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GmmiR156b overexpression delays flowering time in soybean

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Abstract Soybean [*Glycine max* (L.) Merr.] is an important crop used for human consumption, animal feed and biodiesel fuel. Wering time and maturity significantly affect soybean grain yield. In *Arabidopsis thaliana*, miR156 has been proposed to regulate the transition from the juvenile to the adult phase of shoot development, which is accompanied by changes in vegetative morphology and an increase in reproductive potential. However, the molecular mechanisms underlying miR156 function in soybean flowering remain unknown. Here, we report that the overexpression of GmmiR156b delays flowering time in soybean. GmmiR156b may target SPL orthologs and negatively regulate GmSPLs, thereby delaying flowering in soybean under LD and natural conditions. GmmiR156b down-regulates several known flowering time regulators in soybean, such as *GmAP1* (*a*, *b*, *c*), *GmLFY2*, *GmLFY2*, *GmFULs*, *GmSOC1s*, *GmFT5a*, *and GmmiR172*. These data show that a similar miR156-SPL regulatory module was conserved in the soybean flowering pathway. However, *GmFULs*, *GmSOC1a* and *GmSOC1b* were significantly suppressed under LD conditions but not under SD conditions, which is different in Arabidopsis that these genes were down-regulated irrespective of photoperiod. In addition, GmmiR156b was up-regulated by E1, E2 (*GmGI*), E3 and E4, which control flowering time and maturity in soybean, and suppressed *E1* (*E1-Like*) and *E2* (*E2-Like*) genes under LD conditions. These data indicated that the miR156-SPL regulatory module was also with some degree of divergent in soybean flowering pathway.

Keywords Soybean · microRNA · Flowering time · GmmiR156b

Dong Cao, Ying Li and Jialin Wang have contributed equally to this work.

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Introduction

Soybean is a facultative short-day (SD) plant. Day length has an important influence on flowering and growth habit in soybean, and responsiveness to this factor is an important production trait. Ten maturity loci, *E1–E9* and *J*, that control flowering time and maturity in soybean, have previously been identified and characterized at both the phenotypic and genetic levels (Bernard 1971; Buzzell 1971; Buzzell and Voldeng 1980; McBlain and Bernard 1987; Ray et al. 1995; Bonato and Vello 1999; Cober and

Voldeng 2001; Cober et al. 2010; Kong et al. 2014). Among these loci, *E1*, *E3*, *E4* and *E7* are associated with photoperiod sensitivity under different light-quality conditions (Buzzell 1971; Buzzell and Voldeng 1980; McBlain and Bernard 1987; Cober et al. 1996; Abe et al. 2003; Xia et al. 2012). The *E1* locus largely impacts flowering time in soybean, and the E1 protein contains a putative nuclear localization signal and a distantly related B3 domain (Xia et al. 2012). Moreover, *E2* has been identified as an ortholog of the Arabidopsis *GIGANTEA* gene (Watanabe et al. 2011). *E3* and *E4* have been confirmed as *PHYA* homologs (Liu et al. 2008; Watanabe et al. 2009). *FLOWERING LOCUS T* (*FT*) homologs in soybean have a conserved role in promoting flowering (Kong et al. 2010; Sun et al. 2011). Two soybean orthologs of the Arabidopsis *FT* gene, *GmFT2a* and *GmFT5a*, coordinately control the transition to flowering in soybean (Kong et al. 2010). GmFT2a and GmFT5a redundantly and differentially regulate flowering through interactions with the bZIP transcription factor GmFDL19, resulting in the subsequent up-regulation of the latter (Nan et al. 2014). The expression of these two genes is down-regulated through the *E1*, *E2*, *E3* and *E4* loci under LD conditions (Kong et al. 2010; Thakare et al. 2011; Watanabe et al. 2011; Xia et al. 2012). In addition, two *SOC1* homologs, *GmSOC1* and *GmSOC1-like* (Zhong et al. 2012; Na et al. 2013), *GmLFY* (Meng et al. 2007), and the AP1 homolog *GmAP1* (Chi et al. 2011) have been characterized in soybean, and these genes are significantly up-regulated through GmFT2a and GmFT5a in a redundant and differential manner (Nan et al. 2014). Despite the economic importance of soybean, knowledge of the molecular mechanisms underlying flowering in this plant remains limited.

In plants, most members of the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factor family are regulated through miR156, and these proteins influence the transition between the juvenile and adult phases (Schwab et al. 2005; Wu and Poethig 2006; Wang et al. 2009; Xing et al. 2010). In Arabidopsis, the overexpression of miR156 represses the *SPL* transcription, thus reduces apical dominance and delays flowering time, leading to dwarfism and increases in total leaf number and plant biomass (Schwab et al. 2005). Further studies have shown that *SPL3* is a direct upstream activator of *LFY*, *FUL*, *AP1* (Yamaguchi et al. 2009) and other genes encoding MADS box transcription factors (Wang et al. 2009). *SPL9* and *SPL10* mediate the transition from high levels of miR-156 to high levels of miR172 through the direct activation of miR172 expression, thereby promoting the juvenile to adult phase transition (Wu et al. 2009; Fornara and Coupland 2009). In addition, the function of the miR156/SPL system in gene regulation is conserved in other plant species, including *Oryza sativa* (Xie et al. 2006), *Brassica napus* (Wei et al. 2010), *Panicum virgatum* (Fu et al. 2012), *Solanum tuberosum* ssp. Andigena (Bhogale et al. 2014) and *Medicago sativa* (Aung et al. 2014). Although previous studies have attempted to identify soybean microRNAs and their targets (Zhang et al. 2008; Song et al. 2011; Xu et al. 2013a, b), the molecular mechanisms by which miR156 regulates soybean flowering remain unknown.

In this study, we generated transgenic soybean plants overexpressing miR156b and analyzed the expression of flowering-related genes in both wild-type and transgenic soybean plants. Our data showed that the overexpression of miR156b suppress flowering time in soybean and negatively regulate *GmSPLs* and flowering-related genes, including *GmFT2a*, *GmFT5a*, *GmAP1* and *GmLFY*. In addition, GmmiR156b was up-regulated through E1, E2, E3 and E4, and suppressed *E1* (*E1-Like*) and *E2* (*E2-Like*) genes under LD conditions.

Materials and methods

Plant materials and growth conditions

Soybean cultivar Williams 82 (WT) and *35S:MIR156b* transgenic lines #5 and #11 were grown in an artificial climate chamber under either SD (12L/12D) or LD (16L/8D) conditions at 24 °C with an average light fluence rate of 200–300 μmol m^{-2} s^{-1}. Five plants were measured for flowering time (R1), which was defined as the time from emergence to the opening of the first flower (Fehr et al. 1971).

For the analysis of flowering-related gene expression in *35S:MIR156b* transgenic line #5, Williams 82 and Harosoy near-isogenic lines (NILs), soybean plants were grown under LD (16L/8D) or SD (12L/12D) conditions. Each cultivar was planted in three pots, with each pot containing one seedling. Three sets of fully expanded trifoliolate leaves from three individual plants were sampled at 4 h after dawn at 20 days after emergence (DAE) under SD and 50 DAE under LD conditions when the flower bud were appeared in soybean cultivar Williams 82, and the samples were frozen at −80 °C until total RNA extraction.

Plant transformation

A 181-bp stem-loop fragment of the GmmiR156b precursor was amplified through PCR using DNA samples obtained from the soybean cultivar Williams 82 and cloned into the pEASY-T1 vector (Transgene, Beijing, China). XbaI/SacI-digested fragments were subsequently subcloned into the pTF101.1-GmFT2a vector, replacing GmFT2a. This vector, driven by the cauliflower mosaic

virus 35S promoter, was designated pTF101.1-GmmiR156b and subsequently used to transform Williams 82 plants using the cotyledon-node method (Flores et al. 2008). The primers used for PCR are listed in Supplemental Table S3. Glufosinate (160 mg/L) was daubed onto the cotyledons of seedlings to screen T0, T1, T2 and T3 transformants. Herbicide-resistant T3 plants were subjected to molecular and phenotypic analyses.

RNA isolation, cDNA synthesis and quantitative real-time PCR analysis

Total RNA was isolated, and cDNA was synthesized as described in Koseki et al. (2005). Quantitative RT-PCR of flowering-related genes and *Tubulin* (as an internal control) was performed as described by Nan et al. (2014). The primers used for qRT-PCR are listed in Supplemental Table S1. Three biological replicates were set up and subjected to real-time PCR in triplicate. Raw data were standardized as described previously (Willems et al. 2008).

The expression of miR156b and miR172 was analyzed through real-time PCR using the All-in-One^{TM} miRNA qRT-PCR Detection System (Gene Copeia) according to the manufacturer's instructions. Total RNA was extracted from trifoliate leaves using TRIzol reagent (Invitrogen^{TM}) according to the manufacturer's instructions. After RNase-free DNase (TaKaRa Bio, Inc.) treatment, 1 μg of total RNA was reverse transcribed using All-in-One^{TM} miRNA First-Strand cDNA Synthesis Kit (Gene Copeia). Quantitative RT-PCR analyses were performed using All-in-One^{TM} miRNA qPCR Kit (Gene Copeia) and primers specifically designed for miR156b and miR172a/b. The analysis was performed using DNA Engine Opticon 2 System (Bio-Rad). The PCR cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 55–60 °C (depending on the gene) for 20 s, 72 °C for 10 s, and 78 °C for 2 s. Fluorescence quantification was conducted before and after the incubation at 78 °C to monitor the formation of primer dimers. The miRNA expression levels were normalized to those of U6 in the same RNA sample.

Database search and gene identification

The Phytozome database (http://www.phytozome.net/soybean) was used for gene searches. Starting with the Arabidopsis SPL3, SPL9, FUL, and TOE1 protein sequences, TBLASTN searches were performed against the soybean (*Glycine max*) gene index (release 1.0). Phylogenetic trees were constructed using the MEGA 5.0 neighbor-joining (NJ) program.

Results

Overexpression of *GmmiR156b* causes late flowering in soybean

To determine whether GmmiR156b regulates soybean flowering time, two *35S:MIR156b* transgenic lines, #5 and #11, were examined under both SD and LD conditions. The overexpression of *GmmiR156b* caused significantly late flowering of Williams 82 plants under LD conditions (Fig. 1). The soybean cultivar Williams 82 flowered at approximately 54.4 DAE, whereas *35S:MIR156b* transgenic line #5 flowered at approximately 74.6 DAE and line #11 flowered at approximately 70.2 DAE (Fig. 1e). Under SD conditions, the overexpression of *GmmiR156b* also caused slightly late flowering. The Williams 82 plants flowered at approximately 27.6 DAE; in contrast, transgenic line #5 flowered at approximately 30.2 DAE, and line #11 flowered at approximately 28.8 DAE (Fig. 1e). These data suggested that *GmmiR156b* is a flowering suppressor in soybean under LD conditions.

The regulation of *GmFTs* and floral meristem identity genes in *35S:MIR156b* plants

GmFT2a and GmFT5a, are involved in photoperiod-regulated flowering and coordinately control flowering in soybean (Kong et al. 2010). To determine whether GmmiR156b suppresses flowering time in soybean through the regulation of *FT*, the expression levels of *GmFT2a* and *GmFT5a* in the leaves of *35S:MIR156b* transgenic line #5 were determined using quantitative RT-PCR. The expression levels of *GmmiR156b* in the leaves of *35S:MIR156b* plants were higher than those in untransformed Williams 82 plants under LD or SD conditions (Fig. 2a). In contrast to *GmmiR156b* expression, the level of *GmFT5a* mRNA expression was lower in transgenic than in wild-type plants under LD or SD conditions (Fig. 2b). However, the level of *GmFT2a* was decreased only under SD conditions (Fig. 2b). Recently, several genes involved in the determination of flowering time have recently been isolated and characterized in soybean, including *GmAP1*, *GmSOC1*, *GmSOC1-like* and *GmLFY* (Zhong et al. 2012; Na et al. 2013; Meng et al. 2007), and these genes are significantly up-regulated through GmFT2a and GmFT5a in a redundant and differential manner (Nan et al. 2014). Therefore, we next analyzed the expression of *GmAP1(a, b, c)*, *GmSOC1a*, *GmSOC1b* and *GmLFY2* in the shoot apex (SA) region of *35S:MIR156b* plants. As shown in Fig. 2c, the expression levels of *GmAP1* (*a*, *b*, *c*) and *GmLFY2* in the SA region were significantly lower in *35S:MIR156b* than in Williams 82 plants under SD or LD conditions.

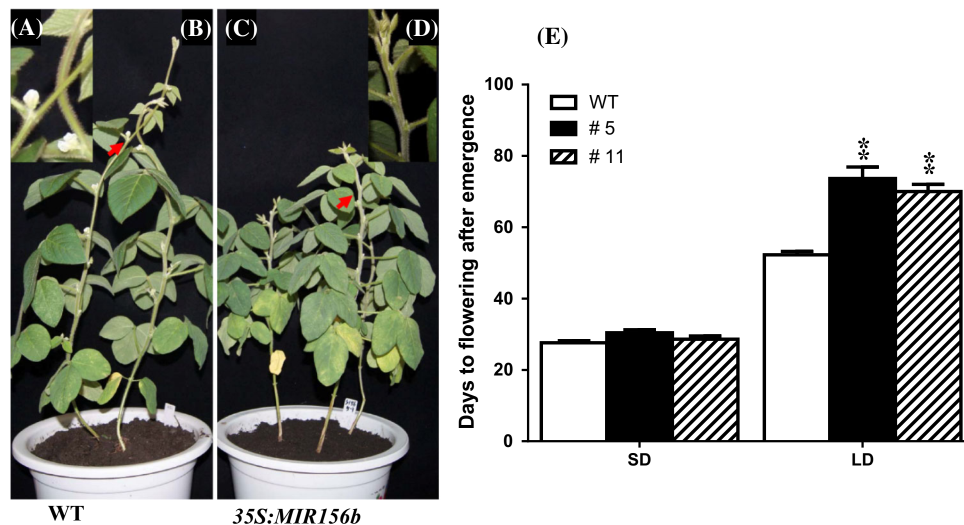

Fig. 1 *GmmIR156* overexpression induces delayed flowering in the soybean cultivar Williams 82. **a** The close-up image of the wild type Williams 82 (WT) plant shown in **b** exhibits flowers at the axils of the trifoliate leaves. **b** A wild type Williams 82 plant showing flowers at the axils of the trifoliate leaves at 53 DAE under LD conditions. **c** *35S:MIR156b* plants. **d** The close-up image of the *35S:MIR156b* plants shown in **c** does not display flowers at the axils of the trifoliate leaves. **e** Days to flowering from the emergence of the transgenic and wild type plants. T3 plants of two *35S:MIR156b* transgenic lines, #5 and #11 were grown for flowering time evaluation. Values represent the average of five replicates +SD. *Double asterisks* indicate significant differences between transgenic and WT plants at $P < 0.01$

GmSOC1a and *GmSOC1b* were significantly suppressed under LD conditions but not under SD conditions (Fig. 4).

Down-regulation of *GmSPLs* in *35S:MIR156b* plants

For Arabidopsis, the miR156-SPL3 module has been identified as a component of a regulatory mechanism that induces flowering (Wang et al. 2009; Yamaguchi et al. 2009). SPL9 and SPL10 mediate the transition from high levels of miR156 to high levels of miR172 through the direct activation of miR172 expression, thereby promoting the juvenile to adult phase transition (Wu et al. 2009). To validate the effects of miR156 overexpression on *SPL* homologs in soybean, we searched for SPL homologs in the soybean genome using Arabidopsis AtSPL3 and AtSPL9 as queries in Phytozome and identified eight high-scoring candidate GmSPL (GmSPL-like) genes (Fig. S1). Expression analyses were performed using RT-PCR for all eight selected *GmSPLs* in the leaves and SAs. In wild-type plants under LD or SD conditions, *GmSPL3b* and *GmSPL3c* transcripts were more abundant in leaves than in the SA, whereas *GmSPL9c* and *GmSPL9d* transcripts were more abundant in the SA than in leaves (Fig. 3). A similar expression pattern was observed for *GmSPLs* in *35S:MIR156b* plants. Nonetheless, *GmSPL3* (*a*, *b*, *c* and *d*) and *GmSPL9* (*a*, *b*, *c* and *d*) transcripts were all reduced in *35S:MIR156b* plants compared with wild-type plants both under LD and SD conditions (Fig. 3). These data demonstrated that the *SPL3/9* orthologs in soybean were down-regulated in *35S:MIR156b* soybean plants (Fig. 3), suggesting that the effects of GmmiR156b on flowering time regulation might reflect the suppression of *SPL3/9* homologs in soybean.

The regulation of *GmFULs*, *GmSOC1a* and *GmSOC1b* in *35S:MIR156b* plants

The overexpression of miR156 in Arabidopsis reduces the expression of *SPL* transcription factors and delays the activation of *FUL* and *SOC1*, which encode MADS box transcription factors expressed in the meristem during the early stages of the floral transition (Wang et al. 2009). As the expression of *SPL* homologs in soybean was down-regulated in *35S:MIR156b* soybean plants (Fig. 3), we next evaluated the effects of miR156 expression on *FUL* and *SOC1* homologs in soybean. We used the Arabidopsis FUL (Ferrandiz et al. 2000) as the query to search for FUL homologs in the soybean genome using Phytozome and identified six high-scoring candidate GmFULs (GmFUL-like) genes (Fig. S2). Expression analyses of *GmSOC1a*, *GmSOC1b* and all six selected *GmFULs* in leaves and the SA were performed using qRT-PCR. The expression of *GmFULs*, *GmSOC1a* and *GmSOC1b* was decreased in the leaves and SA region of *35S:MIR156b* under LD conditions (Fig. 4a); under SD conditions, however, the expression of these genes was not, or at most weakly altered in transgenic plants (Fig. 4b). These results were not consistent with the results obtained in Arabidopsis, whereby irrespective of photoperiod, the expression levels of FUL and SOC1 were decreased in *35S:MIR156*

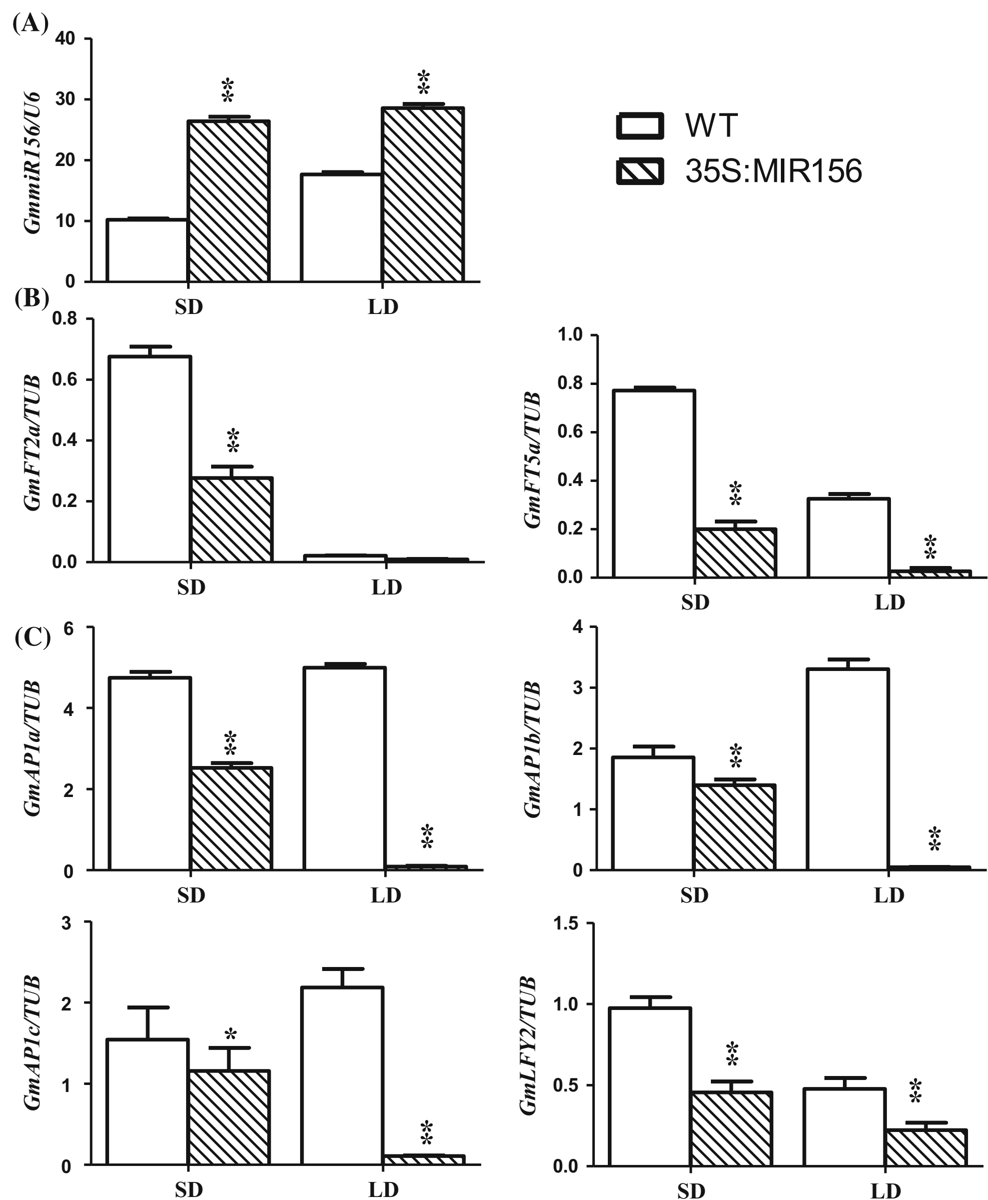

Fig. 2 The expression of *GmFT2a*, *GmFT5a* and flowering-related genes in *35S:MIR156b* plants. The expression of *GmmiR156b* (**a**), *GmFT2a* and *GmFT5a* (**b**), and flowering-related genes (**c**) in the shoot apical meristems of *35S:MIR156b* (line #5) and the wild-type Williams 82 (WT) plants under SD and LD conditions. Relative transcript levels analyzed by quantitative RT-PCR and normalized to *Tubulin*. Values represent the average of three biological replicates +SD. *Asterisks* and *double asterisks* indicate significant differences between transgenic and WT plants at $0.01 < P < 0.05$ and $P < 0.01$, respectively

seedlings (Wang et al. 2009). This discrepancy suggests that the miR156-SPL regulatory module might be divergent in the soybean flowering pathway under SD conditions.

Down-regulation of *GmmiR172* in *35S:MIR156b* plants

In Arabidopsis, miR156 regulates the expression of miR172 via SPL9, which acts redundantly with SPL10 and directly promotes the transcription of miR172b (Wu et al. 2009). Previous studies in soybean have shown that miR156 expression decreases during development, with miR172 expression increasing (Yoshikawa et al. 2013). In our study, we found that the expression of *GmSPL9* (*a*, *b*, *c and d*) was down-regulated in *35S:MIR156b* soybean plants (Fig. 3), suggesting that miR156 might also regulate the expression of miR172 via SPL9 in soybean. To determine whether GmmiR172 acts downstream of miR156, we used real-time PCR to analyze the expression of *GmmiR172* in the leaves and SA region of *35S:MIR156b* soybean plants. *GmmiR172* expression in these plants was decreased in both the leaves and SA region under LD or SD conditions (Fig. 5a). Zhao et al. (2015) demonstrated that *GmTOE4a* is GmmiR172 targets genes and functional as flowering suppressor in soybean. We then analyzed the expression of *GmTOE4a* in *35S:MIR156b* plants. The expression of *GmTOE4a* was up-regulated in the SA region

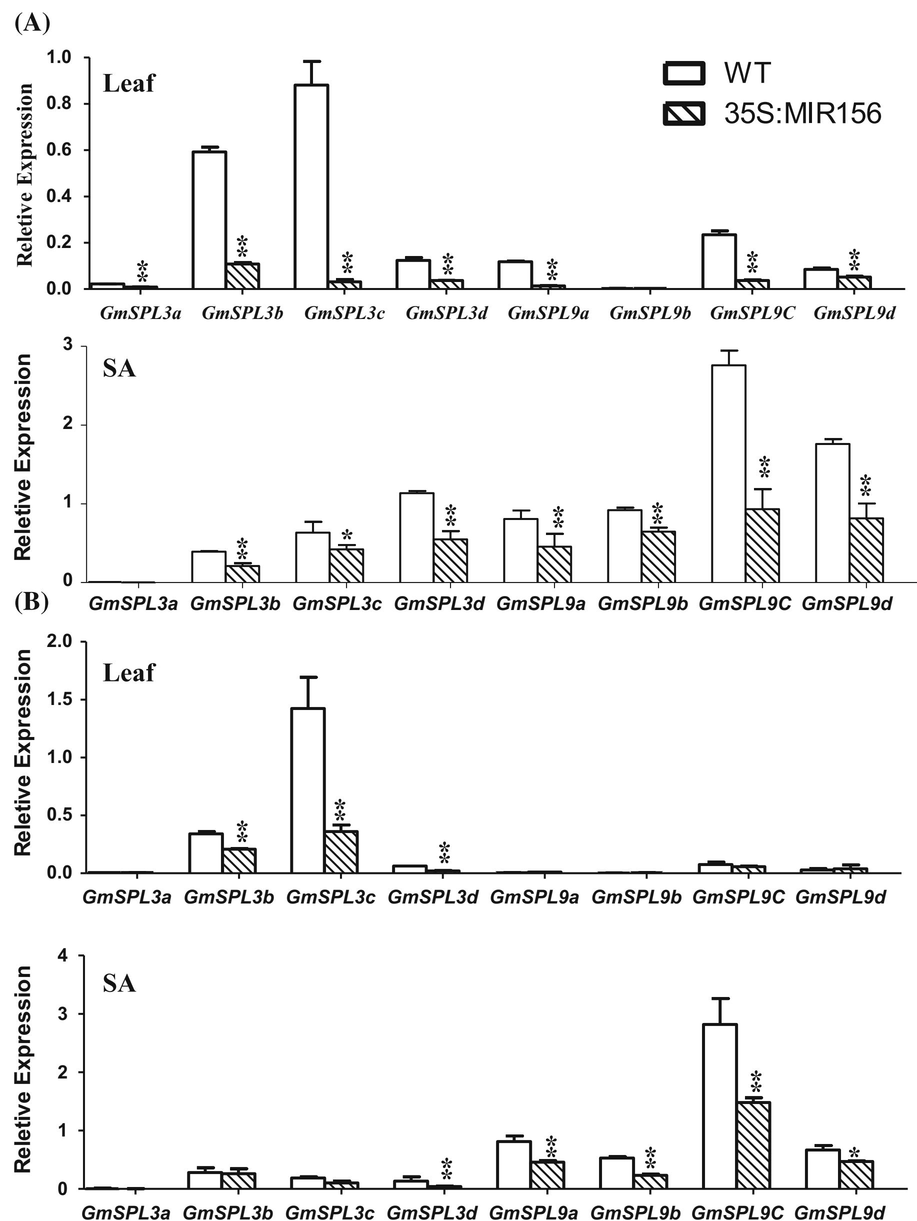

Fig. 3 *GmSPL-like* genes act downstream of GmmiR156b. **a** Expression analyses of eight putative miR156-targeted *GmSPLs* in the leaves and shoot apical meristems of *35S:MIR156b* (line #5) and wild-type Williams 82 (WT) plants under LD conditions. **b** Expression analyses of eight putative miR156-targeted *GmSPLs* in the leaves and shoot apical meristems of *35S:MIR156b* and wild-type Williams 82 (WT) plants under SD conditions. Relative transcript levels were analyzed by quantitative RT-PCR and normalized to *Tubulin*. Values represent the average of three biological replicates +SD. *Asterisks* and *double asterisks* indicate significant differences between transgenic and WT plants at $0.01 < P < 0.05$ and $P < 0.01$, respectively

of *35S:MIR156b* soybean plant (Fig. 5b). These data suggested that GmmiR156b down-regulated GmmiR172 and up-regulated its targets gene, *GmTOE4a*, in soybean.

Regulation of *GmmiR156b* through soybean maturity genes *E1*, *E2*, *E3* and *E4*

Previous studies have suggested that the maturity genes *E1*, *E2*, *E3* and *E4* control soybean flowering time through the down-regulation of *GmFT2a* and *GmFT5a* under LD conditions (Kong et al. 2010; Thakare et al. 2011; Watanabe et al. 2011; Xia et al. 2012). Genetic variations in these four maturity genes affect photoperiod insensitivity and PHYA-regulated post-flowering responses in soybean (Xu et al. 2013a, b). We demonstrated that overexpression of *GmmiR156b* delays flowering in soybean, and we next determined whether the four maturity genes regulate the expression of *GmmiR156b* in soybean under LD conditions. The Harosoy near isogenic lines (NILs) L71L-3004 (*E1E2E3E4*) exhibited the highest *GmmiR156b* expression, whereas OT89-5 (*e1e2e3e4*) showed the lowest expression level. Similar expression levels were observed between Harosoy (*e1e2E3E4*), L68-694 (*E1e2E3E4*) and L64-4584 (*e1E2E3E4*) (Fig. 6a). These data indicated that the expression of *GmmiR156b* was up-regulated through E1, E2, E3 and E4. We evaluated whether the overexpression of *GmmiR156b* regulates *E1* (*E1-like*) and *E2* (*E2-like*) transcription and examined the mRNA levels of these

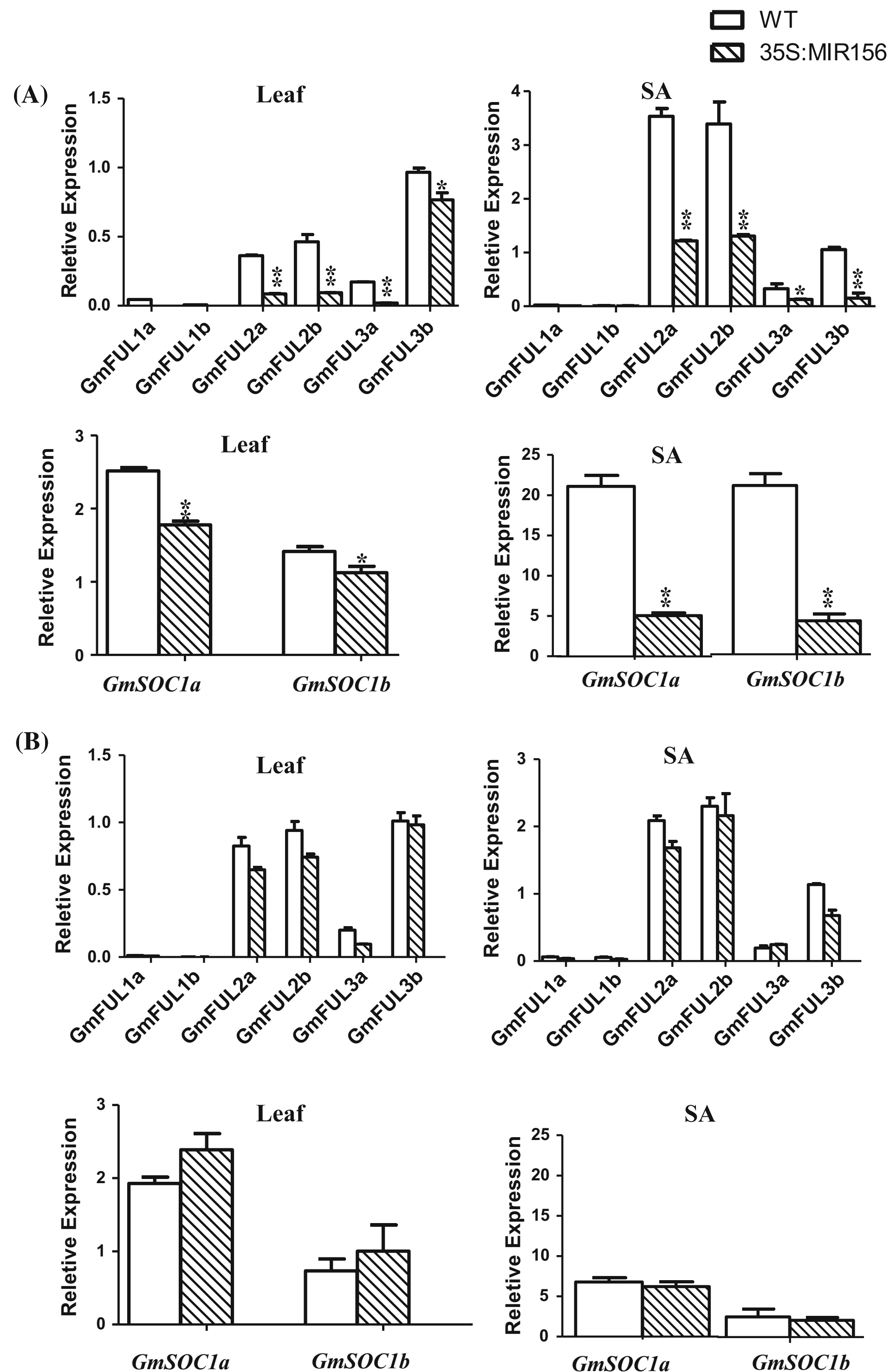

Fig. 4 The expression of *GmFUL-like* genes, *GmSOC1a* and *GmSOC1b* in *35S:MIR156b* plants. **a** Expression analyses of six *GmFUL-like* genes, *GmSOC1a* and *GmSOC1b* in the leaves and shoot apical meristems of *35S:MIR156b* (line #5) and wild-type Williams 82 (WT) plants under LD conditions. **b** Expression analyses of six *GmFUL-like* genes, *GmSOC1a* and *GmSOC1b* in the leaves and shoot apical meristems of *35S:MIR156b* (line #5) and wild-type Williams 82 (WT) plants under SD conditions. Relative transcript levels were analyzed by quantitative RT-PCR and normalized to *Tubulin*. Values represent the average of three biological replicates +SD. *Asterisks* and *double asterisks* indicate significant differences between transgenic and WT plants at $0.01 < P < 0.05$ and $P < 0.01$, respectively

genes in transgenic and wild-type soybean plants under LD conditions. *E1* (*E1-like*) and *E2* (*E2-like*) mRNA levels were suppressed in transgenic plants, indicating that these genes were down-regulated through GmmiR156b under LD conditions (Fig. 6b). Therefore, together with the role of E1 and E2 in the regulation of *GmmiR156b* expression, the interplay between E1 (E2) and GmmiR156b completes a negative feedback loop. We also analyzed *SPL* homologs in Harosoy NILs. NIL L71L-3004 (*E1E2E3E4*) showed the highest *GmmiR156b* expression and the lowest *GmSPL*

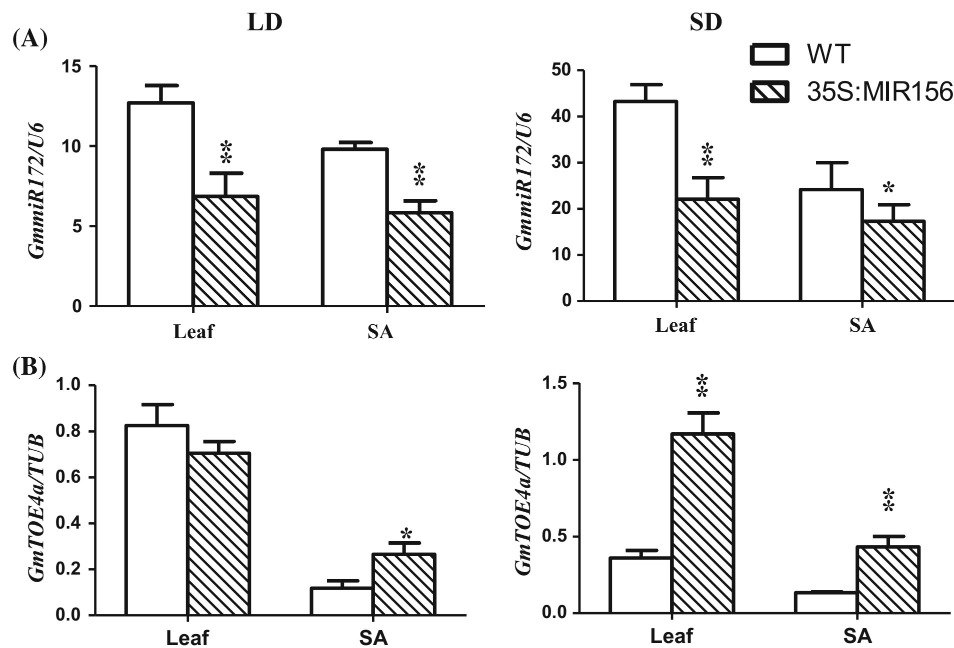

Fig. 5 The expression of *GmmiR172* and *GmTOE1*-like genes in *35S:MIR156b* plants. **a** The expressions of *GmmiR172* in the leaves and shoot apical meristems of *35S:MIR156b* (line #5) and wild-type Williams 82 (WT) plants under LD or SD conditions. **b** The expressions of *GmTOE4a* genes in the leaves and shoot apical meristems of *35S:MIR156b* (line #5) and wild-type Williams 82 (WT) plants under LD or SD conditions. Relative transcript levels were analyzed by quantitative RT-PCR and normalized to *Tubulin*. Values represent the average of three biological replicates +SD. *Asterisks* and *double asterisks* indicate significant differences between transgenic and WT plants at $0.01 < P < 0.05$ and $P < 0.01$, respectively

expression, but there were no significant differences in the expression levels of *GmSPLs* among the other four genotypes, indicating the involvement of other genes in the regulation of *GmSPLs* in soybean (Fig. 6c). Therefore, these data suggested that the maturity genes *E1*, *E2*, *E3* and *E4* regulate maturity and flowering time, at least in part, through GmmiR156b, which might target *GmSPLs* in soybean plants.

Discussion

In plants, the juvenile and adult phases of vegetative development can be distinguished through leaf morphology, and the reproductive phase of development can be distinguished based on flower production (Fornara and Coupland 2009). In soybean, ten maturity loci, *E1* to *E9* and *J*, which control flowering time and maturity, have previously been identified and characterized at the phenotypic and genetic levels (Bernard 1971; Buzzell 1971; Buzzell and Voldeng 1980; McBlain and Bernard 1987; Ray et al. 1995; Bonato and Vello 1999; Cober and Voldeng 2001; Cober et al. 2010; Kong et al. 2014). *E1–E4* have recently been identified and characterized: these genes down-regulate the expression of *GmFT2a* and *GmFT5a*, which redundantly and differentially regulate flowering through interactions with the bZIP transcription factor GmFDL19 for the subsequent up-regulation of the transcription factor in soybean (Liu et al. 2008; Watanabe et al. 2009, 2011; Xia et al. 2012; Nan et al. 2014). Herein, we reveal another flowering regulation pathway in which GmmiR156b regulates floral transition in soybean. The overexpression of GmmiR156b suppresses flowering in soybean (Fig. 1). Under LD conditions, the molecular mechanism involves the GmmiR156b-mediated down-regulation of the genes that promote flowering in soybean, such as *GmFT5a*, *GmAP1* (*a*, *b*, *c*), *GmSOC1a*, *GmSOC1b*, *GmLFY2*, *GmFULs*, *GmSPLs* and *GmmiR172*. These results were consistent with those obtained in Arabidopsis (Schwab et al. 2005; Wu and Poethig 2006; Fornara and Coupland 2009; Wang et al. 2009; Xing et al. 2010), demonstrating that the miR156-SPL module for the regulation of phase transition from juvenile to adult is conserved in soybean. However, some differences between soybean and Arabidopsis were noted. In Arabidopsis, irrespective of photoperiod, the expression levels of FUL and SOC1 are decreased in *35S:MIR156* seedlings (Wang et al. 2009); by contrast, weak or no effect on the expression of *GmFULs*, *GmSOC1a* and *GmSOC1b* was observed in *35S:MIR156b* soybean under SD conditions. In addition, the expression of the four *TOE1* homologs was up-regulated in *35S:MIR156b* plants only under SD conditions

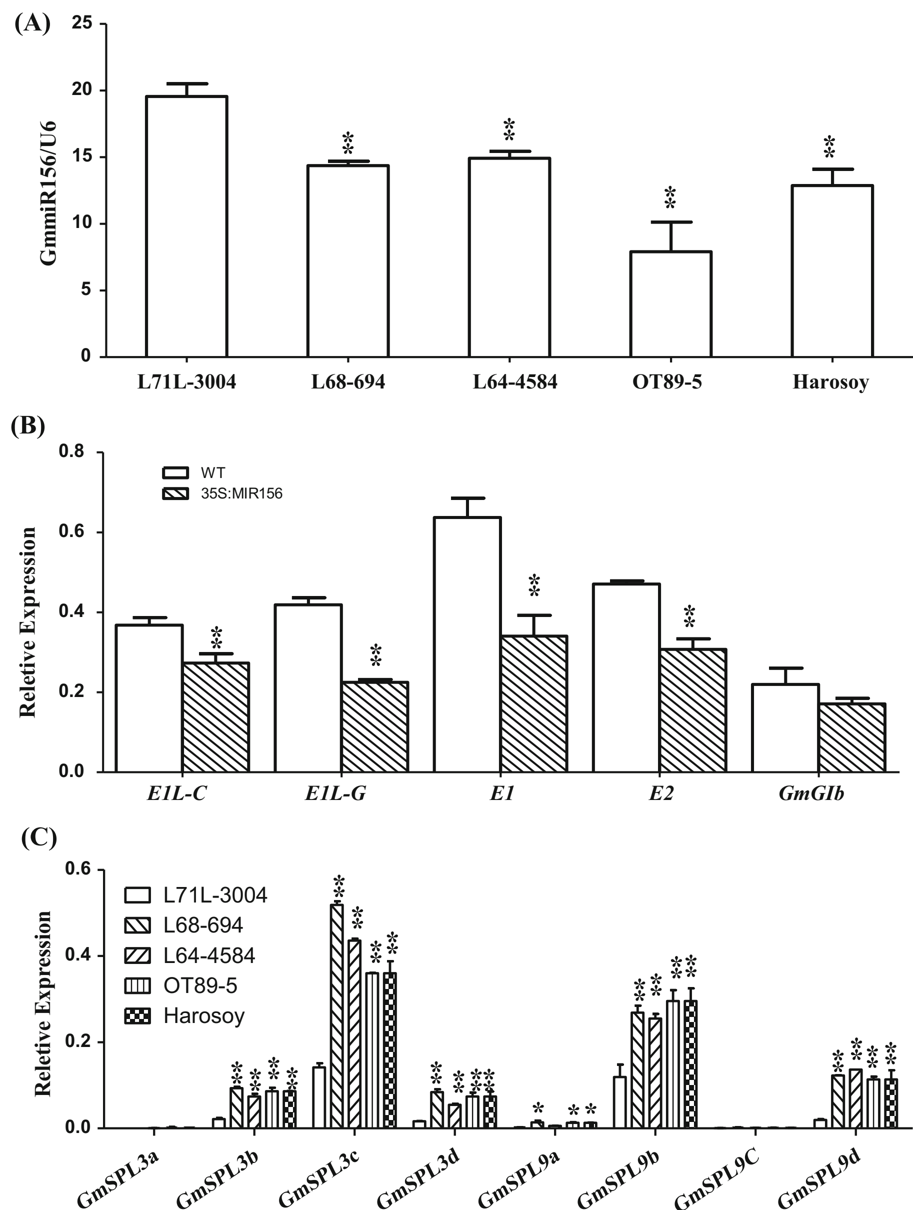

Fig. 6 Regulation between GmmiR156b/*GmSPLs* and E1, E2, E3 and E4. **a** The expression of *GmmiR156b* in the leaves of Harosoy NILs under LD conditions. **b** The exjpression of *E1*, *E1*-likes, *E2* and *E2*-like genes in the leaves of *35S:MIR156b* (line #5) and wild type Williams 82 (WT) plants under LD conditions. **c** The expression of *GmSPLs* in Harosoy NILs under LD conditions. The genotypes of L71L-3004, L68-694, L64-4584, OT89-5 and Harosoy were E1E2E3E4, E1e2E3E4, e1E2E3E4, e1e2e3e4 and e1e2E3E4, respectively. Relative transcript levels were analyzed by quantitative RT-PCR and normalized to *Tubulin*. Values represent the average of three biological replicates +SD. *Asterisks* and *double asterisks* indicate significant differences between transgenic and WT plants at $0.01 < P < 0.05$ and $P < 0.01$, respectively

(Fig. 5b). Furthermore, Xia et al. (2012) described that the *E1* gene, which is a major repressor of flowering time in soybean, has no apparent homolog in either Arabidopsis. The data obtained in the present study showed that GmmiR156b was up-regulated through E1, E2, E3 and E4 (Fig. 6a) and suppressed *E1* and *E2* expression (Fig. 6b), completing a negative feedback loop. However, the expression of *SPL* orthologs was not consistent with the expression of *GmmiR156b* (Fig. 6c), indicating that additional factors might also regulate *SPL* transcription in soybean.

In soybean, two FT homologs, GmFT2a and GmFT5a, play conserved roles in promoting flowering (Kong et al. 2010): these proteins redundantly and differentially regulate flowering through interactions with the bZIP transcription factor GmFDL19, resulting in up-regulation of the latter (Nan et al. 2014). Our data showed that the expression of *GmFT5a* was down-regulated by GmmiR156b under both SD and LD conditions (Fig. 2b). However, the expression levels of *GmFT2a* has no significantly difference between wild-type and *35S:MIR156b* plants under LD conditions (Fig. 2b). These data indicated that the regulation of *GmFT2a* and *GmFT5a* was differentially regulated through GmmiR156b, consistent with the differential roles of *GmFT2a* and *GmFT5a* in the regulation of flowering time in soybean (Nan et al. 2014). Previous studies have suggested that the SPL3 protein directly binds to GTAC motifs within the *FT* promoter (Kim et al. 2012).

Therefore, we analyzed the promoter regions of *GmFT2a* and *GmFT5a* and identified three GTAC motifs 1.5 kb upstream of the *GmFT5a* promoter (Fig. S5). Conversely, no GTAC motifs were detected, even up to 3.0 kb, upstream of the *GmFT2a* promoter region using Phytozome analysis. These data suggested that *GmFT5a*, but not *GmFT2a*, might be down-regulated through GmmiR156b via *SPL3* gene orthologs in soybean. Nonetheless, the expression of *GmFT2a* was also decreased in *35S:MIR156b* plants under SD conditions. As a previous study showed that GmTOE4a down-regulates *GmFT2a* and *GmFT5a* in soybean (Zhao et al. 2015), it is likely that *GmFT2a* is regulated through GmmiR156b via other genes, such as miR172 and the miR172 target TOE1, in soybean under SD conditions. These data suggest that *GmFT2a* and *GmFT5a* are differentially regulated through GmmiR156b and provide further information concerning the roles of *GmFT2a* and *GmFT5a* in the regulation of flowering time in soybean.

In summary, we propose a molecular network for the genetic interactions of GmmiR156b and its role in the regulation of the photoperiodic flowering pathway in soybean under LD conditions (Fig. 7). GmmiR156b was found to be up-regulated through E3/E4, E1 and E2. GmmiR156b may target SPL orthologs and negatively regulate *GmSPLs*, which up-regulate the promoters of flowering-related genes in soybean, thereby delaying flowering time. GmmiR156b also down-regulates E1, E2 and GmmiR172 in soybean.

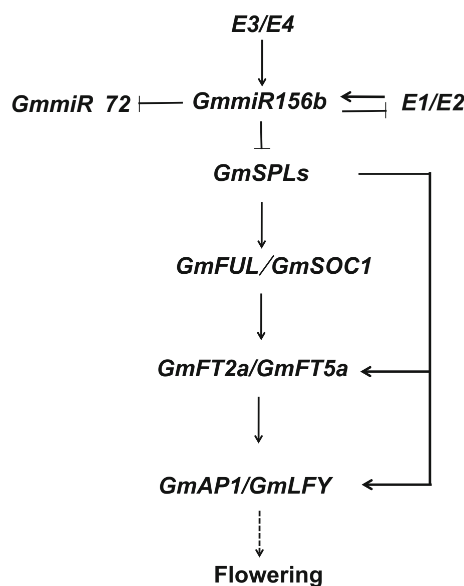

Fig. 7 Proposed model for the involvement of GmmiR156b in the regulation of photoperiodic flowering in soybean under LD conditions. *Arrows* represent stimulation of the gene expression; *T-shaped symbols* represent inhibition of gene expression

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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