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Research

Regulation of flowering time by SPL10/MED25 module in Arabidopsis

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Summary

- Several SQUAMASA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors are involved in plant developmental transition from vegetative to reproductive growth. However, the function of SPL10 in regulating floral transition is largely unknown. It is also not known which Mediator subunit mediates SPL10 transcriptional activity.
- Here, we used overexpression lines and knockout mutants to examine the role of *SPL10* in flowering-time regulation and we investigated possible interactions of SPL10 with several mediator subunits *in vitro* and *in vivo*.
- Plants overexpressing *SPL10* showed precocious flowering, whereas the triple loss-of-function mutants of *SPL10* and its two homologous genes, *SPL2* and *SPL11*, flowered late compared with wild-type plants. We found that SPL10 interacts with MED25, a subunit of the Mediator complex, which bridges transcription factors and RNA polymerase II to facilitate transcription initiation. Genetic analysis showed that *MED25* acts downstream of *SPL10* to execute *SPL10*-regulated floral transition. Furthermore, SPL10 was required for MED25 association with the promoters of two target genes, *FUL* and *LFY*.
- We provide evidence that SPL10 recruits MED25 to the promoters of target genes to regulate flowering time. Our results on the SPL10/MED25 module are relevant to the molecular mechanism of other SPL family members.

Introduction

Flowering time determines phase transition of a plant from the vegetative stage to the reproductive stage, and a proper flowering time is essential for plants to grow and to reproduce in the environment. The timing of floral transition is subjected to precise regulation by six flowering pathways: photoperiod pathway, vernalization pathway, autonomous pathway, GA pathway, temperature pathway, and age pathway (Fornara *et al.*, 2010). The age pathway, representing one of the endogenous flowering-time pathways, is defined by *miR156* and SQUAMASA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors. *MiR156* is a negative regulator of flowering time as it restricts the expression of *SPLs* through messenger RNA cleavage or translational inhibition. The expression of *miR156* decreases with plants' age, allowing the increasing of *SPLs* expression to promote plant phase transition (Wu & Poethig, 2006; Wang *et al.*, 2009; Wu *et al.*, 2009).

Ten out of 16 *SPL* transcription factors are targeted by *miR156* (Preston & Hileman, 2013). The role of SPLs in flowering time has been extensively studied with respect to two clades of *miR156*-targeted *SPL* genes, *SPL3/4/5* and *SPL9/15*, and several downstream targets of these SPLs have been identified: *FUL*, *LFY*, *AP1*, *SOC1*, and *miR172b* (Wang *et al.*, 2009; Wu *et al.*, 2009; Yamaguchi *et al.*, 2009; Xu *et al.*, 2016). By contrast, the contribution of another clade of *SPLs*, *SPL10/11/2*, in flowering-time regulation has not been well studied, largely because of its high genetic redundancy and the unavailability of multiple knockout mutants (Shikata *et al.*, 2009). Using ectopic expression of *SPL10*, Gao *et al.* (2017) have recently characterized the function of SPL10 in flowering time and even identified *AGL79* as a direct target gene. Furthermore, the creation of *spl10/11* double mutant by the CRISPR/Cas9 technique has shed light on the function of *spl10/11/2* clade in flowering-time regulation (Xu *et al.*, 2016).

In eukaryotes, it is known that the Mediator complex links transcription factors with RNA polymerase II to initiate transcription (Malik & Roeder, 2010). The plant mediator complex is composed of *c.* 34 subunits, and the functions of several mediator subunits in plant growth, development, and stress responses have been described (Samanta & Thakur, 2015; Buendia-Monreal & Gillmor, 2016; Dolan & Chapple, 2017). The first mediator subunit identified in flowering-time regulation, MED25, which is also known as PHYTOCHROME AND

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FLOWERING 1 (PFT1), has been reported to promote flowering downstream of phyB (Cerdán & Chory, 2003; Bäckström *et al.*, 2007). Further studies showed that MED25 regulates flowering in cooperation with the photoperiod pathway (Iñigo *et al.*, 2012; Liu *et al.*, 2017). Other than *med25*, several mediator subunit mutants also show a late-flowering phenotype: *med8*, *med12*, *med13*, *med15*, *med16*, *med17*, *med18* and *med20a* (Buendia-Monreal & Gillmor, 2016). In addition, three mediator subunits are involved in age-regulated flowering time; for instance, MED12 and MED13 repress the expression of *miR156*, and MED18 interacts with SPL15 to initiate transcription of flowering-related genes (Gillmor *et al.*, 2014; Hyun *et al.*, 2016). The contributions of other mediator subunits in age-related flowering-time regulation remain to be clarified.

By generating the triple mutant of *spl10/11/2*, we investigated the function of the *SPL10/11/2* clade in flowering-time regulation. More importantly, we showed that SPL10 recruits MED25 to the promoter of downstream genes, *FUL* and *AP1*, and that MED25 is required for SPL10 activation. Our results broaden the understanding of the regulatory mechanism of mediator subunits in flowering time regulation.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild-type (WT) in this study. Mutants *spl2-1*, *spl10-2*, *spl11-1*, and *spl10-3/spl11-1* (*spl10/11*) were described previously (Xu *et al.*, 2016). The double mutant *spl2/spl10* (*spl2/10*) was isolated from the progeny of a cross between *spl2-1* and *spl10-3*. The double mutant *spl2/spl11* (*spl2/11*) was isolated from progeny of a cross between *spl2-1* and *spl11-1*. The triple mutant *spl10/spl11/spl2* (*spl10/11/2*) was isolated from the progeny of a cross between *spl10-3/spl11-1* and *spl2-1*. Mutants *pft1-2* (a *med25* knockout mutant, SALK_129555), *med18-2* (SALK_027178), and *med19a-2* (SALK_034955) were obtained from the Arabidopsis Biological Resource Center. The transgenic lines *PSPL10:rSPL10-VENUS*, *PSPL11:rSPL11*, and *PSPL2:rSPL2* were described previously (Stief *et al.*, 2014; Yu *et al.*, 2015). Full-length genomic DNA and complementary DNA (cDNA) encoding *MED25* were cloned into *pBA002a-DC-HA* and *pBA002-DC-HA* by LR reaction to generate *PMED25::MED25-HA* and *35S:MED25-HA*, respectively. The constructs were transformed into *Agrobacterium* strain GV3101 and then infiltrated into *pft1-2* and WT plants using the floral dip method (Clough & Bent, 1998; Zhang *et al.*, 2006). All primers for Gateway cloning and PCR genotyping are listed in Supporting Information Tables S1 and S2, respectively.

Seeds of WT, mutants, and transgenic lines were grown on Murashige and Skoog (MS) plates with 1% sucrose at 20°C under long-day conditions (16 h : 8 h, light : dark). For soil-grown plants, 7-d-old seedlings were transferred to soil and grown under the same conditions in a growth chamber.

Yeast two-hybrid assay

Full-length cDNAs encoding *SPL2*, *SPL3*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13* and *SPL15* were cloned into *pGAD424-GW* vector using LR Clonase II to generate AD-SPL2, AD-SPL10, and AD-SPL11 vectors. Full-length cDNAs of *MED25*, *MED18*, *MED19a*, and *MED36a* were cloned into *pGBT9-GW* to generate BD-MED25, BD-MED18, BD-MED19a and BD-MED36a, respectively. *pGAD424-GW* and *pGBT9-GW* vectors were described by Deng *et al.* (2016). All constructs were transformed into AH109 strain by the lithium acetate method according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Transformed yeast colonies were examined on SD/-Ade/-His/-Leu/-Trp quadruple dropout medium to test for possible interactions through colony growth.

Bimolecular fluorescence assay

Full-length coding sequences of *SPL10* and *MED25* were cloned into *pBA3136* and *pBA3132* by Gateway cloning to generate MED25-nYFP and SPL10-cYFP vectors, respectively (Seo *et al.*, 2017). Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and the transformed *Agrobacterium* were infiltrated into leaves of 3-wk-old *Nicotiana benthamiana* plants. Yellow fluorescent protein (YFP) fluorescence signals were analyzed using an LSM 510 confocal laser scanning microscope (Zeiss) 3 d after infiltration.

Co-immunoprecipitation using plant extracts

Nuclei were isolated from 2-wk-old transgenic seedlings of *35S:MED25-HA* and *35S:MED25-HA/PSPL10:rSPL10-VENUS* (Fiil *et al.*, 2008). Nuclei were resuspended in immunoprecipitation (IP) buffer (100 mM Tris-Cl (pH 7.4), 75 mM sodium chloride, 10% glycerol, 1 mM EDTA, 0.1% Triton-X-100, and 0.1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktail (Roche) and sonicated. Nuclear proteins were treated with nuclease for 1 h on ice and centrifuged at 16 000 ***g*** for 30 min. The supernatants were incubated with GFP-Trap GFP-Trap®_M (ChromoTek GmbH, Munich, Germany) overnight according to the manufacturer's instructions. After three washes with IP buffer, proteins were eluted from the beads by boiling with 40 µl 2 × Laemmli loading buffer and detected by Western blotting with anti-green fluorescent protein (GFP) and anti-hemagglutinin monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Quantitative real-time PCR

Total RNA was extracted from 10- to 12 d-old Arabidopsis seedlings using RNeasy plant mini kit (Qiagen, Hilden, Germany) followed by DNase I treatment. Total RNA (2 µg) and an oligo$(dT)_{20}$ primer were used for reverse transcription with SuperScript III kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time (qRT)-PCR was performed using SYBR premix (TaKaRa, Kusatsu, Japan) on the Bio-Rad CFX96 system.

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Transcript levels were normalized to *ACT2* expression. Gene-specific qRT-PCR primers are listed in Table S3.

Chromatin IP analysis

Two-week-old seedlings were harvested and crosslinked in 1% formaldehyde for nuclei extraction. Chromatin IP (ChIP) assays were performed as described by Bowler *et al.* (2004). Anti-GFP (Abcam, Cambridge, MA, USA) or anti-MED25 was used to precipitate protein-bound DNA fragments, and the eluted DNA was detected by qRT-PCR. Primer sequences are given in Table S4.

Electrophoretic mobility shift assay analysis

The coding sequence for SPL10 was cloned into the *Bam*HI and *Xba*I sites of the *pCold-TF* vector (TaKaRa) to express His-TF-SPL10 protein in *Escherichia coli* BL21(DE3) cells. The recombinant protein was purified with Ni-NTA resin (Qiagen) according to the manufacturer's instructions. DNA probes were labeled with α-^{32}P-dATP (Amersham, Arlington Heights, IL, USA) in a PCR reaction. DNA gel shift assays were performed as described by Birkenbihl *et al.* (2005). The primer sequences used are given in Table S4.

Antibody generation

The coding sequence for a C-terminal (CT) fragment of the MED25 protein (637–836 aa) was cloned into the *Bam*HI and *Sal*I sites of the pET28a vector (Novagen, Madison, WI, USA) to express His-MED25-CT protein in *E. coli* BL21(DE3) cells. The recombinant protein purified with Ni-NTA resin (Qiagen, Hilden, Germany) was used to raise polyclonal antibodies in mouse. MED25 antibodies purified from mouse serum were used in immunoblots at a final concentration of 1 : 5000.

Results

Effect of *SPL10*-clade genes on flowering-time regulation

Although it is known that the miR156/SPL module defines the age-dependent pathway that controls flowering time, the function of each member of the SPL transcription factor family has not yet been fully elucidated. Xu *et al.* (2016) have previously reported that *spl10* single mutant does not change flowering time; we have confirmed this observation and further showed that single mutants of *spl2* and *spl11* had little effect on flowering time as well. Similar results were obtained from double mutants *spl2/10*, *spl2/11*, and *spl10/11* (Fig. S1). To overcome the issue of functional redundancy, we generated a triple mutant *spl10/spl11/spl2* (*spl10/11/2*) that includes the mutations of *SPL10* and its homologous genes, *SPL11* and *SPL2*. We found that *SPL10/11/2* triple mutant flowered later than WT (Figs 1, S2). By contrast, overexpression of each gene within the clade by the introduction of the *miR156*-resistant form transcribed with the cognate native promoter accelerated flowering (Figs 1, S3). These results indicate that *SPL10* regulates flowering time with functional redundancy with *SPL11* and *SPL2*.

Regulation of flowering-related genes by *SPL10*-clade genes

To elucidate the molecular mechanism of *SPL10*-clade genes in regulating flowering genes, we investigated the expression of flowering-related genes, *FUL*, *LFY*, *AP1*, *FT*, *SOC1*, and *miR172b* in various genotypes. Except for *FT*, expression of all of these genes was decreased in the *spl10/11/2* triple mutant. By contrast, expression of these downstream target genes was increased in plants overexpressing each of the *SPL10*-clade genes (Fig. 2). We assumed that the elevated expression of *FT* in *spl10/11/2* mutant was due to feedback regulation of a lower expression of its downstream genes, such as *FUL*, *AP1*, and *SOC1*. These results suggest SPL10-clade transcription factors function as activators to promote floral transition by upregulating *FUL*, *LFY*, *AP1*, *SOC1*, and *miR172b*.

To further investigate the regulatory mechanism of *SPL10*-clade genes, we performed ChIP analysis using the *SPL10* transgenic line, and *FUL* and *AP1* as representative target genes. We interrogated several regions containing the SPL binding site (GTAC) of the *FUL* and *AP1* genes (Birkenbihl *et al.*, 2005; Fig. 3a). We found that SPL10 was enriched on FUL-2 and AP1-2 regions, which locate in the proximal promoter region of the *FUL* and *AP1* genes (Fig. 3b,c). Electrophoretic mobility shift assays further confirmed the binding of SPL10 to these two regions (Fig. 3d,e). Collectively, these results suggest that *FUL* and *AP1* are direct targets of SPL10.

Interaction of SPL10 with MED25

As the Mediator complex functions as a bridge to link transcription factors with RNA polymerase II to modulate transcription, it is of interest to determine which mediator subunit(s) is needed to communicate the activating function of SPL10. The Mediator complex subunit MED25 has been shown to regulate flowering time in response to photoperiod and temperature signals (Cerdan & Chory, 2003; Iñigo *et al.*, 2012). This observation led us to hypothesize that SPL10 may interact with MED25. We employed a bimolecular fluorescence approach to test whether SPL10/MED25 association also occurs in plants. MED25 and SPL10 were fused to the N-terminal and C-terminal of YFP, respectively; then, after infiltration of these two fusion genes into tobacco leaves, reconstituted YFP signals were detected in the nucleus. These results show that not only was SPL10 associated with MED25 but also the SPL10/MED25 complex was localized in the nucleus, consistent with its role in transcription (Fig. 4a). Next, using a yeast two-hybrid assay we found that SPL10 indeed interacted with MED25 in yeast, and similar results were found for SPL2 (Figs 4b, S4). Finally, the *in vivo* interaction of SPL10 with MED25 in plants was confirmed by co-IP assays (Fig. 4c).

We also explored possible interactions of SPL10 with other mediator subunits such as MED18, MED19a, and MED36a in yeast, and obtained positive results with MED18 and MED19a

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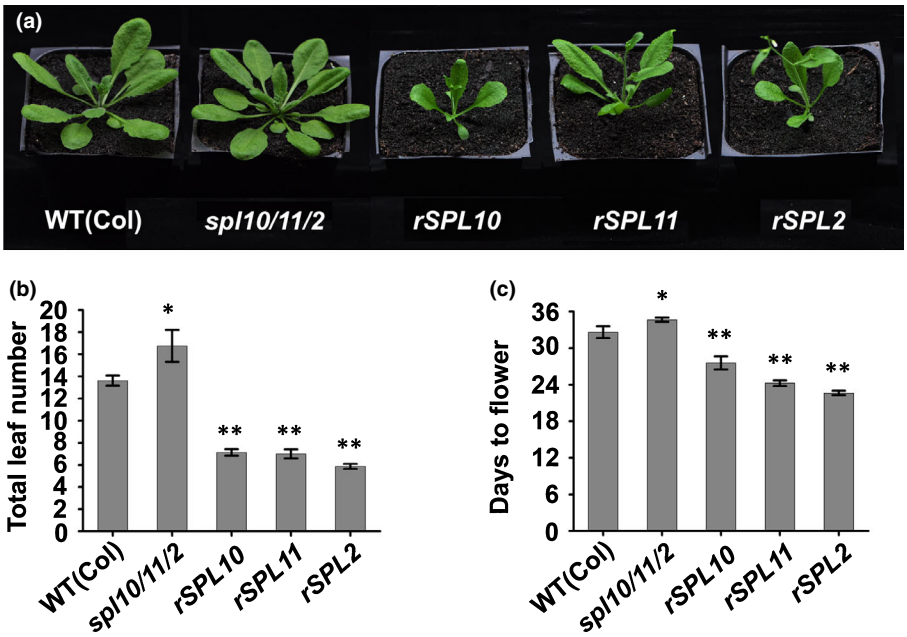


Fig. 1 SPL10/11/2 family members promote floral transition in Arabidopsis. (a) Flowering phenotype of *spl10/11/2* triple mutant and transgenic lines harboring *PSPL10:rSPL10* (*rSPL10*), *PSPL11:rSPL11* (*rSPL11*), and *PSPL2:rSPL2* (*rSPL2*), respectively. (b, c) Leaf number at time of flowering and timing of the first opened flower of *spl10/11/2*, *rSPL10*, *rSPL11*, and *rSPL2*. More than 20 plants ($n > 20$) were used for each experiment and the average value was used. Error bars indicate ± SD of three independent experiments. Asterisks indicate statistically significant difference compared with wild-type (WT) as analyzed by two-tailed *t*-test: *, $P < 0.05$; **, $P < 0.01$.

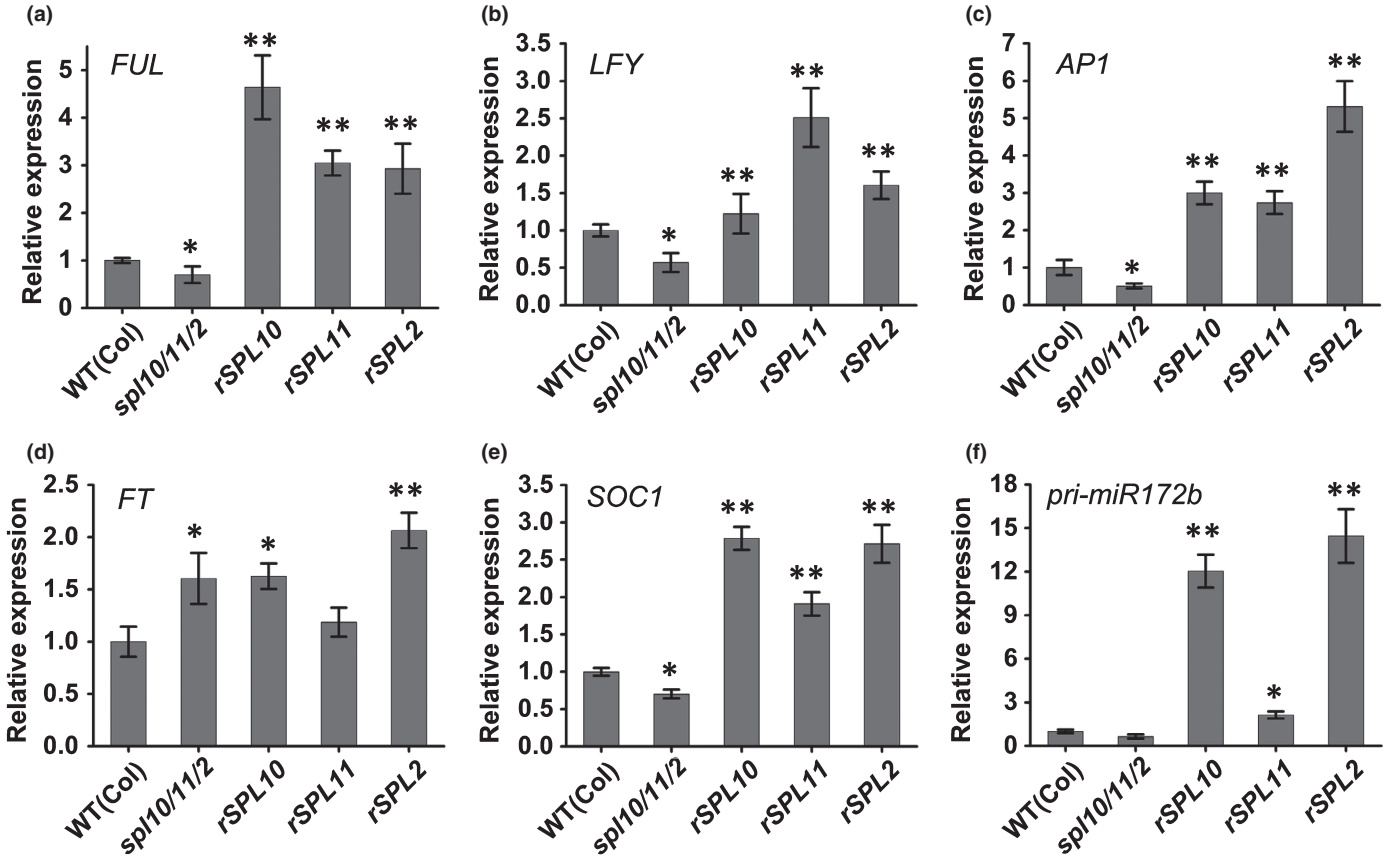


Fig. 2 Regulation of flowering-time-related genes by *SPL10/11/2* in Arabidopsis. Quantitative real-time (qRT)-PCR analysis of expression levels of flowering-related genes, *FUL* (a), *LFY* (b), *AP1* (c), *FT* (d), *SOC1* (e), and *pri-miR172b* (f) in *spl10/11/2*, *rSPL10*, *rSPL11*, and *rSPL2* plants. Total RNA was extracted from 2-wk-old seedlings for qRT-PCR analysis. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). Asterisks indicate statistically significant difference compared with wild-type (WT) as analyzed by two-tailed *t*-test: *, $P < 0.05$; **, $P < 0.01$.

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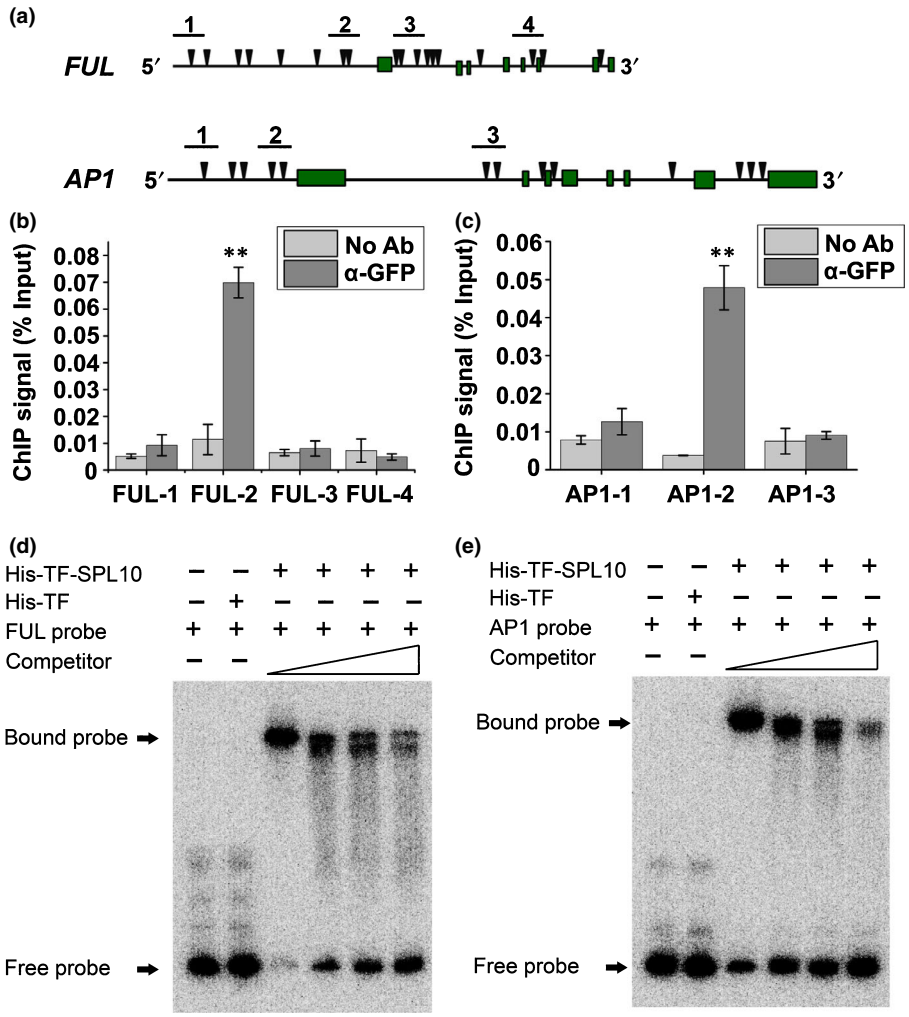


Fig. 3 SPL10 directly binds to the promoters of *FUL* and *AP1* in Arabidopsis. (a) Schematic diagrams of genomic structures of *FUL* and *AP1*. The green boxes indicate exons and the black arrows represent SPL binding motifs. The numbers show amplicons for chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR). (b, c) ChIP–qPCR analysis showing SPL10 enrichment on the gene loci of *FUL* and *AP1*. Chromatin of 2-wk-old *PSPL10:rSPL10-VENUS* plants was immunoprecipitated using anti-green fluorescent protein (GFP) antibody. Precipitated DNA was quantified by qPCR and the results are shown as a percentage of input. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). Asterisks indicate statistically significant difference compared with no antibody control as analyzed by two-tailed *t*-test: **, $P < 0.01$. (d, e) Electrophoretic mobility shift assays showing direct binding of SPL10 protein with the promoter regions of *FUL* and *AP1*. FUL-2 (d) and AP1-2 (e) fragments were used as probes, and competition was performed with 10-, 20- or 30-fold unlabeled probe.

(Fig. S5). These results suggest the possible involvement of these two subunits in SPL10-regulated floral transition. As MED18 has been reported to interact with SPL15, we checked for the possible interactions of MED18 with other SPL family members in yeast. Our results show that MED18 also interacts with SPL2, SPL6, SPL11 and SPL13, highlighting the possible role of MED18 in SPL-regulated floral transition (Hyun *et al.*, 2016; Fig. S6).

Promotion of floral transition by SPL10 requires MED25

The formation of a nuclear SPL10/MED25 complex prompted us to investigate the genetic relationship between these two genes. To this end, we generated the double transgenic/mutant line of overexpressed *SPL10* (*rSPL10*) in the *med25* mutant background. The mutant *pft1-2* has been identified as a *med25* mutant. This mutant showed a delaying flowering phenotype that can be complemented by *MED25* genomic DNA (Kidd *et al.*, 2009; Rival *et al.*, 2014; Fig. S7). Fig. 5 shows that *rSPL10/pft1-2* plants flowered late, similar to *pft1-2* mutant plants (Fig. 5a–c). The inhibition of the early-flowering phenotype of *rSPL10* by *pft1-2* indicates that MED25 is required for SPL10 to promote floral transition.

To understand the functional inhibition of *rSPL10* by *pft1-2*, we first examined the expression level of *SPL10* in *rSPL10/pft1-2*. We found that *SPL10* expression level in *pft1-2* background is comparable to that in WT background (Fig. 5d). We then compared the expression of downstream genes (*FUL*, *LFY*, *AP1* and *FT*) between plants of four genotypes: WT, *rSPL10*, *rSPL10/pft1-2* and *pft1-2*. Consistent with the results of flowering time, the upregulation of *FUL*, *LFY*, *AP1* and *FT* genes in *rSPL10* was found to be dependent on MED25, and the expression of *SOC1*

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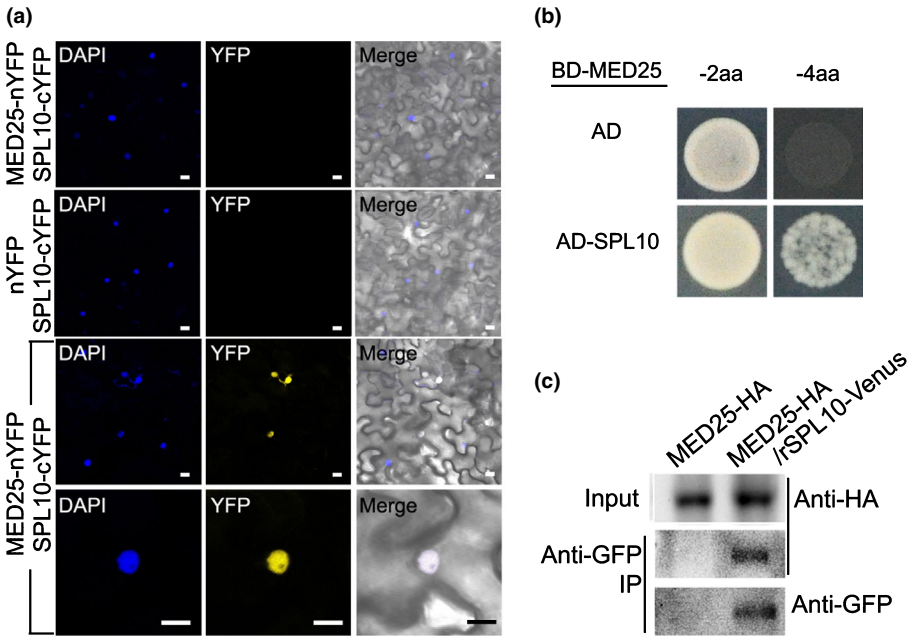


Fig. 4 SPL10 interacts with MED25 *in vitro* and *in vivo*. (a) Bimolecular fluorescence analysis of interaction between SPL10 and MED25 in tobacco cells. The upper two panels are negative control, and the fourth panel is a magnified image of the third panel. Reconstituted yellow fluorescent protein (YFP) signals were detected in nuclei of tobacco leaf cells when MED25-nYFP was transiently co-expressed with SPL10-cYFP in *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium* cell suspensions. Confocal images were taken 2 d after infiltration. Bars, 20 μm. (b) Yeast two-hybrid analysis showing interaction of MED25 with SPL10. Transformed yeast strains were plated on SD medium lacking Leu and Trp (-2aa); and SD medium lacking His, Ade, Leu and Trp (-4aa). (c) Co-immunoprecipitation (IP) showing that SPL10 binds to MED25 in Arabidopsis. Nuclear proteins were extracted from transgenic seedlings co-expressing *PSPL10:rSPL10-VENUS* and *35S:MED25-HA*, or only *35S:MED25-HA*. SPL10-VENUS was then immunoprecipitated with anti-green fluorescent protein (GFP). Immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the immunoblot was detected with anti-hemagglutinin (HA).

and *miR172b* was compromised by the *MED25* mutation (Fig. 6). These results show that MED25 is required for SPL10 to activate flowering-related genes.

Because of the functional redundancy of SPL10 with SPL11 and SPL2, we also examined the genetic relationships of *MED25* with *SPL2* and with *SPL11*. Similar to the case of SPL10, we found the early-flowering phenotype of *rSPL2* and *rSPL11* plants was reversed by the *MED25* mutation, indicating these two other SPL10-clade members may share a similar mechanism of action as SPL10 (Fig. S8). Since MED18 and MED19a also interacted with SPL10 in yeast, we examined the genetic interactions of *SPL10* with *MED18* and *MED19a* in *rSPL10/med18* and *rSPL10/med19a*. The early-flowering phenotype of *rSPL10* was partially inhibited by the *MED18* and *MED19a* mutations, suggesting that MED18 and MED19a partially contribute to SPL10-regulated floral transition (Fig. S9a,b). However, different from *rSPL10/med18*, the expression level of *SPL10* was significantly reduced in *rSPL10/med19a* compared with that of *rSPL10*/WT (Fig. S9c). Taken together, these results suggest that MED18/SPL10 interaction may play a role in SPL10-regulated flowering time.

Enrichment of MED25 on the promoters of *FUL* and *AP1* depends on SPL10/11/2

Because SPL10 directly binds to the promoter regions of *FUL* and *AP1*, the association of SPL10 with MED25 suggests that SPL10 may recruit MED25 to these promoter regions. To investigate this possibility, we performed ChIP experiments with a mouse antibody specific to MED25 (Fig. S10). Fig. 6 shows that MED25 was enriched on the promoter regions of *FUL* and *AP1*, which contain binding sites of SPL10 (Fig. 3). Moreover, enrichment of MED25 on these target promoter regions was considerably decreased in the *spl10/11/2* triple mutant background (Fig. 7a–c). We performed the reciprocal experiments to examine the effect of MED25 on SPL10 binding. We found that the absence of MED25 reduced SPL10 binding by *c.* 20–25%. These results support the notion that SPL10 recruits MED25 to regulate downstream gene expression. On the other hand, the binding ability of SPL10 was slightly decreased in the *pft1-2* mutant, indicating that MED25 is not absolutely required for SPL10 binding to target promoter regions (Fig. 7d,e).

Discussion

The function and molecular mechanism of SPL10-clade transcription factors in flowering-time regulation have not been well studied. Here, we show that SPL10-clade members positively regulate flowering time and provide genetic and biochemical evidence that MED25 interacts with SPL10 to facilitate the transcriptional activity of SPL10.

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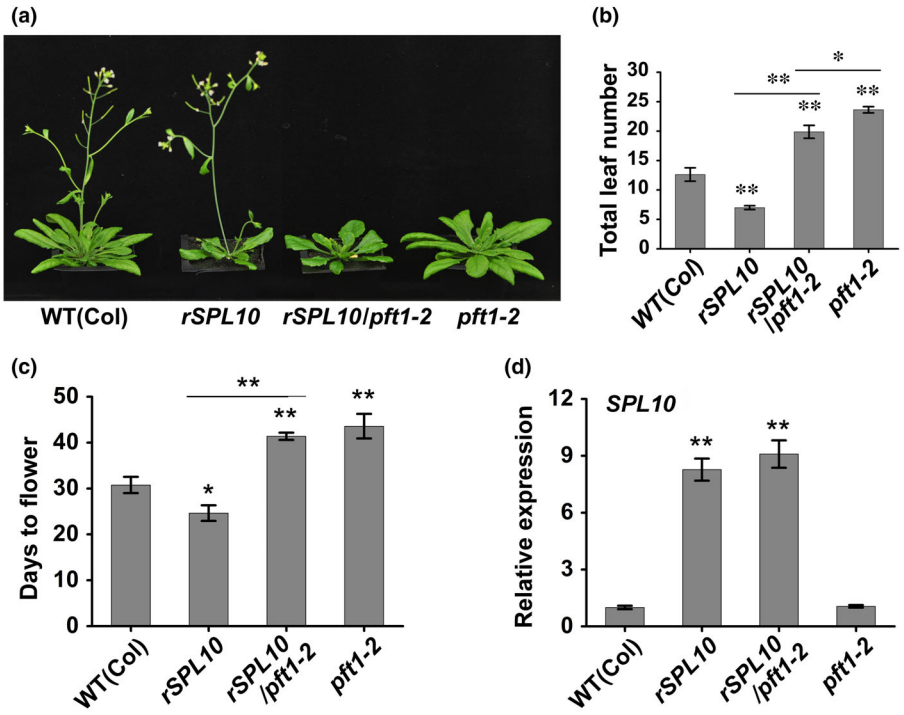


Fig. 5 Genetic relationship between *SPL10* and *MED25* in Arabidopsis. (a) Flowering phenotype of *PSPL10:rSPL10/pft1-2* double mutant. Plants were grown in long-day (LD) (16 h : 8 h, light : dark) conditions for 6 wk. From left to right, wild-type (WT), *PSPL10:rSPL10*, *PSPL10:rSPL10/pft1-2*, and *pft1*-2. (b, c) Leaf number at time of flowering (b) and timing of the first opened flower (c) of *PSPL10:rSPL10*, *PSPL10:rSPL10/pft1-2*, and *pft1*-2. More than 20 plants ($n > 20$) were used for each experiment, and the average value was used. Error bars indicate ± SD of three independent experiments. (d) Quantitative real-time (qRT)-PCR analysis of *SPL10* expression levels in *PSPL10:rSPL10* and *PSPL10:rSPL10/pft1-2*. Total RNA from 2-wk-old seedlings was used for qRT-PCR analysis. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). Asterisks indicate statistically significant difference compared with WT or between indicated values as analyzed by one-way ANOVA followed by *t*-test: *, $P < 0.05$; **, $P < 0.01$.

The SPL10-clade regulates flowering time

Among the three clades of *miR156*-targeted *SPL* genes, *SPL3/4/5* and *SPL9/15* have been studied more extensively than *SPL10/11/2* (Wang *et al.*, 2009; Yamaguchi *et al.*, 2009; Kim *et al.*, 2012; Hyun *et al.*, 2016; Jung *et al.*, 2016). Although SPL10 has been reported to regulate flowering time, single mutant of *spl10* does not have a flowering-time phenotype (Xu *et al.*, 2016; Gao *et al.*, 2017). Consistent with previous studies, our results show that overexpression of *SPL10*, *SPL11* or *SPL2* promoted flowering, whereas *spl10/11/2* triple mutant showed late-flowering phenotype (Fig. 1). These gain-of-function and loss-of-function experiments confirmed the function of *SPL10*-clade genes in flowering-time regulation. Considering that the *spl2/9/10/11/13/15* mutant flowers much later than *spl10/11/2* or *spl9/15* mutants, we think that functional redundancy still exists between *SPL10/11/2* and *SPL9/15* (Xu *et al.*, 2016; Fig. 1).

Identifying the target genes for SPLs is crucial to elucidating their regulatory networks in flowering regulation. Much progress has been made for SPL3, SPL9 and SPL15. SPL3 was found to bind directly to the genomic loci of *FUL, LFY* and *AP1* (Yamaguchi *et al.*, 2009); both SPL9 and SPL15 were confirmed to bind the promoters of *FUL* and *miR172b* (Wu *et al.*, 2009; Hyun *et al.*, 2016). Here, we showed that overexpression of *SPL10* promotes expression of *FUL*, *LFY*, *AP1*, *SOC1* and *miR172b*, and SPL10 binds directly to the promoters of *FUL* and *AP1*. *AGL79*, a MADS-box protein-encoding gene, was also found to be a direct target gene of SPL10 (Gao *et al.*, 2017; Fig. 3). Further analysis of these downstream genes will deepen our understanding of the regulatory network of *SPL10*-clade genes in flowering-time regulation.

Another way to characterize the function of SPL transcription factors is to identify the proteins that they associate with. SPL3, SPL4, and SPL5 have been reported to interact with the FLOWERING LOCUS D–FLOWERING LOCUS T complex and participate in photoperiod-regulated flowering time (Jung *et al.*, 2016). Our study here showed that SPL10 interacts with MED25, which is required for SPL10-clade members to regulate flowering time (Figs 4, S4).

MED25 is required for SPL10 activation

As a transcription factor, SPL10 acts both as an activator and a repressor. For example, SPL10 represses *DFR* expression by recruiting the MYB-bHLH-WD40 complex to inhibit anthocyanin biosynthesis (Gou *et al.*, 2011). Our results pertain to the activator function of SPL10. We showed that this transcription factor is dependent on MED25 to execute its function. However, the binding ability of SPL10 to target genes was only slightly decreased in *pft1-2* background, indicating that MED25 is required for SPL10 transactivating activity but not for its DNA binding activity. MED25 is a subunit of the Mediator complex,

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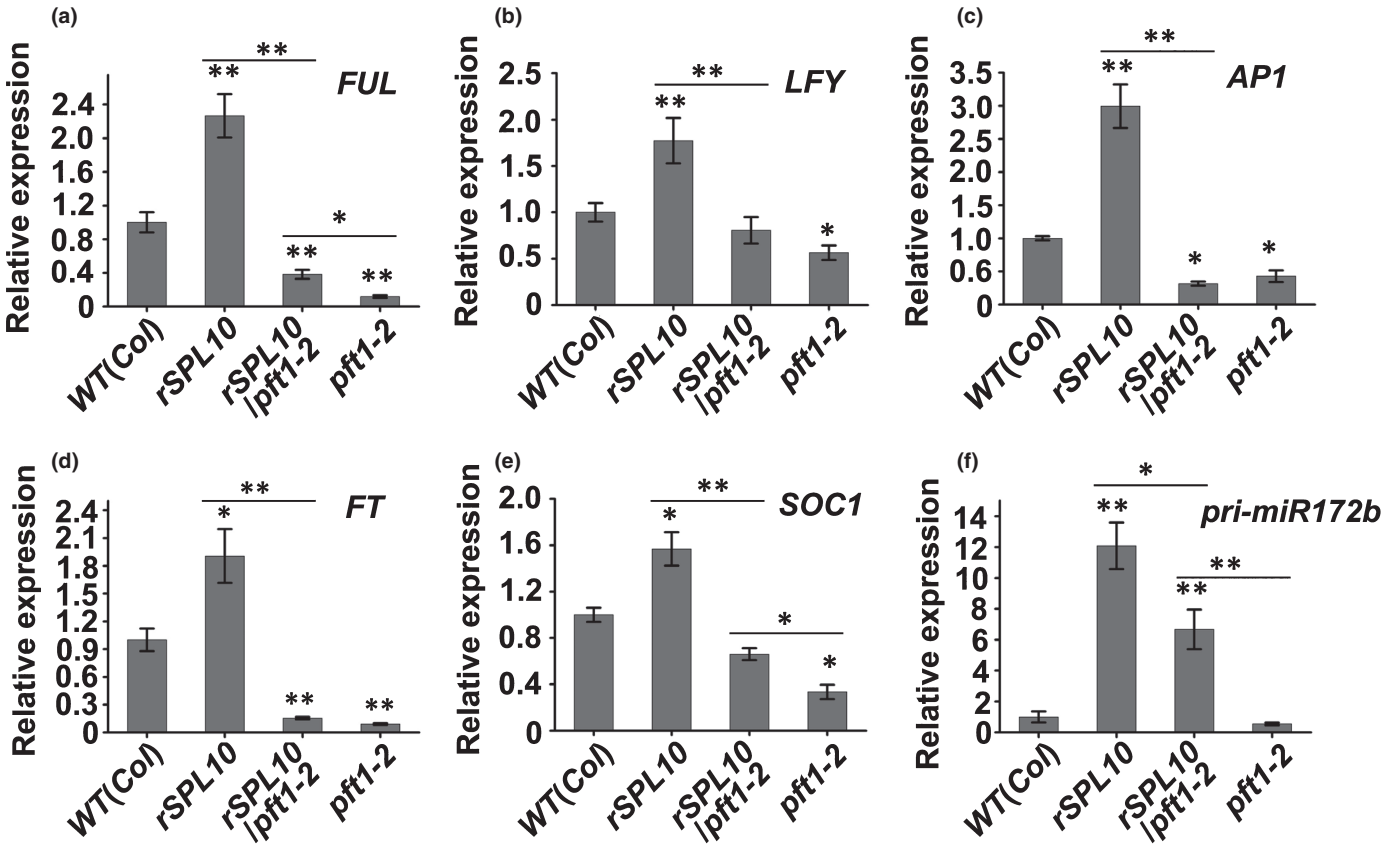


Fig. 6 MED25 is required for SPL10 to regulate the expression of flowering-related genes in Arabidopsis. (a–f) Quantitative real-time (qRT)-PCR analysis of expression levels of *FUL* (a), *LFY* (b), *AP1* (c), *FT* (d), *SOC1* (e), and *miR172b* (f) in *PSPL10:rSPL10*, *PSPL10:rSPL10/pft1-2* and *pft1-2*. Total RNA from 2-wk-old seedlings was used for qRT-PCR analysis. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). Asterisks indicate statistically significant difference compared with wild-type (WT) or between indicated values as analyzed by one-way ANOVA followed by *t*-test: *, $P < 0.05$; **, $P < 0.01$.

which is required for RNA polymerase II recruitment and transcription initiation (Kazan, 2017). Moreover, this mediator subunit contains a conserved glutamine (Q)-rich poly-track domain that may be involved in transcriptional activation by interacting with other activators (Rival *et al.*, 2014). Recently, HISTONE ACETYLTRANSFERASE 1 (HAC1), an evolutionarily conserved coactivator, has been reported to physically and functionally associate with MED25 to activate the expression of jasmonic acid responsive genes by H3K9 acetylation (An *et al.*, 2017). SPL10 also enhances the transcript levels of *miR172b*, *AGL79*, *FUL*, and *AP1* during floral transition and shoot maturation (Shikata *et al.*, 2009; Wu *et al.*, 2009; Gao *et al.*, 2017; Fig. 2). Because the activation of flowering-related genes by SPL10 was impaired by *MED25* mutation, we propose that MED25 is crucial for the transcriptional activating function of SPL10 (Fig. 6). HAC1, or other epigenetic factors, is probably recruited to enhance the expression of target genes, in line with the observation that HAC1 has been reported to promote floral transition (Deng *et al.*, 2007).

Not only SPL10 but also many other *miR156*-targeted SPL transcription factors, such as SPL11, SPL2, SPL3, SPL4, SPL5, SPL9 and SPL15, can act as transcriptional activators (Yamaguchi *et al.*, 2009; Wu *et al.*, 2009; Yu *et al.*, 2012; Jung *et al.*, 2016; Fig. 2). A future challenge is to determine how these transcription factors promote gene expression. For example, SPL9 has been found to interact with DELLA proteins to promote *AP1* expression after the initiation of flowering (Yamaguchi *et al.*, 2014). MED18 has already been reported to participate in SPL15-regulated flowering time under short-day conditions, and our results here show that MED25 is involved in SPL10-regulated flowering time under long-day conditions (Hyun *et al.*, 2016; Fig. 5). Although not investigated in detail as with the SPL10/MED25 module, our genetic results suggest that the activating function of SPL11 and SPL2 on flowering time is also dependent on MED25 (Fig. S8). Therefore, MED25 appears to mediate the transcriptional activities of the three SPLs belonging to the same clade.

MED25 integrates photoperiod and age pathways to regulate flowering time

MED25 was first identified from genetic screening of mutants involved in phytochrome-regulated flowering time, and the gene was characterized as a positive regulator of floral transition downstream of phyB (Cerdán & Chory, 2003). The function of MED25 in flowering time has also been

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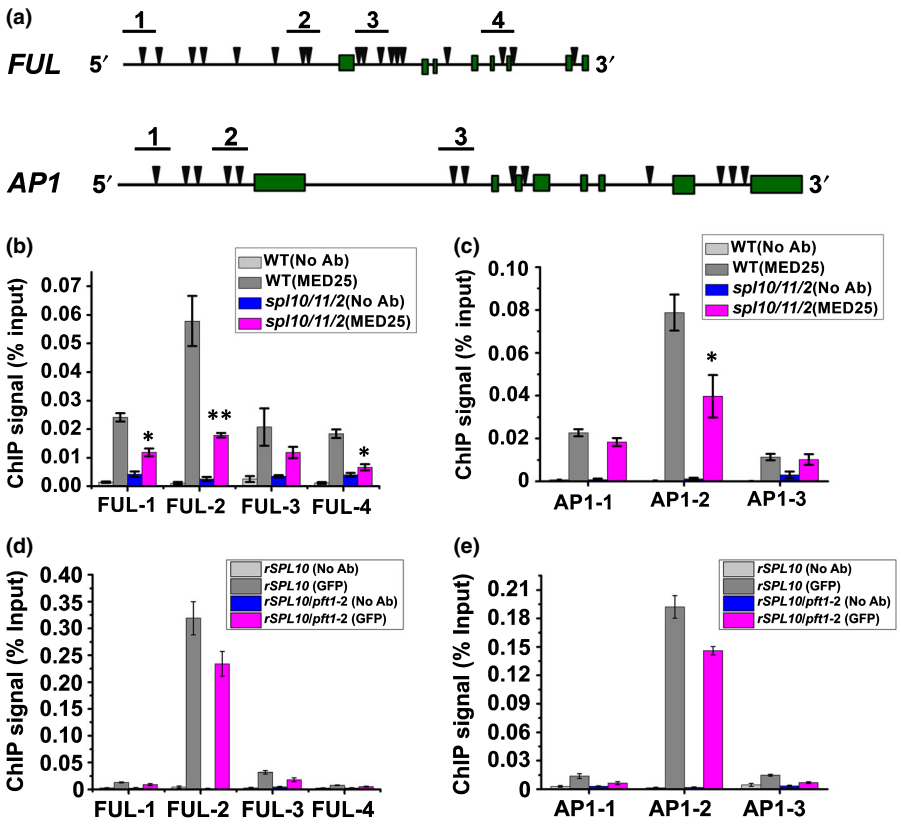


Fig. 7 SPL10 is required for MED25 enrichment on the promoters of *FUL* and *AP1* in Arabidopsis. (a) Schematic diagrams of genomic structures of *FUL* and *AP1*. The green boxes indicate exons, and the black arrows mark the SPL binding motifs. The numbers show amplicons for chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR). (b, c) ChIP–qPCR analysis showing MED25 enrichment on gene loci of *FUL* and *AP1*. Chromatin of 2-wk-old wild-type (WT) and *spl10/11/2* mutant were immunoprecipitated using anti-MED25 antibody. Precipitated DNA was quantified by qPCR and the results were shown as a percentage of input. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). (d, e) ChIP-qPCR analysis showing SPL10 enrichment on gene loci of *FUL* and *AP1*. Chromatin of 2-wk-old *PSPL10::rSPL10-VENUS* and *PSPL10::rSPL10-VENUS/pft1-2* plants were immunoprecipitated using anti-green fluorescent protein (GFP) antibody. Precipitated DNA was quantified by qPCR and the results are shown as a percentage of input. Error bars indicate ± SD of three independent experiments ($n = 3$ independent seedling pools). Asterisks indicate statistically significant difference compared with WT as analyzed by two-tailed *t*-test: *, $P < 0.05$; **, $P < 0.01$.

confirmed by the gain of function of *eid3* mutant, which displays early flowering under both short-day and long-day conditions (Klose *et al.*, 2012).

Two lines of evidence support the view that MED25 plays a role in the photoperiod pathway regulating flowering time. First, loss-of-function *med25* mutant (*pft1-2*) only shows late flowering under long-day but not short-day conditions, suggesting that the function of MED25 in flowering time regulation is photoperiod -dependent (Cerdán & Chory, 2003; Rival *et al.*, 2014). Second, *CONSTANS* (*CO*), which encodes a central component in the photoperiod pathway, has been identified as an important target gene of MED25 (Iñigo *et al.*, 2012; Liu *et al.*, 2017). Recently, two transcription factors, TCP4 and FBH1, have been shown to bind to MED25 and additively enhance *CO* transcription. Moreover, MED25 was found to associate with the promoter region of *CO*, which is also occupied by TCP4 (Liu *et al.*, 2017).

On the other hand, MED25 has also been reported to regulate flowering time though a CO-independent pathway. For instance, MED25 is able to positively regulate *FT* expression through misregulation of *FLC* transcription (Klose *et al.*, 2012). Our results show that the SPL10-targeted flowering-time genes are also regulated by *MED25* mutation, demonstrating that MED25 regulates flowering time by the age pathway (Fig. 6). Moreover, the association of MED25 with SPL10, as well as the occupancy of MED25 on *FUL* and *AP1* promoters, provides a molecular mechanism of how MED25 regulates flowering time (Fig. 7a–c). It will be interesting to investigate whether the expression of *MED25* correlates with age, as it has been reported that *MED25* expression level was higher in older leaves than in younger ones (Xu & Li, 2011).

In conclusion, our study identifies SPL10/MED25 as a transcriptional regulatory module in flowering-time regulation (Fig. 8). The interaction of SPL10 with MED25, a Mediator complex component, recruits RNA polymerase II to initiate the expression of target genes *FUL* and *AP1*. On the other hand, the absence of MED25 or SPL10 will lead to the failure of RNA polymerase II recruitment and much reduced or no transcription of *FUL* and *AP1*. It will be interesting to further investigate whether other chromatin modifiers are also involved in this process.

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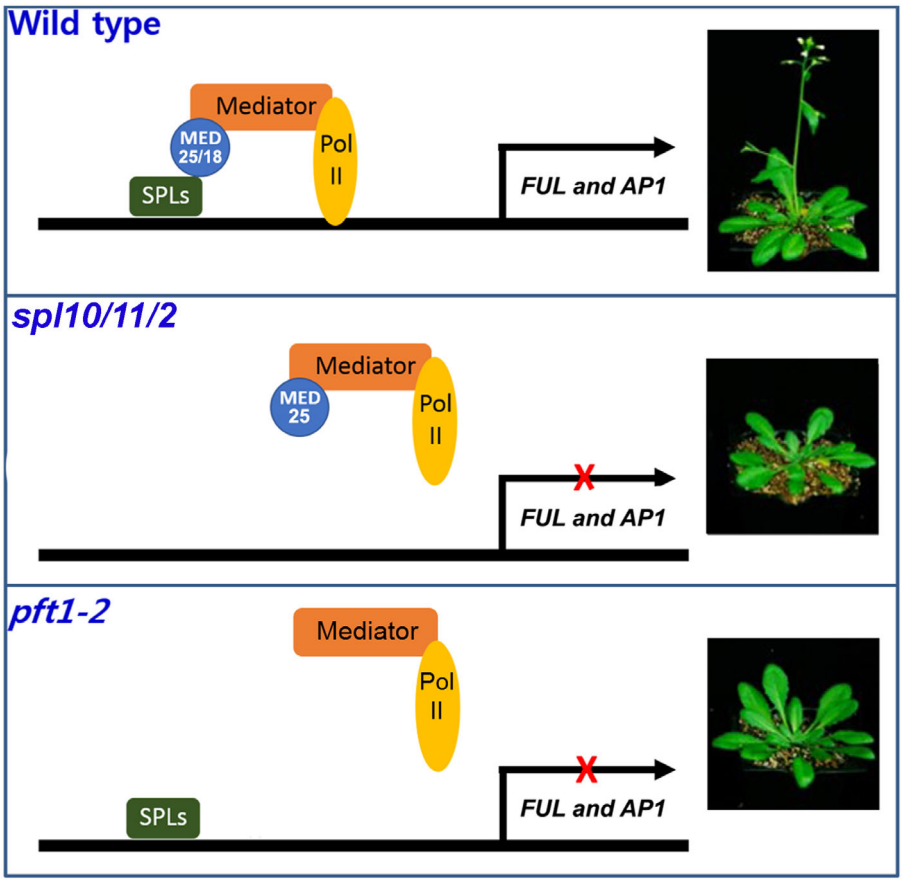


Fig. 8 Working model of the SPL10/MED25 module in controlling flowering time in Arabidopsis. Schematic illustration of the role of SPL10 in flowering-time regulation. At the flowering transition stage, SPL10 protein recruits MED25/MED18 and the Mediator complex to facilitate the transcription of *FUL* and *AP1* by polymerase II (Pol II). In the absence of SPL10/11/2 or MED25, the Mediator complex and Pol II fail to associate the target genes, and thus the downstream genes are not able to be transcribed.

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Author contributions

TY and N-HC designed the experiments; TY, BSP, H-ZM, JSS, NO, YL, NY, NFBM and C-HH performed the experiments; TY and N-HC discussed results and wrote the manuscript, which was read by all authors.

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Supporting Information

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Fig. S1 Gene redundancy of *SPL2*, *SPL10* and *SPL11* in regulating flowering time.

Fig. S2 Genotyping of the *spl2/10/11* triple mutant.

Fig. S3 Expression level analysis of transgenic plants overexpressing *SPL10*, *SPL11* or *SPL2*.

Fig. S4 Interactions of MED25 with SPL transcription factors in yeast.

Fig. S5 Interactions of SPL10 with other mediator components in yeast.

Fig. S6 Interactions of MED18 with SPL transcription factors in yeast.

Fig. S7 Complementation of *pft1-2* mutant.

Fig. S8 Phenotype of *PSPL2::rSPL2/pft1-2* and *PSPL11::rSPL11/pft1-2*.

Fig. S9 Phenotype of *PSPL10::rSPL10/med18* and *PSPL10::rSPL10/med19*.

Fig. S10 Verification of MED25 antibody specificity.

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Table S1 Primers used for gene cloning.

Table S2 Primers used for genotyping.

Table S3 Primer used for real-time qRT-PCR.

Table S4 Primer used for ChIP and EMSA assays.

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