Cloning, Characterization, and Overexpression in *Arabidopsis* to Determine the Function of *SPL* Genes from Woodland Strawberry (*Fragaria Vesca*)

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Research Article

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Abstract

SQUAMOSA promoter binding protein-like (SPL) proteins is a class of plant specific transcription factors that play important roles during plant development. However, the majority of *SPL* genes in strawberry are functionally uncharacterized. In this study, three *SPL* genes, i.e. *FvSPL1*, *FvSPL2*, and *FvSPL11* (*FvSPL1/2/11*), from woodland strawberry were cloned and characterized. Phylogenetic analysis with *SPL* genes from *Arabidopsis*, tomato and chrysanthemum indicated that *FvSPL1/2/11* were clustered into the same group with those of miR156 target site located at the 3'-untranslated region (UTR). Further biochemical analysis indicated that FvSPL1 was exclusively localized in the nucleus. Electrophoretic mobility shift assay demonstrated FvSPL1 could specifically recognized the GTAC motif. Transcriptional activity analysis showed FvSPL1 was a transcriptional activator that could activate the expression of *FvAP1* gene. Finally, all of the transgenic *Arabidopsis* that overexpression the three *FvSPL* genes were exhibited significantly early flowering phenotype. Taken together, our study indicated that *FvSPL1/2/11* similar to their orthologs in *Arabidopsis* mainly functions in regulating plant flowering. These results enriched our understanding to the functions of *SPL* genes in strawberry and might be utilized for strawberry flowering time manipulation in the future.

Key Message

MiR156-targeted woodland strawberry *SPL* genes, *FvSPL1*, *FvSPL2*, *FvSPL11,* regulate plant flowering.

Introduction

Floral transition is the prerequisite for plants to complete reproductive growth, which is regulated by both external environmental factors and internal genetic factors (He et al. 2010). At present, a number of floral induction pathways, such as autonomous pathway (Wu et al. 2020), photoperiod pathway (Song et al. 2015), gibberellin (GA) pathway (He et al. 2020), age pathway (Wang 2014) and ambient temperature pathway (Fornara et al. 2010), have been identified in various plants. Numerous studies have indicated that these floral induction pathways are not separated but act synergistically to regulate plant flowering. Many key integrators that link different pathways have been identified. For example, several studies have demonstrated GA could promote the expression of *FLOWERING LOCUS T* (*FT*), the key regulator of photoperiod pathway, under long day condition (Song et al. 2013; Yamaguchi 2008). In addition, SQUAMOSA promoter binding like proteins (SPLs), the key regulators of age pathway were also found integrated with GA pathway through interaction with DELLA proteins (Yu et al. 2012). Moreover, Jung et al identified several SPLs regulate flowering through integration with photoperiod pathway by directly interaction with FD protein (Jung et al. 2016).

SPL gene family encodes a class of plant-specific transcription factor that have been identified nearly in all plant species. This class of TFs were found play vital roles in many aspects during plant growth and development, such as phase transition i.e. changes from vegetative stage to reproductive stage (Jung et al. 2011), phytohormone signal transduction (Zhang et al. 2007), embryo formation (Unte et al. 2003),

plant architecture (Miura et al. 2010) and fruit development (Manning et al. 2006). In *Arabidopsis*, 11 *SPLs* were found targeted by miR156. Functional analysis indicated that nearly all these miR156 targeted *SPLs* except *AtSPL6* and *AtSPL13* were acted as phase transition accelerators (Wang et al. 2015). In addition, studies have found these miR156 targeted *AtSPLs* can be classed into two groups which represented by *AtSPL3* and *AtSPL9*, respectively (Yu et al. 2012). The *AtSPL3* group contains three relative small genes, i.e. *AtSPL3, AtSPL4* and *AtSPL5* (*AtSPL3/4/5*), that encode proteins less than 200 amino acids residues in length. Beside the size of *AtSPL3/4/5* are relative smaller, the miR156 target site in the three genes was found all located at the 3'-untranslated region (UTR). *AtSPL3/4/5* have been found mainly involved in phase transition. *AtSPL3/4/5* was reported could directly combine to the promoter and activate the expression of floral meristem genes such as *APETALA1* (*AP1*), *FRUITFULL* (*FUL*) and *LEAFY* (*LFY*) to promote plant flowering (Yamaguchi et al. 2009). Latter, AtSPL3/4/5 were found link age pathway and photoperiod pathway to mediate plant flowering through physically interaction with FD (Jung et al. 2016). However, genes in *AtSPL9* group have been found possess diverse functions. For example, *AtSPL9* not only play roles in phase transition (Schwarz et al. 2008; Wu et al. 2006), but also could negatively regulate anthocyanin biosynthesis (Gou et al. 2011). In chrysanthemum, several miR156 targeted *SPL* genes were also been identified play roles in regulation flowering. For instance, Wei et al found silencing a nuclear factor gene, *CmNF-YB8*, leading to down-regulation of miR156, which resulted in up-regulation of several *CmSPLs* such as *CmSPL3*, *CmSPL5* and *CmSPL9*, consequently resulted in early flowering phenotype (Wei et al. 2017).

In our previous study, we identified nine miR156 targeted *FvSPL* genes in woodland strawberry. Further analysis found among the nine *FvSPLs* the miR156 target site of three *FvSPLs*, *FvSPL1, FvSPL2* and *FvSPL11* (*FvSPL1/2/11*), were located in the 3'-UTR and they were highly expressed in flower organs (Xiong et al. 2018). These characteristics were very similar to *AtSPL3/4/5*. As studies in *Arabidopsis* have demonstrated *AtSPL3/4/5* were mainly function in regulating plants flowering (Yamaguchi et al. 2009). Therefore, we functionally analyzed *FvSPL1/2/11* in this study. We first cloned the three genes from woodland strawberry, phylogenetic analysis showed *FvSPL1/2/11* were closely related to the *SPL* genes with miR156 target site at 3'-UTR from *Arabidopsis*, tomato and chrysanthemum. Due to the functional redundancy of *AtSPL3/4/5*, we further analyzed the biological functions of *FvSPL1* in detail as representative. Subcellular localization assay found FvSPL1 was exclusively localized in the nucleus. Both *in vivo* and *in vitro* assays demonstrated that FvSPL1 could recognize and bind to the GTAC motif, further transcriptional activity assay indicated that FvSPL1 is a transcriptional activator. Finally, we ectopically expressed the three *FvSPLs* in *Arabidopsis*, respectively. All the transgenic plants exhibit dramatically early flowering phenotype, several flowering related genes such as *AtAP1*, *AtFUL*, *AtAGL42*, *AtCO* and *AtLFY* were significantly upregulated. These results demonstrated the specific roles of *FvSPL1/2/11* in plant phase transition, which provides valuable information for investigating their role plant flowering and fruit development of strawberry.

Materials And Methods

1. Plant materials and growth conditions

The woodland strawberry ‘Hawaii 4’ seeds were obtained from the National Clonal Germplasm Repository (Corvallis, Oregon). *Nicotiana benthamiana* was used for Agroinfiltration assay. The ecotype Col-0 of *Arabidopsis thaliana* was used for genetic transformation of *FvSPL1/2/11*. All plants were grown in greenhouse at 22℃ in 16h light/8h dark cycles. All plant materials were collected and stored at -80℃ after frozen by liquid nitrogen.

2. RNA extraction and the amplification of *FvSPL* genes

Plant total RNA was extracted using Plant Total RNA Isolation Kit Plus (Foregene, Chengdu, China) following the user manual instruction. RNA quality was evaluated by gel electrophoresis and spectrophotometry. First-strand cDNAs was synthesized by the Prime Script RT reagent kit with gDNA eraser (TAKARA, Dalian, China). Full-length CDS of *FvSPL1/2/11* were amplified and further verified by sequencing. The primers were listed in Supplemental Table 1.

3. Sequence analysis

The protein sequences of FvSPL1/2/11 were identified from *F.vesca* v1.1. The protein sequences of *Solanum lycopersicum* were retrieved from phytozome v12 (https://phytozome.jgi.doe.gov/pz/portal.html). For *Chrysanthemum morifolium*, the protein of SPLs were obtained according to Song et al. (Song et al. 2016). The protein sequences of *Arabidopsis* SPLs were collected from TAIR database (https://www.arabidopsis.org). Then, the SBP domain (PF03110) of SPL proteins were retrieved using Pfam (http://pfam.xfam.org). The phylogenetic tree based on SBP domain was constructed by MEGA7.0 with 1000 bootstrap replicates (Kumar. et al. 2016).

4. Subcellular localization of FvSPL1

The *FvSPL1* CDS was cloned in frame with green fluorescent protein (GFP) tag in the binary vector pJX002. Subcellular localization analysis of FvSPL1 was performed as described by Xiong et al (Xiong et al. 2019). GFP fluorescence signal was observed by laser scanning microscopy (LSM800, Zeiss, Germany) within 2-5 days after injection, transient expression assays were repeated at least three times.

5. Transcriptional activity analysis

The transcriptional activity of FvSPL1 was determined by dual-luciferase assay system in tobacco leaves. Vectors construction and transcriptional activity assay were performed as described by Cheng et al (Cheng et al. 2017).

To analyze the trans-activation of FvSPL1 to *FvAP1* gene. A 1000 bp fragment of the *FvAP1* promoter that immediately upstream from the start codon was inserted into the reporter vector pGreenII 0800-LUC, and *FvSPL1* was cloned into the effector vector pEAQ (Fig. 5a). The trans-activation activity of FvSPL1 to *FvAP1* was analyzed by dual-luciferase system as described by Cheng et al (Cheng et al. 2017).

After two days of co-cultivation, the ratio of LUC/REN was measured using the Dual-Luciferase® Reporter Assay System kit (Promega, USA), the ratio of LUC/REN was detected by GloMax 20 ~ 20 photoluminescence detector (Promega, USA). Each set of data was repeated three times.

6. Protein extraction and Electrophoretic mobility shift assay

The CDS of *FvSPL1* was inserted into prokaryotic expression vector pGEX-4T-1. The FvSPL1-GST fusion protein was induced by 0.3 mM isopropyl thio-β-D-galactoside (IPTG) at 37°C for 4 hours and further purified by glutathione-super flow resin (Fig. 3a).

A 47 bp fragment of *FvAP1* promoter containing the second GTAC motif was chosen as probe to verify the specific binding ability of FvSPL1 to this motif. Probe was labeled by biotin at 3' end. The EMSA experiment was performed using the Chemiluminescent EMSA Kit (GS009) according to the manufacturer's instructions (Beyotime, China). Labeled probes were detected by chemiluminescence method.

7. Overexpression of *FvSPLs* in *Arabidopsis*

Full-length cDNA of *FvSPL1/2/11* was respectively inserted into binary vector pJX001 under the control of 35S promoter to generate overexpression constructs. The constructs were transformed into *Arabidopsis* (Col-0) using floral-dip method (Topping et al. 1995). The seeds of transgenic *Arabidopsis* were harvested and screened on 1/2 MS medium containing 50 mg/L hygromycin. T_3 generation homozygous transgenic lines were selected for further analysis. Primers are listed in Supplemental Table 1.

8. Phenotypic and gene expression analysis of the transgenic *Arabidopsis*

The homozygous T3 generation *FvSPL1/2/11* overexpression plants were used for phenotypic analysis. Flowering time and leaves numbers was counted at the day when the first bud emergence after germination.

Two week olds *Arabidopsis* plants were collected to detect the expression level of flowering-related genes. qRT-PCR was performed with Light Cycler480 II (Roche, Switzerland) using SYBR Premix Ex Taq II (TAKARA, Dalian, China). *Atβ-tubulin2* was used as an internal reference gene to normalize the expression of flowering-related genes. Two-step PCR amplification standard procedure through the following steps: 95℃/30s to predenaturation, 95℃/5s, 60℃/30s, 40 cycles. Relative expression levels of the detected genes were calculated by $2^{-\triangle\triangle Ct}$ method. All quantitative real-time primers were listed in Supplemental Table 1.

9. Statistical analyses

IBM SPSS Statistics v25 was used to analyzed the data by Student's *t*-test. Data were presented as the mean ± SD. * and ** represent $P < 0.05$ and $P < 0.01$ respectively.

Results

1. Sequence analysis of *FvSPL1/2/11*

Our previous study indicated that *FvSPL1/2/11* were highly expressed in flower organs. Therefore, we cloned the three genes from flower of woodland strawberry 'Hawaii 4'. Sequence analysis showed that *FvSPL1/2/11* encodes relative small protein containing 187, 157 and 178 amino acid, respectively. To further identify the evolutionary relationship between *FvSPL1/2/11* and *SPL* genes of other species, the phylogenetic analysis was performed based on SBP domain of the SPL genes from woodland strawberry, chrysanthemum, tomato and *Arabidopsis*. The results indicated that *FvSPL1/2/11* were divided into the same group with *CNR*, *SlySBP3*, *SlySBP4* from tomato, *AtSPL3/4/*5 from *Arabidopsis* and *CmSPL4*, *CmSPL5* from chrysanthemum (Fig. 1).

2. Subcellular localization and transcriptional activity of FvSPL1

In *Arabidopsis*, *AtSPL3/4/5* were found redundantly regulate vegetative to productive phase transition. Here, we toke *FvSPL1*, the homolog of *AtSPL3*, as representative to analyze its biochemical functions. To determine the express location of FvSPL1, we transiently expressed GFP fused FvSPL1 in tobacco leaves through Agroinfiltration. As shown in Fig. 2a, FvSPL1-GFP is exclusively localized in the nucleus, while the GFP control is distributed both at cytoplasm and nucleus. We further analyzed the transcriptional activity of FvSPL1 by dual-luciferase assay system. The results indicated that the value of LUC/REN ratio of FvSPL1 was 1.5 times higher compared to the empty vector control (Fig. 2c). These results demonstrated that FvSPL1 is a nuclear protein possesses transcriptional activation activity.

3. FvSPL1 recognizes and binds to the GTAC motif both in vitro

The first identified two *SPL* genes, *SBP1* and *SBP2*, were isolated from *Antirrhinum majus* as they specifically bind to the core GTAC motif in the promoter of *SQUAMOSA* (Klein et al. 1996). Here, we also observed the existence of the same core motifs in the promoter region of several flowering genes. For example, there are four GTAC motifs in the promoter of *FvAP1* (Fig. 3b). Therefore, we synthesized the second fragment of *FvAP1* promoter that contains the GTAC motif to detect whether FvSPL1 could specifically bind to it by electrophoretic mobility shift assay (EMSA). The results showed that GST-FvSPL1 recombinant protein could specifically recognize and bind to the GTAC motif in the promoter of *FvAP1* in vitro (Fig. 3b).

4. FvSPL1 activates the transcription of *FvAP1* in vivo

As we have confirmed FvSPL1 is a transcriptional activator that could specifically binds to the GTAC motif in the promoter of *FvAP1*. Therefore, we further applied dual-luciferase assay system to determine whether *FvSPL1* could regulate the expression of *FvAP1* in vivo. As shown in Fig. 4b, the value of LUC/REN was significantly higher in sample that co-transformed *FvSPL1* and the promoter of *FvAP1* than those of in empty effector control. These results verified *FvSPL1* could positively regulate the

transcription of *FvAP1* in vivo, and supports the premise that *FvSPL1* has functional role in the regulation plants flowering.

5. Ectopic expression of *FvSPLs* in *Arabidopsis* accelerates plant flowering

To further analyze the biological functions of three *FvSPLs* in plants, we respectively generated overexpression constructs of the three genes that under the control of CaMV35 promoter then further genetically transformed them in *Arabidopsis*. All transgenic lines that overexpression of the three genes exhibited dramatically early floweirng phenotype under long day conditions compared to the wild type (Fig. 5; Supplementary Fig. 1 and Supplementary Fig. 2). For example, transgenic lines overexpression *FvSPL1*, *FvSPL2* and *FvSPL11* flowering approximate at the 16th, 17th and 17th days, while the wild type plants flowering at the 23th days.

As the transgenic plants significantly promoted flowering, we therefore detected the expression levels of several flowering-related genes in *Arabidopsis* by qRT-PCR. As shown in Fig. 5c, the expression levels of *AtFUL*, *AtGL42*, *AtLFY* and *AtAP1* increased significantly in *FvSPL1* overexpression lines, especially *AtAP1*, which was up to 14 times higher than that of the control. However, in *FvSPL1* overexpression lines *AtCO* was only up-regulated about three folds higher. Similarly, all these five detected genes were up regulated in *FvSPL2* and *FvSPL11* overexpression lines, despite the expression level of individual gene was quite different in each lines. For example, the expression level of *AtCO* in *FvSPL2* overexpression lines were much higher than those of in other two lines, but the expression level of *AtAP1* in the three transgenic plant lines were quite similar.

Discussion

As a class of plant specific transcription factors SPLs has been extensively studied in model plants. For example, in *Arabidopsis*, *AtSPL7* was found regulating Cu homeostasis (Zhang et al. 2014). *AtSPL8* play roles in pollen sac development (Unte et al. 2003). *AtSPL1* and *AtSPL12* functions in regulating plant thermotolerance at reproductive stage (Chao et al. 2017), and almost all the miR156 targeted *AtSPLs* play role in phase transition (Wang et al. 2015). In rice, *OsSPL3* regulates crown root development (Shao et al. 2019), *OsSPL13* and *OsSPL16* function in regulating grain size (Si et al. 2016; Wang et al. 2012), and *OsSPL14* was identified play important role in regulating plant architecture (Jiao et al. 2010; Miura et al. 2010). In tomato, *Colorless nonripening* (*CNR*), the ortholog of *AtSPL3*, was found essential for fruit ripening (Manning et al. 2006). However, the function of *SPLs* in strawberry are still largely elusive. In our previous study, we observed *FvSPL1/2/11* were specifically expressed in flower organs. In addition, the target site of miR156 in *FvSPL1/2/11* also located at their 3'-UTR region similar to *AtSPL3/4/5* (Xiong et al. 2018). Therefore, we cloned these three gene from woodland strawberry for further analysis. Phylogenic analysis with *SPL* genes from *Arabidopsis*, tomato and chrysanthemum indicated that all *SPLs* with miR156 target site at 3'-UTR were classed into the same group. This information implying *FvSPL1/2/11* might possess similar functions with *AtSPL3/4/5*.

Due to the functional redundancy between *AtSPL3/4/5*, here, we only take *FvSPL1* as representative for detail analysis. We first detected the subcellular localization of FvSPL1 in tobacco epidermal cells by Agroinfiltration. The results shown that FvSPL1 was exclusively localized in the nucleus (Fig. 2a). Further transcriptional activity assay indicated that FvSPL1 has transcriptional activation capacity (Fig. 2c), which confirmed FvSPL1 is a transcriptional activator that could activate the expression of its downstream genes. *SPL* genes were originally cloned from *A. majus* because they were found binding to the GTAC core motif of the promoter of SQAUMSA gene (Klein et al. 1996). Following studies about *SPL* genes in plants also demonstrated that the SBP domain in required and sufficient binding to the GTAC motif. For example, in *Arabidopsis*, SPL3/4/5 were found could directly bind to GTAC motif in the promoters of several flowering genes such as *LFY*, *FUL* and *AP1* to regulate plant flowering (Yamaguchi et al. 2009). In this study, we also observed the existence of several GTAC motifs in the promoter region of several flowering homolog genes in woodland strawberry. Therefore, we utilized EMSA to verify the specific binding ability of FvSPL1 to the GTAC motif in the promoter of *FvAP1*. In addition, in vivo assay by luciferase system also confirmed FvSPL1 could bind the *FvAP1* promoter and activate its expression.

To explore the biological functions of *FvSPL1/2/11*, we ectopically expressed the three genes in *Arabidopsis*, respectively. As expected, transgenic plants overexpression the three genes were significantly accelerated flowering compared to the wild type plants under long day condition (Fig. 5; Supplementary Fig. 1 and Supplementary Fig. 2). Further qRT-PCR analysis indicated that several flowering genes such as *AtFUL*, *AtCO*, *AtAGL42*, *AtLFY* and *AtAP1* were significantly up regulated in these transgenic lines (Fig. 5c; Supplementary Fig. 1c and Supplementary Fig. 2c), despite the expression level of these genes were a little bit different. These results confirmed that *FvSPL1/2/11* have similar function to *AtSPL3/4/5*, and *CmSPL5*, because up regulation these genes lead to early flowering phenotype in the corresponding plant. Interestingly, no other phenotypic changes were observed in these transgenic *Arabidopsis* lines except early flowering. This phenomenon confirmed that the miR156 targeted small size group *SPL* genes mainly function in regulating plant flowering. As we have not obtained transgenic strawberry plants that overexpression *FvSPL1/2/11*, we could not make conclusions that the three genes have similar function to tomato *CNR* that could regulate strawberry fruit development. It would be worthy to investigating the function of *FvSPL1/2/11* in strawberry plants in the future.

Conclusions

In this study, we cloned and characterized three miR156 targeted *SPL* genes, *FvSPL1/2/11*, from woodland strawberry. Functional analysis indicated that FvSPL1 is a transcription activator that upregulate the expression of flowering genes through specifically binding to the GTAC motif in their promoter. Further genetics analysis confirmed transgenic *Arabidopsis* overexpression the three genes all dramatically promoted flowering by up regulation flowering related genes. These results indicated that *FvSPL1/2/11* specifically function in regulating plant flowering.

Declarations

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Author contribution statement JSX and ZMC conceived and designed research. YBB performed the experiments and data analysis. MRL and CJM helped in experimental works and data analysis. YBB drafted the manuscript. JSX and ZMC revised and finalized the manuscript. All authors reviewed and approved the final manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

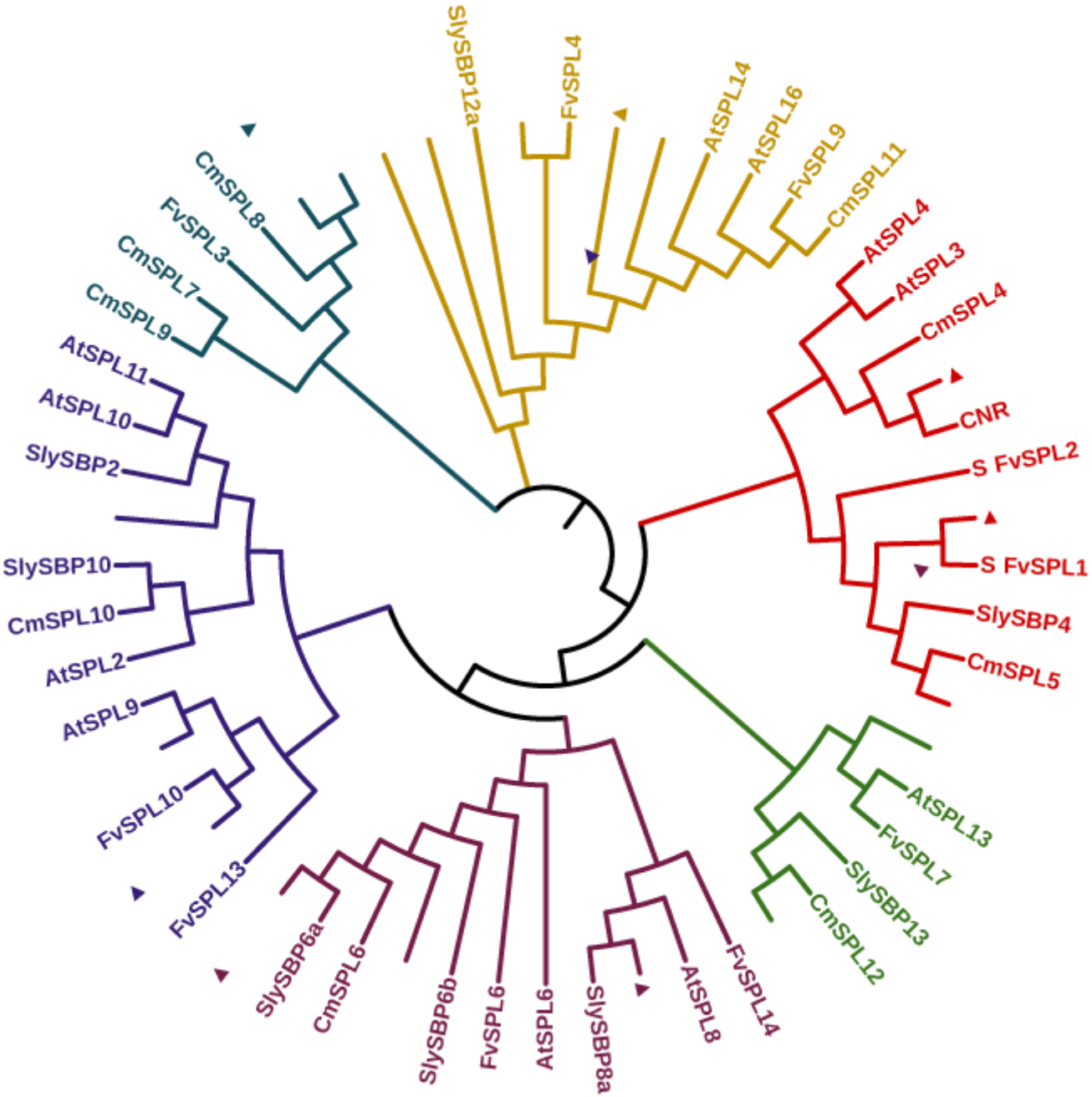

Figure 1

Phylogenetic analysis of FvSPL1/2/11 with SPLs from other plants. Phylogenetic analysis based on SPL domain sequence from five plant species. Woodland strawberry (FvSPL), tomato (CNR or SlySBP), chrysanthemum (CmSPL) and Arabidopsis (AtSPL). Phylogenetic tree was constructed using the neighbor-joining (NJ) method. FvSPL1, FvSPL2, FvSPL11 were represented by red triangles.

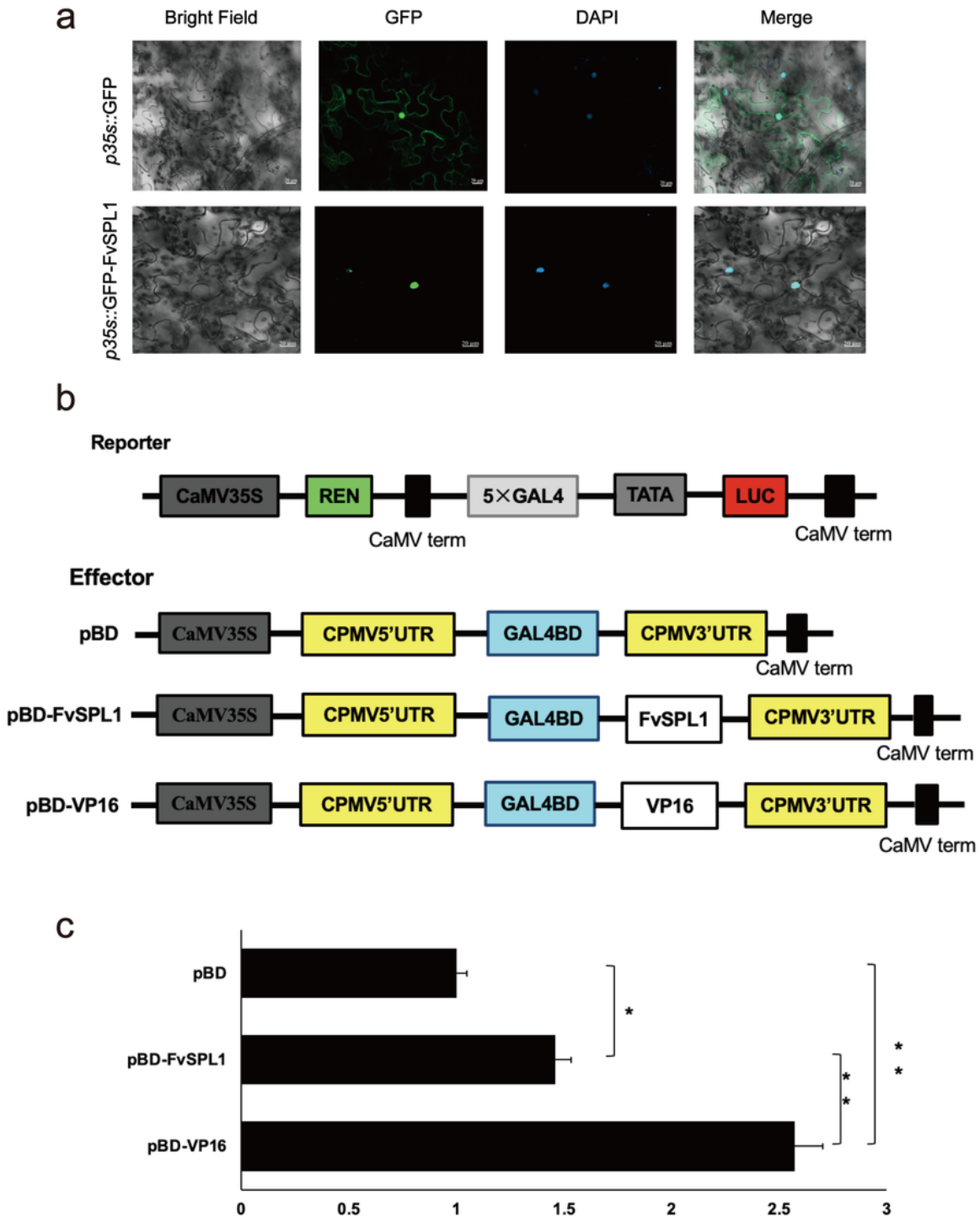

Figure 2

Subcellular localization and transcriptional activity analysis of FvSPL1. (a) Subcellular localization of FvSPL1. Constructs of GFP or GFP fused FvSPL1 that under the control of 35S promoter were expressed in tobacco leaves through Agroinfiltration. Fluorescence was detected at 72 h after infiltration. Nucleus was stained by 4'6-diamidino-2-phenylindole (DAPI). Bar = 20 μm. (b) The structure of reporter and effector for transcriptional activity assay. REN represents renilla luciferase and LUC represents firefly

luciferase. (c) Transcriptional activity analysis of FvSPL1. Reporter vector was co-transformed with each effector into tobacco leaves by Agroinfiltration, respectively. The pBD and pBD-VP16 were used as negative control and positive control respectively. The relative ratio of LUC/REN of the negative control was set as calibrator. Each ratio value represents the means of six biological replicates, * Represents significant difference compared with the negative control by Student's t-test ($P < 0.05$). ** Represents extremely significant difference by Student's t-test ($P < 0.01$).

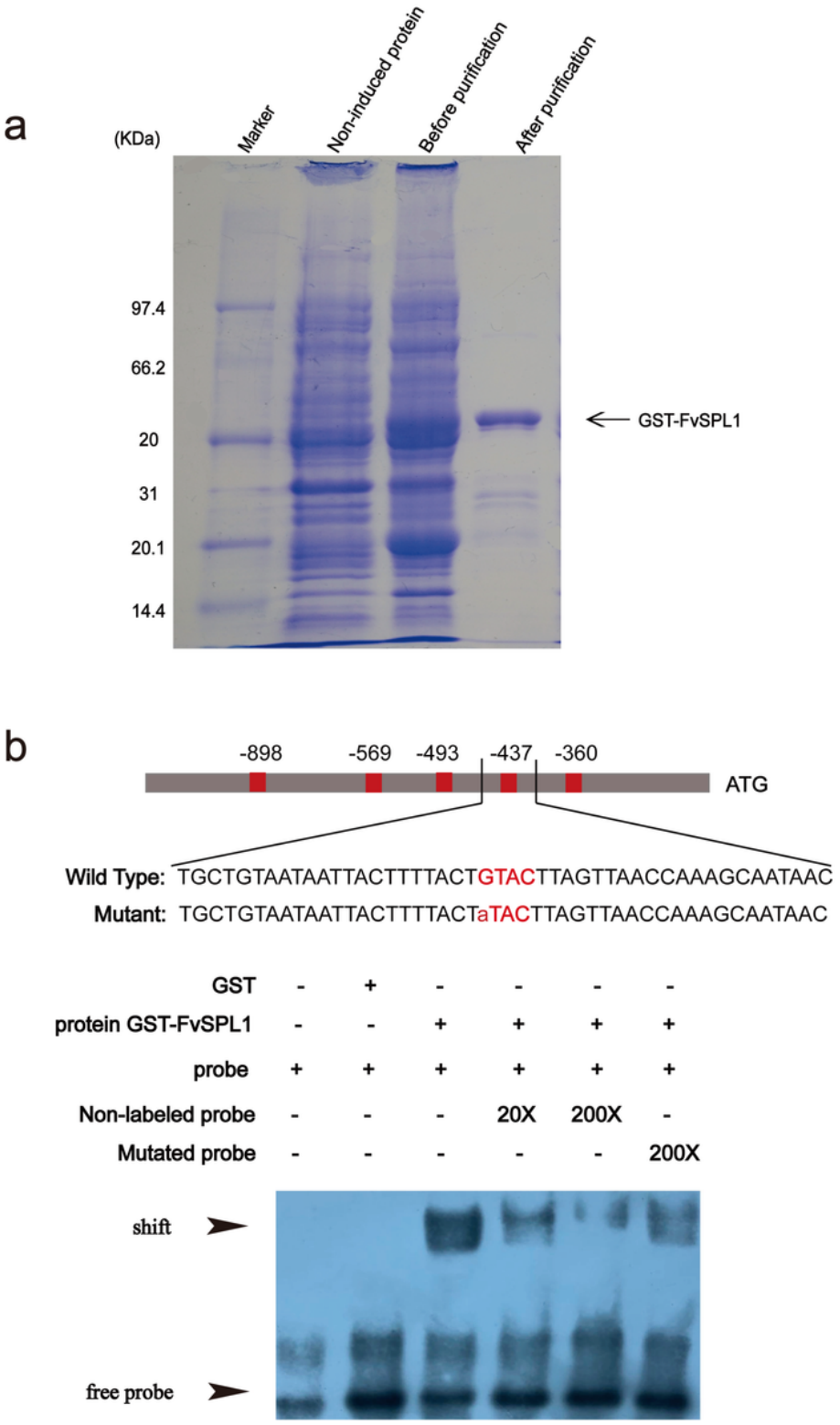

Figure 3

Verification the DNA binding ability of FvSPL1 by EMSA. (a) Expression and purification FvSPL1 fusion protein for EMSA. (b) EMSA detect the binding ability of FvSPL1 to FvAP1 promoter. Schematic diagram of the 1000 bp fragment of FvAP1 promoter that immediately upstream from the start codon and probes utilized in EMSA. The red bars represent the GTAC motifs in the promoter of and number above the bars represent the position of each motif in the promoter of FvAP1. A 47 bp oligonucleotides containing the second GTAC motif of the FvAP1 promoter was synthesized as the probe. Arrows indicate the position of biotin-labeled shifted and free probes, respectively.

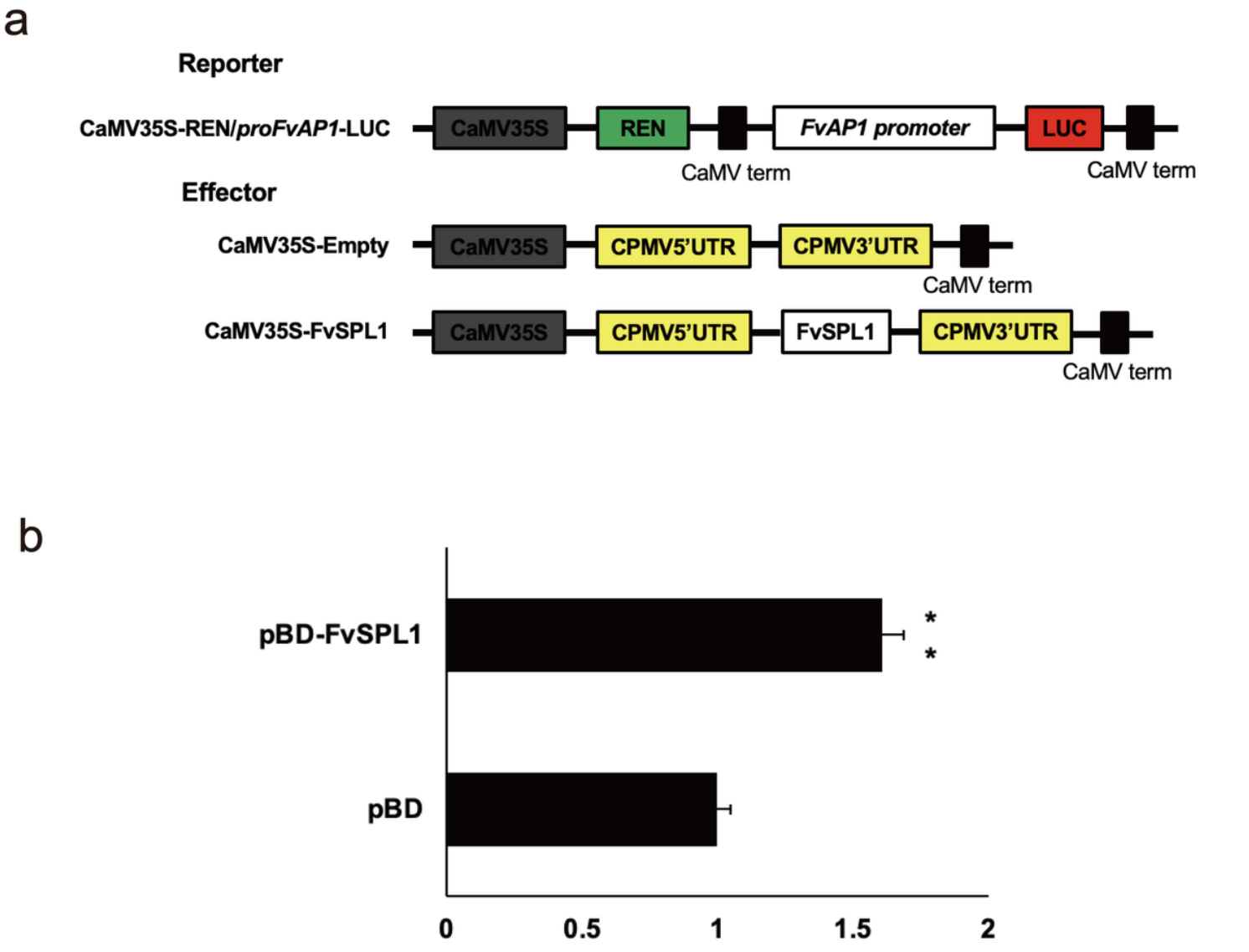

Figure 4

FvSPL1 activates the expression of FvAP1. (a) Structure illustrations used in dual-luciferase assay. REN represents renilla luciferase and LUC represents firefly luciferase. (b) FvSPL1 trans-activates the FvAP1 promoter. The relative ratio of LUC/REN of reporter to empty vector was set as calibrator. Each ratio value represents the means of six biological replicates. ** Indicates extremely significant difference by Student's t-test ($P < 0.01$).

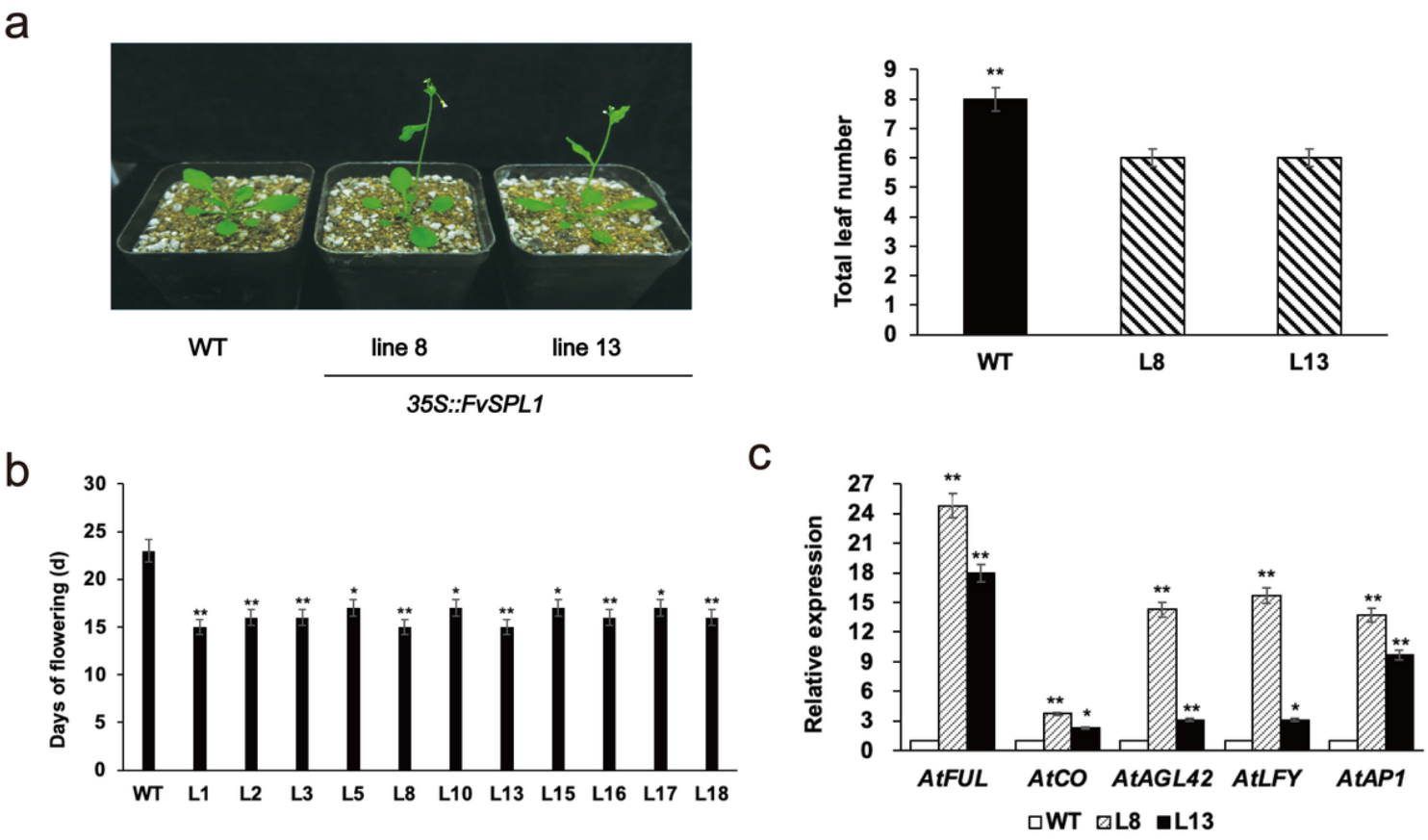

Figure 5

Overexpression of FvSPL1 in Arabidopsis accelerates flowering. (a) Phenotypes of WT and FvSPL1-OE transgenic Arabidopsis plants. Two FvSPL1 overexpression lines, L8 and L13, were selected as representatives. Total leaves numbers were calculated in WT and FvSPL1-OE lines. (b) Comparison of FvSPL1-OE lines and WT in flowering time. Eleven FvSPL1-OE lines were calculated. (c) Relative expression levels of flowering-related genes in WT and FvSPL1-OE lines. Two FvSPL1 overexpression lines, L8 and L13, were selected as representatives. * Represents significant difference by Student's t-test (n= 10, $P < 0.05$). ** Represents extremely significant difference by Student's t-test (n=10, $P < 0.01$).

Supplementary Files

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- SupplementaryTable1.xls
- supplementaryFig.1.tif
- supplementaryFig.2.tif