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# GB_SynP: a modular dCas9-regulated synthetic promoter collection for fine-tuned recombinant gene expression in plants

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An important challenge in genetic engineering is the selection of **promoters that confer precise control of the transgene expression in both time and space**. Here we present **a collection of synthetic promoters that are activated by using a "dead" Cas9 activation system (dCasEV2.1**, Selma et al., 2019). Such tool can be used for **creating synthetic regulatory cascades** where a number of downstream genes (e.g. a whole metabolic pathway) are controlled by a single dCas9 gene at custom expression levels. By **adding as much variability as possible** to each synthetic promoter, issues arising from the use of similar promoters very close to each other's will be avoided.

## 1. Design and testing GB_SynP synthetic parts

GB_SynP is a collection of 32 synthetic promoter parts that can be assembled using the GoldenBraid (GB) strategy to form **more than 500 different dCas9-regulated promoters**. GB_SynP promoters are characterised by a **negligible basal expression and a wide range of expression levels** when activated with the dCasEV2.1 system (Figure 1A).

GB_SynP promoters consist of **3 different promoter parts** (Figure 1B):

- **A1 Distal promoter parts**: randomly-generated sequences used to **adjust the length** of the final promoter.
- **A2 Proximal promoter parts**: determine the activation levels by including the **target sequence(s) of the gRNA(s)** that direct the dCasEV2.1 activation system towards the resulting promoter.
- **A3(-B2) Minimal promoter parts**: created based on the **5'UTR and TATA box region** of different plant and fungal promoters.

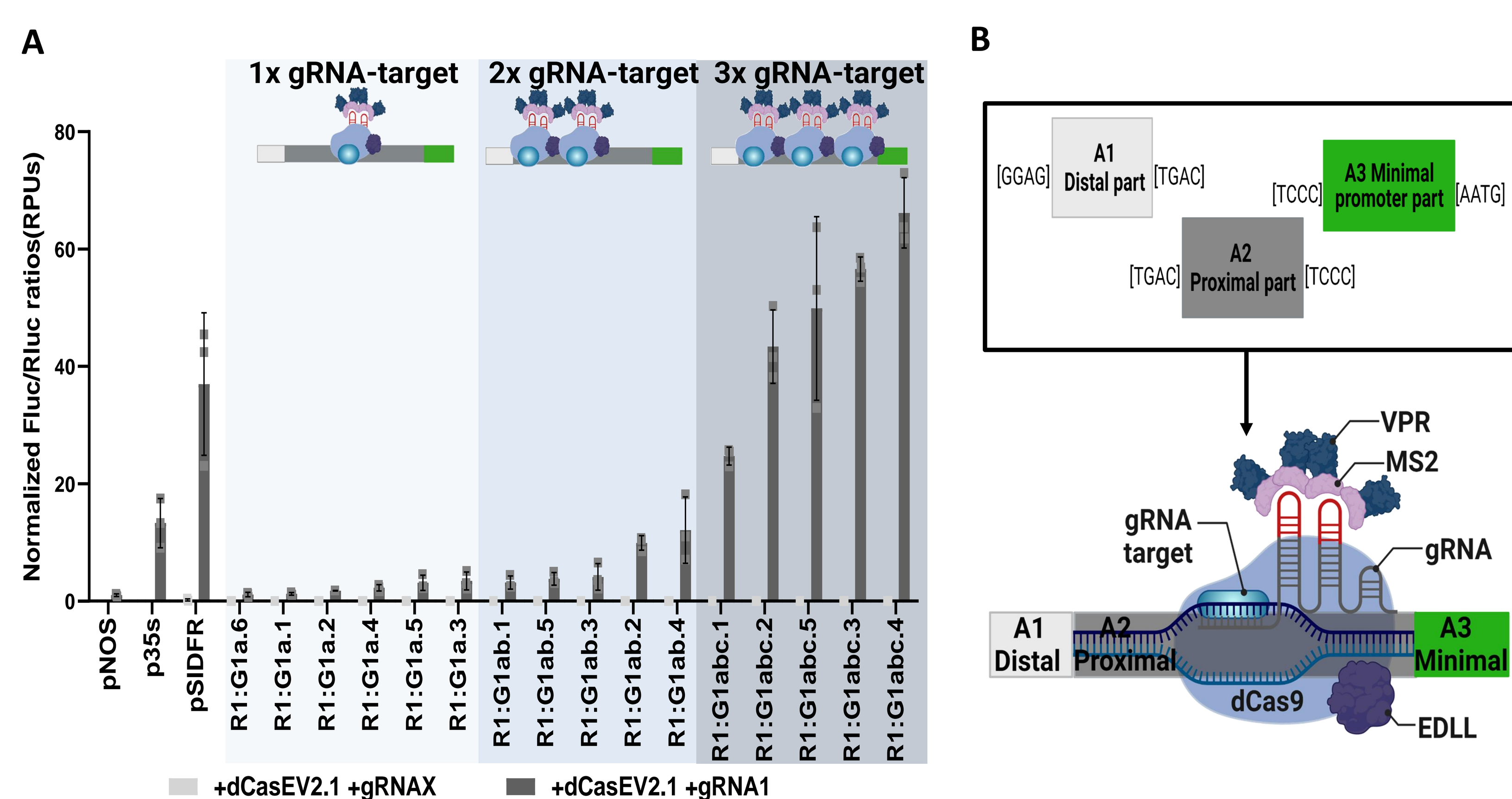


**Figure 1. Design and expression range of dCasEV2.1-responsive GB_SynP promoters.** (A) Normalized expression of GB_SynP promoters with different A2 parts containing the target sequence for the gRNA1 once (G1a.N), twice (G1ab.N) or three times (G1abc.N). Error bars represent the average values ± SD (n=3). (B) Schematic representation of the GB_SynP parts and their assembly with the dCasEV2.1 system.

## 2. Additional configurations of the cis-regulatory region

The target sequences of **different gRNAs can be used as cis-regulatory elements** to control the expression of the GB_SynP promoters. To test whether the position and/or the sequence of gRNA targets affect the expression of the GB_SynP promoter, two A2 proximal parts were designed the exact same sequence but replacing the target sequence of gRNA1 in G1aG2b.7 part by the target sequence of gRNA3 in G3aG2b.1 (Figure 2A). **Both gRNA3 and gRNA1 triggered similar activation levels,** while **gRNA2,** positioned **further from the Transcription Start Site, triggered a lower response** (Figure 2 B).

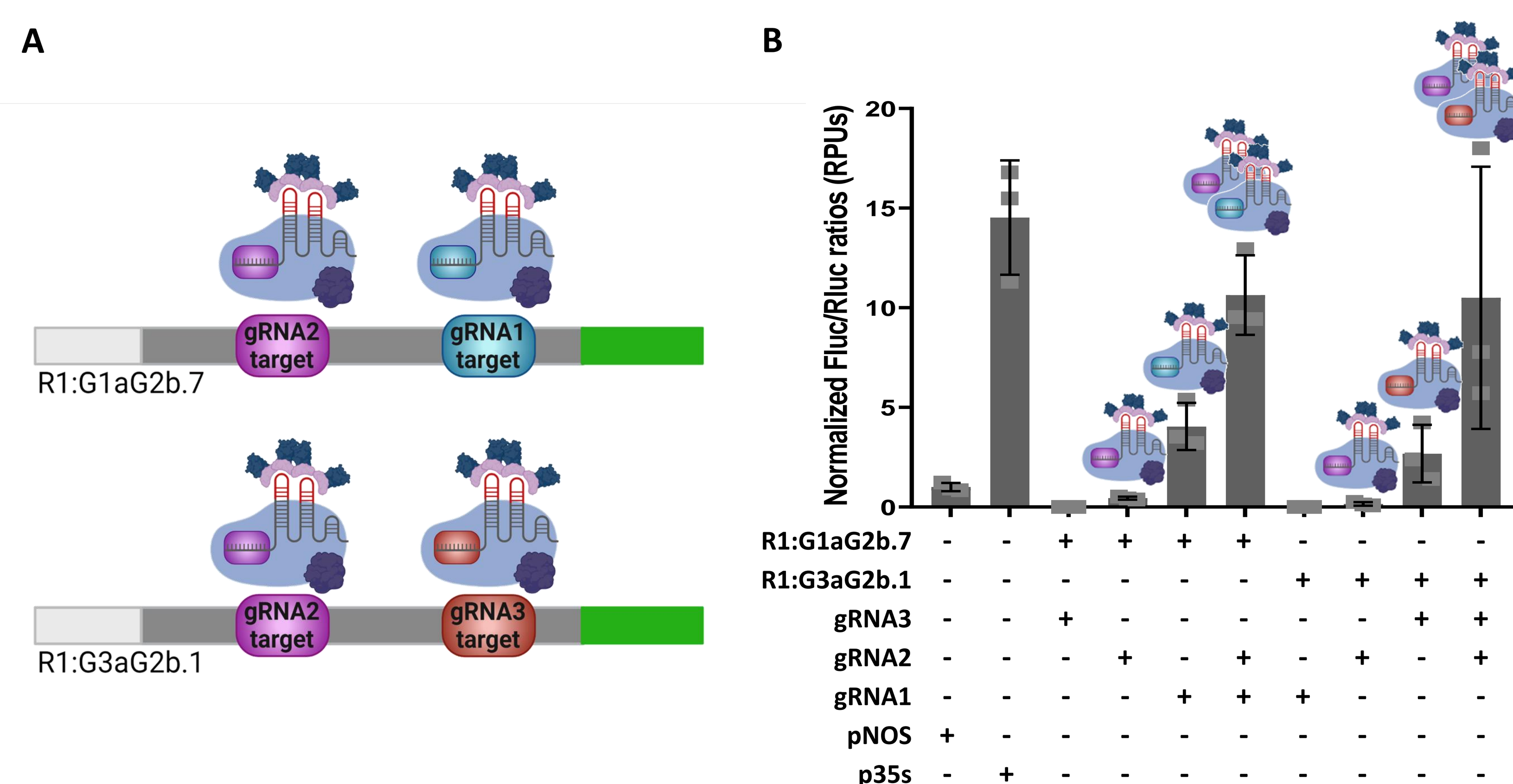


**Figure 2. Variation of the cis-regulatory boxes within the GB_SynP A2 proximal parts.** (A) Schematic design of the GB_SynP promoters including the A2 parts G1aG2b.7 and G3aG2b.1. (B) Normalized expression of GB_SynP promoters containing G1aG2b.7 or G3aG2b.1 parts, activated with dCasEV2.1 system loaded with different gRNAs. Error bars represent the average values ± SD (n=3).

## 3. Biological approach: fine-tuning the expression of a bioluminescence pathway

The combinatorial power and wide expression range of the GB_SynP promoters were used to **optimise a multigene bioluminescence pathway** (Figure 3A). A series of assemblies was thus created to **regulate three genes - *Luz, H3H* and *HispS* - with GB_SynP promoters of different strength** (Figure 3B) and interrogated in a **time-course assay** (Figure 3C). It was found that**:**

- **GB_SynP regulates the genes of the pathway in a predictable and reliable way**, obtaining the lowest output when all genes are under the weakest promoter (1x), and reaching the highest expression when they are driven by the strongest (3x) promoter.
- ***HispS* was found to limit the pathway when expressed at low levels** (red boxes), while the effect of the remaining genes was observed when raising its expression with an intermediate-strength (2x) promoter (yellow boxes).
- **When *Luz* and *HispS* are under the 2x promoter, the pathway is limited by *H3H*** (blue arrow), suggesting that a **reporter system** with an appropriate dynamic range could be made that consists of **constitutively-expressed *Luz* and *HispS*** by a mid-strength promoter **and *H3H* under a variable-strength promoters.**

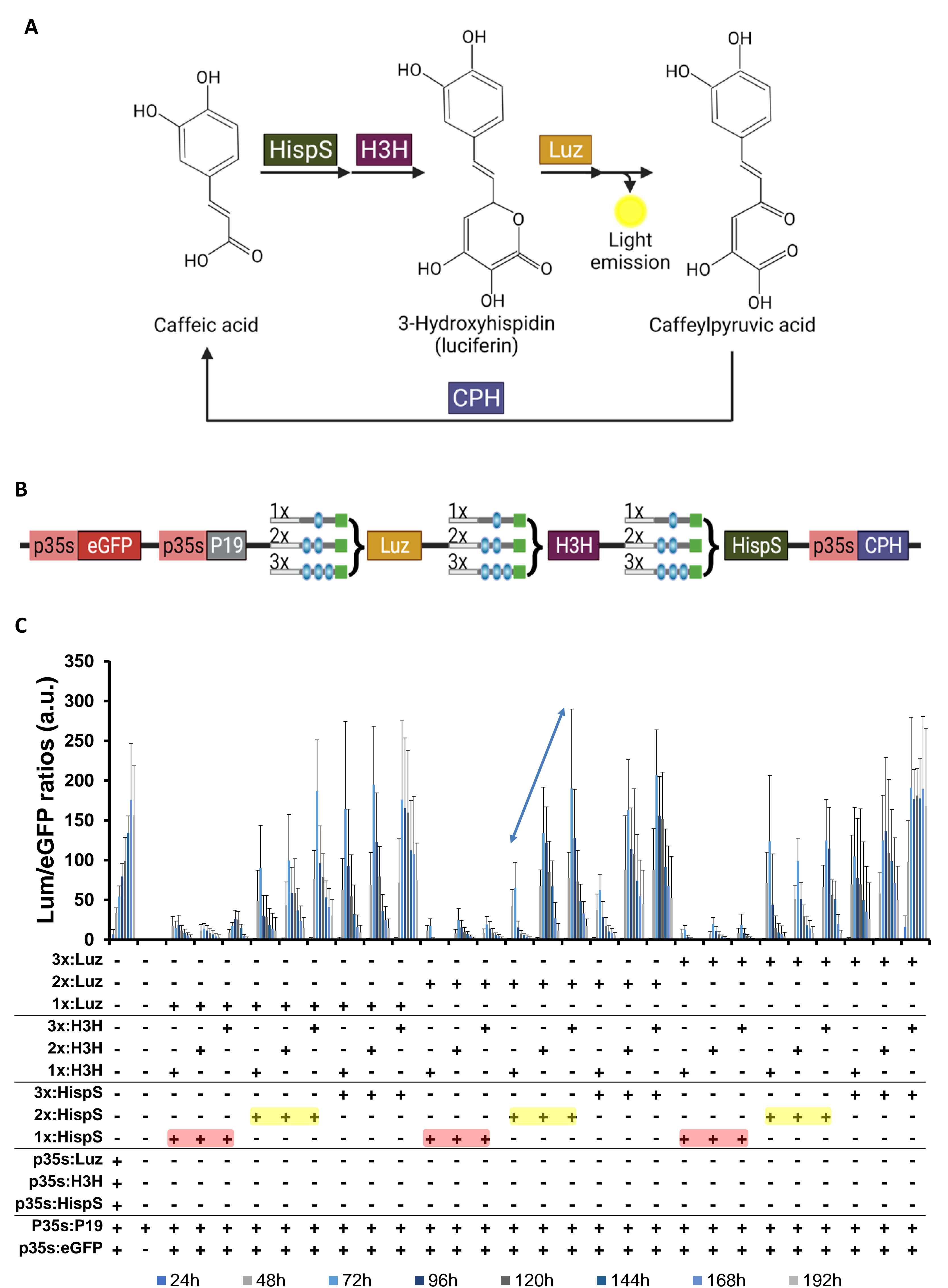


**Figure 3. Regulation of a multigene pathway with GB_SynP promoters.** (A) Schematic representation of the bioluminescence pathway described by Kotlobay et al. (2018). (B) Genetic constructs assembled to express the pathway under the regulation of GB_SynP promoters with either one (1x), two (2x) or three (3x) targets for gRNA1. (C) Time-course expression of the 27 constructs expressing the pathway transiently in *Nicotiana benthamiana* leaves. Error bars represent the average values ± SD (n=3).