# PULSE – Optogenetic control of gene expression in plants in the presence of ambient white light

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## ABSTRACT

Optogenetics, the genetic approach of controlling cellular processes with light, is revolutionizing biological signalling and metabolic studies. It provides unmatched spatiotemporal, quantitative and reversible control, overcoming limitations of chemically-inducible systems. However, optogenetics severely lags in plant research because ambient light required for growth leads to undesired system activation. We solved this issue engineering PULSE (Plant Usable Light-Switch Elements), the first optogenetic tool for reversibly controlling gene expression in plants under ambient light. PULSE combines a blue light-regulated repressor with a red light-inducible switch. Gene expression is only activated under red light and remains inactive under white light/darkness. Supported by a quantitative mathematical model we characterized PULSE in protoplasts achieving high induction rates, and combined it with CRISPR/Cas9-based technologies to target synthetic signalling and developmental pathways. We applied PULSE to control immune responses in plant leaves and generated Arabidopsis transgenic plants. PULSE opens broad experimental avenues for plant research and biotechnology.

## INTRODUCTION

The reversible and orthogonal control of cellular processes with high spatiotemporal resolution is key for quantitatively understanding the dynamics of biological signalling networks as well as for programming desired phenotypes. The optimal stimulus for such cellular control is light as it can be applied with unmatched spatiotemporal precision in a quantitative manner, with minimized toxicity and invasiveness. Accordingly, optogenetics, the control of cellular events by using genetically encoded, light-responsive switches is opening revolutionary avenues in mammalian systems. A non-limiting list of successfully manipulated and regulated cellular and physiological processes with optogenetic switches includes neuromodulation, gene expression, epigenetics, protein and organellar activity, and subcellular localization[1–7].

While similar approaches to address important biological questions are needed in plant research, the use of optogenetics to answer them is limited by the intrinsic need of plants for broad-spectrum light which would erroneously activate the engineered light-responsive switches. We have recently developed and successfully implemented the first two optogenetic systems for the control of gene expression in plant cells. The systems are regulated by red and green light and proved useful for the quantitative manipulation of hormone signalling pathways and recombinant protein expression control[8,9]. However, due to the spectral compatibility limitations described above or the need for co-factors difficult to administer to whole plants, these tools could only be applied in transiently transformed plant cells such as mesophyll protoplasts from *Nicotiana tabacum* or *Arabidopsis thaliana,* and the moss *Physcomitrella patens* which can be kept in the dark prior to the optogenetic experiment[8–10]. Despite their utility for transient signalling studies in cell culture, it is highly desirable to have an optogenetic tool functional in whole plants and being

insensitive to broad-spectrum white light to harness the full potential of optogenetics in the plant kingdom.

Towards this goal, we set here to develop the first optogenetic system for the control of gene expression in plants that is silent under white light and can be activated with monochromatic red light. The system, termed PULSE (Plant Usable Light-Switch Elements), comprises two engineered photoreceptors exerting a combined activity over the regulation of transcription initiation: one actively represses gene expression under blue light ($B_{Off}$, Blue Light-repression) engineered from the EL222 photoreceptor[11], and the second one activates gene expression with red light ($R_{On}$, Red Light-activation) based on a Phytochrome B (PhyB) - PIF6 optoswitch[8,10] (**Fig. 1**).

We first engineered and characterized PULSE in *Arabidopsis thaliana* protoplasts. PULSE provides quantitative and spatiotemporal reversible control over gene expression, achieving high induction rates (up to *ca.* 400-fold) while being Off under white light or in the dark. We developed a mathematical model to quantitatively characterize the dynamic behaviour of the system and guide designing experimental setups. We combined it with a plant transcription factor (TF) or a CRISPR/Cas9-derived gene activator and showed its functionality for the light-controlled activation of both Arabidopsis and orthologous promoters. Furthermore, we applied PULSE to engineer light-inducible immunity *in planta* using *Nicotiana benthamiana* leaves as model system, and tested its functionality in whole Arabidopsis transgenic plants. These results demonstrate the wide applicability of PULSE, opening up novel perspectives for the targeted spatiotemporal and quantitative study and control of plant signalling, genetic and metabolic networks as well as its implementation for biotechnological approaches.

## RESULTS

### Design, implementation, and test of the PULSE system in plant cells

PULSE is an integrated optogenetic molecular device, consisting of two components, a module providing activation of gene expression under red light ($R_{On}$) and a second one ensuring effective transcriptional repression under blue light ($B_{Off}$) (**Fig. 1**). The rationale behind this new conceptual and experimental approach is that the combination of both switches will yield a system that is inactive in ambient growth conditions (light and darkness) and only active upon irradiation with red light. This enables full applicability in plants growing under standard light conditions.

We first constructed a blue light-regulated gene repression switch $B_{Off}$ based on the photoreceptor EL222 from the bacterium *Erythrobacter litoralis*[11] which has a Light-Oxygen-Voltage (LOV) dependent motif and an Helix-Turn-Helix (HTH) domain. Upon blue light it binds as a dimer to the target DNA sequence C120[12]. $B_{Off}$ thus comprises (**Fig. 2a**): i) the constitutively expressed EL222 fused to a transcriptional repressor domain (REP), and ii) a reporter module driving the expression of a reporter gene (*e.g.* Firefly luciferase, FLuc) under the control of a synthetic tripartite promoter. The promoter comprises a quintuple-repeat target sequence for EL222, termed $(C120)_5$, flanked by the enhancer sequence of the CaMV35S promoter and the minimal domain of the constitutive promoter hCMV.

We evaluated three versions of the blue light-repressor module by fusing either of three different known transrepressor domains to the N-terminus of EL222, one from the human Krüppel Associated Box (KRAB)[13,14] protein, and two from Arabidopsis, namely the B3 repression domain (BRD)[15] and the EAR repression domain (SRDX)[15] (**Fig. 2a**). The functionality of the $B_{Off}$ optoswitches was assayed by transient co-transformation with the reporter construct into Arabidopsis protoplasts. Constitutively

expressed Renilla luciferase, RLuc, was included for normalization. The cells were illuminated for 18 h at different light intensities of blue light (0, 0.25, 0.5, 1, 5 and 10 µmol $m^{-2}$ $s^{-1}$), and FLuc/RLuc activity was quantified (**Fig. 2b**). These blue light intensities had no negative effect on protoplast performance. All three versions of the repressor modules were functional although with different efficiencies, yielding a range of repression levels (SRDX, 92%; BRD, 84%; and KRAB, 53%; at 10 µmol $m^{-2}$ $s^{-1}$ blue light). Based on the highest repression level and dynamic range achieved, we decided to use SRDX-EL222 as a trans-repressor module for all subsequent experiments.

To allow gene induction with PULSE, we then combined the novel blue light-repressible ($B_{Off}$) module with our previously developed PhyB – PIF6 red light-inducible split TF switch ($R_{On}$)[8,10] (**Fig. 3a,b**). PULSE thus integrates: i) a constitutively expressed red light-activation module composed of PhyB-VP16 and E-PIF6, ii) a constitutively expressed blue light-repressor module SRDX-EL222, and iii) a synthetic target promoter, $P_{Opto}$, integrating the binding domains for both switches, namely $(C120)_5$ and $(etr)_8$, upstream of a hCMV minimal promoter sequence driving the expression of a gene of interest. In the presence of blue or white light (a combination of blue, green, red and far-red wavelengths as present in ambient light) both photoreceptors PhyB and EL222 bind to $P_{Opto}$. The net result of the recruitment of the transcriptional activator and repressor near to the minimal promoter sets the system to the Off state. This also applies to darkness and far red light conditions, as the red light-switch is rendered inactive under these wavelengths. Under any other illumination condition lacking the blue light component, SRDX-EL222 is unable to bind $P_{Opto}$ and thus to repress transcription. The system is, then, exclusively in the On state upon monochromatic red light treatment when the interaction between PhyB

and PIF6 leads to the recruitment of the activation domain to the minimal promoter inducing gene expression (**Fig. 3a**).

The PULSE system controlling FLuc expression was first introduced and tested in isolated Arabidopsis protoplasts (**Fig. 3c**). The plasmids coding for the $R_{on}$ switch were co-transformed either with or without $B_{Off}$, and the protoplasts were incubated for 18 h under either red, blue, white or far-red light (as described in **Methods**). In the absence of the repressor module (equivalent to $R_{On}$), efficient activation of PhyB was observed by red light but also under blue and white, as UV and blue light (300 - 460 nm) also activate PhyB[16,17]. Upon addition of the $B_{Off}$ repressor module (PULSE system) we observed induction under red light treatment only, showing a high dynamic range, with up to 396.5-fold induction rates relative to darkness, and a very low basal level of expression in blue and white light (1.7- and 1.6-fold, respectively).

## Development and application of a quantitative model to describe and predict the PULSE activity

In order to quantitatively understand the dynamics and functional characteristics of PULSE and to guide the experimental design of future applications concerning optimal light quality, intensity, and duration, we developed an ordinary differential equation (ODE)-based quantitative mathematical model. The **Supplementary Information** provides a detailed derivation of the model equations, error measurements, system parameters and uncertainty analysis performed. To parameterize the model, On-Off kinetic studies of the PULSE system were performed in protoplasts by monitoring FLuc protein and mRNA levels (**Extended Data Fig. 1a,b**). The experiments demonstrate the reversibility of the system. In order to further characterize thresholds of time and light intensity for protein production, end point measurements and dose-response experiments were performed (**Supplementary**

**Fig. 1a,b,c**). Next, we used the parameterized model to predict the experimental gene expression outcomes of the system as a function of different light intensities, wavelengths and illumination times. Heat maps were generated based on simulations of the dynamic behaviour of PULSE (**Extended Data Fig. 1c, Supplementary Fig. 2**) which will aid in the experimental design by guiding the targeted selection of conditions to obtain a given expression level of interest. To illustrate this, PULSE was tested for combinations of red light intensities and illumination durations selected from the heatmap. A strong correspondence between predicted and experimentally determined activities was observed (**Extended Data Fig. 1c,d**). This indicates the applicability of the model to determine the experimental conditions needed to achieve a tight control over the levels of gene expression with PULSE.

**PULSE-controlled expression of CRISPR/Cas9-derived gene activator and plant TFs to regulate orthologous and plant promoters in Arabidopsis protoplasts**

We next set out to customize PULSE to achieve quantitative and temporally resolved control over the expression of genes from any given promoter of interest, be it orthologous, synthetic or endogenous (downstream activation). For this we devised two approaches applying PULSE: i) to induce the synthesis of a CRISPR/Cas9-derived gene activator, or ii) to induce expression of an endogenous TF. These expressed transcriptional activators, in turn, activate expression from target orthologous (**Fig. 4a,b**) or Arabidopsis promoters (**Fig. 4c-f**).

To achieve optogenetic and customizable control of potentially any target promoter, PULSE was set to control expression of a nuclease-deficient *Streptococcus pyogenes* Cas9 protein fused to a strong activation domain (termed dCas9TV)[18,19]. In a first proof of principle application, PULSE-induced dCas9-TV was used to drive expression from an orthologous promoter, the *Solanum lycopersicum* dihydroflavonol

4-reductase promoter ($P_{SlDFR}$), using FLuc as a quantitative readout in Arabidopsis protoplasts (**Fig. 4a**). To target the promoter, a gRNA against the -150 bp region relative to the transcription start site (TSS) of $P_{SlDFR}$ was used[19]. PULSE-controlled dCas9-TV led to activation of the promoter only upon red illumination, achieving 24.5- and 40.0-fold induction rate compared to blue light and dark treatments, respectively (**Fig. 4b**). Constitutive expression of dCas9-TV served as a positive control yielding the maximum activation capacity of $P_{SlDFR}$, 105.1-fold induction relative to the configuration without dCas9-TV **(Supplementary Fig. 3a).** In a second set up, optogenetically-induced dCas9-TV targeted the promoter of the Arabidopsis gene APETALA1 ($P_{AtAP1}$) which includes the 5'UTR and 2,781 bp upstream of the TSS fused to the reporter FLuc ($P_{AtAP1}$-FLuc) in a plasmid. A gRNA was designed to target the -100 bp region relative to the TSS of $P_{AtAP1}$ (**Fig. 4c**). Red light induction of dCas9-TV yielded 17.9- and 14.1-fold FLuc induction rates from the $P_{AtAP1}$-FLuc construct compared to blue and dark illumination (**Fig. 4e**). Constitutive expression of dCas9-TV yielded a 28.6-fold induction relative to the configuration without dCas9-TV (**Supplementary Fig. 3b**).

We next configured PULSE to drive the expression of the Arabidopsis TF LEAFY (LFY) that is known to bind $P_{AtAP1}$ and promote the expression of AP1[20]. LFY and AP1 are involved in Arabidopsis flowering and both are expressed in the floral primordia. LFY was fused to the transactivator VP16 and RLuc using a self-cleaving 2A sequence, which yields equimolar amounts of both proteins from a single transcript[21] ($P_{Opto}$-LFY-VP16-2A-RLuc). RLuc allows the indirect quantification of the amount of LFY protein synthesized (**Fig. 4d**). The PULSE plasmids were co-transformed in Arabidopsis protoplasts either with or without the optogenetically inducible LFY, and a $P_{AtAP1}$-FLuc target plasmid**.** RLuc values indicate expression of LFY-VP16 upon red light treatment, while only basal levels were obtained upon blue

light or dark treatment (17.5- and 26.6-fold induction, respectively). The red light-induced expression of LFY-VP16 led to activation of $P_{AtAP1}$ and, therefore, FLuc expression achieving 31.4- and 7.4-fold induction rates compared to blue and darkness conditions, respectively (**Fig. 4f**, controls in **Supplementary Fig. 3c**).

### *In planta* optogenetic control of gene expression with PULSE

We next set to evaluate the functionality of PULSE in plants. For this, a new set of vectors was engineered for transformation via *Agrobacterium tumefaciens* with all necessary components in binary plasmids. The vectors comprise a reporter gene under the control of PULSE ($P_{Opto}$), PULSE expressed under a constitutive promoter (either $P_{CaMV35S}$ or $P_{AtUbi10}$), and optionally, a constitutively expressed reporter gene as a normalization element and a plant selection cassette (full description of vectors in **Supplementary Table 1**).

*N. benthamiana* leaves were transiently transformed with a construct having PULSE, a fluorescent protein gene as a reporter (Venus fused to histone H2B for nuclear localization, $P_{Opto}$-Venus-H2B) and constitutively expressed Cerulean fused to a nuclear localization sequence (NLS) as a normalization element. The plants showed an increase in nuclear Venus/Cerulean fluorescence ratio over time when treated with red light, reaching 28.7-fold induction after 9 h and keeping background levels in blue, dark and white light, demonstrating activation of the system *in planta* (**Fig. 5a,b** and **Supplementary Fig. 4**). Additionally, PULSE control over a β-glucuronidase gene ($P_{opto}$-GUS) is shown in **Supplementary Fig. 5**.

### *In planta* optogenetic induction of immunity and conditional subcellular fluorescent targeting of receptors

In plants, signal integration of extracellular stimuli is predominantly mediated by membrane-resident receptor and transport complexes. To mechanistically understand their function, we require non-invasive inducible systems that allow transcriptional induction or complex formation with high temporal precision in order to reconstitute these functional entities in homologous as well as heterologous systems. To test this, we asked whether PULSE allows the generation of immune-competent leaf epidermal cells by introducing a heterologous pattern recognition receptor.

In *Arabidopsis*, the recognition of the bacterial microbe-associated molecular pattern (MAMP) elf18 by the plant innate immune EF-Tu Receptor (EFR) results in a fast and transient increase in cellular reactive oxygen species (ROS)[22]. By contrast, Solanaceae species such as *N. benthamiana* are devoid of EFR and therefore unable to perceive the elf18 peptide. However, genetic transformation of *N. bethamiana* and *S. lycopersicum* with *At*EFR allows these plants to recognize elf18 and confers increased resistance against phytopathogens such as *Ralstonia solanacearum*[22,23]. To achieve optogenetically controlled induction of immunity we expressed an EFR-GFP fusion protein under the control of PULSE ($P_{Opto}$-EFR-GFP) in *N. benthamiana* leaf epidermal cells (**Fig. 6a**). Red light treatment of leaves for 16 h resulted in a clear GFP signal at the cell periphery indicating that EFR-GFP was successfully localized to the plasma membrane (**Supplementary Fig. 6**). To test whether optogenetically controlled EFR provides susceptibility of these cells towards elf18, we applied 1 μM of the elf18 ligand. Indeed, a strong and transient production of ROS was observed *ca.* 10 min after elf18 application in leaves that have been red light-treated (red filled circles; **Fig. 6b**). Quantitative assays showed around 10-fold lower ROS burst triggered in white light-grown plants (black filled circles; **Fig. 6b**), demonstrating light-repression by PULSE under ambient light conditions. No responses were found in untransformed tissue and leaves expressing EFR, but

incubated in the absence of elf18. It should be noted that MAMP-triggered ROS production also relies on a self-amplifying mechanism. ROS spread to neighbouring cells where they induce calcium fluxes leading to the activation of the ROS-producing protein respiratory burst oxidase homolog protein D (RBOHD)[24,25]. Thus, ROS will be detected even at very low background levels of EFR in this system. These data show that PULSE can be used for inducing physiological responses *in planta* in a time-controlled manner.

Next, we set to test the applicability of PULSE for conditional targeting of receptors using nanobodies. In mammalian cells, receptor complexes have been reconstituted and modulated using genetically encoded nanobodies[26,27]. Given their small size and their high-affinity binding characteristics, nanobodies can be used to subcellularly relocalize proteins in a stimulus-dependent manner or to visualize endogenous proteins (using fluorophore-tagged nanobodies). We constitutively expressed the immune receptor EFR-GFP in *N. benthamiana* leaf epidermal cells and co-transformed a genetically encoded GFP nanobody (GFP binding protein, GBP) that binds GFP[28]. To monitor localization, we additionally fused GBP to mCherry and placed it under the control of PULSE ($P_{Opto}$-GBP-mCherry). (**Fig. 6c**). Red light-induction of GBP-mCherry expression in EFR-deficient cells resulted in a cytosolic localization of the soluble protein. By contrast, red light-induction in cells constitutively expressing EFR-GFP showed an almost exclusive targeting of the fluorescently-tagged nanobody to the plasma membrane (**Fig. 6d**). This illustrates potential applications using PULSE-driven genetically encoded specific nanobodies to conduct time-resolved conditional precision targeting of plasma membrane-localized proteins, *e.g.* targeting proteins for degradation or inhibition similarly to what has been described in animal cells[26,27,29]. This approach could thus provide novel opportunities to non-invasively control signalling processes in plants.

## PULSE functionality in stable Arabidopsis transgenic lines

To test the functionality of PULSE in whole plants, transgenic Arabidopsis lines were generated using the plasmid coding for PULSE under the control of the $P_{CaMV35S}$ promoter and $P_{Opto}$-FLuc as a reporter (BM00654). Seedlings of homozygous T3 plants were grown in a multi-well plate for 7 days, before incubation with luciferin. The luminescence was quantified while the plate was subjected to different light treatments as indicated in **Fig. 6e**. The results for two independent PULSE lines, #4-4 and #6-3, show that the system is functional with activation levels ranging from 10- to 21-fold, respectively (determined after 12 h of red light, $t_{36h}$, compared to right before the induction, $t_{24h}$). Transfer from white light to red light led to activation of expression, and subsequent inactivation was achieved when the plants were moved back to white light (**Fig. 6e**), demonstrating reversibility of the system, which was verified also in a second cycle. This is the first example of an optogenetic tool controlling gene expression in whole plants, opening up unforeseen opportunities for plant research and biotechnology.

## DISCUSSION

In order to study and understand cellular processes, it is required to be able to achieve a precise spatiotemporal and quantitative control over their regulation. Genetically encoded chemical-inducible systems have been widely employed for the targeted manipulation of gene expression and other signalling events in prokaryotic and diverse eukaryotic organisms, including plants[30–32]. However, they suffer from intrinsic drawbacks including limited temporal and spatial resolution, diffusion effects, and constrains to deactivate the system after the application of the inducer, in addition to potential pleiotropic activity and toxicity. Some of these experimental

constraints can be solved by using light as an inducer. A plant´s requirement for light to grow, however, limits the implementation of optogenetic approaches, as ambient light leads to undesired activation of most currently available light-controlled systems. Consequently, most of the advantages of optogenetics which have been recently revolutionizing animal and microbial research are simply not applicable in plants. A recent optogenetic approach challenged a plant intrinsic physiological conundrum, namely, how to conserve water under hydric stress by minimizing transpiration without limiting $CO_2$ uptake, two processes directly regulated by stomatal aperture. Papanatsiou *et al.*[33] resorted to a synthetic, blue light-gated $K^+$ channel (BLINK1), engineered for the control of $K^+$ conductance in animal cells[34]. Guard cell-specific expression of BLINK1 in Arabidopsis led to accelerated kinetics of ion fluxes (full activation after 2 min blue light), with reduction of mean stomatal opening and closure half-life times by 40-70% in comparison to wild type controls. Faster stomatal movements improved gas exchange efficiency under fluctuating light conditions, resulting in a more efficient water use without a trade-off in carbon assimilation. This tool profits from the fact that it is applied to a process that is photosynthesis-dependent therefore occurring already naturally under ambient light.

Towards a more generalized application of optogenetic in plants, creative engineering approaches are needed. We set here to design an optogenetic device for the control of gene expression in plants that overcomes the intrinsic challenges, namely, that is non-responsive to ambient illumination conditions and can be only activated by illuminating with a specific, narrow wavelength spectrum. The novel concept implements the design of a dual-wavelength optogenetic switch combining a blue light-regulated repressor with a red light-inducible gene expression switch. PULSE introduces the superior experimental assets of optogenetic systems into plants. The system showed a high dynamic range in Arabidopsis protoplasts with *ca.*

345 400-fold (red light vs. darkness) induction, reversibility and no toxicity. PULSE is
346 applicable for the targeted study of signalling and metabolic networks by, in principle,
347 allowing the control of any endogenous or synthetic promoter of interest as
348 exemplified with the light-driven expression of a plant TF or of a CRISPR/Cas9-
349 derived transcriptional activator. *In planta*, implementation of PULSE demonstrated
350 tight temporal control over subcellular conditional protein targeting, and the capability
351 to induce immunity in *N. benthamiana* leaves. The system is functional in Arabidopsis
352 plants, showing high dynamic range of transgene expression when activated with red
353 light and reversibility when the plants were returned to white light. PULSE could in
354 the future be combined with tissue-specific promoters for organ or developmentally
355 specific expression and activity, as currently done for genetically encoded biosensors
356 and other tools. When using different promoters, the dynamic range of induction
357 might be affected, therefore usage-specific optimizations might be necessary.

358 By using only the N-terminus of PhyB (amino acids 1-650) and the first 100 amino
359 acids of PIF6, we intend to minimize potential interactions of the system with
360 endogenous plant components (EL222 is of bacterial origin, therefore we do not
361 expect any considerable effect on plant signalling). However, we cannot rule out a
362 possible PULSE cross-talk with the endogenous signalling (PhyB) pathway. This is
363 an unavoidable cost to pay in exchange of getting a new functionality as it is also the
364 case when using chemically inducible switches[30,31] or genetically encoded
365 biosensors, *e.g.* some hormone sensors can lead to hormone hypersensitivity
366 phenotypes, as previously exemplified and discussed[35].

367 The strategy here presented, based on engineering and combining switches
368 sensitive to different wavelengths, can be expanded to inspire the engineering of
369 other optogenetic tools compatible with the plant's growth needs. These will likely not
370 be restricted to transcriptional regulation but could also be extended to the

application of selected mammalian optogenetic systems with a high transfer interest to the plant community, *e.g.* to control cellular receptors, kinase activity, ion and metabolite transporters, among other cellular processes[1,36]. For example, signalling proteins could be engineered for red light-regulated recruitment to sub-cellular locations where they activate a signalling cascade, *e.g.* to the plasma membrane as described in mammalian cells[37,38]. To prevent activation under white light, the same signalling protein could additionally be targeted for degradation under blue light by fusing it to a blue light-inducible degron[14,39,40]. Alternatively it could be sequestered to the nucleus under white light by fusing it to the blue light-responsive LINuS[41] or LANS systems[42]. Hence, only under exclusive red light treatment, the protein would be targeted to the site of activity in the cytoplasm or plasma membrane and exert its function.

In this work, we pioneer the optogenetic control of gene expression in plants under ambient light, reflecting the ground-breaking opportunities for plant fundamental and biotechnological fields provided by optogenetics. Due to the quantitative modulation, spatiotemporal resolution and the reversible control capabilities provided, we think that a generalized application of PULSE will facilitate the targeted manipulation and study of biological processes including development, metabolism, hormone signalling, and stress responses.

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## AUTHOR CONTRIBUTIONS

R.O., N.B.A., L.A.K., B.M., and S.B. designed and cloned the constructs. S.B. performed preliminary tests and R.O.F. conducted all Arabidopsis protoplasts experiments. F.W. and R.E. developed the mathematical model. R.O., N.B.A., J.S., and L.A.K. contributed to the establishment of PULSE *in planta*. N.B.A. conducted the conditional targeting and immunity induction *in planta*. R.O.F. and G.G. generated the transgenic Arabidopsis PULSE lines and performed the experiments. R.O., N.B.A., T.O., R.S., and M.D.Z. designed the experiments. J.T., W.W., T.O., R.S., M.D.Z. supervised the research. T.O., R.S., and M.D.Z. analyzed the data and

discussed results. M.D.Z. planned and directed the project. R.O. and M.D.Z. designed the system and wrote the initial manuscript with input from all authors. All authors contributed to editing and read the final version of the manuscript.

## ETHICS DECLARATION

The authors declare no competing interests.

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## FIGURE LEGENDS

**Fig. 1.** Design of PULSE, a functional optogenetic system for the control of gene expression in plants grown under standard light/dark cycles. Plants require light to grow and this poses an experimental challenge to the implementation of optogenetic switches in plants as they will be activated under ambient conditions. To avoid this issue, we designed PULSE (Plant Usable Light Switch-Element), an optogenetic tool that combines a blue light-regulated repressor ($B_{Off}$) with a red light-inducible gene-expression switch ($R_{On}$). In this way gene expression is active only upon illumination with monochromatic red light, while remaining inactive in darkness and under blue, far-red, and white light, hence being applicable to plants grown under standard day/night cycles. (+), presence; (-), absence.

**Fig. 2**: Design and characterization of the blue light-regulated gene repression switch ($B_{Off}$) in Arabidopsis protoplasts**. (a)** Constructs and mode of function. The components engineered and characterized in plant cells are: i) the blue light-responsive *E. litoralis* photoreceptor EL222 fused to either of three different repressor (REP-EL222) domains: KRAB, BRD, SRDX and placed under the control of the constitutive promoter $P_{CaMV35S}$, ii) a synthetic promoter composed of the enhancer region of $P_{CaMV35S}$, five repeats of C120 - $(C120)_5$ - and a minimal promoter $P_{hCMV}$, driving the expression of the reporter gene FLuc, and iii) $P_{CaMV35S}$ driving the constitutive expression of the normalization element RLuc. The transcription factor EL222 has a Light-Oxygen-Voltage (LOV) dependent domain and a Helix-Turn-Helix (HTH) domain. The photoreceptor is folded in the dark due to a flavin-protein adduct and incapable of binding to the $(C120)_5$ element. As a result, expression of FLuc is constitutively active. Upon blue light illumination REP-EL222 unfolds allowing the formation of dimers binding to the $(C120)_5$ element via the HTH. As a result, the initiation of FLuc transcription is repressed. **(b)** Characterization of the system. Arabidopsis protoplasts were transformed with the reporter module (pROF402) and the blue light-responsive element (photoreceptor, EL222) fused to either repressor: KRAB (pROF018), BRD (pROF050), and SRDX (pROF051) or without the optoswitch (Ø, stuffer plasmid). Constitutively expressed RLuc (GB0109) was included for normalization. After transformation, protoplasts were kept in darkness or illuminated with different intensities of blue light (0.25, 0.5, 1, 5, 10 µmol $m^{-2}$ $s^{-1}$), and FLuc and RLuc were determined after 18 h. Shown data are FLuc/RLuc ratios of distinct protoplasts samples ($n$ = 6), bars are the mean ratios and error bars indicate standard error of the mean (SEM). RLU = Relative Luminescence Units. NLS = Nuclear Localization Sequence.

**Fig. 3**: Molecular design and functional characterization of PULSE in Arabidopsis protoplasts. **(a,b)** Mode of function of PULSE and constructs engineered: i) blue light-photoreceptor EL222 fused to the SRDX repressor domain ($B_{Off}$), ii) red light-activated/far-red light-inactivated (reversible) split switch comprising the first 650 amino acids of the PhyB photoreceptor ($PhyB_{1-650}$) fused to the VP16 transactivation domain, and the DNA-binding protein E 8mphR(A) fused to the first 100 amino acids of PIF6 ($PIF_{1-100}$)[8] ($R_{On}$). The $B_{Off}$ and $R_{On}$ modules are constitutively expressed (promoter $P_{CaMV35S}$), iii) synthetic promoter $P_{Opto}$ comprising target sequence of the protein E, $(etr)_8$, $(C120)_5$, and the minimal promoter $P_{hCMVmin}$, driving expression of the reporter FLuc, iv) normalization element RLuc expressed constitutively ($P_{CaMV35S}$). Under white/ambient or blue light, SRDX-EL222 binds to $(C120)_5$, and PhyB is also active ($PhyB_{fr}$) due to the blue and red light components of white light[16,17], and therefore interacts with PIF6, which is bound to $(etr)_8$ through the E protein. In consequence both VP16 and SRDX are recruited to the minimal promoter, resulting in no expression of FLuc as the repressor has a dominant effect on gene expression (left). In darkness or in far-red light EL222 and PhyB are inactive ($PhyB_r$), therefore not binding to $P_{Opto}$, resulting in no FLuc transcription (middle). There is FLuc expression only under monochromatic red light, in which EL222 is inactive and PhyB is active (right). **(c)** Functional characterization of PULSE. Arabidopsis protoplasts were transformed with the normalization element, reporter $P_{Opto}$-FLuc, $R_{On}$ module and either with the $B_{Off}$ module (PULSE system complete) or without $B_{Off}$ (stuffer plasmid, equivalent to the $R_{On}$ system alone). Protoplasts were kept in the dark or illuminated with white LEDs, or 10 µmol $m^{-2}$ $s^{-1}$ of $red_{\lambda max\ 655nm}$, $blue_{\lambda max\ 461nm}$, or far-$red_{\lambda max\ 740nm}$ light. FLuc/RLuc ratios of distinct protoplast samples ($n$ = 6) determined 18 h after illumination, mean and SEM are plotted. RLU = Relative Luminescence Units. NLS = Nuclear Localization Sequence.

582 **Fig. 4**: PULSE-controlled expression of a Cas9-derived gene activator (dCas9-TV)
583 and an Arabidopsis transcription factor (LFY) in Arabidopsis protoplasts. **(a,b)**
584 PULSE drives dCas9-TV expression ($P_{Opto}$-dCas9-TV) under red light. dCas9-TV
585 targets the orthologous $P_{SlDFR}$ promoter activating FLuc expression in Arabidopsis
586 protoplasts**. (c-f)** Optogenetic control of an Arabidopsis promoter from a plasmid
587 construct ($P_{AtAP1}$-FLuc) via two approaches: i) PULSE drives dCas9-TV expression
588 ($P_{Opto}$-dCas9-TV). dCas9-TV activates expression from $P_{AtAP1}$-FLuc (c,e); ii) PULSE
589 drives expression of LFY-VP16 ($P_{Opto}$-LFY-VP16-2A-RLuc). Co-expressed RLuc via
590 a self-cleaving 2A peptide serves as proxy of LFY-VP16 expression. LFY-VP16
591 activates expression from the Arabidopsis promoter $P_{AtAP1}$ ($P_{AtAP1}$-FLuc) (d,f). RLuc
592 and FLuc determinations: $P_{Opto}$-LFY-VP16-2A-RLuc (stripped bars) and $P_{AtAP1}$-FLuc
593 (solid bars) (f). Protoplasts were incubated in darkness, red or blue light, and
594 luminescence determined after 18 h. Data shown are means of FLuc/RLuc of distinct
595 protoplast samples (*n* = 4) (b,e), and RLuc and FLuc means, background values
596 (configuration without $P_{Opto}$-LFY-VP16-2A-RLuc) subtracted for FLuc (*n* = 6 distinct
597 protoplast samples) (f), SEM. RLU = Relative Luminescence Units.

598 **Fig. 5**: Implementation and characterization of PULSE in *Nicotiana benthamiana*
599 leaves. **(a,b)** Plants *Agrobacterium*-infiltrated with PULSE, $P_{Opto}$-Venus and a
600 constitutively expressed Cerulean cassette (pROF346) were kept in dark for 2.5 days
601 prior to light treatment for 2 h, 6 h, 9 h (10 µmol $m^{-2}$ $s^{-1}$ of red light, 10 µmol $m^{-2}$ $s^{-1}$ of
602 blue light, white light, or darkness). Samples were taken at indicated time points from
603 three different areas of the leaf of two plants for each illumination condition for
604 fluorescence confocal microscopy observation. At least 6 images, with 2 to 8 nuclei
605 per image, were taken for each condition. Representative images are shown (a). The
606 images were used to quantify the ratio of nuclear Venus and Cerulean fluorescence
607 intensities (b). Data is presented as box plot with the median (center line),
608 interquartile range (box) and the minimum to maximum values (whiskers),
609 $12 \leq n \leq 34$ nuclei. The statistical significance is determined by a one way-ANOVA
610 and Dunnett's multiple comparison test. *p*-values are 0.9696, 0.0001, and 0.0001, for
611 2, 6 and 9 h, respectively for red light treatment; 0.3828, 0.0020, and 0.0071, for 2, 6
612 and 9 h, respectively for white light treatment; 0.0643, 0.0727, 0.9989, for 2, 6 and 9
613 h, respectively for blue light treatment; 0.5051, 0.5251, and 0.7580, for 2, 6 and 9 h,
614 respectively for dark treatment (**$p < 0.01$, ***$p < 0.001$, ****$p \leq 0.0001$, *ns* not
615 significant).

**Fig. 6**: *In planta* optogenetic heterologous induction of immunity and conditional subcellular targeting of receptors, and PULSE functionality in Arabidopsis transgenic lines. **(a,b)** PULSE-controlled conditional gain of immunity *in planta*. *N. benthamiana* leaves were *Agrobacterium*-infiltrated with PULSE and $P_{Opto}$-EFR-GFP. Disks were collected from two different plants and treated with 1 µM elf18 or mock previous to ROS quantification over time. Luminescence mean values (*n* = 8 leaf disks), SEM. **(c,d)** Conditional targeting of receptors by optogenetically controlled expression of a nanobody (GBP-mCherry) observed by confocal microscopy. *N. benthamiana* leaves were infiltrated with PULSE, $P_{Opto}$-GBP-mCherry, and $P_{CaMV35S}$-EFR-GFP constructs (control: without $P_{CaMV35S}$-EFR-GFP). **(b,d)** Plants were kept in standard growth conditions (16 h white light – 8 h dark) for 2 d prior to induction with 10 µmol $m^{-2}$ $s^{-1}$ red light for additional 16 h (control: white light). **(e)** PULSE functionality in Arabidopsis plants. Seedlings of wild type plants (*n = 6* seedlings) and two independent Arabidopsis homozygous T3 lines (#4-4, #6-3) transformed with PULSE controlling $P_{Opto}$-FLuc (*n* = 26 seedlings, each line) were grown for 8 d, subsequently illuminated as indicated and luminescence determined over time. Plotted data are mean values (background values from wild type seedlings subtracted), SEM. RLU = Relative Luminescence Units.

**Extended Data Fig. 1.** Model-based functional characterization, and prediction and validation of PULSE function. **(a,b)** Quantitative characterization of On-Off FLuc expression kinetics. Protoplasts of Arabidopsis were transformed with PULSE and first kept in the dark, 12 h for protein (a) and 16 h for mRNA (b) determination assays. Samples were afterwards illuminated with either 10 µmol $m^{-2}$ $s^{-1}$ of red or blue light, or kept in darkness for the indicated time periods. Arrows indicate the time point where the samples were split into different illumination conditions for response and reversibility analyses, *e.g.* red to dark, red to blue (On-Off), red to blue to red (On-Off-On). Samples were collected every 3 h for 15 h for FLuc and RLuc determinations in a plate reader (a); and at 15 min, 30 min, 1 h, 2 h, 4 h, 4 h 15 min, 4 h 30 min, 6 h, 7 h for RT-qPCR determinations of mRNA production (b). The curves are the fits to the ODE-based model. The shaded areas represent the error bands as calculated in 95% confidence intervals with a constant Gaussian error model using the profile likelihood method. Depicted are the FLuc/RLuc ratios for protein expression kinetics of distinct protoplast samples ($n$ = 6) (a), and the ratio between starting quantity (SQ) of FLuc and the geometric mean of EF, TIP41L (internal normalization controls) transcripts, of two technical replicates for each transcript (b). **(c)** Model aided prediction of PULSE-controlled protein expression levels as a function of red light intensities and illumination times. The calibrated model yields estimated FLuc/RLuc expression ranges (heatmap). **(d)** Experimental validation of the model predictions of the operating range of PULSE. Selected model simulated expression levels at different red light intensities and illumination times as indicated in (c) were experimentally tested and the resulting FLuc/RLuc ratios (2xSEM, $n$ = 6 distinct protoplast samples) were compared to the predicted values (error bars calculated as in (a,b)). RLU = Relative Luminescence Units.

## ONLINE METHODS

### Plasmid construction

A description of the plasmid construction can be found in **Supplementary Table 1.** DNA fragments were released by restriction from existing plasmids, amplified by PCR using primers synthesized by Sigma Aldrich or Eurofins genomic (listed in **Supplementary Table 2**), or synthesized by GeneArt, Invitrogen. The PCR reactions were performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs). Gel extractions were performed using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), or Zymoclean Gel DNA Recovery Kit (Zymo Research). Assemblies were performed using either Gibson[43], AQUA[44], GoldenBraid[45] or Golden Gate[46,47] cloning methods prior to transformation into chemically competent *Escherichia coli* strain 10-beta (NEB) or TOP10 (Invitrogen). The plasmid purifications were performed using Wizard® Plus SV Minipreps DNA Purification Systems (Promega), NucleoBond® Xtra Midi kit (Macherey-Nagel) or GeneJET Plasmid Miniprep Kit (Thermo Scientific). All preparations were tested by restriction enzyme digests and sequencing (GATC-biotech/SeqLab). All restriction enzymes were purchased from New England Biolabs or Thermo Scientific.

### Arabidopsis protoplast isolation and transformation

Protoplasts were isolated from two- to three-week old *Arabidopsis thaliana* plantlet leaves, grown on 12 cm square plates containing SCA medium (0.32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 4 mM $MgSO_4 \cdot 7H_2O$, 43.8 mM sucrose and 0.8% (w/v) phytoagar in $H_2O$, pH 5.8, autoclaved, 0.1 % (v/v) Gamborg B5 Vitamin Mix (bioWORLD), in a 23 °C, 16 h light - 8 h dark regime. A floatation method was employed for isolation and the plasmids were transferred by polyethylene glycol-mediated transformation as described before[10]. Shortly, plant leaf material was sliced with a scalpel and incubated in dark at 23 °C overnight in MMC

solution (10 mM MES, 40 mM $CaCl_2 \cdot H_2O$, mannitol 85 g $L^{-1}$, pH 5.8, sterile filtered) containing 0.5 % cellulase Onozuka R10 and macerozyme R10 (SERVA Electrophoresis GmbH). After release of the protoplasts with a pipette, the suspension was transferred to a MSC solution (10 mM MES, 0.4 M sucrose, 20 mM $MgCl_2 \cdot 6H_2O$, 85 g $L^{-1}$ mannitol, pH 5.8, sterile filtered) and overlaid with MMM solution (15 mM $MgCl_2$, 5 mM MES, 85 g $L^{-1}$ mannitol, pH 5.8, sterile filtered). The protoplasts were collected at the interphase and transferred to a W5 solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 5 mM glucose, pH 5.8, sterile filtered) prior to counting in a Rosenthal chamber. Mixtures of the different plasmids, as described in the figures, to a final amount of 30-35 µg DNA were used to transform 500,000 protoplasts by dropwise addition of a PEG solution (4 g $PEG_{4000}$, 2.5 mL of 0.8 M mannitol, 1 mL of 1 M $CaCl_2$ and 3 mL $H_2O$). After 8 min incubation, 120 µL of MMM and 1,240 µL of PCA (0.32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWorld)), 2 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, 0.342 mM L-glutamine, 58.4 mM sucrose, 80 g $L^{-1}$ glucose, 8.4 µM Ca-panthotenate, 2 % (v/v) biotin from a biotin solution 0.02 % (w/v) 0.1 % (v/v) in $H_2O$, pH 5.8, sterile filtered, 0.1 % (v/v) Gamborg B5 Vitamin Mix, 64.52 µg $\mu L^{-1}$ ampicillin) were added to get a final volume of 1.6 mL of protoplast suspension.

After transformation, protoplasts were then divided in different 24-well plates in 960 µL aliquots (300,000 protoplasts-necessary to measure six technical replicates for both FLuc and RLuc) or in 640 µL aliquots (200,000 protoplasts-necessary to measure 4 technical replicates for both FLuc and RLuc). Afterwards, the plates were either illuminated with LED arrays with the appropriate wavelength and intensity (as indicated in the figures) for 18 - 20 h at 19 - 23 °C unless indicated otherwise.

**Illumination conditions**

Custom made LED light boxes were used as described before[10,48]. The panels contain LEDs from Roithner: blue (461 nm), red (655 nm), far-red (740 nm) and white LEDs (4000K). For blue, red or far-red light treatment, the intensity was adjusted to 10 µmol $m^{-2}$ $s^{-1}$ unless indicated otherwise. White LEDs were supplemented with blue and far-red LEDs in order to have an equivalent ratio of blue, red and far-red light similar to the sunlight spectra (simulated white light)**.** The intensity of the white light LED was adjusted to 10 µmol $m^{-2}$ $s^{-1}$ for the following wavelength ranges: blue 420 - 490 nm, red 620 - 680 nm, and far-red 700 - 750 nm[49] (see spectra shown in **Supplementary Fig. 7)**. For the *Nicotiana benthamiana* GUS experiment the plants were kept, prior light treatment, in the plant incubator with fluorescent tubes (cool daylight, OSRAM). Cell- and plant- handling and sampling was done, when needed, under green LED (510 nm) light which does not affect the PULSE system. Spectra and intensities were obtained with a spectroradiometer (AvaSpec-ULS2048 with fiber-optic FC-UVIR200-2, AVANTES).

**Luciferase protoplasts assay**

Firefly (FLuc) and Renilla luciferase (RLuc) activities were quantified in intact protoplasts as detailed elsewhere[10]. Six technical replicates of 80 µL protoplast suspensions (approximately 25,000 protoplasts) were pipetted into two separate 96-well white flat-bottom plates (Costar) for simultaneous parallel quantification of both luciferases. Addition of 20 µL of either FLuc substrate (0.47 mM D-luciferin (Biosynth AG), 20 mM tricine, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $EDTA \cdot 2H_2O$, 33.3 mM dithiothreitol, 0.52 mM adenosine 5′-triphosphate, 0.27 mM acetyl–coenzyme A, 5 mM NaOH, 264 µM $MgCO_3 \cdot 5H_2O$, in $H_2O$, pH 8), or RLuc substrate (0.472 mM coelenterazine stock solution in methanol, diluted directly before use, 1:15 in phosphate buffered saline, PBS) was performed prior luminescence determination in a plate reader (determination of 20 min kinetics, integration time 0.1 s). RLuc

luminescence was measured with a BertholdTriStar2 S LB 942 multimode plate reader and FLuc luminescence was determined with a Berthold Centro XS3 LB 960 microplate luminometer. When applicable, FLuc/RLuc was determined and the average of the replicates and SEM was plotted ($n$ = 4 - 6).

**RNA isolation and quantitative RT-qPCR**

Protoplasts were isolated and transformed as described before. The protoplasts were kept in the dark, at room temperature for 16 h prior illumination treatment. At the indicated time point and illumination condition, samples containing *ca.* $10^6$ protoplasts were collected by centrifugation (10 min, 100 *g*) and were frozen in liquid $N_2$ for posterior RNA extraction. The RNA was extracted with a PeqGold Plant RNA kit following the user specifications. The samples were treated with DNase I (Thermo Scientific). The cDNA was synthesized from 500 ng of the RNA samples, using the Revert Aid Reverse Transcriptase (Thermo Scientific) and diluted 1:100 prior to qPCR. Expression levels on the samples were measured in duplicates using SYBR® Green Master Mix (Bio-Rad) with specific primer pairs in a Real-time PCR cycler CFX96 (Bio-Rad) as described before[50]. A DNA mass standard for each gene was prepared in serial dilutions of $10^2$ to $10^7$ copies and measured in parallel with the samples. The genes TIP41-like family protein, TIP41L (*At4g34270*), and Elongation Factor, EF (*At5g19510*), were used as an internal reference genes. Starting quantity values of the samples were calculated using the mass standard curve and normalized with the internal reference gene. Primer pairs used to amplify the DNA mass standard are oROF422/oROF423 for FLuc, oROF518/oROF519 for TIP41L, and EF STD 5'/3'[50] for EF. Specific primer pairs used for the qPCR are oROF424/oROF425 for FLuc cDNA, oROF514/oROF515 for TIP41L cDNA, and EFc RT 5'/3'[50] for EF cDNA (**Supplementary Table 2**).

***Agrobacterium tumefaciens* transformation**

Electro-competent *Agrobacterium tumefaciens* strains C58 (pM90), GV3101 (pM90), containing pSOUP helper plasmid, or AGL1 was transformed with the plasmid of interest. Clones growing in YEP media (10 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ bacto peptone, 5 g $L^{-1}$ NaCl, pH 7.0) supplemented with appropriate antibiotics were selected and each transcriptional unit was confirmed by colony PCR using Q5 DNA polymerase (New England Biolabs).

**Transient transformation of *Nicotiana benthamiana* plants**

*A. tumefaciens* cultures were adjusted to $OD_{600nm}$ = 0.1 - 0.2 in infiltration medium (10 mM $MgCl_2$,10 mM MES, 200 µM acetosyringone, in $H_2O$, pH 5.6). The cultures were mixed in a volume ratio 1:1 with an *A. tumefaciens* culture coding for the RNA silencing suppressor p19. The cultures were incubated for 3 h at room temperature in the dark prior infiltration through the adaxial part of leaves from 4- to 5-week old *N. benthamiana* grown in the greenhouse as described before[51]. The plants were incubated for 2-3 days in the indicated illumination conditions prior to light treatment and analysis by microscopy or enzymatic GUS reporter assay.

**GUS reporter assay in *Nicotiana benthamiana* leaves**

After the illumination of the plants as depicted in the **Supplementary Fig. 5**, two disks of 0.8 cm diameter from different leaves for each illumination treatment were cut and incubated on GUS substrate (100 mM $Na_2HPO_4$, 100 mM $NaH_2PO_4$, adjusted to pH 7.0, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 2 mM X-Gluc, 0.20 % Triton X-100, in $H_2O$) for 3 h at 37°C in dark[52]. The stained disks were washed several times with 70% ethanol to remove the chlorophylls and the pictures were taken with a Nikon D3200 camera.

**Confocal imaging of *Nicotiana benthamiana* leaf material**

For the experiments of optogenetically controlled Venus, leaves of one to two plants for each condition were transiently transformed and incubated for 2.5 days in the

dark, and afterwards illuminated for 2 h, 6 h or 9 h with the appropriate wavelength as indicated in **Fig. 5a,b.** Samples were taken at indicated time points from three different areas of the leaves of the two plants and imaged with a LSM 780 Zeiss laser scanning confocal microscope. The constitutive Cerulean was excited with a Diode 405-30 at 405 nm. The optogenetically controlled Venus expression was excited with an Argon laser at 514 nm. The emission was detected at 440-500 nm for Cerulean and 516-560 nm for Venus. For each condition at least 6 images, with 2 to 8 nuclei per image, were generated. The fluorescence intensities of nuclei were quantified using ImageJ. For each nucleus, an area was selected by using the elliptical selection tool and the mean grey values of the Cerulean and Venus channels were measured, respectively. The ratio of Venus and Cerulean was calculated and expressed in percentage, and plotted for 12 - 34 nuclei (see Life Science reporting summary for detailed information).

For the experiments of conditional targeting and immunity control, *N. benthamiana* were grown for 2 d in 16 h simulated white light – 8 h dark cycle (see **Supplementary Fig. 7**), hereafter half of the plants were grown for 16 h in red light only to induce expression (red light-induced), the other half were grown in simulated white light for 16 h (white light control). The white light control plants were further grown for 16 h after the experiments in red light to induce expression as control for successful transformation. Samples were taken for confocal observation. Confocal laser scanning microscopy was performed with a Leica SP8 confocal microscope using a 20×/0.75 HC PL APO CS IMM CORR lens with a scanning speed of 200 Hz. EFR-GFP and GBP-mCherry were excited with a white light laser at 488 nm and 561 nm, respectively. The emission was detected at 500 - 550 nm for GFP and 575 - 630 nm for mCherry.

**Reactive oxygen species (ROS) burst assay**

Samples were collected from *N. benthamiana* leaves transformed with the indicated constructs or only infiltration buffer (two plants were used for each illumination treatment). ROS production was determined using a BMG CLARIOstar plate reader and following the protocol by Trujillo[53] for Arabidopsis leaves with the following modifications: samples were prepared with a 4 mm biopsy puncher and placed in 150 µL sterile tap water for 3 h in dark to get rid of any ROS production originating from the sample harvest before elf18 or control treatment. Approximately 20 min before addition of 1 µM elf18, water was removed from leaf samples and replaced with reaction solution[53], incubated for *ca.* 3 min before background measurement of ROS production was performed for *ca*. 15 min followed by addition of reaction solution with elf18 or without (mock control).

**Stable transformation of *Arabidopsis thaliana***

Four to five week old *A. thaliana* ecotype Columbia plants grown in a plant chamber (16 h light – 8 h dark, 22°C) were transformed via *Agrobacterium tumefaciens* by floral dip as described earlier[54] with minor modifications. Agrobacterium cells transformed with the corresponding constructs (described in **Supplementary Table 1**) were grown to $OD_{600nm}$ values between 0.6 and 0.9, centrifuged and gently resuspended in 2.4 g/L Murashige & Skoog medium including vitamins (Duchefa Biochemie), 5% (w/v) sucrose, 0.05% (v/v) Silwet L-77 (bioWORLD) and 222 nM 6-Benzylaminopurine (Duchefa Biochemie).

Transformants were selected by seeding in SCA plates (0.32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 4 mM $MgSO_4 \cdot 7H_2O$, 43.8 mM sucrose, 0.8 % (w/v) phytoagar, 0.1 % (v/v) Gamborg B5 Vit Mix (bioWORLD), pH 5.8) containing 30 µg $mL^{-1}$ kanamycin (Duchefa Biochemie) and 150 µg $mL^{-1}$ ticarcillin disodium/potassium clavulanate (Duchefa Biochemie). The positive T1 plants were

checked for expression of the reporter/normalization gene when possible, and the T2 seeds were collected and selected in kanamycin containing media. The lines exhibiting a segregation ratio 3:1 (resistant to sensitive) were propagated until a T3 generation and homozygous lines were selected and used for further experiments. The transgenic PULSE lines are functional and viable.

**Luciferase assay in *Arabidopsis thaliana* plants**

Seeds from the *A. thaliana* lines ($n$ = 26 for the PULSE lines, $n$ = 6 for the wild type controls) were seeded in individual wells of white 96-well white flat-bottom plates (Costar), containing 200 µL of 2.4 g $L^{-1}$ Murashige & Skoog medium including vitamins (M0222, Duchefa Biochemie ) and 0.8 % (w/v) phytoagar (bioWORLD). They were kept for 3 - 4 days at 4°C in the dark, and illuminated for 1 h with simulated white light (see spectra in **Supplementary Fig. 7**) on the fourth day. Then the plate was placed in simulated white light with photoperiod (16 h light – 8 h dark) for 4 days. Addition of 20 µL of FLuc substrate 1.667 mM D-luciferin (from a 20 mM stock in DMSO, Biosynth AG) and 0.01 % Triton in $H_2O$ was performed on the fourth day prior starting the measurements. The plate was sealed with an optically clear film (Sarstedt) thinly perforated. Luminescence was measured, 1 - 2 days after addition of the substrate, in a Berthold Centro XS3 LB 960 microplate reader every hour during several days (1 min delay, 0.5 integration time) while being illuminated as indicated. The background readout levels of Arabidopsis wildtype seedlings were averaged, and the value was subtracted from the rest of the lines for each time point.

**Sample size, replication and statistics**

Data shown in the figures are representative experiments from at least two independent experiments (see Life Science Reporting Summary for detailed information). The sample number per experiment is indicated in each corresponding

figure. Plotting and statistical tests were performed with GraphPad or MATLAB software.

## DATA AND MATERIAL AVAILABILITY STATEMENT

Source data for the figures are available (Source Data .xls files). The raw and associated data that support the findings of this study, and biological material and plasmid maps are available from the corresponding author upon request.

## CODE AVAILABILITY STATEMENT

The numerical integration, fitting process and identifiability analysis with the profile likelihood method were performed in MATLAB using the freely available Data2Dynamics software. Details relative to the equations used can be found in the **Supplementary Information**.

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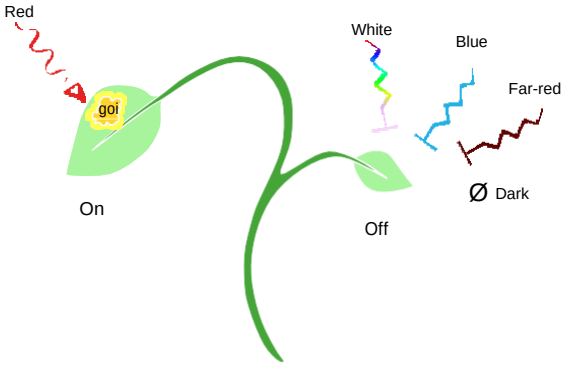
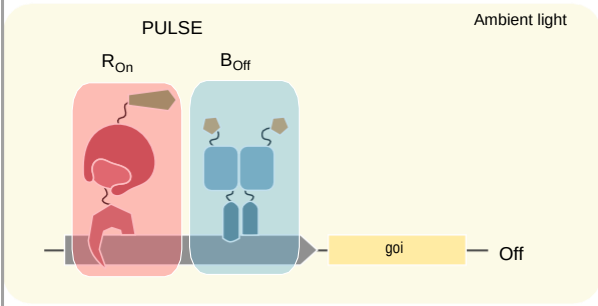
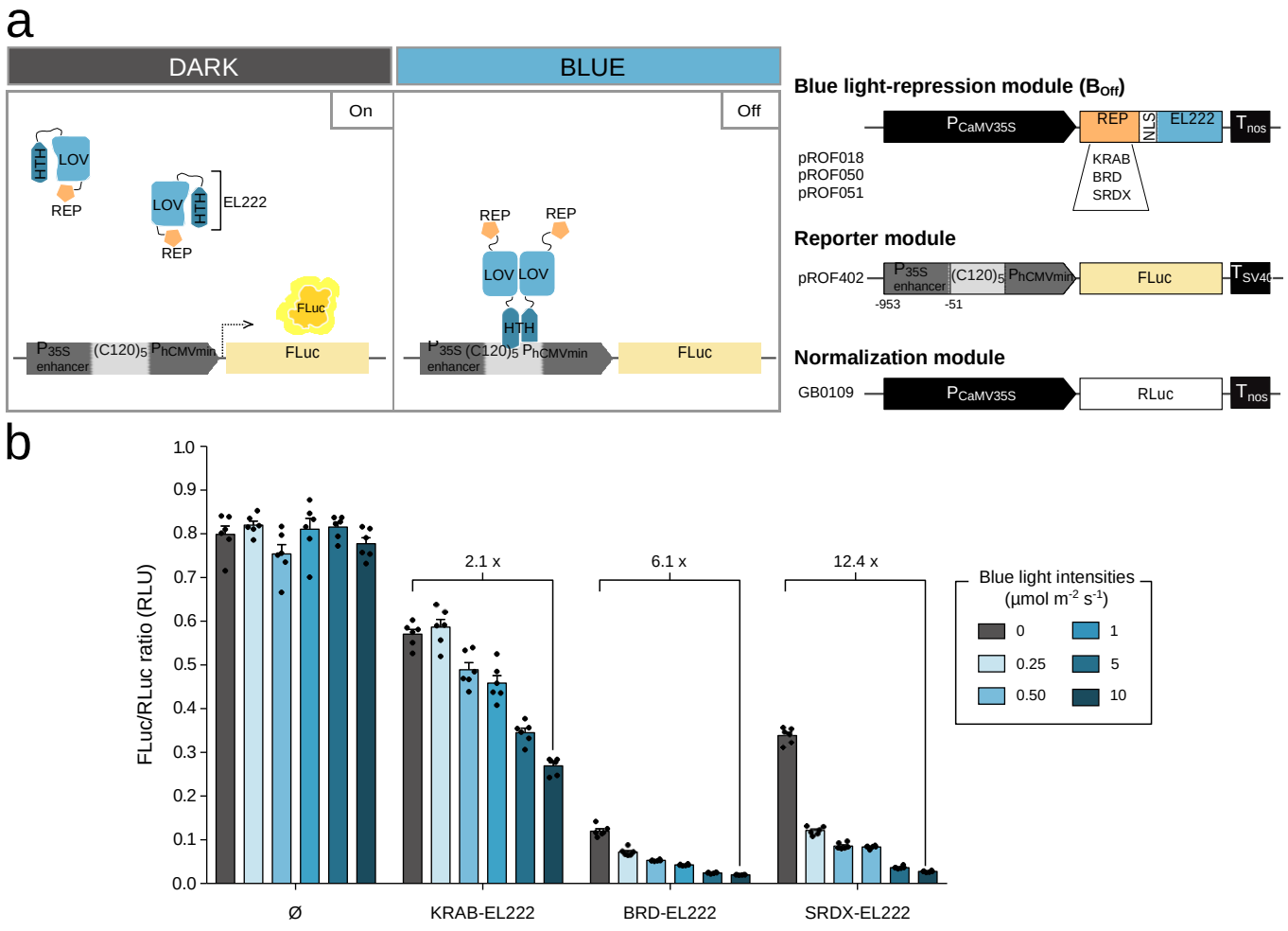
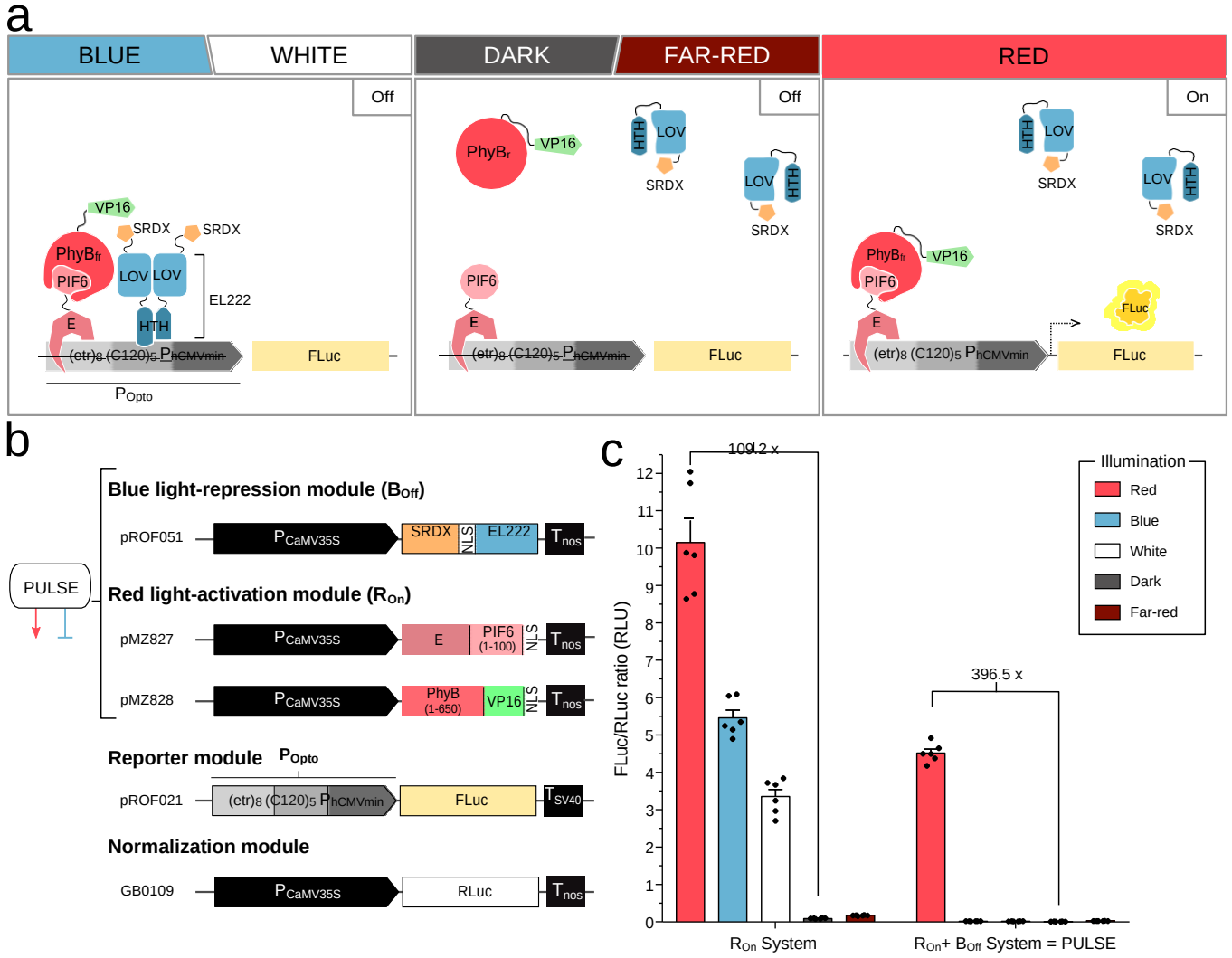


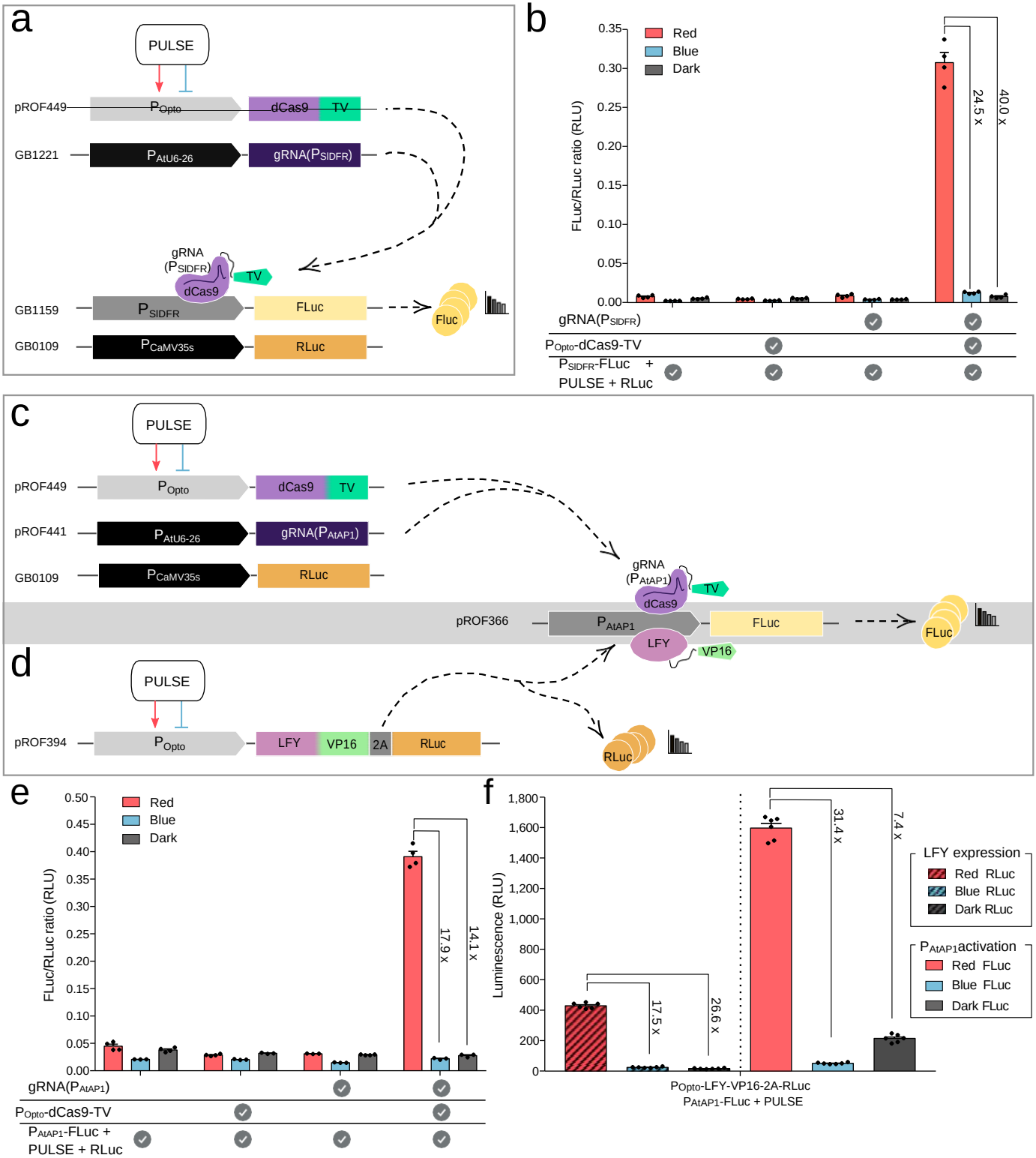



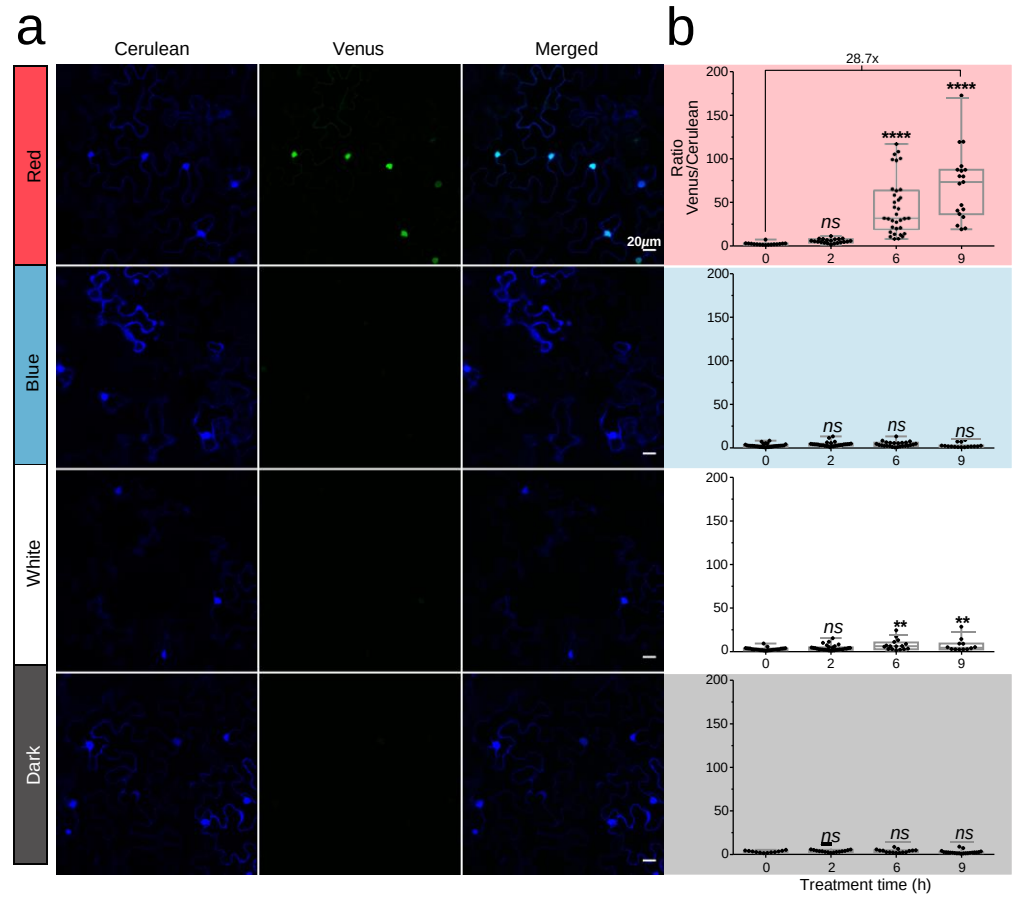

Illumination conditions

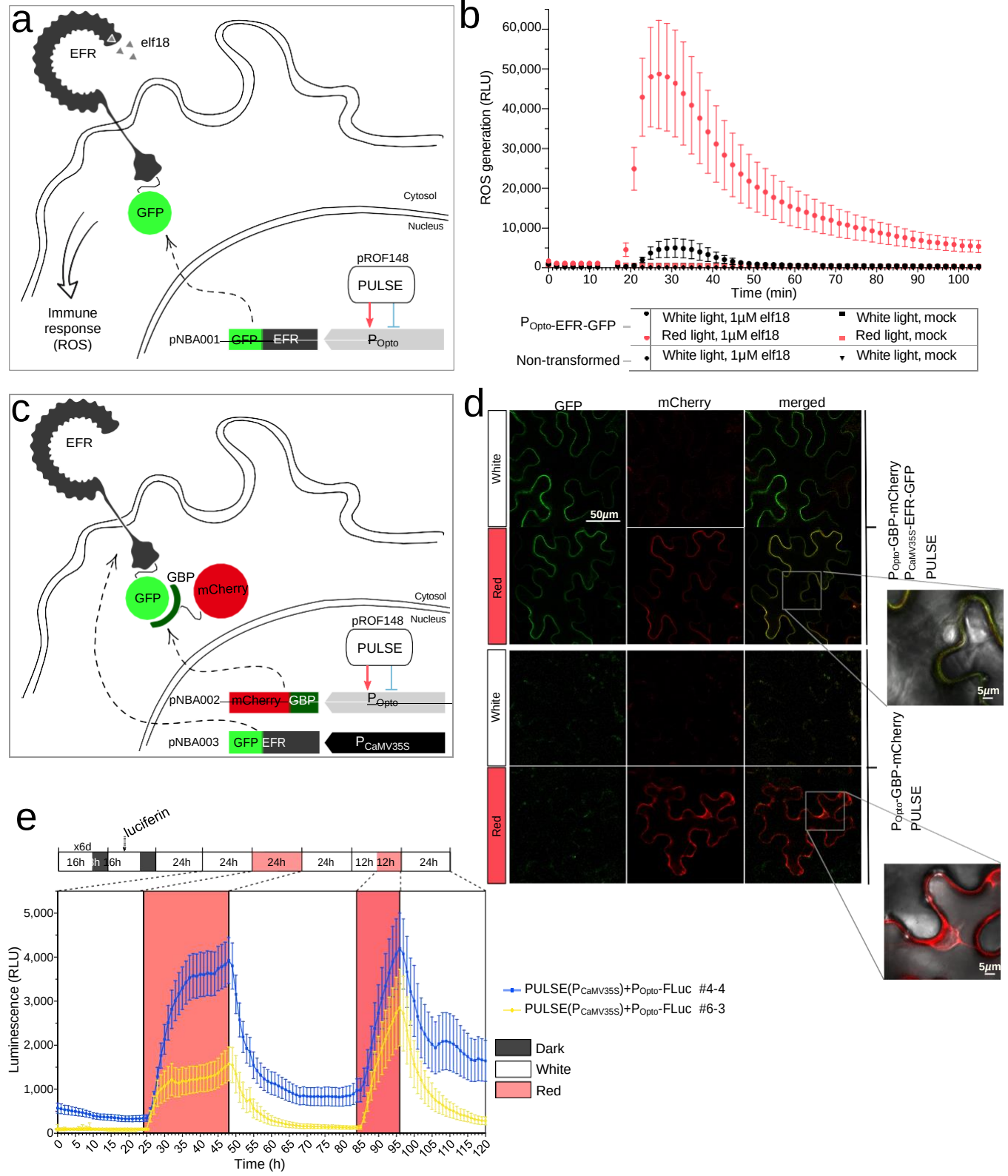
| | Wavelength composition of light | | | Gene expression |
|---|---|---|---|---|
| | Blue | Red | Far-red | |
| W | + | + | + | - |
| B | + | - | - | - |
| R | - | + | - | + |
| FR | - | - | + | - |
| D | - | - | - | - |

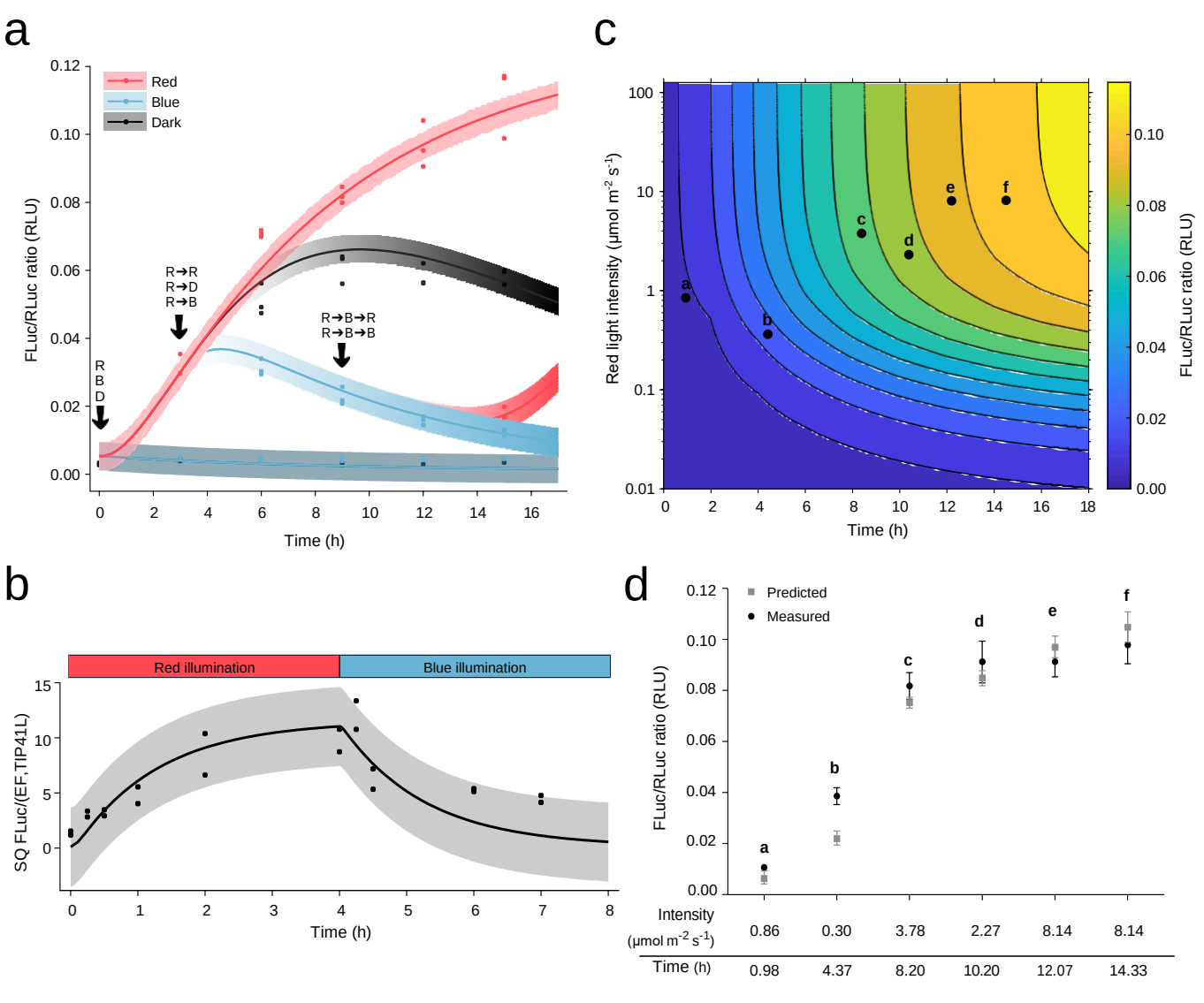

a
DARK
BLUE
On
Off
HTH
LOV
REP
EL222
FLuc
P35S enhancer
(C120)5
PhCMVmin
Blue light-repression module (BOff)
PCaMV35S
REP
NLS
EL222
Tnos
pROF018
pROF050
pROF051
KRAB
BRD
SRDX
Reporter module
pROF402
P35S enhancer
(C120)5
PhCMVmin
FLuc
TSV40
-953
-51
Normalization module
GB0109
PCaMV35S
RLuc
Tnos
b
FLuc/RLuc ratio (RLU)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
2.1 x
6.1 x
12.4 x
Blue light intensities
(µmol m-2 s-1)
0
0.25
0.50
1
5
10
Ø
KRAB-EL222
BRD-EL222
SRDX-EL222


a
BLUE
WHITE
DARK
FAR-RED
RED
Off
Off
On
VP16
SRDX
SRDX
PhyBfr
PIF6
LOV
LOV
EL222
E
HTH
(etr)8 (C120)5 PhCMVmin
FLuc
POpto
PhyBr
HTH
LOV
SRDX
LOV
HTH
SRDX
PIF6
E
(etr)8 (C120)5 PhCMVmin
FLuc
HTH
LOV
SRDX
LOV
HTH
SRDX
PhyBfr
VP16
PIF6
E
FLuc
(etr)8 (C120)5 PhCMVmin
FLuc
b
PULSE
Blue light-repression module (BOff)
pROF051
PCaMV35S
SRDX
NLS
EL222
Tnos
Red light-activation module (ROn)
pMZ827
PCaMV35S
E
PIF6 (1-100)
NLS
Tnos
pMZ828
PCaMV35S
PhyB (1-650)
VP16
NLS
Tnos
Reporter module
POpto
pROF021
(etr)8 (C120)5 PhCMVmin
FLuc
TSV40
Normalization module
GB0109
PCaMV35S
RLuc
Tnos
c
109.2 x
396.5 x
FLuc/RLuc ratio (RLU)
0
1
2
3
4
5
6
7
8
9
10
11
12
Illumination
Red
Blue
White
Dark
Far-red
ROn System
ROn+ BOff System = PULSE


a
PULSE
pROF449
P Opto
dCas9
TV
GB1221
P AtU6-26
gRNA(P SlDFR)
gRNA (P SlDFR)
dCas9
TV
GB1159
P SlDFR
FLuc
Fluc
GB0109
P CaMV35s
RLuc
b
Red
Blue
Dark
FLuc/RLuc ratio (RLU)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
24.5 x
40.0 x
gRNA(P SlDFR)
P Opto-dCas9-TV
P SlDFR-FLuc + PULSE + RLuc
c
PULSE
pROF449
P Opto
dCas9
TV
pROF441
P AtU6-26
gRNA(P AtAP1)
GB0109
P CaMV35s
RLuc
gRNA (P AtAP1)
TV
dCas9
pROF366
P AtAP1
FLuc
FLuc
LFY
VP16
d
PULSE
pROF394
P Opto
LFY
VP16
2A
RLuc
RLuc
e
Red
Blue
Dark
FLuc/RLuc ratio (RLU)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
17.9 x
14.1 x
gRNA(P AtAP1)
P Opto-dCas9-TV
P AtAP1-FLuc + PULSE + RLuc
f
Luminescence (RLU)
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
17.5 x
26.6 x
31.4 x
7.4 x
LFY expression
Red RLuc
Blue RLuc
Dark RLuc
P AtAP1activation
Red FLuc
Blue FLuc
Dark FLuc
P Opto-LFY-VP16-2A-RLuc
P AtAP1-FLuc + PULSE


a
Cerulean
Venus
Merged
Red
Blue
White
Dark
20μm
b
28.7x
Ratio
Venus/Cerulean
****
****
ns
ns
ns
ns
**
**
0
2
6
9
Treatment time (h)


a
EFR
elf18
GFP
Cytosol
Nucleus
pROF148
PULSE
Immune response (ROS)
pNBA001
GFP
EFR
P Opto
b
ROS generation (RLU)
60,000
50,000
40,000
30,000
20,000
10,000
0
0 10 20 30 40 50 60 70 80 90 100
Time (min)
P Opto-EFR-GFP
White light, 1µM elf18
Red light, 1µM elf18
White light, mock
Red light, mock
Non-transformed
White light, 1µM elf18
White light, mock
c
EFR
GBP
GFP
mCherry
Cytosol
pROF148
Nucleus
PULSE
pNBA002
mCherry
GBP
P Opto
pNBA003
GFP
EFR
P CaMV35S
d
GFP
mCherry
merged
White
Red
50µm
P Opto-GBP-mCherry
P CaMV35S-EFR-GFP
PULSE
5µm
White
Red
P Opto-GBP-mCherry
PULSE
5µm
e
luciferin
x6d
16h
16h
24h
24h
24h
24h
12h
12h
24h
Luminescence (RLU)
5,000
4,000
3,000
2,000
1,000
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Time (h)
PULSE(P CaMV35S)+P Opto-FLuc #4-4
PULSE(P CaMV35S)+P Opto-FLuc #6-3
Dark
White
Red


a
Red
Blue
Dark
FLuc/RLuc ratio (RLU)
Time (h)
R
B
D
R→R
R→D
R→B
R→B→R
R→B→B
b
Red illumination
Blue illumination
SQ FLuc/(EF,TIP41L)
Time (h)
c
Red light intensity (μmol m-2 s-1)
Time (h)
FLuc/RLuc ratio (RLU)
d
Predicted
Measured
FLuc/RLuc ratio (RLU)
Intensity (μmol m-2 s-1)
0.86
0.30
3.78
2.27
8.14
8.14
Time (h)
0.98
4.37
8.20
10.20
12.07
14.33