

# A Small Natural Light-induced Bidirectional Promoter of Rapeseed (*Brassica Napus*)

**Ruijia Zhu**

Sichuan University - Wangjiang Campus: Sichuan University

**Li Zhang**

Sichuan University - Wangjiang Campus: Sichuan University

**Tao Wei**

Sichuan University - Wangjiang Campus: Sichuan University

**Xuefei Jiang**

Sichuan University - Wangjiang Campus: Sichuan University

**Maolin Wang** (✉ [mlwang@scu.edu.cn](mailto:mlwang@scu.edu.cn))

Sichuan University - Wangjiang Campus: Sichuan University

---

## Research Article

**Keywords:** Brassica napus, bidirectional promoter, light-induced promoter, GUS, EGFP

**Posted Date:** March 30th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-339409/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

A bidirectional promoter is an intergenic region located between a pair of adjacent and oppositely transcribed genes ('head-to-head' genes) that concurrently promote both genes expression. In the genome of *Brassica napus*, we identified a bidirectional promoter (265bp long), named  $P_{Bn265}$ .  $P_{Bn265}$  was located between the transcription initiation codons (ATG) of two genes that encode the homeodomain protein SHH2, and chloroplast GROUP II intron splicing factor CFM3. Its bidirectional promoting activity was verified by transient expression of *Nicotiana benthamiana* leaf tissue via Agrobacterium-mediated transformation of  $P_{Bn265}$ -F::EGFP and  $P_{Bn265}$ -R::mCherry. The expression of both reporter genes, EGFP linked to one end and GUS on the other end of the  $P_{Bn265}$  sequence, was observed in the various tissues of the transgenic *Arabidopsis thaliana* using histochemical staining and fluorescence microscopy. Furthermore, we also found that the promoting activity of this sequence was regulated by illumination. Considering its short sequence length and light inducible regulation, this promoter likely has application potential in bioengineering and agricultural molecular breeding.

## 1. Introduction

A promoter, as an important *cis*-element, is a special DNA sequence located upstream of its associated gene. The promoter sequence contains the core regulatory information of gene expression, such as *cis*-elements like TATA-box, as well as defines gene transcription initiation, transcription efficiency, and transcription temporal-spatial specificity [1]. Promoters can be roughly divided into three types according to their function, constitutive promoters [2] inducible promoters [3] and tissue-specific promoters [4]. A single promoter can also have two characteristics at the same time [5]. They can respond to environmental factors, and inducible promoters can be divided into biological-stress-induced promoters, physical-stress-induced promoters, and chemical-stress-induced promoters [6]. Inducible promoters can avoid excessive consumption of plant energy resulting from continuous target gene expression, in addition to eliminating damage caused by the accumulation of gene products to the plant itself [7]. Therefore, the identification of inducible promoters has recently become a research priority in plant genetic engineering [8].

Bidirectional promoters specify an intergenic region between two genes that are adjacent but in opposite directions of transcription, often with less than 1,000 bp between transcription initiation sites [9]. These two genes are called bidirectional gene pairs or "head-to-head" gene pairs. The first plant seed-specific bidirectional promoter of the oleosin gene in *Brassica napus*, included an expression module that was ABA-inducible at the forward orientation and ethylene-inducible at the reverse orientation [10]. Given that the bidirectional promoter can drive two genes at the same time, it has great potential in genetic engineering and metabolic engineering research involving multi-gene expression. Furthermore, when multiple genes are expressed, repeated utilization of constitutive promoters can easily lead to gene silencing. The application of bidirectional promoters can avoid these unwanted negative effects [11].

In recent years, bioinformatics studies have identified a large number of bidirectional gene pairs in humans [12], Arabidopsis [13], rice [14] and populus [15]. There are many intergenic regions between “head-to-head” genes that are potentially active bidirectional promoters [16]. Currently, only a few bidirectional promoters in Arabidopsis and rice have been verified using biological experiments [17]. These include the Arabidopsis chlorophyll *a/b* binding protein gene promoter [18], antioxidant protein B gene promoter [19], rice chymotrypsin inhibitor gene promoter [20], and heat shock protein gene promoter [21]. The Arabidopsis chlorophyll *a/b* binding protein gene promoter has light-inducible properties, while the rice chymotrypsin inhibitor gene promoter was induced by drought [18]. Both have already exhibited application value in the field of genetic engineering [21].

In this study, we identified that LOC106347029 and LOC106347039 were a pair of “head-to-head” genes in the *Brassica napus* genome. The gene annotation from the NCBI website revealed that the protein encoded by LOC106347029 gene was a chloroplast group II intron splicing factor (*CFM3*), and LOC106347039 encoded the protein SHH2. *CFM3* and *SHH2* genes are adjacent and arranged oppositely on the two complementary DNA strands. The distance between the transcription initiation codons was only 265bp. Therefore, we hypothesized that the intergenic region between *CFM3* and *SHH2* may be a bidirectional promoter that can simultaneously drive the expression of genes on both sides. To validate this hypothesis, we analyzed the promoter sequence using Plantcare website software, and conducted a series of characterization experiments to test bidirectional activity of this sequence, the expression level of native gene pairs (*CFM3* and *SHH2*), and reporter gene pairs (*EGFP* and *GUS*). Here we report one of the shortest natural bidirectional and light induced promoters for *Brassica napus*, to our knowledge. Therefore, the promoter has broad application prospects in plant genetic improvement and genetic engineering [22, 23].

## 2. Materials And Methods

### 2.1 Sequence analysis and PCR amplification

The arrangement of the genes on chromosome A1 of *Brassica napus* (ZS11) was observed using the NCBI genome data viewer (<https://www.ncbi.nlm.nih.gov/>). The DNA sequence between “head-to-head” genes was downloaded from GenBank as a candidate for bidirectional promoters. The *cis*-elements were analyzed by Plantcare software. CpG island prediction was made by MethPrimer software. Specific primers were designed according to the sequences of both ends. In order to amplify the sequence (named *P<sub>Bn265</sub>*) from the genomic DNA of *Brassica napus* (KeleYou), the PCR reaction procedure was as follows: 95°C for 3min; 34 cycles of 95°C for 10s, 55°C for 10s and 72°C for 15s; and a final extension at 72°C for 5min.

### 2.2 Construction of vectors

In this study, five recombinant plasmids were constructed, namely pBI121-*P<sub>Bn265</sub>F*::EGFP, pEG-*P<sub>Bn265</sub>R*::mCherry, pDX2181-*P<sub>Bn265</sub>* (pDX2181 vector was provided by Yongjun Lin [24], pBI221-*SHH2* and

pBI221-*CFM3*. Plasmid pBI121- $P_{Bn265}F::EGFP$  contained the forward orientation promoter that drove the *EGFP* reporter gene expression. Plasmid pEG- $P_{Bn265}R::mCherry$  contains the reverse promoter that drove the expression of *mCherry* reporter gene.

Vector pDX2181- $P_{Bn265}$  contained a fused pair of reporter genes *GUS* and *EGFP* which are “head-to head” genes (Fig. 4a), and a promoter  $P_{Bn265}$  was inserted between them to promote the two genes simultaneously, the forward orientation drove the expression of *GUS* and the reverse orientation drove the expression of *EGFP*. pBI221-*SHH2* and pBI221-*CFM3* were used for subcellular localization of *SHH2* and *CFM3* genes, these two genes were ligated to the vector pBI221 before the *EGFP* reporter gene and driven by CaMV35S promoter. All restriction sites and primers are reported (Supplementary Table S1).

## 2.3 Tobacco instantaneous expression experiment

We constructed and transformed the recombinant plant binary expression vectors, pBI121-*EGFP* and pEG-*mCherry*, which contained  $P_{Bn265}F::EGFP$  and  $P_{Bn265}R::mCherry$  respectively (Fig. 1b,c), into *Agrobacterium tumefaciens* strain GV3101. Then we selected positive clones from LB medium containing 50 $\mu$ M kanamycin and 20 $\mu$ M rifampicin after cultivating for 36 h at 28°C, and identified them by PCR using specific primers (Supplementary Table S1). Positive clones were inoculated into 20mL LB liquid medium containing the same antibiotics at 28°C for 16h at 200rpm in an incubator shaker. When the OD600 reached 0.8, we centrifuged it at 2500 $\times$ g for 10min by the differential centrifuge at room temperature, and then resuspended bacteria to OD600 at 1.0 by Agromix Buffer [10 mM MES (pH 5.8), 10mM MgCl<sub>2</sub>, 200M acetosyringone]. We selected *Nicotiana benthamiana* with 6 to 8 true leaves, and injected the bacterial suspension into the young leaves closest to the top. Plants inoculated with bacterial suspension were incubated in darkness at 70% humidity for 16h, and then placed in 70% humidity for three days, with alternating 16 h of light and 8 h of darkness. Fluorescent signals were then viewed with a positive fluorescence microscope (Leica microsystems DM4 B).

## 2.4 Subcellular localization assay

We obtained the *N. benthamiana* protoplast through PEG-mediated transfection [25], and gently mixed 10 $\mu$ L recombinant plasmid DNA into 100 $\mu$ L protoplast solution in a 2mL round bottom tube, before adding and gently mixing 110 $\mu$ L freshly prepared PEG solution (PEG4000, 0.2M mannitol, 0.1M CaCl<sub>2</sub>). This solution was left at room temperature for 15min before adding W5 solution [5mM KCl, 125mM CaCl<sub>2</sub>, 154mM NaCl, and 2mM MES (pH 5.7)] and inverting it several times, in order to completely terminate the transfection process. After centrifugation at 100g for 3min, the supernatant was removed, and 1mL WI solution [4mM MES (pH5.7), 20mM KCl, and 0.5M mannitol] was added to resuspend the protoplasts. Finally, we cultured the transfected protoplasts in the darkness for 16h at room temperature, and the *EGFP* signals were observed and photographed under a fluorescence microscope (Leica microsystems DM4 B).

## 2.5 Arabidopsis transformation and selection of the transformants.

In order to explore the efficiency of  $P_{Bn265}$ , the recombinant vector pDX2181- $P_{Bn265}$  with *GUS* and *EGFP* reporter genes, was transformed into *Arabidopsis thaliana* ecotype Col-0 by floral-dip method. The transformants were selected on 1/2MS solid medium with 20µg/mL hygromycin. DNA was extracted from all T1 transformants, and transgenesis was identified through PCR using  $P_{Bn265}$ -F/R primer (Supplementary Table S1). The T2 transgenic seedlings were selected by 1/2MS solid medium with 30µg/mL hygromycin. We repeated the previous steps until the homozygous transformants of T3 generation were obtained.

The *GUS* assay was performed by immersing tissues in the *GUS* staining solution (Solarbio) for 4h in the dark at 37°C, before soaking them in 70% ethanol and observing them under the microscope (Leica MZFLIII). In addition, the fluorescence of different tissues was detected by fluorescence microscope (Leica microsystems DM4 B).

## 2.6 Synthesis of cDNA and Real-Time qPCR analysis.

We extracted RNA from the roots, stems, leaves, flowers, and siliques of transgenic *Arabidopsis thaliana*, and obtained cDNA, before quantitatively analyzing the expression of  $P_{Bn265}::GUS$  and  $P_{Bn265}::EGFP$  genes by RT-qPCR. We then divided transgenic plants into two groups, treatment group and control group. The transgenic *Arabidopsis thaliana* seedlings in treatment groups were maintained in continuous darkness for 12, 24, 36 and 48 h, and relighted for 12, 24, 36 and 48 h, while the control group continued with its regular light conditions (16h light/8h darkness). Tissues from the roots, stems, and leaves were collected every 12 h for RNA extraction. Quantitative analysis of  $P_{Bn265}^F::GUS$  and  $P_{Bn265}^R::EGFP$  activity was conducted three times, each treatment had three biological replicates and each replicate was made from a pool of 5 *Arabidopsis* plants. All of the data were normalized to the ratio of *GUS* and *EGFP* genes expression in treatment group, to its counterpart in control group over treatment time.

RNA was extracted by using Plant Total RNA Isolation Kit (FOREGENE). Then the first-strand cDNA was obtained through reverse transcription by using One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech). Primers At.actin (Supplementary Table S1) were used to detect the quality of cDNA and as an endogenous control to normalize the variance between samples. RT-qPCR was performed with qPCR SYBR Green Master Mix (YEASEN Biotech). Procedure of RT-qPCR as follow: qPCR cycle parameters were set as 95°C for 30s, 39 cycles of 5s at 95 °C, 15s at 57 °C, a final melting curve from 65°C to 95°C with an increment of 0.5°C.

## 3. Results

### 3.1 Sequence analyze of promoter $P_{Bn265}$ from *Brassica napus*

According to the reference genome of *Brassica napus* (ZS11), we identified an intergenic region between the initiation codon ATG of the two "head-to-head" gene pairs (Fig. 1a), SAWADEE HOMEODOMAIN

HOMOLOG 2 (*SHH2*) and Containing Factor of CRM domain (*CFM3*). It was only 256bp long and was named  $P_{Bn265}$ . The primers  $P_{Bn265}$ -F/R were designed (Supplementary Table S1) to amplify the complete 265bp sequence from the *Brassica napus* (Keleyou) genome. We stipulated that the forward orientation controls transcription of *SHH2* and the reverse for *CFM3*.

The sequence of  $P_{Bn265}$  was analyzed by Plantcare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), and we located approximately 10 *cis*-elements (Fig. 2), including AE-box, ATCT-motif, CAAT-box, DRE1, G-box, GCN4-motif, MRE, MYC, SP1 and TATA-box. Some of these *cis*-elements are in the forward orientation, such as SP1 (GGGCGG, a light responsive element), GCN4-motif (TGAGTCA, *cis*-regulatory element involved in endosperm expression), MRE (AACCTAA, MYB binding site involved in light responsiveness) and two TATA-box (TA-rich sequences, core promoter element around -30 of transcription start). While the others are in the reverse orientation, including G-box (TCCACATGGCA, *cis*-acting regulatory element involved in light responsiveness), ATCT-motif (AATCTAATCC, part of a conserved DNA module involved in light responsiveness) and AE-box (AGAAACAA, part of a module for light response). Due to the identity of these *cis*-elements, we hypothesized that  $P_{Bn265}$  was most likely affected by light.

## 3.2 The function of promoter $P_{Bn265}$ was preliminarily verified by transient expression assays.

In order to verify whether  $P_{Bn265}$  regulated promotion in plant tissue, we conducted tobacco instantaneous expression experiments by introducing the promoter  $P_{Bn265}$  ( $P_{Bn265}$ -F::*EGFP* and  $P_{Bn265}$ -R::*mCherry*) into *N. benthamiana* leaf tissue by Agrobacterium-mediated transformation for transient expression. These results revealed that both forward and reverse promoters could drive the expression of the corresponding reporter genes, and green (*EGFP*) or red (*mCherry*) fluorescence was observed in transformed tobacco leaves (Fig. 1b, c). Therefore, we successfully illustrated that this  $P_{Bn265}$  sequence has bidirectional promoter activity in both rapeseeds and tobacco.

## 3.3 *Cis*-elements and subcellular localization of *SHH2* and *CFM3*.

The SAWADEE domain has been verified as a novel chromatin-binding module, which is used to probe the methylation state of the histone H3 tail [26]. SHH protein can also interact with Pol IV (RNA polymerase IV) and is important for the location of Pol IV at some target sites [27]. Therefore, *SHH2* should have been located in the nucleus, and the promoter,  $P_{Bn265}$ -F, which regulates its transcription should be a constitutive promoter. The CRM domain is one of a group II intron splicing factors which is involved in chloroplasts [28]. However, many of them have lost their capacity for self-splicing, resulting in some pseudo genes and they will not transcribe and translate into proteins [29], such as LOC106451789.

In accordance with the characterizations and functions of *SHH2* and *CFM3* mentioned above, the results of subcellular localization of *SHH2* and *CFM3* revealed that *SHH2* was located in the nucleus, and *CFM3*

was most likely to be located in chloroplasts (Fig. 3). These results were also consistent with previous research reports and the predictions of *cis*-acting elements of this promoter (Fig. 2).

### 3.4 *GUS* and *EGFP* gene expression analysis in transgenic plants

The reporter genes, *GUS* and *EGFP*, were introduced into *Arabidopsis thaliana* (Fig. 4a), and real-time fluorescence quantitative analysis of  $P_{Bn265-F}::GUS$  and  $P_{Bn265-R}::EGFP$  in transformants showed that  $P_{Bn265-F}::GUS$  was expressed in root, stem, leaf, flower and silique (Fig. 4b), and *GUS* staining was also observed in these tissues (Fig. 4c). Measurement of *GUS* staining intensity supported the RT-qPCR results. However, fluorescence determined by microscope images indicate that  $P_{Bn265-R}::EGFP$  was almost exclusively expressed in stem, leaf, flower and silique, with its highest expression level in green tissues like the leaf and stem, while barely detectable in the root (Fig. 4d). These results were similar to the analysis of RT-qPCR (Fig. 4b).

### 3.5 RT-qPCR of $P_{Bn265-F}::GUS$ and $P_{Bn265-R}::EGFP$ in light-treat transformants verified that $P_{Bn265}$ is induced by light.

The different reporter gene expressions driven by the same promoter with different orientations indicated that the forward and reverse orientations of the  $P_{Bn265}$  promoter were regulated by different factors. To verify this, we conducted an illumination experiment. Transformants were grown under darkness for 64h and then shifted into a light room for an additional 64h.

Quantitative analysis revealed that the relative expression level of  $P_{Bn265-R}::EGFP$  in the root was significantly lower than the other tissues (Fig. 4b), and the relative expression level of  $P_{Bn265-R}::EGFP$  in the root, stem and leaf decreased significantly under constant darkness. On the other hand, the relative expression level of  $P_{Bn265-F}::GUS$  in the root did not show a regular change. In the stem, however, the relative expression level of  $P_{Bn265-F}::GUS$  significantly decreased after dark treatment for 36 h. Changes of  $P_{Bn265-F}::GUS$  in the leaf were similar to those in the stem (Fig. 5a). After resumption of light, both  $P_{Bn265-F}::GUS$  and  $P_{Bn265-R}::EGFP$  relative expression was elevated in the stem and leaf. Interestingly, the relative expression level of  $P_{Bn265-F}::GUS$  was persistently decreased (Fig. 5b).

In order to obtain a more accurate trend of the gene variation in transgenic plants under the change of light, we recalculated the quantitative data of treatment group and control group (described in method 2.6). As can be seen in the processed data, in the root, the changing trend of  $P_{Bn265-R}::EGFP$  under illumination treatment was not regular, while  $P_{Bn265-F}::GUS$  expression showed a downward trend as a whole, and there was no obvious trend of gene expression recovery even after restoration of light. In the stem and leaf, the changing trend of both  $P_{Bn265-F}::GUS$  and  $P_{Bn265-R}::EGFP$  decreased as darkness continued and then gradually recovered after restoration of light (Fig. 5c).

## 4. Discussion

In this study, we identified a potential bidirectional promoter from the genome of *Brassica napus* (Fig. 1a). Through the cloning and functional analysis of the promoter  $P_{Bn265}$  of *Brassica napus*, we have drawn the following preliminary conclusions: the promoter has transcriptional activity in both two directions (Fig. 1b,c); the promoter may be a bidirectional promoter, containing a variety of *cis*-acting elements related to light response (Fig. 2); the  $P_{Bn265}$ -*F* has activity in the root, stem, leaf, flower and silique in transgenic *Arabidopsis thaliana* (Fig. 4), and that *SHH2* may also express in all tissues in *Brassica napus*. On the other hand, the expression level of  $P_{Bn265}$ -*R* was relatively high in green tissues such as stem and leaf, but significantly lower or not even present in the roots. In stem and leaf tissues, the expression level of *EGFP* and *GUS* decreased significantly in the absence of light. After resumption of light, the gene expression level rose gradually, and with the prolonging of the light, the gene expression level increased too. Similar patterns were not observed in the root. Therefore, we hypothesized that *CFM3* is mainly expressed in chloroplasts, as there are fewer chloroplasts in roots. Based on these results, when  $P_{Bn265}$  simultaneously drives the expression of both two genes,  $P_{Bn265}$ -*R* may affect the function of  $P_{Bn265}$ -*F* (Fig. 5).

The leaf is the photosynthesis organ of the plant, playing an important role in energy fixation and utilization. The study of light-induced promoters has important theoretical significance and application value for the further study of genes for which light regulates expression [30]. Light-induced promoters with bidirectional driving function have a potentially broad application in plant genetic improvement and genetic engineering. In seven species of *Brassicaceae*, the arrangement of the structure, “CFM3-bidirectional promoter-SHH2”, has a high degree of conservation of synteny (Supplementary Table. S2). This evolutionarily conservative arrangement may have some unknown biological significance and these promoters may all be bidirectional promoters. Two genes that share a short promoter can potentially save on resource usage, such as time and space, energy, and RNA polymerase. This is highly conducive to an organism's growth and development. Therefore, the evolutionary origin of this promoter deserves further study.

It is worth noting that, in previous studies on bidirectional promoters, only 9% of them have TATA-box [31]. It was speculated that the regulation mechanism of bidirectional promoters without TATA-box may be different from directional promoters [32]. However, this short (265bp) sequence contains two TATA-box and rich *CpG* islands, but low GC content. Among the promoters randomly selected from the human genome, the average GC content is 53% and about 70.8% of them have GC content higher than 60%, significantly higher than average [33]. We found that  $P_{Bn265}$  has only 42% GC content and does not contain *CpG* islands (Supplementary Fig. S1). This may be a new discovery of the characteristics of bidirectional promoters, indicating that there may be new regulation mechanisms for bidirectional promoters, which will help future generations to further study the regulatory mechanism of bidirectional promoters.

In summary, the light-induced bidirectional promoter found in this study is the shortest among the natural inducible bidirectional promoters as far as we know, and it also has some characteristics different from other promoters, containing two TATA-boxes, having low GC content and lacking *CpG* islands. In addition, considering the promotion activity and the applicability in heterologous species, the promoter has broad application potential in plant genetic improvement and genetic engineering.

## Declarations

### Ethical Approval

Not applicable.

### Consent to Participate

All authors have given consent to participate.

### Consent to Publish

All authors have given approval of the manuscript to be published.

### Authors Contributions

Ruijia Zhu: Conceptualization, Methodology, Writing - Original Draft, Investigation. Li Zhang: Data Curation, Formal analysis, Validation. Tao Wei: Methodology, Writing – Review & Editing. Xuefei Jiang: Methodology. Maolin Wang: Investigation, Resources, Writing – Review & Editing, Project administration, Funding acquisition.

### Funding

This work was supported by The National Key Research and Development Plan (2016YFD0100202 and 2018YFD0100501) and Sichuan Province Breeding Project (2016NYZ0031).

### Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. No conflict of interest exists in the submission of this manuscript.

### Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

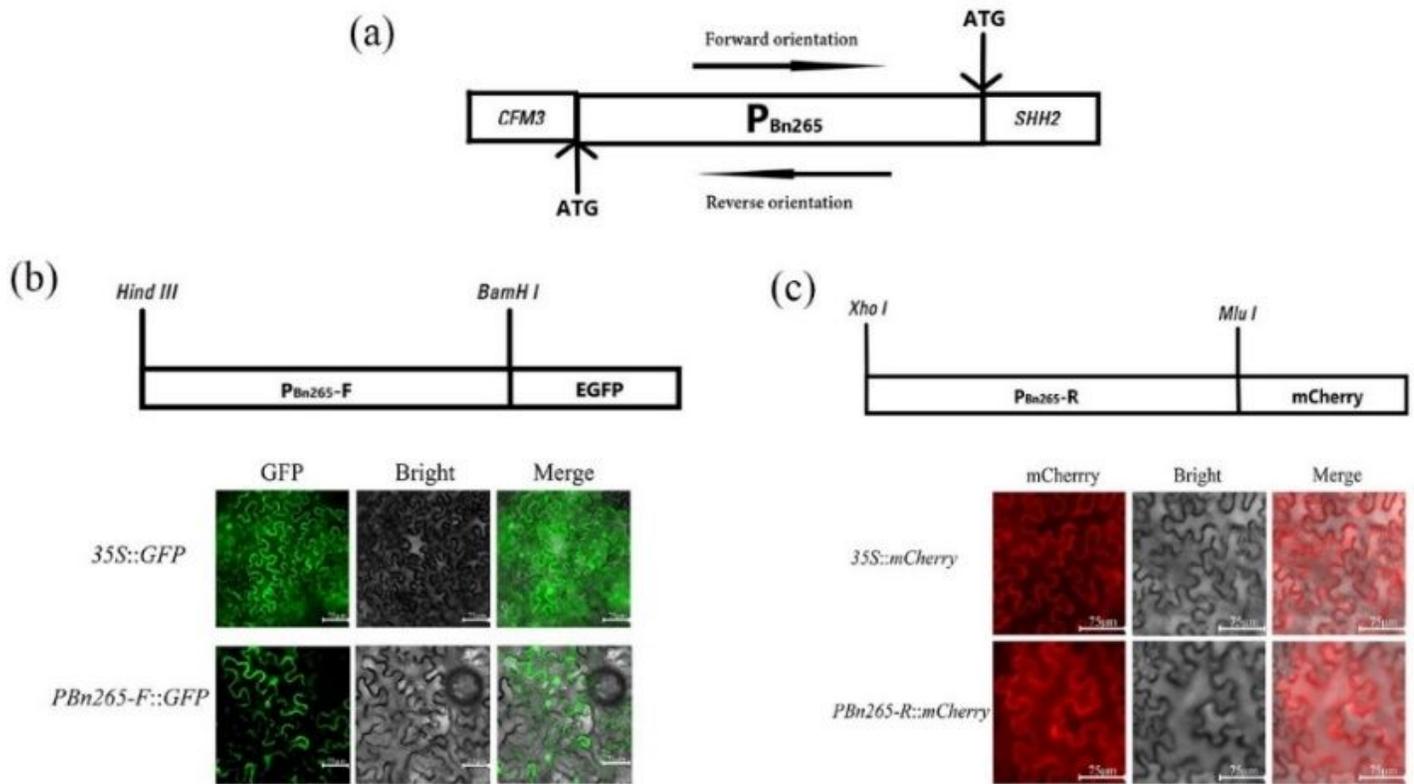
## References

1. Novina, C. D., & Roy, A. L. (1996). Core promoters and transcriptional control. *Trends in genetics : TIG*, *12*, 351-355. [https://doi.org/10.1016/S0168-9525\(96\)80017-5](https://doi.org/10.1016/S0168-9525(96)80017-5).
2. Su-Hyun, P. Yi, N., Kim, Y. S., Jeong, M. H., Bang, S. W., Choi, Y. D., & Kim, J. K. (2010). Analysis of five novel putative constitutive gene promoters in transgenic rice plants. *Journal of Experimental Botany*, *61*, 2459-2467. <https://doi.org/10.1093/jxb/erq076>.
3. Delatorre, C. A., Cohen, Y., Liu, L., Peleg, Z., & Blumwald, E. (2012). The regulation of the SARK promoter activity by hormones and environmental signals. *Plant science*, *193-194*, 39-47. <https://doi.org/10.1016/j.plantsci.2012.05.005>.
4. Nain, V., Verma, A., Kumar, N., Sharma, P., Ramesh, B., Kumar, & P. A. (2008). Cloning of an ovule specific promoter from *Arabidopsis thaliana* and expression of beta-glucuronidase. *Indian journal of experimental biology*, *46*, 207-211.
5. Xi, Y. L., Zhou, P., Song, M. F., Li, Z. Y., Meng, F. H., & Yang, J. P. (2012). Expression Patterns of *Arabidopsis* RBCS-1A Gene in Response to Light Treatments and Application Evaluation of Its Promoter in Transgenic Engineering. *Acta Agronomica Sinica*, *38*, 1561-1569. <https://doi.org/10.3724/SP.J.1006.2012.01561>.
6. Wakano, C., Byun, J. S., Di, L. J., & Gardner, K. (2012). The dual lives of bidirectional promoters. *Biochimica et biophysica acta*, *1819*, 688-693. <https://doi.org/10.1016/j.bbagr.2012.02.006>.
7. Holtorf, S., Apel, K., & Bohlmann, H. (1995). Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. *Plant molecular biology*, *29*, 637-646. <https://doi.org/10.1007/BF00041155>.
8. Vogl, T., Kickenweiz, T., Pitzer, J., Sturmberger, L., Weninger, A., Biggs, B. W., Köhler, E. M., Baumschlager, A., Fischer, J. E., Hyden, P., Wagner, M., Baumann, M., Borth, N., Geier, M., Ajikumar, P. K., & Glieder, A. (2018). Engineered bidirectional promoters enable rapid multi-gene co-expression optimization. *Nature Communications*, *9*, 3589-3617. <https://doi.org/10.1038/s41467-018-05915-w>.
9. Trinklein, N. D., Aldred, S. F., Hartman, S. J., Schroeder, D. I., Otilar, R. P., & Myers, R. M. (2004). An abundance of bidirectional promoters in the human genome. *Genome research*, *14*, 62-66. <https://doi.org/10.1101/gr.1982804>.
10. James, K., Tsiantis, M., Piffanelli, P., Cella, R., Hatzopoulos, P., & Murphy, D. J. (1994). A seed-specific *Brassica napus* oleosin promoter interacts with a G-box-specific protein and may be bi-directional. *Plant Molecular Biology*, *24*, 327-340. <https://doi.org/10.1007/BF00020171>.
11. Alsibai, K. D., Vacher, S., Meseure, D., Nicolas, A., Lae, M., Schnitzler, A., Chemlali, W., Cros, J., Longchamp, E., Cacheux, W., Pignot, G., Callens, C., Pasmant, E., Allory, Y., & Bieche, I. (2019). High Positive Correlations between ANRIL and p16-CDKN2A/p15-CDKN2B/p14-ARF Gene Cluster Overexpression in Multi-Tumor Types Suggest Deregulated Activation of an ANRIL-ARF Bidirectional Promoter. *Non-Coding RNA*, *5*, 44. <https://doi.org/10.3390/Ncrna5030044>.
12. Li, Y. Y., Yu, H., Guo, Z. M., Guo, T. Q., Tu, K., Li, Y. X. (2006). Systematic analysis of head-to-head gene organization: evolutionary conservation and potential biological relevance. *PLoS computational biology*, *2*, e74. <https://doi.org/10.1371/journal.pcbi.0020074>.

13. Liu, S. J., Yue, Q. J., & Zhang, Wei. (2015). Structural and functional analysis of an asymmetric bidirectional promoter in *Arabidopsis thaliana*. *Journal of integrative plant biology*, *57*, 162-170. <https://doi.org/10.1111/jipb.12308>.
14. Fang, Y., Wang X. M., Wang, L., Pan, X. C., Xiao, J., Wang, X., Wu, Y. F., & Zhang W. L. (2016). Functional characterization of open chromatin in bidirectional promoters of rice. *Scientific reports*, *6*, 32088. <https://doi.org/10.1038/srep32088>.
15. Dhadi, S. R., Krom, N., & Ramakrishna, W. (2009). Genome-wide comparative analysis of putative bidirectional promoters from rice, *Arabidopsis* and *Populus*. *Gene*, *429*, 65-73. <https://doi.org/10.1016/j.gene.2008.09.034>.
16. Elison, G. L., Xue, Y., Song R. J. & Acar, M. (2018). Insights into Bidirectional Gene Expression Control Using the Canonical GAL1/GAL10 Promoter. *Cell Reports*, *25*, 737-748. <https://doi.org/10.1016/j.celrep.2018.09.050>.
17. Joydeep, B., Sahoo, D. K., Dey, N., Houtz, R. L., & Maiti, I. B. (2013). An Intergenic Region Shared by At4g35985 and At4g35987 in *Arabidopsis thaliana* Is a Tissue Specific and Stress Inducible Bidirectional Promoter Analyzed in Transgenic *Arabidopsis* and Tobacco Plants. *Plos One*, *8*, e79622. <https://doi.org/10.1371/journal.pone.0079622>.
18. Mitra, A., Han J. G., Zhang, Z. Y. J., & Mitra, A. (2009). The intergenic region of *Arabidopsis thaliana* cab1 and cab2 divergent genes functions as a bidirectional promoter. *Planta*, *229*, 1015-1022. <https://doi.org/10.1007/s00425-008-0859-1>.
19. Mishra, R. C., & Grover, A. (2014). Intergenic Sequence between *Arabidopsis* Caseinolytic Protease B-Cytoplasmic/Heat Shock Protein100 and Choline Kinase Genes Functions as a Heat-Inducible Bidirectional Promoter. *Plant Physiology*, *166*, 1646-1658. <https://doi.org/10.1104/pp.114.250787>.
20. Huang, Y. M., Xiao, B. Z., & Xiong, L. Z. (2007). Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. *Planta*, *226*, 73-85. <https://doi.org/10.1007/s00425-006-0469-8>.
21. Sharma, P., Kumar, V., Singh, S. K., Thakur, S., Siwach, P., Sreenivasulu, Y., Srinivasan, R., & Bhat, S.R. (2017). Promoter Trapping and Deletion Analysis Show *Arabidopsis thaliana* APETALA2 Gene Promoter Is Bidirectional and Functions as a Pollen- and Ovule-Specific Promoter in the Reverse Orientation. *Appl Biochem Biotech*, *182*, 1591-1604. <https://doi.org/10.1007/s12010-017-2420-9>.
22. Yang, J. T., Wang, X. J., Hasi, A., Wang, Z. X., (2018). Structural and Functional Analysis of a Bidirectional Promoter from *Gossypium hirsutum* in *Arabidopsis*. *International journal of molecular sciences*, *19*, 3291-3307. <https://doi.org/10.3390/ijms19113291>.
23. Wang, R., Y. Y., Zhu, M. L., Yang, M., Zhou, F., Chen, H., & Lin, Y. J. (2016). Isolation and Functional Characterization of Bidirectional Promoters in Rice. *Frontiers in plant science*, *7*, 766. <https://doi.org/10.3389/fpls.2016.00766>.
24. Bai, J. Y., Wang, X., Wu, H., Ling, F., Zhao, Y., Lin, Y. J., & Wang, R., (2020). Comprehensive construction strategy of bidirectional green tissue-specific synthetic promoters. *Plant Biotechnology Journal*. *18*, 668-678. <https://doi:10.1111/pbi.13231>.

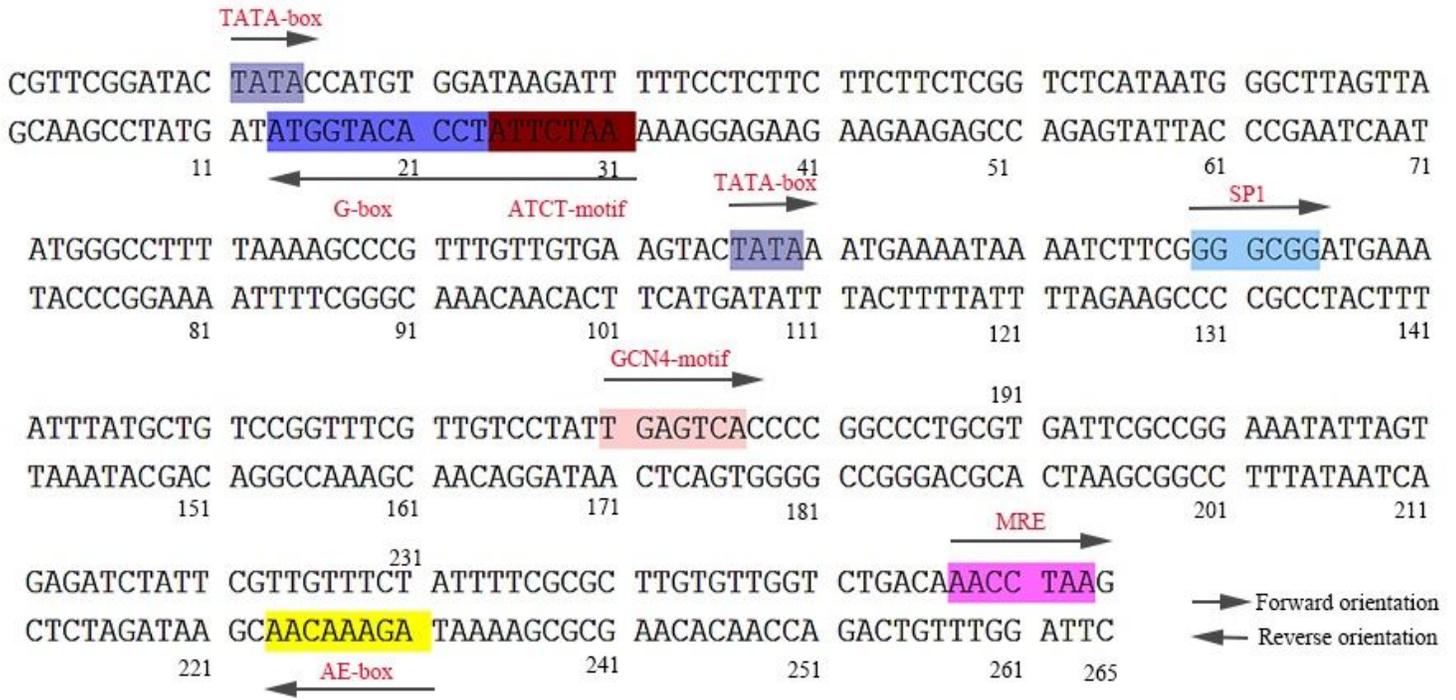
25. Im, J. H., & Yoo, S. D. (2014). Transient expression in Arabidopsis leaf mesophyll protoplast system for cell-based functional analysis of MAPK cascades signaling. *Methods in molecular biology*, 1171, 3-12. [https://doi.org/10.1007/978-1-4939-0922-3\\_1](https://doi.org/10.1007/978-1-4939-0922-3_1).
26. Law, J. A., Du, J. M., Hale, C. J., Feng, S.H., Krajewski, K., Palanca, A. M. S., Strahl, B. D., Patel, D. J., Jacobsen, & S. E. (2013). Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. *Nature*, 498, 385-389. <https://doi.org/10.1038/nature12178>.
27. Law, J. A., Ausin, I., Johnson, L. M., Vashisht, A. A., Zhu, J. K., Wohlschlegel, J. A., & Jacobsen, S.E. (2010). A protein complex required for polymerase V transcripts and RNA- directed DNA methylation in Arabidopsis. *Current biology : CB*, 20, 951-956. <https://doi.org/10.1016/j.cub.2010.03.062>.
28. Yukari, A., & Barkan, A. (2007). A CRM Domain Protein Functions Dually in Group I and Group II Intron Splicing in Land Plant Chloroplasts. *The Plant Cell*, 19, 3864-3875. <https://doi.org/10.1105/tpc.107.055160>.
29. Morden, C. W., Wolfe, K. H., dePamphilis, C. W., & Palmer, J. D. (1991). Plastid translation and transcription genes in a non-photosynthetic plant: intact, missing and pseudo genes. *The EMBO journal*, 10, 3281-3288. <https://doi.org/10.1089/dna.1991.10.695>.
30. Huang, H. Q., L. Y. J. (2007). Cloning and Functional Analysis of the Rice rbcS Gene Promoter. *Journal of Agricultural Biotechnology*, 15, 451-458.
31. Adachi, N., & Lieber, M. R. (2002). Bidirectional gene organization: a common architectural feature of the human genome. *Cell*, 109, 807-809. [https://doi.org/10.1016/s0092-8674\(02\)00758-4](https://doi.org/10.1016/s0092-8674(02)00758-4).
32. Takai, D., & Jones, P. A. (2004). Origins of Bidirectional Promoters: Computational Analyses of Intergenic Distance in the Human Genome. *Molecular Biology & Evolution*, 21, 463-467. <https://doi.org/10.1093/molbev/msh040>.
33. Yang, M. Q., & Elnitski, L. (2008). Diversity of core promoter elements comprising human bidirectional promoters. *BMC Genomics*, 9, S3. <https://doi.org/10.1186/1471-2164-9-S2-S3>.

## Figures



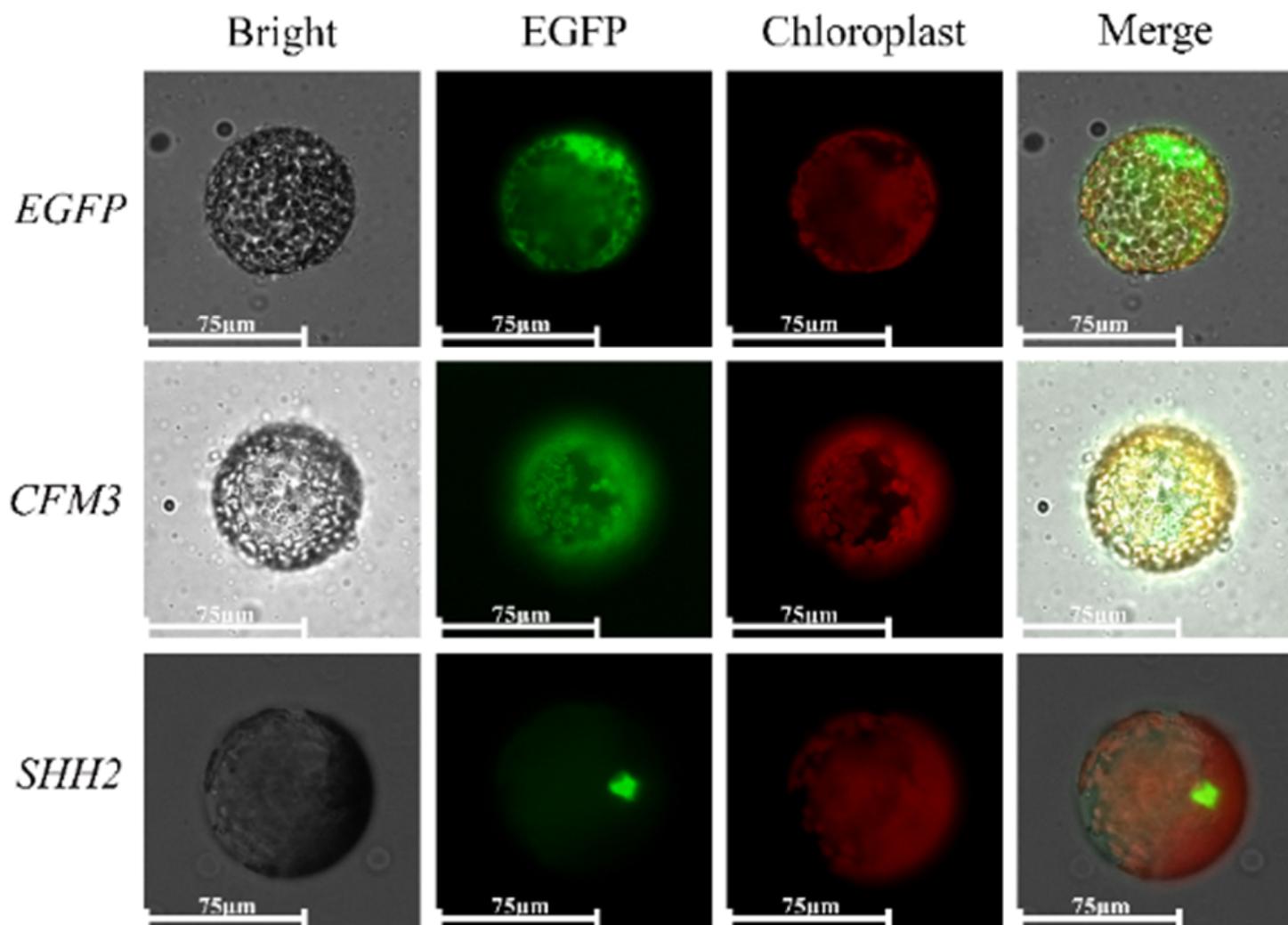
**Figure 1**

A schematic presentation of  $P_{Bn265}$ , promoter::reporter constructs and transient expression analysis using two reporter genes (EGFP and mCherry) in both orientations. a A schematic presentation of the bidirectional promoter ( $P_{Bn265}$ ) located in Brassica napus chromosome A1, drives two "head-to-head" genes, *CFM3* (27969316..27973484) and *SHH2* (27973609..27976028), expressing in different orientations. b, c Schematic diagram of bidirectional promoter::reporter gene constructs:  $P_{Bn265F}::EGFP$  and  $P_{Bn265R}::mCherry$  for transient Agro-infiltration experiments. Below each construct a representative assay of transient EGFP and mCherry expression detected in fluorescence imaging by *Agrobacterium tumefaciens* infiltration assay in *N. benthamiana* leaf are shown for respective promoter  $P_{Bn265-F}$  and  $P_{Bn265-R}$  activities. (bar represents 75  $\mu$ m)



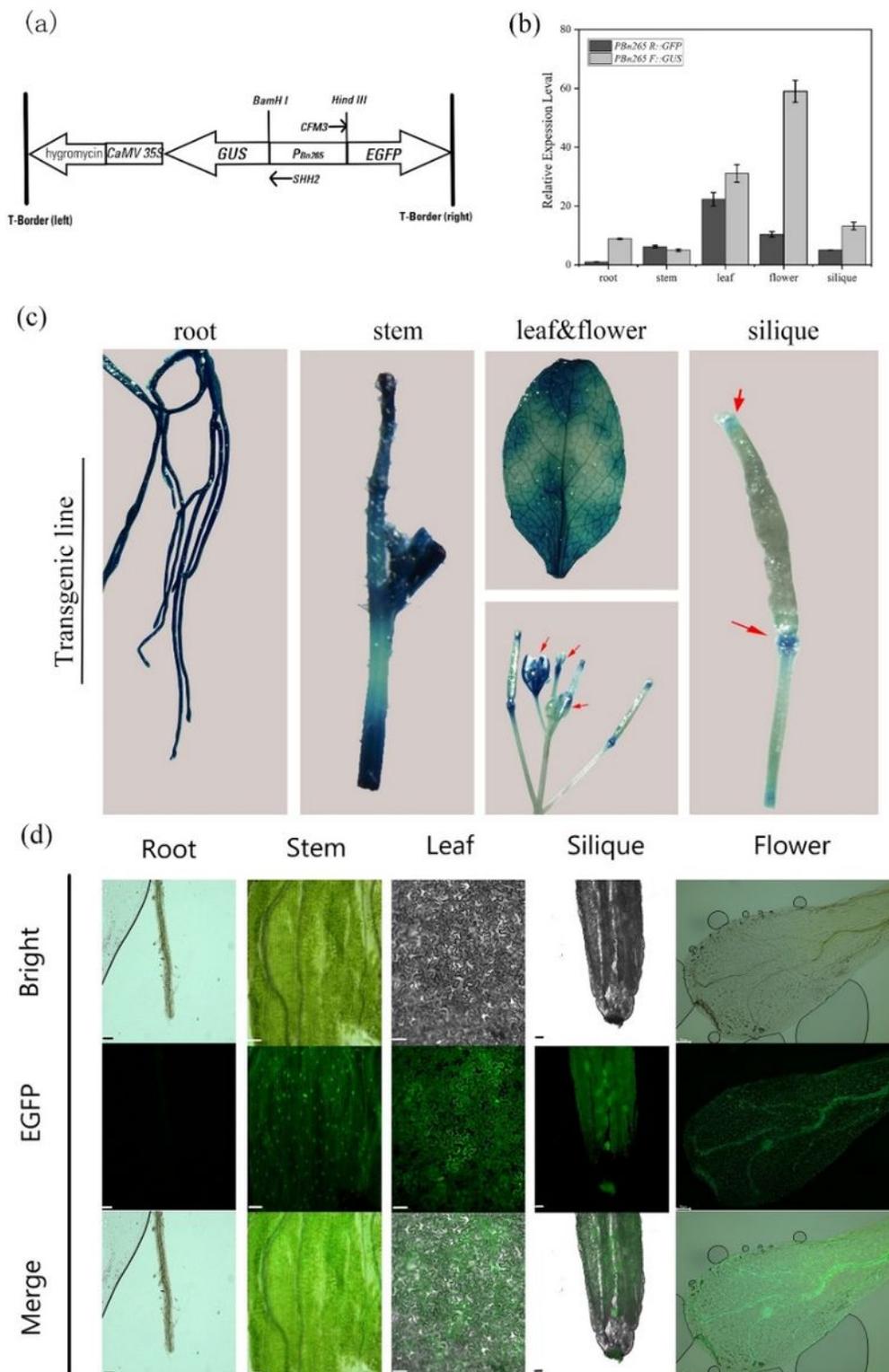
**Figure 2**

The double strand sequence of PBN265. The cis-elements found in the analyzed region are indicated in the figure. The forward orientation is upstream of SHH2 and the reverse orientation is upstream of CFM3 and the immediate upstream nucleotide of the start codon of CFM3 is designated as position 1 and the immediate upstream nucleotide of the start codon of SHH2 is designated as position 265. Two TATA boxes have been identified. G-box is cis-acting regulatory element involved in light responsiveness, ATCT-motif is part of a conserved DNA module involved in light responsiveness, SP1 is a light responsive element, GCN4-motif is cis-regulatory element involved in endosperm expression, MRE is binding site involved in light responsiveness and AE-box is part of a module for light response



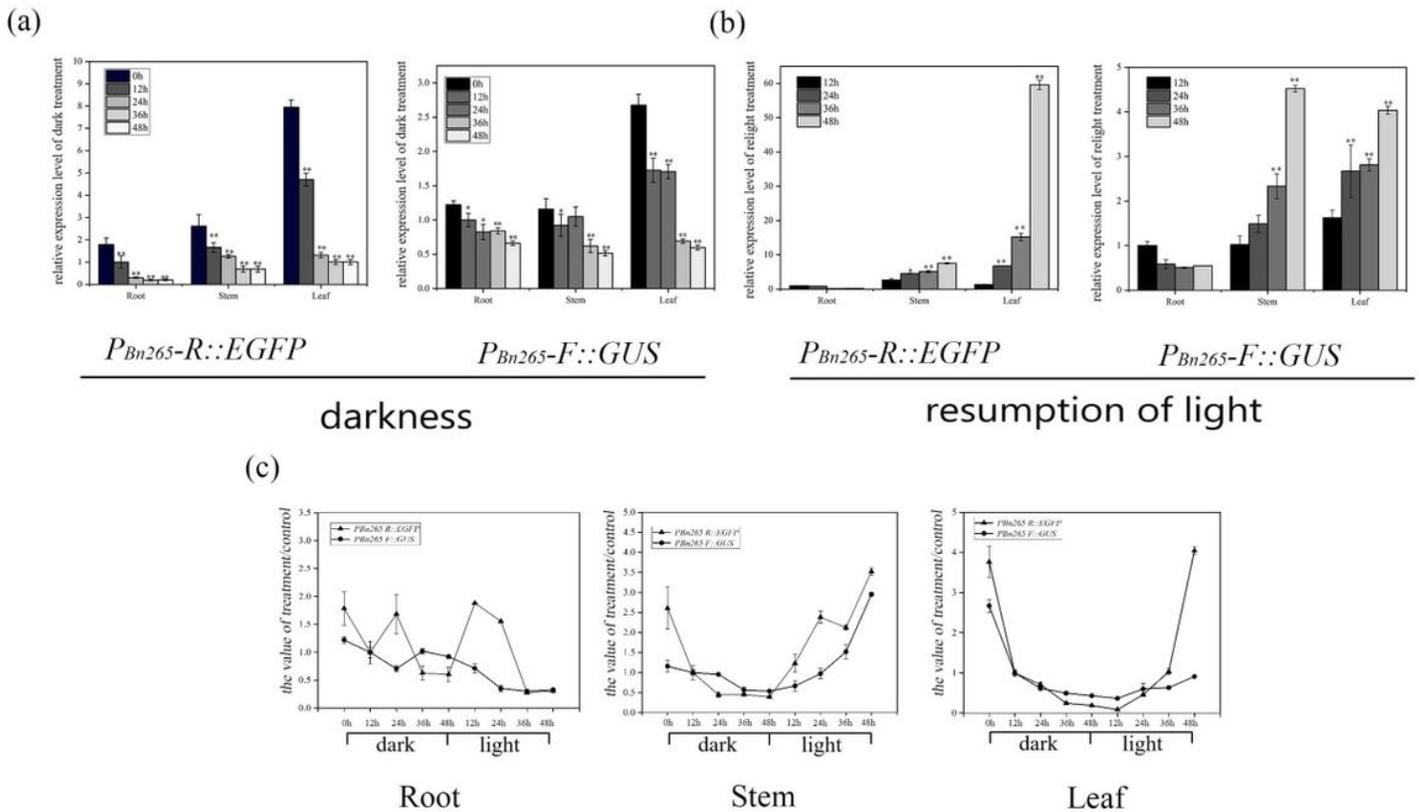
**Figure 3**

Subcellular localization of SHH2 and CFM3. Transient EGFP expression detected in fluorescence imaging of tobacco protoplast using pBI221-CFM3 and pBI221-SHH2 (bar represents 75µm)



**Figure 4**

Expression of EGFP and GUS in transgenic Arabidopsis. a A schematic presentation of pDX-EGFP::PBn265::GUS. b relative expression level of GUS and EGFP in root, stem, leaf, flower and silique at flowering stage in transgenic plants. c Histochemical staining of root, stem, leaf, flower and silique, red arrows point to the area which dyed blue. d Fluorescence detection of root, stem, leaf, flower and silique at mature stage in transgenic plants. Scale bars=75 $\mu$ m



**Figure 5**

The relative expression level and changing trend of GUS and EGFP in transgenic plants after darkness and relight treatment. a Relative expression level of GUS and EGFP in transgenic line's root, stem and leaf under a continuous darkness. EGFP/GUS relative expression level was standardized by the EGFP/GUS expression in root which was treated for 12h. The Actin2 was used as an internal control. Significance analysis was conducted by independent-samples t-test. \*  $p < 0.05$ ; \*\*  $p < 0.01$ . Error bars indicate standard error based on three independent biological replicates. b Relative expression level of GUS and EGFP in transgenic line's root, stem and leaf under resumption of light. EGFP/GUS relative expression level was standardized by the EGFP/GUS expression in root which was treated for 12h. The Actin2 was used as an internal control. Significance analysis was conducted by independent-samples t-test. \*  $p < 0.05$ ; \*\*  $p < 0.01$ . Error bars indicate standard error based on three independent biological replicates. c The change trend of EGFP and GUS in transgenic line after darkness and relight treatment. Continuous darkness treatment for 0, 12, 24, 36 and 48 h and then relight for 12, 24, 36 and 48 h. All the data were normalized by the ratio of GUS and EGFP genes expression level in treatment group to the counterpart in control along treatment time

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementarymaterials.docx](#)