTRrbc2 Insert was amplified from pOD082 with oligos oOD333 and oOD334. Backbone was digested with AatII and EcoRV. Both fragments were assembled using AQUA cloning.	FLUC(ChRe+)	pOpt_gLuc_Hyg	This work
pOD184	Par::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}:3'UTRrbc2 Par::HygR(APHVII):3'UTRrbc2 Insert was amplified from pOD120 with oligos oOD321 and oOD322. Backbone was digested with NdeI and EcoRV. Both fragments were assembled using AQUA cloning.	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pOpt_gLuc_Hyg	This work
pOD186	(PIR)₃ (Pos. A1) Insert was amplified from pKT365 with oligos oOD326 and oOD327. BsmBI restriction-ligation reaction (GoldenBraid).	(PIR) ₃	pUPD2	This work
pOD195	(C120)₅-PhCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}:3'UTRrbc2 Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}:Tpsad Par::HygR(APHVII):3'UTRrbc2 Insert was amplified from pROF021 with oligos oOD361 and oOD362. Backbone was digested with NdeI and NotI. Both fragments were assembled using AQUA cloning.	(C120) ₅ -PhCMVmin	pOD203	This work
pOD196	Ppsad::MIC10(ChRe)-GFP(ChRe+):Tpsad Par::ParoR(APHVIII):3'UTRrbc2 Insert was amplified from pOD177 with oligos oOD350 and oOD351. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.	CrMIC10	pOD175	This work
pOD198	Par (Pos. A1-B3) Insert was amplified from pCM0-008 with oligos oOD353 and oOD354. BsmBI restriction-ligation reaction (GoldenBraid).	AR promoter	pUPD2	This work
pOD199	NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B6) Insert was amplified from pOD184 with oligos oOD355 and oOD356. BsmBI restriction-ligation reaction (GoldenBraid).	NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pUPD2	This work
pOD200	VP16(ChRe+) (Pos. B4-B5) Insert was amplified from pOD061 with oligos oOD357 and oOD358. BsmBI restriction-ligation reaction (GoldenBraid).	VP16(ChRe+)	pUPD2	This work
pOD201	Tpsad (Pos. C1) Insert was amplified from pCM0-114 with oligos oOD359 and oOD360. BsmBI restriction-ligation reaction (GoldenBraid).	PsaD terminator	pUPD2	This work

pOD202	Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pOD198, pOD200, pOD199, pOD201	pDGB 3alpha1	This work
pOD203	Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD202 with oligos oOD377 and oOD378. Backbone was digested with NruI and BamHI. Both fragments were assembled using Gibson cloning.	Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad	pOpt_gLuc _Hyg	This work
pOD204	Par::SPcCA-FLUC(ChRe+)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD047 with oligos oOD333 and oOD334. Backbone was digested with AatII and EcoRV. Both fragments were assembled using AQUA cloning.	FLUC(ChRe+)	pOpt_gLuc _Hyg	This work
pOD205	P35S::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD125 with oligos oOD363 and oOD364. Backbone was digested with NdeI and SpeI. Both fragments were assembled using AQUA cloning.	35S promoter	pOpt_gLuc _Hyg	This work
pOD206	Pβtub2::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Insert was amplified from pOD127 with oligos oOD365 and oOD366. Backbone was digested with NdeI and SpeI. Both fragments were assembled using AQUA cloning.	βTUB2 promoter	pOpt_gLuc _Hyg	This work
pOD207	Ppsad::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Insert was amplified from pOD128 with oligos oOD367 and oOD368. Backbone was digested with NdeI and SpeI. Both fragments were assembled using AQUA cloning.	PsaD promoter	pOpt_gLuc _Hyg	This work
pOD216	Par::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pOD059, pCM0-114	pDGB 1alpha1	This work
pOD217	Pβtub2min (Pos. A3-B2) Insert was amplified from pCM0-021 with oligos oOD387 and oOD388. BsmBI restriction-ligation reaction (GoldenBraid).	βTUB2 minimal promoter	pUPD2	This work
pOD218	Pβtub2enh (Pos. A1) Insert was amplified from pCM0-021 with oligos oOD389, oOD390, oOD391 and oOD392. BsmBI restriction-ligation reaction (GoldenBraid).	βTUB2 proximal promoter	pUPD2	This work
pOD220	Pβtub2enh-(C120)₅-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pOD218, pROF054, pOD217, pOD108, pCM0-107, pCM0-114	pDGB 1alpha1	This work
pOD225	(C120)₅-PhCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-	SRDX(ChRe+)-NLS(SV40)(Ch	pOD195	This work

	{Irbcs2.2}::3'UTRrbc2 Par::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD216 with oligos oOD399 and oOD400. Backbone was digested with AgeI and SbfI. Both fragments were assembled using AQUA cloning.	Re+)-EL222(ChRe+): :Tpsad		
pOD226	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Par::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+):Tpsad Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD220 with oligos oOD401 and oOD402. Backbone was digested with NotI and NdeI. Both fragments were assembled using AQUA cloning.	Pβtub2enh-(C120) _s -Pβtub2min	pOD225	This work
pOD227	Ppsad::MIC10(ChRe)-spacer-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-HAtag-spacer-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD177 with oligos oOD403 and oOD404. Backbone was digested with NdeI. Both fragments were assembled using AQUA cloning.	Spacer-MAC	pOD196	This work
pOD228	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+):Tpsad Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD196 with oligos oOD405 and oOD406. Backbone was digested with AgeI and BamHI. Both fragments were assembled using Gibson cloning.	PsaD promoter	pOD226	This work
pOD234	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 MCS Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD227 with oligos oOD410 and oOD411. Backbone was digested with BamHI and SbfI. Both fragments were assembled using AQUA cloning.	MCS (Multiple cloning site)	pOD226	This work
pOD235	Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD216 with oligos oOD412 and oOD414. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)	pJR38	This work
pOD242	EcoRV-NLUC(ChRe+)-EcoRV (Pos. B2-B5) Insert was amplified from pOD236 with oligos oOD423 and oOD424. BsmBI restriction-ligation reaction (GoldenBraid).	EcoRV-NLUC(ChRe+)-EcoRV	pUPD2	This work
pOD245	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-HAtag (Pos. B3-B5)	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(Ch	pUPD2	This work

	Insert was amplified from pOD238 with oligos oOD426 and oOD427. BsmBI restriction-ligation reaction (GoldenBraid).	Re+)- {Irbcs2.1}- {Irbcs2.2}- HAtag		
pOD247	PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-HAtag (Pos. B3-B5) Insert was amplified from pOD063 with oligos oOD428 and oOD429. BsmBI restriction-ligation reaction (GoldenBraid).	PHYB(ChRe+)- {Irbcs2.1}- {Irbcs2.2}- VP16(ChRe+)- NLS(SV40)(Ch Re+)- {Irbcs2.1}- {Irbcs2.2}- HAtag	pUPD2	This work
pOD253	Pβtub2::FLUC(v2)::3'UTRrbc2 Insert was amplified from L0-wtFLUC with oligos oOD432 and oOD433. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	FLUC(v2). FLUC with 3 introns	pOD206	This work
pOD254	Pβtub2::FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Insert was amplified from pOD179 with oligos oOD432 and oOD434. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	FLUC(v1): FLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pOD206	This work
pOD276	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+):Tpsad Pβtub2::FLUC(v2)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD435 and oOD436. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD228	This work
pOD280	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Pβtub2::FLUC(v2)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD490 and oOD436. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD234	This work
pOD282	SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}#noSTOPcodon (Pos. B3-B4) Insert was amplified from pOD059 with oligos oOD458 and oOD459. BsmBI restriction-ligation reaction (GoldenBraid).	SRDX(ChRe+)- NLS(SV40)(Ch Re+)- EL222(ChRe+)- {Irbcs2.1}- {Irbcs2.2} without STOP codon	pUPD2	This work
pOD284	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}#noSTOPcodon (Pos. B3-B4) Insert was amplified from pOD065 with oligos oOD426 and oOD460. BsmBI restriction-ligation reaction (GoldenBraid).	E(ChRe+)- PIF6(ChRe+)- NLS(SV40)(Ch Re+)- {Irbcs2.1}- {Irbcs2.2}	pUPD2	This work

		without STOP codon		
pOD286	PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}#noSTOPcodon (Pos. B3-B4) Insert was amplified from pOD063 with oligos oOD428 and oOD460. BsmBI restriction-ligation reaction (GoldenBraid).	PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2} without STOP codon	pUPD2	This work
pOD295	ePDZb(ChRe+)-{Irbcs2.1} (Pos. B3) Exons were amplified from pOD055 with oligos oOD210, oOD217, oOD220 and oOD224. Intron was amplified from pOpt_gLuc_Hyg with oligos oOD218 and oOD219. BsmBI restriction-ligation reaction (GoldenBraid).	ePDZb(ChRe+) with intron 1 from rbc2	pUPD2	This work
pOD302	Pβtub2enh-(C120)s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)::Tpsad 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD483 and oOD484. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD228	This work
pOD306	Pβtub2enh-(C120)s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD488 and oOD484. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD234	This work
pOD308	3'UTRrbc2::FLUC(v2)::Pβtub2 Pβtub2enh-(C120)s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)::Tpsad Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD485 and oOD486. Backbone was digested with NotI. Both fragments were assembled using Gibson cloning.	3'UTRrbc2::FLUC(v2)::Pβtub2	pOD228	This work
pOD317	AOC5(ChRe) (Pos. B3-B4) Insert was amplified from <i>C. reinhardtii</i> cDNA with oligos oOD471, oOD500, oOD501 and oOD480. BsmBI restriction-ligation reaction (GoldenBraid).	AOC5(ChRe)	pUPD2	This work
pOD327	EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B4-B5) Insert was amplified from pOD199 with oligos oOD536 and oOD537. BsmBI restriction-ligation reaction (GoldenBraid).	EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pUPD2	This work

pOD328	Ppsad::NLS(SV40)(ChRe+)-KRAB(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-016, pOD139, pOD327, pCM0-114	pDGB 3alpha1	This work
pOD329	Ppsad::NLS(SV40)(ChRe+)-KRAB(ChRe+)-EL222(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD328 with oligos oOD538 and oOD565. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using Gibson cloning.	NLS(SV40)(ChRe+)- KRAB(ChRe+)- EL222(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pJR38	This work
pOD333	AOC5(ChRe)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B4) Exons were amplified from pOD317 with oligos oOD471, oOD504, oOD507, oOD511, oOD528 and oOD529. Introns were amplified from pOpt_gLuc_Hyg with oligos oOD505, oOD506, oOD512 and oOD513. BsmBI restriction-ligation reaction (GoldenBraid).	AOC5(ChRe)- {Irbcs2.1}- {Irbcs2.2} with introns 1 and 2 from Rbcs2	pUPD2	This work
pOD341	Pβtub2::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD206 with oligos oOD548 and oOD549. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	Pβtub2::SPcCA	pOD306	This work
pOD346	Pβtub2min (Pos. A3-B2) Insert was amplified from pOD217 with oligos oOD387 and oOD560. BsmBI restriction-ligation reaction (GoldenBraid).	Pβtub2min	pUPD2	This work
pOD361	Pβtub2enh-(C120)s- Pβtub2min::AOC5(ChRe)-{Irbcs2.1}-{Irbcs2.2}-STOPcodon::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pOD218, pROF054, pOD217, pOD333, pCM0-101, pCM0-114	pDGB 3alpha1	This work
pOD364	Pβtub2enh-(C120)s- Pβtub2min::AOC5(ChRe)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Ppsad::SRDX(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+):Tpsad 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD361 with oligos oOD570 and oOD571. Backbone was digested with NotI and EcoRV. Both fragments were assembled using Gibson cloning.	Pβtub2enh-(C120)s- Pβtub2min::AOC5(ChRe)- {Irbcs2.1}- {Irbcs2.2}	pOD302	This work
pOD380	Par::FLUC(v2)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD598 and oOD433. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	FLUC(v2)	pOpt_gLuc_Hyg	This work
pOD381	Par::FLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2	FLUC(v1): FLUC(ChRe+)-	pOpt_gLuc_Hyg	This work

	Insert was amplified from pOD254 with oligos oOD599 and oOD434. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	{Irbcs2.1}- {Irbcs2.2}		
pOD382	Pβtub2::SPcCA-GLUC(ChRe+)-{Irbcs2.1}- {Irbcs2.2}::3'UTRrbc2 Pβtub2::FLUC(v2):: 3'UTRrbc2 Par::HygR(APHVII)::3'UTRrb cs2 Insert was amplified from pOD206 with oligos oOD548 and oOD549. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	Pβtub2::SPcCA	pOD280	This work
pOD383	P35S::FLUC(v2)::3'UTRrbc2 Par::HygR(A PHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD600 and oOD601. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	FLUC(v2)	pOD205	This work
pOD384	Ppsad::FLUC(v2)::3'UTRrbc2 Par::HygR(A PHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD602 and oOD601. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	FLUC(v2)	pOD207	This work
pOD385	Pβtub2min::SPcCA-GLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC (v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrb cs2 Insert was amplified from pOD306 with oligos oOD549 and oOD603. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	Pβtub2min::SPc CA	pOD306	This work
pOD386	PhCMVmin::SPcCA-GLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC (v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrb cs2 Insert was amplified from pOD004 with oligos oOD549 and oOD604. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	PhCMVmin::SP cCA	pOD306	This work
pOD387	Pβtub2enh::SPcCA-GLUC(ChRe+)- {Irbcs2.1}- {Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC (v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrb cs2 Insert was amplified from pOD306 with oligos oOD401 and oOD605. Backbone was digested with NotI and AgeI. Both fragments were assembled using Gibson cloning.	Pβtub2enh	pOD386	This work
pOD390	GAL4(ChRe+) (Pos. B3) Insert was amplified from pOD141 with oligos oOD608 and oOD609. BsmBI restriction-ligation reaction (GoldenBraid).	GAL4(ChRe+)	pUPD2	This work
pOD396	Pβtub2enh2ndhalf-Pβtub2min::SPcCA- GLUC(ChRe+)-{Irbcs2.1}- {Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC	Pβtub2enh2ndh alf	pOD385	This work

	(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD306 with oligos oOD615 and oOD616. Backbone was digested with NotI. Both fragments were assembled using Gibson cloning.			
pOD397	Pβtub2enh1sthalf-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 3'UTRrbcs2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD306 with oligos oOD401 and oOD617. Backbone was digested with NotI. Both fragments were assembled using Gibson cloning.	Pβtub2enh1sthalf	pOD385	This work
pOD399	Pβtub2enh2ndhalf-(C120)₅-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 3'UTRrbcs2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD306 with oligos oOD549 and oOD616. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	Pβtub2enh2ndhalf-(C120) ₅ -Pβtub2min::SPcCA	pOD306	This work
pOpt_gLuc_Hyg	Par::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 Par::HygR(APHVII)::3'UTRrbcs2			Lauersen et al., 2015
pJR38	Ppsad::GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbcs2			Neupert et al., 2009
pAES14	PARS2::RLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-Hs6tag::3'UTRrbcs2			Markus Heitzer (https://www.chlamycollection.org)
L0-wtFLUC	FLUC(v2) (Pos. B3-B4)			Kindly provided by Michael Schroda
pROF021	(etr)₈-(C120)₅-PhCMVmin::FLUC::TSV40			Ochoa-Fernandez et al., 2020
pROF053	(etr)₈ (Pos. A1)			Ochoa-Fernandez et al., 2020
pROF054	(C120)₅ (Pos. A2)			Ochoa-Fernandez et al., 2020
pROF059	PhCMVmin (Pos. A3-B2)			Ochoa-Fernandez
pROF060	PhCMVmin (Pos. A2-B1)			Ochoa-Fernandez

Table S2. Oligonucleotides used for cloning.

Name	Sequence (5' → 3')
oOD007	GCGCCGTCTCGCTCGTGACCTGTTTAAATAGCCAGGCCC
oOD008	GCGCCGTCTCGCTCAATGGTTTGCGGGTTGTGACTGAAAC
oOD031	GGGCTGCGCGCAGGCTTGACAGATCTGACGTCTGGTCTCAAATGGTGTTCACCC
oOD032	CTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCGGTCTCAAAGCTTAGGCCAGG
oOD043	GCGCCGTCTCGCTCGAATGACACAAGAATCCCTGTTAC
oOD050	GCGCCGTCTCGCTCAACCTGCGGCGCCGGGGGCGGTGTCCG
oOD051	GCGCCGTCTCGCTCGTACTATGACACAAGAATCCCTGTTAC
oOD052	GCGCCGTCTCGCTCAATGGCCGGCGCCGGGGGCGGTGTC
oOD053	GCGCCGTCTCGCTCGCCATGCCCCGGTGAAGCAGACCCT
oOD054	GCGCCGTCTCGCTCACATTCCGGGGCCCCGGGTTGCTCTC
oOD055	GCGCCGTCTCGCTCGAGGTATGGATTATGGAGGCGCCCT
oOD056	GCGCCGTCTCGCATCTCGACCTCAATCTCAG
oOD057	GCGCCGTCTCGGATGCTGGTGGAGGACGAGG
oOD058	GCGCCGTCTCGCTCACGAACCCTTGCCGGTGCCCTTGTTGA
oOD063	GCGCCGTCTCGCTCGAGGTATGTACAGCAACGGC
oOD064	GCGCCGTCTCGCGTGGCGGATCTCGGCGATC
oOD065	GCGCCGTCTCGCACGGTGAGTCGACGAGCAAGCCC
oOD066	GCGCCGTCTCGTCGCCTGCAAATGGAAACGGCGACG
oOD067	GCGCCGTCTCGGCGACATCTTCCACAGCGCC
oOD069	GCGCCGTCTCGGAAGGTGAGTCGACGAGCAAGCCC
oOD072	GCGCCGTCTCGCGGCCTCCTCCTCGGCG
oOD074	GCGCCGTCTCGCTGCCTGCAAGCAAGGGGATGAAGGG
oOD075	GCGCCGTCTCGGCAGCTACTTCATCAGCGCC
oOD076	GCGCCGTCTCGCTCACGAAGGCGGCCAGCACC
oOD077	GCGCCGTCTCGCTCGAATGATGGAGGACGCCAAGAAC
oOD082	GCGCCGTCTCGTCACCATGATGATGATCTTCTGGATGATGG
oOD083	GCGCCGTCTCGGTGAGTCGACGAGCAAGCCC
oOD084	GCGCCGTCTCGTGTCTGCAAATGGAAACGGCGACG
oOD085	GCGCCGTCTCGGACAGCAAGACCGACTACCAGG
oOD090	GCGCCGTCTCGTGGTCTCGGTCAGGCCGTAG
oOD091	GCGCCGTCTCGACCACCAGCGCCATCCTGAT
oOD092	CTAACCCTGCGTCGCCGTTTCCATTTGCAGGATGCATATGATGCTCGAGATGGGTGCC CTC
oOD093	GTTACTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCTTACGTATGGTGGTGGT ATGGTGG
oOD096	GCGCCGTCTCGCTCGAATGGCCCCTGGAGCGGTGCCCTCCTGAT
oOD097	GCGCCGTCTCGCTCGTACTGCCCTGGAGCGGTGCCCTCCTGAT
oOD100	GCGCCGTCTCGGGGCCTGCAAATGGAAACGGCGACG
oOD102	GCGCCGTCTCGCACGAACACCACGCCGCC

oOD103	GCGCCGTCTCGCGTGGTGAGCTTGCGGGGTTGC
oOD104	GCGCCGTCTCGCGTCCTGCAAGCAAGGGGATGAAGGG
oOD105	GCGCCGTCTCGGACGAGGTGCCCAAGGGC
oOD106	GCGCCGTCTCGCTCAAAGCTTACACGGCGATCTTGC
oOD107	GCGCCGTCTCGCTCGAATGATGACCACCATGCCC
oOD108	GCGCCGTCTCGCGCAGCGCTTCAGCACCAC
oOD109	GCGCCGTCTCGTGCGGTGAGTCGACGAGCAAGCCC
oOD110	GCGCCGTCTCGGCCCATCGAGTTCACCCTG
oOD113	GCGCCGTCTCGCTGCAAATGGAAACGGCGACG
oOD115	GCGCCGTCTCGCTGGGTGTCGCCCAGGTTGG
oOD116	GCGCCGTCTCGCCAGGTGAGCTTGCGGGGTTGCG
oOD117	GCGCCGTCTCGCCACCTGCAAGCAAGGGGATGAAGGG
oOD118	GCGCCGTCTCGGTGGTGCCCCAGAGCCAC
oOD119	GCGCCGTCTCGCTCAAAGCTTACACCTTGC
oOD120	GCGCCGTCTCGCTCGAATGATGACCACCATGGTGAG
oOD121	GCGCCGTCTCGCAGTCTCCCAGGGCTGGCTG
oOD122	GCGCCGTCTCGACTGCCGAGATGGACGCCAT
oOD124	GCGCCGTCTCGGCTGGTGAGTCGACGAGCAAGCCC
oOD126	GCGCCGTCTCGCACGATCATGCGCACGCG
oOD127	GCGCCGTCTCGCGTGGTGAGTCGACGAGCAAGCCC
oOD128	GCGCCGTCTCGAGTCCTGCAAATGGAAACGGCGACG
oOD129	GCGCCGTCTCGGACTGCAACGCCACCCCC
oOD131	GCGCCGTCTCGGGTCCTGCAAATGGAAACGGCGACG
oOD133	GCGCCGTCTCGCTTGAAGCTGTCGCGCAGGATC
oOD134	GCGCCGTCTCGCAAGGTGAGCTTGCGGGGTTGCG
oOD135	GCGCCGTCTCGCTGCAAGCAAGGGGATGAAGGG
oOD136	GCGCCGTCTCGGCAGGAGAGCGAGGCCGCCATG
oOD137	GCGCCGTCTCGCTCAACCTGCCAGCTCGTC
oOD138	GCGCCGTCTCGTAGTCTCGGTCAGGGCGCGG
oOD139	GCGCCGTCTCGACTATCATGTTGACGACGC
oOD140	GCGCCGTCTCGGAGTCTCCTCGTCCAGGGGC
oOD141	GCGCCGTCTCGACTCACGCCGGCGAGGACGT
oOD142	GCGCCGTCTCGCTCGAATGATGCTGCTGCTGC
oOD147	GCGCCGTCTCGCAGTTGCGGTTACGGTGTGG
oOD148	GCGCCGTCTCGACTGGTGAGTCGACGAGCAAGCCC
oOD150	GCGCCGTCTCGGCAGGTACAGCGACGCCGACGTG
oOD154	GCGCCGTCTCGCATCACGTGGGCGATGAAGGTC
oOD155	GCGCCGTCTCGGATGGTGAGCTTGCGGGGTTGC
oOD156	GCGCCGTCTCGAGGCCTGCAAGCAAGGGGATGAAGGG

oOD157	GCGCCGTCTCGGCCTTCGCCGCCTGCCTG
oOD158	GCGCCGTCTCGCTCAAAGCTTAGCCGGGGTGGG
oOD160	GCGCCGTCTCGCTCGAGGTGAGTTCGACAGCG
oOD161	GCGCCGTCTCGCAGCAGGCCCTCGATGGTG
oOD162	GCGCCGTCTCGGACCTGCCCGACGACGAC
oOD163	GCGCCGTCTCGCGTAGGGGGCGCTGTCGTG
oOD164	GCGCCGTCTCGTACGGTGAGCTTGCGGGGTTGCG
oOD165	GCGCCGTCTCGGCAGGCGCCCTGGACATGGCCG
oOD166	GCGCCGTCTCGCTCACGAAGCCGCCGTACTCG
oOD167	GCGCCGTCTCGGAGTCTCGTACTGCTTCTCG
oOD168	GCGCCGTCTCGACTCTGGAGAACCAGCTGGC
oOD169	GCGCCGTCTCGGGAGTCTCGCGCTCCTCCTT
oOD170	GCGCCGTCTCGCTCCCACCACCGACAGCATG
oOD171	GCGCCGTCTCGCTCGAATGATGAGCCGCCTGG
oOD172	GCGCCGTCTCGCAGCTTGCGGGTGGTCAGG
oOD173	GCGCCGTCTCGGCCAGAAAGCTGGGCGTG
oOD174	GCGCCGTCTCGCTGGTGGTCGAACAGCTCGATG
oOD175	GCGCCGTCTCGGCAGGGCGCCGAGCCCGCCTTC
oOD176	GCGCCGTCTCGCTCAACCTGCGGCTGTAGGCGC
oOD177	GCGCCGTCTCGCTCGAATGATGCTGGACCTGGAG
oOD181	GCGCCGTCTCGCTTCTTGTAGTTCAGGATCTCCACC
oOD182	GCGCCGTCTCGCGTCCTGCAAATGGAAACGGCGACG
oOD183	GCGCCGTCTCGGACGGCACCCCTTCCGC
oOD184	GCGCCGTCTCGTCACCGCGGTGCATCTTACGGTC
oOD185	GCGCCGTCTCGGTGAGCTTGCGGGGTTGCG
oOD186	GCGCCGTCTCGGCCTGGTGATGGAGAAGCTGAAC
oOD187	GCGCCGTCTCGCTCAAAGCTTAGATGCCGGCCTC
oOD188	GCGCCGTCTCGCTCGAATGATGAGCCGCGGC
oOD189	GCGCCGTCTCGCAGGGGTGGCGCACCAGC
oOD190	GCGCCGTCTCGGCAGGAGCAGCCGCCTGGTGGG
oOD191	GCGCCGTCTCGTCACCGCGGGCGGCCATGATGTC
oOD192	GCGCCGTCTCGCCTGGTGAGTCGACGAGCAAGCCC
oOD193	GCGCCGTCTCGCCGCCTGCAAGCAAGGGGATGAAGGG
oOD194	GCGCCGTCTCGGCGGCGACACCGTGGCCG
oOD195	GCGCCGTCTCGCTCAACCTGGCCTGCTCCACCATG
oOD196	GCGCCGTCTCGCTCGAATGATGGACCCCAAGAAG
oOD200	GCGCCGTCTCGCTTGAAGATGCTGCGGCTGC
oOD201	GCGCCGTCTCGTGTCTGCAAGCAAGGGGATGAAGGG
oOD202	GCGCCGTCTCGGACAAGCAGAGCTGCGACATCAAG

oOD203	GCGCCGTCTCGCTCAACCTGAACTCCCACAGGTCG
oOD204	GCGCCGTCTCGCTCGAATGATGAAGCTGCTGACCAG
oOD208	GCGCCGTCTCGCGCTCACGGTCAGCTGGC
oOD209	GCGCCGTCTCGAGCGGTGAGTCGACGAGCAAGCCC
oOD210	GCGCCGTCTCGCTCGAATGATGCCCGAGCTGG
oOD211	GCGCCGTCTCGGCAGGCACCGGCGGCAGCGGCG
oOD212	GCGCCGTCTCGCTCCAGCTGCACGCCGATGAAATA
oOD213	GCGCCGTCTCGGACGGTGAGCTTGCGGGGTTGCG
oOD214	GCGCCGTCTCGGTGCCTGCAAGCAAGGGGATGAAGGG
oOD215	GCGCCGTCTCGGCACCGAGCACGTGCGCG
oOD216	GCGCCGTCTCGCTCAACCTTTACACCCAGGTGTCC
oOD217	GCGCCGTCTCGCGTCGTCGTCGGGGCGGAAG
oOD218	GCGCCGTCTCGGACGGTGAGTCGACGAGCAAGCCC
oOD219	GCGCCGTCTCGATGCCTGCAAATGGAAACGGCGACG
oOD220	GCGCCGTCTCGGCATCTTCGTGACCCGCGTG
oOD221	GCGCCGTCTCGTCACCGCTGATGGTGGCGGTGC
oOD222	GCGCCGTCTCGCTCGCCATGCCCCCCCC
oOD223	GCGCCGTCTCGGCCTGAAGCCCGGCGTG
oOD224	GCGCCGTCTCGCTCAACCTGGTGCGGTAGTTGATGC
oOD226	GCGCCGTCTCGCCAGCCCCAGCCGTACAG
oOD227	GCGCCGTCTCGCTGGGTGAGTCGACGAGCAAGCCC
oOD228	GCGCCGTCTCGCTCGTTCGCAGTACCTGCCCCG
oOD229	GCGCCGTCTCGGCAGGGCCGCTACGGCAACCTG
oOD230	GCGCCGTCTCGCAGGCGAACACGTTGTTGCG
oOD231	GCGCCGTCTCGCCTGGTGAGCTTGCGGGGTTGCG
oOD232	GCGCCGTCTCGGCCCTGCAAGCAAGGGGATGAAGGG
oOD233	GCGCCGTCTCGGGGCCGCGGCACCAACGG
oOD234	GCGCCGTCTCGCTCACATTGCCGTCCACGCTCAG
oOD235	GCGCCGTCTCGTCACCGCTGAAGGGGCTCTTCTTCATG
oOD236	GCGCCGTCTCGGCCCCACCGACCCCCGCC
oOD239	GCGCCGTCTCGCGCTCAGCAGGCCGTTGG
oOD240	GCGCCGTCTCGAGCGGTGAGCTTGCGGGGTTGCG
oOD241	GCGCCGTCTCGCTCAACCTCCGGGGCCCCGGGTTGCTCTC
oOD242	GCGCCGTCTCGCTCGAGGTGCGAGTTCGACAGCGCCGGC
oOD243	GCGCCGTCTCGGCAGGCGACGAGGACTTCAGCAGC
oOD244	GCGCCGTCTCGCTCAAAGCTTACTTGTCGTCGTCGTC
oOD245	GCGCCGTCTCGCTCAAAGCTTACACCTTGCGCTTCTTCTTGGGGCCGCGTACTCGTC GATGC
oOD247	GCGCCGTCTCGCTCGAATGCTCGAGATGGGTGCCCT
oOD248	GCGCCGTCTCGTAGTCTCCACGAAGAAGTTGT

oOD249	GCGCCGTCTCGACTATGCTGCCCAGCAAGAT
oOD250	GCGCCGTCTCGCTCAAAGCTTACGTATGGTGGTGGTGATG
oOD251	GCGCCGTCTCGCTCGAATGATGATGACCACCATGCCCCG
oOD252	GCGCCGTCTCGCTCAACCTGTTGATCAGGCTGTAGGCGC
oOD253	GCGCCGTCTCGCTCGTTCGATGGAGGACGCCAAGAACAT
oOD254	GCGCCGTCTCGTAGTCTCGGTCAGGCCGTAG
oOD255	GCGCCGTCTCGACTACCAGCGCCATCCTGAT
oOD266	GCGCCGTCTCGCTCGTTCGATGCTGCTGCTGCTGCTGCT
oOD268	GCGCCGTCTCGCTCGAATGGCGCGTACTGGCGCTCTACT
oOD269	GCGCCGTCTCGCTCACGAACCGCAAGCCTGCGCGCAGCC
oOD270	GCGCCGTCTCGCTCGAATGGCCCCCTCCGAACCTCGCT
oOD271	GCGCCGTCTCGTAGTCTCGAACAACCTGGTCAT
oOD272	GCGCCGTCTCGACTACGCTGCGGCGGACGGT
oOD273	GCGCCGTCTCGCTCAACCTGCCTTGGTCTTCAGCAGCTCCG
oOD274	GCGCCGTCTCGCTCGGGAGGCTGAGGCTTGACATGATTG
oOD275	GCGCCGTCTCGCTCAGTCATTGTAAAAAAGTAAAGAACATAGGCC
oOD278	GCGCCGTCTCGCTCGAATGGCGCGTACTGGCGCTCTACTCCTGGTCGCGCTGGCGCTT GCGGGCTGCGCGCAGGCTTGCGGAGGACGCCAAGAACAT
oOD279	GCGCCGTCTCGCTCGAATGGCGCGTACTGGCGCTCTACTCCTGGTCGCGCTGGCGCTT GCGGGCTGCGCGCAGGCTTGATGCTGCTGCTGCTGCTG
oOD280	GCGCCGTCTCGCTCAAAGCTTACTTCTCGAACTGCGGGT
oOD293	GCGCCGTCTCGCTCACATTGGTGGACTAGTGGATCTCGA
oOD295	GCGCCGTCTCGCTCGGGAGAGCTTGATGCCTGCAGGTC
oOD296	GCGCCGTCTCGCTCAGGGACCCTCTAGAGTCTCCGCTCG
oOD299	GGACCCCACTGCTACTCACAAACAAGCCCATATGGATTATGGAGGCGCCCTG
oOD303	CTAGGACCCCACTGCTACTCACAAACAAGCCCAATGGCCCCTCCGAACCTCGCTTACAA AG
oOD313	GCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGGC CATAGGCTTGCCGGTGCCCTTGTTG
oOD314	GCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGGC CATAGGGCGGGCGCCGTCGTCCTC
oOD321	CCCTGCGTCGCCGTTTCCATTTGCAGGATGCATATGATGCTGGACCTGGAGCTGCG
oOD322	TACTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCTTAGATGCCGGCCTCCACGG
oOD326	GCGCCGTCTCGCTCGGGAGAGGAGGATATCGAAATAGCGC
oOD327	GCGCCGTCTCGCTCAGTCACCTGCAGGGTACGGTGTACG
oOD333	CTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCCTTACACGGCGATCTTGCC
oOD334	GCTTGCGGGCTGCGCGCAGGCTTGAGATCTGACGTCATGGAGGACGCCAAGAAC
oOD350	CGCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGG CCATATGACCTGCCTTGGTCTTCAGCAG
oOD351	CTAGGACCCCACTGCTACTCACAAACAAGCCCATAGATATCATGGCCCCTCCGAACCTC G
oOD353	GCGCCGTCTCGCTCGGGAGGGATCCGCTGAGGCTTGACATGATTG
oOD354	GCGCCGTCTCGCTCAACCTACCGGTTTAAGATGTTGAGTGACTTCTCTTG
oOD355	GCGCCGTCTCGCTCGGCTTTGTACACCCAAGAAGAAGCGCAAGG

oOD356	GCGCCGTCTCGCTCATACCTTAGATGCCGGCCTCCACGG
oOD357	GCGCCGTCTCGCTCGAGGTATGAGGTGCGAGTTCGACAGC
oOD358	GCGCCGTCTCGCTCAAAGCGCCGCCGTA CTGTCGATGC
oOD359	GCGCCGTCTCGCTCGGGTAAGCAGCTGGACCGCCTGTAC
oOD360	GCGCCGTCTCGCTCAAGCGCCTGCAGGGGCATTGCACAGTCACGCTG
oOD361	CTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGATGCTAGCCTCGAGTAG GTAGCC
oOD362	CGCGACCAGGAGTAGAGCGCCAGTACGCGCCATATGGCTAGCGCCAGCGCAGCC
oOD363	CGCGGTGGCGGCCGCTCTAGAACTAGACTAGTACTAGAGCCAAGCTGATCTC
oOD364	GCGACCAGGAGTAGAGCGCCAGTACGCGCCATATGTCTGACTAGAATAGTAAATTGTA ATG
oOD365	CACCGCGGTGGCGGCCGCTCTAGAACTAGACTAGTCTGGCACTTTCTTGCGCTAT
oOD366	CGACCAGGAGTAGAGCGCCAGTACGCGCCATATGTTTGGGGTTGTGACTGAAAC
oOD367	CACCGCGGTGGCGGCCGCTCTAGAACTAGACTAGTCCCGTCTGCCTGACAGGAAG
oOD368	CGACCAGGAGTAGAGCGCCAGTACGCGCCATATGGGCTTGTTGTGAGTAGCAGTGG
oOD377	TCCATGGGCGGGCTGGGCGTATTTGAAGCGGGATCCGCTGAGGCTTGACATG
oOD378	AAGCCTCAGCACGCGTATTTAAATTACGTACCTGCAGGGGCATTGCAC
oOD387	GCGCCGTCTCGCTCGTCCCCTGTTTAAATAGCCAGGCCC
oOD388	GCGCCGTCTCGCTCACATTTTGGCGGGTTGTGACTGAAAC
oOD389	GCGCCGTCTCGCTCGGGAGCTGGCACTTTCTTGCGCTAT
oOD390	GCGCCGTCTCGCAGTCTCGCAGCCCGCCCTA
oOD391	GCGCCGTCTCGACTGCTTCCCGGCGCTGCAT
oOD392	GCGCCGTCTCGCTCAGTCACGCTCGCCCTGGAGCGGCAT
oOD399	TTTTACAAGAGAAGTCACTCAACATCTTAAACCGGTATGCTGGACCTGGAGCTG
oOD400	AAGCCTCAGCACGCGTATTTAAATTACGTACCTGCAGGGGCATTGCACAGTCACGC
oOD401	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGATGCTAGCCTCGAGGGAG CTGGCACTTTCTTGCG
oOD402	CAGCGCGACCAGGAGTAGAGCGCCAGTACGCGCCATATGTCTGTTTTGCGGGTTGTG ACTGAAACG
oOD403	CACCGGTGAACAGCTCCTCGCCCTTGGCCATATGAGAGCCAGAGCCAGAGCCAGCGT AATCTGGAACATCGTATGGGTAGCGGGCGCCGTCGTCCTC
oOD404	GTCCCGGAGCTGCTGAAGACCAAGGCAGGTTCTGGCTCTGGCTCTCTGATC GTGGACCAGTTCGAGGAGG
oOD405	TCCATGGGCGGGCTGGGCGTATTTGAAGCGGGATCCGATCCCACACACCTGCC
oOD406	AAGCCCAGGCGCAGCTCCAGGTCCAGCATACCGGTTATGGGCTTGTTGTGAGTAGCA G
oOD410	TCCATGGGCGGGCTGGGCGTATTTGAAGCGGGATCCAGAGCCAGAGCCAGAGCC
oOD411	AAGCCTCAGCACGCGTATTTAAATTACGTACCTGCAGGTACCCATACGATGTTCCAG ATTACG
oOD412	TCCATGGTACAGGCGGTCCAGCTGCTGCCAGAATTCTTAGATGCCGGCCTCCAC
oOD414	CTAGGACCCCACTGCTACTCACAACAAGCCCATATGATGCTGGACCTGGAGCTG
oOD423	GCGCCGTCTCGCTCGCCATGATATCATGGTGTTCACCCTGGAGGA
oOD424	GCGCCGTCTCGCTCAAAGCTTAGATATCGGCCAGGATGCGCTCGCACA
oOD426	GCGCCGTCTCGCTCGAATGACCACCATGCCCCGCC

oOD427	GCGCCGTCTCGCTCAAAGCTTAAGCGTAATCTGGAACATCGTATGGGTACACCTTGC GCTTCTTCTTGGGGTC
oOD428	GCGCCGTCTCGCTCGAATGACCACCATGGTGAGCGG
oOD429	GCGCCGTCTCGCTCAAAGCTTAAGCGTAATCTGGAACATCGTATGGGTACACCTTGC GCTTCTTCTTGGG
oOD432	TTCTCTTCGTTTCAGTCACAACCCGCAAACATATGGAGGACGCCAAGAACATC
oOD433	CGGTTACTTCTCGAACTGCGGGTGGCTCCAGAATTCCGAACCCACGGCGATCTT
oOD434	CGGTTACTTCTCGAACTGCGGGTGGCTCCAGAATTCCACGGCGATCTTGCCGC
oOD435	GAGACAGGGGGAGACAGCGTGACTGTGCAATGCCCCTGCAGGGGGCCGCTCTAGAAC TAGACTAGT
oOD436	CATACGCACCAATCATGTCAAGCCTCAGCACGCGTATTTAAATTTAACCGTACGTTGC AAGGATCC
oOD458	GCGCCGTCTCGCTCGAATGCTGGACCTGGAGCTGCG
oOD459	GCGCCGTCTCGCTCACGAACCGATGCCGGCCTCCACGGCGA
oOD460	GCGCCGTCTCGCTCACGAACCCACCTTGCCTTCTTCTTGG
oOD471	GCGCCGTCTCGCTCGAATGGCTTCGCGTCTAGGTGG
oOD480	GCGCCGTCTCGCTCACGAAGCGGCTCTCGTGGCCTCCG
oOD483	AGGGGGAGACAGCGTGACTGTGCAATGCCCCTGCAGGTAAACCGTACGTTCGAAGGA TCC
oOD484	GCACCAATCATGTCAAGCCTCAGCACGCGTATTTAAATGGCCGCTCTAGAACTAGAC TAG
oOD485	ATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTTAACCGTACGTTCGAAGGA TCC
oOD486	AAGAAAGTGCCAGCTCCCTCGAGGCTAGCATCGCGGCCGCGCTCTAGAACTAGAC TAG
oOD488	CCAGCGTAATCTGGAACATCGTATGGGTACCTGCAGGTAAACCGTACGTTCGAAGGA TCC
oOD490	CAGAGCCAGCGTAATCTGGAACATCGTATGGGTACCTGCAGGGGGCCGCTCTAGAACT AGACTAGT
oOD500	GCGCCGTCTCGCATCTCCACAGACTCGTCAG
oOD501	GCGCCGTCTCGGATGGGCACCTCCGGGGACC
oOD504	GCGCCGTCTCGCAATGAGGGTCGCGAAGTAGG
oOD505	GCGCCGTCTCGATTGGTGAGTCGACGAGCAAGCCC
oOD506	GCGCCGTCTCGTTGCCTGCAAATGGAAACGGCGACG
oOD507	GCGCCGTCTCGGCAAGGACCCCGATTTCTTTGTG
oOD511	GCGCCGTCTCGCTCTGCGGAGGCTGAGGAC
oOD512	GCGCCGTCTCGAGAGGTGAGCTTGCGGGGTTGC
oOD513	GCGCCGTCTCGCGGCCTGCAAGCAAGGGGATGAAGGG
oOD528	GCGCCGTCTCGGCCGCCCAAGCAGGTGGTGCCGCTCAAGGCGGAGGCC
oOD529	GCGCCGTCTCGCTCACGAAGCGGCTCTCGTGGCCTCCGCGTCTGCGGCCTCCGCCTTG AGCGG
oOD536	GCGCCGTCTCGCTCGAGGTGGGGCGCCGACGACACCCGCGT
oOD537	GCGCCGTCTCGCTCAAAGCTTAGATGCCGGCCTCCACGG
oOD538	TCTCCATGGTACAGGCGGTCCAGCTGCTGCCAGAATTCTTAGATGCCGGCCTCCAC
oOD548	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTGGCACTTTCTTGCGCTA TG
oOD549	GTTGTTCTCCGTGCGCTTCCTGCCCTCGATGACGTCAGATCTGCAAGCCTGCGC

oOD560	GCGCCGTCTCGCTCACATTGACGTCTTTGCGGGTTGTGA
oOD565	CTAGGACCCCACTGCTACTCACAACAAGCCCATATGGACCCCAAGAAGAAGCGCAA GGTGGACCCCAAGAAGAAGCGC
oOD570	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCCTGGCACTTTCTTGCGCT
oOD571	CTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCCGAAGCGGCTCTCGTGGCCT
oOD598	CCTGCGTCGCCGTTTCCATTTGCAGGATGCATATGGAGGACGCCAAGAACATCAAGA AG
oOD599	CCTGCGTCGCCGTTTCCATTTGCAGGATGCATATGGAGGACGCCAAGAACATCAAGA AGG
oOD600	AACAACATTACAATTTACTATTCTAGTCGACATATGGAGGACGCCAAGAACATCAAG
oOD601	CGGTTACTTCTCGAACTGCGGGTGGCTCCAGAATTCCGAACCCACGGCGATCTTG
oOD602	CTAGGACCCCACTGCTACTCACAACAAGCCCATATGGAGGACGCCAAGAACATCAAG AAG
oOD603	ATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCCTGTTTAAATAGCCAGGCCC CC
oOD604	ATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCCCTATATAAGCAGAGCTCGT TTAG
oOD605	CAGGAGTAGAGCGCCAGTACGCGCCATGGACCGGTCGCTCGCCCTGGAGCGGCATCG G
oOD608	GCGCCGTCTCGCTCGAATGAAGCTGCTGACCAGCAT
oOD609	GCGCCGTCTCGCTCAACCTGCTCACGGTCAGCTGGCGCT
oOD615	CAATCGGGGGCCTGGCTATTTAAACAGGCGGCCCGCTCGCCCTGGAGCGGCA
oOD616	ATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGATGCTAGCCTCGAGGGAG AACACCGATGATGCTTCGACCCCCC
oOD617	TTGCAATCGGGGGCCTGGCTATTTAAACAGGCGGCCAGCGCCCATGCAGCCCCG

Table S3. Overview of the CrBOff plasmids generated and screened. Final CrBOff plasmids are shaded in yellow. T, target module; LS, light switch; NM, normalization module; hCMVmin, cytomegalovirus minimal promoter; β TUB2, β 2-tubulin promoter; β TUB2min, β TUB2 minimal promoter; β TUB2enh, β TUB2 enhancer region; PSAD, PsaD promoter; AR, HSP70A-RBCS2 promoter; AREnh, HSP70A-RBCS2 proximal promoter; ORF, open reading frame; SRDX, SRDX repressor domain; KRAB, KRAB repressor domain.

Plasmid name	Transcriptional units	Target (T) promoter	Light switch (LS) promoter	Normalization module (NM)	Orientation of NM	
pOD124	T-LS	AREnh-hCMVmin	AR	No		Single plasmid (target + light switch)
pOD226	T-LS	β TUB2enh- β TUB2min	AR	No		
pOD228	T-LS	β TUB2enh- β TUB2min	PSAD	No		
pOD276	T-LS-NM	β TUB2enh- β TUB2min	PSAD	Yes	Forward	
pOD302	T-LS-NM	β TUB2enh- β TUB2min	PSAD	Yes	Reverse (ORF3)	
pOD308	NM-T-LS	β TUB2enh- β TUB2min	PSAD	Yes	Reverse (ORF1)	
pOD234	T	β TUB2enh- β TUB2min		No		Target
pOD280	T-NM	β TUB2enh- β TUB2min		Yes	Forward	
pOD306	T-NM	β TUB2enh- β TUB2min		Yes	Reverse	
pOD399	WeakerT-NM	Weaker β TUB2enh- β TUB2min		Yes	Reverse	
pOD235	LS (SRDX)		PSAD	No		Light switch
pOD329	LS (KRAB)		PSAD	No		

4.2 Optogenetic switches for transcriptional activation in *C. reinhardtii*:

CrBlueOn and *CrRedOn* systems

Optogenetic systems for controlling gene expression can be divided in two main categories depending on the outcome of their activation: systems that result in activation of gene expression and those designed for gene repression (Baaske et al., 2018; Blomeier et al., 2021; Christie & Zurbriggen, 2021; Konrad et al., 2023; Müller, Engesser, Schulz, et al., 2013; Ohlendorf & Möglich, 2022). In parallel to the implementation of the blue-light repression system (*CrBOff*, see manuscript 2), two light-inducible transcriptional activation systems were engineered for its establishment in *C. reinhardtii*: a blue-light-inducible system, *CrBlueOn*, and a red-light-inducible system, *CrRedOn* (Fig. 1). The *CrBlueOn* system utilizes the EL222 photoreceptor, the same component employed in the *CrBOff* system, but is fused to a transactivation domain (AD) instead of a repression domain. On the other hand, the *CrRedOn* system for *C. reinhardtii* was adapted from a previously established system in mammalian cells and plants, which is based on the red-light-dependent interaction between *A. thaliana* phytochrome B and its interacting factor PIF6 (Müller, Engesser, Metzger, et al., 2013; Müller et al., 2014). All components were codon-optimized and intronized to ensure proper expression in *C. reinhardtii*.

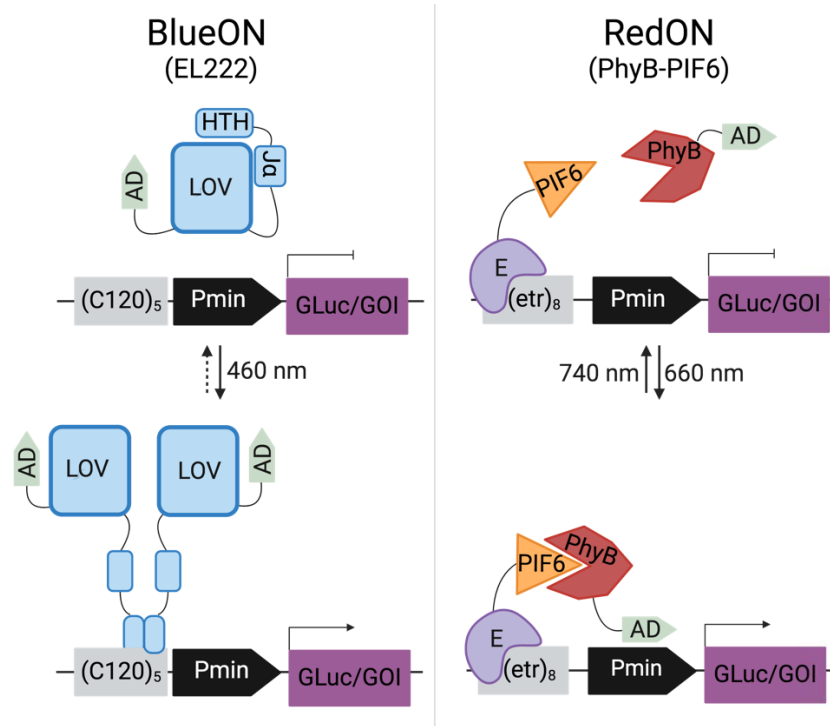


Figure 1. Mode of function of the gene activation optogenetic systems engineered for use in *Chlamydomonas*: *CrBlueOn* and *CrRedOn*. AD, transactivation domain; Pmin, minimal promoter; GLuc, Gaussia luciferase; GOI, gene of interest; EL222, *E. litoralis* photoreceptor; HTH, helix-turn-

helix domain; J α , J alpha helix; LOV, light-oxygen-voltage sensing domain; PhyB, *A. thaliana* phytochrome B; PIF6, phytochrome B interacting factor 6.

Blue-light-inducible optogenetic system

To generate the *CrBlueOn* system, EL222 was combined with the transactivation domain VP16. The system comprises two different parts: i) the target and ii) the light-switch modules (Fig. 2a). The light-switch module consists of EL222 fused to VP16. Whereas the target module contains the Gaussia luciferase reporter, which expression is regulated by the β TUB2 minimal promoter positioned downstream of five copies of the EL222 binding sequence, C120. Additionally, the target module includes a normalization module, in which Firefly luciferase is under the control of the β TUB2 constitutive promoter. Two versions of the target module were tested: one with the normalization module in forward direction with the GLuc transcriptional unit, and another with it in reverse. The transformation of the target and light-switch modules was carried out independently in two sequential steps, according to the two-phases transformation workflow (see manuscript 2), that allows to generate a control line containing only the target module. This control line is used to verify that any differences in gene expression observed in the line with the light switch are attributable to the light switch itself and not to variations in cell physiology or genome positioning effects. The plasmids tested are summarized in Table 1.

Table 1. Overview of the *CrBlueOn* plasmids generated and screened. T, target module; LS, light switch; NM, normalization module; β TUB2min, β 2-tubulin minimal promoter; PSAD, PsaD promoter.

Plasmid name	Transcriptional units	Target (T) promoter	Light switch (LS) promoter	Normalization module (NM)	Orientation of NM	
pOD279	T-NM	β TUB2min		Yes	Forward	Target
pOD305	T-NM	β TUB2min		Yes	Reverse	
pOD267	LS		PSAD	No		LS

To test the functionality of the *CrBlueOn* system, the target module (pOD305) was transformed into UVM4. Gaussia and Firefly luciferase activities were quantified in 96 independent transgenic lines, revealing generally low GLuc values, with 25 clones showing Firefly expression indicating successful transformation (Fig. 2b,c). Out of these 25, 8 clones with varying GLuc values (low GLuc: clones 28, 50, 60; medium GLuc: clones 17, 47, 69; medium-

high GLuc: 25; high GLuc: 48) were selected. These clones were further evaluated for GLuc and FLuc expression under red or blue light to identify those with no significant differences between the two light conditions, since they have not been transformed with the light switch yet and, therefore, no differences in gene expression between different light conditions should be observed. Based on the results, 3 clones (48, 50 and 69) with distinct GLuc values and no significant expression differences under red and blue light were selected for transformation with the light-switch (Fig. 2c). From each transformation of the selected target-lines with the light-switch, 94 clones were selected and cultivated, together with the *CrBlueOn*-target line, under blue and red light. Firefly and Gaussia luciferase activities were measured, and the luciferase ratio under both light conditions was compared between the *CrBlueOn*-target line and the light-switch expressing lines (Fig. 2d). Overall, no significant differences in gene expression under blue and red light were observed between the lines transformed with the light-switch and those containing only the target module. Despite this, three clones with the complete *CrBlueOn* system (4, 10, and 94), originated from the target-line 50 and exhibiting a higher ratio between blue and red, were selected for an additional light experiment. Clones 4, 10, and 94 were grown in triplicates in 6-well plates under red or blue light before measuring luciferase activity. Although higher gene expression levels of GLuc were found under blue light than under red light, the dynamic range was very similar between the lines containing the light-switch and the line lacking it, therefore the functionality of the *CrBlueOn* system could not be confirmed (Fig. 2e).

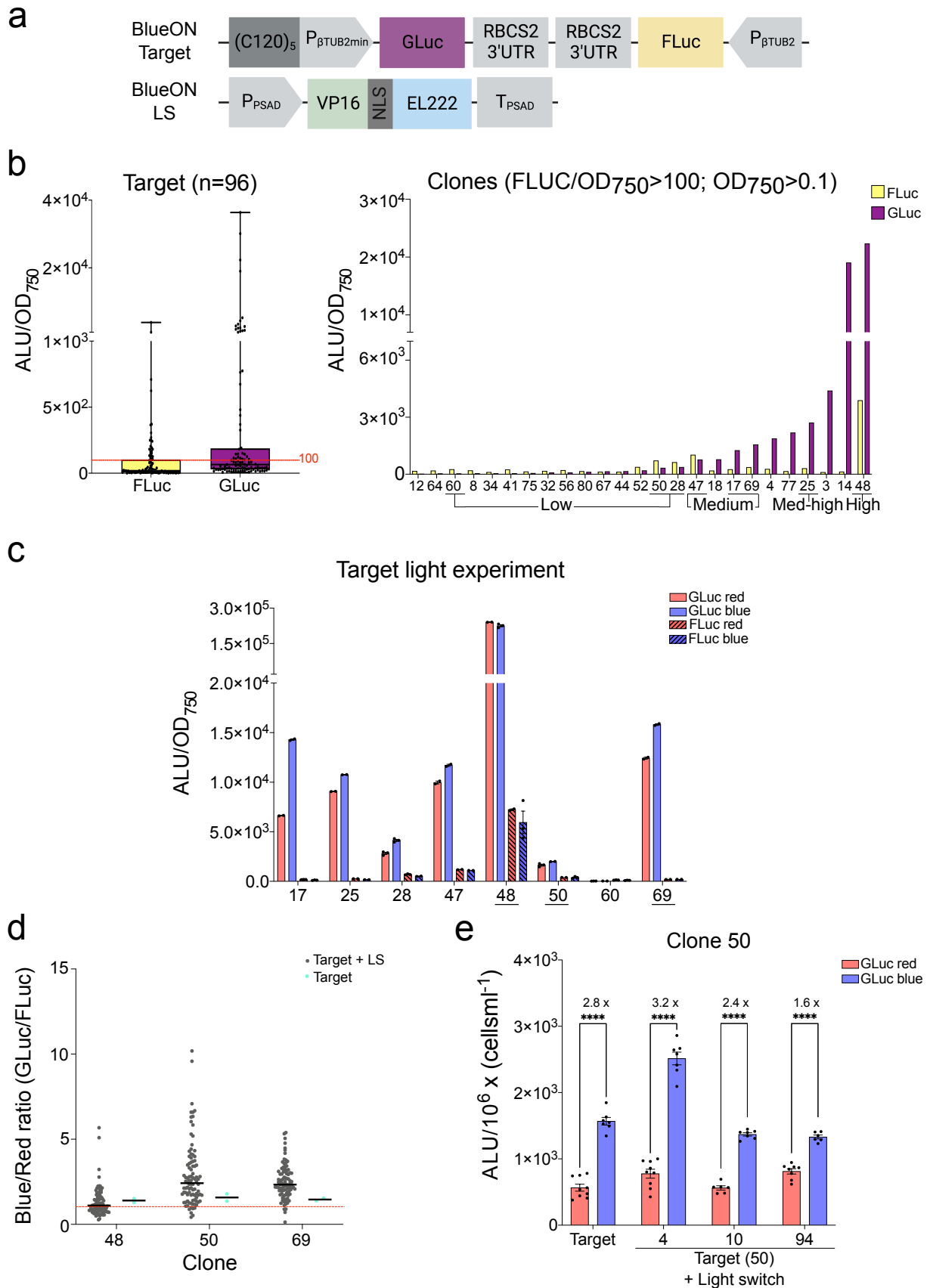


Figure 2. Implementation of a blue light-regulated gene activation switch (BlueOn) in *C. reinhardtii*. (a) Constructs design. The BlueOn target module contains a synthetic promoter composed of five repeats of the binding sequence of EL222, C120, and the minimal core promoter of β TUB2 that

drives the expression of GLuc. The β TUB2 promoter drives the constitutive expression of the normalization reporter FLuc. The light switch construct consists on the blue-light responsive element fused the activation domain VP16, under the control of the PsaD promoter. **(b)** Screening of 96-UVM4 clones transformed with the target of the BlueON system. Left, quantitative overview of the luciferase activity for each independent clone ($n=96$), whiskers delineate the minimum and maximum values. Right, detailed depiction of GLuc and FLuc activity of selected clones with values of FLuc/OD₇₅₀ higher than 100 and OD₇₅₀ higher than 0.1. Clones selected for further experiments are underlined and classified in low, medium, medium-high or high, based on the luciferase activity. **(c)** Light experiment with clones selected in (b). Selected clones are underlined. Mean and standard error of the mean are plotted for $n=3$ samples. **(d)** Testing of the BlueOn switch. Selected clones in (c) were transformed with the blue-light responsive element. For each selected target clone, 94 clones were screened. The ratio between the normalized GLuc and FLuc was calculated. The blue/red ratio for each clone is plotted, for the target clones two samples were measured ($n=2$), the whiskers delineates the minimum and maximum values. **(e)** Light experiment with candidate clones expressing all the components of the BlueOn system. Ratio between blue and red is indicated. Mean and SEM of three independent algae cultures sampled in triplicates is represented ($n=9$) for each line. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant (multiple t -test). Luciferase activity is expressed as absolute luminescence units (ALU) normalized to OD₇₅₀, unless otherwise indicated. In (c), (d) and (e) algae strains were kept in darkness for two days before the experiment, followed by illumination with red or blue light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 24 h GLuc and FLuc activities were determined. P $_{\beta\text{TUB2}}$, β 2-tubulin promoter; P $_{\beta\text{TUB2min}}$, β TUB2 minimal promoter; UTR, untranslated region; P $_{\text{PSAD}}$, PsaD promoter; NLS, nuclear localization sequence; T $_{\text{PSAD}}$, PsaD terminator; EL222, *E. litoralis* photoreceptor; GLuc, Gaussia luciferase; FLuc, Firefly luciferase.

Red-light-inducible optogenetic system

The CrRedOn system was constructed by fusing the first 650 amino acids of the Phytochrome B to the transactivation domain VP16, denoted as light-switch component 1, and the first 100 amino acids of PIF6 to the DNA-binding E protein, designated as light-switch component 2 (Müller, Engesser, Metzger, et al., 2013; Müller et al., 2014). To engineer this system, it was divided into two parts (target and light switch modules), and the two-phase transformation workflow was employed. Numerous variants of this system were tested and are summarized in Table 2. In brief, different minimal promoters (cytomegalovirus and β 2-tubulin minimal promoters) were employed, the linker between the minimal promoter and the reporter gene was modified, and the position and orientation of the normalization module were changed. However, across all these versions, GLuc levels were consistently low in the 96 clones screened after transformation with the target module.

Low GLuc values were expected since no endogenous transcriptional factor should bind to the E-protein DNA-binding sequence (etr) and induce gene expression. However, distinguishing between clones where GLuc was silenced, or even not integrated into the genome, and those that could potentially express it after transformation with the light-switch module was

challenging. To address this, NLuc, a more sensitive reporter, was tested. Unfortunately, the results obtained were not more promising than those presented in this subchapter.

Table 2. Overview of the *CrRedOn* plasmids generated and screened. T, target module; LS, light switch; LSC, light switch component; NM, normalization module; CMVmin, cytomegalovirus minimal promoter; β TUB2min, β 2-tubulin minimal promoter; PSAD, PsaD promoter; AR, HSP70A-RBCS2 promoter; ORF, open reading frame; GLuc, Gaussia luciferase; NLuc, Nanoluciferase.

Plasmid name	Transcriptional units	Target (T) promoter	Light switch (LS) promoter	Reporter	Normalization module (NM)	Orientation of NM	
pOD014	T	CMVmin		GLuc	No		Target
pOD237	T	β TUB2min		NLuc	No		
pOD278	T-NM	β TUB2min		NLuc	Yes	Forward	
pOD310	NM-T	β TUB2min		NLuc	Yes	Reverse (ORF1)	
pOD324	T-NM	β TUB2min		GLuc	Yes	Reverse (ORF3)	
pOD326	T-NM	β TUB2min		NLuc	Yes	Reverse (ORF3)	
pOD394	T-NM	β TUB2min bigger linker		GLuc	Yes	Reverse (ORF3)	
pOD118	LSC1-LSC2		AR-AR		No		Light switch
pOD240	LSC1-LSC2		PSAD-PSAD		No		

In the first *CrRedOn* variant tested both light-switch components were driven by the synthetic AR promoter. The target module in this variant lacked the normalization component and had the CMV minimal promoter upstream of the Gaussia luciferase gene in the inducible synthetic promoter. Figure 3 shows the comparison of the GLuc expression levels between a clone transformed with the full RedOn system and its origin target line without the light switch. Expression levels were analysed at different time points (24, 48, and 72 h) and under varying red-light intensities (0, 5, 10, 20 and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The data reveals no significant differences in the red/dark ratios between the line with only the target and the line with the complete RedOn system after 24 h of incubation. These differences however, become more

pronounced with increasing incubation time; however, the maximum gene expression achieved with the light switch, compared to the target, is only 3.6 times higher. This increase occurs with $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 72 h of incubation, where small variations between cultures or experimental errors (e.g., increased cell lysis during pipetting) may have accumulated.

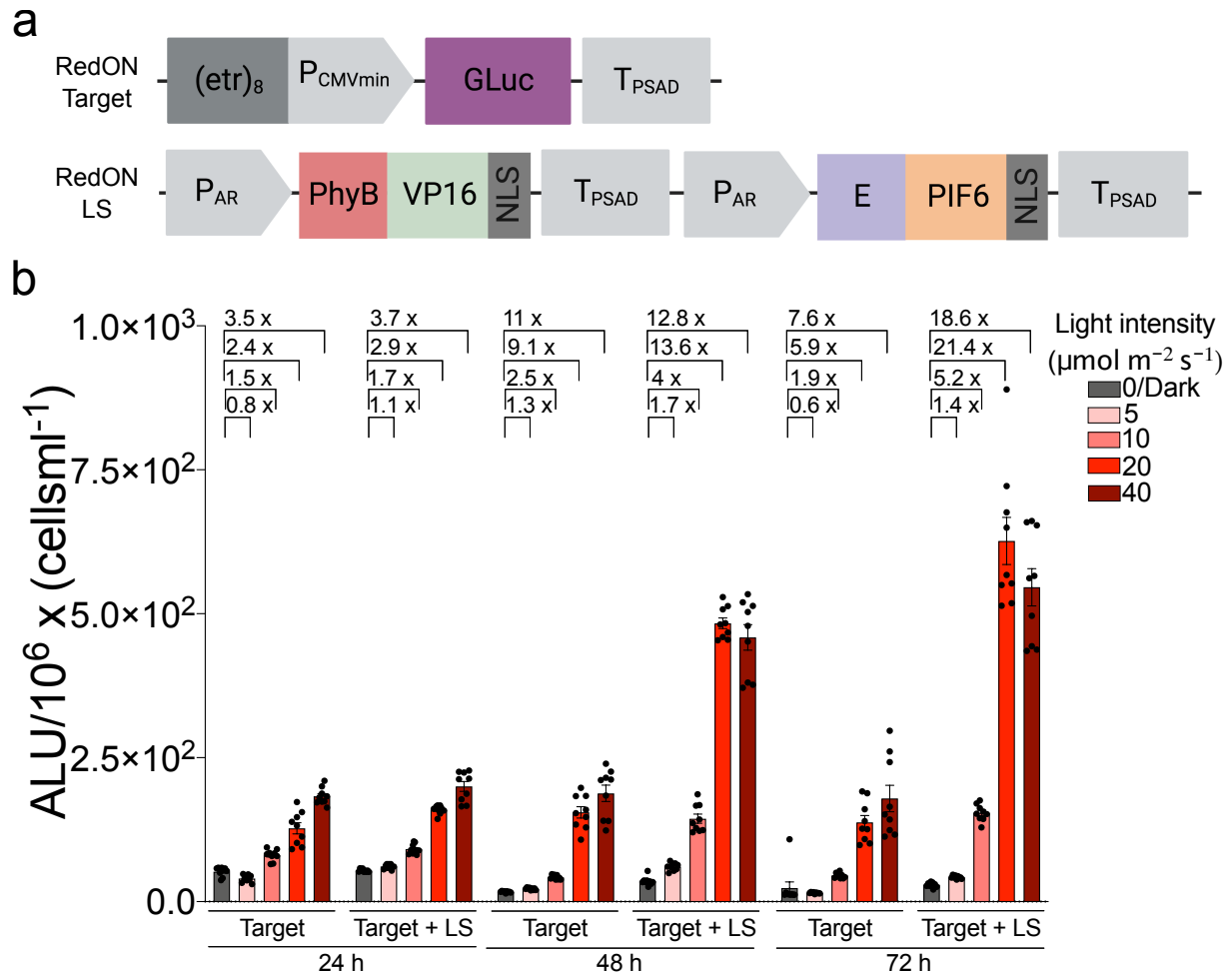


Figure 3. Molecular design and characterization of the first version of the red light-regulated gene activation switch (*CrRedOn*). (a) Constructs scheme. In this version of the *CrRedOn* system, in the target plasmid (pOD014), GLuc is placed under the control of the light regulated promoter, consisting on eight repetitions of the target sequence of protein E, *etr*, and the CMV minimal promoter. The red-activated light switch components are under the control of the constitutive AR promoter (pOD118). One component comprises the first 650 amino acids of the PhyB photoreceptor fused to the activation domain VP16, the second component consist on the fusion of the macrolide repressor DNA-binding protein E fused to the first 100 amino acids of PIF6. (b) Luciferase activity, expressed as absolute luminescence units (ALU) normalized to OD₇₅₀, of one *Chlamydomonas* clone transformed either only with the target or with the target and the light switch of the RedOn system, under different red light intensities (0, 5, 10, 20, 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and times (24, 48, 72h). Cultures were kept in dark before illumination. Error bars indicate standard error of the mean (SEM), n=9. NLS, nuclear localization sequence; PhyB, phytochrome B; PIF6, phytochrome interacting factor 6; P_{AR}, HSP70A-RBCS2 promoter; P_{CMVmin}, cytomegalovirus minimal promoter; T_{PSAD}, PsaD terminator; GLuc, Gaussia luciferase; E, macrolide repressor DNA-binding protein E; LS, light switch.

In the latest tested version of *CrRedOn*, the AR promoter, whose activity we observed to be more dependent of light than that of other promoters (see manuscript 2), was replaced with the PsaD promoter to drive the expression of the light switch components. Additionally, a normalization module was introduced, and the CMV minimal promoter was substituted with the β TUB2 minimal promoter. As a light-independent control, the synthetic transcription factor formed by fusing the E protein directly to VP16 was used (Fig. 4a).

UVM4 was transformed with the target module (pOD324) of the *CrRedOn* system, which is also the target of the light-independent control. In this transformation, 96 clones were screened for GLuc and FLuc: 42 clones expressed Firefly, while the GLuc levels were generally very low and sometimes indistinguishable from the background. This low GLuc levels are expected and preferred to obtain systems with low leakiness, however it makes it harder to distinguish between clones that are expressing GLuc in low levels and clones in which GLuc is silenced or even untransformed. Only one clone had GLuc/OD₇₅₀ levels over 100 (Fig. 4b). This particular clone and 6 additional clones were selected and incubated under red light or blue light supplemented with far-red to identify differences in gene expression upon illumination with different light wavelengths (Fig. 4c). Three clones with similar GLuc values under both blue + far-red, and red light were selected. Additionally, despite differences in GLuc activity under blue + far-red and red light, the clone with GLuc/OD₇₅₀ values over 100 was included. These four clones (16, 62, 81, and 89) were then transformed with either the RedOn light switch or the light-independent control construct.

For the four selected *CrRedOn*-target lines transformed with the light-independent control, 94 clones were selected from each transformation event and cultured under white light before Firefly and Gaussia luciferases activity determination (Fig. 4d). GLuc values indicated that, in general, there was no increase in gene expression when the synthetic transcription factor, E-VP16, is transformed, compared to the target lines. This observation might indicate a lack of activity of the E-VP16 fusion in *C. reinhardtii*.

In a similar manner, the RedOn light switch was transformed into the target lines, and 94 clones were grown under blue + far-red or red light before determining GLuc and FLuc activities. The luciferase ratio under both light conditions was then compared between the target line and the 94 lines containing the light-switch (Fig. 4e). Overall, no significant differences were observed.

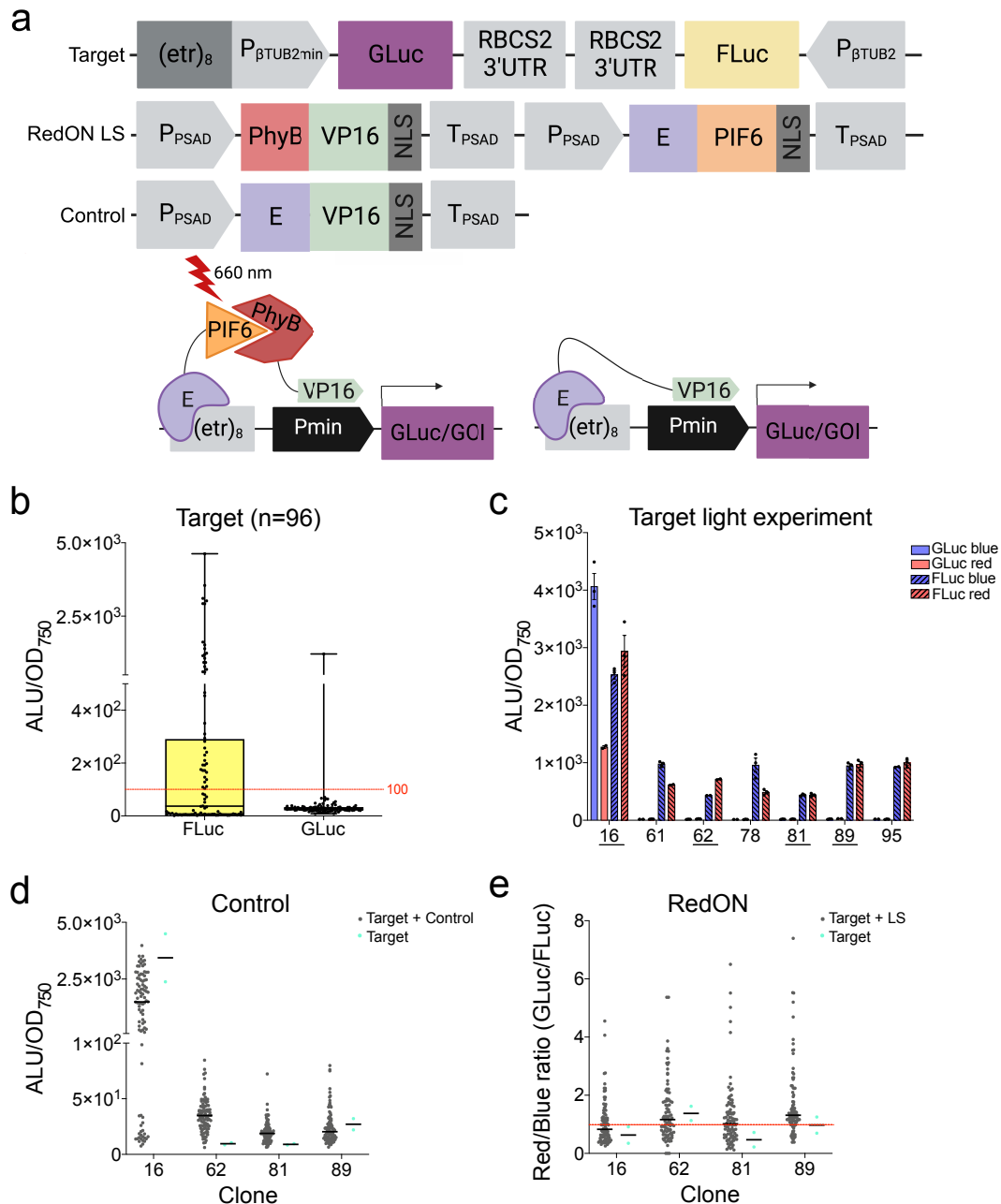


Figure 4. The reengineered version of the red light-regulated gene activation switch (CrRedOn) in *C. reinhardtii*. (a) Mode of function and design of the constructs for the RedOn target (pOD324) and light switch (pOD240), and the light-independent control (pOD388). In the RedOn target module, eight repeats of the binding sequence of the E protein, etr, and the minimal core promoter of βTUB2 drive the expression of GLuc. In the reverse orientation, the βTUB2 promoter regulates the expression of the normalization reporter FLuc. The RedOn light switch construct contains the PhyB(1-650)-VP16 and the E-PIF6(1-100) components under the control of the PsaD promoter. The same PsaD promoter is used in the control construct to drive the expression of the E-VP16 fusion protein. (b) Quantitative overview of the luciferase activity of 96-UVM4 clones transformed with the target of the RedOn system. Whiskers delineate the minimum and maximum values. (c) Light experiment with selected clones from (b). Mean and standard error of the mean are plotted for n=3 samples. Clones selected for transformation with the light-switch or the control are underlined. (d,e) Screening of the target lines selected in (c) transformed with the light-independent control (d) or the RedOn light switch (e). For each target clone, 94 clones transformed with the control or the light switch were screened. In (d) clones

were grown under white light conditions and GLuc activity was measured after 24h. In (e) before measuring, the clones were cultivated in red or blue light supplemented with far-red light, the ratio between normalized GLuc and FLuc was calculated, and the red/blue ratio for each clone is plotted. For the target clones, two samples were measured ($n=2$), the whiskers depict the minimum and maximum values. Luciferase activity is expressed as absolute luminescence units (ALU) normalized to OD_{750} , unless otherwise indicated. In (c) and (e) the microalgae lines were kept in darkness for two days before illumination with red ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$), or blue light supplemented with far-red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of each light wavelength). After 24 h incubation GLuc and FLuc activities were determined. $P_{\beta\text{TUB}2}$, β 2-tubulin promoter; $P_{\beta\text{TUB}2\text{min}}$, $\beta\text{TUB}2$ minimal promoter; UTR, untranslated region; P_{PsaD} , PsaD promoter; NLS, nuclear localization sequence; T_{PsaD} , PsaD terminator; GLuc, Gaussia luciferase; FLuc, Firefly luciferase; GOI, gene of interest; PhyB, first 650 amino acids of phytochrome B; PIF6, first 100 amino acids of phytochrome interacting factor 6; E, macrolide repressor DNA-binding protein E.

The binding of the E-protein to its cognate sequence, *etr*, is modulated by the antibiotic clarithromycin. This property has been widely utilized in the past for the development of chemical-inducible systems (Müller et al., 2014; Weber et al., 2002), where the addition of the antibiotic leads to the repression of transgene expression. To confirm the lack of activity of the synthetic transcription factor E-VP16, we examined certain lines generated in *C. reinhardtii* containing the light-independent control (E-VP16) and its target. We analysed GLuc activity levels in the presence and absence of clarithromycin (Fig. 5). For this, lines derived from the target line 16 and one from the target line 62 were used. The data demonstrates a lack of response to the addition of clarithromycin, this also points to the non-functionality of the generated E-VP16 construct for *C. reinhardtii*.

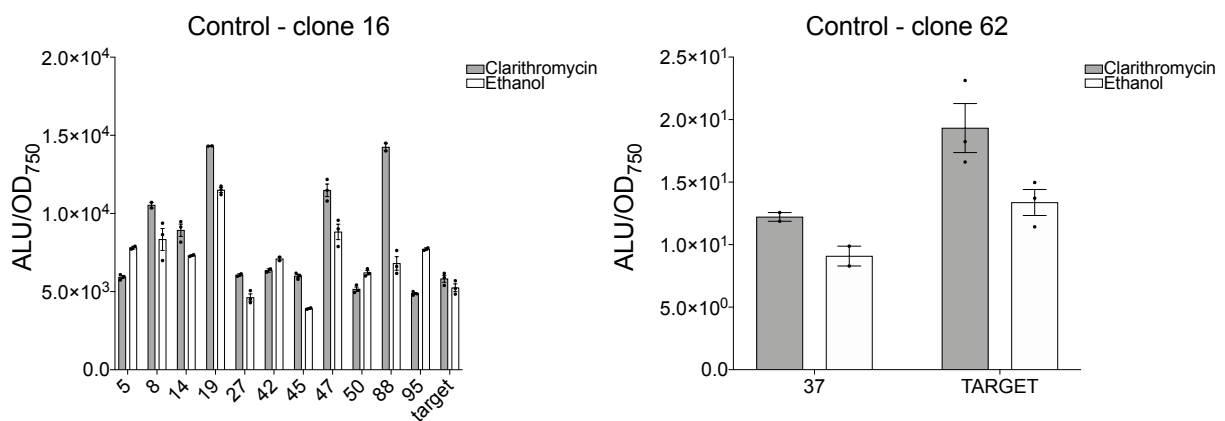


Figure 5. Evaluating the functionality of the light-independent control (E-VP16) in *C. reinhardtii* through its response to the chemical clarithromycin. Comparison of GLuc gene expression in clones transformed with the light-independent control into the target lines 16 (left) or 62 (right), in the presence or absence of clarithromycin. GLuc quantification was performed after a 24-hour incubation with $100 \mu\text{g mL}^{-1}$ clarithromycin or ethanol (blank) and is expressed as ALU normalized to OD_{750} . Mean and SEM are presented ($n=3$).

The outcomes from the light-independent control (E-VP16) experiments, along with the RedOn and BlueOn results, strongly suggest a probable lack of functionality of the VP16 transactivation domain. Since VP16 has been previously used in *C. reinhardtii* (Wang et al., 2017), we hypothesize that the intronized configuration, codon-optimization, or promoter-VP16-terminator combination that we employed might not be conducive to proper expression of the VP16 gene. To discriminate whether the issue lies in transcription or translation, a qPCR analysis could be performed to examine whether transcription of E-VP16 is taking place. Alternatively, a different transactivation domain such as p65, which has already been codon-optimized and intronized for *C. reinhardtii* (see manuscript 2) could be used. Achieving a functional RedOn system would open the way for implementing the PULSE system (Ochoa-Fernandez et al., 2020) in *C. reinhardtii*. Combining it with the already functional *CrBOff* system would enable the control of gene expression in cultures grown not only in heterotrophic conditions but also in photoautotrophic conditions.

Materials and methods

E. coli and *C. reinhardtii*-UVM4 (Neupert et al., 2009) transformation and growth conditions, luminescence analysis, light experiments details, software used, statistical methods, and plasmid generation procedures were performed as described in manuscript 2. Details regarding the plasmids and oligonucleotides designed, constructed, and used in this section can be found in tables S1 and S2, respectively.

Clarithromycin treatment

C. reinhardtii lines were grown in TAP medium under white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 4-7 days. Liquid cultures were diluted to the same OD_{750} and transferred to 12-well plates. $100 \mu\text{g mL}^{-1}$ clarithromycin (purchased from Sigma and prepared as 2 mg mL^{-1} stock solution in ethanol) was added to the cultures, ethanol was used as mock.

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Supplementary information

Table S1. Plasmids design and used. All the pCM0 plasmids are from the *C. reinhardtii* MoClo-kit (Crozet et al., 2018). pUPD2, pDGB and GB plasmids were acquired from the Golden Braid collection (Sarrion-Perdigones et al., 2013; <https://gbcloning.upv.es>). ChRe, genes coming from *C. reinhardtii*; ChRe+, genes codon optimized for *C. reinhardtii*.

ID	Description and cloning procedure	Insert	Backbone	Reference
pOD004	(etr) ₈ -P _{CMV} min::SPcCA-GLUC(ChRe+)-{Irbcs2.2}::Tpsad			Manuscript 2
pOD012	Par-{Iβtub2.1}::HygR(APHVII)::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pCM0-062, pCM0-092, pCM0-114	pDGB 3alpha2	This work
pOD013	Par-{Iβtub2.1}::ParoR(APHVIII)::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pCM0-062, pCM0-091, pCM0-114	pDGB 3alpha2	This work
pOD014	(etr) ₈ -P _{cmv} min::SPcCA-GLUC(ChRe+)-{Irbcs2.2}::Tpsad Par-{Iβtub2.1}::HygR(APHVII)::Tpsad BsmBI restriction-ligation reaction (GoldenBraid).	pOD004, pOD012	pDGB 3omega1	This work
pOD017	Pβtub2min (Pos. A2-B1)	Pβtub2min	pUPD2	Manuscript 2
pOD061	VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B4-B5)			Manuscript 2
pOD062	PHYB(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5)			Manuscript 2
pOD063	Par::PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pOD062, pOD061, pCM0-114	pDGB 3alpha1	This work
pOD065	E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3-B5)			Manuscript 2
pOD071	E(ChRe+)-{Irbcs2.1}-{Irbcs2.2} (Pos. B3)			Manuscript 2
pOD072	Par::E(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pOD071, pOD061, pCM0-114	pDGB 3alpha1	This work
pOD088	Par::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-017, pOD065, pCM0-114	pDGB 3alpha2	This work
pOD089	Par::PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Par::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsmBI restriction-ligation reaction (GoldenBraid).	pOD063, pOD088	pDGB 3omega1	This work
pOD117	Par-{Itub2.1}::ParoR(APHVIII)::Tpsad BsmBI restriction-ligation reaction (GoldenBraid).	GB0106, pOD013	pDGB 3omega2	This work
pOD118	Par::PHYB(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Par::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Par-{Itub2.1}::ParoR(APHVIII)::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pOD089, pOD117	pDGB 3alpha1	This work

pOD195	(C120)_s-Pcmvmin::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::HygR(APHVII)::3'UTRrbcs2			Manuscript 2
pOD220	Pβtub2enh-(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad			Manuscript 2
pOD227	Ppsad::MIC10(ChRe)-spacer-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-HAtag-spacer-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbcs2			Manuscript 2
pOD229	(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 Par::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD220 with oligos oOD407 and oOD402. Backbone was digested with NdeI and NotI. Both fragments were assembled using Gibson cloning.	(C120) _s -Pβtub2min	pOD195	This work
pOD232	(C120)_s-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbcs2 MCS Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD227 with oligos oOD410 and oOD411. Backbone was digested with BamHI and SbfI. Both fragments were assembled using AQUA cloning.	MCS (Multiple cloning site)	pOD229	This work
pOD233	Ppsad::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::Tpsad Par::ParoR(APHVIII)::3'UTRrbcs2 Insert was amplified from pOD195 with oligos oOD412 and oOD413. Backbone was digested with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.	VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+)	pJR38	This work
pOD236	(etr)_s-Pβtub2min::SPcCA-NLUC(ChRe+):Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pROF053, pOD017, pCM0-051, pCM0-078, pCM0-114	pDGB 3alpha1	This work
pOD237	(etr)_s-Pβtub2min::SPcCA-NLUC(ChRe+):3'UTRrbcs2 Par::HygR(APHVII)::3'UTRrbcs2 Insert was amplified from pOD236 with oligos oOD415 and oOD416. Backbone was digested with NotI and EcoRI. Both fragments were assembled using AQUA cloning.	(etr) _s -Pβtub2min::SPcCA-NLUC(ChRe+)	pOpt_gLuc_Hyg	This work
pOD238	Ppsad::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-016, pOD065, pCM0-114	pDGB 3alpha1	This work
pOD239	Ppsad::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Ppsad::GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbcs2	Ppsad::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(	pJR38	This work

	Insert was amplified from pOD238 with oligos oOD417 and oOD418. Backbone was digested with NotI and SpeI. Both fragments were assembled using AQUA cloning.	ChRe+)- {Irbcs2.1}- {Irbcs2.2}:: Tpsad		
pOD240	Ppsad::E(ChRe+)-PIF6(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::<tpsad ppsad::phyb(chre+)-vp16(chre+)-nls(sv40)(chre+)-{irbcs2.1}-{irbcs2.2}::<tpsad par::paror(aphviii)::3'utrrbc2< b=""> Insert was amplified from pOD063 with oligos oOD419 and oOD420. Backbone was digested with EcoRI and NdeI. Both fragments were assembled using Gibson cloning.</tpsad ppsad::phyb(chre+)-vp16(chre+)-nls(sv40)(chre+)-{irbcs2.1}-{irbcs2.2}::<tpsad par::paror(aphviii)::3'utrrbc2<>	PHYB(ChRe+)- {Irbcs2.1}- {Irbcs2.2}- VP16(ChRe+)- NLS(SV40)(ChRe+)- {Irbcs2.1}- {Irbcs2.2}	pOD239	This work
pOD253	Pβtub2::FLUC(v2)::3'UTRrbc2			Manuscript 2
pOD267	Ppsad::VP16(ChRe+)-NLS(SV40)(ChRe+)-EL222(ChRe+){Irbcs2.1}-{Irbcs2.2}::<tpsad par::paror(aphviii)::3'utrrbc2< b=""> Insert was amplified from pOD233 with oligos oOD412 and oOD441. Backbone was digested with BsrGI and EcoRI. Both fragments were assembled using AQUA cloning.</tpsad par::paror(aphviii)::3'utrrbc2<>	NLS(SV40)(ChRe+)- EL222(ChRe+){Irbcs2.1}- {Irbcs2.2}	pOD233	This work
pOD278	(etr)₈-Pβtub2min::SpcCA-NLUC(ChRe+)::3'UTRrbc2 Pβtub2::FLUC(v2)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD489 and oOD436. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD237	This work
pOD279	(C120)₅-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::<3'UTRrbc2 Pβtub2::FLUC(v2)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD490 and oOD436. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD232	This work
pOD305	(C120)₅-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::<3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD488 and oOD484. Backbone was digested with SbfI and SwaI. Both fragments were assembled using Gibson cloning.	Pβtub2::FLUC(v2)::3'UTRrbc2	pOD232	This work
pOD306	Pβtub2enh-(C120)₅-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::<3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2			Manuscript 2
pOD310	3'UTRrbc2::FLUC(v2)::Pβtub2 (etr)₈-Pβtub2min::SpcCA-NLUC(ChRe+)::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD253 with oligos oOD485 and oOD491. Backbone was digested	3'UTRrbc2:: FLUC(v2):: Pβtub2	pOD237	This work

	with NotI. Both fragments were assembled using Gibson cloning.			
pOD324	(etr)₈-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD236 with oligos oOD532 and oOD534. Backbone was digested with NotI and AatII. Both fragments were assembled using Gibson cloning.	(etr) ₈ -P β tub2min::SPcCA	pOD306	This work
pOD326	(etr)₈-Pβtub2min::SPcCA-NLUC(ChRe+):3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD236 with oligos oOD532 and oOD535. Backbone was digested with NotI and EcoRV. Both fragments were assembled using Gibson cloning.	(etr) ₈ -P β tub2min::SPcCA-NLUC(ChRe+)	pOD306	This work
pOD386	PCMVmin::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2			Manuscript 2
pOD388	Ppsad::E(ChRe+)-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD072 with oligos oOD606 and oOD607. Backbone was digested with EcoRI and NdeI. Both fragments were assembled using AQUA cloning.	E(ChRe+)-{Irbcs2.2}-VP16(ChRe+)-NLS(SV40)(ChRe+)-{Irbcs2.1}-{Irbcs2.2}	pJR38	This work
pOD394	(etr)₈-Pβtub2min::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 3'UTRrbc2::3'UTRrbc2::FLUC(v2)::Pβtub2 Par::HygR(APHVII)::3'UTRrbc2 Insert was amplified from pOD324 with oligos oOD532 and oOD613. Backbone was digested with NotI and AgeI. Both fragments were assembled using Gibson cloning.	(etr) ₈ -P β tub2min	pOD386	This work
pOpt_gLuc_Hyg	Par::SPcCA-GLUC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}::3'UTRrbc2 Par::HygR(APHVII)::3'UTRrbc2			Lauersen et al., 2014
pJR38	Ppsad::GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2			Neupert et al., 2009
pROF053	(etr)₈ (Pos. A1)			Ochoa-Fernandez et al., 2020

Table S2. Oligonucleotides used for cloning.

Name	Sequence (5' → 3')
oOD007	GCGCCGTCTCGCTCGTGACCTGTTTAAATAGCCAGGCCC
oOD008	GCGCCGTCTCGCTCAATGGTTTTCGGGTTGTGACTGAAAC
oOD402	CAGCGCGACCAGGAGTAGAGCGCCAGTACGCGCCATATGTCGGTTTTTCGGGTTGT GACTGAAACG
oOD407	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGCTAGCCTCGAGTAGGTA GC
oOD410	TCCATGGGCGGGCTGGGCGTATTTGAAGCGGGATCCAGAGCCAGAGCCAGAGCC
oOD411	AAGCCTCAGCACGCGTATTTAAATTACGTACCTGCAGGTACCCATACGATGTTCCAG ATTACG
oOD412	TCCATGGTACAGGCGGTCCAGCTGCTGCCAGAATTCTTAGATGCCGGCCTCCAC
oOD413	CTAGGACCCCACTGCTACTCACAACAAGCCCATATGAGGTGCGAGTTCGACAGC
oOD415	CTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGCAATTCGTCTCAGGAG GTTTAAAC
oOD416	CGGTTACTTCTCGAACTGCGGGTGGCTCCAGAATTCGGCCAGGATGCGCTCGCA
oOD417	GTCAGGCAGACGGGCAGGTGTGTGGGATCCACTAGTGTCTCATGACAGCGGCATTG
oOD418	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCAATTCGTCTCAGGAGCAC ACAC
oOD419	TCCATGGTACAGGCGGTCCAGCTGCTGCCAGAATTCCTACACCTTGCGCTTCTTCTTG GG
oOD420	CTAGGACCCCACTGCTACTCACAACAAGCCCATATGACCACCATGGTGAGCGGC
oOD436	CATACGCACCAATCATGTCAAGCCTCAGCACGCGTATTTAAATTTAACCGTACGTTC GAAGGATCC
oOD441	GGGCATCGACGAGTACGGCGGCGCTTTGTACCCCAAGAAGAAGCGCAAGG
oOD484	GCACCAATCATGTCAAGCCTCAGCACGCGTATTTAAATGGCCGCTCTAGAACTAGAC TAG
oOD485	ATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTTAACCGTACGTTCGAAGG ATCC
oOD488	CCAGCGTAATCTGGAACATCGTATGGGTACCTGCAGGTTAACCGTACGTTCGAAGG ATCC
oOD489	AGCGGGATCCTTCGAACGTACGGTTAACTCGCGACCTGCAGGGGCCGCTCTAGAAC TAGACTAGT
oOD490	CAGAGCCAGCGTAATCTGGAACATCGTATGGGTACCTGCAGGGGCCGCTCTAGAAC TAGACTAGT
oOD491	TTCAATCGTTTAAACCTCCTGAGACGAATTCGGCGGCCGCGCTCTAGAACTAGAC TAG
oOD532	TATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGCAATTCGTCTCAGGAGG TTTAAAC
oOD534	GTTGTTCTCCGTCGGCTTCCTGCCCTCGATGACGTCCATTCTGCAAGCCTGCGC
oOD535	CTTCTCGAACTGCGGGTGGCTCCAGAATTCGATATCATCGGCCAGGATGCGCTC
oOD606	TTCTCCATGGTACAGGCGGTCCAGCTGCTGCCAGAATTCTTACACCTTGCGCTTCTTC TTGGG
oOD607	TAGGACCCCACTGCTACTCACAACAAGCCCATATGACCACCATGCCCCGCC
oOD613	CCAGGAGTAGAGCGCCAGTACGCGCCATGGACCGGTTTTTCGGGTTGTGACTGAAA C

4.3 Manuscript 3: Towards optogenetic control of mitochondrial membrane potential in *C. reinhardtii*

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Author contribution:

O.D.-R., wrote the manuscript, and designed, performed, analysed the data and supervised all the experiments despite the *C. reinhardtii* experiments related to the characterization of the physiological switch, the mitochondria isolation, and the MIC10 prediction bioinformatic analysis, which were performed by P.K.R. and conceptualized and supervised by S.B.G. M.D.Z. designed, conceptualized and supervised the project, analysed the data and wrote the manuscript.

Abstract

Mitochondria are central in various cellular processes, including energy production and Ca^{2+} signaling, which rely on the mitochondrial membrane potential ($\Delta\psi_m$). Optogenetic strategies utilizing opsins have been extensively employed to manipulate cellular membrane potential and, more recently, also to modulate the mitochondrial membrane potential with high specificity and reversibility. While optogenetic methods for $\Delta\psi_m$ modulation have been successfully implemented and used for studying different key cellular processes in mammalian cells, *C. elegans*, and *Drosophila sp.*, they have yet to be implemented in organisms performing photosynthesis. In this study, we take the initial steps toward implementing opsin-based tools in mitochondria of *C. reinhardtii* and, to a lesser extent, *A. thaliana*. Specifically, we targeted the ChR2 variant SSFO and the proton pump MAC into the mitochondria membrane. For this we fused it with inner mitochondrial membrane proteins. The opsins SSFO was selected with the intention of generating a light-controlled depolarization tool, mito-OFF, while MAC was chosen for generating a hyperpolarization tool, mito-ON. Additionally, we established a protocol for mitochondria isolation and $\Delta\psi_m$ measurement using the TMRE dye in *C. reinhardtii*, capable of detecting fast changes in $\Delta\psi_m$. This work lays the groundwork for investigating the impact of mitochondrial membrane potential on cellular processes, opening avenues for future research employing mitochondrial optogenetics in photosynthetic organisms ranging from algae up to plants.

Introduction

Mitochondria are organelles involved in numerous critical cellular processes, such as Ca^{2+} signaling, reactive oxygen species (ROS) production, cell signaling, and energy production (Abate et al., 2020; Shen et al., 2022). The inner mitochondrial membrane (IMM) houses the electron transport chain (ETC), which is responsible for the generation of a proton electrochemical gradient across the IMM. This protonmotive force is required to generate ATP and maintain mitochondrial functions (Chen et al., 2022; Zorova et al., 2018).

However, despite the importance of these organelles and the impact of the protonmotive force, methods to modulate the mitochondrial membrane potential ($\Delta\psi_m$) used to rely on uncoupling agents, like carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP), which irreversibly depolarize the mitochondria and lack cell specificity (Demine et al., 2019). Only seven years ago, an optogenetic tool for $\Delta\psi_m$ modulation was established in mammalian cells (Tkatch et al., 2017). This optogenetic approach utilizes opsins, channels and pumps controlled by light, targeted to the inner mitochondrial membrane. Light-activation of these proteins led to ion transport and therefore changes in membrane potentials. After that, various opsins have been employed in different organisms, including mammalian cells, *C. elegans* and *Drosophila* (Amitrano et al., 2021; Berry, Baldzizhar, et al., 2020; Berry, Trewin, et al., 2020; Berry & Wojtovich, 2020; Chen et al., 2022; Ernst et al., 2019; Imai et al., 2019; Tkatch et al., 2017).

Chlamydomonas reinhardtii, a green microalga, can grow under both aerobic and anaerobic conditions. Anaerobic growth of this microalga has attracted researcher's attention due to its association with a boost in H_2 production by a [Fe-Fe]-hydrogenase (A. C. Hemschemeier, 2005; A. Hemschemeier & Happe, 2005). During anaerobic conditions, there is a drop in mitochondrial membrane potential, that could potentially impact processes that depend on $\Delta\psi_m$ like mitochondrial protein import or mitochondrial dynamics (Ham & Raju, 2017; Hao et al., 2023; W. Neupert, 2015). The study of organisms tolerant to oxygen deprivation, like *C. reinhardtii*, could provide valuable insights into processes related to hypoxia, a common physiological stress (Sokolova et al., 2019).

Anaerobic conditions can be induced by growing the microalga in a sealed container or bioreactor in the absence of light and the presence of a carbon source. After some days, oxygen is consumed leading to anaerobiosis (Fig. 1a). Alternatively, sulfur deprivation in the media results in a decrease in the activity of photosystem II. Therefore, oxygen consumption by respiration is higher than production by photosystem II leading to a decrease in oxygen

concentration (Fouchard et al., 2005; A. Hemschemeier & Happe, 2005). The drop in oxygen levels is accompanied by a reduction in mitochondrial membrane potential (Fig. 1b). This allows the study of mitochondrial membrane potential-related processes by simply changing the physiological conditions of the cell. However, when the physiology is altered, many more factors are affected besides the mitochondrial membrane potential. This makes it difficult to make direct association between processes and changes in the $\Delta\psi_m$ (Hemschemeier, 2005; Mortensen & Gislerød, 2015; Mus et al., 2007). In order to have a tool to precisely study processes related to changes in mitochondrial membrane potential independently of the physiological state of the cell, we engineered lines in which opsins were targeted to the inner mitochondrial membrane. Opsins are channels and pumps capable of transporting ions in response to light. These generated lines would allow to modify the mitochondrial membrane potential without changing the growing conditions (i.e., effect of the lack of $\Delta\psi_m$ in certain processes when cells are grown in aerobiosis) (Fig. 2a).

Here, we targeted two different opsins to the inner mitochondrial membrane of *C. reinhardtii*: SSFO, a blue-light-activated channelrhodopsin-2 variant with longer open states due to two mutations in D156 and C128, and the orange-light-induced proton pump MAC from *Leptosphaeria maculans* (Waschuk et al., 2005; Yizhar et al., 2011). SSFO activation should lead to dissipation of existing gradients by cation transport in favor of the gradient (mito-OFF tool), while MAC would hyperpolarize the mitochondria by actively transporting protons to the intermembrane space (mito-ON tool). The transport direction is determined by the orientation of the pump within the membrane (Fig. 2b). The SSFO channel was previously used to modify mitochondrial membrane potential in mammalian cells (Tkatch et al., 2017). Similarly, the MAC pump was previously targeted to mitochondria in *C. elegans* and mammalian cells (Amitrano et al., 2021; Berry, Baldzizhar, et al., 2020; Berry, Trewin, et al., 2020). In these animal models, supplementation with the opsin cofactor, all-trans retinal (ATR), was required. However, this compound is naturally present in *C. reinhardtii*. Opsins were N-terminally-fused to *C. reinhardtii* MIC10 protein for IMM targeting.

Once the opsins have been targeted to the mitochondria, their correct functioning must be verified by analysing their ability to induce changes in the mitochondrial membrane potential in response to light. To monitor changes in the mitochondrial membrane potential, we established a protocol for mitochondria isolation and $\Delta\psi_m$ measurement with TMRE in *C. reinhardtii*, based on the methods implemented in mammalian cell cultures (Amitrano et al., 2021; Creed & McKenzie, 2019).

In brief, our aim is to generate optogenetic tools for manipulating mitochondrial membrane potential with light, independent of the physiological state of the cell. We also aim to establish protocols for monitoring changes in $\Delta\psi_m$ to be able to test the correct activity of the mito-targeted opsins. This would allow us to subsequently characterize the opsins and employ these new opto-tools to study processes affected by changes in $\Delta\psi_m$ by precisely modifying it with light. Our objective is to establish these opto-tools in the oxygen-deprivation-tolerant organism *C. reinhardtii*. Due to the complexity of expressing heterologous genes in this alga (Schroda, 2019), we initially tested the correct design of the opto-tools and the mitochondrial membrane potential measurement method using other model organisms such as *A. thaliana* and mammalian cell cultures.

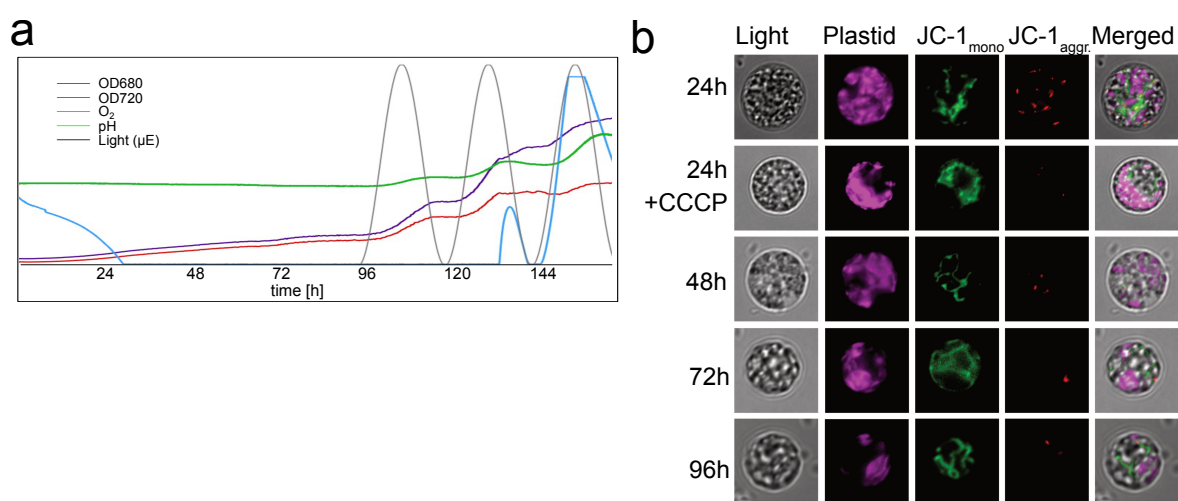


Figure 1. Characterization of the mitochondrial membrane potential physiological switch in *C. reinhardtii*. **(a)** Induction of anaerobic conditions. Oximetry of *C. reinhardtii* shows a drop in oxygen levels after 24 h culture in a sealed bioreactor in the absence of light. **(b)** Effect of anaerobic conditions in $\Delta\psi_m$. Mitochondrial membrane potential staining with JC-1 reveals a drop in $\Delta\psi_m$ when anaerobiosis is reached or a mitochondria uncoupler (carbonyl cyanide m-chlorophenyl hydrazine, CCCP) is added. JC-1 exhibits green fluorescence in its monomeric form (mono) when $\Delta\psi_m$ is low, while it exhibits red fluorescence when it aggregates (aggr.) in the presence of high $\Delta\psi_m$.

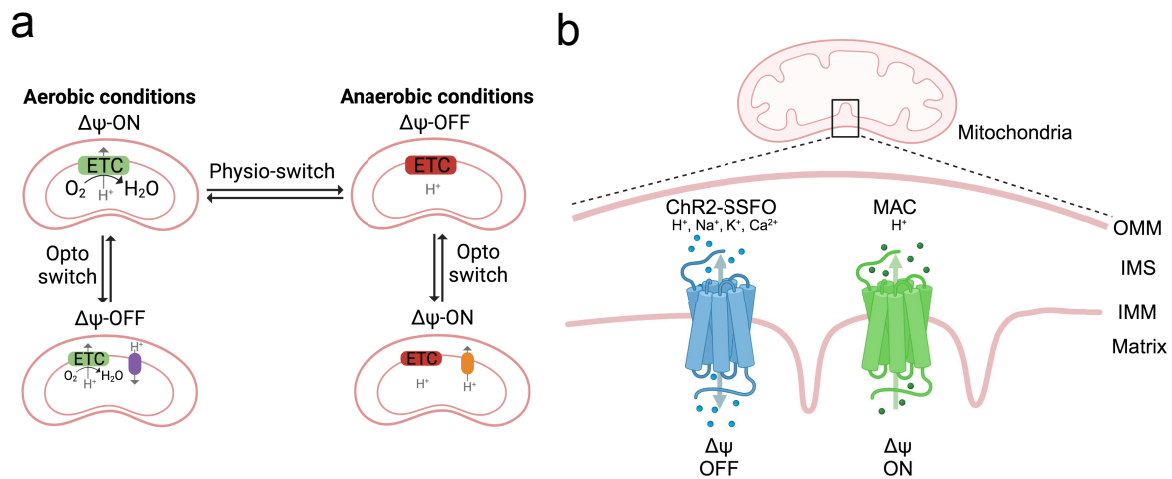


Figure 2. Depiction of the optogenetic molecular strategy to modify mitochondrial membrane potential ($\Delta\psi_m$). (a) Physiological and optogenetic switches of $\Delta\psi$ in *C. reinhardtii*. The physiological switch is based on the $\Delta\psi_m$ reduction that occurs when the alga is grown in anaerobic conditions. The lack of oxygen impedes the electron transport chain and, therefore, the generation of a proton gradient. The optogenetic switch is based on opsins that transport protons, thereby modifying the proton gradient. Two different opsins are employed: one enables the polarization of mitochondria in cells grown under anaerobic conditions by actively transporting protons outside the matrix, restoring the proton gradient. The other opsin facilitates the dissipation of the existing gradient in mitochondria of cells grown under aerobic conditions, thereby depolarizing the mitochondria. (b) Scheme of the selected opsins to generate the $\Delta\psi_m$ optoswitches: ChR2-SSFO and MAC, targeted to the IMM. Light activation of the channel ChR2-SSFO dissipates existing gradients, while MAC will hyperpolarize the mitochondria by actively transporting protons from the matrix to the IMS. ETC, electron transport chain; $\Delta\psi$, membrane potential; OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane.

Materials and methods

Plasmid generation

A detailed description of plasmids and oligonucleotides designed, constructed and used in this work are listed in tables S1 and S2, respectively. Heterologous genes were codon-optimized for *C. reinhardtii*: gene design was done with Intronserter (Jaeger et al., 2019), synthesis of the intronized and codon optimized genes was performed by GeneArt Thermo Fisher Scientific. DNA fragments of interest were amplified according to table S1 using polymerase chain reaction (PCR), with oligonucleotides provided by Sigma Aldrich or Integrated DNA Technologies, using Q5 High-Fidelity DNA Polymerase with GC enhancer solution (New England Biolabs, NEB). 1 % agarose gel was used to separate DNA fragments. For gel extractions, NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used. Plasmids were assembled using Golden Braid, Gibson assembly or AQUA cloning (Beyer, Gonschorek, et al., 2015; Gibson et al., 2009; Sarrion-Perdigones et al., 2011) and transformed into chemically competent *Escherichia coli* strain 10-beta (New England Biolabs, NEB). Plasmids were isolated from *E. coli* using Wizard Plus SV Miniprep DNA Purification Systems (Promega) and NucleoBond Xtra Midi kit (Macherey-Nagel). Newly constructed plasmids were verified by restriction enzyme (New England Biolabs, NEB) digestion and DNA sequencing (Microsynth).

E. coli and *C. reinhardtii* strains, transformation and growth conditions

E. coli strain 10-beta (New England Biolabs, NEB) was grown at 37°C in LB broth supplemented with agar (4 % m/V) and kanamycin (25 µg/mL), ampicillin (100 µg/mL), chloramphenicol (25 µg/mL) or spectinomycin (100 µg/mL) when required. Plasmid were transformed into chemically competent cells by heat shock as described elsewhere (Froger & Hall, 2007).

C. reinhardtii strain UVM4 (J. Neupert et al., 2009) was routinely grown in Tris-acetate-phosphate (TAP) medium (Gorman & Levine, 1965) supplemented with agar (1.2 % m/v), ampicillin (0.05 mg/mL) and, when required, paromomycin (10 µg/mL). Liquid cultures were grown at 160 rpm under 50 µmol photons m⁻² s⁻¹ long day 16:8 h cycle, at 22 °C. When indicated, liquid cultures were grown in Sueoka's high salt (HS) media (Sueoka, 1960).

For *Chlamydomonas* transformation, the glass bead method was followed (Kindle, 1990; Lauersen et al., 2015). A TAP culture of 1-3 x 10⁶ cells/mL was set to a final concentration of 3 x 10⁸ cells/mL. 1 x 10⁸ cells were mixed with 2 µg of linearized plasmid and 300 mg of 0.5 mm glass beads, prior to 15 s vortexing at high speed. Transformations were spread on TAP-

agar plates with appropriate antibiotics. 20 h incubation in darkness was followed by 5-7 days of constant white light illumination ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Mammalian cell cultivation and transfection

Human epithelioid cervix carcinoma cells (HeLa; DSMZ, Braunschweig, Germany) were cultivated in Dulbecco's Modified Eagle Medium (DMEM, PAN, cat. no. P04-03550) supplemented with 10 % tetracycline-free fetal bovine serum (FBS, PAN, cat. no. P30-3602) and 1 % penicillin/streptomycin (PAN, cat. no. P06-07100) in a 5 % CO_2 atmosphere at 37 °C. Cells were transiently transfected as described elsewhere (Beyer, Juillot, et al., 2015; Müller et al., 2013) in 24-well (3.5×10^4 cells in 500 μL DMEM, in each well) plates (Corning) using polyethyleneimine (PEI; Polysciences Inc. Europe, Hirschberg, Germany; no. 23966-1).

***A. thaliana* mesophyll protoplast isolation and transformation**

Growth of *A. thaliana* plants, material preparation, protoplast isolation and subsequent transformation was performed according to Ochoa-Fernandez et al., 2016. If not indicated otherwise, all plasmids were transformed in equal amounts (weight-based) and a total of 30 μg of DNA was transformed into 500,000 protoplasts.

Mammalian, *A. thaliana* and *C. reinhardtii* sample preparation and fluorescent microscopy

HeLa cells were seeded on glass coverslips. 24h after transfection, cells were incubated for 30 min with 300 nM of Mitotracker Red CMXRos #9082 (1 mM stock solution in DMSO; Cell Signaling Technology) for mitochondria staining and fixed at 37 °C with paraformaldehyde as described elsewhere (Beyer, Juillot, et al., 2015).

A. thaliana transformed protoplasts were kept 24 h in darkness before addition of 200 nM of Mitotracker Red CMXRos #9082 (1 mM stock solution in DMSO; Cell Signaling Technology) to the protoplast suspension in PCA media (0.32% (wt/vol) Gamborg's B5 basal salt powder with vitamins (bioWorld), 58.4 mM sucrose, 3.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mM MES, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.342 mM l-glutamine, 444 mM glucose, 8.4 μM calcium pantothenate, 2% (vol/vol) biotin from a biotin solution 0.02% (wt/vol) 0.1% (vol/vol) in H_2O , pH 5.8, sterile filtered, 0.1% (vol/vol) Gamborg's B5 Vitamin Mix, 64.52 $\mu\text{g} \mu\text{L}^{-1}$ ampicillin). 15 min after, protoplasts were transferred to a 4-well cell culture chamber on glass slide (Sarstedt) and imaged.

For *C. reinhardtii* transgenic lines samples preparation, 75 nM of Mitotracker Red CMXRos #9082 (50 μM stock solution in TAP media; Cell Signaling Technology) was added to 1 mL

of culture. Samples were shaken at 160 rpm and 22 °C for 15 min. Cells were centrifuged at 3,000 rpm during 3 min, and the pellet was resuspended in 100 µL of fresh media. For cell imaging, cell suspensions were embedded in low melting agarose 1.2 % (w/v).

Mammalian and *A. thaliana* confocal imaging was performed with an Eclipse Ti2 microscope (Nikon, Tokyo, Japan) equipped with a C2plus confocal laser scanner and a 60x oil objective (NA 1.4). EGFP and Mitotracker Red CMXRos #9082 fluorescence were visualized using an excitation laser of 488 and 561 nm and emission filters from 500-550 and 570-1000 nm, respectively. Image acquisition was performed with NIS-Elements Viewer (Nikon instruments, version 4.11.0) and image analysis with Omero.

For *C. reinhardtii* confocal imaging a TCS SP8 STED 3x (Leica Microsystems) equipped with an 93x glycerol (NA 1.3) objective was used. For excitation a White Light Laser (WLL) was employed. EGFP was excited with 488 nm, while Mitotracker Red CMXRos #9082 and chlorophyll fluorescence were excited with 580 nm. Detection was carried out using Hybrid (HyD) detectors in the spectral range of 500-530 nm, 590-610 nm, and 670-710 nm, respectively. Additionally, deconvolution of images was performed.

Protein extraction and cell subfractionation of *C. reinhardtii*

For total protein extraction from *C. reinhardtii* cells, 1 mL of liquid culture $1-5 \times 10^6$ cells/mL was centrifuged at 3,000 rpm for 5 min. Cell pellets were resuspended in RIPA buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.1 % NP40, 5 mM EDTA) supplemented with 1 tablet of Protease Inhibitor Cocktail (cOmplete, Mini EDTA-free Protease Inhibitor Cocktail, Roche), and stirred at 700 rpm for 15 min. The solution was further centrifuged and the supernatant was stored at -20 °C.

For cell subfractionation, $1-5 \times 10^9$ cells were collected from a liquid culture by centrifuging at 1,000 g during 3 min at 4 °C. Two washing steps were performed in 50 mL of 20 mM HEPES-KOH, pH 7.2, before resuspending the pellet with a cut pipette tip to a final concentration of 0.5 g/mL in breaking buffer (50 mM HEPES-KOH-pH7.2, 5 mM EDTA, 0.25 M Sorbitol, 4 mM cysteine, 0.5 % PVP40, 0.1 % BSA). Cells were lysed by passing the solution through a 27 Gauge needle at a flow rate of 0.06 mL/s. Intact cells were spun down at 3,000 g for 5 min at 4 °C. The supernatant was collected and centrifuged at 15,000 g for 15 min at 4 °C. Subsequently the supernatant was centrifuged at 60,000 g during 2 h at 4 °C. From here, the supernatant was taken as cytosolic fraction and the pellet as membrane fraction.

Mitochondria isolation from mammalian and *C. reinhardtii* cells

Mitochondria isolation from mammalian cells protocol is based on Amitrano et al., 2021 and Lampl et al., 2015 procedures with minor modifications. Briefly, 2 10-cm petri dishes plates with 1.2×10^6 cells HeLa cells were used for transfection. 24 h later, mitochondria isolation was performed. Cells were washed twice with PBS, prior to scraping and collection of the cells. Cell suspension was centrifuged at 4 °C for 5 min at 700 g. Cell pellet was resuspended in 2 mL of mitochondria isolation buffer (10 mM HEPES, 1 mM EDTA, 320 mM sucrose, pH 7.2) and homogenized with a pestle for 60 passes in a Dounce homogenizer. The homogenized solution was centrifuged (700 g, 8 min, 4 °C). The supernatant was collected and further centrifuged at 4 °C for 11 min at 17,000 g. The pellet containing the mitochondria was resuspended in mitochondria isolation buffer.

C. reinhardtii mitochondria isolation was performed from a $1-3 \times 10^6$ cells/mL culture grown in TAP media for 36-40 h. 1.5 L of culture were centrifuged at 1,000 g during 3 min at 4 °C. Cells were washed twice with 50 mL of 20 mM HEPES-KOH, pH 7.2. The pellet was resuspended with cut pipette tips in breaking buffer (50 mM HEPES-KOH-pH7.2, 5 mM EDTA, 0.25 M Sorbitol, 4 mM Cysteine, 0.5 % PVP40, 0.1 % BSA), 1 mL of breaking buffer was used for every 0.5 g of pellet. Solution was passed through a 27 Gauge needle at a flow rate of 0.06 mL/s and centrifuged at 2,000 g during 5 min at 4 °C. The supernatant was further centrifuged at 2,000 g during 10 min at 4 °C. The supernatant was collected and centrifuged in an Optima XPN-80 ultracentrifuge (Beckman Coulter GmbH) at 12,000 g during 10 min at 4 °C. Pellet was resuspended in 8 mL assay buffer (10 mM KH_2PO_4 -pH 7.2, 0.25 M Sorbitol, 10 mM KCl, 5 mM MgCl_2 , 0.1 % BSA) with 0.5 % PVP40 and centrifuged at 2,000 g for 10 min at 4 °C. Supernatant was transferred to a glass potter in which a plunger was slowly passed once. Mitochondria suspension was divided in two and loaded on 10 mL of 20 % percoll solution (20 % Percoll, 0.25 M Sorbitol, 10 mM Mops-KOH-pH7.2, 0.1 % BSA) and centrifuged in a Sorvall LYNX 4000 Superspeed-centrifuge (Thermo Scientific) at 15,000 g for 40 min at 4 °C. 2 mL from the bottom were collected and washed twice with 5-8 mL washing buffer (10 mM KH_2PO_4 -pH 7.2, 0.25 M Sorbitol, 1 mM EDTA, 0.1 % BSA) by centrifuging at 12,000 g for 15 min at 4 °C. Pellet was resuspended in 25-100 μL of assay buffer.

Western blot

Samples were boiled for 10 min at 95 °C with 4x SDS buffer (Sigma Aldrich) and proteins were separated by SDS-PAGE on a 10-12 % polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories), that were subsequently blocked

for 1 h with blocking solution (5 % milk powder in PBS + 0.1 % Tween20) and incubated overnight with primary antibodies at 4 °C. Washing with PBS + 0.1 % Tween20 was performed prior to membrane incubation with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (7074 S and 7076 S, respectively, Cell Signaling) for 1 h at room temperature. After incubation, membranes were washed and incubated for 1-5 min with WesternBright™ Sirius (Advansta). Chemiluminescence was visualized using an Amersham Imager 680 (GE Healthcare) system.

Primary antibodies used: anti-GFP (TP-401, Torrey Pines Biolabs), anti-coxIIb (AS06 151, Agrisera), anti-NAB1 (AS08 333, Agrisera), anti-GAPDH (Cell Signaling Technology, 2118), anti-TOM20 (sc-114115, Santa Cruz Biotechnology).

TMRE assay

Similar to Amitrano et al., 2021, isolated mitochondria were diluted to a final concentration of 0.4-0.5 mg/mL in mitochondria respiration buffer (120 mM KCl, 25 mM sucrose, 5 mM MgCl₂, 5 mM KH₂PO₄, 1 mM EGTA, 10 mM HEPES, 1 mg/mL FF-BSA, pH 7.35) with 20 nm Tetramethylrhodamin-Ethylester (TMRE, Thermo Fisher Scientific). When indicated, rotenone was added at a concentration of 1.7 µM. This mitochondria suspension was stirred at 700 rpm for 15 min at 22 °C, protected from light. Samples were transferred to black 96-well flat-bottom plates (100 µl/well; Costar). TMRE fluorescence was measured in a BMG Labtech CLARIOstar (BMG Labtech GmbH) in intervals of approximately 1 minute (Ex: 570-15 nm/Em: 620-20 nm). During the measurement, 6-12.5 mM succinate was added to some of the samples. 2-15 µM of FCCP was added to all of the samples as a control at the end of the measurement.

Software

Data was analysed using GraphPad Prism 9 for MacOS version 9.0.0 and Microsoft Excel (version 16.16.27 for Mac). Benchling [Biology Software] was used for molecular cloning design. Inkscape 1.2.2 (b0a8486, 2022-12-01) and BioRender.com were employed for graphical design.

Results

Construction, expression and targeting of mito-opsins: mammalian cells, *A. thaliana* protoplasts and *C. reinhardtii*

To generate a mito-ON tool for *C. reinhardtii*, we used the proton pump MAC from *L. maculans*, previously employed to modify mitochondrial membrane potentials in *C. elegans* and mammalian cells (Amitrano et al., 2021; Berry, Baldzizhar, et al., 2020; Berry, Trewin, et al., 2020). The expression of heterologous proteins in *C. reinhardtii* is known to be complex and inefficient, mainly attributed to the silencing of transgenes (Schroda, 2019). Additionally, the need to generate stably transformed lines by integration of DNA into the genome impedes rapid evaluation of constructs as it is usually done with transient set ups in protoplasts or mammalian cell cultures. For this reason, when developing new synthetic biology tools, it might be worth considering testing the correct design in an alternative model organism. In order to confirm the correct expression and targeting of the MAC pump to the IMM in our hands, and to establish the methods for later verification of opsins functionality in *C. reinhardtii*, we tested the pBJB28 construct generated by Amitrano et al., 2021 in transiently transfected HeLa cells. In this construct, MAC is targeted to the IMM through fusion with the first 187 amino acids from MIC60, a homologue of mitofilin, and fused to EGFP for visualization. Overlapping of GFP signal with Mitotracker CMXRos, visualized by fluorescence microscopy, verifies correct targeting of MAC to the mitochondria (Fig. 3a,b). Furthermore, we tested it in *A. thaliana*, a model organism more similar to *C. reinhardtii*, to validate correct targeting to the mitochondria in the presence of chloroplasts. *A. thaliana* protoplasts were transformed with pOD193, in which the mitofilin-MAC-EGFP fusion protein is placed under the control of the CaMV 35S promoter. Staining with Mitotracker CMXRos and imaging shows correct mitochondrial targeting (Fig. 3d). Moreover, we designed and assembled a plasmid suitable for generating stable *A. thaliana* lines expressing the IMM-targeted MAC pump. This construct is intended for transformation into *A. thaliana* Ret-eYFP lines capable of synthesizing the ATR cofactor (Zhou et al., 2021; Fig. 3c).

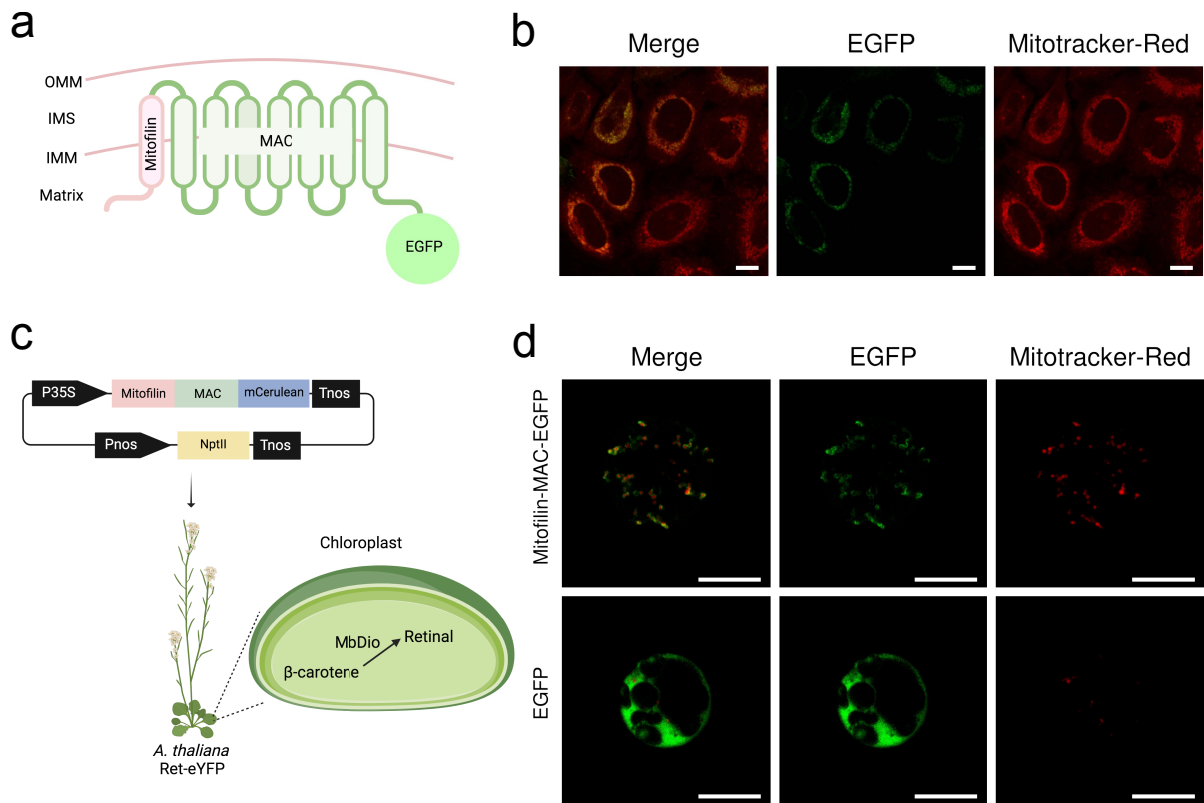


Figure 3. MAC pump in HeLa cells and *A. thaliana* protoplasts. (a) Molecular depiction of the targeting strategy developed by Berry et al. (Amitrano et al., 2021; Berry, Trewin, et al., 2020) to target MAC to the IMM. (b) Expression and colocalization of MAC in mitochondria in HeLa cells. 24 h after transfection with pBJB28, mitochondria were stained and cells were fixed with PFA, prior to confocal fluorescence microscopy imaging. (c) Molecular construct scheme to generate stable Ret-eYFP *A. thaliana* lines expressing an IMM-targeted MAC. Ret-eYFP *A. thaliana* lines produce MAC chromophore retinal (Zhou et al., 2021). (d) Confocal microscopy colocalization analysis of mitofilin-MAC-EGFP (top) or cytosolic EGFP (bottom) with mitochondria in *A. thaliana* protoplasts. Imaging was performed 24 h after protoplasts transformation with pOD193 or PGEN16. (b, d) 300 or 200 nM of Mitotracker Red CMXRos was used to stain mitochondria from HeLa cells or *A. thaliana* protoplasts, respectively. Scale bar 10 μ m. OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane; P35S, CaMV 35S promoter; Tnos, *Agrobacterium tumefaciens* nopaline synthase terminator; Pnos, *A. tumefaciens* nopaline synthase promoter; MbDio, β -carotene 15,15'-dioxygenase.

To ensure the proper expression and mitochondrial targeting of the MAC pump in *C. reinhardtii*, MAC was codon optimized for *C. reinhardtii* and intronized, and the mammalian MIC60 protein was substituted with *C. reinhardtii* MIC10. The fusion construct was expressed under a promoter-terminator combination previously proven effective in *C. reinhardtii* (Crozet et al., 2018) and transformed in the strain UVM4 (J. Neupert et al., 2009). The orientation of the pump within the membrane depends on the number of transmembrane helices (TMH). As MAC has 7 TMH, it is likely to orient itself into mito-OFF orientation. Therefore, fusion with an IMM protein with a single TMH, such as MIC10, was necessary.

MIC10, a small component of the MICOS complex, was identified through bioinformatic prediction of its homologue in yeast, followed by in-silico validation of its structural similarity to well-characterized MIC10 proteins (Alkhaja et al., 2012). The DeepTMHMM bioinformatic tool (Hallgren et al., 2022) was employed to predict the final construct's topology, confirming its correct insertion within the IMM, which would result in transport of protons from the matrix to the intermembrane space upon light activation (Fig. 4a). Western blots against GFP and confocal microscopy were used to verify the correct protein size and co-localization with mitochondria. Two transgenic lines (#12 and #54) were analysed, along with two controls: GFP and MIC10 fused to GFP (Fig. 4b,c). The localization of MIC10 alone to mitochondria offers initial evidence of the localization of this potentially functional MICOS subunit. However, a perfect overall merge between Mitotracker CMXRos and GFP is not observed in the mito-ON lines. Therefore, to further validate the mitochondrial membrane localization of the MIC10-MAC-GFP construct, we optimized a method to significantly enrich the membrane fraction from total protein of *C. reinhardtii*. This method was employed to separate proteins from the cytosolic/matrix and the membrane fractions. The comparison between the cytosolic/matrix fraction and the membrane enriched fraction showed a stronger signal of MIC10-GFP, MIC10-MAC-GFP, and the known IMM protein COXII (IMM control) in the membrane fraction. In contrast, GFP alone and a known cytosolic protein NAB1 (cytosolic control) were only faintly visible in the membrane fraction (Fig. 4d). This, in conjunction with confocal microscopy data, suggests that MIC10-MAC-GFP is localized to one of the mitochondrial membranes and highlights that MIC10 can serve as a mitochondrial membrane targeting sequence.

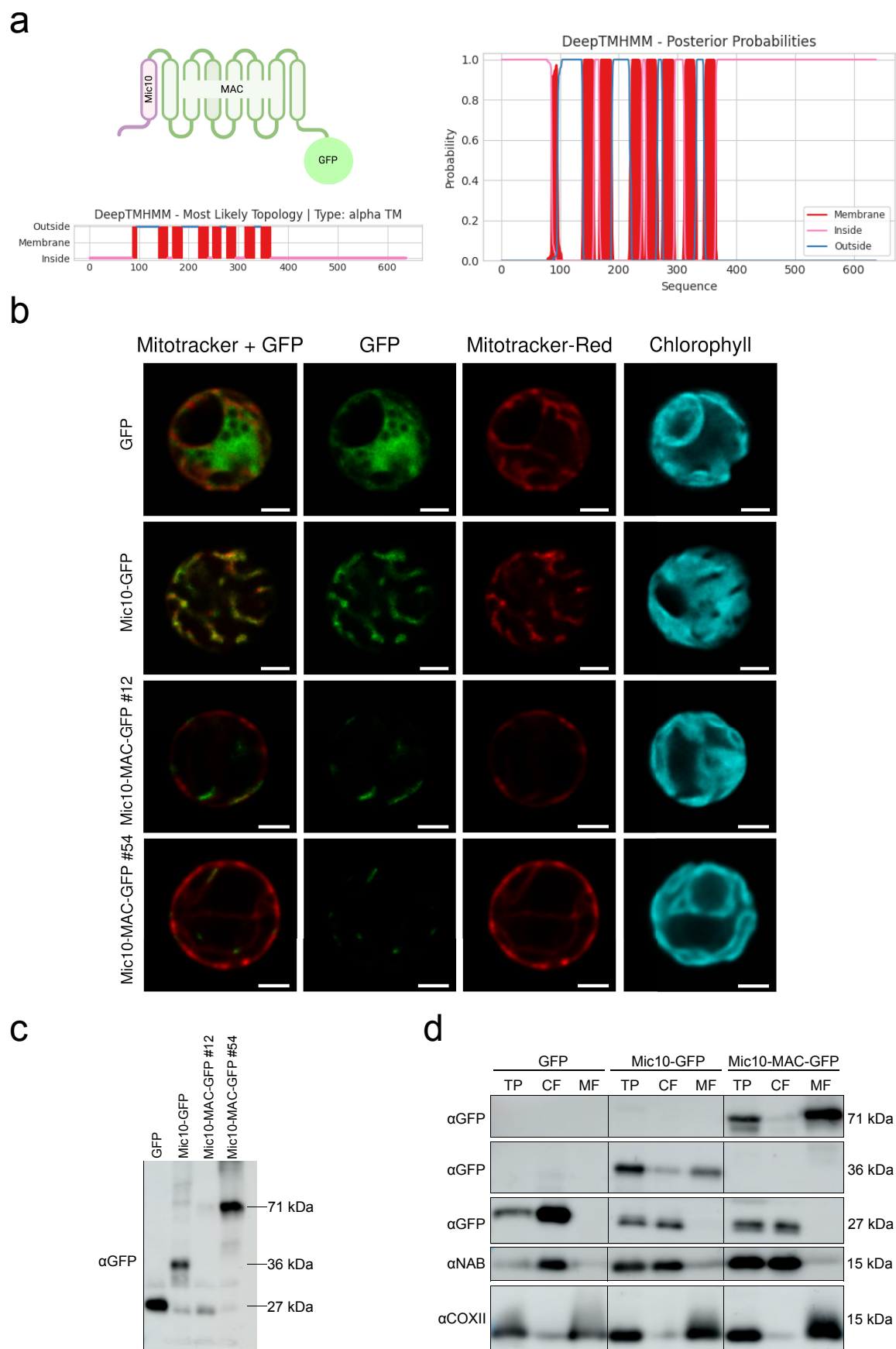


Figure 4. Targeting of MAC pump into the inner mitochondrial membrane of *C. reinhardtii*. (a) Molecular scheme of the approach to target MAC to the IMM by its fusion to *C. reinhardtii* Mic10. Topology prediction of the resulting fusion (Mic10-MAC-GFP), made with the bioinformatic tool

DeepTMHMM. **(b)** Confocal fluorescence microscopy imaging of intracellular IMM-targeted MAC. Two *C. reinhardtii* lines (12 and 54) expressing Mic10-MAC-GFP (pOD177) and the control lines expressing GFP (pJR38) or IMM-targeted GFP (Mic10-GFP; pOD196) were stained with 75 nM MitoTracker Red CMXRos to analyse colocalization with mitochondria (mitotracker-Red). Liquid cultures were grown in HSM, in the absence of acetate. Scale bar 2 μm . **(c)** Western blot detection of GFP in the lines described in (b) using an anti-GFP antibody. Cytosolic GFP, 27 kDa; Mic10-GFP, 36 kDa; Mic10-MAC-GFP, 71 kDa. **(d)** Analysis of MAC localization by cell subfractionation. Immunoblot comparing total protein with cytosolic and membrane fractions. Mic10-GFP and Mic10-MAC-GFP are enriched in the membrane fraction. The anti-NAB1 and anti-CoxIIb antibodies from the cytosolic and inner mitochondrial membrane proteins, respectively, are used as controls.

To engineer the mito-OFF tool, the Channelrhodopsin-2 variant, SSFO, devoid of the 24 first amino acids responsible for its targeting to the cell membrane, was fused with the previously validated *C. reinhardtii* mitochondrial membrane targeting peptide, MIC10 (Fig. 5a). In a functional mitochondrion, blue light activation of the IMM-targeted SSFO is predicted to result in the flow of cations into the matrix, leading to dissipation of the existing $\Delta\psi_m$. As for the mito-ON tool, the MIC10-SSFO-GFP fusion protein was placed under the control of a specific promoter-terminator combination, and the final construct was transformed into *C. reinhardtii* UVM. Out of ninety-six screened clones, four exhibited positive GFP expression. In two of these lines (#26 and #47), fluorescence microscopy showed an enhanced GFP signal in the mitochondrial regions (Fig. 5b). Immunoblotting against GFP displayed a band of the expected protein size (68 kDa), although with more potential GFP truncations compared to the mito-ON lines (Fig. 5c).

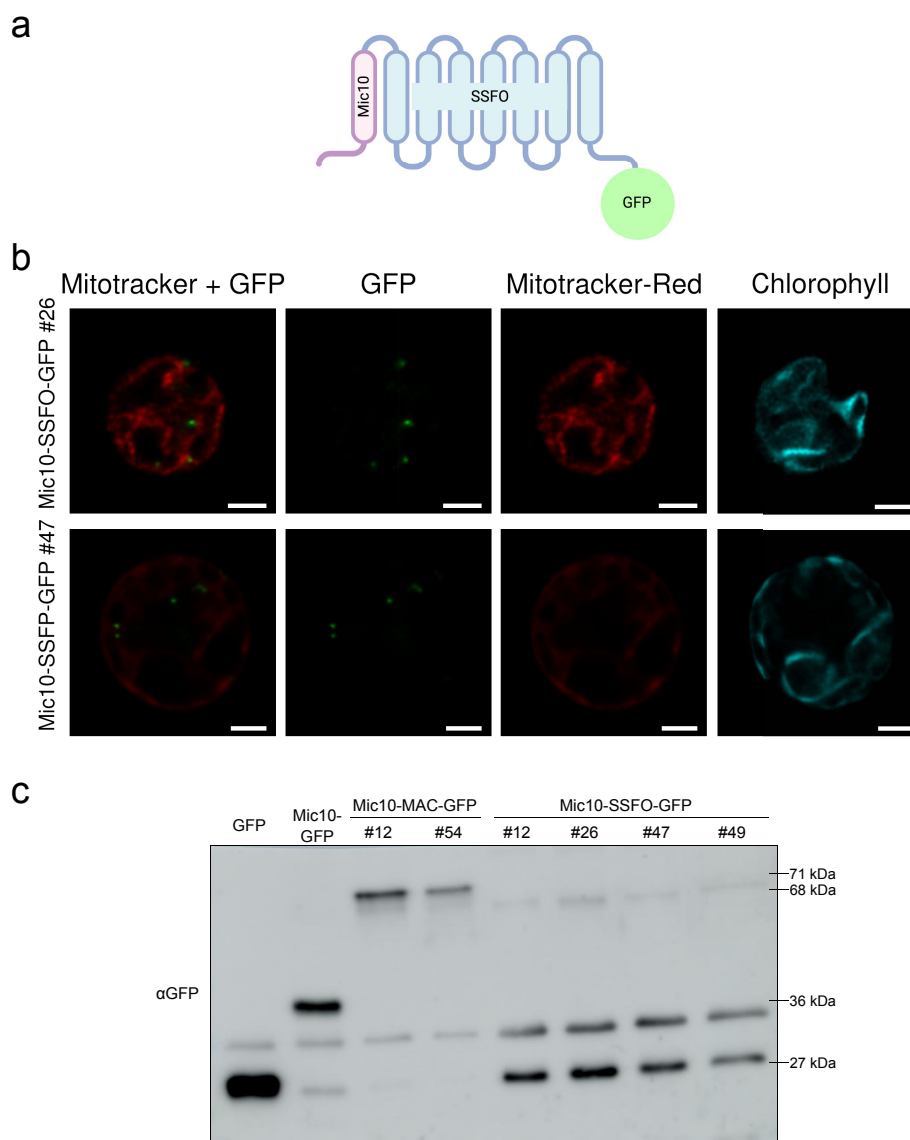


Figure 5. Targeting of Chr2-SSFO into the inner mitochondrial membrane of *C. reinhardtii*. (a) Illustration of the molecular strategy to target SSFO to the IMM by the fusion with Mic10. (b) Confocal fluorescence microscopy colocalization analysis of Mic10-SSFO-GFP with mitochondria (mitotracker-Red). Two *C. reinhardtii* lines (26 and 47) transformed with pOD366 were grown TAP liquid cultures in the absence of light, mitochondria were stained with 75 nM Mitotracker Red CMXRos. Scale bar 2 μ m. (c) Western blot comparison of GFP in the control and Mic10-MAC-GFP lines described in Figure 4b, and four lines transformed with pOD366, using the anti-GFP antibody. Cytosolic GFP, 27 kDa; Mic10-GFP, 36 kDa; Mic10-MAC-GFP, 71 kDa; Mic10-SSFO-GFP, 68 kDa.

Monitoring changes in mitochondrial membrane potential in *C. reinhardtii*

TMRE assay: establishment in mammalian cells and implementation in *C. reinhardtii*

To validate the functionality of the mito-ON and mito-OFF tools generated for *C. reinhardtii*, a quantitative method for measuring changes in $\Delta\psi_m$ is required. We opted for the tetramethylrhodamine ethyl ester (TMRE), a cationic dye that accumulates in the mitochondrial matrix proportionally to the magnitude of the $\Delta\psi_m$ (Fig. 6). The fluorescence signal output of

this dye varies depending on the concentration of dye used. High concentrations (>50 nM), known as “quenching” conditions, lead to high accumulation of dye in polarized mitochondria, resulting in dye aggregation, quenching of the signal, and low fluorescence. Dye aggregation does not occur when low dye concentrations (0.5-30 nM), referred to as nonquenching conditions, are used. In nonquenching conditions, increased TMRE retention in the matrix leads to a stronger fluorescence signal (Creed & McKenzie, 2019; Perry et al., 2011). At the end of every experiment, to confirm whether the dye was employed in quenching or nonquenching conditions, addition of an uncoupling agent as FCCP is recommended. This addition would result in either a decrease or increase in fluorescence, depending on whether the dye was in nonquenching or quenching conditions, respectively. TMRE assays can be performed in whole cells or in isolated mitochondria. To test if the IMM-targeted MAC pump is functional, rotenone is added to disrupt existing $\Delta\psi_m$ by inhibiting complex-I of the electron transport chain. The subsequent regain of $\Delta\psi_m$ upon light activation of the MAC pump would be an indicator of its functionality. Conversely, for SSFO, after rotenone addition, succinate is added to regain $\Delta\psi_m$ by transferring protons to the ETC from complex II onwards. Activation of the SSFO channel is expected to dissipate the proton gradient under such condition (Fig. 6).

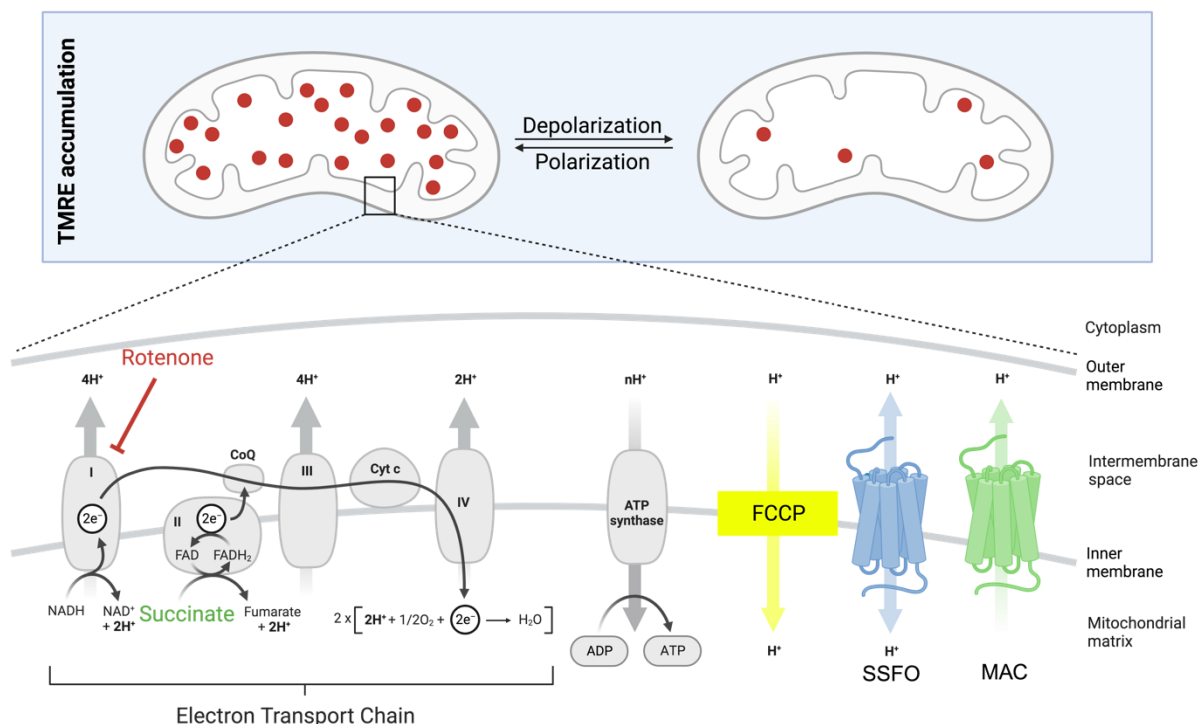


Figure 6. Schematic of the TMRE assay employed to monitor changes in the mitochondrial membrane potential caused by opsins. Top, differential $\Delta\psi_m$ -dependent accumulation of the cationic dye TMRE in mitochondria. Gain and loss of $\Delta\psi_m$ can be measured by the fluorescence of TMRE, which is proportional to $\Delta\psi_m$, when the dye is used in non-quenching concentrations. Bottom, depiction of the effect of two opsins (SSFO and MAC), and different chemicals (rotenone, succinate and FCCP) on the proton gradient across the inner mitochondrial membrane. The proton gradient generation by the

ETC is suppressed by the addition of rotenone, a complex-I inhibitor. Addition of succinate can restore the proton gradient through the oxidation of succinate by complex II. FCCP acts as an uncoupling agent of the ETC by transporting protons across the inner mitochondrial membrane. IMM-targeted opsins can induce reversible changes in the $\Delta\psi_m$, activation of a channelrhodopsin like SSFO result in dissipation of the proton gradient, while the latter increases upon activation of the MAC pump in outward orientation. ETC, electron transport chain.

Establishing TMRE assays to test opsin activity in isolated mitochondria requires first isolating the mitochondria. Given the complexity of mitochondria isolation in *C. reinhardtii*, mainly due to the necessity of separation from chloroplasts, the TMRE assay protocol was first established and optimized using isolated mitochondria from HeLa cells transiently transfected with the mito-ON construct. Following mitochondria isolation, the integrity of the outer mitochondrial membrane (OMM) was assessed by immunoblotting against the TOM20, an OMM protein, ensuring that not only mitoplasts were isolated during the process (Fig. 7a). Subsequently, the TMRE assay was performed, in which the $\Delta\psi_m$ of rotenone-treated mitochondria was regained by the addition of succinate. The addition of FCCP, which disrupts $\Delta\psi_m$, confirmed the nonquenching concentration of the TMRE dye (Fig. 7b). The response to the addition of succinate and FCCP demonstrated the functionality of the TMRE assay in monitoring fast changes in $\Delta\psi_m$.

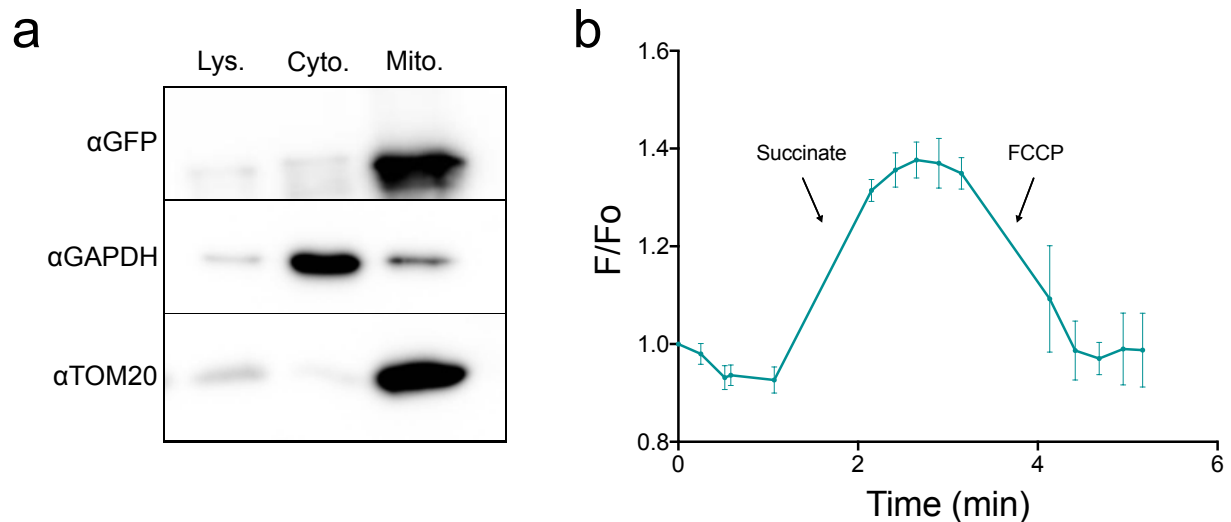


Figure 7. Mitochondria isolation and TMRE assays in HeLa cells transiently transfected with the proton pump MAC targeted to the IMM and fused to GFP. (a) Analysis of the integrity of isolated mitochondria and localization of MAC. Western blot comparing mitochondria (Mito.) with cytosolic fraction (Cyto.) and whole cell lysate (Lys.). MAC, detected with and anti-GFP antibody, is enriched in mitochondria. To examine mitochondria presence and integrity an outer mitochondrial membrane protein antibody, anti-TOM20, was used. Anti-GAPDH antibody was employed as a cytoplasm marker. **(b)** $\Delta\psi_m$ measurement from purified mitochondria by TMRE assay. Rotenone was added to disrupt existing $\Delta\psi_m$ by blocking complex-I of the electron transport chain. $\Delta\psi_m$ is regained by the addition of succinate. Proton gradient is dissipated by the addition of FCCP.

Following the optimization of the TMRE assay in mitochondria derived from mammalian cells, we extended the application of mitochondria isolation and TMRE assays to *C. reinhardtii*. Mitochondria were separated from chloroplasts using a percoll gradient, an overview of the isolation method is illustrated in Figure 8a. The TMRE assays conducted with algal mitochondria were carried out in the same way as for mammalian mitochondria. The resulting curve confirmed proper TMRE fluorescence changes in response to succinate and FCCP (Fig. 8b). The successful adaptation of this methodology in *C. reinhardtii* opens up the way to test the activity of mitochondrial-targeted opsins.

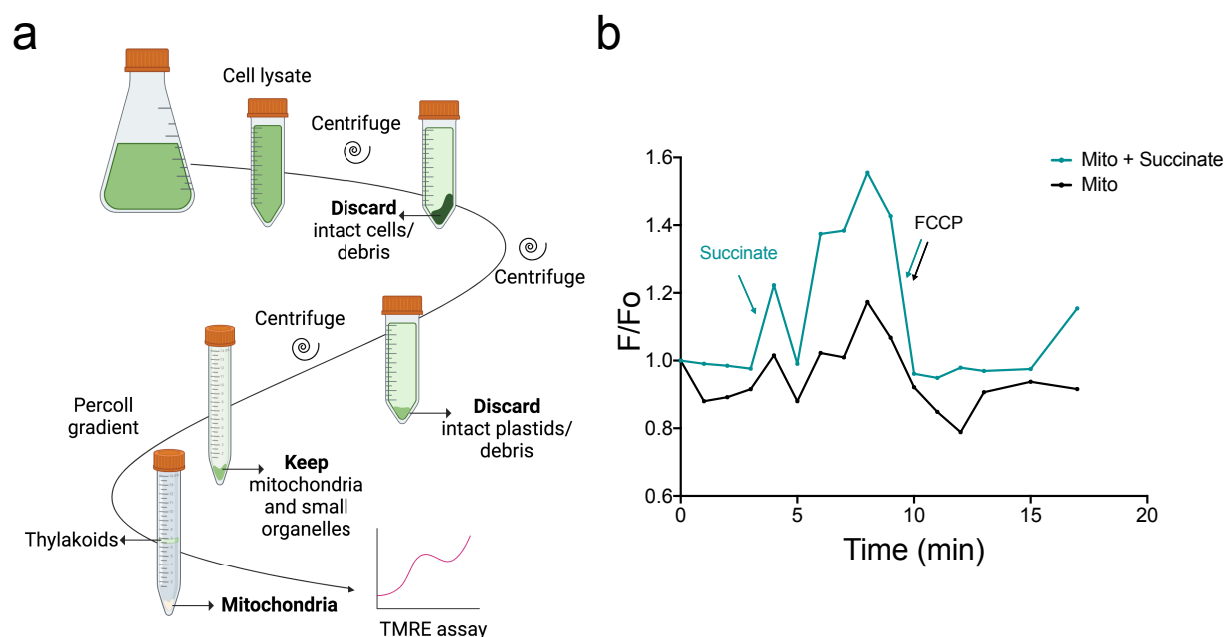


Figure 8. Mitochondria isolation and TMRE assays in *C. reinhardtii*. (a) Schematic overview of the optimized protocol to purify mitochondria from *C. reinhardtii* cells to perform TMRE assays. (b) Monitoring changes in the $\Delta\psi_m$ of isolated mitochondria from UVM4 by TMRE assay. Two mitochondria samples were treated with rotenone to disrupt existing $\Delta\psi_m$. The latter was regained in the sample in which succinate was added. FCCP addition resulted in a depolarization of the $\Delta\psi_m$.

Discussion

Opsin-based optogenetics provides a valuable opportunity to investigate processes related to the electrochemical activity of cells or organelles by activating opsins targeted into different cellular membranes. Upon light-activation, opsins lead to ion transport, thereby modifying membrane potential. The magnitude of these modifications strongly depends on the specific opsin used, as well as the intensity and duration of light exposure. This methodology enables the examination of how changes in membrane potential impact different cellular processes, independently of other influencing factors (Deisseroth, 2015; Entcheva & Kay, 2021; Ferenczi et al., 2019; Zhang et al., 2022; Zhou, Ding, Nagel, et al., 2021). Given the close link between mitochondrial activity and changes in mitochondrial membrane potential, optogenetics emerges as a valuable tool for studying mitochondrial-related processes such as mitochondrial autophagy or cellular energy sensing (Amitrano et al., 2021; Berry, Baldizhar, et al., 2020; Berry, Trewin, et al., 2020; Berry & Wojtovich, 2020; Chen et al., 2022; Ernst et al., 2019; Imai et al., 2019; Tkatch et al., 2017).

Although opsins have been successfully targeted to the inner mitochondrial membrane of different organisms, including mammalian cells, *Drosophila sp.*, and *C. elegans* (Berry, Baldizhar, et al., 2020; Berry, Trewin, et al., 2020; Imai et al., 2019), they have not been targeted to mitochondria of any photosynthetic organism to date. In this study, we aimed to target two different opsins, the MAC pump from *L. maculans* and the SSFO channel (a ChR2 variant), to the mitochondria of *C. reinhardtii*. This photosynthetic model organism presents an advantage for investigating mitochondrial membrane potential changes, as the loss of mitochondrial membrane potential can be induced physiologically without adverse effects by culturing the algae under anaerobic conditions.

Considering the lack of opsins targeted to the mitochondria of photosynthetic organisms, which possess both mitochondria and plastids, we first employed transiently transformed *A. thaliana* protoplasts as a simplified model to assess our capability to specifically target opsins to the mitochondria. Utilizing a fusion of the MAC pump with the inner mitochondrial membrane protein mitofilin, previously shown to be effectively targeted to the inner mitochondrial membrane of mammalian cells (Amitrano et al., 2021), we confirmed exclusive targeting to the mitochondria.

In *C. reinhardtii*, we confirmed the mitochondrial localization of the bioinformatically predicted homologue of mitofilin (MIC60), MIC10. Furthermore, we demonstrated successful expression in *C. reinhardtii* of the fusion of MIC10 to the codon optimized and intronized

MAC or SSFO. Although solid experimental evidence for colocalization of the fusions with stained mitochondria was not observed, its proximity and enrichment in the membrane fraction of cellular lysates suggest mitochondrial membrane localization of the targeted opsins.

Furthermore, we implemented a protocol for mitochondrial isolation and measurement of mitochondrial membrane potential changes using the dye TMRE in isolated mitochondria of *C. reinhardtii*. We demonstrated that mitochondria activity was retained during the process of mitochondrial purification, and the correct detection of changes resulting from the addition of chemicals that alter mitochondrial membrane potential, such as succinate or the uncoupling agent FCCP, using TMRE staining.

This established method will enable the investigation of whether and under which experimental conditions the mitochondrial-targeted opsins are active in *C. reinhardtii* by monitoring changes in mitochondrial membrane potential resulting from the activation of the opsins with the corresponding light wavelength. According to the topology prediction studies made, activation of the MAC pump should lead to an increase in mitochondrial membrane potential, while activation of SSFO should result in depolarization.

Once their activity is demonstrated, these newly developed optogenetic tools for *C. reinhardtii* could be utilized to explore the relationship between mitochondrial membrane potential and certain cellular processes, such as protein targeting, independently of the physiological state of the alga.

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Supplementary information

Table S1. Plasmids design and used. All the pCM0 plasmids are from the *C. reinhardtii* MoClo-kit (Crozet et al., 2018). pUPD2, pDGB and GB plasmids were acquired from the Golden Braid collection (Sarrion-Perdigones et al., 2013; <https://gbcloning.upv.es>). ChRe, genes coming from *C. reinhardtii*; ChRe+, genes codon optimized for *C. reinhardtii*.

ID	Description and cloning procedure	Insert	Backbone	Reference
pOD038	F2A(ChRe+) (Pos. B2)			Manuscript 2
pOD039	ChR2[p.1-315](ChRe) (Pos. B4) Insert was amplified from <i>C. reinhardtii</i> gDNA with oligos oOD055, oOD056, oOD057 and oOD058. BsmBI restriction-ligation reaction (GoldenBraid).	ChR2[p.1-315]	pUPD2	This work
pOD113	HygR(APHVII)#noSTOPcodon (Pos. B1)			Manuscript 2
pOD114	MIC10(ChRe)#noSTOPcodon (Pos. B3) Insert was amplified from <i>C. reinhardtii</i> cDNA with oligos oOD270, oOD271, oOD272 and oOD273. BsmBI restriction-ligation reaction (GoldenBraid).	CrMIC10 without STOP codon	pUPD2	This work
pOD115	Par::HygR(APHVII)-F2A(ChRe+)-MIC10(ChRe)-ChR2[p.1-315](ChRe)-mVenus(ChRe+):Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-008, pOD113, pOD038, pOD114, pOD039, pCM0-102, pCM0-114	pDGB 3alpha1	This work
pOD136	Par::HygR(APHVII)-F2A(ChRe+)-MIC10(ChRe)-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-mVenus(ChRe+):Tpsad BsaI restriction-ligation reaction (GoldenBraid).	pCM0-008, pOD113, pOD038, pOD114, MAC synthesized in pos. B4, pCM0-102, pCM0-114	pDGB 3alpha1	This work
pOD175	Ppsad::ChR2[p.1-315](ChRe)-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD115 with oligos oOD313 and oOD299. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.	ChR2[p.1-315]	pJR38	This work
pOD177	Ppsad::MIC10(ChRe)-MAC(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD136 with oligos oOD314 and oOD303. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.	CrMIC10-MAC(ChRe+)	pJR38	This work
pOD192	P35S::MITOFILIN-MAC-EGFP::Tnos Insert was amplified from pBJB28 with oligos oOD340 and oOD323. Backbone was digested with AfeI and EcoRI. Both fragments were assembled using AQUA cloning.	MITOFILIN-MAC	pEP028	This work
pOD193	P35S::MITOFILIN-MAC-EGFP::Tnos Insert was amplified from pBJB28 with oligos oOD347 and oOD348. Backbone was digested	MITOFILIN-MAC-EGFP	pMZ827	This work

	with NdeI and EcoRI. Both fragments were assembled using AQUA cloning.			
pOD196	Ppsad::MIC10(ChRe)-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from pOD177 with oligos oOD350 and oOD351. Backbone was digested with NdeI and AgeI. Both fragments were assembled using AQUA cloning.	CrMIC10	pOD175	This work
pOD209	P35S::MITOFILIN-MAC-CERULEAN::Tnos Insert was amplified from pOD211 with oligos oOD379 and oOD380. Backbone was digested with BsaI. Both fragments were assembled using AQUA cloning.	P35S::MITO FILIN- MAC- CERULEA N::Tnos	pDGB 3alpha2	This work
pOD211	P35S::MITOFILIN-MAC-CERULEAN::Tnos Insert was amplified from pROF349 with oligos oOD383 and oOD384. Backbone was digested with BamHI and EcoRI. Both fragments were assembled using AQUA cloning.	CERULEA N	pOD192	This work
pOD213	Pnos::NptII::Tnos P35S::MITOFILIN-MAC-CERULEAN::Tnos BsmBI restriction-ligation reaction (GoldenBraid).	pOD209, GB0226	pDGB 3omega1	This work
pOD366	Ppsad::MIC10(ChRe)-SSFO(ChR2[p.25-315])(ChRe+)-{Irbcs2.1}-{Irbcs2.2}-GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2 Insert was amplified from a synthesized plasmid by Genewiz with oligos oOD572 and oOD573. Backbone was digested with NdeI. Both fragments were assembled using Gibson cloning.	SSFO(ChR2 [p.25-315]- D156- C128)(ChRe +)- {Irbcs2.1}- {Irbcs2.2}	pOD196	This work
pBJB28	Pcmv::MITOFILIN-MAC-EGFP::Tpa			Amitrano et al., 2021
pGEN16	P35S::EGFP::Tnos			Samodelov et al., 2016
pJR38	Ppsad::GFP(ChRe+):Tpsad Par::ParoR(APHVIII)::3'UTRrbc2			Neupert et al., 2009
pMZ827	P35S::E-PIF6(1-100)-NLS::Tnos			Müller 2014
pROF349	Tnos::KanR::Pnos Tnos::PIF6-E::P35s Tnos::AsiSI-cerulean-NLS-NruI::PAAtUbq10(short) Tnos::VP16-PhyB::P35S Tnos::EL222-SDRX::P35S T35s::Sbfl-venus-H2B-SpeI::PCMVmini-(C120)₅-(etr)₈			Ochoa-Fernandez (unpublished)
pEP028	P35S::CRF3-EGFP::Tnos			Pavesi (unpublished)

Table S2. Oligonucleotides used for cloning.

Name	Sequence (5' → 3')
oOD055	GCGCCGTCTCGCTCGAGGTATGGATTATGGAGGCGCCCT
oOD056	GCGCCGTCTCGCATCTCGACCTCAATCTCAG
oOD057	GCGCCGTCTCGGATGCTGGTGGAGGACGAGG
oOD058	GCGCCGTCTCGCTCACGAACCCTTGCCGGTGCCCTTGTTGA
oOD270	GCGCCGTCTCGCTCGAATGGCCCCCTCCGAACCTCGCT
oOD271	GCGCCGTCTCGTAGTCTCGAACAACCTGGTCAT
oOD272	GCGCCGTCTCGACTACGCTGCGGCGGACGGT
oOD273	GCGCCGTCTCGCTCAACCTGCCTTGGTCTTCAGCAGCTCCG
oOD299	GGACCCCACTGCTACTCACAACAAGCCCATATGGATTATGGAGGCGCCCTG
oOD303	CTAGGACCCCACTGCTACTCACAACAAGCCCAATGGCCCCTCCGAACCTCGCTTACAA AG
oOD313	GCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGGC CATAGGCTTGCCGGTGCCCTTGTTG
oOD314	GCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGGC CATAGGGCGGGCGCCGTGCTCCTC
oOD323	GCCCTTGCTCACCATACTGCCACTGCCGGCGAATTCTCGGGCGCCGTGCTCCTCG
oOD340	GAAGTTCATTTTCATTTGGAGAGAAACACGGGGACTCTAGCGCTACCGGTGCGCCACCAT GCTGCGGGCCTGTCAG
oOD347	GGTAGGTAAGCTTGGTACCACCTGAACGACGCATATGATGCTGCGGGCCTGTCAG
oOD348	CGGTGGATCCAAGCTTCTCGAGCCCGGGGAATTCTTACTTGTACAGCTCGTCCATGCC
oOD350	CGCCGTCCAGCTCCACCAGGATGGGGACCACACCGGTGAACAGCTCCTCGCCCTTGG CCATATGACCTGCCTTGGTCTTCAGCAG
oOD351	CTAGGACCCCACTGCTACTCACAACAAGCCCATAGATATCATGGCCCCTCCGAACCTC G
oOD379	TGTGGTGTAACATAACAAGCTTCGTCTCAGTCATTAATTAAGAGCTCGCATGCCTGC
oOD380	GCCAATATATCCTGTCAAAGCTTCGTCTCAAGCGTATGTTGTGTGGAATTGTGAGCGG
oOD383	ATCAGGATCGGCGAGGACGACGGCGCCCGAGAATTCCCGGTAGTAGCAGTGAGCAA GGGCGAGGAG
oOD384	TCGCCTCGAGATCAGTTATCTAGATCCGGTGGATCCTTACTTGTACAGCTCGTCCATG C
oOD572	CCGGTGAACAGCTCCTCGCCCTTGCCCATAGGCTTGCCGGTGCCCTTGTTTC
oOD573	CTTGTCCCGGAGCTGCTGAAGACCAAGGCAGGTGCCATAGTGCTGGTGCCCGAGGTG

5 CHAPTER III: Optogenetic tools for mitochondrial research

The review “The optogenetic landscape of the mitochondrion” (manuscript 4), recently published as part of the book “The Dynamic Nature of Mitochondria: from Ultrastructure to Health and Disease” edited by Andreas S. Reichert, offers an overview of optogenetic tools available for mitochondrial research. I authored the opsin-based optogenetics section within it, which explores the targeting of opsins to mitochondria to modulate the mitochondrial membrane potential in different organisms. Additionally, I contributed to the writing of the introduction and discussion, and the reviewing of the non-opsin-based section of the article.

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5.1 Manuscript 4: The optogenetic landscape of the mitochondrion

9 The Optogenetic Landscape of the Mitochondrion

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Hannes M. Beyer*, and Matias D. Zurbriggen*

INTRODUCTION

Optogenetics – a collection of methods to interface biological systems with optical stimulation – has considerably evolved during the past decade. Originating from the use of opsins for controlling neuron activity through light-gated ion channels and pumps, optogenetics transitioned into a discipline with broad application in cell biology across kingdoms. Deisseroth defined optogenetics as “the combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue”¹. Nowadays, the term “optogenetics” refers to any genetically encoded system or product thereof that performs specialized light-mediated functions². The past decade has witnessed ambitious progress in the development of optogenetic tools and photochemical solutions to control the excitability and signaling of cells^{3–7}. These tools increasingly employ natural and engineered photosensitive switches, rewired to diverse biological functions within cells or the extracellular space, giving rise to non-opsin-based optogenetics. A broad scope of innovation emerged to control diverse cellular functions spatially and temporally. Some of these advances have prompted the discovery of photosensory signaling mechanisms^{8–11}, the development of photoreceptor engineering strategies^{12–15}, and the emergence of high-resolution structures of natural and engineered light-activated proteins^{16–19}. Additionally, major advances in the development of required hardware, i.e., light sources and spatially resolved illumination technologies, accelerated the implementation of optogenetic approaches into biological systems^{20,21}. Light-controlled protein-protein interactions and the use of photosensors that respond to photoexcitation with pronounced conformational changes, as well as photoregulated enzymes, constitute next-generation optogenetic components. These tools enable accurate regulation and analysis of dynamic protein activities in living cells combined with precise sample illumination strategies. All these efforts aim to inform how to construct photo-inducible signaling solutions from natural proteins, resulting in modern biotechnological tools that will enhance our understanding of diverse and dynamic (sub)cellular events²².

Recently, optogenetics has further yielded strategies to specifically control cell organelle function, providing new insights into fundamental processes occurring within a cell. The study of mitochondrial biology, for example, would much benefit from the precision provided by optogenetic methods in order to disentangle their role as endosymbiotic cellular constituents and the balanced crosstalk with the cytosol. Mitochondria are crucial cell organelles, given their pivotal role in adenosine triphosphate (ATP) generation, regulation of cell fate decisions, calcium homeostasis, and cellular metabolism^{23,24}. Several optogenetic techniques have been applied to mitochondria for regulating their membrane potential, protein localization, as well as to control apoptosis^{15,25–31} and mitophagy^{32,33}. Due to the spatiotemporal precision intrinsic to optogenetic approaches, such applications will likely grow in number shortly and assist in exploring diverse functions within the cellular mitochondrial network. In this review, we summarize recent advancements and applications of optogenetic approaches in mitochondria, structured in two main parts: (1) non-opsin-based optogenetics and (2) opsin-based

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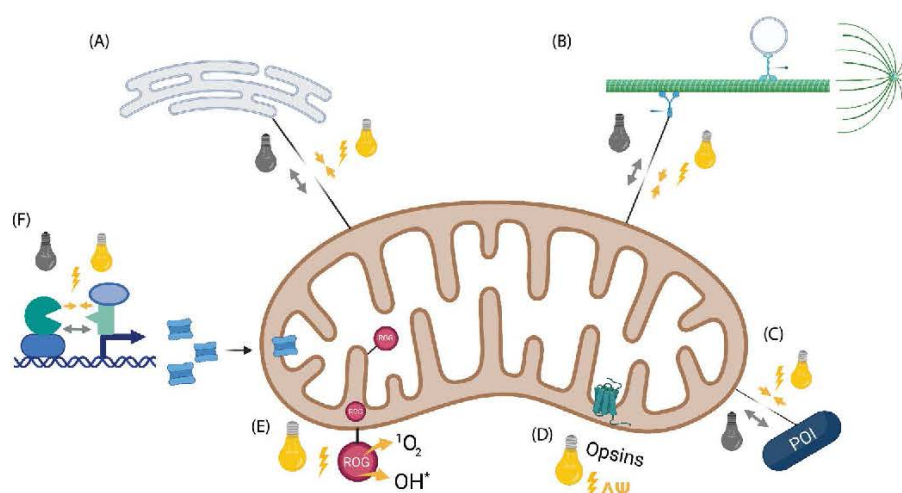


FIGURE 9.1 Overview of optogenetic systems used in mitochondria research. (A) Light-activated attachment of the outer mitochondrial membrane (OMM) to the ER. (B) Light-activated attachment of the OMM to mitotic motor proteins. (C) Light activated recruitment of target proteins (POI, proteins of interest), for example, BAX, AMBRA-1 to the OMM. (D) Insertion of light-activated ion channels and pumps into the inner mitochondrial membrane (IMM). (E) Targeting of light-activated reactive oxygen generating (ROG) proteins to the OMM, IMM, or intermembrane space (IMS). (F) Light-activated gene expression of an ion channel targeted to the IMM. (Created with [BioRender.com](https://www.biorender.com).)

optogenetics. We provide a comprehensive picture of the current optogenetic toolkit for use in mitochondria and discuss possible avenues for future optogenetic investigations (Figure 9.1).

NON-OPSIN-BASED OPTOGENETICS

Non-opsin-based optogenetics (also referred to as next-generation optogenetics or optogenetics 2.0) involves both naturally occurring and synthetically engineered photosensitive switches that can influence various biological functions within cells or the extracellular space. Existing optogenetic tools to control cellular processes can be classified in a variety of ways, such as by the origin of the utilized photoreceptors, the wavelength of induction, signaling mechanisms, or the scope of application. In this review, we primarily discuss applications involving membrane-mediated signaling events, constituting the major field of optogenetic applications pursued in mitochondria to date. In eukaryotic cells, some approaches for optically inducing intracellular signaling mediated *via* integral membrane and membrane-associated proteins include membrane receptors (OPN4, OptoXR, JellyOp)^{34–36}, direct membrane lipid binding (*BcLOV4*)⁹, clustering (LINC, CLICR, LARIAT)^{37–39}, single-chain photoswitching (Dronpa, PA-Rac, Lumitox)^{40–42}, and hetero-dimer association and dissociation (CRY2:CIBN, iLID, TULIPs, Magnets, FKFI:Gigantea, BphP1:Pps-R2, LOVTRAP, PhyB:PIF6)^{13–15,22,43–48}. As many of these systems have previously been thoroughly reviewed^{2,22,24,49}, we wish to briefly introduce the most impactful assets to the optogenetic toolbox, before shifting the focus toward the potential of optogenetics for the study of mitochondria.

PHOTOSWITCHES EMPLOYED AS OPTOGENETIC TOOLS AND THEIR MECHANISMS

The targeted interaction of proteins represents a major mechanistic principle involved in a wide range of biological pathways. Consequently, molecular switches regulating protein-protein interactions upon light stimulation form the basis of various developed optogenetic principles employing a range of photosensitive proteins originating from plants, bacteria, cyanobacteria, and fungi. The most

common of these include light-oxygen-voltage-sensing (LOV) domains, cryptochromes, phytochromes, and cyanobacteriochromes, which have been extensively exploited for modulating signaling pathways and cell membrane dynamics^{2,22,24,49}. The comprehensive online resource “Optobase” (www.optobase.org) hosts an ever-growing resource bank of optogenetic switches, applications, and related literature, guiding interested researchers on the choice of the optogenetic components for implementation into various biological systems⁵⁰. In the following, we describe the mode of function of common optogenetic switches and their applications.

LOV Domains

LOV domains are constituents of blue light photoreceptors found in organisms from various kingdoms of life including plants, bacteria, and fungi^{51,52}. The photoactivity of LOV domains is mediated by light-dependent interactions with a flavin such as flavin adenine dinucleotide (FAD), a cofactor abundant in eukaryotic cells. The LOV2 domain of *Avena sativa* phototropin 1 is widely used for designing synthetic single- and dual-chain photoswitches. In its natural context, it regulates the activity of a serine/threonine protein kinase. AsLOV2 possesses a C-terminal α -helix which undocks from the core domain. A conformational shift in the overall protein tertiary structure initiated by blue light absorption causes the reversible generation of a flavin-cysteine adduct in the hydrophobic core of the LOV sensor, which ultimately converts the photosensory input into biochemical signaling outputs^{8,53}. This conformational shift has been utilized as the molecular switching event in engineered photoswitches, for example, used to control numerous signaling outputs including signaling cascades, the activity of DNA binding proteins, small GTPases, membrane-bound Ca^{2+} channels, and cell death^{9,41,54–56}. Figure 9.2A and Table 9.1 describe a multitude of LOV domain-based tools that are also employed in optogenetic applications related to mitochondria. The most common ones (iLID, TULIP, LOVTRAP) are all based on the AsLOV2 domain (Figure 9.2, Table 9.1).

The improved light-inducible dimer (iLID), for example, constitutes a pair of optical heterodimers based on an engineered AsLOV2 variant caging SspA and its adaptor protein SspB¹³. The iLID/SspB combination has been designed to allow for interactions with a tandem-dimer SspB construct for improved photodimerization and functional utility.^{13,49,56} Similarly, the tunable light-inducible dimerization tags (TULIPs) are based on the interaction of the AsLOV2 domain-fused tag and an engineered cognate PDZ domain (ePDZ), which can recruit proteins to a variety of cellular structures with high spatial precision⁴⁷. Further, LOV domains, such as the one from aureochrome 1 of *Vaucheria frigida* (VfAU1-LOV), which homodimerize when exposed to blue light and can activate various distantly related receptor tyrosine kinases, have found various optogenetic applications like controlling signaling pathways or targeted gene expression^{57–59}. BcLOV4, a *Botrytis cinerea* photoreceptor, directly binds plasma membrane (PM) lipids in response to blue light stimulation via an amphipathic helix-mediated electrostatic interaction^{9,60}. This LOV-domain-based single-component system has fast membrane association/dissociation kinetics and is ideal for high-performance control. Vivid (VVD) is another blue-light LOV domain-containing photoreceptor derived from *Neurospora crassa*, that can be very useful for guiding protein-protein interactions as it switches from a monomeric to a homodimeric state when exposed to blue light^{14,61}. Magnets are additional versatile tools for optogenetically influencing molecular processes. These are re-engineered variants of VVD with better dimerization properties, enabling the formation of heterodimers initiated by engineered electrostatic interactions¹⁴.

Other optogenetic tools involving the AsLOV2 domain include LOVTRAP^{15,62}, LINuS⁶³, and LEXY⁶⁴. LOVTRAP enables precise and reversible control of protein activity, by using the protein Zdark (Zdk) which selectively binds to the dark state of AsLOV2. A protein of interest (POI) can be sequestered away from the region of action in the dark by being fused to either AsLOV2 or Zdk. However, Zdk separates from AsLOV2 when exposed to light, enabling the POI to return to its initial location⁶². Another LOV domain-based tool that uses Zdk is called Z-lock. This tool allows for reversible, light-controlled steric inhibition of protein active sites. When fused to the termini of

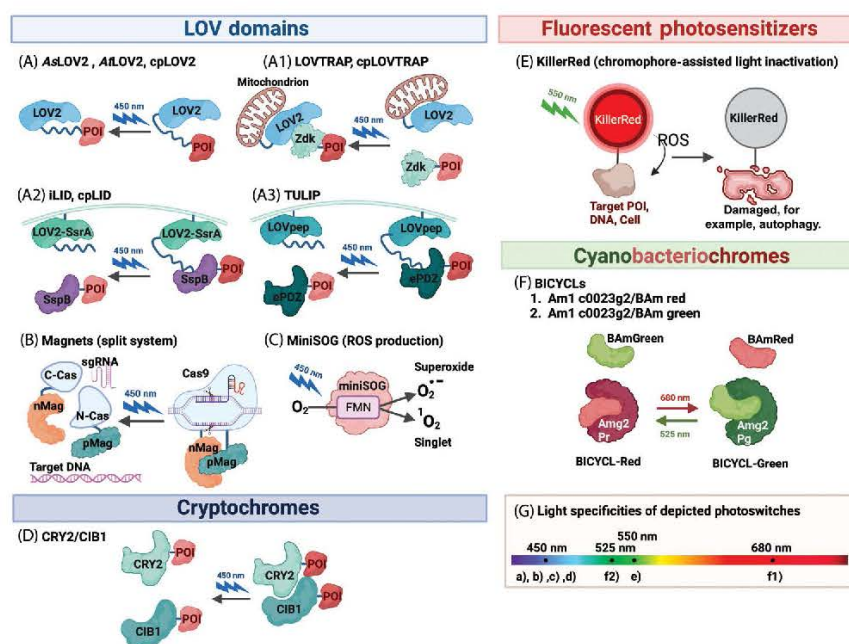


FIGURE 9.2 Schematic modes of function of the most common photoswitches with possible applications in mitochondria. (A) *Avena sativa* (*AsLOV2*), *Arabidopsis thaliana* (*AtLOV2*) light-oxygen-voltage domain, and the circularly permuted *AsLOV2* domain (*cpLOV2*) undergo blue light-induced conformational changes which have been used to recruit a protein of interest (POI) to the outer mitochondrial membrane (OMM). (A1) *LOVTRAP* and *cpLOVTRAP* use *Zdark* (*Zdk*) which selectively binds to the dark state of the *AsLOV2* domain protein enabling precise and reversible control of protein activity and have been used to recruit a POI to the OMM. (A2) *iLID* and *cpLID* constitute pairs of optical heterodimers based on an engineered *AsLOV2* variant caging *SspA* and its adaptor protein *SspB*. They have been used for endoplasmic reticulum (ER)-mitochondria tethering. (A3) Tunable, light-controlled interacting protein tags (*TULIPs*) are based on the interaction of the *AsLOV2* domain and an engineered cognate PDZ domain (*ePDZ*), which can recruit proteins to a variety of structures, like the OMM, with high spatial precision. (B) *Magnets* are re-engineered variants of *Vivids* (*VVD*) with better dimerization properties, enabling the formation of heterodimers initiated by electrostatic interactions. They have been used to recruit POIs to organelles or the cellular membrane. (C) *Mini-singlet oxygen generator* (*miniSOG*), a flavoprotein for correlative light and electron microscopy studies, enables tagging proteins genetically with a fluorophore and also plays a major role in ROS production *in vitro* and *in vivo*. It has been used to target the OMM and induce cellular ablation. (D) *Cryptochrome 2/calcium- and integrin-binding protein 1* (*CRY2/CIB1*), a cryptochrome-based photoswitch, has been used to control *Bcl-2*-associated X protein (*BAX*) recruitment to the OMM. (E) *KillerRed*, the first intended reactive oxygen generator protein that generates reactive oxygen species (ROS) upon irradiation with green light, has been used to target the OMM for reversible mitochondrial fragmentation (see the “Reactive Oxygen Species Generators” section). (F) Bidirectional, cyanobacteriochrome-based light-inducible dimers (*BICYCLs*) can be used for controlling protein subcellular localization. (G) Spectral distribution of light specificities of photoswitches from ultraviolet (UV) to far-red light. (Table 9.1 complements the figure with further details and optogenetic applications.) (Created with [BioRender.com](#).)

a target protein, the *AsLOV2* domain and *Zdk* sterically block the active site of the protein in the dark state. Illumination with blue light resolves the sterical block⁶⁵.

The light-inducible nuclear localization signal (*LINuS*), another tool based on *AsLOV2*, uses blue light to reversibly activate the import of proteins into the cell nucleus. In this case, the *AsLOV2* domain cages a nuclear localization signal (NLS) within its C-terminal α -helix. *LINuS* facilitates nuclear translocation upon blue light illumination of fused POIs⁶³. Controlling protein nuclear

TABLE 9.1
Non-Comprehensive List of Recent Non-Opsin-Based Optogenetic Tools
for Mitochondrial Manipulation

Category	Light	Tool	Target	Description	Reference
Autophagy	Blue	CRY2-CIB1	OMM	BAX recruitment to OMM	(Hughes <i>et al.</i> , 2015 ²⁷ , Godwin <i>et al.</i> , 2019 ⁸⁴)
	Blue	miniSOG	OMM	ROS-induced cell ablation	(Xu & Chisholm, 2016 ¹²¹)
Mitophagy	Blue	iLID	OMM	AMBRA-1 recruitment to OMM	(D'Acunzo <i>et al.</i> , 2019 ³³)
	Green	KillerRed	OMM	Reversible mito fragmentation	(Williams <i>et al.</i> , 2013 ¹²²)
	Green	KillerRed	Matrix	ROS-induced Parkin- mediated autophagy	(Choubey <i>et al.</i> , 2011 ¹²³) (Yang & Yang, 2011 ¹²⁴)
	Red/Far-red	MG2I-dl5 ^{***}	COX4 COX8	ROS-induced neuronal mitochondria ablation	(Xie <i>et al.</i> , 2020 ¹²⁵)
Protein inactivation	Green/Yellow	Supernova	SDHB-1 SDHC-1	Inactivation of Complex II	(Trewin <i>et al.</i> , 2019 ¹²⁶)
	Blue	miniSOG	SDHC-1	Inactivation of Complex II	(Wojtovich <i>et al.</i> , 2016 ¹²⁷)
Protein localization	Blue	LOVTRAP	OMM	POI (mCherry) recruitment to OMM	(Wang <i>et al.</i> , 2016 ¹⁵) (Zhen <i>et al.</i> , 2020 ¹²⁸)
	Blue	cpLOV2	OMM	POI (mCherry) recruitment to OMM	(He <i>et al.</i> , 2021 ¹²⁸)
	Red	BYCICLE-green	OMM	POI (mCherry) recruitment to OMM	(Jang <i>et al.</i> , 2023 ¹⁰²)
	Green	BYCICLE-red	OMM	POI (mCherry) recruitment to OMM	(Jang <i>et al.</i> , 2023 ¹⁰²)
	Blue	iLID	OMM	Recruitment of mitotic spindle proteins to OMM	(Milas <i>et al.</i> , 2018 ¹¹⁶)
Position and shape	Blue	iLID	OMM	ER-tethering	(Shi <i>et al.</i> , 2018 ³²)
	Blue	LOVpep	OMM	OMM attached to kinesin or dynein	(Harterink <i>et al.</i> , 2016 ¹²⁰)
	Blue	CRY2-CIB1	OMM	OMM attached to kinesin or dynein	(Duan <i>et al.</i> , 2015 ⁸²)
	Blue	CRY2/CIB1	OMM	OMM attached to kinesin	(Song <i>et al.</i> , 2022 ¹¹⁹)
Membrane depolarization	Blue	CRY2/CIB1	IMM	Expressing K ⁺ channels Kev and Kmp _{17T} targeted to IMM	(Engel <i>et al.</i> , 2020 ¹²⁹)

Notes: *** Approaches applied to mitochondria.

Abbreviations: IMM/OMM, inner/outer mitochondrial membrane; ROS, reactive oxygen species; SDHB-1/SDHC-1, succinate dehydrogenase subunits B/C; ER, endoplasmic reticulum; POI, protein of interest.

export optogenetically has also been achieved with AsLOV2, as exemplified with LEXY. In contrast to LINuS, LEXY harbors a nuclear export signal (NES) within the C-terminal J α -helix of AsLOV2. Blue light excitation leads to the exposure of the NES and hence to receptors that specifically assist active nuclear protein export through the nuclear pores⁶⁴.

In another study, a circular permutation technique was employed to re-engineer the AsLOV2 domain and provide photoswitchable control of the C-terminus of a polypeptide⁶⁶. As AsLOV2 features the light-regulated J α -helix at the C-terminus, it requires the fusion of target proteins to its C-terminus,

which might affect the N-terminus of the target factor. Therefore, AsLOV2 is often suitable for caging factors whose functions require a free N-terminus. However, caging the C-terminus might be the better choice for some target factors which, for example, harbor C-terminal residues that are crucial for activity. The circularly permuted AsLOV2 domain (cpLOV) has reengineered termini which positions the α -helix close to the N-terminus. Therefore, cpLOV suits the latter caging requirements but may also be used in combination with AsLOV2, enabling dual caging strategies^{66,67}. cpLOV has been tested for various applications, thus broadening the scope of optogenetic tool designs⁶⁶.

EL222 is a transcription factor isolated from *Erythrobacter litoralis* HTCC2594 that can bind to DNA in a light-dependent way. It contains a C-terminal helix-turn-helix (HTH) DNA-binding domain in addition to the N-terminal LOV domain. The interactions between the LOV and HTH domains prevent DNA binding in the dark state, whereas blue light illumination resolves these interactions, releasing the LOV and HTH domains from each other and allowing EL222 dimerization to recognize its target DNA sequences. This conformational change is crucial for EL222 to bind DNA and control gene expression^{68,69}. In one application, a VP-EL222 chimeric protein was created to activate gene expression by blue light, utilizing a VP16 transcriptional activation domain (AD) fused to EL222⁷⁰. StrongLOV, a mutant version of EL222 developed in more recent applications in budding yeast, requires lower light intensities for the same degree of activity and may thereby result in lower phototoxicity⁷¹. As EL222 is widely used across kingdoms of life, such as in mammalian cells⁷², bacteria⁷³, and plants⁷⁴, this mutation could be helpful for light-controlled experiments in various areas of synthetic biology.

Cryptochromes

Cryptochromes (e.g., plant CRY2) are photoreceptor proteins that play an important role in plant and animal circadian rhythms⁷⁵. Although they were first discovered in *Arabidopsis*, cryptochromes were since found in many other organisms, ranging from bacteria to animals and even humans^{76,77}. Cryptochromes evolved from photolyases that engage in DNA repair. Their evolution likely occurred multiple times independently leading to blue light perception through a conserved N-terminal photolyase-homologous region (PHR)⁷⁶. Cryptochromes are composed of two domains: the N-terminal PHR domain that binds the FAD chromophore, and the CRY C-terminal extension (CCE) domain, which seems naturally unstructured but is crucial to cryptochrome function and control⁷⁸ (Figure 9.2D). Cryptochrome 2 (CRY2) from *Arabidopsis thaliana* uses FAD to sense light. The photoexcited CRY2 can both undergo homo-oligomerization and heterodimerization with its interaction partner calcium- and integrin-binding protein 1 (CIB1)^{44,79,80}. Directional design and rational mutagenesis approaches yielded CRY2 variants with considerably enhanced^{37,81,82}, or suppressed⁸², homo-oligomerization properties. The induction of clustering can result in the physical sequestration of CRY2-fused target proteins, inhibiting or activating relevant biological functions^{38,39,49}. One of the most popular heterodimerization modules for inducing protein interactions is CRY2/CIB1. Upon blue light activation, photoexcited CRY2 changes conformation and can quickly heterodimerize with CIB1, within milliseconds of light exposure. The CRY2-CIB1 pair dissociates with a half-life time of around 5.5 min and this interaction can be repeatedly generated through numerous cycles⁸⁰. This module requires no exogenous chromophore addition and is an advantageous tool for inducing protein translocation, transcription, and DNA recombination⁴⁴. Capitalizing on CRY2 homo-oligomerization and CRY2/CIB1 heterodimerization, the photoswitch has found multiple applications, ranging from controlling organelle transport to positioning to the more recent induction of autophagy in cells^{27,83,84}.

Phytochromes

Phytochromes (Phys) are an important class of sensory (photochromic) biliproteins involved in regulating critical physiological processes across biological kingdoms. They can be found in all plants, the majority of cyanobacteria, most algae, some fungi, and many prokaryotes^{85,86}. Phys are activated with red or far-red to near-infrared (NIR) light and rely on tetrapyrrole chromophores.

Typically, they are photochromic in the range of 600–750 nm, transitioning from the red light-absorbing ground state Pr to the far-red light absorbing Pfr state. Far-red light changes Pfr back to Pr. Plant Phys rely on phytochromobilin (PΦB) or phycocyanobilin (PCB) as chromophores, while bacterial phytochromes, such as bathy phytochromes, use biliverdin IX α (BV) instead^{43,85,87,88}. In the presence of the cofactor PCB, the cyanobacterial phytochrome 1 (Cph1) from *Synechocystis* dimerizes under red light and monomerizes under far-red light, representing an immediate photo-switch for inducible homodimer formation⁸⁵.

The red light-induced binding capability of the plant photoreceptor phytochrome B (PhyB) to the interaction factor PIF3 and the reversal of this binding triggered by far-red light was used to engineer a split transcription factor for the control of gene expression in yeast cells⁸⁹. Upon light activation, PIF3, a basic helix–loop–helix protein, interacts exclusively with the Pfr form (representing the active form of the photoreceptor) of both PhyA and PhyB⁸⁹. Since then, many other Phy-based optogenetic applications ensued. In 2009, Levskaia *et al.* demonstrated how to deploy a new genetically encoded light-control system based on an improved, reversible PhyB/PIF6 protein-protein interaction switch derived from *Arabidopsis thaliana* components⁴⁸. Plant PhyB covalently binds an exogenously supplied PCB chromophore in mammalian cells. This optoswitch can be utilized to precisely and reversibly translocate target proteins to the plasma membrane with high spatial resolution and within the timeframe of seconds to induce cell signaling responses. Shortly after, the first red/far-red light-triggered gene switch for mammalian cells was developed, which enables the spatiotemporal control of gene expression⁴⁸. The authors used the PhyB/PIF6 red light-induced interaction and light illumination regimes at 660 and/or 740 nm to reversibly toggle the system between stable on and off states⁹⁰. One year later, they adapted this concept into a red light-controlled synthetic gene expression switch for plant systems⁹¹. Plant phytochrome-based optogenetic tools have also been used in vertebrates *in vivo*, for instance, in 2015 Beyer *et al.* engineered a red light-inducible and far-red light-reversible synthetic system, based on the PhyB/PIF3 interaction, for controlling nucleo-cytoplasm shuttling of proteins in mammalian cells and zebrafish embryos⁹².

DrBphP, a bacterial phytochrome from *Deinococcus radiodurans*, forms a head-to-head parallel dimer that may alter conformation reversibly in milliseconds when illuminated with far-red or near-infrared light without the use of any exogenous cofactor, as BV is already endogenously present in bacteria and mammalian cells⁹³. Under red light illumination (660 nm), bacterial phytochromes transmit the light signal from the photosensory module (PSM) to a C-terminal output domain. However, some bacterial phytochromes have an inverted photocycle with a far-red light-absorbing state (Pfr) as the resting state. DrBphP, for instance, possesses a C-terminal histidine kinase domain, but other phytochromes employ, for example, protein domains called GGDEF-EAL with phosphodiesterase (PDE) or diguanylate cyclase (DGC) activity in engineered and truncated variants⁹⁴. These domains approach each other in the dark or near-infrared light (740–780 nm), allowing for the homo-interaction of proteins fused to the C-termini^{49,95–97}.

Cyanobacteriochromes

Cyanobacteriochromes (CBCRs) constitute a class of photoreceptor proteins found in cyanobacteria with color sensitivities that extend over the full visible light spectrum from ultraviolet to far-red light^{98–101}. The majority of these proteins consist of tandemly linked subdomains called GAF domains (cGMPphosphodiesterase/adenylate cyclase/FhlA). A conserved cysteine in the GAF domain forms a thioether bond with a bilin, usually phycocyanobilin (PCB), and upon radiation exposure, this chromophore undergoes E/Z isomerization of the C15 double bond¹⁰⁰.

The recently engineered bidirectional light-inducible dimers (BICYCLs) are the first CBCR-based devices that allow for reliable, adaptable control of protein-protein interactions *in vitro*, in yeast, and animal cells. They can be employed in regulating transcriptional processes, mitochondrial outer membrane protein localization, and can be used for multiplexing with the currently available blue-light tools LEXY, LINuS, and TULIPs¹⁰².

BOX 9.1 OPTOGENETIC SYSTEMS AT THE PLASMA MEMBRANE

Due to similarities in applications between the cellular plasma membrane (PM) and the outer mitochondrial membrane (OMM), we provide here a short prospective introduction of optogenetic systems targeting the PM. The PM is a well-defined, two-dimensional subcellular unit engaged in highly regulated processes relevant to cell physiology. As a result, a wide range of optogenetic methods for dynamically controlling protein localization to the plasma membrane have been developed. These methods allow the optogenetic modulation of signaling cascades that start at the PM or change cell membrane dynamics, usually involving the recruitment of a protein-of-interest, or clustering at the membrane to regulate cell signaling. Previous reviews provide an extensive overview of these strategies^{22,24,103,104}, here we will only touch on a few relevant examples.

The most general strategy for modulating biological activities at the PM relies on the recruitment of various signaling components. For instance, the iLID system was recently used to enrich the red-fluorescent protein mCherry near macrophage plasma membranes¹⁰⁵. A local increase in mCherry levels causes PM deformation, which in turn causes cells to move in the opposite direction. Another study reported the development of a blue light-inducible method for generating nanoscale membrane curvature in live cells. The authors used the Bin/Amphiphysin/Rvs (BAR) domain proteins to achieve a system that is controllable, reversible, and capable of precise spatial and temporal manipulation¹⁰⁶. Cell motility is another process that could be modulated optogenetically. Various membrane structures important for cellular motility, such as lamellipodia, filopodia, and ruffles, arise from actin-dependent cell membrane motions¹⁰⁴. Using the CRY2-CIB system, researchers showed that phosphatidylinositol-3,4,5-triphosphate (PIP3) production can be modulated to affect membrane dynamics. Phosphatidylinositol-3-kinase (PI3K) is recruited to the cell membrane by blue light-induced dimerization of CRY2 and CIBN, where it transforms local phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3, thereby increasing cellular motility and causing the development of apical ruffles¹⁰⁴. Another approach is to use photoactivated binders for protein recruitment to the PM. Optogenetic nanobodies (OptoNBs) were used to modulate Ras activity *in vivo* by targeted recruitment of the SOS catalytic domain (SOScat) to the PM using both light-induced and light-inhibited OptoNBs¹⁰⁷. SOS, which is mostly found at the plasma membrane, acts as a positive regulator of Ras¹⁰⁸. Another study combined dual-wavelength control with actin-targeted intrabodies to attract actin to the plasma membrane and the nucleus. Here, they used BphP1/Q-PAS1, a phytochrome-based switch. The mCherry-tagged Q-PAS1 is recruited to the target protein bound to the membrane in a light-induced manner. Irradiation with far-red light boosted actin targeting to the PM, whereas illumination with blue light enhanced nuclear intrabodies targeted to actin levels. Their sequestration to the PM reduces cytoskeleton-driven cellular motility⁹⁵.

Employing such optogenetic techniques enables precise spatial and temporal control and modulation of large cellular structures without the use of pharmaceutical effectors²⁴. Understanding how these systems work to control the plasma membrane can help us further understand how to employ optogenetic techniques on organelle membranes, such as the OMM discussed in this review.

NON-OPSIN-BASED OPTOGENETICS INVOLVING MITOCHONDRIA

Mitochondria play a pivotal role in metabolic homeostasis and the regulation of various cellular pathways, and research on functional and regulatory aspects of these organelles can certainly profit from implementing optogenetic approaches. Maintaining stable homeostasis allows mitochondria to regulate cellular health as well as death processes. Mitochondria are the main source of ATP and reactive oxygen species (ROS) production. They are also important for second messenger generation

and production of biosynthetic precursors for macromolecules, some of which also function as second messengers involved in regulating vital processes like cellular aging^{109,110}. The function and regulation of the cellular mitochondrial network is quite complex. Therefore, utilizing the spatial and temporal regulation possibilities of optogenetic approaches represents one of the most attractive strategies to gain access to the study of mitochondrial physiology. Optogenetically regulating diverse mitochondrial functions opens additional avenues to the study of intricate cellular programming and processes, such as autophagy, mitophagy, and apoptosis (regulated cell death), which can assist in revealing new insights into the function of the complex organelle. Optogenetics could in the future provide, for example, novel strategies to study the role of mitochondria in disease etiology and their key role in metabolic engineering^{23,24}. In this chapter, we discuss optogenetic solutions, highlighting applications in mitochondria-related processes including autophagy, apoptosis, mitophagy or mitochondrial positioning, and shape modulation.

Autophagy

Autophagy is a crucial biological process in which cells sequester internal constituents such as organelles and proteins and transfer them to lysosomes for the breakdown and recycling of macromolecule precursors. It is a key cell survival mechanism that enables cells to respond to metabolic stress by degrading and recycling intracellular components¹¹¹. Channelrhodopsins (ChRs), traditionally used to optogenetically modify the activity of neurons, now offer an interesting solution to change the potential of mitochondrial membranes. Disruption of the electrochemical gradient alters mitochondrial calcium mobilization and storage. Light stimulation of mitochondrial membrane-localized ChRs causes strong impacts on cell survival (a more detailed review on channelrhodopsins is presented in [Chapter 2: Energetics/Opsin-Based Optogenetics](#))^{25,26}.

Apoptosis

Apoptosis is a form of programmed cell death with characteristic features, such as chromatin condensation, nuclear disintegration, and plasma membrane blebbing¹¹². Before autophagic cell death and programmed necrosis (necroptosis) were described, the terms apoptosis and programmed cell death were frequently used interchangeably. Optogenetics also allows for the regulation of apoptosis *via* the targeted control of the localization of apoptosis-regulating proteins such as Bcl-2-associated X protein (BAX)²⁷. Optogenetic recruitment of CRY2-BAX fusion proteins to CIB monomers attached to the outer mitochondrial membrane (OMM) *via* translocase of OMM 20 (TOM20) sequences could, for example, promote cell death in response to blue light. BAX targets the mitochondrial membrane and causes the release of cytochrome *c*, which in turn triggers apoptosis²⁴.

Mitophagy

Mitophagy, or autophagy-mediated degradation of mitochondria, is another process that has been opened for optogenetic manipulations. This specific kind of autophagy is responsible for the turnover of damaged mitochondria by selectively destroying them. Mitophagy acts often independently of the signaling that regulates basal autophagy and primarily serves as a quality control mechanism by identifying and removing malfunctioning mitochondria that could otherwise contribute to death signaling activation. The pathway is further responsible for the elimination of healthy mitochondria during cell differentiation¹¹³. D'Acunzo and his colleagues presented a bimodular system to reverse the light-dependent induction of mitophagy and rescue cells from apoptosis³³. iLIDs have been implemented to attract autophagy and beclin-1 regulator 1 (AMBRA1) to the mitochondrial surface, thereby activating mitochondrial recycling and mitophagy in the presence of blue light. This design utilizes the fusion of SsrA to an OMM linker sequence from actin assembly-inducing protein (ActA). Furthermore, Shi *et al.* were able to employ light-triggered dimerization of iLIDs to induce mitochondrial-ER tethering with high spatiotemporal precision, which is required for mitophagy³².

Protein Localization

Optogenetic tools employed for the control of subcellular protein localization are usually customized for the recruitment of proteins to the mitochondrial surface (Figure 9.2). In fact, some examples of protein localization have already been introduced before, for instance, the implementation of iLIDs in combination with AMBRA1 (application in mitophagy) uses protein localization to attract the protein to the mitochondrial surface. Furthermore, LOVTRAP-induced protein sequestration at the OMM has been employed to modulate intracellular protein accumulation at the OMM of mammalian cells¹⁵.

Another tool with applications in mitochondrial protein localization are BICYCLs, the smallest green/red optogenetic tools known so far, which were used in combination with LEXY⁶⁴ and LINuS⁶³ to control subcellular protein localization and gene expression¹⁰². In the dark, LEXY-tagged Amg2 (the photoreceptor utilized in BICYCLs) was mostly localized in the nucleus and accumulated at the mitochondria upon red light illumination; green light reversed this mitochondrial accumulation. Exploiting the bidirectional switching behavior of phytochromes enhances spatial localization in contrast to, for example, LOV-based photoswitches, where dark reversion kinetics control the relaxation into the ground state^{102,114,115}. Mitochondrial sequestration of Zdk fusion proteins and their optical release has been realized with AsLOV2 anchored to the OMM using a sequence derived from TOM20. LOVTRAP, like other optogenetic tools, usually requires customization and optimization when transferred to other cell systems. In particular, portability to yeast cells where the high levels of mitochondrial AsLOV2 required for successful protein trapping was challenging, as it affected cellular development³¹. Milas *et al.* introduced an optogenetic tool based on iLID for rapid and reversible translocation of proteins in human cells using blue light¹¹⁶. Proteins were removed from the mitotic spindle to the mitochondria and turned back to the mitotic spindle on a minute-by-minute scale, allowing regulation of protein activity in different periods of mitosis and in individual cells. Optogenetically exploring the complicated nature, dynamics, and function of mitotic proteins bears the potential to further investigate the mechanisms underlying mitosis¹¹⁶.

Position and Shape

Organelle transport and distribution play a significant role in a variety of cellular processes. However, the paucity of tools to alter molecular motors within the complicated intracellular environment makes it difficult to draw a direct link between organelle distribution and cellular functions. The use of optogenetic strategies represents a potential solution to gain access to such methods, enabling a deeper investigation of these aspects. Duan *et al.* showed that it is possible to redistribute organelles like mitochondria, peroxisomes, and lysosomes in the cell in response to light⁸². Using a light-induced CRY2/CIB1 interaction, they recruited molecular motors to the outer membrane of organelles, thereby achieving optical control of directional transport and redistribution of organelles in living cells.

Mitochondria are also affected by mechanical stimuli, which directly impact and regulate their shape and function. Mechanical strains applied to cells have been shown to cause mitochondrial elongation, a decrease in mitochondrial membrane potential, and the generation of ROS^{117,118}. Recently, Song *et al.* demonstrated the possibility of optogenetically controlling mitochondria shape and motility through light-activated protein-protein hetero-dimerization¹¹⁹. They were able to implement different optogenetic modules (CRY2/CIBN, iLID/SspB, and LOVpep/ePDZ) into their system to trigger mitochondria deformation by recruiting molecular motors to the OMM^{119,120}.

Reactive Oxygen Species (ROS) Generators

The processes involved in the oxidative phosphorylation at the mitochondrial respiratory chain give rise to a significant source of cellular ROS. ROS play a crucial role in cellular signaling, stress, and mitophagy where they engage as second messengers. The intended induction of ROS in a precise spatiotemporal manner may help to understand cellular signaling, as well as apoptotic and

necroptotic pathways in whole organisms^{130,131}. ROS generators (ROGs) can be used to investigate second messengers in the cytosol, ROS-induced ROS release¹³², to mimic biological ROS production and mitophagy^{121,123–125}, as well as for cell ablation experiments¹²¹. Using genetically encoded light-activated ROGs has one main advantage over chemical light-activated ROGs. The production and thus the location of ROGs can be spatially controlled within the cellular compartments by genetically encoded regulative principles. Furthermore, it is possible to attach ROGs to proteins of interest *in vitro* and *in vivo*.

Formation of Reactive Oxygen Species

Activating a chromophore by the light of a specific wavelength within the excitation range leads to the molecule reaching a higher energetic state S_1 (Figure 9.3, blue arrow). The return to the basal S_0 state occurs *via* several mechanisms, including the non-radiative dissipation of energy (called internal conversion, gray wavy arrows) and emission of photons, fluorescence, which can occur from the S_1 state (green arrow). Another very rare and quantum mechanically “forbidden” state is the triplet state T_1 , reached by intersystem crossing (ISC). Deactivation from this state may either occur *via* phosphorescence (yellow arrow) or the production of ROS that can occur through one of the two different types of reactions (Figure 9.3B). Since the triplet state has a lifetime of microseconds to minutes, molecules reaching this state can perform random energy transfer collisions. When colliding directly with substrates like lipids, proteins, or nucleic acids, the activated molecule tends to transfer an electron (type I reaction). The so-generated radicals themselves react with oxygen, forming ROS. The direct transfer of an electron to oxygen by collision is also possible, yielding the anion superoxide ($O_2^{\bullet-}$). Superoxide dismutases generate H_2O_2 and O_2 while the hydrogen peroxide can be further catalyzed by copper or iron ions to the extremely reactive hydroxyl radical, $HO^{\bullet}$ (Fenton reaction). Type II reaction is the energy transfer from a triplet state chromophore to a ground state oxygen (3O_2) yielding singlet oxygen, 1O_2 . It has to be stated that ROS production does not always follow just one reaction type. Since singlet

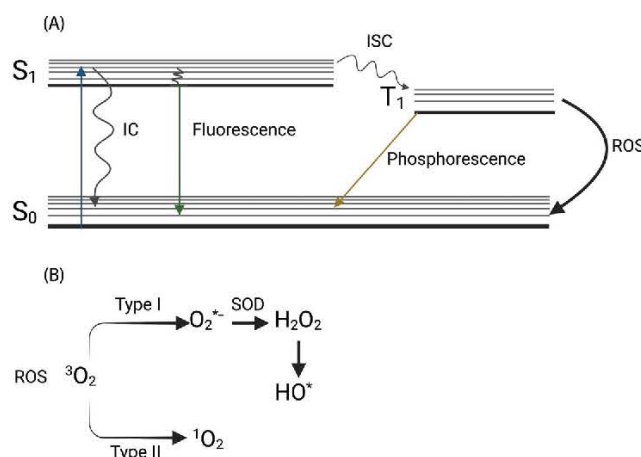


FIGURE 9.3 ROS production. (A) Jablonsky scheme, ROGs are excited to the high-energy singlet state S_1 upon photon absorption (blue arrow). Relaxation into the ground state follows either internal conversion (IC), fluorescence, or intersystem crossing (ISC), followed by phosphorescence or ROS generation. (B) ROS mechanisms, type I: Collision of the excited ROG leads to a single electron transfer. The superoxide dismutase (SOD) converts the superoxide radical $O_2^{\bullet-}$ to hydrogen peroxide. In the presence of metal cations, H_2O_2 can give rise to highly reactive hydroxyl radicals, $HO^{\bullet}$, *via* the Fenton reaction. Type II: Energy transfer by a direct collision between ROS and oxygen molecules.

TABLE 9.2
Photophysical Properties of a Series of ROGs

Name	λ_{ex} (nm)	λ_{em} (nm)	$\Phi_{\Delta}^1O_2$ (i)	$\Phi_{\Delta}^1O_2$ (ii)	$\Phi_{\Delta}^1O_2$ (iii)
KillerRed	585	610	0 ¹³⁰	n.d.	0.0076 ¹³⁶
KillerOrange	512	555	n.d.	n.d.	n.d.
Supernova (red)	579	610	n.d.	n.d.	0.022 ¹³⁶
SupernovaGreen	440/480	510/560	n.d.	n.d.	n.d.
mCherry	587	610	n.d.	n.d.	0.0057 ¹³⁶
miniSOG	501	526	0.42 ¹³³	0.0 ¹³⁷ 0.03 ^{133,138}	n.d.
miniSOG Q103V	n.d.	n.d.	n.d.	0.39 ¹³⁹	n.d.
miniSOG2	n.d.	n.d.	n.d.	n.d.	n.d.
SOPP	488	515	n.d.	0.25 ¹³⁷ 0.23 ¹⁴⁰ 0.19 ¹³⁸	n.d.
SOPP2	491	513	n.d.	0.51 ¹⁴⁰	n.d.
SOPP3	490	514	n.d.	0.61 ¹⁴⁰	n.d.
FMN	444	531	n.d.	0.57 ¹³⁷ 0.51 ¹⁴¹ 0.49 ¹³⁸	n.d.
MG-2I-d15 ^{***}	666	693	0.13 ¹³⁰	0.18 ¹³⁴	n.d.

*** Singlet oxygen quantum yields $\Phi_{\Delta}^1O_2$ determined by (i) use of anthracene dipropionic acid (ADPA), (ii) phosphorescence measurement at 1,275 nm, or (iii) singlet oxygen sensor green (SOSG). The wavelength of the excitation and emission maximum are indicated by λ_{ex} and λ_{em} respectively.

oxygen (type II) is highly reactive and plays a major role in phototoxic effects, the quantum yield of 1O_2 is the mainly used reference value to compare ROGs^{133,134}. It has to be mentioned that the indirect measurements of singlet oxygen *via* loss of fluorescence signal due to photooxidation by scavengers like anthracene dipropionic acid (ADPA) or singlet oxygen sensor green (SOSG) is shown to be not 1O_2 exclusive^{133,135}. Therefore, the direct measurement of 1O_2 phosphorescence can be seen as the most reliable detection method^{133,134}. Table 9.2 shows the respective data for all mentioned ROGs.

KillerRed

KillerRed was the first intended ROG protein described, which was developed by Bulina *et al.* from the hydrozoan chromoprotein anm2CP, a homolog of the green fluorescent protein (GFP)¹⁴². Unlike GFP, KillerRed provides a water-filled channel to the chromophore which most likely is the reason for the photosensitizing effect. KillerRed generates ROS upon irradiation with green light mostly *via* the type I mechanism (Figures 9.2C and 9.3). Producing superoxide anions, which are the major ROS species in the cellular environment, makes KillerRed and all of its variants an attractive tool assisting in the study of ROS signaling and stress.

KillerOrange was described by Sarkisyan *et al.* in 2015¹⁴³. The new variant is phototoxic upon green and blue light exposure. Furthermore, the ability of KillerRed and KillerOrange to form dimers affects the cellular localization and perturbed mitotic cell division. To face this problem, the monomeric variants Supernova red¹⁴⁴ and Supernova green¹⁴⁵ were subsequently developed.

MINISOG

MiniSOG was initially described as a new tool for correlative light and electron microscopy (CLEM) studies. Shu *et al.* used its ability to produce ROS for catalyzing the conversion of diaminobenzidine into a product stainable with osmium tetroxide¹⁴⁶. Thus, miniSOG acts as a fluorophore tag and as an ROS production tag. This makes it possible to localize a protein of interest, which is genetically tagged with miniSOG and irradiated with the ROS-specific wavelength under the electron microscope when providing diaminobenzidine as substrate. MiniSOG not only revolutionized CLEM staining but also plays a major role in ROS production *in vitro* and *in vivo*. The 106-residue long miniSOG is the smallest ROG. It is derived from the *AtLOV2* domain and depends on the availability of intracellular FMN as a cofactor¹⁴⁶.

The mechanism of ROS production is not yet fully understood. Nevertheless, one may assume that miniSOGs can produce superoxide as well as singlet oxygen (type I and II mechanism, Figure 9.3). The production of $^1\text{O}_2$ likely causes cellular damage rather than ROS signaling. Therefore, miniSOG is well suited for loss of function and ablation experiments (Figure 9.3)¹³³. In recent years, many improved variants were developed with increased triplet state lifetime and shifted ratios toward the production of $^1\text{O}_2$ (type II) rather than $\text{O}_2^{\bullet-}$ (type I), including SOPP¹³⁸, SOPP2¹⁴⁰, SOPP3¹⁴⁰, miniSOG Q103V¹³⁹, and miniSOG2¹⁴⁷.

THE USE OF ROG IN THE CONTEXT OF MITOCHONDRIA

Even though ROS production and investigation of mitochondria seem to be a perfect match, only a few mitochondrial targeting approaches are described in the literature. Here we will briefly describe some approaches exemplarily to state the possibilities provided by ROGs.

Mitophagy

Induction of mitophagy is by far the most widely used approach of ROGs in the context of mitochondria research, as exemplified by the activation and monitoring of the Parkin-mediated mitophagy pathway^{123,124}. In 2011, Choubey and Yang & Yang addressed this pathway optogenetically by targeting KillerRed to the mitochondrial matrix and further fusing Parkin to a fluorophore of a different excitation color than the light used for the excitation of the ROG, to avoid bleaching. After excitation of the ROG, the impaired mitochondria membrane produces ROS, and Parkin-mediated autophagy is initiated through the assembly of Parkin at illuminated mitochondria. Later, Tan and Tu *et al.* fused miniSOG to PINK1, and mCherry to Parkin to investigate the role of RhoA¹⁴⁸ and HK-II¹⁴⁹ in Parkin-mediated mitophagy.

The choice of the ROG used affects the result not just quantitatively but also qualitatively. Additionally, the capacity to buffer/detoxify ROS in different cellular environments plays a major role in ROG experiments. The fusion of KillerRed to the OMM of muscle cells in *C. elegans* did not result in cell death. However, when fusing KillerRed to the plasma membrane, the cells effectively died. Since KillerRed produces superoxide, which is the most common natural ROS in cells, the mitochondria were able to buffer the effect and fully recover within 48 h¹²². In contrast, fusing miniSOG, a type II ROG, to the OMM of neuronal mitochondria in *C. elegans* led to apoptotic cells. Still, a direct comparison revealed an efficacy of just about 10% when miniSOG was fused to the OMM rather than to the plasma membrane, most likely due to the different ROS buffering capacities within the cellular sub-environments¹²¹.

Chromophore-Assisted Light Inactivation

Chromophore-assisted light inactivation (CALI) relies on the oxidation of the protein of interest fused to the ROG. Since ROS are highly active and the intracellular environment is quite dense, the produced ROS have a very small reaction radius. Trewin *et al.* attached the Supernova-ROG to the Complex II succinate dehydrogenase subunits B and C, monitoring the effect of ROS on the inner

mitochondrial membrane and in the intermembrane space¹²⁶. Wojtovich *et al.* inactivated the SDHC subunit of the Complex II by fusing it to miniSOG¹²⁷.

Recent Chemo-Optogenetic Approaches

While setting the primary focus of this review article to genetically encoded gene optogenetic applications in mitochondria, we wish to mention one recent chemo-optogenetic hybrid system with a broad scope for use in mitochondria. Chemo-optogenetic hybrid systems utilize chemical compounds, e.g., a chemical dye, induced or activated by an optogenetic system. Optogenetic ROGs that have been used so far in mitochondria share a relatively low quantum yield in comparison to chemical ROGs. Furthermore, the spectral limitation to blue and green excitation wavelengths compromises their use for applications, that require a relatively deep tissue penetration, for example, in whole animals without causing cytotoxic effects by the excitation light. The chemo-optogenetic FAP-TAP (fluorogenic-activated protein-targeted activated photosensitizer) system addresses these issues^{130,150}. Fluorogenic-activating proteins (FAP) are single-chain antibodies that activate and stabilize chemical dyes in their triplet state^{125,130,151}. The here-used FAP, dl5^{***}¹⁰³ activates TAP (targeted activated photosensitizer)-fluorescence approximately 1,000-fold¹⁵⁰. Malachite green, modified by substitution with two iodines, acts as TAP. The heavy atom insertions (2I) additionally shift the emission spectra toward longer wavelengths and increase the probability of intersystem crossing (Figure 9.3), thus leading to a higher quantum yield. The ROS production follows mainly the type II mechanism and is, therefore, more destructive than KillerRed and its derivatives. Fusing the antibody genetically to a protein of interest leads to a highly specific ROS production tag. In comparison to KillerRed, the FAP-TAP system dl5^{***} – Malachite Green (MG2I) required a 60-fold lower light dose for effective cell ablation in *C. elegans* (90 s vs. 90 min of illumination)¹³⁰. A recent study triggered mitophagy of neuronal mitochondria in zebrafish larvae, which led to a massive loss of neuronal function in combination with either apoptosis or necroptosis¹²⁵. MG2I can penetrate membranes in its non-activated form so that very low concentrations suffice. ROS production of inactive TAP is usually not detectable; therefore, FAP-TAP systems are in principle well suited for studies in entire organisms¹³⁰.

Even though the actual singlet oxygen quantum yield might be lower than SOPP and its variants, MG-2I-dl5^{***} possesses a significantly stronger phototoxic effect on mitochondria than KillerRed^{130,134}. Additionally, unlike miniSOG, SOPP and their derivatives do not depend on FMN, which is not evenly distributed over the different sub-environments. Furthermore, the absorption spectra do not overlap with free FMN which is itself known to have a quite high singlet oxygen quantum yield (Table 9.2) and provides a well-spread background in ROS production in every illuminated cell¹³⁴.

Since fully genetically encoded ROGs with activation wavelengths in red or far-red light are urgently needed, the excitation maximum of MG2I-dl5^{***} between 660 and 670 nm makes it a very promising tool for deep tissue experiments in red/far-red light conditions^{125,130}.

ENERGETICS/OPsin-BASED OPTOGENETICS

During the past two decades, opsin-based optogenetics has increasingly developed and matured into a powerful research approach. For a long time, optogenetics was synonymous with neuronal optogenetics, used to control the plasma membrane potential in neurons. Nowadays, the expanding opsin-based optogenetics approaches strongly impact not only neurobiology but also cardiology for the control of the PM potential in myocytes and other related application areas^{3,152,153}. Recently, opsins were also used to control the membrane potential in plants to study electrical signaling networks^{154–157}. While opsin-based optogenetics is widely employed to modify the PM potential in the abovementioned environments, its use to influence the activity of mitochondria remained unexplored until very recently^{25,26,28–30,158,159}. Conventionally, one would resort to the use of chemical solutions for altering the mitochondrial membrane potential ($\Delta\psi_m$). Carbonyl

cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP), for instance, represent two of the most frequently used protonophores to control $\Delta\psi_m$. However, they also affect the PM potential and are cytotoxic¹⁶⁰. Moreover, the difficulty of applying them in tissues and organs, the lack of spatiotemporal and quantitative control resolution, and their low reversibility hamper the use of these drugs for therapeutic purposes. Recent studies that successfully applied opsin-based optogenetics in mitochondria suggest an alternative and precise methodology which overcomes the mentioned bottlenecks^{25,26,28–30,159}.

Opsins are transmembrane proteins that respond to light. Their activation leads to ion transport, resulting in changes in the membrane potential. Their use as optogenetic tools offers the possibility to manipulate membrane potentials with high spatiotemporal resolution, on the millisecond scale, and with minimized toxicity^{161,162}. Natural opsins generally belong to one of two types, microbial or animal opsins, also called type I and type II rhodopsins, respectively¹⁶³. Microbial rhodopsins act as channels and pumps which, in response to light, control the potential across membranes by transporting ions. Animal opsins possess a different functional mechanism and act as G protein-coupled receptors (GPCRs)¹⁶⁴. In this section, we review the use of microbial rhodopsins to artificially control membrane potentials, specifically in mitochondria.

Type I rhodopsins have been identified in algae, archaea, bacteria, and fungi¹⁶⁵. All type I rhodopsins share a seven-transmembrane alpha-helical topology and require *all-trans*-retinal (ATR) as a cofactor¹⁶⁶. Upon light illumination, ATR isomerizes to 13-*cis* retinal and promotes structural changes of the opsin leading to ion fluxes. Note that 13-*cis* retinal reversion to ATR in the dark inactivates the opsin, making the stimulation reversible¹⁶⁷. Type I rhodopsins in mammalian neurons do not require ATR supplementation, although when used in other cell types and organisms, ATR supplementation may enable or boost opsin function¹⁶⁸. In addition to the strong general light dependence of plants, the absence of retinal makes it difficult to use opsins as optogenetic tools. The recently engineered ATR production in *Arabidopsis thaliana*, obtained by expression of a β -carotene 15,15'-dioxygenase from a marine bacterium that converts β -carotene into retinal, might enable the generalized use of opsins for plant research in the future¹⁵⁷.

Over the past decades, several opsins with different properties have been discovered and further engineered to generate characteristic variants, which give rise to a broad and diverse opsin-based optogenetic toolbox. Table 9.3 summarizes some of the most used opsins, which have been described elsewhere^{10,25,156,161,164,169–211}, indicating the ones used for mitochondrial research.

Microbial rhodopsins further form two subgroups, light-gated ion channels and light-driven ion pumps²¹². Pumps actively transport ions in a unidirectional way, even against an existing electrochemical gradient, dependent on energy input. In this case, each absorbed photon results in the transport of a single ion. Therefore, high light intensities and expression levels are generally required to significantly alter the membrane potential²¹³. Light-driven ion pumps belong to four main groups based on the nature of the transported ion and the direction of the transport: Inward chloride pumps, outward sodium pumps, and inward or outward hydrogen pumps¹⁶⁷ (Figure 9.4). Bacteriorhodopsin from *Halobacterium salinarum* (HsBR) was the first discovered microbial rhodopsin²¹¹. It belongs to the outward hydrogen pumps; this subgroup induces membrane hyperpolarization by pumping protons into the extracellular space. Other bacteriorhodopsins and archaeorhodopsins of this subgroup include archaeorhodopsin-3 (Arch) from *Halorubrum sodomense*, which is the most frequently used pump for inducing hyperpolarization, and the fungal light-driven proton pump from *Leptosphaeria maculans* (MAC)²⁰⁵, which is used for mitochondrial membrane potential control^{28,29,159}. Contrasting the broad availability of outward proton pumps, very few inward proton pumps are known. PoXeR, from *Parvularcula oceani*, was the first one characterized¹⁸⁷. Inward proton pumps could potentially be used for the depolarization of membranes. Although their capacity does not reach that of the light-gated ion channels, their activity does not depend on the ion gradient. Halorhodopsins are inward chloride pumps that trigger hyperpolarization by the intrusion of negative charges. Unlike proton pumps, halorhodopsins do not cause pH changes. NpHR, from *Natronomonas pharaonic*, for instance, is a chloride pump

TABLE 9.3
Commonly Used Natural Microbial Rhodopsins and Engineered Variants

PUMPS	Name(s)	Description	Ion	Activation Wavelength (nm)	Function	Used in Mitochondria	Reference
	Arch/aR-3	Archaeorhodopsin-3 from <i>Haloarabrum sodomense</i> . Widely used pump with rapid kinetics and high dynamic range	H ⁺ (outward)	552	Hyperpolarization	No	(Mukohata <i>et al.</i> , 1999) ¹⁹⁷
	eArch 3.0	Arch variant with high photocurrents				No	(Mattis <i>et al.</i> , 2012) ¹⁹⁵
	Arch M128A/S151A/A225T	Arch blue-shifted variant (550 nm)				No	(Sudo <i>et al.</i> , 2013) ²⁰³
	ArchT	Archaeorhodopsin from <i>Haloarabrum</i> strain TP009, higher light sensitivity than Arch	H ⁺ (outward)	566	Hyperpolarization	No	(Han <i>et al.</i> , 2011) ¹⁸²
	HsBR	Bacteriorhodopsin from <i>Halobacterium salinarum</i> . First-discovered microbial rhodopsin	H ⁺ (outward)	560	Hyperpolarization	No	(Oesterhelt <i>et al.</i> , 1971) ²¹¹
	Ace/AR	BR-like rhodopsin from <i>Acetabularia acetabulum</i> . First eukaryotic proton pump reported	H ⁺ (outward)	518	Hyperpolarization	No	(Tsunoda <i>et al.</i> , 2006) ²⁰⁴
	dR	Deltarhodopsin from <i>Haloterrigena</i> sp. Arg-4	H ⁺ (outward)	550	Hyperpolarization	Yes	(Ihara <i>et al.</i> , 1999) ¹⁸⁵
	MAC/LR/Ops	From <i>Leptospira maculans</i> , lower photocurrent than Arch	H ⁺ (outward)	555	Hyperpolarization (also used for depolarization)	Yes	(Waschuk <i>et al.</i> , 2005) ²⁰⁵
	PoXeR	Xenorhodopsin from <i>Parvularcula oceanii</i>	H ⁺ (inward)	567	Depolarization	No	(Inoue <i>et al.</i> , 2016) ¹⁸⁷
	NsXeR	Xenorhodopsin from <i>Nanosalina</i>	H ⁺ (inward)	565	Depolarization	No	(Shevchenko <i>et al.</i> , 2017) ²⁰²
	HsHR/HR	Halorhodopsin from <i>Halobacterium salinarum</i> , poor heterologous expression	Cl ⁻ (inward)	580	Hyperpolarization	No	(Zhang <i>et al.</i> , 2007) ²⁰⁰
	NpHR/Halo	Halorhodopsin from <i>Natronomas pharaonis</i> with low photocurrents	Cl ⁻ (inward)	580	Hyperpolarization	No	(Zhang <i>et al.</i> , 2007) ²⁰⁰
	eNpHR 3.0	NpHR variant with high photocurrents (20 times stronger than NpHR)				No	(Gradinaru <i>et al.</i> , 2010) ¹⁸⁰
	Sa/HO	Halorhodopsin-like chloride pump from <i>Salinibacter ruber</i>	Cl ⁻ (inward)	582	Hyperpolarization	No	(Mongodin <i>et al.</i> , 2005) ¹⁹⁶

(Continued)

TABLE 9.3 (Continued)
Commonly Used Natural Microbial Rhodopsins and Engineered Variants

Name(s)	Description	Ion	Activation Wavelength (nm)	Function	Used in Mitochondria	Reference
Jaws	Crxhalorhodopsin from <i>Haloraccula (Halobacterium) salinarum</i>	Cl ⁻ (inward)	632	Hyperpolarization	No	(Chuong <i>et al.</i> , 2014) ¹⁷²
KR2	Rhodopsin 2 from <i>Krokinobacter eikastus</i>	Na ⁺ (outward), H ⁺ in the absence of Na ⁺	524	Hyperpolarization	No	(Inoue <i>et al.</i> , 2013) ¹⁸⁶
GrCCR4	Cation channelrhodopsin from <i>Guillardia theta</i> with high light sensitivity and rapid desensitization	CCR (H ⁺ and Na ⁺)	540	Depolarization	No	(Yamauchi <i>et al.</i> , 2017) ²¹⁰
C+ChR1	Cation channelrhodopsin-1 from <i>Chlamydomonas reinhardtii</i>	CCR (highly permeable to H ⁺)	495	Depolarization	No	(Nagel <i>et al.</i> , 2002) ¹⁸⁸
VChR1	Cation channelrhodopsin from <i>Volvox carterii</i> , poor expression in mammalian cells	CCR	570	Depolarization	No	(Zhang <i>et al.</i> , 2008) ²⁰⁸
CaChR1/ CaCCRI	Cation channelrhodopsin from <i>Chlamydomonas augustae</i>	CCR	520	Depolarization	No	(Hou <i>et al.</i> , 2012) ¹⁸⁴
Chronos /ShChR	Cation channelrhodopsin from <i>Stigeoclonium helveticum</i> with fast kinetics and high light sensitivity	CCR	480	Depolarization	No	(Klapoetke <i>et al.</i> , 2014) ¹⁸⁹
SdChR	Cation channelrhodopsin from <i>Scherffelia dubia</i> with high light sensitivity	CCR	460	Depolarization	No	(Klapoetke <i>et al.</i> , 2014) ¹⁸⁹
CheRiff	SdChR variant with fast kinetics than <i>sd</i>	ChR and high light sensitivity			No	(Hochbaum <i>et al.</i> , 2014) ¹⁸³
PsChR/ PsChR2	Cation channelrhodopsin from <i>Platymonas subcordiformis</i> . More blue-shifted variant available together with TsChR	CCR	445	Depolarization	No	(Govorunova <i>et al.</i> , 2013) ¹⁷⁶
PsChR D139H	PsChR variant with five-fold enhanced photocurrent and enhanced Na ⁺ and Ca ²⁺ conductance				No	(Duan <i>et al.</i> , 2019) ¹⁷⁴
TsChR	Cation channelrhodopsin from <i>Tetraselmis striata</i> . More blue-shifted variant available together with PsChR	CCR	445	Depolarization	No	(Klapoetke <i>et al.</i> , 2014) ¹⁸⁹
DChR1	Cation channelrhodopsin from <i>Dunaliella salina</i> , H ⁺ selective	CCR (H ⁺ selective)	475	Depolarization	No	(Zhang <i>et al.</i> , 2011) ¹⁶⁴

(Continued)

TABLE 9.3 (Continued)
Commonly Used Natural Microbial Rhodopsins and Engineered Variants

Name(s)	Description	Ion	Activation Wavelength (nm)	Function	Used in Mitochondria	Reference
Chrimson (<i>CnChR1</i>)	Cation channelrhodopsin from <i>Chlamydomonas noctigama</i> , highly H ⁺ selective	CCR (H ⁺ selective)	590	Depolarization	No	(Klapoetke <i>et al.</i> , 2014) ¹⁸⁹
ChrimsonR	Chrimson variant with fast off kinetics				No	(Klapoetke <i>et al.</i> , 2014) ¹⁸⁹
vf-Chrimson	Chrimson variant with very fast kinetics (comparable to Chronos)				No	(Mager <i>et al.</i> , 2018) ¹⁹³
Chrimson-SA	Chrimson mutant with red-shifted activation spectra (605 nm)				No	(Oda <i>et al.</i> , 2018) ²²⁴
ChRmine (RICCR1)	Cation channelrhodopsin from <i>Tiarina fusus</i> with high conductance and light sensitivity	CCR	600	Depolarization	No	(Marshall <i>et al.</i> , 2019) ¹⁹⁴
CxChR2	Cation channelrhodopsin-2 from <i>Chlamydomonas reinhardtii</i> with fast recovery. Widely used	CCR (H ⁺ > Na ⁺ > K ⁺ >> Ca ²⁺)	470	Depolarization	Yes	(Nagel <i>et al.</i> , 2003) ¹⁹⁹
Chr2	Chr2 variant with long-lived open states and enhanced light sensitivity. It remains open after activation with a short light pulse and can be closed with orange light				No	(Bemdt <i>et al.</i> , 2011) ¹⁶⁹
C128X(X=T/A/S)	Most used Chr2 variant with slow channel closure, improved conductivity, and modified ion selectivity				Yes	(Nagel <i>et al.</i> , 2005) ²⁰⁰
Chr2 HI34R	Chr2 variant with slow inactivation				Yes	(Tkatch <i>et al.</i> , 2017) ²⁵
Chr2 C128A/HI34R					No	(Dawydow <i>et al.</i> , 2014) ¹⁷³
SSFO	Chr2 D156C mutant with large photocurrents and high light sensitivity (even at low retinal concentrations)				Yes	(Yizhar <i>et al.</i> , 2011) ²³⁹
SOL	Chr2 C128S/D156A mutant with extremely slow τ -off and high light sensitivity				No	(Gong <i>et al.</i> , 2020) ¹⁷⁵
CHETA	SSFO variant with ultra-high light sensitivity				No	(Gunaydin <i>et al.</i> , 2010) ¹⁸¹
CatCh	Fast-cycling Chr2-E123T/A mutant with low light sensitivity and photocurrent				No	(Bemdt <i>et al.</i> , 2011) ¹⁶⁹
	Chr2 L132C mutant with weak inactivation, fast response kinetics, large photocurrents and light sensitivity, and increased Ca ²⁺ and Mg ²⁺ conductance				No	(Kleinlogel <i>et al.</i> , 2011) ¹⁹⁰
Chr2 T159C	Chr2 variant with increased photocurrent and light sensitivity				No	(Bemdt <i>et al.</i> , 2011) ¹⁶⁹
ChomeQ	Chr2 variant with improved photocurrent and reduced Ca ²⁺ and H ⁺ conductance				No	(Cho <i>et al.</i> , 2019) ¹⁹¹
ChloC	Chloride-conducting Chr2 variant (still some H ⁺ conductance)				No	(Wietek <i>et al.</i> , 2014) ²⁰⁶
iChloC	Optimized ChloC with high Cl ⁻ selectivity				No	(Wietek <i>et al.</i> , 2015) ²⁰⁷

(Continued)

TABLE 9.3 (Continued)
Commonly Used Natural Microbial Rhodopsins and Engineered Variants

Name(s)	Description	Ion	Activation Wavelength (nm)	Function	Used in Mitochondria	Reference
C1V1	Chimera of ChR1 and VChR1 with high light sensitivity	CCR	540	Depolarization	No	(Yizhar <i>et al.</i> , 2011) ²³⁹
ReaChR	Chimera of ChR2, VChR1, and VChR2	CCR	590	Depolarization	No	(Lin <i>et al.</i> , 2013) ¹⁹²
bReaChES	ReaChR variant with improved trafficking and kinetics	CCR			No	(Rajasekharan <i>et al.</i> , 2015) ²⁴¹
C1C2	Chimera of C1ChR1 and C2ChR2 with large photocurrent and small desensitization	CCR	470	Depolarization	No	(Kato <i>et al.</i> , 2012) ¹⁶¹
iC1C2	Chloride-conducting C1C2 variant (still some H ⁺ conductance)				No	(Berndt <i>et al.</i> , 2014) ¹⁷⁰
iC++	Optimized iC1C2 with high Cl ⁻ selectivity				No	(Berndt <i>et al.</i> , 2016) ¹⁷¹
SwiChR++	Optimized iC++ with higher photocurrents				No	(Berndt <i>et al.</i> , 2016) ¹⁷¹
GiACR1	Natural anion channelrhodopsin-1 from <i>Gaillardia theta</i> with high photocurrent	ACR	515	Hyperpolarization/Depolarization	No	(Govornova <i>et al.</i> , 2015) ¹⁷⁷
FLASH	GiACR1 variant with fast kinetics and large photocurrent				No	(Kato <i>et al.</i> , 2018) ¹⁸⁸
ACR1.2.0	GiACR1 variant with large photocurrents and increased expression and plasma membrane trafficking in plants				No	(Zhou <i>et al.</i> , 2021b) ¹⁵⁷
GiACR2	Natural anion channelrhodopsin-2 from <i>Gaillardia theta</i> with faster kinetics than GiACR1	ACR	470	Hyperpolarization/Depolarization	No	(Govornova <i>et al.</i> , 2015) ¹⁷⁷
AIACR1	Natural anion channelrhodopsin from <i>Aurantiochytrium</i> sp. KH105 (rubyACR)	ACR	590	Hyperpolarization/Depolarization	No	(Govornova <i>et al.</i> , 2020) ¹⁷⁸
HcKCR1	Potassium channelrhodopsin from <i>Hyphochytrium catenoides</i> with rapid kinetics and robust photocurrent	CCR (K ⁺)	540	Hyperpolarization	No	(Govornova <i>et al.</i> , 2021) ¹⁷⁹

Notes: Outward proton pumps are shaded in red, inward proton pumps in green, inward chloride pumps in yellow, outward sodium pumps in purple, cation channelrhodopsins in blue, and anion channelrhodopsins in orange.

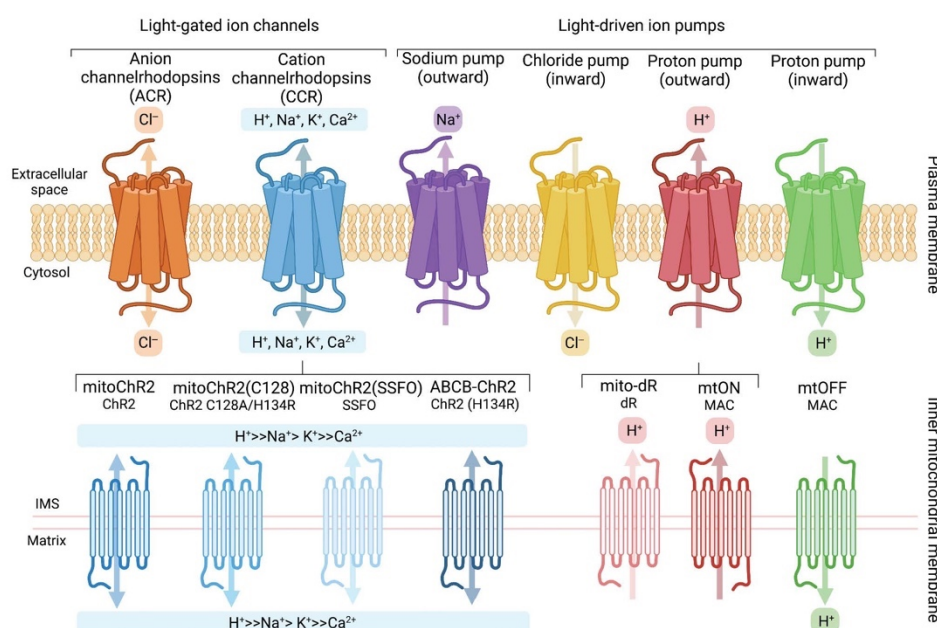


FIGURE 9.4 Types of microbial rhodopsins and variants used for mitochondrial optogenetic control. Upper part, a scheme of the different types of light-gated ion channels and light-driven ion pumps available. Lower part, channels and pumps used in mitochondrial applications: *Wild-type* and different variants of the cation channelrhodopsin 2 (ChR2) (ChR2 C128A/H134R, SSFO, ChR2 H134R) and two proton pumps (MAC in two different orientations and dR). (Created with [BioRender.com](#).)

halorhodopsin (HR) that has been used *in vitro* and *in vivo* in primates for optogenetic control of neural activity^{3,214,215}. Hyperpolarization can also be triggered by the use of the outward sodium pump of *Krokinobacter eikastus* KR2¹⁸⁶, as shown by its use in rat hippocampal neurons²¹⁶. It is important to note that, depending on the orientation of the pump within the membrane, ion transport will take place in one or another direction. This means that the function of pumps employed for optogenetic approaches (hyperpolarization or depolarization) directly depends on their topology at the membrane. Therefore, preliminary experiments to analyze the pump orientation are highly recommended at the initial stages of an experiment¹⁵⁸.

Unlike proton pumps, channelrhodopsins (ChRs) passively transport ions along the electrochemical gradient, thereby dissipating energy¹⁶⁷. Light absorption triggers the opening of the channel resulting in the bidirectional transport of ions. This multiple ion permeation makes channels more efficient than pumps. Two types of channelrhodopsins can be distinguished: Non-selective cation channels (CCR) and non-selective anion channels (ACR)¹⁶⁷. Channelrhodopsin 1 (ChR1) and Channelrhodopsin 2 (ChR2) from *Chlamydomonas reinhardtii* were the first discovered cation-channelrhodopsins^{198,199}. Not surprisingly, ChR2 has increasingly found application as cation-channelrhodopsins due to its fast response to blue light²¹⁷. A great number of CCRs were since identified or engineered, displaying different ion selectivities, kinetics, photocurrent, and response spectra. However, *in vivo* applications would strongly benefit from activation using light of the red portion of the spectrum, as red light is far less toxic compared to light of shorter wavelength, and it also penetrates deeper into tissue than blue light, which is absorbed, for example, by hemoglobin or melanin²¹⁸. Therefore, there is a quest to develop bathochromatically

shifted opsins which absorb light of longer wavelengths than the original opsin. Engineering strategies to obtain “bathy-opsins” commonly involve mutagenesis and alteration of the proteinaceous chromophore environment, to match the therapeutic window of tissue light penetration. For instance, the recently engineered channelrhodopsin NeoR, with a maximum absorption peak at 690 nm, might enable non-invasive therapies²¹⁹. Other red-shifted variants include, for example, the chimera ReaChR, C1V1, or ChrimsonR. The latter was recently used for partially restoring vision in a blind patient²²⁰. ChR2 kinetics were also modified to generate variants, like C128¹⁶⁹, which has long open states, or ChETA¹⁸¹, with very fast response kinetics. Protein engineering has also yielded opsins with altered ion selectivity, as in the case of CatCh, where Ca^{2+} and Mg^{2+} conductance has been improved¹⁹⁰. Another example of an engineered opsin is ChR2-XXL, which has lower retinal requirements, making it a good candidate for opsin use in plants, as shown in Reyer *et al.*¹⁵⁴.

Recently, potassium channelrhodopsins (KCR) were reported for the first time¹⁷⁹. These light-gated cation channels have a 20-fold higher selectivity for K^+ than for Na^+ . This new family of cation channelrhodopsins open up perspectives for the treatment of different diseases, such as epilepsy or mitochondrial channelopathies found in aging in which K^+ signaling is affected²²¹.

The opening of anion channelrhodopsins generates chloride fluxes that can result in hyperpolarization or depolarization depending on the existence of a high or low chloride gradient, respectively. These chloride gradients vary depending on the cell type and the organism. In 2014, two new ChR2 variants highly permeable to chloride were engineered, ChloC from ChR2²⁰⁶ and iC1C2 from the C1C2 chimera¹⁷⁰. Natural ACRs have some advantages in comparison to ACR derived from ChR2 since engineered ACRs still transport some cations and are sometimes pH-dependent. The natural *GtACR1* and *GtACR2* were discovered from *Guillardia theta* in 2015¹⁷⁷. *GtACR1* has been successfully used in plants for growth regulation^{156,157}.

Despite the difference in the number of ions transported by channels and pumps, one needs to consider further aspects when selecting the ideal candidate for an optogenetic experiment from the extensive toolkit of available opsins. Characteristics that deem particular attention involve their function, spectral sensitivity, response kinetics, and photocurrent^{195,222}. Molecular engineering efforts supported by structural information enabled the tuning of opsins for their ion selectivity. Depending on the transported ion and the opsin orientation within the membrane (in the case of pumps), opsin activation either leads to hyperpolarization or depolarization. An inward flow of cations or an outward flow of anions causes depolarization, whereas the opposite transport will trigger hyperpolarization of the membranes.

The maximum excitation peak of the different opsins ranges from 445 to 690 nm. Utilizing opsins with different spectra allows multichannel optical control, as demonstrated by Vierock *et al.*²²³, who combined a blue anion channel, *GtACR2*, with a red cation channel, Chrimson, to bidirectionally control the activity of a population of neurons. When combining different rhodopsins, one has to consider that red-shifted variants respond with a significant activity to blue light. One of the best choices, when using more than one opsin, is currently Chrimson-SA, a red-shifted mutant of Chrimson (*CnChR1*)²²⁴. Moreover, the different rhodopsins also vary in light sensitivity, which influences the photocurrent of the opsin¹⁹⁵.

Another important aspect that has to be contemplated is the kinetics of the light-activated proteins, which determines how fast the membrane potential changes, which therefore directly influences the possible temporal resolution.

The repertoire of opsins continues to expand as the redshift engineering is still in progress and increasingly peculiar rhodopsins are discovered, as for instance OLPVR1I, a pentameric light-gated ion channel encoded by a virus²²⁵, or enzyme-rhodopsins, opsins that modulate, e.g., cyclic nucleotide levels due to their fusion to one of the following catalytic domains: histidine kinases, guanylyl cyclases, or phosphodiesterases²²⁶. The exciting field of opsin-based optogenetics can look forward to the utilization of an increasing number of opsins with particular properties. These will likely expand the potential of the field beyond their use in regulating neuronal activities in the future.

OPsin-BASED OPTOGENETICS IN MITOCHONDRIA

ATP and ROS production, mitochondrial protein import, calcium homeostasis, oxygen consumption, and many signaling pathways depend on the state of the mitochondrial membrane potential^{227,228}. For this reason, precise manipulation of $\Delta\psi_m$ by the use of optogenetic tools opens up the possibility to control cell metabolism and study mitochondrial function in great detail.

Until six years ago, the possibility of using optogenetics to precisely regulate the mitochondrial membrane potential had not been explored. Nowadays, opsins represent a straightforward tool for use within the inner mitochondrial membrane to modify the membrane potential. The first step in developing such a system requires targeting the opsin to the inner mitochondrial membrane rather than to the plasma membrane, their natural homing environment. The following targeting peptides have been used for this purpose: one or four repeats of the mitochondria-targeting sequence of subunit VIII of human cytochrome *c* oxidase (cox-8)^{25,30}, the mitochondrial leading sequence (MLS) of ABCB10^{26,229}, 144 N-terminal amino acids of the rat SDHC1 protein²⁹, and the mitochondrial targeting sequence (MTS) of IMMT1^{28,159}.

Once the channel or pump has been correctly targeted to the inner mitochondrial membrane, changes in mitochondrial membrane potential due to opsin activation can be monitored, for example, with $\Delta\psi_m$ -dependent fluorescent dyes, such as tetramethylrhodamine methyl ester perchlorate (TMRE), tetramethylrhodamine ethyl ester perchlorate (TMRE), MitoView 633, JC-1, or Rhod123. Up to date, TMRE and TMRM have been used in opsin-based optogenetic studies in mitochondria^{25,26,28–30,159,229}. These two lipophilic cationic dyes undergo a potential-dependent accumulation in mitochondria: intact or hyperpolarized mitochondria accumulate more dye than depolarized mitochondria. The behavior of these dyes directly depends on their concentration. Under quenching mode conditions, when high concentrations (50–100 nM) are used, the dye forms aggregates in the matrix and inner membrane of intact or hyperpolarized mitochondria, resulting in the quenching of fluorescence (auto-quenching). Similarly, depolarization causes the dye to diffuse into the cytoplasm, lowering the concentration within the mitochondria and preventing aggregate formation, thus increasing the fluorescence signal. Low, non-quenching concentrations (0.5–30 nM) are not sufficient to produce dye aggregation, even in hyperpolarized mitochondria.

In conclusion, non-quenching mode fluorescence intensity increases upon polarization, since it is proportional to dye accumulation in the inner membrane of mitochondria, whereas quenching mode fluorescence signals increase upon depolarization. The addition of uncouplers that dissipate $\Delta\psi_m$ at the end of the experiment, such as FCCP or CCCP, assists in reliably determining the behavior of the dye^{230,231}.

Opsin activation and assays of dynamic mitochondrial membrane potential changes have been performed either in mammalian and *Drosophila* cells^{25,26,30,31} or isolated mitochondria^{28,29,159}. Dye accumulation can be detected by fluorescence microscopy and bulk-quantified using fluorescent plate readers. Dye excitation profiles often overlap with the spectra of opsins. Therefore, decreasing the energy of the excitation source and the frequency of image acquisition might be necessary in these cases to avoid undesired opsin activation.

Mitochondrial Hyperpolarization and Energization

Mitochondria not only provide energy to the cell but also engage in regulating key cell functions, such as apoptosis. However, the lack of reversible tools for modulating mitochondrial function has severely hampered the study of this organelle and its associated diseases in the past. Optogenetics offers ways to increase the electrochemical gradient at membranes reversibly. During the last few years, opsins have increasingly been used to artificially generate a proton motive force, leading to an “energized-mitochondrion”. Two different proton pumps were used for the hyperpolarization of this organelle: the deltarhodopsin (dR) from *Haloterrigena* sp. *Arg-4*³⁰ and the fungal light-driven proton pump MAC^{28,159}.

The first mitochondria hyperpolarizing tool, mito-dR, was generated by Imai³⁰. It is based on the genetic fusion of a single COX8A targeting sequence to the dR proton pump, which, upon green

light illumination, transports protons out of the mitochondrion. They reported that activation of mito-dR boosts mitochondrial functions such as ATP production or Ca^{2+} metabolism while decreasing the generation of ROS. One of the main causes of Parkinson's disease is a defective mitochondrial respiratory chain. In a Parkinson's disease *Drosophila sp.* model, activation of the mito-dR pump decreased the effects of the disease (reduced alpha-synuclein accumulation, dopaminergic neuron loss, degeneration of mitochondrial cristae, and brain lipid peroxidation were reported), resulting in improved locomotor activity.

A second strategy to hyperpolarize the mitochondrial membrane was devised by Berry *et al.* using the light-driven proton pump from *Leptosphaeria maculans* (MAC)¹⁵⁹. The authors fused the proton pump to a mitochondrial targeting sequence (MTS) to localize it to mitochondria. They made use of one transmembrane domain of MIC60/IMMT1, resulting in the so-called mtON tool. mtON transports protons out of the mitochondria upon 555 nm light absorption, which leads to an increase in the proton motive force (PMF) and thus, the pH of the mitochondrial matrix. As expected, the activation of mtON increased ATP synthesis independent of oxygen consumption in *C. elegans*. mtON could drive the synthesis of ATP even when mitochondrial electron-transport-chain inhibitors of Complex I, III, or IV were added. In *C. elegans*, they showed that mtON can alter metabolic pathways such as the sensing of food availability through AMP-activated protein kinase (AMPK) signaling²⁹. Amitrano *et al.* further confirmed the performance of mtON in mammalian cells, where it was used to improve CD8⁺ T cell-based immunotherapy in solid tumors²⁸. The immunosuppressive tumor microenvironment has detrimental effects on CD8⁺ T cells by impeding their energy production. The authors showed that supporting mitochondrial energy metabolism by activation of mtON using 590 nm light increased CD8⁺ T cell migration and effector function.

In conclusion, optogenetic manipulations of mitochondria hyperpolarization enable the regulation of the PMF and matrix pH, and improve ATP synthesis and calcium metabolism.

Mitochondria Depolarization and “De-Energization”

Light-dependent energization of mitochondria through innovative optogenetic solutions represents a promising starting point for the study and treatment of diseases derived from mitochondrial dysfunction. However, optogenetic dissipation of the PMF likewise serves as a tool for gaining a deeper understanding of mitochondrial activity and function.

Mitochondrial membrane depolarization through optogenetic methods that initiate PMF dissipation has been successfully achieved by Tkatch *et al.*²⁵, Ernst *et al.*²⁶, and Berry *et al.*¹⁵⁹ by using the light-gated ion channel ChR2^{25,26} or the proton pump MAC²⁹. Tkatch *et al.* used ChR2 (mitoChR2) and two ChR2 variants with long open states: ChR2 C128A/H134R (mitoChR2 C128) and ChR2 SSFO (mitoChR2(SSFO))²⁵. Targeted to the inner mitochondrial membrane *via* four repeats of the COX8A-derived MTS and the deletion of the first 24 residues of ChR2, both variants effectively decreased the mitochondrial membrane potential of mammalian cells quantitatively. Pulses of high light intensity (10 or 22 mW/mm²) caused a stronger depolarization than lower light intensities (0.18 or 3.1 mW/mm²). Moreover, they described how the channel can be switched off with long pulses of orange light (595 nm), suggesting that mitoChR2 causes a weaker depolarization and has a faster recovery than the two ChR2 variants. The activation of the channel by 10 s of blue light illumination of 2 mW/mm² caused a decrease in Ca^{2+} uptake and an increase in O_2 consumption. The use of mitoChR2(SSFO) could further suppress the spontaneous beating of cardiomyocytes in cell culture and prevent ATP production in pancreatic β -cells that were exposed to high glucose concentrations. Tkatch and collaborators also demonstrated the high spatial resolution achieved by the optogenetic approach. They depolarized a restricted group of mitochondria within a single cell by using a laser illumination source²⁵.

A similar approach was devised by Ernst *et al.* with the ChR2(H134R) variant fused to the ABCB10 MTS to generate ABCB-ChR2, which is capable of regulating cell fate decision and function^{26,229}. Sustained light illumination for 24 h with 0.5 mW/mm² blue light caused cell death *via* apoptosis and mitophagy. Due to these findings, the authors concluded that IMM permeabilization

itself can trigger apoptosis. Although increasing light intensities and exposure time have a detrimental effect on cell viability, mitochondrial pretreatment with 2 h of low-intensity (0.2 mW/mm²) blue light showed a cytoprotective effect and could prevent cell death.

Berry and colleagues reused the MAC proton pump placed in the inverted direction to generate a depolarization tool called mtOFF. They fused MAC to the 144 N-terminal residues of the rat SDHC1 protein, which includes the mitochondrial targeting sequence and two transmembrane regions achieving the opposite topology to the mtON version²⁹. mtOFF thus adopts an orientation that pumps protons into mitochondria, which induces depolarization upon light illumination. The authors showed, in *C. elegans*, that a light-induced decrease in the proton motive force reduces oxidative damage during oxygen starvation (hypoxia), thereby increasing the survival rate of the worm. In addition, they found that, upon activation of the opsin, oxygen consumption increased by around 70% while ATP production decreased. Food depletion, which represents a high-energy-demand condition in *C. elegans*, increased the AMPK activity, resulting in increased locomotion. In this regard, PMF dissipation through mtOFF activation can increase AMPK activity and locomotion in *C. elegans* independently of food availability.

Unlike hyperpolarizing tools, optogenetic depolarization of mitochondria decreases PMF, ATP production, and calcium uptake and increases oxygen consumption.

PERSPECTIVES AND DISCUSSION

Optogenetic technologies, once devised out of the need to differentially excite neuronal cells in order to study their function, developed at a rapid pace after the first reports appeared. The concept instantly found application in a broad range of cell biological disciplines, likewise benefitting from the excellent precision light stimulation offers. Light-regulated molecular switches hence enable versatile applications not limited to single-cell control but even extending to the regulation of cellular subcompartments. After gene-expression control in the nucleus and signaling pathway modulations at the plasma membrane and in the cytosol saw an extensive asset of optogenetic solutions²², it is now less surprising to see the technology supporting the field of mitochondria research^{158,229}. However, mitochondrial optogenetics is still at its earliest stages, as we are just starting to see how optogenetic switches and strategies transition to the study of mitochondria biology. We expect that new optogenetic approaches will be applied in mitochondria, for example, utilizing multi-color control of specific functions, or tracing and stimulating individual mitochondria within the cellular mitochondrial network. Thanks to these new strategies, complex pathways that, for example, lead to mitophagy can now be studied at high spatiotemporal resolution. By multiplexing the orthogonal optogenetic color palette, one may assist in detangling sequential physiological events at the mitochondrial membrane. Furthermore, by providing temporal control over enzymatic clustering, light-sensitive programming of synthetic mitochondria could boost metabolic engineering efforts.

Throughout this review, we have seen the different optogenetic technologies so far applied to mitochondria. The approaches include both opsin- and non-opsin-based tools, together aiming to better study the metabolism of mitochondria and the various cellular processes mitochondria engage in, such as mitophagy and apoptosis. Opsin-based tools constantly evolve and increasingly mature, enabling precise control of membrane potential. During the last decade, inward proton pumps, outward sodium pumps, potassium channelrhodopsins, and anion channelrhodopsins were identified, enriching the rapidly growing opsin toolbox. These newly identified variants, together with the engineered ones, have expanded the applications of opsin-based optogenetics. Moreover, *in vivo* limitations of blue light were overcome by the engineering of red-shifted variants, such as NeoR²¹⁹. Opsin-based mitochondrial optogenetics successfully applied proton pumps and channelrhodopsins to hyperpolarize or depolarize the inner mitochondrial membrane and assisted in demonstrating the role of the mitochondrial membrane potential in Parkinson's disease³⁰ or in apoptosis^{26,229}, among other processes^{25,28,29,159}. However, many strategies remain yet to be explored, such as the combination of opsins with different excitation wavelengths to hyper or depolarize mitochondria in a

light-dependent manner with a millisecond-resolution, or the use of non-proton selective pumps or channels, such as anion and potassium channels, that do not cause pH changes and could help to shed light on the role of mitochondrial channelopathies in aging²²¹.

Reports of enzyme rhodopsins open another avenue for developing novel strategies of opsin-based optogenetics. Application of these opsins could lead to the control of biological functions by converting light inputs into enzyme functions. For instance, a fungi rhodopsin-guanylyl cyclase (RhGC) described by Scheib *et al.* is capable of producing cyclic guanosine monophosphate (cGMP) in mammalian neurons²³². One could speculate about the potential uses of these enzyme rhodopsins to treat diseases such as hypertension, upon rebalancing the cGMP pathway at the outer mitochondrial membrane²³³. In terms of light-assisted protein inactivation, mitochondria research is still in its early stages. The promising ROS generators SOPP, SOPP2, and SOPP3 with their outstanding high $^1\text{O}_2$ quantum yields are not yet applied in CALI studies.

Finally, optogenetic transcriptional control and genome editing technologies, both targeting nuclear and mitochondrial genes, represent methods that have not yet been applied to the study of mitochondria. While genome editing using TALEN and CRISPR/Cas9-based methods has already yielded new insights into mitophagy and hypoxia²³⁴, optogenetic control would enable the study of individual cells and mitochondria, offering a venue for controlling complex processes pertaining to the organelle. Together with the potential of ROS generators and the steady evolution of versatile methods such as OptoNBs, we foresee a bright future for the field.

BOX 9.2 WHAT'S HOLDING BACK OPTOGENETICS?

Optogenetic techniques are constantly shedding fresh light on numerous dynamic cell processes, even though they have only been available for a relatively short time. However, implementing optogenetic approaches requires sophisticated instruments and a suitable light-shielded experimental environment. Most optogenetic experiments can be performed simply by using fluorescent light microscopes. However, to achieve true spatial control, equipment such as laser-scanning or digital micromirror devices are needed. Another experimental limitation of using optogenetic tools is that the spatial precision with which an area of interest can be exposed to light in a microscope is physically limited by diffraction. Photochemistry kinetics further restrict the accuracy of intracellular photoactivation². Thermal decay causes blue light-sensitive regions to revert to the dark state, and the pace at which this happens is crucial for generating a crisp photoactivation boundary. Slow dark state reversal permits photoactivated molecules to diffuse deeper, allowing the boundary between activated and non-activated regions to be widened. The AsLOV2 photoactivated state has a half-life of about 5–50 s, which is long enough to form intracellular gradients of easily diffusible molecules²³⁵. On the other hand, the half-life of photoactivated CRY2 is in the range of minutes, which is still fast enough to form gradients if diffusion is limited. Furthermore, fast dark state reversal requires frequent exposure to photoactivation light, which can cause phototoxicity even at low intensities. Bennedetti *et al.* provide a detailed comparison of the three main blue light-sensitive dimerizers and their performance in live cells. Magnets produced the best spatial confinement in comparison with iLID/nano and CRY2/CIBN. The authors imply that this is because both parts of the Magnet dimerizer must be photoactivated, restricting interaction within the light-exposed region²³⁶. A more “stable” system is PhyB, which photoconverts between two very stable states in mammalian cells using either red or infrared light. Longer wavelengths are less harmful to cells and penetrate deeper into biological samples. However, perfect spatial control of the PhyB/PIF interaction necessitates complementary 650–670 and 740–760 nm illumination patterns^{13,235}.

The recently published BICYCL systems enable tight spatial and temporal control due to their bi-stable nature. However, the requirement for a green light may be a drawback for *in vivo* applications. The switch also requires the external addition of chromophores (PCB) to function, which could be improved by further engineering to enhance phycocyanobilin and/or biliverdin absorption or by simply using endogenous biliverdin¹⁰¹.

Another point to keep in mind is that the inactive and active states of photosensors are in a chemical equilibrium, thus dark is never completely dark. For instance, even in the dark, a very low percentage of LOV2 molecules (1.5%) are still activated, with a dissociated J α -helix²³⁷, which means individual LOV2 molecules have a limited dynamic range. This is to a greater degree the case for Phys, due to the spectral overlap of the two forms (Pfr/Pr) in the red region.

A major challenge arises when combining optogenetics and fluorescence microscopy because of overlapping excitation spectra, as this may cause unwanted photoactivation. For example, blue light-sensitive domains can often only be combined with red or far-red fluorescent proteins²³⁵. For PhyB, both blue and red/far-red light cause its photoconversion, making YFP one of the only possible combinations^{2,238}.

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6 Conclusion

The main objective of this Thesis was to develop a broad spectrum optogenetic toolbox for controlling cellular processes with high spatiotemporal and quantitative resolution in the model organisms *P. patens* and *C. reinhardtii*. This involves optimizing and characterizing molecular components, generating opto-tools, and utilizing them to control cellular processes with high quantitative and spatiotemporal resolution.

For the moss *P. patens*, the lack of an optogenetic system for controlling gene expression suitable for plants growing under white light was addressed. The PULSE optogenetic system was established and quantitatively characterized in *P. patens* protoplasts. Additionally, CRISPRa technology was implemented for the first time in this moss, and combined with PULSE, demonstrating light-controlled activity of CRISPRa. This work lays the groundwork for the precise control of endogenous promoters with light in *P. patens*.

In the model alga *C. reinhardtii*, transgene expression is often inefficient, requiring researchers to make greater efforts to establish molecular technologies. In this work, significant advances were made in the quantitative characterization of commonly used promoters and reporters, and the dual luciferase assay was established. Optimal orientation of transcriptional units within plasmids was studied, contributing to improve the expression efficiency when large plasmids are transformed in *C. reinhardtii*. We also performed a detailed characterization of the different parts composing the *C. reinhardtii* β TUB2 promoter and engineered two weaker promoters from it. All this was done to create an optogenetic toolbox with the elements needed for implementing optogenetic tools to control gene expression. A protocol for implementing these opto-tools in the alga was also established. This optogenetic platform, which allows faster and standardized implementation of optogenetic systems, was used to establish the first light-controlled repression system in *C. reinhardtii*, named *CrBOff*. This system allows repression of gene expression up to 8.1 times under blue light. Additionally, canavanine was established as a novel marker for *C. reinhardtii* and combined with *CrBOff* for light-controlled resistance to the antibiotic, allowing growth in the presence of canavanine only under blue light.

Efforts to establish an optogenetic system for transcriptional activation were made with little success. Results point to a deficient expression of the transactivation domain used, VP16. Substitution with another transactivation domain, like P65, which is also available in the generated opto-toolbox, or optimization of intronization or promoter-transgene/promoter-

terminator combinations could be tested to generate a functional optogenetic system for activation of gene expression.

Regarding the generation of a light-controlled mitochondria, a method for mitochondrial isolation and monitoring of mitochondrial membrane potential changes in *C. reinhardtii* was established. Additionally, two different opsins were targeted to the mitochondrial membrane, predicted to modify the mitochondrial membrane potential in opposite directions. Combining these advances will allow testing whether these opsins can modify *C. reinhardtii* mitochondrial membrane potential in a light-dependent manner.

This work lays the foundation for broad implementation of optogenetics in *C. reinhardtii* and opens up the possibility of controlling biological processes with high resolution in *P. patens* and *C. reinhardtii*. It addresses the lack of tools in these organisms for precise manipulation of cellular processes at the highest quantitative, spatial and temporal resolution. These processes include gene expression and mitochondrial activity, which are crucial for fundamental research and numerous biotechnological applications.

