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Cloning and expression analysis of *GmGAL1*, *SOC1* homolog gene in soybean

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Abstract A MADS box gene *AGL20/SOC1* is a main integrator in *Arabidopsis* flowering pathway whose structure and function are highly conserved in many plant species. A soybean MADS box gene *GmGAL1* (*Glycine max AGAMOUS Like 1*) as a homolog of *AGL20/SOC1*, was cloned from soybean cultivar Kennong18 and its function was investigated in transgenic *Arabidopsis* lines. Sequence comparisons showed that the closest homolog gene to *GmGAL1* is *AGL20/SOC1* in *Arabidopsis* and *VuSOC1* in *Vigna unguiculata*. We investigated the expression level of *GmGAL1* using quantitative real-time PCR, and the result showed that *GmGAL1* was regulated by a circadian mechanism and its expression oscillated at a cycle of 24 h. The expression level of *GmGAL1* was fluctuated in diverse tissues/organs and developmental stages. Considering its expression can be detected in different tissues throughout the life cycle and its protein localized in cytoplasm in *Arabidopsis* protoplasm, we proposed that *GmGAL1* may be a multifunctional gene in the context of the soybean development. Ectopic expression of *GmGAL1* in *Arabidopsis* enhanced flowering under long-day condition and partially rescued *soc1* late flowering type.

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Introduction

MADS-box genes belong to a large multifunctional family which controls the identity of the apex meristem, development of lateral organs, and flowering time of plants [1–8]. In *Arabidopsis*, *AGAMOUS*-like (*AGL*) genes belong to a sub-family of MADS-box genes which play important roles especially in regulating flowering pathways. For example, *FLOWERING LOCUS C* (*FLC*, *AGL25*) acts as a repressor of flowering [9–12]. *APETALA1* (*AP1*, *AGL7*) promotes flowering and controls the identity of meristem and sepals [13, 14] and *AGL12* regulates both flowering time and root development [15].

Another *AGL* gene, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), is a critical integrator of different flowering pathways which promotes flowering in *Arabidopsis* [16–20]. Its expression was detected in several organs include leaves, root, steam apex, etc. [19, 21]. In development stages, mRNA abundance of *SOC1* increases after seed germination and further accelerates in flower meristem during flower organs transition [19, 21]. Ectopic expression of the *SOC1* gene is sufficient to induce the early flowering in both long-day and short-day conditions [22], while lose-of-function of *SOC1* results in late flowering. Although *soc1* mutant show obviously late flowering time phenotype, it's flower organs develop almost normally because it is functionally redundant with *AGL24* and *SHORT VEGETATIVE PHASE* (*SVP*) in the control of flower organ identity [21, 23].

SOC1 genes are widely expressed in various tissues. Another *SOC1* like gene *ETL* (*Eucalyptus TM3* Like,

Eucalyptus globules ssp. bicostata), is also expressed in both vegetative and reproductive organs, including shoot meristems, roots, and floral organ primordia [24]. In monocotyledons, genes similar to *SOC1/TM3* are also broadly expressed and regulate floral transition or floral development, such as *OsSOC1* (*Oryza sativa*) [17, 25], *ZmMADS1* (*Zea may*) [26] and *TrcMADS1* (*Trillium camtschatcense*) [27].

Although *SOC1* genes are highly conserved and have similar expression patterns among dicots and monocots, their functions can be divergent in Angiosperms [22]. Recent studies have uncovered some interesting functions of *SOC1s*. For example, the constitutive expression of *UNS* (*UNSHAVEN*), an ortholog gene of *SOC1* in *Petunia hybrid*, results in ectopic trichome formation on floral organs and the reversion of petals into organs with leaf-like features, besides early flowering phenotype [28]. Therefore, further research is required to uncover the functional divergence of *SOC1s* among plant species.

The time of flowering significantly affects the yield of soybean. As a short-day plant, flowering of soybean is sensitive to day length which makes soybean an important model plant for photoperiod research [29]. Previously, we have cloned and analyzed a MADS-box gene *GmGAL2* from soybean [30]. In this work, another soybean MADS-box gene *GmGAL1* (*Glycine max AGAMOUS Like 1*) was cloned by rapid-amplification of cDNA ends (RACE). The expression patterns of *GmGAL1* in soybean at different stage were investigated. We further demonstrated that ectopic expression of soybean *GmGAL1* accelerated flowering in *Arabidopsis* and partially rescued *soc1* late flowering phenotype.

Materials and methods

Plant materials

The soybean cultivar used in this study was *Glycine max* L. "KN 18". The seeds were sown in pots (diameter 18 cm, height 16 cm) with mixed field soil, turf soil, and vermiculite with a ratio of 2:2:1. The *Arabidopsis* Col-0 and Ler were used as Wild-type plants. *soc1-1* was in the Ler background. The soybean and *Arabidopsis* grown condition were as described previously [30].

RNA preparation and gene cloning

For RNA preparation, the leaves of soybean were harvested in long-day and short-day conditions (16 h/8 h, light/dark, light was provided from 8:00 a.m. to 0:00 a.m.; 8 h/16 h, light/dark, light was provided from 8:00 a.m. to 16:00 p.m. everyday) at 0:00, 4:00, 8:00, 12:00 a.m., 16:00 and 20:00 p.m. Different soybean organs were sampled at different stages respectively. The fully expanded new leaves at unifoliolate or trifoliolate stages were sampled respectively for *GmGAL1* expression analysis. Samples were collected from more than three soybean plants. RNA was prepared with Trizol (Invitrogen) and reversed transcribed to cDNA with M-MLV (Invitrogen).

We detected a set of ESTs that showed high sequence similarity to *AGL20* (*AGAMOUS*-LIKE 20, accession number NP_182090) by blast *AGL20* DNA sequence against the NCBI nucleotide collections (nr/nt) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome). Among them, BQ611561.1 and BU926043.1 were used as the candidate sequences for cloning the full length *GmGAL1* gene [30]. The resultant clones were then sequenced for confirmation.

RT-PCR and quantitative PCR (q-PCR)

RT- and q-PCR were performed with the primers shown in Table 1 according to the manufacturer's instructions (ABI). Total cDNA was used as the template for RT- and q-PCR. All experiments were replicated in triplicate. The expression level of the *GmACT11* or *AtUBQ* gene was used as the internal control for target genes in soybean or *Arabidopsis* respectively (http://rsb.info.nih.gov/ij/).

Constructing expression vectors and plant transformation

The ORF of the *GmGAL1* gene (primers shown in Table 1) was cloned into Entry clone pDONR201 by BP clonase (Invitrogen), and transferred to destination vector by LR clonase (Invitrogen). The resultant expression vector was a binary vector in which *GmGAL1* was driven by the CaMV 35S promoter. It was transferred into *Arabidopsis* Col-0 via floral dipping approach mediated by *Agrobacterium* strain GV3101 (pMP 90RK) [31].

Sub-cellular localization of GmGAL1

Confocal microscopy was performed with a Leica TCS SP5 laser scanning confocal microscope. The well-expanded rosette leaves of Col-0 plants grown for 4 weeks in short-day conditions were collected for the protoplast isolation. Protoplasts was transformed with 10 μg of plasmid DNA (prepared using the Axygen Plasmid Midi Kit, http://www.axygen.com/) and incubated at 22°. After 12–16 h of transformation, the sub-cellular localization of GFP:GmGAL1 fusion proteins in protoplasts were observed with the confocal laser scanning microscope.

Table 1 Primers used in this study

Reaction	Name of primers	Sequence (5′–3′)
Cloning for GmGAL1	F1	TTTAAATTTCATTCTCCAAAACTC
Cloning for GmGAL1	R1	GACTCTGATGCTGACAATGACTTT
q-PCR for GAL1	qGmACT11-580F	ATCTTGACTGAGCGTGGTTATTCC
q-PCR for GAL1	qGmACT11-705R	GCTGGTCCTGGCTGTCTCC
q-PCR for GAL1	qGmGAL1-F	AACTTTCTGTTCTTTGTGATGC
q-PCR for GAL1	qGmGAL-1R	CTCCTGTTATGCCTGCGGTA
RT-PCR for GmGAL1	RT-AtUBQ-F	GATCTTTGCCGGAAAACAATTGGAGGATGG
RT-PCR for GmGAL1	RT-AtUBQ-R	CGACTTGTCATTAGAAAGAAAGAGATAACAGG
RT-PCR for GmGAL1	RT-GmGAL1-F	ACGCTACCGCAGGCATAACA
RT-PCR for GmGAL1	RT-GmGAL-1R	TTGCTGGCTGTGGCTGGATA
Gateway BP reaction	GW-GAL1-F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCT ATG ACG GCG TAC CAA TCG GAG C
Gateway BP reaction	GW-GAL1-R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA CAC TAA CTG GAG AGC GGT TTG GT

Phylogenetic analysis

Phylogenetic analysis of protein sequences was carried out using the amino acid sequence alignment generated by CLUSTAL-W. A Neighbor-Joining tree was built using the software of MEGA version 3.1. Support for the tree was assessed using the bootstrap method with 1000 bootstrap replicates. The numbers at each node represent the bootstrap support (percentage).

Results

Cloning of *GmGAL1* gene

We detected a set of ESTs that showed high sequence similarity with *SOC1* (*AGL20*, TAIR:AT2G45660) by BLAST search in NCBI database. Based on the sequence of ESTs, specific RACE primers for the full-length candidate gene were designed (Table 1). Using the RACE approach, we cloned a MADS box gene, *GmGAL1* (*Glycine max Agamous-Like1*) and sequence alignment analysis indicated that *GmGAL1* was a homolog gene of Arabidopsis *AGL* family (Fig. 1). Full length cDNA of *GmGAL1* (Genebank ID: DQ355801) was 801 bp including 630 bp of coding DNA sequence (CDS) which coded 209 amino acids. As shown in Fig. 1a, GmGAL1 had a MADS-MEF2-like domain at N terminal and a K-box domain at middle of the protein. GmGAL1 protein sequence had the highest similarity (93% identity) to VuSOC1 of *Vigna unguiculata* (data not shown) and it had 69.9% identity with AtAGL20 (Fig. 1b–d). Phylogenetic analysis (www.ncbi.nlm.nih.gov/blast) showed that *GmGAL1* had relatively lower sequence similarity with the *AGL20* homologs in monocots

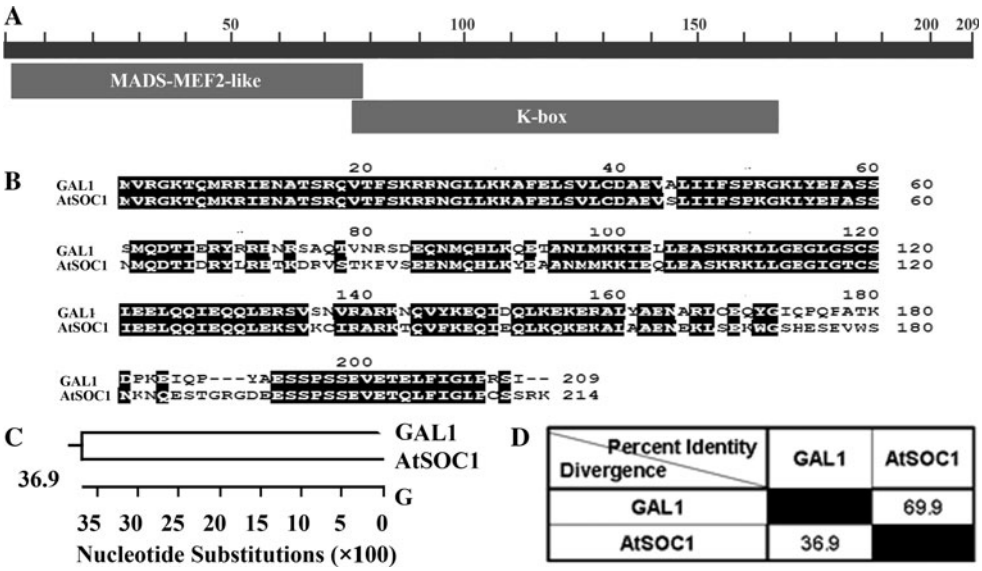

Fig. 1 Protein structure of GmGAL1 and alignment to SOC1 in *Arabidopsis thaliana*. **a** Protein structure of GmGAL1. **b** Alignment of GmGAL1 and SOC1. *Black block* represented identical or high similarity of amino acid in these two sequences. **c**, **d** Phylogenetic distance and identity between GmGAL1 and SOC1

and other species of gymnosperm, suggesting that *GmGAL1* and *AGL20* belong to the same dicot *SOC1* family (Fig. 2a, b).

Characteristic of *GmGAL1* expression at vegetative and reproductive stages of soybean

SOC1 expressed in multiple organs including leaves, roots and stem meristems in *Arabidposis*. *GmGAL1* also expressed in several organs and at different development stages in soybean (Fig. 3a–c). The *GmGAL1* mRNA abundance was low in unifoliolates of 7 and 14 day old seedings and increased a little bit in 21 day old ones. But in 28 day seedings, mRNA abundance of *GmGAL1* increased rapidly in unifoliolates (Fig. 3a). These results suggested that the expression level of *GmGAL1* at vegetative stage gradually increase according to development period and reach the highest level near to flowering. Using fully expanded unifoliolates and the first to the fourth trifoliolates as detection templates, we obtained the same result as using unifoliolates (Fig. 3b). Comparing to vegetative stage, the highest expression level appeared in stem of flowering phase. Its expression was low in flower and then became high in pod at reproductive stage (Fig. 3c).

Circadian clock analysis

The *GmGAL1* circadian clock was obtained by checking the mRNA abundance in fully open unifoliolates of soybean adapted with 24 h period of either short-day or long-day, followed by 48 h continuous light conditions (Fig. 4). In long-day adapted condition, *GmGAL1* expression increased gradually, peaks at 8 h after light, decreased slowly till at dusk and peaked again at 8 h after light of next period. This circadian cycle oscillated at a cycle of about 24 h and continued in subsequent 1 day of continuous light. But at the third day of continuous light, the peak showed up 4 h earlier in the period, indicating the oscillating period of *GmGAL1* become shorter in continuous light condition. The similar results showed in short-day and short-day to continuous light conditions.

GmGAL1 promotes flowering in *Arabidopsis*

To study the function of *GmGAL1* in soybean development, we over-expressed *GmGAL1* via the CaMV-35S promoter in *Arabidopsis*. The transgenic plants were analyzed by RT-PCR (Fig. 5) to confirm the presence and expression level of the transferred gene. Two transgenic lines with ectopic expression of *GmGAL1* showed earlier flowering phenotype compared with the wild-type control and the expression level of *GmGAL1* was positively correlated with the flowering severity of flowering phenotype (Fig. 5). To further recapitulate its function, we over-expressed *GmGAL1* in *soc1-1* mutant. As shown in Fig. 6, *GmGAL1*-OX in *soc1-1* mutant partially rescued the late flowering phenotype.

The sub-cellular localization of GmGAL1

We next examined the sub-cellular localization of GmGAL1 through an expression vector containing *GFP:GmGAL1* fusion gene driven by CaMV-35S promoter which was constructed and introduced into protoplast of Arabidopsis. After 12–16 h transformation, the result showed *GmGAL1* localized in cytoplasm as shown in Fig. 7.

Discussion

In this study, we cloned an *AGL20/SOC1* homolog from soybean, *GmGAL1*. Its sequence shows high similarity to *AGL20/SOC1* genes in many plants and it can be classed into the *AGL20* group of dicots. The sequence of GmGAL1 is most similar to SOC1 protein in *Arabidopsis* (with 69.9% identity) and Legume plant (*V. unguiculata*, with 93% identity). These data suggest that *SOC1* might originate from the same ancestor, but evolve independently in dicots, monocots, gymnosperm; long-day plants, and short-day plants. Thus, *SOC1* has different functions between long-day and short-day plants, although they share some conserved functions. The sequence of *GmGAL1* shows typical characters of MADS-box proteins, such as a highly conserved MADS-box at the N-terminus and a K-box in the middle of the MADS-box. These data suggest that *GmGAL1* is a MADS-box gene and a putative *SOC1* homolog in soybean.

SOC1, as a member of MADS-box proteins, its main function is as the integrator of multiple flowering signals. It has additionally interesting functions which have been uncovered by recent research [32]. But it is also proved that *SOC1* evolves to become functional divergence in many species [24, 25, 27, 28, 33, 34]. It is speculated that *SOC1/TM3*-like genes in dicots are widely expressed in various tissues and the regulatory functions of these genes may be more diversified [22]. Our results of *GmGAL1* expression in different development stages and organs are probably an evidence of this speculation. Soybean is a typical short-day plant and sensitive to photoperiod. mRNA and protein of GmCRY1/CRY2 show obvious circadian clock [29] and putative central clock genes *GmLCL1*, *GmLCL2* (*Glycinemax LHY/CCA1 Like1* and *2*) and *GmTOC1* also oscillate with circadian rhythms in several conditions [35]. As a main direct target gene of *CO*, we also detect that *GmGAL1* gene expresses in a circadian manner of 24 h. In continuous light condition, the circadian period becomes

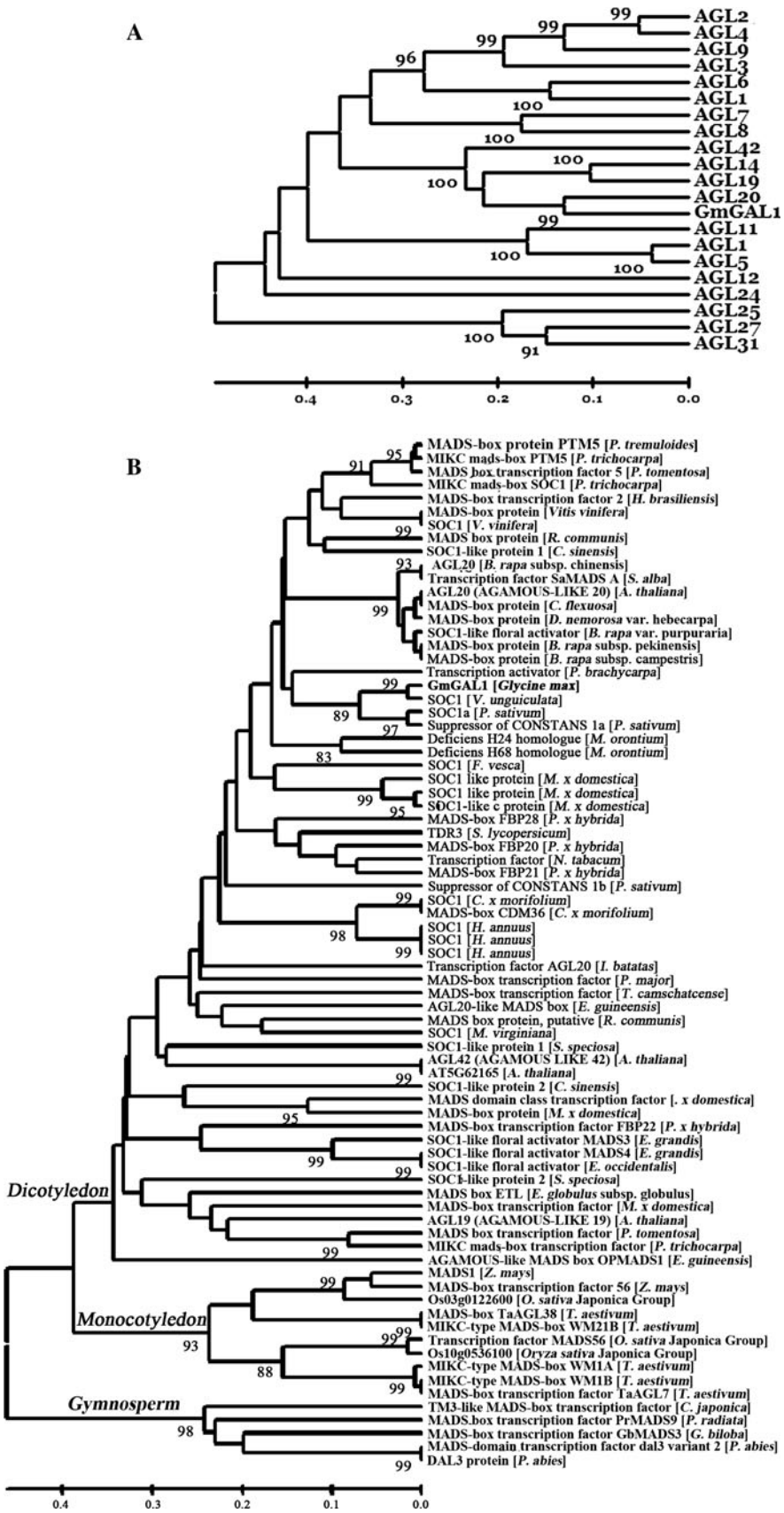

Fig. 2 Alignments of GmGAL1 and AGL family and SOC1 homologs in other plant species. *Numbers on the branches* were bootstrap values. **a** Alignment of GmGAL1 and AGL family genes in Arabidopsis. **b** Alignment of GmGAL1 and SOC1 homologs in different plant species. Dicotyledon, monocotyledon and gymnosperm were in the same branch separately

shorter than 24 h (Fig. 4). Previously study has proved that the loss function of *CCA1* or *LHY* in *Arabidopsis* conferring a shorter oscillator period [36] implies one of the possible explanations for our observation: the function of the central oscillator of soybean cultivar (KN18) used in this report may be as lesion as that of *cca1* or *lhy* mutant of *Arabidopsis* and cannot keep its regular 24 h period when the conditions changed from light–dark cycle to continue light. These results imply that *GmGAL1* is regulated by clock genes.

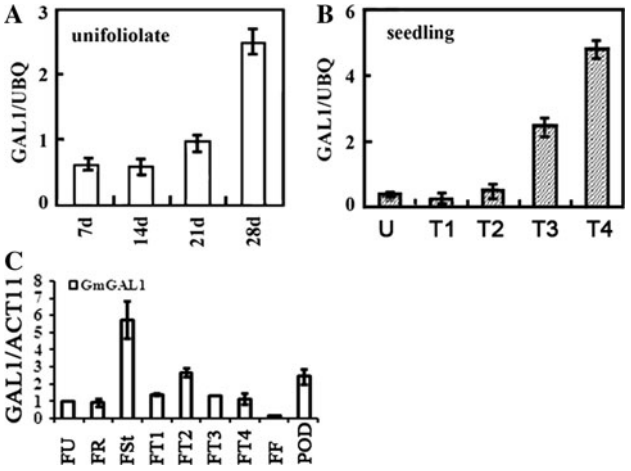

Fig. 3 *GmGAL1* expression pattern in different development stages of *Glycine max*. **a** *GmGAL1* mRNA abundance in unifoliolate of 7, 14, 21 and 28 day old samples. **b** *GmGAL1* mRNA abundance in unifoliolate, 1st (*T1*), 2nd (*T2*), 3rd (*T3*) and 4th (*T4*) trifoliolate. **c** *GmGAL1* mRNA abundance in different organs/tissues at flowering stage. *FU* unifoliolate at flowering, *FR* root at flowering, *FSt* stem at flowering, *FT1–FT4* 1–4 trifoliolate, *FF* flower at flowering. All leaves used are fully open. *Error bars* standard error

Because *SOC1* is functionally redundant with *AGL24* and *SVP* in control of flower organ identity [23], no obvious morphology changes are detected in *SOC1* deletion mutant, although *SOC1* plays important role in flowering pathway. In consistent, overexpression of *GmGAL1* in wild-type *Arabidopsis* or in *soc1* mutant accelerates flowering (Figs. 5, 6) but the transgenic lines show no obviously morphological change. These results suggest that the major role of *GmGAL1* gene is to play as a critical integrator of flowering pathway, which is similar as its homolog gene *SOC1* in *Arabidopsis*.

SOC1 localizes in cytoplasm and then in nuclear when it combines with AGL24 [37]. We don't obtain the homolog of *AGL24* gene from *Glycine max*, but the localization of GmGAL1 in cytoplasm of protoplast is similar to the SOC1 localization in *Arabidopsis* [37].

In summary, *GmGAL1* is a homolog of *Arabidopsis thaliana SOC1/AGL20* in soybean. It has conserved, but

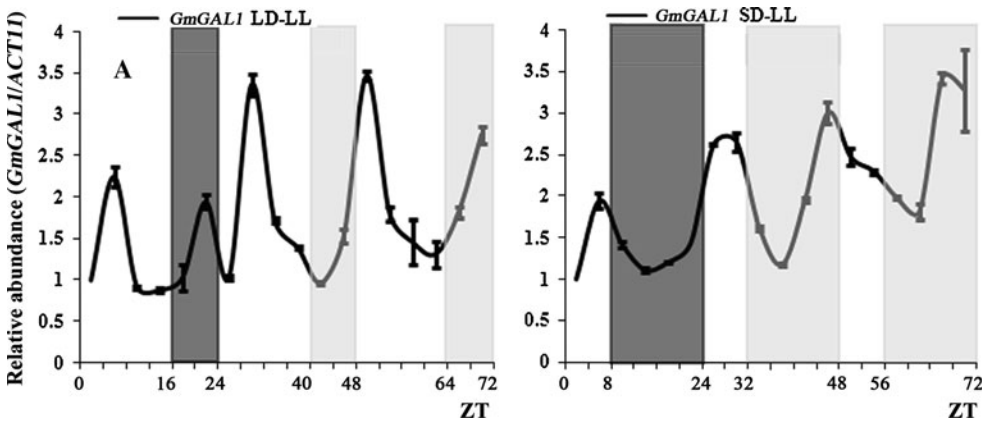

Fig. 4 The expression of *GmGAL1* in response to either long-day (16 h light/8 h darkness) or short-day (8 h light/16 h darkness) condition for 24 h, followed by 48 h continuous light (LD-LL or SD-LL). *Dark gray* darkness, *light gray* light instead of darkness. All fully open unifoliolates were collected from more than three soybean plants. *Error bars* standard error

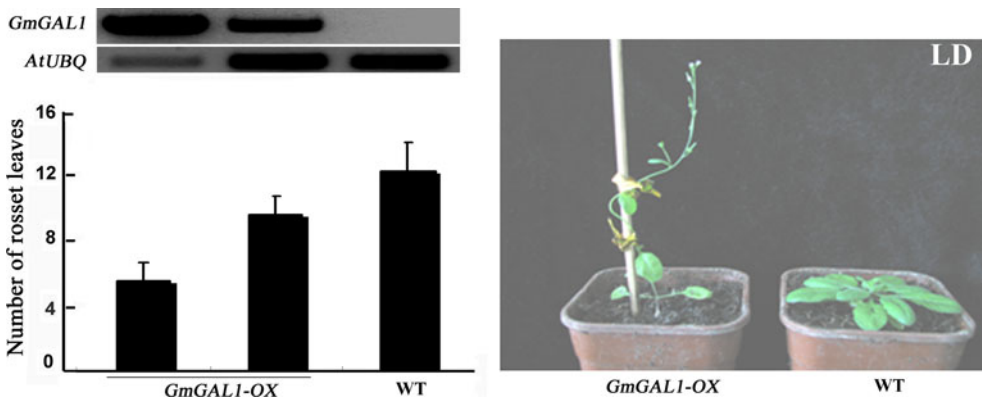

Fig. 5 Over-expression of *GmGAL1* enhances flowering in Arabidopsis in long-day condition and has fewer leaves. Transgenic plants were confirmed by RT-PCR. *UBQ* was used as the loading control for PCR. *LD* long-day condition

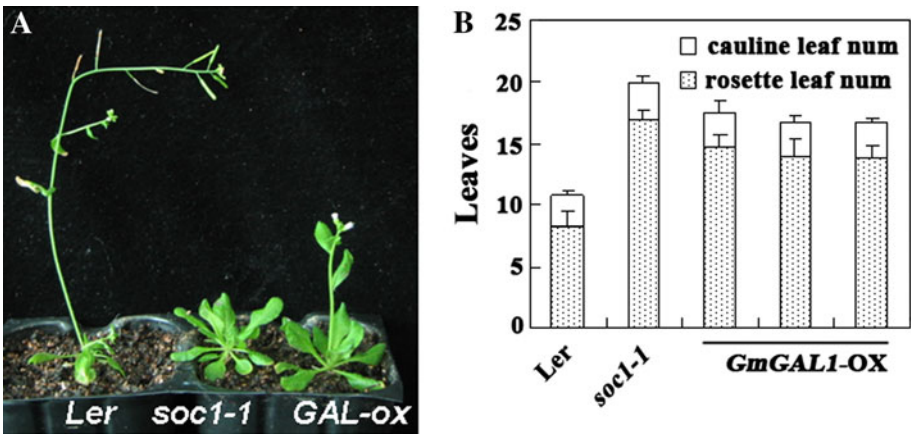

Fig. 6 Over-expression of *GmGAL1* rescues late flowering type of *soc1-1* mutant partially in long-day condition. **a** Phenotype of Ler, *soc1-1* and *GmGAL1*-OX. **b** Leaf numbers of these lines when flowering in long-day condition. *LD* long-day condition

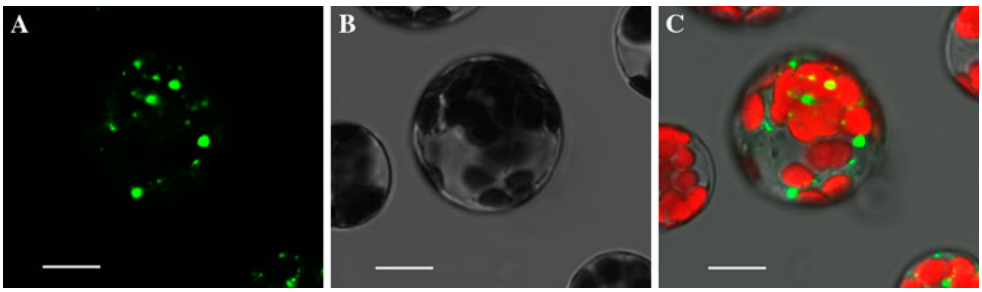

Fig. 7 GmGAL1 sub-cellular localization in cell. The construct was transformed into protoplasm of *Arabidopsis* and fluorescence visualized by confocal microscope. **a** The GFP:GmGAL1 fusion proteins were localized in the cytoplasm as green signal shown. **b** Bright field image of GFP:GmGAL1. **c** Merge of **a** and **b**. The *red* signal was from the autofluorescence of chloroplasts. The *scale bars* at the bottom represented 10 μm. (Color figure online)

not identical, functions on plant development compared with its homolog in other plants.

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