

A quantitative autonomous bioluminescence reporter system with a wide dynamic range for Plant Synthetic Biology

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Summary

Plant Synthetic Biology aims to enhance the capacities of plants by designing and integrating synthetic gene circuits (SGCs). Quantitative reporting solutions that can produce quick, rich datasets affordably are necessary for SGC optimization. In this paper, we present a new, low-cost, and high-throughput reporter system for the quantitative measurement of gene expression in plants based on autonomous bioluminescence. This method eliminates the need for an exogenous supply of luciferase substrate by exploiting the entire *Neonothopanus nambi* fungal bioluminescence cyclic pathway to build a self-sustained reporter. The *HispS* gene, the pathway's limiting step, was set up as the reporter's transcriptional entry point as part of the new system's design, which significantly improved the output's dynamic range and brought it on par with that of the gold standard FLuc/RLuc reporter. Additionally, transient ratiometric measurements in *N. benthamiana* were made possible by the addition of an enhanced GFP as a normalizer. The performance of new NeoLuc/eGFP system was extensively validated with SGCs previously described, including phytohormone and optogenetic sensors. Furthermore, we employed NeoLuc/eGFP in the optimization of challenging SGCs, including new configurations for an agrochemical (copper) switch, a new blue optogenetic sensor, and a dual copper/red-light switch for tight regulation of metabolic pathways.

Keywords: Autonomous bioluminescence, ratiometric reporter system, transcriptional regulation, *Nicotiana benthamiana*.

Introduction

Plant Synthetic Biology (PSB) aims to create plants with augmented capacities by engineering new metabolic pathways and robust synthetic gene circuits (SGCs) operating new-to-nature genetic programs. The development of SGCs can be speeded up by adopting the recursive design-build-test-learn (DBTL) framework (Liu and Stewart, 2015). Quantitative reporter systems are essential in the testing steps of the DBTL cycle (McCarthy and Medford, 2020). Reporter genes act as final circuit actuators, generating easily scoreable readouts of fluorescence, luminescence or absorbance that proxy the transcriptional activity generated by the upstream elements in the circuit, facilitating the acquisition of numeric values that feed mathematical models (Khosla *et al.*, 2020; Wang *et al.*, 2013). In plants, engineering through DBTL cycles is restricted by the lack of experimental platforms that support SGC testing in a fast and high throughput manner. The dual-ratiometric Firefly luciferase (FLuc)/Renilla luciferase (RLuc) reporter (FLuc/RLuc) is, owing to its wide dynamic range, ease of use and low endogenous activity, the current golden standard for SGC evaluation in plants (Schauberg *et al.*, 2016; Vazquez-Vilar *et al.*, 2017). In this system, the promoter transactivation of FLuc serves as a transcriptional input, which is proportionally converted into luminescence readouts by the FLuc enzyme, maintaining a high dynamic range. In parallel, the luminescence of a constitutively-expressed RLuc serves as an output normalizer. Despite its advantages, FLuc/RLuc involves laborious protein extraction, and most importantly, it requires the

external addition of expensive luciferase substrates, limiting its use as a throughput data-rich reporter system in PSB.

The recently discovered bioluminescence pathway from the fungus *Neonothopanus nambi* has opened new possibilities for development of optimal reporter systems in PSB. The fungal bioluminescence pathway (FBP) is a cyclic metabolic route comprising four enzymes with no functional homologues in plants (see Figure 1a). The fungus employs caffeic acid as the substrate of the first committed step in the pathway. Caffeic acid is a common intermediary metabolite in plants; therefore, plant cells can use their own metabolic pool of caffeic acid to feed the FBP autonomously, as it was recently demonstrated (Mitiouchkina *et al.*, 2020). The third enzyme in the pathway, LUZ, is the actual luciferase, converting the metabolite 3-hydroxyhispidin into caffeoylpyruvic acid and releasing a photon in the reaction. The facility for detection and the fact that there is no need to supply exogenously luciferin make FBP a promising reporter for plants (Khakhar *et al.*, 2020). However, to serve as a robust quantitative system in PSB, several improvements and adaptations are required. One of the main caveats lies in the low dynamic range that results from linking circuit transcriptional outputs to the expression of the fungal luciferase gene. Furthermore, in the absence of a second reporter gene, the variations in transfection efficiency cannot be accounted for.

Here we report the development of a wide dynamic range, high-throughput reporter system for fast gene circuit testing based on FBP. This system combines fungal luminescence as model circuit actuator with eGFP as normalising signal and

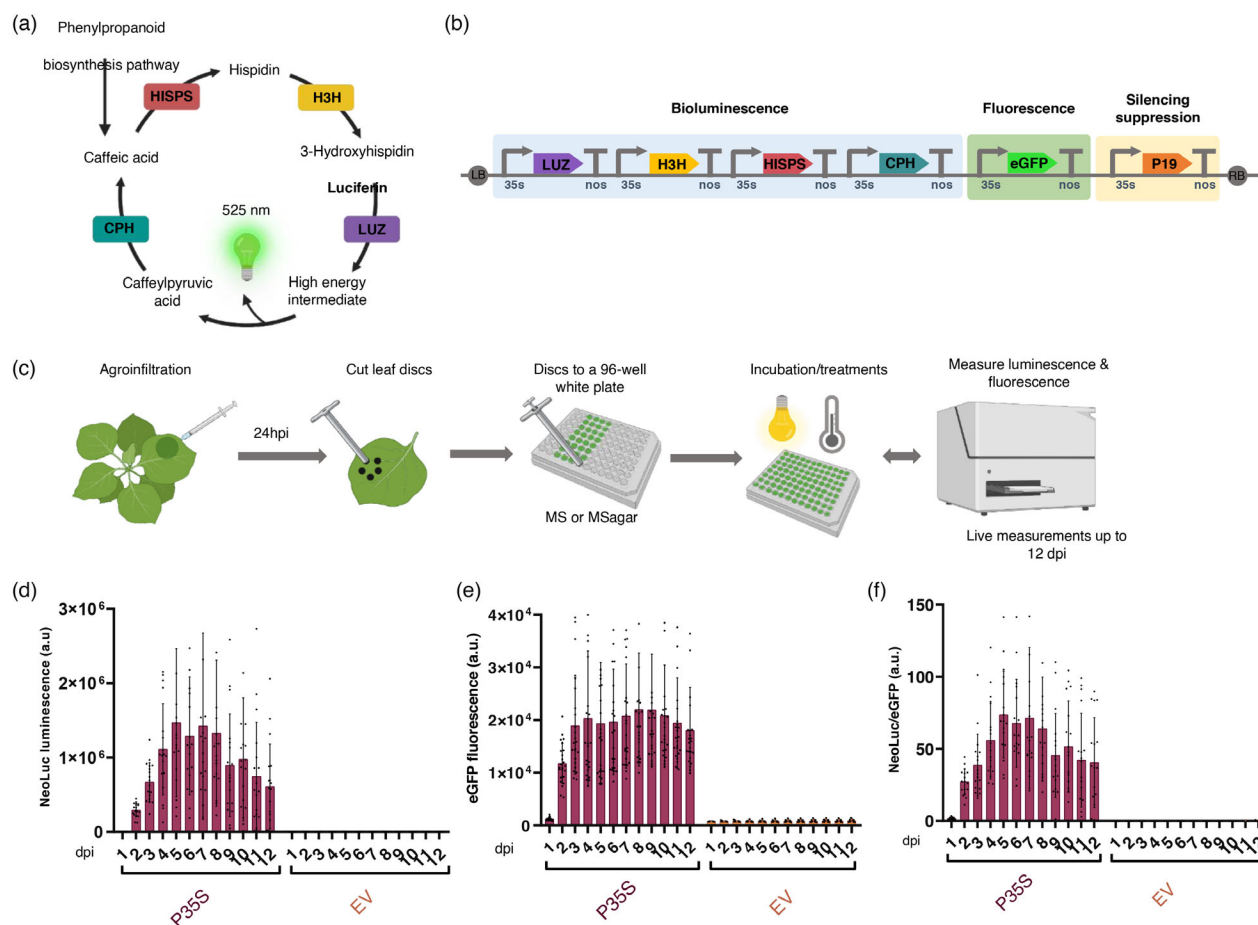


Figure 1 A quantitative bioluminescence reporter system for *N. benthamiana*. (a) Schematic representation of the *N. nambi* fungal bioluminescence pathway (FBP). (b) Scheme of T-DNA construct used for the dual reporter system, including the transcriptional units for the constitutive expression of the FBP genes (*Luz*, *H3H*, *HispS*, *CPH*), the enhanced GFP gene (*eGFP*) and the *p19* silencing suppressor gene. (c) Schematic representation of the experimental procedure used for luminescence and fluorescence measurements. (d) NeoLuc signal (a.u.) and (e) eGFP fluorescence signal (a.u.) of *N. benthamiana* leaf discs transiently expressing the reporter module depicted in (b) (P35S) or an empty vector (EV). (f) NeoLuc/eGFP ratios calculated with the luminescence and fluorescence values plotted in (d) and (e). Error bars indicate SD ($n = 18$).

employs transiently transformed *N. benthamiana* leaf discs as in vivo chassis. A key improvement in this new system is the employ of *HispS* gene, the enzyme catalysing the first committed step in the pathway, as the gene that connects the reporter module with the upstream elements in the circuit. The use of *HispS* instead of *Luz* results in a wider dynamic range of the reporter, up to levels equivalent to the FLuc/RLuc system. The simultaneous measurements of LUZ-driven fungal luminescence (NeoLuc) and green fluorescence (eGFP) showed no interference using standard equipment. By employing this new methodology, we rapidly characterized several SGCs involving hormone and optogenetic sensors. Furthermore, we show the optimization of new copper switches and the *de novo* development of a dual-input control circuit for metabolic pathways.

Materials and methods

GoldenBraid cloning

DNA constructs used in this work were assembled using GoldenBraid (GB) (Sarrion-Perdigones *et al.*, 2013). Coding sequences of the *N. nambi* bioluminescence genes *Luz*, *H3H*,

CPH and *HispS* previously codon optimized for expression in *N. tabacum* by Mitiouchkina *et al.* (2020) were ordered as gBlocks™ from IDT and domesticated at www.gbcloning.upv.es/do/domestication/. Level 0, Level 1 and Level > 1 assemblies were performed using GB as previously described (Sarrion-Perdigones *et al.*, 2013). Level 0 assemblies were confirmed by restriction analysis and Sanger sequencing, and Level 1 and Level > 1 assemblies were verified by restriction analysis. All plasmids used in this work are listed in Table S1.

N. benthamiana transient expression

For transient expression assays, plasmids were transferred to *A. tumefaciens* strain GV3101 or EHA105 (see Table S1 for details). Five to six weeks old *N. benthamiana* plants grown at 24 °C and 16 h (light)/20 °C and 8 h (darkness) conditions were used. For agroinfiltration, overnight *A. tumefaciens* cultures were pelleted and resuspended in agroinfiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂ and 200 μM acetosyringone) to an optical density of 0.1 at 600 nm (OD₆₀₀). Bacterial suspensions were incubated for 2 h at room temperature on a horizontal rolling mixer, and then mixed for co-expression experiments, in which more than

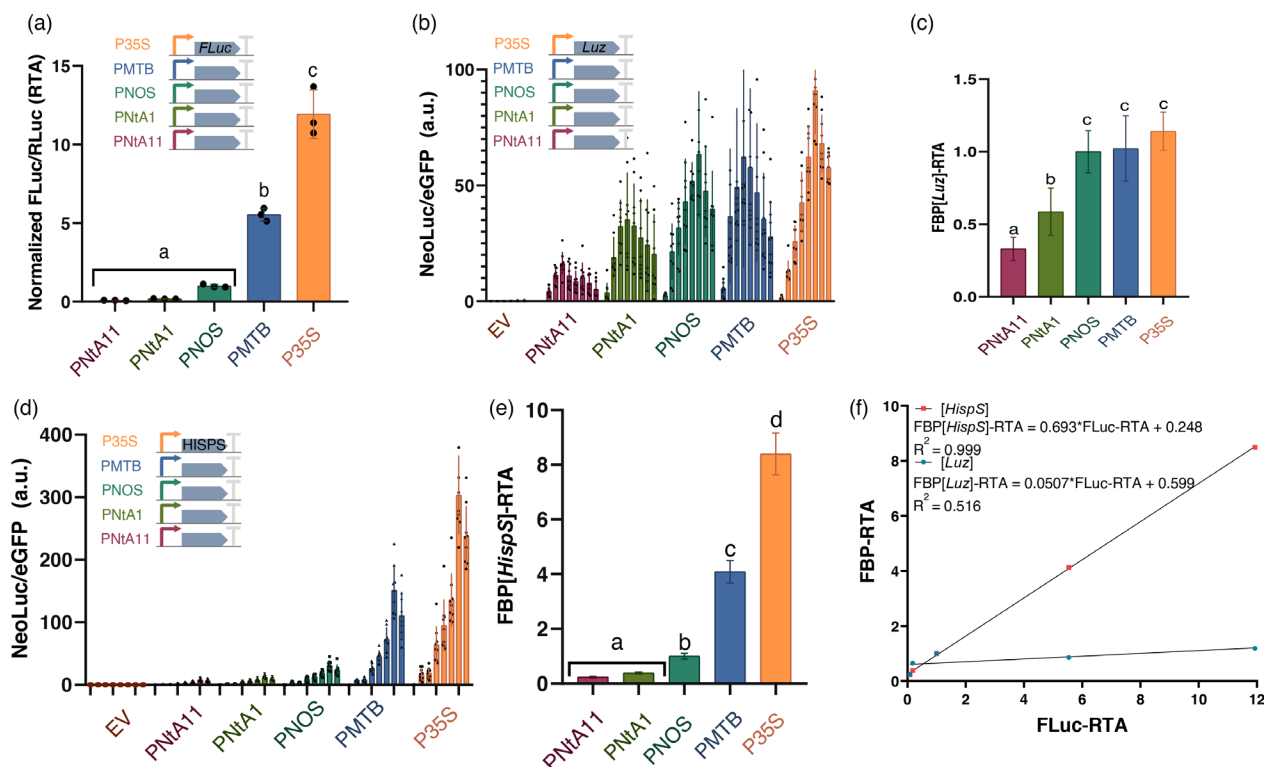


Figure 2 Optimization of the FBP/eGFP reporter employing five constitutive promoters. (a) Relative transcriptional activities (RTA) of four different constitutive promoters (PNTA11, PNTA1, PMTB and P35S) transiently expressed in *N. benthamiana*, calculated as FLuc/RLuc ratios for each promoter and normalized with the FLuc/RLuc ratios conferred by the constitutive PNOS promoter. (b) NeoLuc/eGFP ratios and (c) FBP[Luz]-RTA values of *N. benthamiana* leaf discs transiently expressing the FBP reporter with the *Luz* gene driven by four different constitutive promoters. (d) NeoLuc/eGFP ratios and (e) FBP [HisP]-RTA values of *N. benthamiana* leaf discs transiently expressing the FBP reporter with *HisP* gene driven by four constitutive promoters. (f) Correlation of normalized FLuc/RLuc ratios (FLuc-RTA) depicted in (a) with FBP[Luz]-RTA and FBP[HisP]-RTA values depicted in (c) and (e), respectively. An empty vector (EV) was used as a negative control in all experiments. Error bars indicate SD (a, b and d) and SE (c and e); $n = 9$. Statistical analyses were performed using One-way ANOVA (Tukey's multiple comparisons test, P -value ≤ 0.05). Variables within the same statistical groups are marked with the same letters.

one GB element was used. Finally, agroinfiltrations were carried out through the abaxial surface of the three youngest fully expanded leaves of each plant with a 1-mL needle-free syringe. Detailed information of the experimental design can be found in the Methods S1 section.

NeoLuc luminescence and eGFP fluorescence measurements and calculation of relative transcriptional activity

Leaf disc samples were collected at 24 h post infiltration using a cork borer with a diameter of 0.5 cm. Two or three discs per agroinfiltrated leaf were excised. Leaf discs were transferred to a white 96-well microplate containing sucrose-free MS agar (or liquid) and incubated at 16 h light/8 h dark, 25 °C, 60%–70% humidity, 250 $\mu\text{mol}/\text{m}^2/\text{s}$ photons. Luminescence and fluorescence were recorded on each leaf disc every 24 h (from 1 to 8–12 dpi). All measurements were carried out with a GloMax®-Multi Detection System (Promega). *N. nambi* luciferase (NeoLuc) activity was determined with the luminescence module using an integration time of 10 s. eGFP fluorescence was determined using the blue optical kit (Ex: 490 nm, Em: 510–570 nm). NeoLuc/eGFP ratios were calculated for each leaf disc at each timepoint. The FBP[Luz]-RTA and FBP[HisP]-RTA values were calculated by dividing the area under the curve (AUC) of the

NeoLuc/eGFP ratios produced by each sample from 2 to 8 dpi by the AUC produced by a PNOS reference calculated in an equivalent manner and assayed in parallel. The AUC were calculated using GraphPad with the default parameters. GB4148 (PNOS:Luz) was used for normalization of data displayed in Figure 2c, GB4807 (PNOS:HisP) was used for normalization of data displayed in Figure 6g and GB4401 (PNOS:HisP) was used for normalization of the rest of the experiments in this work.

FLuc and RLuc activity assays and calculation of relative transcriptional activity

For the FLuc/RLuc assay, leaf samples were collected at 4 dpi. One 0.8 cm diameter disc per agroinfiltrated leaf was excised using a cork borer. Leaf discs were frozen in liquid nitrogen and subsequently homogenized. The Dual-Glo® Luciferase Assay System (Promega) was used for sample analysis. Briefly, 180 μL of Passive Lysis buffer were added to the homogenized sample, vortexed and centrifuged for 15 min at 14 000 g at 4°C. Crude extract (10 μL) was mixed with 40 μL of LARII and FLuc activity was determined using a GloMax®-Multi Detection System (Promega) with a 2 s delay and a 10 s integration times. After the measurements, 40 μL of Stop&Glo reagent were added per sample and RLuc activity was determined using the same protocol. Sample FLuc/RLuc ratios were calculated as the average

value of the three independent agroinfiltrated leaves. Relative transcriptional activities (RTAs) were calculated as previously described (Vazquez-Vilar *et al.*, 2017). Briefly, the FLuc/RLuc ratios in each sample normalized with the FLuc/RLuc ratios produced by a PNOS reference (GB1116) assayed in parallel.

Sample size and statistical analysis

The sample number and statistical analysis considerations for each experiment are indicated in the corresponding figure. For FLuc/RLuc assays, three biological replicates at a single timepoint were analyzed. For NeoLuc/eGFP determination, at least five biological replicates and 7 to 11 timepoints were analyzed. Statistical analyses were performed using One-way ANOVA (Tukey's multiple comparisons test, P -Value ≤ 0.05). Plotting and statistic tests were all performed with GraphPad.

N. benthamiana stable transformation

For *N. benthamiana* stable transformation, *A. tumefaciens* LBA4404 was used. Transformation was performed following a standard protocol. Briefly, fully expanded leaves of *N. benthamiana* were sterilized with 5% commercial bleach (40 g of active chlorine per litre) for 10 min followed by four consecutive washing steps with sterile demi-water. Leaf discs ($d = 0.8$ cm) were cut with a cork borer and incubated overnight in co-culture plates [4.9 g/L Murashige and Skoog medium (MS) supplemented with vitamins (Duchefa), 3% sucrose (Sigma-Aldrich), 0.9% Phytoagar (Duchefa), 1 mg/L BAP (Sigma-Aldrich), 0.1 mg/L NAA (Sigma-Aldrich), pH 5.7]. Leaf discs were incubated for 15 min with the *Agrobacterium* culture ($OD_{600} = 0.3$). Then, the discs were returned to the co-culture plates and incubated for 2 days in darkness. Next, discs were transferred to selection medium [4.9 g/L MS supplemented with vitamins (Duchefa), 3% sucrose (Sigma-Aldrich), 0.9% Phytoagar (Duchefa), 1 mg/L BAP (Sigma-Aldrich), 0.1 mg/L NAA (Sigma-Aldrich), 500 mg/L carbenicillin, 100 mg/L kanamycin, pH 5.7]. Discs were transferred to fresh medium every 7 days until shoots appeared (4–6 weeks). Shoots were cut and transferred to rooting medium [4.9 g/L MS supplemented with vitamins (Duchefa), 3% sucrose (Sigma-Aldrich), 0.9% Phytoagar (Duchefa), 500 mg/L carbenicillin, 100 mg/L kanamycin, pH 5.7] until roots appeared. Growing conditions were in all steps 16 h light/8 h dark, 25 °C, 60%–70% humidity, 250 $\mu\text{mol}/\text{m}^2/\text{s}$ photons.

Results

Setting up an easy-to-use quantitative bioluminescence reporter system

To establish the basis for a dual reporter system, we first assembled a T-DNA containing the transcriptional units (TUs) for the 35SCaMV-driven constitutive expression of the four genes of the pathway (*Luz*, *H3H*, *CPH*, *HispS*) together with the silencing suppressor P19 and an enhanced GFP (eGFP) reporter (Figure 1b). When agroinfiltrated in *N. benthamiana* leaves, eGFP showed a strong normalizing signal with minimal interference with *N. nambii* LUZ emission and therefore this construct was employed in the search for high throughput experimental procedures that minimized sample manipulation while producing consistent NeoLuc and eGFP measurements. We opted for a process consisting in the agroinfiltration of the FBP/eGFP module in intact *N. benthamiana* leaves followed, after 24 h, by the excision and transfer of the leaf discs to 96-well plates containing sucrose-free MS medium (Figure 1c). Next, dual NeoLuc/eGFP measurements

were performed in plates from 1 dpi onwards using standard luminometer equipment. Leaf disks showed measurable expression levels up to 12 dpi (Figure 1d–f). Based on these results, we built a FBP/eGFP reporter module, initially setting the *Luz* gene under the control of the query promoter, as previously described by Khakhar *et al.* (2020). We compared then the performance of this initial configuration (referred as FBP[*Luz*]/eGFP), with the standard dual FLuc/RLuc system employing five constitutive promoters covering a wide range (~150 fold) of relative transcriptional activities (RTAs): the 35SCaMV promoter (P35S), the *Solanum lycopersicum* metallothionein-like protein type 2B promoter (PMTB), the Nopaline Synthase promoter (PNOS) and the promoters of the genes Nitab4.5_0000037g0360.1 and Nitab4.5_0006225g0030.1 of *Nicotiana tabacum* cv. K326 (named as PNTA1 and PNTA11, respectively). In the standard FLuc/RLuc system, RTA is calculated by normalizing FLuc/RLuc output ratios of a problem circuit with those provided by aPNOS in pre-defined standard experimental conditions (see Figure 2a). To facilitate comparisons between the two reporter systems, we calculated for each promoter the area under the curve (AUC) of the NeoLuc/eGFP values measured through the time course of the experiment (Figure 2b), and normalized this value against the AUC generated by the PNOS promoter, obtaining the final FBP[*Luz*]-RTA values. This parameter was thereafter employed to represent the overall transcriptional output of each SGCs. Unfortunately, as can be observed by comparing Figure 2a,c, there was a clear saturation in the FBP[*Luz*]-RTA values that severely affected stronger promoters, thus limiting the applicability of the reporter system in its current configuration.

We reasoned that the observed signal saturation could be due to the choice of *Luz* as the connection point for the transcriptional output, as we earlier determined that the *HispS* gene and to a lesser extent the *H3H* gene, are the transcriptionally limiting steps in the pathway (Moreno-Giménez *et al.*, 2022; see Figure S1). We therefore changed the reporter conformation, setting the query promoter position driving *HispS* expression instead of *Luz* (referred to as FBP[*HispS*]/eGFP configuration). Using this new setup, signal saturation was eliminated for stronger promoters (Figure 2d), thus generating FBP[*HispS*]-RTA values that showed a strong lineal correlation ($R^2 = 0.9997$) to the classical RTA data observed with FLuc/RLuc system, also for strong promoters (Figure 2e,f). In general, we observed that FBP[*HispS*]-RTA estimations are higher than FLuc/RLuc for weak promoters (PNTA11, PNTA1), probably reflecting a higher sensitivity, but somehow lower for stronger promoters, possibly due to some remaining signal saturation. Given the strong lineal correlation between the two systems, a conversion factor of 0.7 could be applied as a rule of thumb for comparisons of strong promoters if required. Although the sensitivity of FBP[*HispS*]-RTA in the *N. benthamiana* agroinfiltration system seems sufficient to cover the whole range of promoter strengths usually employed in PSB, we also assayed the introduction of a fifth enzyme, NPGA, known to activate HISPS (Khakhar *et al.*, 2020). The co-transformation with NPGA significantly increased the NeoLuc/eGFP values for all promoters assayed (Figure S2), offering additional sensitivity, if required.

Phytohormone sensors with the FBP[*HispS*]/eGFP reporter

As a first immediate application of the new reporter, we designed new circuits to detect changes in phytohormone concentrations in the incubation media. A first device was set up to respond to

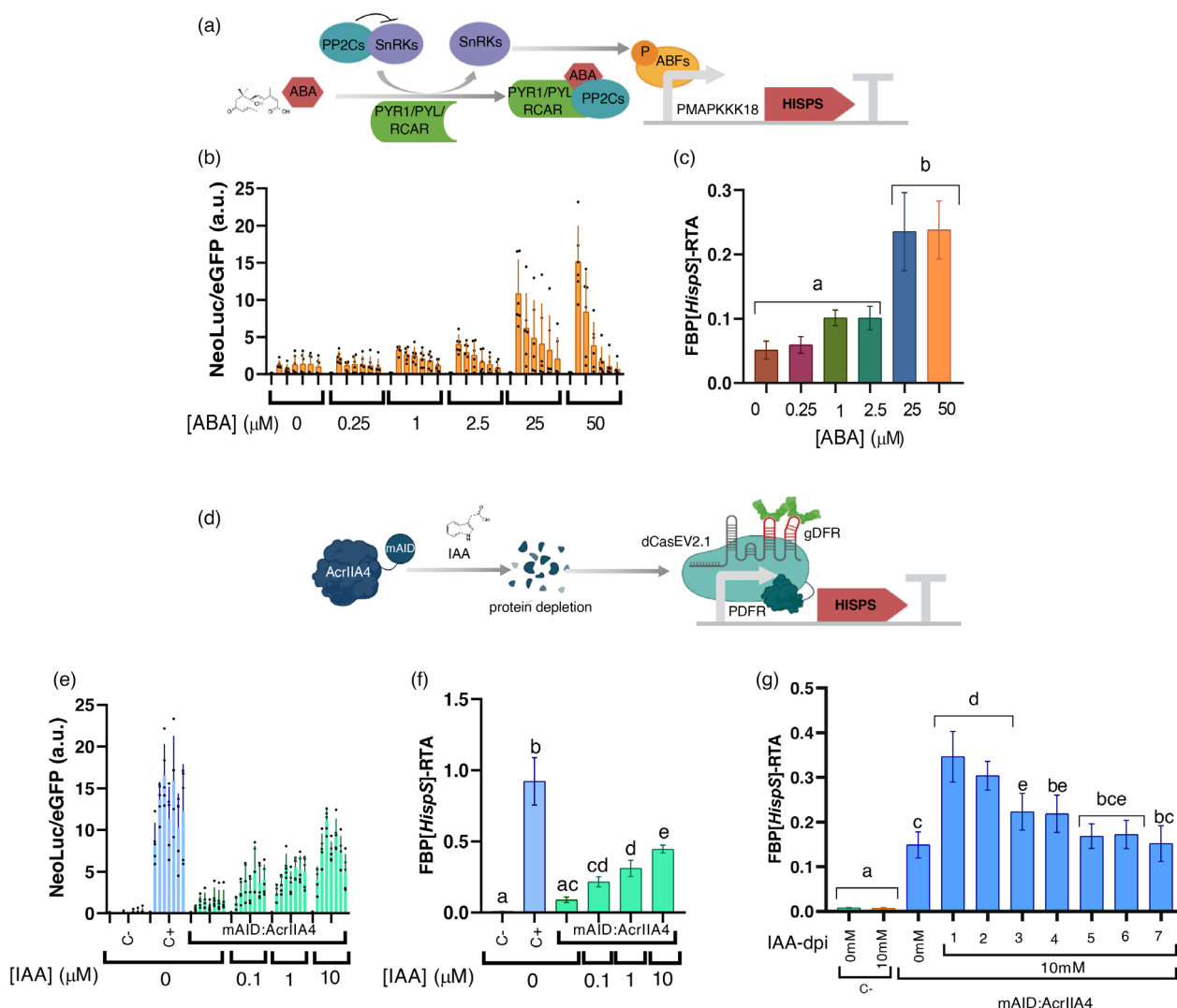


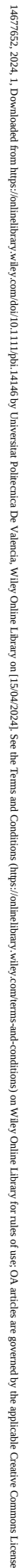
Figure 3 Phytohormone sensors with the FBP[Hisps]/eGFP reporter. (a) Schematic representation of *Hisps* gene activation driven by the ABA inducible promoter P_{MAPKKK18} (ABA sensor). (b) Neoluc/eGFP ratios and (c) FBP[Hisps]-RTA values of the ABA sensor in *N. benthamiana* leaf discs treated with different ABA concentrations. (d) Schematic representation of an IAA mediated AcrIIA4 protein depletion switch for the regulation of the dCasEV2.1-mediated reporter activation. (e) Neoluc/eGFP ratios and (f) FBP[Hisps]-RTA values of the IAA-mediated depletion switch in *N. benthamiana* leaves expressing dCasEV2.1 with an unspecific gRNA (C−) or the specific gRNA gDFR without (C+) or with mAID:AcrIIA4 treated with different IAA concentrations. (g) FBP[Hisps]-RTA values of the IAA-mediated depletion switch in *N. benthamiana* leaves expressing dCasEV2.1 with an unspecific gRNA (C−) or the specific gRNA gDFR with mAID:AcrIIA4. Samples were either left untreated (0 mM), or treated with 10 mM IAA at different time points. Error bars indicate SD (b and e) and SE (c, f and g); $n = 6$ for (b, c and g) and $n = 5$ for (e, f). Statistical analyses were performed using one-way ANOVA (Tukey's multiple comparisons test, P -value ≤ 0.05). Variables within the same statistical groups are marked with the same letters.

increasing ABA concentrations using the plant endogenous hormone receptor components and an ABA-responsive promoter (p_{MAPKKK18}) driving the expression of *Hisps* (Figure 3a). As shown (Figure 3b,c), this simple sensor detected the presence of ABA with high confidence at concentrations above 25 μM . A second, more sophisticated and fully synthetic phytohormone detection system was next established for auxin sensing. In this design, we employed an anti-Cas protein, AcrIIA4, fused to the auxin degron mAID. The mAID:AcrIIA4 fusion functioned as a negative regulator of the CRISPR-based transcriptional activation (CRISPRa) mediated by the previously described dCasEV2.1 programmable transcription factor (Selma et al., 2019). dCasEV2.1 strongly activates *Hisps* by binding the PDFR promoter that contains a target site for a specific gRNA (gDFR) (Figure 3d).

dCasEV2.1 activity is strongly repressed by mAID:AcrIIA4, but in the presence of IAA, mAID:AcrIIA4 is degraded, releasing the activation of the transcriptional signal. As shown in Figure 3e,f, a concentration of 0.1 μM IAA in the medium is sufficient to produce significant changes in FBP[Hisps]-RTAs values. We also determined that the excised leaf disks retain the capacity to respond to the IAA up to 2 dpi (Figure 3g and Figure S3). At later time points, the system's responsiveness to the hormone is sharply lost.

Diversifying copper switches in plants using the FBP/eGFP reporter system

Repurposing agrochemicals such as copper sulphate into signaling molecules is an attractive option in PSB since agrochemicals



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different configurations of a copper switch taking advantage of the new data-rich FBP[*HispS*]/eGFP reporter. In its simplest configuration, the copper-responsive processor comprised a synthetic promoter containing CBS and the minimal promoter of the DFR gene (mDFR). As reflected in Figure 4b and Figure S4a, copper-dependent transcriptional activation was detected for this circuit at all assayed concentrations (from 0.1 to 5.0 mM), however the best activations were observed at the lowest concentration. These results suggest certain toxicity effects of CuSO₄, something consistent with the development of necrotic symptoms and the lower levels of fluorescence recorded for the samples at the two highest tested concentrations (Figure S4b). However, at 0.1 and 0.5 mM CuSO₄, fluorescence levels are maintained over time and are equivalent to those of the control treatment indicating the viability of the leaf discs in these conditions in the timeframe of the experiment (Figure S4b). The system responsiveness to copper was maintained for 4 dpi, although after the first 24 h responsiveness declines sharply (Figure 4c and Figure S4c). The second configuration assayed comprised an intermediary step involving dCasEV2.1 activation. The involvement of dCasEV2.1 (CRISPRa) provides two potential advantages to the copper switch. On one hand it enables multiplexing of downstream targets, and on the other hand, it offers additional control points, since the expression of all three components comprising the programmable activator (the gRNA, dCas9:EDLL and the MS2:VPR fusion, see Figure 4d) can be regulated, jointly or independently, by one or more triggers. To reinforce copper control of the switch, we introduced a configuration where both the dCas9:EDLL and the MS2:VPR are regulated by copper (Figure 4d). This regulation is important because it has been earlier observed that the introduction of intermediary CRISPRa elements raise background expression levels as side effect (Garcia-Perez *et al.*, 2022). As can be observed in Figure 4e, by introducing the new two copper control points, we obtained a CRISPRa-mediated copper switch with negligible background expression levels and with a dose–response curve similar to that exhibited by the simple (not multiplexable) switch (see also Figure S4d).

Finally, we used FBP[*HispS*]/eGFP to study a new copper switch in which we introduced an ‘expression lock’ mediated by a phage recombinase. For this, we inserted a terminator (OCS) within the mDFR promoter driving *HispS* expression, thus interrupting any residual activity conferred by the upstream CBS operator. The terminator was flanked by site-specific recombinase sites and therefore it was removable upon the presence of the recombinase, following a design earlier proposed by Lloyd *et al.* (2022). As depicted in Figure 4f, we generated two alternative versions of the OCS-mDFR ‘locked’ promoter, containing recombination sites for either the PhiC31 Integrase (att sites) or the *S. cerevisiae* Flipase (FLP), (FRT sites). The copper sensing module comprising the CUP2:Gal4 transactivator was functionally linked to the FBP/eGFP actuator through the ‘locked’ promoter and was co-agroinfiltrated either with a constitutively expressed recombinase (single copper ‘key’ configuration) or with a copper-controlled one (double ‘key’ configuration). As shown in Figure 4g and Figure S4e, only when all elements of the circuit are present, and in the occurrence of copper sulphate, bioluminescence is produced. The FLP single and double ‘key’ circuits produced output RTAs comparable to those produced by the simplest ‘unlocked’ circuit, with lower background levels on average. Single and double key configurations of the PhiC31 version of the lock also maintained low background levels but the overall RTA

levels in the presence of copper were significantly lower. For both types of SGCs, no significant differences were observed between single and double key configurations.

Dual chemical-optogenetic control of the FBP

We next decided to explore new control mechanisms in plants that integrate two independent inputs, namely a chemical and an optogenetic signal. Rather than designing an AND logic gate as described elsewhere (Brophy *et al.*, 2022; Khan *et al.*, 2022), we took advantage of the fact that FBP is a metabolic pathway to assay an *in-series* control mechanism where each input signal controls a different step in the pathway. First, we built and assayed two different optogenetic control circuits. The blue light (BL) sensor, assayed in plants for the first time, relies on the interaction of *A. thaliana* CRY2 and CIB1 proteins with BL. By linking the *E. coli* Etr DNA-binding domain (EBD) to one of the proteins (CRY2:EBD) and an activation domain to the second interactor (CIB1:VPR), activation of FBP[*HispS*] only occurs in the presence of BL (Figure 5a). Similarly, in the red-light (RL) sensor earlier characterized in plants (Ochoa-Fernandez *et al.*, 2020), PIF6:EBD and PHYB:VP16 interact upon illumination with RL, activating transcription of downstream genes (Figure 5b). Both circuits were assayed in two alternative configurations, each one using a different minimal promoter upstream the *HispS* gene (mDFR or mCMV). As can be observed when comparing Figure 5c,d with Figure 5e,f, the behaviour of the RL circuit was superior to the BL both in terms of background expression and in terms of maximum expression levels. In both systems, the employ of the minimal DFR promoter resulted in larger induction rates. The remarkable performance of the RL/mDFR circuit prompted us to employ it in combination with the single copper switch described in Figure 4a to create the final double-input auto-bioluminescent circuit. To this end, we generated two FBP reporter modules containing (i) the *HispS* gene driven by the copper responsive promoter and (ii) the *H3H* gene driven by the RL responsive promoter, and vice versa (Figure 5g). *N. benthamiana* plants were then co-infiltrated with agrobacterium cultures containing the FBP reporter module plus either one or both sensing modules (PIF-PHYB for RL or CUP2 for copper). Excised discs were subsequently placed onto MS 96-well plates with or without 0.1 mM CuSO₄ and incubated in red light or in darkness. As observed in Figure 5h,i, bioluminescence was only detected in the presence of both sensor modules and in RL/copper combined treatments. In all other circumstances, autonomous bioluminescence remained in an OFF state, with very low basal levels. Configuration II, linking *H3H* with copper and *HispS* with RL modules, respectively, resulted in significantly higher RTA levels, while maintaining similar background expression in single-input treatments.

Generation of stable reporter plant lines for FBP[*HispS*]/eGFP

In the pursuit of reporter system simplification, we decided to generate stable transgenic *N. benthamiana* plants carrying three (*H3H*, *Luz* and *CPH*, named as FBPΔ*HispS*) (Figure 6a) or two (*CPH* and *Luz*, named as FBPΔ*HispS*Δ*H3H*) (Figure 6b) of the four genes of the *N. nambi* luminescence metabolic pathway. Transformed plants were screened by transiently expressing the missing pathway genes plus the eGFP. As observed in Figures 6c,d, several positive plants were recovered in which both NeoLuc and eGFP were readily detected. As a negative control, the same plants were infiltrated with an empty vector (EV) showing

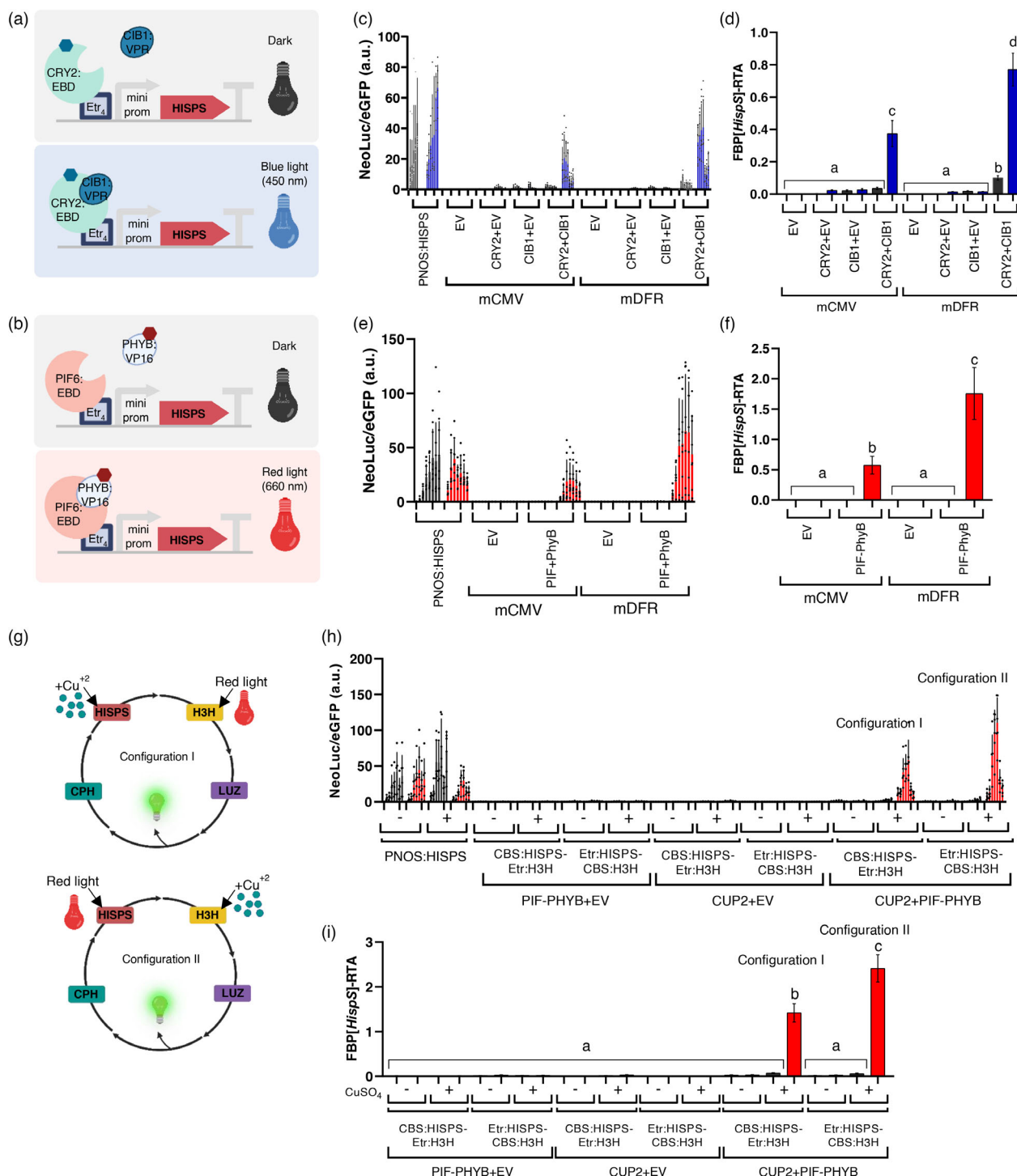


Figure 5 Dual optogenetic and chemical control of the fungal bioluminescence pathway (FBP). (a) Schematic representation of the CRY2:EBD-CIB1:VPR blue light (BL)-dependent *HispS* activation. (b) Schematic representation of the PIF6:EBD-PHYB:VP16 red light (RL)-dependent *HispS* activation. (c) NeoLuc/eGFP ratios and (d) FBP[*HispS*]-RTA values of the BL switch depicted in (a) expressed in *N. benthamiana* leaves, with or without CRY2:EBD and CIB1:VPR. Two minimal promoters driving *HispS* were assayed: mDFR and mCMV. Blue bars are BL treatments; black bars are dark treatments. (e) NeoLuc/eGFP ratios and (f) FBP[*HispS*]-RTA values of the RL switch depicted in (b) assayed in *N. benthamiana*, with or without PIF6:EBD and PHYB:VP16. Red bars are RL treatments; black bars are dark treatments. (g) Schematic representation of the two alternative configurations assayed for the dual RL and copper control of the FBP. (h) NeoLuc/eGFP ratios and (i) FBP[*HispS*]-RTA values of the circuits depicted in (g) in Configurations I and II. (–) are water treatments and (+) represents 0.1 mM CuSO₄ treatments. CBS:HisSp-Etr:H3H represents Configuration I of the circuit. Etr:HisSp-CBS:H3H represents Configuration II. CUP2 + PIF-PHYB are samples containing the entire circuit. PIF-PHYB+EV and CUP2 + EV represent control samples where one of the sensor modules was missing and substituted by an empty vector. PNOS:HisSp is a normalizing control. EV samples were agroinfiltrated with an empty vector. Error bars indicate SD (c, e and h) and SE (d, f and i); $n = 9$ for (c), (d), (e) and (f) and $n = 6$ for (h) and (i). Statistical analyses were performed using one-way ANOVA (Tukey's multiple comparisons test, P -value ≤ 0.05). Variables within the same statistical groups are marked with the same letters.

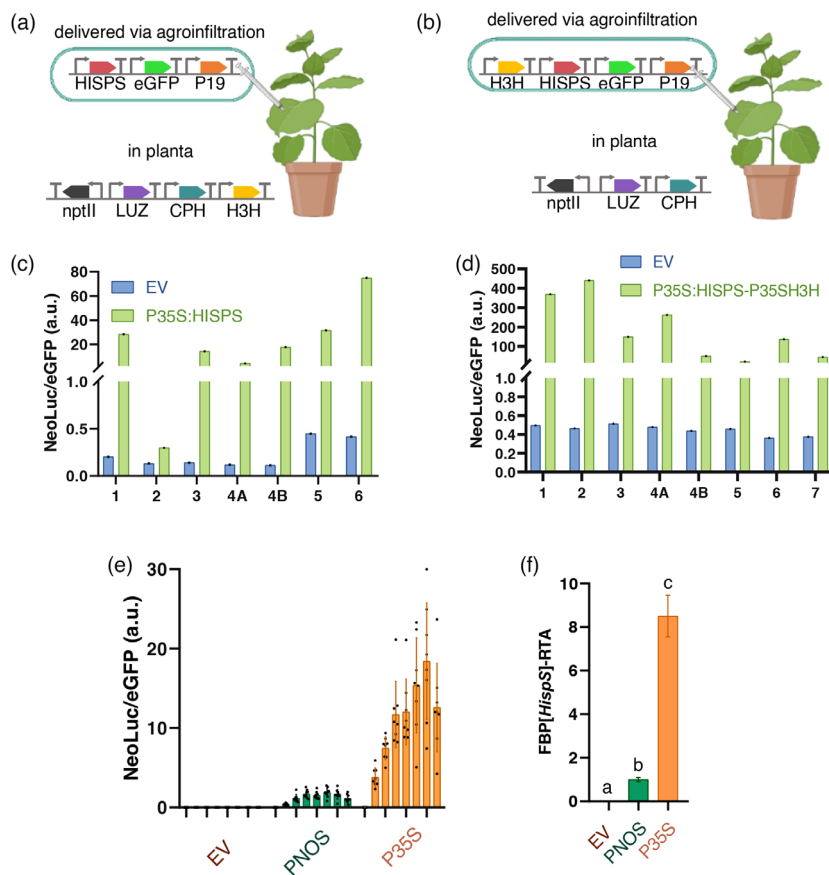


Figure 6 *N. benthamiana* plants expressing components of the FBP reduce the size of the plasmids delivered via agroinfiltration. Schematic representation of the FBP genes incorporated in the plant and those delivered via agroinfiltration in (a) the [ΔHISPS] plants and (b) the [ΔHISPS, ΔH3H] plants. NeoLuc/eGFP ratios of (c) seven independent T0 [ΔHISPS] and (d) eight independent T0 [ΔHISPS, ΔH3H] *N. benthamiana* plants transiently expressing either an empty vector (EV) or the missing components of the FBP reporter in each plant. (e) NeoLuc/eGFP ratios and (f) FBP[*HispS*]-RTA values of T0 [ΔHISPS] line 1 transiently expressing either an EV or the *HispS* gene driven by the PNOS or P35S promoters. Error bars indicate SD (e) and SE (f): $n = 9$. Statistical analyses were performed using one-way ANOVA (Tukey's multiple comparisons test, P -value ≤ 0.05). Variables within the same statistical groups are marked with the same letters.

negligible levels of luminescence and fluorescence. NeoLuc/eGFP levels were up to 10 times higher in the FBPΔHispSΔH3H configuration, which could be related to the number of T-DNA copies per cell delivered via agroinfiltration. We next calibrated the FBPΔHispS line #1 (which delivered the best luminescence levels) by comparing NeoLuc/eGFP readings of P35S and PNOS promoters driving *HispS*. As observed in Figure 6e,f, the P35S/PNOS ratios in *HispS*-complemented FBPΔHispS plants were very similar to those observed with the same promoters when all FBP genes are delivered transiently (P35S RTA = 7.62 ± 0.78 for all-transient system and 7.19 ± 1.06 for FBPΔHispS plants). This indicates that the stable integration of the final enzymes in the pathway is a suitable strategy to alleviate the cargo of the transiently expressed modules while preserving the dynamic range of the reporter system.

Discussion

One of the engineering principles in Synthetic Biology consists in advancing innovations through the design–build–test–learn (DBTL) cycle, a recursive loop used to develop new synthetic biological devices. Plant genetic engineers are progressively adopting DBTL paradigm despite the many plant-specific

experimental obstacles. In the last decade, building DNA constructs was the main bottleneck in the cycle, however modular cloning methods have enormously facilitated multigene SGCs building (Marillonnet and Grütznér, 2020), moving the constraints to the testing steps. Here, we have developed a new auto-bioluminescence/fluorescence reporter system that can significantly contribute to bypass this second DBTL bottleneck. We demonstrate that, by linking the circuit transcriptional outputs to *HispS*, and by including eGFP as normalizer, the FBP/eGFP reporter overcomes the limitations of previously described FBP reporters (Khakhar *et al.*, 2020) and serves as a reliable and highly sensitive alternative for FLuc/RLuc in SGC characterization. The sensitivity of the FBP/eGFP reporter for low-strength promoters could be further improved by incorporating NPGA and its precision for quantification could be upgraded by using polyketide synthases that are independent of NPGA.

A potential constrain for the use of FBP as reporter is the dependence on the plant's endogenous metabolic status, in particular the intracellular concentration of caffeic acid. Depletion of the cell's caffeic acid pool is unlikely, as FBP is a cyclic pathway and this metabolite is recycled. However, fluctuations in substrate availability, or uncharacterized feedback mechanisms may occur, and will need to be considered in experimental designs. Actually,

substrate availability issues are not exclusive of FBP, as differential tissue permeability also affects luminescent reporters in other in vivo systems where the substrate luciferin needs to be added exogenously (Shinde *et al.*, 2006). However as shown extensively in this work, the interferences of the plant's metabolic status, if any, can be easily neutralized by a careful standardization of the testing procedures in the *N. benthamiana* agroinfiltration process, as proposed earlier (Vazquez-Vilar *et al.*, 2017). *N. benthamiana* leaf agroinfiltration has gained great popularity among plant biotechnologists, and it is also the system of choice for plant-based bioproduction at industrial level (COVID vaccines grow on leaves, 2021). Furthermore, the rich multi-omic resources made available recently completes the requirements for *N. benthamiana* to become a standard chassis for fast DBTL engineering (Kurotani *et al.*, 2023; Ranawaka *et al.*, 2022), without precluding the adaptation of FBP/eGFP to other experimental systems as tobacco/*Arabidopsis* protoplasts or seedlings (Furuhata *et al.*, 2020).

In the experiments described here we have generated a total of 1500 experimental points to characterize nine different plant SGCs, employing significantly less effort and much less resources than with any previous reporter system. For convenience, we have used AUC to condense the time-course data points in a single RTA value. We consider this parameter much more informative of promoter/circuit transcriptional activity than single time-point measurements usually offered in FLuc/RLuc experiments. Furthermore, by recording circuit behaviour as time-course, any abnormal behaviour attributed, for instance, to circadian clock interferences, can be easily accounted for and eventually corrected. Some of the SGCs described here recapitulated previous designs as proof of the performance of FBP/eGFP (Calvache *et al.*, 2021; Garcia-Perez *et al.*, 2022; Selma *et al.*, 2019), whereas others are new to plants. Among them, we have developed new circuit configurations for agrochemical (copper) control of gene expression with direct application in crops, and we have designed a new dual optogenetic/agrochemical control system that could be applied to the tight regulation of metabolic pathways in sensible metabolic engineering approaches. The expanded testing potential of the FBP/eGFP reporter is one example of the many new opportunities that the long-time pursued autonomous luminescence can offer to plant biotechnology.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

Sequence information of all plasmids used in this work is available at <https://gbcloning.upv.es/>. Luminescence and fluorescence values are deposited in Zenodo (<https://doi.org/10.5281/zenodo.7704409>).

References

- Brophy, J.A.N., Magallon, K.J., Duan, L., Zhong, V., Ramachandran, P., Kniazev, K. and Dinneny, J.R. (2022) Synthetic genetic circuits as a means of reprogramming plant roots. *Science*, **377**, 747–751.
- Calvache, C., Vazquez-Vilar, M., Selma, S., Uranga, M., Fernández-Del-Carmen, A., Daròs, J.-A. and Orzáez, D. (2021) Strong and tunable anti-CRISPR/Cas activities in plants. *Plant Biotechnol. J.* **20**, 399–408.
- COVID vaccines grow on leaves (2021) COVID vaccines grow on leaves. *Nat. Biotechnol.* **39**, 649.
- Furuhata, Y., Sakai, A., Murakami, T., Nagasaki, A. and Kato, Y. (2020) Bioluminescent imaging of *Arabidopsis thaliana* using an enhanced Nano-lantern luminescence reporter system. *PLoS One*, **15**, e0227477.
- García-Perez, E., Diego-Martin, B., Quijano-Rubio, A., Moreno-Giménez, E., Selma, S., Orzáez, D. and Vazquez-Vilar, M. (2022) A copper switch for inducing CRISPR/Cas9-based transcriptional activation tightly regulates gene expression in *Nicotiana benthamiana*. *BMC Biotechnol.* **22**, 12.
- Khakhar, A., Starker, C.G., Chammess, J.C., Lee, N., Stokke, S., Wang, C., Swanson, R. *et al.* (2020) Building customizable auto-luminescent luciferase-based reporters in plants. *eLife*, **9**, e52786.
- Khan, M.A., Herring, G., Oliva, M., Fourie, E., Zhu, J.Y., Johnston, B., Pflüger, J. *et al.* (2022) CRISPRi-based circuits for genetic computation in plants. *BioRxiv*. <https://doi.org/10.1101/2022.07.01.498372>
- Khosla, A., Rodríguez-Furlan, C., Kapoor, S., Van Norman, J.M. and Nelson, D.C. (2020) A series of dual-reporter vectors for ratiometric analysis of protein abundance in plants. *Plant Direct*, **4**, e00231.
- Kurotani, K.-I., Hirakawa, H., Shirasawa, K., Tanizawa, Y., Nakamura, Y., Isobe, S. and Notaguchi, M. (2023) Genome sequence and analysis of *Nicotiana benthamiana*, the model plant for interactions between organisms. *Plant Cell Physiol.* **64**, 248–257.
- Liu, W. and Stewart, C.N. (2015) Plant synthetic biology. *Trends Plant Sci.* **20**, 309–317.
- Lloyd, J.P.B., Ly, F., Gong, P., Pflueger, J., Swain, T., Pflueger, C., Fourie, E. *et al.* (2022) Synthetic memory circuits for stable cell reprogramming in plants. *Nat. Biotechnol.* **40**, 1862–1872.
- Marillonnet, S. and Grützner, R. (2020) Synthetic DNA assembly using golden gate cloning and the hierarchical modular cloning pipeline. *Curr. Protoc. Mol. Biol.* **130**, e115.
- McCarthy, D.M. and Medford, J.I. (2020) Quantitative and predictive genetic parts for plant synthetic biology. *Front. Plant Sci.* **11**, 512526.
- Mitiouchkina, T., Mishin, A.S., Sommermeier, L.G., Markina, N.M., Chepurnyh, T.V., Guglya, E.B., Karataeva, T.A. *et al.* (2020) Plants with genetically encoded autoluminescence. *Nat. Biotechnol.* **38**, 944–946.
- Moreno-Giménez, E., Selma, S., Calvache, C. and Orzáez, D. (2022) GB_SynP: a modular dCas9-regulated synthetic promoter collection for fine-tuned recombinant gene expression in plants. *ACS Synth. Biol.* **11**, 3037–3048.
- Ochoa-Fernandez, R., Abel, N.B., Wieland, F.-G., Schlegel, J., Koch, L.-A., Miller, J.B., Engesser, R. *et al.* (2020) Optogenetic control of gene expression in plants in the presence of ambient white light. *Nat. Methods*, **17**, 717–725.
- Ranawaka, B., An, J., Lorenc, M.T., Jung, H., Sulli, M., Aprea, G., Roden, S. *et al.* (2022) A multi-omic *Nicotiana benthamiana* resource for fundamental research and biotechnology. 2022.12.30.521993.
- Sarrion-Perdigones, A., Vazquez-Vilar, M., Palaci, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J. *et al.* (2013) GoldenBraid 2.0: a comprehensive DNA

- assembly framework for plant synthetic biology. *Plant Physiol.* **162**, 1618–1631.
- Schaumberg, K.A., Antunes, M.S., Kassaw, T.K., Xu, W., Zalewski, C.S., Medford, J.I. and Prasad, A. (2016) Quantitative characterization of genetic parts and circuits for plant synthetic biology. *Nat. Methods*, **13**, 94–100.
- Selma, S., Bernabé-Orts, J.M., Vazquez-Vilar, M., Diego-Martin, B., Ajenjo, M., Garcia-Carpintero, V., Granell, A. *et al.* (2019) Strong gene activation in plants with genome-wide specificity using a new orthogonal CRISPR/Cas9-based programmable transcriptional activator. *Plant Biotechnol. J.* **17**, 1703–1705.
- Shinde, R., Perkins, J. and Contag, C.H. (2006) Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays. *Biochemistry*, **45**, 11103–11112.
- Vazquez-Vilar, M., Quijano-Rubio, A., Fernandez-Del-Carmen, A., Sarrion-Perdigones, A., Ochoa-Fernandez, R., Ziarsolo, P., Blanca, J. *et al.* (2017) GB3.0: a platform for plant bio-design that connects functional DNA elements with associated biological data. *Nucleic Acids Res.* **45**, 2196–2209.
- Wang, Y.-H., Wei, K.Y. and Smolke, C.D. (2013) Synthetic biology: advancing the design of diverse genetic systems. *Annu. Rev. Chem. Biomol. Eng.* **4**, 69–102.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Analysis of the dynamic range of different combinatorial configurations of the FBP.

Figure S2 NPGA enhances luminescence production allowing increased sensitivity of the FBP[HisP5] reporter.

Figure S3 Phytohormone sensors with the FBP[HisP5]/eGFP reporter.

Figure S4 Alternative designs for copper switches assayed with the FBP[HisP5]/eGFP reporter.

Table S1 List of GoldenBraid plasmids used in this work.

Methods S1 The tables provided below show the *Agrobacterium* cultures co-infiltrated in the same mix for each experiment.