Identification and Characterization of *DIGI* Promoter Involved in Photoperiod, Light Intensity, Hormone, and DIELF4 Response from Longan

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ADDITIONAL INDEX WORDS. circadian rhythm, *Dimocarpous longan*, ELF4, flowering induction, GI

ABSTRACT. In fruit trees, flowering is a key event followed by fruit development and seed production. *Gigetea* (*GI*), a clock-associated gene, is known to contribute to photoperiodic flowering and circadian clock control in *Arabidopsis thaliana*. However, its functions in woody fruit trees remain unclear. In this study, a 2000 bp promoter fragment of the longan (*Dimocarpous longan*) *DIGI* gene was isolated from the genomic DNA of longan ‘Honghezi’ by polymerase chain reaction amplification. The *DIGI* promoter contained two main types of potential *cis*-acting elements: light-responsive and hormone-responsive elements. The promoter was fused with the β -glucuronidase (*GUS*) reporter gene of pBI121 to generate the pDIGI:GUS construct. *GUS* histochemical staining of transgenic *A. thaliana* revealed that *DIGI* might play a role in different developmental phases of longan. Exposure of transgenic *A. thaliana* to varying light intensities showed that the *GUS* activity increases with increased light intensity. Transient expression of pDIGI::GUS in *Nicotiana benthamiana* showed that the *GUS* activity was higher and reached peak a few hours earlier under short-day (SD) than long-day conditions. Exposure to different hormonal treatments revealed that the transcript level of *GUS* was activated by gibberellin (GA₃) and indoleacetic acid (IAA) but suppressed by abscisic acid and methyl jasmonate treatment. In addition, *N. benthamiana* transient assay and dual-luciferase assay revealed that the presence of *early flowering 4* (*ELF4*) homologs of longan (*DIELF4-1* and *DIELF4-2*) significantly activated the *DIGI* promoter. The positive response of *DIGI* promoter to high light-intensity, SD photoperiod, GA₃ and IAA signals, and *DIELF4* transcription factor suggest that *DIGI* may function as a circadian clock and play a role in responding to SD conditions and other signals in flower initiation of longan.

Dimocarpus longan (longan) is an evergreen fruit tree that belongs to the Sapindaceae family and grows well in many tropical and subtropical regions of the world. China is top-ranked in terms of the plantation area and fruit production of longan (Matsumoto, 2006; Wu, 2008). Flowering is a crucial developmental phase of plant life, especially in the case of fruit trees, for which the transition of flowering is essential for the fruit set and ripening process (Peng et al., 2008). Generally, longan trees have a single spring flowering period. However, one cultivar of longan, Sijimi, originating in China (Guangxi province), has a perpetual flowering trait and has been successfully used to produce off-season fruit without any additional environmental stimuli (Jia et al., 2014). RNA sequencing results revealed that there were several flowering-time homologs in longan, including *gigetea* (*GI*) and *early flowering 4* (*ELF4*), which could be involved in the perpetual flowering traits of ‘Sijimi’ (Jia et al., 2014). Moreover, our previous studies showed that the longan *GI* gene (*DIGI*) has a higher expression level during the floral bud physiological differentiation stage (Huang et al., 2017). *DIGI* is supposed to be involved in the regulation of physiological differentiation of floral buds, inducing floral initiation by affecting endogenous auxin synthesis and transport in longan (Huang et al., 2017).

Multiple flowering time pathways are controlling the vegetative-to-reproductive phase transition, and *GI* is proposed to have essential functions in promoting the photoperiodic flowering pathways (Mishra and Panigrahi, 2015). In *Arabidopsis thaliana*, *GI* plays a vital role in the regulation of flowering time by controlling the daily endogenous rhythms, also known as circadian rhythms (Dodd et al., 2005). *GI* was found to induce flowering in long-day (LD) photoperiods by regulating miR172 accumulation (Jung et al., 2007). Plants having a defect in maintaining circadian rhythm usually show altered flowering phenotypes (Imaizumi, 2010), and defects in circadian clock components have been shown to affect *GI* transcription (Mishra and Panigrahi, 2015). The overexpression of *GI* exhibited early flowering phenotypes (Mishra and Panigrahi, 2015). While mutation in *GI* delays flowering under LD conditions (Park et al., 1999), short circadian periods (Mizoguchi et al., 2005), long hypocotyls (Araki and Komeda, 1993), and sensitivity to low-temperature exposures (Cao et al., 2005).

Recent studies demonstrated that internal and external signals affect *GI* expression in *A. thaliana* (Berns et al., 2014). The transcription of the *GI* gene is activated by exposure to light and regulated by the circadian clock (Patiel et al., 2006; Sawa and Kay, 2011). *GI* participates in flowering induction by extending photoperiods; therefore, *GI* is a key component in the mechanism leading to day-length discrimination (Sawa et al., 2007). Mutations within the components of the morning loop, such as CCA1 and LHY, result in *GI* transcription to occur earlier in the circadian cycle, suggesting that these proteins repress or delays the *GI* transcription when they are expressed in the morning (Mizoguchi et al., 2002). The CCA1 and LHY bind to several

Received for publication 13 May 2020. Accepted for publication 16 June 2020.
Published online 10 September 2020.

This work was supported by The Construction of Plateau Discipline of Fujian Province (102/71201801101), China.

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motifs that are closely associated in sequence, including “evening element” (EE) (Harmer and Kay, 2005; Mikkelsen and Thomashow, 2009). In addition to light regulation, EE is also found in close proximity of abscisic acid (ABA) ABREL motifs (Mikkelsen and Thomashow, 2009). Berns et al. (2014) suggested a composite effect of ABREs and EE that contributes to generating the observed pattern of *GI* transcription in *A. thaliana* in the evening. *ELF4*, another essential component of circadian clock input pathways (McWatters et al., 2007). Overexpression of *ELF4* shows delayed flowering signs in *A. thaliana*, while early flowering was observed in *ELF4*-deficient mutants (McWatters et al., 2007). In *A. thaliana*, *ELF4* has been proposed to interact with *GI* to control the hypocotyl elongation and flowering time (Kim et al., 2012). *ELF4* is epistatic to *GI* during hypocotyl elongation control, whereas *GI* is epistatic to *ELF4* regarding flowering time regulation (Kim et al., 2012). A study in pea (*Pisum sativum*) supposed that *Late bloomer 1* (*LATE1*) and *Die neutralis* (*DNE*), orthologs of *ELF4* and *GI*, respectively, interact genetically to regulate flowering time (Liew et al., 2009).

Flowering induction in perennial fruit trees is much different from annual plants. Perennial plants sense the seasonal changes and initiate flowering. However, up to now, understanding of the functions of *GI* homologs in perennial fruit trees have been limited. On the basis of previous research, we speculate that *DIGI* may play a role in longan physiological differentiation of floral buds by sensing internal and external signals. It would be interesting to investigate the upstream regulatory factors of *DIGI*. Therefore, in this study, the promoter of *DIGI* gene was cloned, and bioinformatics analysis, *A. thaliana* stable transformation, *Nicotiana benthamiana* transient cotransformation, and dual luciferase assay were conducted. The results show that *DIGI* responded to photoperiod, light intensity, hormones, and *DIELF4* genes. These findings will contribute to a better understanding of *DIGI* functions and the molecular mechanism of flowering induction in perennial fruit trees.

Materials and Methods

PLANT MATERIAL. The seeds of *N. benthamiana* were grown in pots containing sterilized compost mix and maintained in a growth chamber. Young *N. benthamiana* leaves were used for transient expression. The *A. thaliana* ecotype Columbia (Col-0) seeds were directly grown in plug trays containing compost mix and maintained in a growth chamber at 22 to 25 °C, 50% to 70% humidity, and a 16-h photoperiod.

ISOLATION OF *DIGI* PROMOTER AND BIOINFORMATIC ANALYSIS. The *DIGI* promoter sequence was amplified (for primers, see Supplemental Table 1) from longan genomic DNA that had been extracted from leaf samples using the cetyltrimethylammonium bromide method (Turaki et al., 2017). The polymerase chain reaction (PCR) reaction for *DIGI* promoter was executed as per the following protocol: initial preheating for 30 s at 94 °C; 35 cycles of 94 °C for 30 s, 61 °C for 90 s, and 72 °C for 2 min; and a final elongation step of 72 °C for 5 min. The amplified fragment was cloned into the pMD-18-T vector, and positive colonies were selected at random and sent for sequencing to Biosune, Shanghai, China. PLACE (Higo et al., 1998) and PlantCARE (Lescot et al., 2002) databases were used for predicting the regulatory elements and potential core sequences of *DIGI* promoter.

PLASMID CONSTRUCTION. The *DIGI* promoter was cloned into the plant expression vector pBI121, which contained *GUS* as a reporter gene, using an In-Fusion HD cloning kit (Clontech

Laboratories, Beijing, China). The pBI-121 plasmid was digested with restriction endonuclease *HindIII* and *EcoRI*. The cut product was recovered by the gel electrophoresis and ligated with the *DIGI* promoter to obtain a recombinant plasmid pDIGI::GUS. The verified pDIGI::GUS construct was transformed into a *Agrobacterium tumefaciens* GV3101. The pBI121-*DIELF4-1* and pCambia2300-*DIELF4-2* plasmids (Fu et al., 2018) were also transformed into *A. tumefaciens* GV3101.

***A. THALIANA* TRANSFORMATION.** The pDIGI::GUS transgenic *A. thaliana* lines were established using the floral dip method on the development of inflorescence stems, described by Zhang et al. (2006). The putative transformants were selected on Murashige and Skoog (MS) medium with (50 mg·L⁻¹) kanamycin. Cetyltriethylammonium was used to extract genomic DNA from the leaf tissues of transformed plants (Wang et al., 1996). PCR analysis was performed to verify the transgenic plantlets by using *DIGI* promoter specific-primers (F: 5' CGCGACTAACATGTATCAATGGTC-3'; R: 5'-CCGCGTATAGGAAAA-CAAAGAGG-3'). Plants corresponding to the T3 generation were selected for further experiments.

***GUS* ACTIVITY ASSAY.** *GUS* activity was visualized histochemically using the chromogenic substrate 5-bromo-4-chloro-3-indole-β-D-glucuronide (X-Gluc) for staining, according to the method described by Jefferson et al. (1987). Transgenic *A. thaliana* plants were incubated in *GUS*-staining solution (2 mM K₃Fe(CN)₆, 1 mM X-Gluc, 2 mM K₄Fe(CN)₆, 100 mg·mL⁻¹ chloramphenicol in 50 mM NaH₂PO₄ buffer (pH 7.0), 0.1% (v/v) Triton X-100; Sigma-Aldrich, St. Louis, MO), for 2 to 3 d at 37 °C. Plant material was then incubated in 100% ethanol for 4 to 5 h to remove chlorophyll from the plant material.

ACTIVITY OF *DIGI* PROMOTER IN *A. THALIANA*. We selected six independent T2 transgenic lines (genetically unique) for each treatment. The T3 seeds (six lines of each antibiotic-resistant line) obtained were further selected on MS medium supplemented with 50 mg·L⁻¹ kanamycin to obtain plants that were homozygous. Ten-day-old T3 resistant seedlings were transferred into the pots containing the compost mix for further expression analysis at lateral developmental stages and incubated them in the *GUS* substrate as previously described. *GUS* activity sites were observed by a stereo fluorescence microscope (SZX16; Olympus, Tokyo, Japan).

RESPONSE OF *DIGI* PROMOTER TO DIFFERENT INTENSITIES OF LIGHT. The 7-d-old T3 transgenic *A. thaliana* plants were kept in growth chambers for 3 d at 22 °C under different light intensities, including high light (1100 μmol·m⁻²·s⁻¹), weak light (400 μmol·m⁻²·s⁻¹), and dark conditions.

***N. BENTHAMIANA* TRANSIENT TRANSFORMATION.** For photoperiod treatment, *N. benthamiana* plants leaves were infiltrated with *A. tumefaciens* harboring vector pDIGI::GUS and kept either in LD (16 h/8 h light-dark) conditions with the photoperiod starting at 0800 HR and ending at 0000 HR, or in SD (8 h/16 h light-dark) with the photoperiod starting at 0800 HR and ending at 1600 HR, for 72 h at 22 °C. To observe the effect of different hormones on the transcriptional activity of *GUS*, *N. benthamiana* leaves were infiltrated with *A. tumefaciens* harboring pDIGI::GUS construct and sprayed with hormone treatment [34.6 μM gibberellin (GA₃), 8.6 μM IAA, 75.7 μM ABA, 100 μM methyl jasmonate (MeJA)] and sterile water for the control.

To investigate the regulation of *DIELF4* by *DIGI* promoter, *A. tumefaciens* strains containing either of *DIELF4* genes or pDIGI::GUS were separately cultured and diluted to measure the absorbance at a wavelength at A₆₀₀ of 0.6 to 0.8.

Stress-related elements were also found in the promoter region, for example, LTR, a *cis*-acting element involved in low-temperature responsiveness.

SPATIOTEMPORAL EXPRESSION OF THE *DLGI* PROMOTER IN *A. THALIANA*. To examine the activity of *DLGI* promoter in T3 transgenic *A. thaliana*, the 2000-bp promoter fragment driven by the *GUS* reporter gene was monitored using histochemical staining during different developmental stages of plant growth. Six-day-old transgenic *A. thaliana* seedlings grown on MS medium were strongly stained, and the whole plantlet showed the *GUS* activity except roots (Fig. 2A). Similarly, *GUS* activity was observed in 14-

d-old plants (Fig. 2B). *GUS* activity was also detected in the flower buds of 21-d-old transgenic *A. thaliana* (Fig. 2C). Strong *GUS* staining was observed in the sepals, petals, and stigma of opened flowers of transgenic *A. thaliana*, but no *GUS* staining was observed in the anthers (Fig. 2D). *GUS* staining was also found in the siliques of 40-d-old transgenic *A. thaliana* (Fig. 2E). As expected, *GUS* staining was not observed in wild-type *A. thaliana* plants (Fig. 2F).

RESPONSE OF *DLGI* PROMOTER TO DIFFERENT INTENSITIES OF LIGHT. Bioinformatical analysis of the *DLGI* promoter sequence revealed that it contains many motifs that are well characterized for their response to light (Table 1), suggesting that different

Fig. 2. Histochemical localization of *GUS* activity in transgenic *Arabidopsis thaliana* plants carrying the pDLGI::GUS fusion construct: (A) *GUS* staining localized in the cotyledons of 7-d-old seedlings; (B) 15-d-old plant, showing strong *GUS* expression in the cotyledons, hypocotyls, and leaves; (C) *GUS* staining observed in 21-d-old flower bud; (D) *GUS* staining localized in flowers (sepals, petals, and stigma) of a 26- to 30-d-old plant; (E) 40-d-old plant siliques showing *GUS* expression; and (F) no expression in wild type *A. thaliana*.

Table 1. Prediction of regulatory elements in the 5-flanking sequence of *DLGI* promoter.

Regulatory element	Function	No.	Sequence
UTR Py-rich stretch	<i>Cis</i> -acting element conferring high transcription levels	1	TTTCTTCTCT
ABRE	<i>Cis</i> -acting element involved in the abscisic acid responsiveness	2	ACGTG
ARE	<i>Cis</i> -acting regulatory element essential for the anaerobic induction	2	AAACCA
Box 4	Part of a conserved DNA module involved in light responsiveness	2	ATTAAT
Box I	Light responsive element	1	TTTCAAA
CGTCA-motif	<i>Cis</i> -acting regulatory element involved in the meja-responsiveness	3	CGTCA/CGTCA
G-box	<i>Cis</i> -acting regulatory element involved in light responsiveness	3	CACGTA
GAG-motif	Part of a light responsive element	1	GGAGATG
GARE-motif	Gibberellin-responsive element	2	AAACAGA
GT1-motif	Light responsive element	2	GTGTGTGAA
Sp1	Light responsive element	1	CC(G/A)CCC
LTR	<i>Cis</i> -acting element involved in low-temperature responsiveness	1	CCGAAA
TGA-box	Part of an auxin-responsive element	1	TGACGTGGC
box II	Part of a light responsive element	1	TCCACGTGGC
O2-site	<i>Cis</i> -acting regulatory element involved in zein metabolism regulation	1	GATGACATGG
EE-motif	Evening elements	1	AAAATATCT
Skn-1-motif	<i>Cis</i> -acting regulatory element required for endosperm expression	1	GTCAT

intensities of light might affect the activity of the *DIGI* promoter. To validate this hypothesis, pDIGI::GUS activity was measured under different light intensities. Strong *GUS* activity was detected in seedlings grown under high light intensity, except for the root apex, which did not show any activity (Fig. 3A). Seedlings from reduced light intensity also showed *GUS* activity in cotyledons and the hypocotyl, but the activity was weaker compared with plants grown in high light intensity (Fig. 3B). Reduced *GUS* activity appeared in the seedlings grown in dark conditions (Fig. 3C). Wild-type *A. thaliana* was used as a negative control and did not show any *GUS* activity when grown under a high light intensity (Fig. 3D).

EFFECT OF DIFFERENT PHOTOPERIODS ON *DIGI* PROMOTER. *GI* is considered to be an essential clock gene due to its significance in circadian clock regulation. The transient assays were performed to detect the pDIGI::GUS expression under different photoperiods. The results showed that *GUS* expression was relatively higher in SD conditions compared with LD photoperiods (Fig. 4A). *GUS* expression was also measured at different points of the day under both LD and SD photoperiod systems. We found that pDIGI::GUS expression peaks at 1200 HR in SD conditions, whereas in LD, the highest expression was observed ≈ 1600 HR. Under the LD photoperiod system, *GUS* expression was delayed by a few hours (i.e., the peak was found at a later time point) compared with the SD photoperiod system. Under both photoperiods, the expression levels were lowest in the morning but increased during the day (before dusk) to a

peak value and then decreased to a minimum during the dark period (Fig. 4B). These results depict that variation in daily rhythms and photoperiods has definite effects on the expression of *DIGI*.

EFFECT OF DIFFERENT HORMONES ON *DIGI* PROMOTER. Inspection of the *DIGI* promoter sequence revealed that it contains a few *cis*-acting elements that are responsive to hormones, such as gibberellin (GARE-motif), auxin (TGA-box), ABA (ABRE), and MeJA (CGTCA-motif). We performed qPCR to detect the pDIGI::GUS expression under different hormonal treatments. GA₃ showed the highest *GUS* expression compared with other hormone treatments, with a 3-fold higher change than the control. *GUS* expression was also induced by IAA, with an up to 1.5-fold higher change compared with the control (Fig. 5). In contrast, *GUS* expression was repressed by ABA and MeJA (Fig. 4).

***DIELF4* INCREASES THE TRANSCRIPTIONAL ACTIVITY OF *DIGI* PROMOTER.** The *ELF4* gene is known to be involved in many of the same physiological processes as *GI*. We examined whether there is any direct interaction between *DIELF4* and *DIGI*. Two *DIELF4* genes, *DIELF4-1* and *DIELF4-2*, were injected with pDIGI::GUS, respectively, and *GUS* expression was assayed by qPCR. Results showed that both *DIELF4* genes significantly increased the transcriptional activity of the *DIGI* promoter, especially *DIELF4-2* (Fig. 6A). Furthermore, a dual luciferase assay was performed to verify the interaction between *DIGI* promoter and *DIELF4*. The results were consistent with the

Fig. 3. *GUS* expression in transgenic *Arabidopsis thaliana* seedlings under different light conditions: (A) high light ($1100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); (B) weak light ($400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); (C) dark conditions; (D) *GUS* staining in wild type *A. thaliana* (negative control); (E) level of *GUS* transcript in cotyledon, hypocotyle, and root under different intensity of light. Bars indicate the SE.

Fig. 4. Effect of different photoperiod on the activity of *DIGI* promoter infiltrated in *Nicotiana benthamiana* leaves: (A) *DIGI* promoter activity under long day (16/8 h light/dark) and short day (8/16 h light/dark) photoperiod; (B) *DIGI* promoter activity measured at different points of the day. The bars indicate the SE of three biological replicates. The level of significant differences is indicated with an asterisk (*) and were assessed by *t* test (* $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$).

Fig. 5. Response of the *DIGI* promoter to different hormones. Leaves of *Nicotiana benthamiana* were infiltrated with the construct pDIGI::GUS under the treatments of 8.6 μM auxin, 34.6 μM gibberellin, 100 μM methyl jasmonate, 75.7 μM abscisic acid, and water, as a control (CK). Bars indicate the SE of three biological replicates. Letters represents a significant difference at the level of $P < 0.05$ using least significant difference.

GUS activity assay, showing that both *DIELF4* genes increase *DIGI* promoter activity: *DIELF4-2* induced 5-fold higher activity and *DIELF4-1* induced 3-fold higher luciferase activity than the control (Fig. 6B).

Discussion

The plant clock uses daily rhythms and recognizes photoperiods in various developmental processes, from seedling growth to flowering (Kim et al., 2012). The expression profile of the *DIGI* promoter in transgenic *A. thaliana* suggested that

Fig. 6. *DIELF4* increase the transcriptional activity of *DIGI* promoter: (A) pDIGI::GUS coinfiltrated with *DIELF4-1* and *DIELF4-2* separately in *Nicotiana benthamiana* leaves. (B) The firefly luciferase (LUC) and *Renilla* luciferase (REN) assay to study the interaction between *DIGI* promoter and *DIELF4* genes. The bars indicate the SE of three biological replicates. Letter represents a significant difference at the level of $P < 0.05$ using least significant difference statistical analysis.

DIGI might play a broader role in different organs and developmental phases of plant growth in longan. A similar kind of *GI* expression pattern has been observed in *A. thaliana* (David et al., 2006), *Oryza sativa* (Hayama et al., 2002), and *Glycine max* (Li et al., 2013), suggesting *DIGI* acts as a circadian clock in longan. However, in contrast to *GI* in *A. thaliana*, *DIGI* has a higher expression level in SD conditions, and the expression level reaches its peak earlier in the day under SD conditions compared with LD, suggesting that the expression levels of *DIGI* are greatly affected by SD-length. Huang et al. (2017) found that *DIGI* might be involved in the regulation of the physiological differentiation of floral buds. The initiation of flowering in longan occurs during autumn in China, when the photoperiod becomes shorter; therefore, the promoter of *DIGI* has higher activity under SD conditions, suggesting that *DIGI* may play a role in responding to SD conditions and inducing flower initiation in longan. Variation in *GUS* activity under different photoperiods indicates that the conserved motif presented upstream of *DIGI* may contribute to maximizing the expression at a precise time of the day, thereby exerting control over flowering time. The EE motif present in the promoter represses the transcription of *GI* during the morning

and increases the amplitude of expression in the evening (Berns et al., 2014). The ABREs are predicted to be recognized by bZIP transcription factors, several of which are implicated in light induction of gene transcription (Chattopadhyay et al., 1998). Thus, the contribution of such elements provides a clue in controlling the distinct subsets of a gene by function, possibly due to differing requirements at different times in the day–night cycle.

Light is a well-known environmental factor that plays a substantial role in controlling flowering time in different plants through applied photoperiod, which depends upon the light quality (wavelength) and intensity (Cerdán and Chory, 2003; Lu et al., 2017). Early flowering was observed in 11 herbaceous plant species when they were subjected for 18 h daily to high-intensity lamps, in contrast to plants grown in an ambient environment (Erwin and Warner, 2000). Light quality has a significant role in flower bud initiation and the transition from vegetative to reproductive state in many fruit crops (Wilkie et al., 2008). Several studies in *A. thaliana* have revealed that the light quantity and quality affect the *GI* transcription (Fowler et al., 1999; Mishra and Panigrahi, 2015). Our results showed that pDIGI::GUS activity was predominantly induced by high-intensity light, compared with low light and dark conditions (Fig. 3A–C), suggesting the expression level of *DIGI* is upregulated by high light intensity. Recent research on fruit crop flowering has indicated that clock genes involved in flower initiation may generally be mediated by both light quality and quantity (Jackson et al., 2011). Flower bud formation in *Malus × domestica* is affected by different intensities of light, as fewer flower buds were formed when the light intensity was reduced for 7 weeks (Tromp, 1983). However, the relationship between light intensity and flowering remains unknown in longan and needs further investigation. Light-responsive *cis*-elements upstream of *DIGI* may play crucial roles in the response of *GUS* gene expression in the transgenic seedlings. Studies related to promoters of light-regulated genes have revealed the presence of different LREs, such as GATA, GT1, and G-box, which play vital roles in light-regulated transcription activity (Yadav et al., 2002). The two most common light-responsive *cis*-elements in the promoter sequence of *DIGI* were the G-box, also found in the promoter of photosynthesis-related genes involved in light regulation (López-Ochoa et al., 2007), and the GT-1 motif, which is involved in the regulation of light responses and is usually found clustered with I-box (Escobar et al., 2004).

Some reports have suggested that auxin plays an essential role in the circadian rhythm of flowering stem growth (Jouve et al., 1999). This is demonstrated by the fact that auxin response is linked to processes controlled by the circadian clock and that some circadian clock functions can be influenced by hormone signals (Covington and Harmer, 2007). In this study, GA₃ and IAA were found to be upregulated and enhanced the transcriptional activity of *GUS*. The gibberellin class of phytohormones is involved in the control of flowering in many species (Hedden, 2017). GA₃ is linked with other floral pathways via GA₃-regulated *DELLA* proteins, creating versatile interacting modules for signaling proteins (Conti, 2017). In longan, the concentration of some hormones, such as IAA, was increased during the floral bud physiological differentiation stage (Yimei, 1997). *DIGI* expression was also found to be upregulated at the same stage (Huang et al., 2017). The time of change in the concentration of hormones and the change in expression of *DIGI* coincides with the time when terminal

shoots begin flower bud initiation in longan. At the same time, *DIGI* was predicted to induce floral initiation by affecting endogenous auxin synthesis and transport in longan (Huang et al., 2017); therefore, there may be an IAA–*DIGI* regulatory loop in longan during flowering induction. However, this hypothesis requires more concrete evidence. Recent studies show that in *A. thaliana*, exogenous application of ABA has exhibited late-flowering phenotype (Achard et al., 2004), also found to participate in circadian clock function and flowering time control (Hanano et al., 2006). Early-flowering phenotypes have been observed in ABA-deficient mutants, showing that ABA acts as an inhibitor of floral transition (Barrero et al., 2005). MeJA was found to inhibit the flowering of short-day plants such as *Chenopodium rubrum* (Albrechtova and Ullmann, 1994). *Pharbitis nil* seedlings placed in a solution of MeJA for a period of 24 h led to a dramatic reduction in the number of formed flower buds (Maciejewska and Kopcewicz, 2002). In this study, abscisic acid and methyl jasmonate were found to reduce the transcriptional activity of the *GUS*, indicating that they might be inhibitors of flowering in longan.

GI and *ELF4* have a similar circadian phase, and they also interact genetically, resulting in identical circadian outcomes in plant growth and flowering time (Kim et al., 2012). Kolmos et al. (2009) found that *ELF4* suppresses *GI* transcription during circadian clock regulation in *A. thaliana*. Jia et al. (2014) demonstrated that the expression level of *DIELF4* (UniGene 4309) was higher in ‘Sijimi’ compared with ‘Lidongben,’ which suggests that *ELF4* may be an essential gene involved in perpetual flowering traits in longan. The data presented in this study demonstrate that *DIELF4* genes significantly increase the activity of the *DIGI* promoter (Fig. 6A and B), suggesting that *DIELF4* works upstream of *DIGI* and promotes *DIGI* expression (Jia et al., 2014). *DIELF4* seems to have different functions from *ELF4* in *A. thaliana*, which is known to destabilize *GI* protein and be a repressor of flowering. It was reported by Fu et al. (2018) that the overexpression of longan *DIELF4* genes in *A. thaliana* exhibited late flowering and adventitious root phenotypes, suggesting that *DIELF4* genes, and in particular *DIELF-2*, may regulate hormone biosynthesis. Therefore, *DIELF4* may regulate hormone biosynthesis by upregulating *DIGI* and could be involved in longan flower initiation. However, this hypothesis needs further study. Taken together, the *DIGI*–*DIELF4* interaction provides an additional novel mechanism that can contribute to the currently known genetic and molecular interactions which control photoperiodic flowering.

Conclusions

In this study, the *GI* homolog gene was identified as a circadian rhythms gene in the woody fruit tree for the first time. Longan *DIGI* responds to external changes in photoperiod and light intensity, activated by SD conditions and high light intensity. In addition, *DIGI* responds to hormones such as GA₃ and IAA, and there may be an IAA–*DIGI* regulatory loop during flowering induction in longan. Moreover, *DIELF4* was found to be working upstream of *DIGI* and activating *DIGI* expression. The *DIELF4*–*DIGI* interaction may provide an additional novel mechanism to the currently known genetic and molecular interactions that control photoperiodic flowering. Our findings not only increase our understanding regarding the upstream regulatory network of *DIGI* (Supplemental Fig. 1)

but also provide more knowledge of molecular mechanisms of flowering induction in woody fruit trees.

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Supplemental Fig. 1. Different factors including gibberellic acid (GA3), auxin (IAA), methyl jasmonate (MEJA), and abscisic acid (ABA) affect the activity of gigentea (*GI*) promoter.

Supplemental Table 1. Putative *cis*-acting elements in the *DIG1* promoter.

Motif	Function	Stand	Position	Sequence (5'–3')
ARE	<i>Cis</i> -acting regulatory element essential for the anaerobic induction	–	–1076 –1734 –241; –268; –463 –1835	AAACCA TATATA
Box 4	Part of a conserved DNA module involved in light responsiveness	–	–461 –1812	TATATAAA ATTAAT
CAT-box	<i>Cis</i> -acting regulatory element related to meristem expression	+	–696 632	GCCACT
CGTCA-motif	<i>Cis</i> -acting regulatory element involved in the meja-responsiveness	+	–996; –40	CGTCA
ABRE	<i>Cis</i> -acting element involved in the abscisic acid responsiveness	+	–1086 –1088 –1130	ACGTG
G-box	<i>Cis</i> -acting regulatory element involved in light responsiveness	+	–1128	GCCACGTGGA
GT1-motif	Light responsive element	–	–1086 –1130 –1597	CACGTC TACGTG GGTTAA
LTR	<i>Cis</i> -acting element involved in low-temperature responsiveness	+	–1485 –1739	GTGTGTGAA CCGAAA
MBS	Myb binding site involved in drought-inducibility	–	–809	CAAACTG
O2-site	<i>Cis</i> -acting regulatory element involved in zein metabolism regulation	+	–1084	GATGATGTGG
TGACG-motif	<i>Cis</i> -acting regulatory element involved in the meja-responsiveness	+	–1086 –40; –996 –1702	TGACG
GAG-motif	Part of a light responsive element	–		AGAGAGT
Box I	Light responsive element	+	–1229	TTTCAAA
G-Box	<i>Cis</i> -acting regulatory element involved in light responsiveness	+	–1130	CACGTA
CE3	<i>Cis</i> -acting element involved in aba and vp1 responsiveness	–	–534	GACGCGTGTC
GARE-motif	Gibberellin-responsive element	+	–1785; –1792	AAACAGA
TGA-box	Part of an auxin-responsive element	+	–1085	TGACGTGGC
GC-motif	Enhancer-like element involved in anoxic specific inducibility	–	1009	GCCCCG
Skn-1 ₁ -motif	<i>Cis</i> -acting regulatory element required for endosperm expression	+	–1080	GTCAT
CAAT-box	Common <i>cis</i> -acting element in promoter and enhancer regions	+	–15; –22; –88; –312; –660; –707; –870; –950; –962; –1013; –1028; –1261; –1563	CAAT

Continued next page

Supplemental Table 1. Continued.

Motif	Function	Stand	Position	Sequence (5'-3')
TATA-box	Core promoter element $\approx$ -30 of transcription start	-	-423; -504; -560; -1125; -1263; -1645; -1679; -1714; -1748; -1799	CAAT
		+	-196; -604; -670; -961; -1012; -1027; -1205; -1279; -1562	CCAAT
		-	-393; -1844; -1905; -1946; -1953	CCAAT
		+	-109; -157; -167; -243; -270; -296; -327; -465; -484; -675; -726; -783; -914	TATA
		-	-1837; -1972; -1992	TATA
		-	-166; -326; -674; -725; -782; -913	TATAA
		+	-242; -240; -325; -267; -295; -241; -156; -268; -461; -463; -464; -673; -781	ATTATA
		-	-461; -724; -1834; -1835	ATTATA
		+	-315	TACAAAA
		-	460; -462; -723	TACAAAA
		-	459	taTATAAAAc

Supplemental Table 2. Primers used in quantitative polymerase chain reaction.

Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')
GUS	TACCGTACCTCGCATTACCC	CTGTAAGTGCGCTTGCTGAG
Tobacco Actin	TCCTGATGGGCAAGTGATTAC	TTGTATGTGGTCTCGTGGATTCT
Arabidopsis Actin	GCAGCATGAAGATCAAGGTGG	GTAACAAACTCACCACCACG