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A CIB1-LIKE transcription factor GmCIL10 from soybean positively regulates plant flowering

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CRYPTOCHROME-INTERACTING basic helix-loop-helix 1 (CIB1) is a well characterized transcriptional factor which promotes flowering through the physical interaction with the blue light receptor CRYPTOCHROME 2 (CRY2) in *Arabidopsis*. However, the role of its counterpart in crop species remains largely unknown. Here, we describe the isolation and characterization of a *CIB1* homolog gene, *Glycine max CIB1-LIKE10* (*GmCIL10*), from soybean genome. The mRNA expression of *GmCIL10* in the unifoliate leaves shows a diunal rhythm in both long day (LD) and short day (SD) photoperiod, but it only oscillates with a circadian rhythm when the soybean is grown under LDs, indicating that the clock regulation of *GmCIL10* transcription is LD photoperiod-dependent. Moreover, its mRNA expression varies in different tissue or organs, influenced by the develpomental stage, implying that *GmCIL10* may be involved in the regulation of multiple developmental processes. Similar to CIB1, GmCIL10 was evident to be a nuclei protein and ectopically expression of *GmCIL10* in transgenic Arabidopsis accelerates flowering under both LDs and SDs, implying that CIBs dependent regulation of flowering time is an evolutionarily conserved mechanism in different plant species.

photoperiod, flowering time, soybean, *Glycine max CIB1-LIKE10*

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Cryptochrome is a type of blue light receptor regulating photomorphogenesis in plant and circadian clock in both plants and animals [1–4]. Plant genomes encode at least two kinds of cryptochrome: cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2). In Arabidopsis, CRY1 primarily mediates blue light-dependent de-etiolation [5], while CRY2 majorly regulates photoperiodic flowering [6]. Arabidopsis CIB1, which is the first identified blue light-dependent CRY2-interacting protein, is a basic/helix-loop-helix (bHLH) transcriptional factor, acting as a CRY- and blue light-dependent regulator to stimulate floral initiation [7–9]. Hitherto, bHLH transcription factors have been well characterized to control a diversity of life processes in eukaryotes [9–12]. Members of this family contain a conserved bHLH signature domain, which consists of two functionally distinct regions. The basic region is involved in binding to E-box (CANNTG), and the HLH region functions as a dimerization domain [13–16]. CIB1 binds to the G-box (CACGTG), a canonical E-box, *in vitro* [7]. However, CIB1 can form heterodimers with other CIB1-related proteins and targets to the no-canonical E-box (CANNTG) of the *FLOWERING LOCUS T* (*FT*) promoter *in vivo* to regulate transcription [9]. CIBs function in mediating CRY2-CIB signal transduction pathway to activate floral initiation [7–9]. CRY2-CIB blue light-dependent interaction has also been tested in soybean (*Glycine max*). GmCIB1 interacts

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with the E-box-containing promoter sequences of the senescence-associated gene *WRKY DNA BINDING PROTEIN 53b* (*WRKY53b*) to activate leaf senescence, whereas photo-excited GmCRY2a physically interacts with GmCIB1 to suppress its DNA binding activity and GmCRY2a acts antagonistically with GmCIB1 to mediate light suppression of leaf senescence in soybean [17]. These findings indicated that CRY-CIB signaling mechanism is evolutionarily conserved in plants, although this mechanism in different plant species may mediate various aspects of plant development in response to light.

Flowering promotion is a major physiological function of CIB1 in Arabidopsis which is a primary model organism for the study of photoreceptor signal transduction. However, whether CIB-related proteins are involved in the flowering time regulation in crops is hitherto unclear [18]. In this study, we isolated a *CIB1* homolog gene *GmCIL10* in soybean, and analyzed its expression profiles in response to photoperiod or in various tissues at different development stages. Moreover, we investigated the subcellular localization of GmCIL10 protein in Arabidopsis protoplasts and evaluated its role in flowering time regulation through ectopic overexpression of *GmCIL10* in Arabidopsis. These results provided support a hypothesis that GmCIL10 in soybean is a positive regulator of flowering time and CIB mediated flowering time regulation is a conserved mechanism in plant species.

1 Materials and methods

1.1 Plant materials and growth conditions

An elite soybean (*Glycine max*) cultivar Ken-nong 18 (KN18) was cultured in environmentally controlled growth rooms with a defined photoperiod, short day (SD, 8 h light/16 h dark) or long day (LD, 16 h light/ 8 h dark), at 25 to 28°C. Cool white fluorescent lights (TLD 18W/54, Philips) were used as a white light source (200 to 300 mmol m^{-2} s^{-1} above the plant canopy). Samples were collected as described before with some modifications [19–23]. For spatial and temporal expression analysis, the plants were grown under SDs and all samples were collected 0.5 h after light on. The unifoliolates, and the first to fourth trifoliolates were collected at indicated growth stages. Developing seeds were collected at 7, 14 and 21 days after flowering and the mature seeds were collected when the seeds became yellow. For the circadian rhythm analysis, the fully expanded unifoliolates of the 2-week-old soybeans were sampled every 2 h for 2 days under LD or SD photoperiod and for additional 2 days in continuous light or dark. At least five individual plants per sample were harvested and all samples were immediately frozen in liquid nitrogen and stored at −80°C before being analyzed. All experiments were repeated three times under consistent conditions.

1.2 RNA isolation, gene cloning and vector construction

Total RNA was extracted by Trizol reagent (Invitrogen, USA) and cDNA synthesis was performed according to Revert Aid first-strand cDNA synthesis kit manual (Promega, USA). The Glyma1 6g10820 (*GmCIL10*) coding DNA sequence (CDS) was amplified using the primer pair GmCIL10-F and GmCIL10-R (Table 1), and then cloned into the pGWC vector [24]. The ectopic expression vector pLeela-*GmCIL10* and the subcellular localization vector pENSG-*YFP-GmCIL10* were constructed respectively by LR recombination reaction (Invitrogen, USA). The recombinant protein expression vector pCold-*GmCIL10* was constructed by insertion of *GmCIL10* CDS into the pCold^{TM}TF DNA (TaKaRa, Japan) vector at the *XhoI* restriction site by In-fusion recombination reaction (Clontech, USA).

1.3 Bioinformatics analysis

The alignment of amino acid sequence was performed using Clustal W software with default parameters. The Neighbor-joining phylogenetic tree was constructed by MEGA software (version 4.0) using bootstrap analysis (1000 replicates). The schematic diagrams were constructed using Adobe Illustrator CS6 software, based on the genome sequences from the database (http://www.phytozome.net/ *Glycine max v1.1*).

1.4 Real-time quantitative reverse transcription PCR

Expression profiles were investigated by Real-time quantitative reverse transcription PCR (RT-qPCR), using SYBR Premix Ex Taq polymerase kit (TaKaRa, Japan) and ABI StepOne Real-Time PCR system (Applied Biosystems, USA). Each 15-μL reaction system consisted of 5 μL template, 7.5 μL 2X SYBR Premix, 200 nmol L^{-1} of each primer, and 0.3 μL ROX. The specific primers used for the quantification of *GmCIL10* *GmFT2a*, *GmFT5a*, and the reference genes were listed in Table 1. The relative expression unit (REU) was calculated following the formula: $REU= 2^{(Cta-Ctb)}$, where Cta and Ctb are the average Ct (Cycle threshold) values of the reference and target genes respectively [25]. Each experiment was repeated at least three times.

1.5 Subcellular localization analysis

The subcellular localization vector pENSG-*YFP-GmCIL10* and the nuclear-marker vector pENSG-*CFP-AHL22* were co-transformed into Arabidopsis mesophyll protoplasts following the previous procedure [26, 27]. The protoplast transformed with pENSG-*YFP* vector was used as control. The transformed protoplasts were incubated in dark for 12–14 h at 23°C, and then analyzed under a confocal mi-

Table 1 The sequence of Primers used in this study

Primer Name	Sequence (5′ to 3′)
GmCIL10-F	ATGGAAAACCAGTTCTTTCTGGCTT
GmCIL10-R	TCAGAGCTCAACTTTCATCTGTG
GmCIL10-pCold-F	TCAAGCTTGTCGACCTGCAGATGGAAAACCAGTTCTTTCTGGCTT
GmCIL10-pCold-R	ATTACCTATCTAGACTGCAGTCAGAGCTCAACTTTCATCTGTG
GmCIL10-QF	CTTCTCAACTCCAACCCAATCAAC
GmCIL10-QR	GTAGCAGTAGCAATGTGGCAAAG
GmACT11-F	ATCTTGACTGAGCGTGGTTATTCC
GmACT11-R	GCTGGTCCTGGCTGTCTCC
GmSKIP16-F	GAGCCCAAGACATTGCGAGAG
GmSKIP16-R	CGGAAGCGGAAGAACTGAACC
GmUKN1-F	TGGTGCTGCCGCTATTTACTG
GmUKN1-R	GGTGGAAGGAACTGCTAACAATC
pLeela-F	GTTATGGGTCAACGGTTTC
ACT2-F	AAGCTCTCCTTTGTTGCTGTT
ACT2-R	GACTTCTGGGCATCTGAATCT
GmFT2a-QF	GGTTCTGGTGGAAGGAGGTTATAC
GmFT2a-QR	ACTACTAAAGAGTGTGGGAGATTGC
GmFT5a-QF	GGACAGAAGCAAAATTAAAGCAGATG
GmFT5a-QR	ACTATATACTATGATGTTTGTGTTTGGG
E-box-F	TCACTCATTAGGGCAAACTGTGGGTCAATAATCAGGAACTAGGAGAGTGG
E-box-R	AGGGCCTGAGATCGGCTTGTAATCGCGCAATGCAAAAGAGCGCANNTGGCCCACTCTCCTAGTTCCTGAT
G-box-R	AGGGCCTGAGATCGGCTTGTAATCGCGCAATGCAAAAGAGCGCACGTGGCCCACTCTCCTAGTTCCTGAT
Em-box-R	AGGGCCTGAGATCGGCTTGTAATCGCGCAATGCAAAAGAGCGAAAAAAGCCCACTCTCCTAGTTCCTGAT
E-box-QF	CATTAGGGCAAACTGTGGGTC
E-box-QR	CCTGAGATCGGCTTGTAATC

croscope (Leica TCS SP2).

1.6 Transgenic Arabidopsis preparation and phenotype analysis

Agrobacterium tumefaciens strain GV3101 (pMP90RK) harboring pLeela-*GmCIL10* vector was used for the transformation of Col-4 Arabidopsis ecotype through the floral dip method. The transgenic lines were screened using 100 mg L^{-1} glufosinate ammonium and further confirmed by PCR using the primers (GmCIL10-R and pLeela-F) specific to *GmCIL10* gene and pLeela vector backbone (Table 1). The mRNA expression analysis of *GmCIL10* in the transgenic lines was performed using the primers (GmCIL10-QF and GmCIL10-QR) specific to *GmCIL10* CDS (Table 1). T2 plants were grown under LDs or SDs in growth chambers for the measurement of flowering time and leaf numbers as described before [26].

1.7 Quantitative PCR-based DNA binding assay

The quantitative PCR-based DNA binding assay experiments were performed as previously described with minor modifications [17]. The CDS of *GmCIL10* was inserted into pCold^{TM} TF vector and the recombinant construct was transformed into *E.coli* (BL21). The expression of recombinant protein His-TF-GmCIL10 was induced. The *E.coli* lysate expression the His-TF-GmCIL10 protein or the His-TF control protein was prepared respectively in lysis buffer [50 mmol L^{-1} Tris (pH 7.8), 500 mmol L^{-1} NaCl, 0.5% Triton X-100, 1 mmol L^{-1} PMSF, 5 mmol L^{-1} DTT, 1 tablet/50 mL of protease inhibitor cocktail], mixed with Dynabeads His-Tag Isolation & Pulldown (#10104D, Novex) for 15 min at room temperature, and then washed six times with the wash buffer (50 m mol L^{-1} Tris, pH 7.8, 500 m mol L^{-1} NaCl, 0.1% Triton X-100, and 1 mmol L^{-1} PMSF) and collected with the DynaMag^{TM}-Spin apparatus (Novex, USA). His-TF-GmCIL10 or His-TF protein binding with dynabeads was collected and diluted to 0.5 μg μL^{-1}, with the concentration determined using the Bradford method. The Ewt, Gwt and Em DNA sequences were prepared by the annealing and extending reactions with respective primer pairs (Table 1): E-box-F/E-box-R for Ewt, E-box-F/G-box-R for Gwt, and E-box-F/Em-box-R for Em. Each reaction consisted of 25 μL 2X Prime STAR GC Buffer, 10 μL (10 μmol L^{-1}) of each primer, 4 μL dNTP Mixture, 0.5 μL Prime STAR HS DNA Polymerase (TaKaRa). PCR reaction was programmed as following: 100°C, 2 min; Slowly ramp to 45°C, and hold on for 10 min; 72°C, 10 min. Each 15-μL DNA binding reaction consisted of 5 μL dynabeads, 0.4 μmol L^{-1} DNA, 2.5 μL 5X DNA binding buffer [20% Glycerol, 2.5 mmol L^{-1} DTT, 250 mmol L^{-1} KCl, 1 mg mL^{-1} BSA, 50 mmol L^{-1} Tris-HCl (pH 7.5), 5 mmol L^{-1} $MgCl_2$], kept in room temperature for 15 min, and then washed six times with 1 X DNA binding

buffer. The precipitated products were eluted with 50 μL elution buffer [50 mmol L^{-1} Tris-HCl (pH 7.8), 300 mmol L^{-1} NaCl, 300 mmol L^{-1} Imidazole (pH 7.5)], and diluted 50-fold prior to quantitative PCR analysis using primer pairs E-box-QF/E-box-QR (Table 1). The DNA binding activity was calculated by the formula: 2^{-Ct}. Ct (cycle threshold) represents the number of cycles required for the fluorescence signal to exceed background level.

2 Results

2.1 GmCIL10 is a member of the bHLH transcription factor family

We analyzed the soybean (*Glycine max* cv. Williams 82) genome deposited in the Phytozome database (http://www.phytozome.org) and identified 33 homologous proteins shared conserved bHLH domain with AtCIBs or AtCILs (AtCIB1-LIKEs). Phylogenetic analysis indicated that these proteins were classed into two groups and that bHLH proteins in Group I are more closely related to AtCIB1 than those proteins in Group II (Figure 1A). The protein encoded by Glyma11g12450 locus was previously designed as GmCIB1 because it is evolutionally close to AtCIB1 in the phylogenetic tree and has been experimentally proofed to interact with GmCRY2a [17]. To keep consistent, other bHLH proteins in Group I were designed as GmCIL1 (GmCIB1-LIKE1) to GmCIL13 respectively, according to their relative evolution distance to GmCIB1.

GmCIL10, encoded by locus Glyma16g10820, belongs

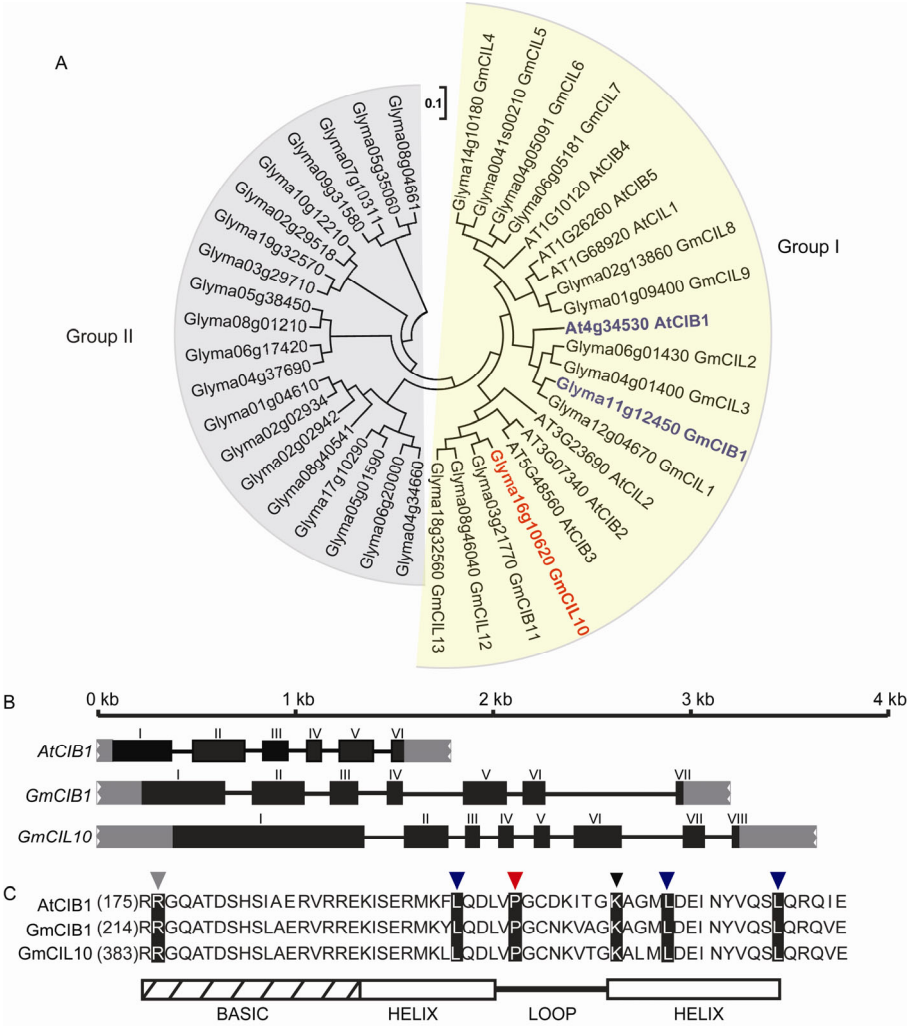

Figure 1 (color online) Phylogenetic analysis and comparisons of the gene structures. (A) Phylogenetic tree demonstrating the evolutionary relationships between Arobidopsis CIBs and soybean homologous proteins. Soybean protein sequences were downloaded from Phytzome V9.1 (http://www.phytozome.net). At, *Arabidopsis thaliana*; Gm, *Glycine max*. The scale bar indicates substitutions per site (1,000 replicates). (B) Diagram representation of gene structures of *AtCIB1*, *GmCIB1* and *GmCIL10*. The numbers on the top of the line segments indicate the length of the DNA sequences.The black boxes represent exons;The lines represent introns; The grey boxes represent UTRs. (C) Alignment of the amino acid sequences (by ClustalX with a manual adjustment) of bHLH domains of AtCIB1, GmCIB1 and GmCIL10. The key amino acids of bHLH domain (black) are shown. A diagram depicting the boundaries of basic and helix- loop-helix motifs is included at the bottom.

to a sub-group which is least close to the sub-group of AtCIB1 and GmCIB1 within group I. We were curious whether GmCIL10 is functionally conserved or not to AtCIB1 or GmCIB1. Therefore, we cloned *GmCIL10* coding DNA sequence (CDS) from cv. Kennong18 (KN18), which encodes a 590-residue polypeptide. We aligned the CDSs of *AtCIB1*, *GmCIB1*, and *GmCIL10* with their genomic DNA sequences (Figure 1B). The result showed that the genomic sequence of *GmCIL10* is the largest and *AtCIB1* is the smallest among the three genes. Besides, *GmCIL10* gene consists of 8 exons while *AtCIB1* and *GmCIB1* comprise 6 and 7 exons respectively. Although their gene structures are different from each other, peptide alignment of their bHLH domains demonstrated that the amino acid sequences are highly conserved, especially those representing residues of the BASIC, HELIX, LOOP or HELIX motifs, which are completely consistent (Figure 1C), implying these proteins may share conserved function. To test this hypothesis, we analyzed if GmCIL10 is able to bind E-box (CANNTG) cis-element like many other bHLH transcriptional factors [7]. The results demonstrated that GmCIL10 interacted with a higher affinity *in vitro* to E-box, and especially to a canonical E-box, name G-box (CACGTG) (Figure 2).

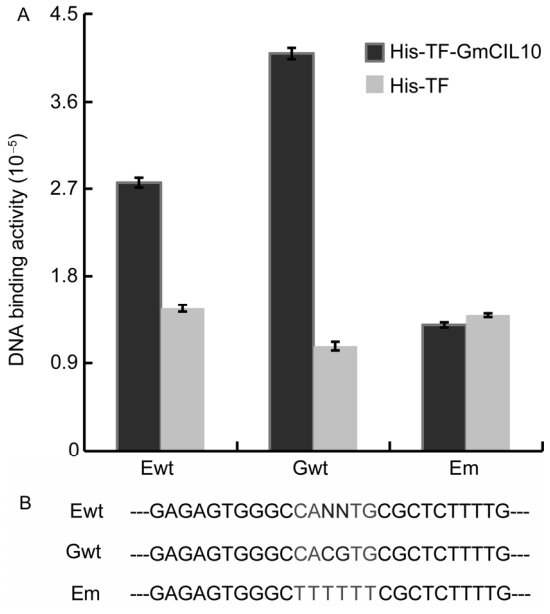

Figure 2 GmCIL10-DNA interaction *in vitro*. (A)The DNA binding assay shows the interaction of GmCIL10 protein with wild-type E-box (Ewt), G-box (Gwt) or mutant E-box (Em) DNA sequence. His-TF-GmCIL10 fusion protein or His-TF protein (used as control) expressed in *E.coli* BL21 was purified using Dynabeads His-Tag Isolation & Pulldown kit, then incubated with the Ewt, Gwt or Em DNA fragment for 15 min at room temperature. After six times of washing with 1X binding buffer, the bound DNA was eluted by the elution buffer and subjected to quantitative PCR. The DNA binding activity was calculated as in method. standard deviation (SD, n=3) was shown. (B) The DNA sequences of Ewt, Gwt and Em used in (A) was shown.

2.2 Diurnal or circadian expression pattern of *GmCIL10* in response to photoperiod

A previous study reported that GmCRY1a is a major regulator of photoperiodic flowering in soybean and the oscillated abundance of GmCRY1a protein significantly correlates with latitudinal distribution of soybean cultivars and their photoperiodic flowering time [22]. Taking that CIB1 physically interact with CRY2 in a blue light specific manner and promotes flowering in Arabidopsis, we reasoned that if GmCIL shows a similar rhythmic expression patterns as GmCRY1a, it may coordinate with GmCRY1a in flowering time regulation in soybean. To test this hypothesis, we analyzed the mRNA expressions of *GmCIL* genes and found that *GmCIL10* is expressed in a photoperiod-dependent manner. In this experiment, we grew soybean in short days (SDs, 8 h light/16 h dark) or long days (LDs, 16 h light /8 h dark) for two weeks until the unifoliolates are fully developed, and collected the unifoliolates every 2 h for two successive 24 h cycles. The plants were then transferred to continuous light (LL) or continuous darkness (DD), and the samples were collected every 2 h for additional 48 h. RT-qPCR results showed that, under SDs, the *GmCIL10* mRNA oscillated with a diurnal rhythm with a peak 4 h after dawn (ZT4) (Figure 3A and B). Such a diurnal expression pattern is similar to that of *GmCRY1a* mRNA [22], implying that GmCIL10 may coordinate with GmCRY1a to regulate photoperiodic flowering. However, the oscillation of *GmCIL10* mRNA became random in subsequent LL or DD (Figure 3A and B), and the amplitude increased in LL (Figure 3A) but decreased in DD (Figure 3B). In contrast to that under SDs, *GmCIL10* mRNA under LDs oscillated with a diunal rhythm with a peak 4 h after dusk and such a rhythm was well maintained in LL or DD (Figure 3C and D)，although the amplitude decreased in DD, demonstating that the circadian oscillation of *GmCIL10* mRNA is LD-photoperiod dependent. It's interesting that *GmCIL10* expression peaking at different time under SDs and LDs. Such kind of inconsistent gene expression pattern has been observed in photoperiodic flowering regulation which is mediated by co-operation between internal circadian clocks and external environmental stimuli, like diurnal photoperiod. For example, the transcription level of *CONSTANS* (*CO*), the key regulator determining the photoperiodic flowering in Arabidopsis, peaks and fluctuates differently in LDs and SDs. It has been illustrated that transcriptional regulation of *CO* gene by the interaction between circadian clock and environmental photoperiod is the most crucial mechanisms for day-length measurement in photoperiodic flowering of Arabidopsis. The inconsistent expression patterns of *GmCIL10* under LDs and SDs imply that internal and external signals may co-regulate *GmCIL10* expression throught multiple transcriptional regulators, and *GmCIL10* may play important role in modulating photoperiodic flowering or other life history trait.

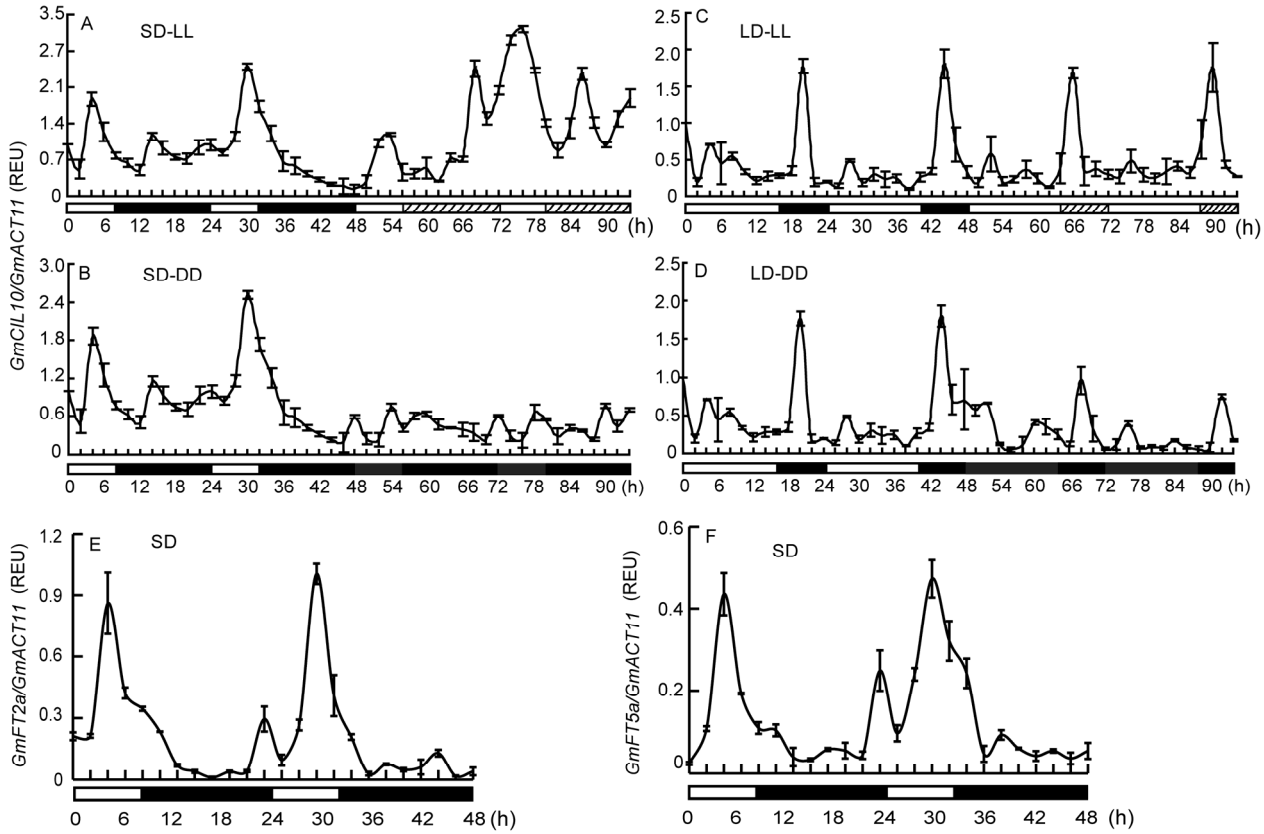

Figure 3 Quantitative PCR (RT-qPCR) showing mRNA expressions in response to different photoperiod treatments. Unifolioate leaves were collected every 2 h as indicated. White box, objective light phase; Dark box, objective dark phase; Striped box, subjective dark phase; Gray box, subjective light phase. (A to D) mRNA expressions of *GmCIL10*. (A and B) Soybean plants grown under short day photoperiods (SDs) for 14 days were transferred to continuous white light (LL) (A) or continuous dark (DD) (B) for 2 days. (C and D) Samples grown under long day photoperiods (LDs) were treated and collected as in (A and B). (E and F) mRNA expressions of *GmFT2a* (E) or *GmFT5a* (F) under SDs.

2.3 Spatial and temporal expression patten of *GmCIL10*

FLOWERING LOCUS T (*FT*) is a key flowering integrator that encodes florigen in Arabidopsis. Two of the *FT* homologs, *GmFT2a* and *GmFT5a*, were reported to coordinately control flowering time in soybean [28]. Similar to *GmCIL10* and *GmCRY1a*, *GmFT2a* and *GmFT5a* show diurnal expression patterns with a peak 4 h after dawn under SDs (Figure 3E and F). Taking that CIB1 and CRY2 physically interact and directly promote *FT* transcription in Arabidopsis, we surmised that *GmCIL10* may associate with *GmCRY1a* to up-regulate *GmFT2a* and *GmFT5a*, and then trigger flowering in soybean. To test this possibility, we investigate the spatial and temporal expression profile of *GmCIL10* in the unifolioates and trifoliolates of soybeans for a consecutive growth stages grown under SDs (Figure 4). To avoid improper interpretation of its expression levels in various tissues at different stages, RT-qPCR was performed using two stably expressed reference genes, *GmSKIP16* (Figure 4A) and *GmUKN1* (Figure 4B) [29]. The levels of *GmCIL10* transcripts in the unifolioates and trifoliolates under SDs increased gradually to their maximum levels prior to the initiation of flowering, and then decreased sharply when the time of flowering approached (Figure 4). Such an expression feature of *GmCIL10* mRNA resembles that of *GmFT2a* or *GmFT5a* in soybeans grown under SDs [28]. In addition, we observed that the level of *GmCIL10* transcripts increased gradually with the development of the seed, reaching a peak 21 days after pod initiation and thereafter decreased at maturation (Figure 4), indicating GmCIL10 may also play a role during pod development.

2.4 Subcellular localization of GmCIL10 protein

To investigate the subcellular compartment where GmCIL10 functions, YFP-GmCIL10 fusion protein was transiently expressed in Arabidopsis protoplasts , and then analyzed by YFP fluorescence under the confocal microscopy (Leica TCS SP2). The result showed that YFP-GmCIL10 protein was co-localized with the nuclear protein CFP-AHL22 in the nucleus (Figure 5A), while the YFP protein alone present in both nucleus and cytoplasm (Figure 5B) [26].This result suggests that GmCIL10 may function as a transcription factor.

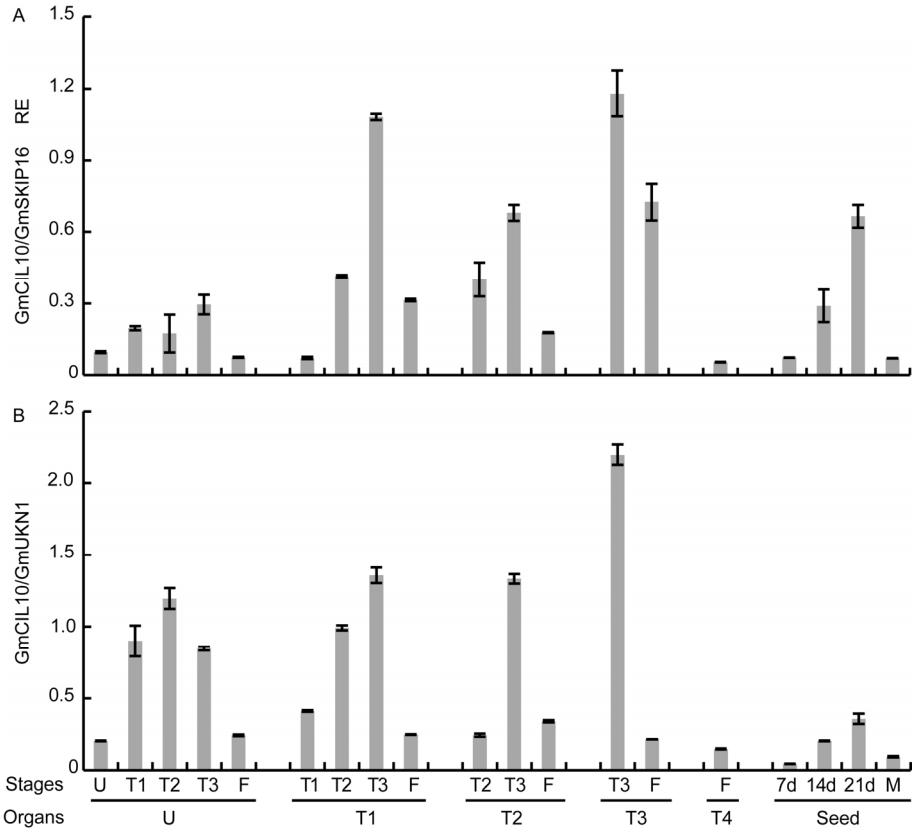

Figure 4 The mRNA expression profiles of *GmCIL10* in leaves or seeds at different growth stages. The indicated samples were collected from the plants grown in SDs at different developmental stages. Organs: U, unifoliolates; T1 to T4, the 1st to 4th trifoliolates. Stages: U, T1,T2, T3 or T4 indicates the time when the unifoliolates or the first to fourth trifoliolates are fully opened respectively; F, flowering; 7 d,14 d, and 21 d indicate the days after flowering respectively; M indicated the seed mature stage. The mRNA level of *GmSKIP16* (A) or *GmUKN1* (B) was used as the internal control respectively. The RT-qPCR results shown are average (±SD) of three biological repeats.

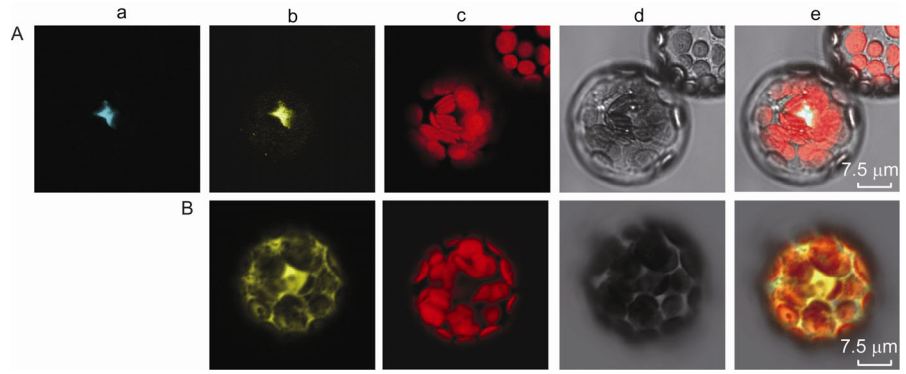

Figure 5 (color online) Subcellular localization of GmCIL10 protein in Arobidopsis mesophyll protoplasts. (A) GmCIL10 protein co-localized with a nuclear protein AHL22 in nucleus of mesophyll protoplasts. The plasmids encoding the YFP-GmCIL10 or CFP-AHL22 protein were co-transformed into the mesophyll protoplasts of 4-week-old plants grown under LDs , incubated for 12-14 h prior to the confocal microscope analysis. Image a, CFP fluorescence; Image b, YFP fluorescence; image c, Auto fluorescence; image d, Bright field; image e, Merge of images from a to d. Bar, 7.5 μm. (B) The images of protoplast expressing YFP protein alone were used as control.

2.5 Ectopic expression of GmCIL10 promotes flowering in Arabidopsis

To investigate if GmCIL10 has an activation activity in floral initiation, we prepared transgenic Arabidopsis that expressed the *35S:GmCIL10* transgene. Multiple transgenic lines showed similar early flowering time phenotype under both LDs and SDs (Figure 6A and B, two independent lines were shown). In consistent with the early flowering phenotype, the ectopic overexpression of *GmCIL10* in three independent lines (OX-1, OX-2 and OX-3) was verified by RT-qPCR (Figure 6C). Statistical analysis indicated that the

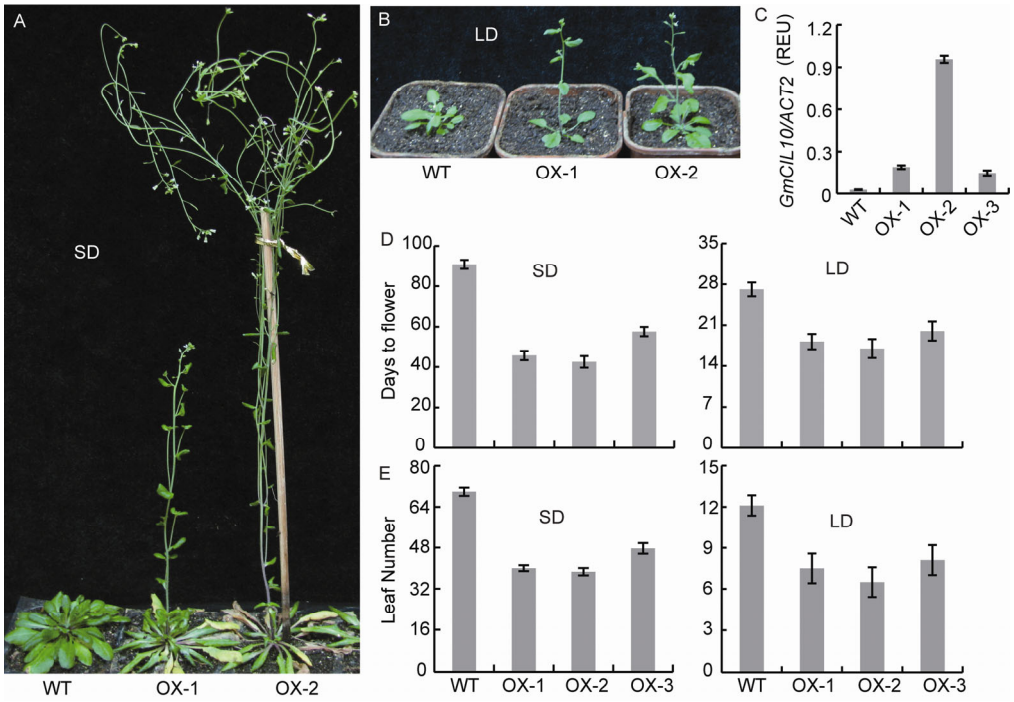

Figure 6 (color online) Flowering phenotypes of Arabidopsis plants grown under SDs or LDs. (A and B) Representative images of indicated plants grown under SDs (A) or LDs (B). (C) Analysis of the *GmCIL10* mRNA expression by RT-qPCR in indicated genotypes. (D and E) The statistical analysis of flowering days (D) and rosette leaf number (E) at the time of flowering. The means and standard deviations ($n \geqslant 20$) are shown. OX-1, OX-2 and OX-3 represents three independent *GmCIL10* overexpression transgenic lines respectively.

days to flowering and rosette leaf numbers of three transgenic lines are significant less than those of WT grown under both SDs and LDs (Figure 6D and E), which supports the hypothesis that GmCIL10 acts as a positive regulator of flowering time.

3 Conclusion

Arabidopsis CIB1 is a type of bHLH transcription factor, specifically interacting with CRY2 in response to blue light to activate the transcription of *FT* and trigger flowering initiation. However, it is unclear whether the CRY-CIB signaling mechanism in the regulation of flowering time is evolutionarily conserved in plants species. In this study, we showed that the diurnal mRNA expression rhythm of *GmCIL10* under SD photoperiods resembles that of *GmCRY1a*, which is a major regulator of photoperiodic flowering in soybean. In addition, the levels of *GmCIL10* transcripts in the unifolioates and trifoliolates under SDs increased gradually with leaf development and then decreased after the initiation of flowering (Figure 4). Such an expression pattern is consistent with that of *GmFT2a* and *GmFT5a*, which encode florigens and coordinately control flowering in soybean. Furthermore, ectopic expression of *GmCIL10* in Arabidopsis accelerates flowering of transgenic plants grown under both LDs and SDs. Taking together with the observation that GmCIL10 protein localizes in the nucleus of Arabidopsis protoplasts, we argue that GmCIL10 in soybean may function as a transcription factor as CIB1 in Arabidopsis in the aspect of flowering time regulation. However, it remains to be investigated whether GmCRY physically interacts with GmCIL10 to regulate flowering in soybean like CRY2-CIB1 complex does in Arabidopsis in response to blue light. In contrast to Arabidopsis, which is a long-day plant flowering earlier under LDs than in SDs, Soybean in nature is a typical short-day crop flowering earlier under SDs than under LDs. Because the flowering time of soybean is commonly hypersensitive to ambient photoperiods, soybean cultivars in general can only be cultivated in a limited latitudinal zone according to individual photoperiodic flowering habit [22]. Therefore, additional studies are needed to elucidate the role of GmCIL10 as well as other CIB homologs underlying photoperiodic control of soybean flowering time in order to improve the eurytopicity of soybean cultivars in different regions by molecular breeding.

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