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Competence to flower : Age-controlled sensitivity to environmental cues

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Running title: Competence to flower

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Introduction: Physiology of competence to flower

Flowering occurs when the shoot apical meristem (SAM) transitions from forming vegetative organs to giving rise to flowers. This switch to flowering represents the most obvious transition in the growth of the shoot and initiates reproductive development. However, even during vegetative development the plant shoot transitions through different phases, often referred to as juvenile and adult (Poethig, 1990). The vegetative organs formed sequentially on the flanks of the SAM can differ markedly during each of these phases, for example, the morphology, physiology and epidermal characteristics of leaves formed during the juvenile phase differ from those formed later during the adult phase (Hackett, 1985; Poethig, 2003). The progressive and sequential transition through these phases was described initially in perennial species (Hackett, 1985) and more recently in detail in genetic model systems, particularly maize and *Arabidopsis thaliana* (Poethig, 1990; Bergonzi and Albani, 2011; Huijser and Schmid, 2011). In *A. thaliana*, the capacity of rosette leaves to form abaxial trichomes is a robust indication of the transition from juvenile to adult vegetative phase (Chien and Sussex, 1996; Telfer et al., 1997). In addition to these vegetative features, the propensity of plants to flower and initiate reproduction also increases with age, and older shoots are described as acquiring competence to flower. This process can be most clearly demonstrated in plants that show an obligate requirement for exposure to an environmental stimulus to undergo floral transition. Plants that have not yet acquired competence to flower will remain vegetative when exposed to stimuli such as photoperiod or vernalization, while competent plants exposed to the same environmental cue are induced to flower. Some of the first examples of this phenomenon were in perennial woody plants such as blackcurrant or ivy, and were reviewed extensively by Hackett (1985).

The extent to which vegetative phase change and competence to flower are causally interlinked is important in considering these processes. Early genetic and physiological experiments on maize exploited the *teopod 2* (*tp2*) mutant to address these issues (Bassiri et al., 1992). This mutant shows a greatly extended juvenile vegetative phase, but acquired competence to flower in response to exposure to short photoperiods at a similar stage in shoot development to wild-type plants (Bassiri et al., 1992). Therefore, this experimental approach suggested that vegetative phase change and competence to flower are not dependent on one another. Nevertheless, more recent work suggests that the underlying mechanisms controlling both processes are related, because microRNA156 (miR156) and its downstream targets the SQUAMOSA PROMOTER BINDNG PROTEIN LIKE (SPL) transcription factors control both vegetative phase change (Poethig, 2013) and competence to flower (Huijser and Schmid, 2011; Bergonzi et al., 2013; Zhou et al., 2013). In this short review we focus on the emerging evidence that miR156/SPL control competence to flower as well as vegetative phase change and discuss their relationship to the growth regulator gibberellin (GA). Finally, we describe other genetic systems that have been implicated in regulating competence to flower and discuss how these are related to miR156/SPL.

miR156 and miR172 contribute to the control of flowering time

Many different factors influence the time after germination at which plants initiate flowering. Genetic analysis of flowering time in *A. thaliana* defined several regulatory pathways controlling this transition, including those mediating responses to the seasonal environmental cues of photoperiod and vernalization and others influenced by endogenous factors such as the growth regulator GA (Fornara et al., 2010; Srikanth and Schmid, 2011; Andres and Coupland, 2012). Therefore, to study specifically the basis of competence to flower, factors centrally involved in controlling age-related competence must be distinguished from those conferring environmental responses or participating in general endogenous pathways. During the last 10 years, interest has focused on miR156 and miR172 because their abundance is dependent on the age of the shoot and they influence flowering

time. In this section, we briefly review the discovery of these miRNAs and their involvement in controlling flowering time.

Initially, miR156 was identified in *A. thaliana* by sequencing small RNAs predicted to be processed by DICER (Reinhart et al., 2002), and based on computational approaches the targets of these miRNAs were identified as mRNAs of genes encoding SPL transcription factors (Rhoades et al., 2002). These miRNAs are encoded by 8 loci in *A. thaliana* (Morea et al., 2016) and therefore their activities were initially difficult to dissect by loss of function genetics. However, overexpression of miR156 has effects on leaf morphology and causes reduced apical dominance, shorter plastochron and later flowering (Schwab et al., 2005). Furthermore, detailed analysis of vegetative phase change showed that overexpression of miR156 delayed the transition from juvenile to adult phase (Wu and Poethig, 2006; Wu et al., 2009). Similarly, in maize the *corngrass* mutation, which extends juvenile phase, is caused by insertion of a retrotransposon upstream of a *MIR156* precursor gene leading to overexpression of miR156 (Chuck et al., 2007).

More recently, the phenotypic effects of reducing miR156 activity were described. Overexpression of a *MIM156* transgene was used to reduce miR156 activity through sequestration (Franco-Zorrilla et al., 2007), and these transgenic *A. thaliana* plants showed opposite phenotypes to the miR156 overexpressors: accelerated vegetative phase transition and reduced number of leaves at flowering, which might be caused by a longer plastochron rather than accelerated flowering time (Wang et al., 2009; Wu et al., 2009; Todesco et al., 2010). Furthermore, double mutant plants carrying T-DNA insertions in two of the miR156 precursor genes, *MIR156a* and *MIR156c*, also accelerated adult vegetative phase transition, flowered with fewer leaves and flowered slightly earlier under long days (Yang et al., 2013; Yu et al., 2013).

Mature miR156 levels are lower in older than younger plants. In RNA samples extracted from whole young *A. thaliana* plants or specifically from their apices, miR156 levels are higher than in samples derived from similar tissues of older plants (Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009). Although miR156 is encoded by 8 precursors, *MIR156a* and *MIR56c* were the only precursor genes whose transcripts reduced in abundance between successive leaf primordia and these are major contributors to the pool of mature 20 nucleotide (nt) miR156, although a 21 nt form seems to be expressed from other precursors (Yang et al., 2013; Yu et al., 2013). These data suggest that in *A. thaliana* progressive reduction of miR156 in leaves that develop successively on the shoot confers the gradual transition from juvenile to adult phase, while this miRNA also accelerates plastochron and causes variable but reproducible delays in flowering time.

As described for miR156, miR172 was initially identified by random sequencing of small RNAs (Park et al., 2002), but soon after was characterized by forward and reverse genetics (Aukerman and Sakai, 2003; Chen, 2004). Based on homology, miR172 was predicted to target mRNAs encoding a small set of transcription factors consisting of APETALA2 (AP2) and closely related proteins (Park et al., 2002). Reverse genetic approaches showed that overexpression of miR172 caused a floral phenotype similar to *ap2* mutants and that a transgene expressing *AP2* mRNA containing a disrupted miR172 recognition sequence caused severe floral defects related to those of *agamous* (*ag*) mutants, consistent with AP2 repressing *AG* transcription (Chen, 2004). Similarly, an early-flowering mutant *early activation tagged* (*eat*) with floral defects was identified in a T-DNA activation tagging screen, and shown to be caused by overexpression of miR172 (Aukerman and Sakai, 2003). The latter result suggested that the AP2-like transcription factors targeted by miR172 are likely to be repressors of the floral transition, and this was confirmed in the same genetic screen by recovery of a late-flowering activation tagged mutant *target of eat 1* (*toe1*) in which one of the *AP2*-like genes targeted by miR172 was overexpressed (Aukerman and Sakai, 2003). Loss of function genetic analysis of the six

AP2-like transcription factors targeted by miR172 showed that they act redundantly to repress flowering, and that the hextuple mutant in which all of the genes are inactivated is extremely early flowering (Mathieu et al., 2009; Yant et al., 2010). Based on misexpression studies and analysis of its binding sites, SCHLAFMÜTZE (SMZ) was proposed to inhibit flowering mainly by binding to and repressing transcription of the floral promoter *FLOWERING LOCUS T* (*FT*) in the leaves (Mathieu et al., 2009). Thus, miR172 is an activator of flowering and floral development whose targets are mRNAs encoding AP2 and five other AP2-like transcription factors.

The transcription of miR172 precursors is regulated by the age of the plant and is part of the same network as the miR156/SPL module. The abundance of miR172 shows the opposite temporal pattern to accumulation of miR156, so that in plants grown under long days it is present at low levels two to five days after germination and progressively increases to accumulate at high levels in plants around 16 days after germination (Aukerman and Sakai, 2003). Some of this increase is likely to be due to older plants forming flowers, where miR172 is highly expressed (Chen, 2004), but the miR172/AP2-like module is also involved in controlling vegetative phase change (Wu et al., 2009). The *toe1 toe2* double mutant and plants overexpressing miR172 prematurely undergo the transition to adult vegetative phase. Finally miR172 acts downstream of miR156/SPL so that higher levels of miR156 lead to reduced expression of miR172, while SPL transcription factors, particularly SPL9 and SPL15 but probably also SPL2, SPL10, SPL11 and SPL13, directly bind to and activate transcription of the miR172 precursor gene *MIR172b* (Wu et al., 2009; Hyun et al., 2016; Xu et al., 2016). This interaction contributes to the inverse relationship of miR156 and miR172 abundance in apices of the same plants (Wu et al., 2009), and these patterns are strikingly conserved in distantly related species including maize (Chuck et al., 2007). Although the details are not yet clear, whole-genome analyses suggest that the network of interactions among the miR156/SPL and miR172/AP2 modules is likely to be intricate and complex because AP2 also directly binds to *MIR172* genes as well as to *MIR156* loci and levels of both miRNAs are altered in *ap2* mutants (Yant et al., 2010).

In summary, reduction in miR156 levels as plants age allows increased expression of specific SPL transcription factors and these in turn activate transcription of *MIR172* genes. The resulting inverse temporal expression patterns of the microRNAs confer their opposing effects on vegetative phase change and presumably flowering time.

MicroRNAs and competence to flower

The observations that the abundance of miR156 declines with the age of the plant and that it regulates flowering time through repression of SPL transcription factors suggested that miR156 might play a central role in controlling competence to flower. Decline of miR156 levels and expression of SPL transcription factors were correlated with initiation of flowering of older *A. thaliana* plants that were not exposed to promotive environmental signals such as long photoperiods (Wang et al., 2009). Also, miR156 overexpressor plants and *spl9 spl15* double mutants were less sensitive to short exposures of one or three long days given three weeks after germination (Schwarz et al., 2008). However, whether this effect was due to impairing age-related competence to flower or more directly to reducing responsiveness to long days was not tested. Overall, testing competence to flower is difficult in *A. thaliana* because it responds to inductive environmental cues extremely early after germination (Mozley and Thomas, 1995) and reference accessions such as Columbia do not exhibit an obligate requirement for these stimuli.

By contrast, flowering and acquisition of competence are delayed in perennial Brassicaceae relatives of *A. thaliana*, and analysis of their obligate vernalization response demonstrated that in these systems miR156 levels act as the timer in controlling competence to flower (Figure 1) (Bergonzi et al., 2013; Zhou et al., 2013). Some accessions of perennial *Arabis alpina* and *Cardamine flexuosa* exhibit

Figure 1. Hyun et al.

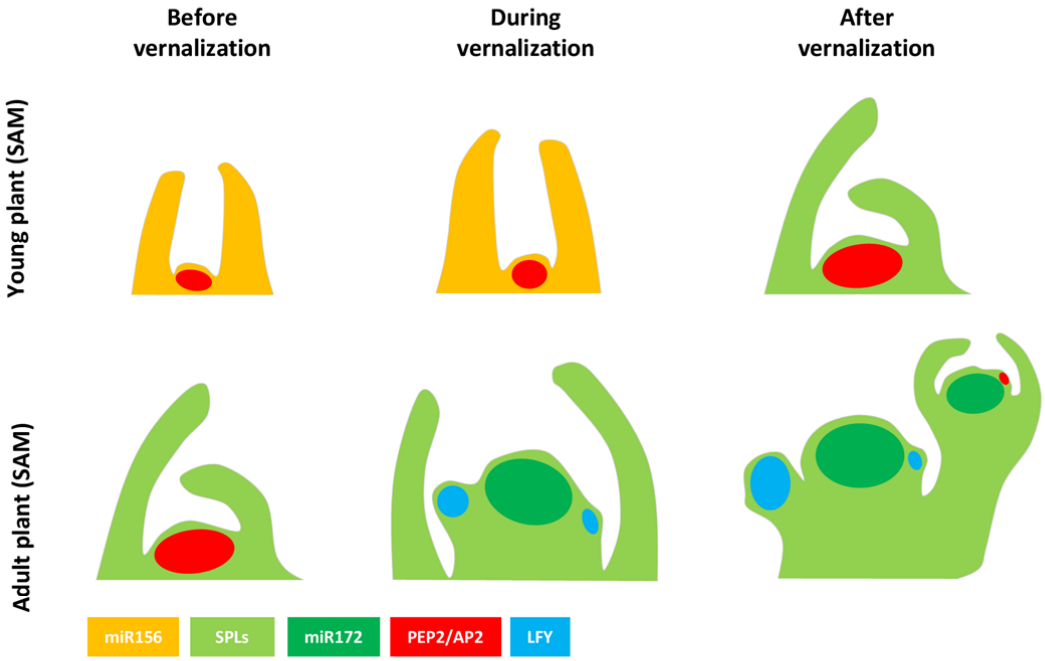

Figure 1. Age-related responsiveness to vernalization controlled by the miR156/SPL and miR172/AP2-like modules in *Arabis alpina*, a perennial relative of *A. thaliana*. Top row. Young plants that have not achieved competence to flower are exposed to vernalization. In the shoot apex miR156 is broadly expressed across the SAM and leaves, and the abundance of miR156 remains high during vernalization. Flowering does not occur during vernalization. After vernalization, miR156 is downregulated and SPL encoding genes are expressed but flowering does not proceed until the plants are vernalized. The *AP2* orthologue *PEP2* is expressed at all stages throughout the plant and is shown here at the SAM. Bottom row. Older plants that have achieved competence are exposed to vernalization. The age-related downregulation of miR156 has occurred allowing expression of SPL encoding genes at the shoot apex. The level of miR172 is markedly Increased at the SAM during vernalization presumably through the activity of SPLs. The increase of miR172 at the SAM inhibits the accumulation of the floral repressor PEP2 in the center of the meristem. Flowering is induced during vernalization and floral meristem identity genes such as *LFY* are expressed. In another perennial Brassicaceae species *Cardamine flexuosa*, the miR172 level was shown to be increased during growth of adult plants even before vernalization. The miR172/AP2 module plays a role also in the floral meristem formed on the flanks of the SAM to determine the identity of developing floral organs. Base d on data from (Bergonzi et al., 2013; Zhou et al., 2013).

obligate vernalization response, and these flowered only if exposed to cold when several weeks old, but remained vegetative if vernalized as younger plants (Wang et al., 2011; Bergonzi et al., 2013; Zhou et al., 2013). In each species the stage in development that miR156 reached trough levels correlated with the time that the plant became sensitive to vernalization to induce flowering. Analysis of transgenic plants of these species supported a causal relationship between the downregulation of miR156 and acquisition of competence to flower in response to vernalization. In *A. alpina* overexpression of miR156 from the *CaMV 35S* promoter prevented flowering in response to vernalization, whereas overexpression of MIM156 caused plants to respond to vernalization sooner after germination (Bergonzi et al., 2013). Similarly, in *C. flexuosa* miR156 and miR172 levels were found to be inversely related, as described above for other species, and overexpression of miR172

caused plants to flower without vernalization (Zhou et al., 2013). These experiments suggested that repression of miR156 and the resulting increase in expression of miR172 in older plants confer an age-related response to vernalization in *C. flexuosa*. Evidence for a role for miR172 was also obtained in *A. alpina*, because mutations in the orthologue of *AP2* caused flowering without vernalization (Figure 1) (Bergonzi et al., 2013). Overall, these results support the idea that in perennial Brassicaceae, acquisition of competence to flower in response to vernalization is conferred by age-related downregulation of miR156.

SPL transcription factors that regulate flowering time

SPL transcription factors were originally identified in a biochemical screen for proteins that bind to the promoter of *SQUAMOSA*, a gene that encodes a MADS box protein involved in floral development of *Antirrhinum majus* (Klein et al., 1996). They were originally called SQUAMOSA BINDING PROTEINs (SBP), and abundance of the mRNAs encoding SBP1 and SBP2 increased as the plant aged, were expressed prior to *SQUAMOSA* and exhibited specific spatial expression patterns in the shoot apical meristem and floral primordia (Klein et al., 1996). Genes encoding proteins related to SBPs were then isolated from *A. thaliana* and named *SPL*s (Cardon et al., 1997). Subsequently this class of transcription factor was identified in all species examined in the green plant lineage, and is defined by a 79 amino acid highly conserved region that represents the DNA-binding domain (Klein et al., 1996; Guo et al., 2008). The *SPL* family comprises 16 genes in *A. thaliana*, and 11 of these were reported to contain miRNA recognition sites (Guo et al., 2008). Of these 11 *SPL*s, *SPL3*/*SPL4*/*SPL5* and *SPL9*/*SPL15*, which represent two clades in the family, contain recognition sequences for miR156 and have been associated most strongly with the floral transition. Therefore, we focus particularly on these five members of the family.

The *SPL3*/*SPL4*/*SPL5* genes have a simpler structure than other members of the family and encode only two exons (Cardon et al., 1999). In this group the miR156 recognition sequence is located in the 3' UTR (Rhoades et al., 2002; Wu and Poethig, 2006; Gandikota et al., 2007). The mRNAs of all three genes are strongly expressed in inflorescences (Figure 2) (Cardon et al., 1999). *SPL4* and *SPL5* mRNAs are strongly increased in abundance under inductive long days through activity of the photoperiodic flowering pathway, and have distinct spatial patterns of expression with *SPL4* mRNA being mainly expressed in the rib meristem and later in floral primordia while *SPL5* mRNA is expressed on the flanks of the inflorescence meristem and in floral primordia (Figure 2) (Cardon et al., 1999; Schmid et al., 2003; Jung et al., 2012; Torti et al., 2012). *SPL3* mRNA has been detected widely in the meristem, shoot and flowers (Cardon et al., 1997; Wang et al., 2009; Wu et al., 2009), in vegetative meristems (Wu et al., 2009) and been reported to be upregulated during floral transition (Wang et al., 2009). Furthermore, miR156 inhibits translation of *SPL3* mRNA when expressed from the CaMV 35S promoter (Wu and Poethig, 2006; Gandikota et al., 2007). Several reverse genetic experiments based on overexpression and biochemical analyses suggest that *SPL3*/*SPL4*/*SPL5* contribute to the promotion of flowering. Transgenic plants carrying a fusion of *SPL3* open reading frame to the *CaMV 35S* promoter were early flowering (Cardon et al., 1997), which was enhanced if the miRNA recognition sequence was removed from the transgene, and these plants also prematurely underwent the transition from juvenile to adult vegetative phase (Wu and Poethig, 2006; Gandikota et al., 2007). Similar phenotypes as for *SPL3* were observed when *SPL4* and *SPL5* were overexpressed without their miRNA156 recognition sites, supporting the idea that these genes show functional redundancy (Wu and Poethig, 2006). However, recent analysis demonstrated that a *spl3 spl4 spl5* triple mutant was not delayed in flowering time compared to wild-type plants, although it did form slightly higher numbers of cauline leaves. Thus these genes were proposed not to promote floral transition but to act relatively late during the flowering process in conferring floral identity on the developing primordium (Xu et al., 2016). The mechanism by which SPL3/SPL4/SPL5 confer floral

Figure 2. Hyun et al.

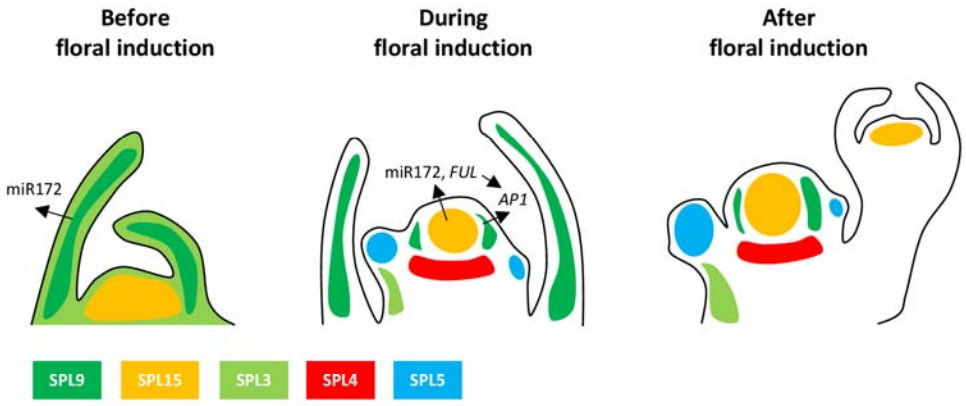

Figure 2. Spatially distinct roles of *SPL genes* in the *Arabidopsis* SAM. Left panel. Two closely-related genes *SPL9* and *SPL15* are expressed before floral induction in leaves and the SAM, respectively. *SPL9* is expressed in leaves where in adult plants after reduction in miR156 it participates in the accumulation of miR172 to promote the transition to adult leaf morphology. Middle panel. During floral induction under SDs, the accumulation of miR172 and mRNA of the floral activator *FUL* at the SAM requires the function of *SPL15*. During and after floral induction, *SPL9* mRNA appears on the flanks of the meristem and the protein activates the floral identity gene *AP1* in cooperation with DELLA and LFY. *SPL3, SPL4* and *SPL5* mRNAs are expressed at the shoot apex. Right panel. After floral induction. *SPL15* is expressed in the floral meristem and the inflorescence meristem. *SPL3*, *SPL4* and *SPL5* are expressed in specific patterns at the apex. See text for references.

meristem identity might involve direct activation of genes involved in the early stages of floral development. In transgenic plants expressing from the *CaMV 35S* promoter a SPL3-GFP fusion protein lacking the miR156 binding site in the 3' UTR or GFP-SPL3 expressed from the endogenous *SPL3* promoter, the fusion protein was found to bind directly to the promoters of *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*) and the *CaMV 35S* transgene caused increased expression of the target genes (Yamaguchi et al., 2009). These genes were also increased in expression in transgenic plants overexpressing from the *CaMV 35S* promoter *SPL4* or *SPL5* transcripts lacking the miR156 binding site (Yamaguchi et al., 2009). Also in the apices of *spl3 spl4 spl5* triple mutants grown for 11 long days, *LFY* and *AP1* transcripts were present at lower levels than in wild-type plants (Xu et al., 2016). Thus the expression patterns of *SPL3*, *SPL4* and *SPL5*, their target genes and the phenotypic effects of their mutation suggest that they act late in the flowering process to contribute to the early steps in floral development.

The closely related *SPL9* and *SPL15* genes represent one clade within the *A. thaliana SPL* family and have distinct functions in floral induction and floral development (Schwarz et al., 2008; Wang et al., 2009; Yamaguchi et al., 2014; Hyun et al., 2016). Although they are closely related paralogues, the *SPL9* and *SPL15* genes exhibit distinct mRNA expression patterns in the shoot meristem (Figure 2) (Wang et al., 2009; Wu et al., 2009; Hyun et al., 2016). *SPL9* mRNA appears not to be expressed in the shoot meristem prior to floral transition, but rises on the flanks of the meristem during the transition before emergence of floral primordia (Hyun et al., 2016). In vegetative plants *SPL9* mRNA is expressed in leaf primordia and leaves (Wang et al., 2009; Wu et al., 2009; Hyun et al., 2016). *SPL15* mRNA is expressed in the vegetative meristem prior to floral induction, throughout the meristem during induction and in the inflorescence meristem (Hyun et al., 2016). During the vegetative phase,

SPL15 mRNA is present in leaves in a more restricted pattern than *SPL9* mRNA (Hyun et al., 2016). Gain of function transgenic alleles of *SPL9* and *SPL15* in which the miRNA recognition site is mutated without affecting the protein sequence, both cause early flowering (Wang et al., 2009; Hyun et al., 2016) and the *SPL9* transgene causes early transition to the vegetative adult phase (Wu et al., 2009). Also, a dominant EMS-induced mutation of *SPL15* that affected the miRNA156 binding site caused premature transition from juvenile to adult vegetative phase and reduced cell size in leaves (Usami et al., 2009). These experiments, which rely on gain of function approaches, suggest that SPL9 and SPL15 have similar functions in controlling flowering time and vegetative phase transition. However, loss of function alleles of *SPL9* and *SPL15* have also been described and seem to distinguish between the activities of the genes (Schwarz et al., 2008; Wu et al., 2009; Hyun et al., 2016). The *spl15* single mutant and *spl9 spl15* double mutants were slightly later flowering than wild-type plants under long days (Schwarz et al., 2008). However, under non-inductive short days, the *spl15* mutant and *spl9 spl15* double mutant showed severe late flowering, whereas the *spl9* mutant did not (Hyun et al., 2016). This phenotype of *spl15* was much weaker and more variable in the studies of Xu et al (2016), suggesting that additional environmental variables such as light quality or intensity that have not yet been identified play an important role in determining the phenotype of the mutant. When observed, the late-flowering phenotype of *spl15* under short days was similar but not quite as severe as that of *35S:miR156* plants, suggesting that most of the late flowering caused by overexpression of miR156 is through inhibition of *SPL15* activity (Hyun et al., 2016). The *spl9* mutant also showed delayed transition to the adult vegetative phase that was enhanced in the *spl9 spl15* double mutant, which also showed reduced leaf plastochron (Schwarz et al., 2008; Wu et al., 2009). Thus the expression patterns and loss of function phenotypes of *SPL9* and *SPL15* suggest that *SPL15* plays the larger role in floral induction and is particularly important under non-inductive short days, whereas *SPL9* plays a more significant role in vegetative phase change and acts in floral primordia after floral induction.

Specific functions of SPL9 and SPL15 in controlling flowering and their regulation by GA

In the floral primordium, SPL9 has specific roles in the activation of genes required for early flower development (Yamaguchi et al., 2014). Analysis of DNA binding showed that SPL9 binds to functionally important regions in the *AP1* promoter (Wang et al., 2009; Yamaguchi et al., 2014), and a constitutively expressed chemically inducible form of SPL9 increases *AP1* transcription synergistically with inducible LFY (Yamaguchi et al., 2014). In addition, analysis of transgenic plants expressing miR156-resistant *SPL9* mRNA showed that SPL9 binds to *FUL* and *SOC1* (Wang et al., 2009), which, considering the expression pattern of *SPL9*, might also be most relevant in wild-type plants during the early stages of floral development. In addition to being regulated by miR156 at the post-transcriptional level, SPL9 is also regulated at the post-translational level by DELLA proteins (Yu et al., 2012) (Box 1). These proteins directly interact with and regulate the activity of transcription factors and are degraded in the presence of GA, providing a mechanism by which this plant growth regulator controls gene expression (Xu et al., 2014). A series of experiments indicated that SPL9 recruits the DELLA protein RGA to the *AP1* promoter and DELLA binding enhances the ability of SPL9 to activate *AP1* transcription in the floral primordium (Box 1) (Yamaguchi et al., 2014). By contrast, interaction of RGA and SPL9 represses the ability of SPL9 to activate transcription of *SOC1* and *MIR172b* (Yu et al., 2012). Thus the effect of the interaction between SPL9 and DELLA appears to differ among target genes or tissues, leading to activation of transcription of some targets such as *AP1* and repression of others including *SOC1* and *MIR172b* (Yu et al., 2012; Yamaguchi et al., 2014).

SPL15 promotes floral transition under non-inductive short days. Fluorescent protein fusions to SPL15 expressed from endogenous regulatory sequences accumulated in the meristem and were directly regulated by miR156 (Hyun et al., 2016), suggesting that the protein acts in the meristem to promote flowering. In agreement with this conclusion, the level of *FT* mRNA, which is the output of

Figure 3. Hyun et al.

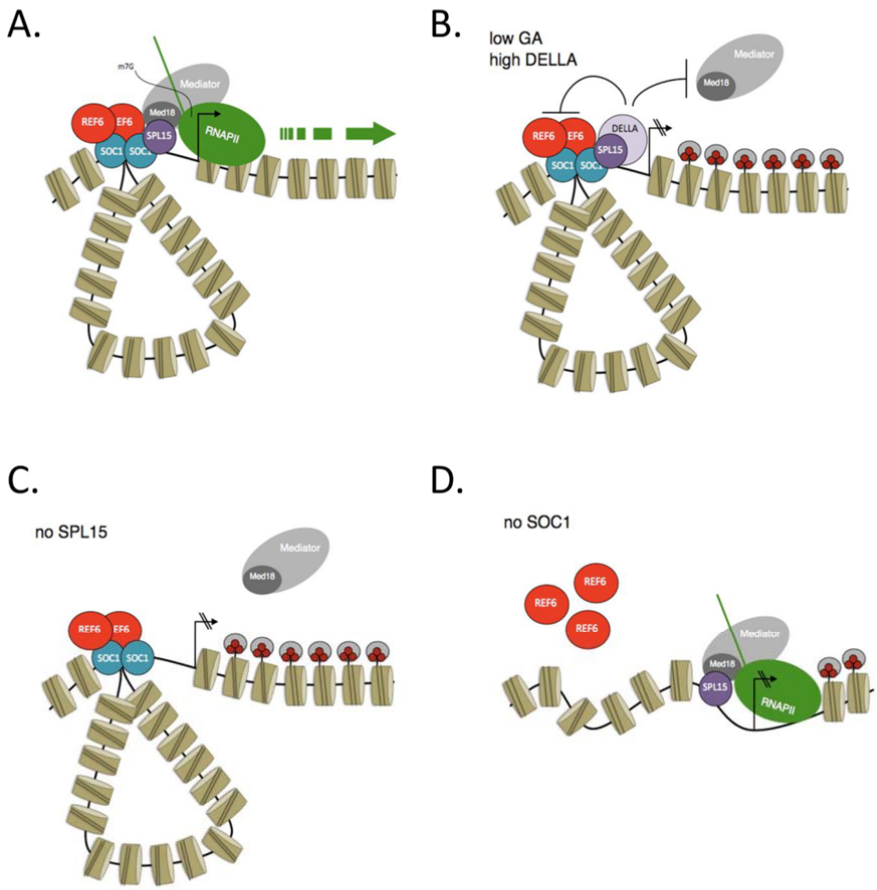

Figure 3. Mechanism by which SPL15 activates its target genes *FUL* and *MIR172b* during floral induction under SDs. A. Activation of transcription requires SPL15 and the MADS box transcription factor SOC1. SOC1 recruits the histone H3 K27me3 demethylase REF6 allowing removal of repressive chromatin marks from the target gene. SPL15 interacts with the Mediator complex to promote transcription through RNA PolII. B. If GA levels are low at the meristem, DELLA protein levels are high. DELLA interacts with SPL15 at the target gene promoter, preventing interaction of SPL15 with the Mediator complex. No transcription occurs. C. In the absence of SPL15, SOC1 binds to the target gene and REF6 is recruited but no transcription occurs. The Mediator complex is not recruited to the target gene. D. In the absence of SOC1, SPL15 binds to the target gene but REF6 is not recruited. The repressive chromatin mark H3K27me3 remains on the gene and no transcription occurs. Data from (Hyun et al., 2016).

flowering pathways that act in the leaves, was the same in *spl9 spl15* double mutants and wild-type plants (Hyun et al., 2016). Also, although overexpression of *SPL9* from heterologous promoters in leaves did promote *FT* transcription and early flowering, the effect was less strong than when *SPL9* was expressed in the shoot meristem (Wang et al., 2009). Therefore, in wild-type plants these SPLs probably act exclusively in the meristem to promote flowering. SPL15 binds directly to the *FUL* and *miR172b* genes and is required for their activation in the shoot meristem under short days. Genetic experiments in which GA was depleted from the shoot meristem by overexpression of a GA catabolic enzyme supported the idea that interaction of RGA with SPL15 prevents the activation of SPL15

target genes such as *MIR172b* and *FUL* (Hyun et al., 2016) (Box 1). These results are consistent with the roles of SPL15 and GA in promoting floral transition under short days (Wilson et al., 1992; Hyun et al., 2016), and suggest that one way in which GA promotes flowering under short days is by stimulating degradation of DELLA allowing SPL15 to activate its target genes in the meristem. Thus among *A. thaliana* SPL transcription factors, SPL15 seems to play the major role in floral transition. Interestingly, the mechanism by which SPL15 activates transcription of its target genes involves cooperativity with known regulators of flowering, particularly the MADS box transcription factor SOC1 (Figure 3), which acts early during the floral transition (Borner et al., 2000; Samach et al., 2000).

The genetic and molecular analyses of *SPL9* and *SPL15* clearly implicate them in the early stages of reproductive development, and suggest that if miR156 acts to regulate competence to flower, as suggested by the experiments in perennial Brassicaceae, then it likely does so by repressing the activity of *SPL15* and probably *SPL9*. Also the observation that DELLAs and GA act to regulate SPL9 and SPL15 activity is consistent with early observations that this growth regulator is implicated in vegetative phase change (Chien and Sussex, 1996; Telfer et al., 1997; Poethig, 2003) as well as floral induction under non-inductive conditions (Wilson et al., 1992; Hyun et al., 2016).

Regulation of the miR156 timer

The temporal regulation of vegetative phase change and competence to flower described above ultimately relies on the gradual reduction of miR156 levels. This process is widely conserved in higher plants (Wu and Poethig, 2006; Chuck et al., 2007; Bergonzi et al., 2013; Zhou et al., 2013), but the precise age-related mechanisms by which miR156 levels are regulated remain unclear. Eight genes encode miR156 in *A. thaliana* (Rhoades et al., 2002; Morea et al., 2016). The precise spatial and temporal expression patterns of these precursor genes have not been described, and it remains unclear, for example, which are expressed in the meristem and what their temporal patterns of expression are. However, two of them, *MIR156A* and *MIR156C*, are highly expressed in the shoot of young plants and express most of the mature miR156 detected at this stage (Yang et al., 2013; Yu et al., 2013). The abundance of these precursor RNAs falls in successive leaf primordia and shows a similar regulation to mature miR156 (Yang et al., 2013). Thus the temporal pattern of reduction in miR156 levels appears to be conferred at least in large part by transcriptional regulation of these precursors, and this conclusion was further supported by analyzing fusions of the regulatory sequences of these precursor genes to the ß-glucuronidase marker gene (Xu et al., 2016a).

The transcriptional downregulation of *MIR156A* and *MIR156C* is regulated by chromatin modification. Trimethylation of lysine 27 on histone 3 (H3K27me3) is a chromatin mark associated with repression of transcription (Derkacheva and Hennig, 2014), and this mark accumulates to higher levels on the *MIR156A* and *MIR156C* genes in apices of 5-week old plants than of 1 or 2 week old plants (Xu et al., 2016a). Deposition of this mark on these genes involves the SWINGER methyl transferase and the chromatin remodeler PICKLE, which was previously shown to associate with genes rich in H3K27me3 modification (Zhang et al., 2012). Both of these proteins regulate vegetative phase change, bind directly to the *MIR156* precursor genes and contribute to the accumulation of H3K27me3 on *MIR156A* and *MIR156C* (Xu et al., 2016a). However, these proteins are part of the general enzyme machinery that contributes to H3K27me3 deposition across the genome and the mechanisms by which they are recruited to *MIR156A* and *MIR156C* in an age-dependent manner is unclear.

However, several experiments suggest that endogenous sugar levels may act earlier in the process to repress transcription of *MIR156* precursor genes (Wahl et al., 2013; Yang et al., 2013; Yu et al., 2013), perhaps by increasing deposition of the H3K27me3 mark. *MIR156* transcriptional repression in vegetative phase change was shown to be promoted by a signal produced in leaf primordia (Yang et

al., 2011). The possible role of sugars as this signal was then tested in a range of genetic and physiological experiments, based on classical work suggesting sugars regulate maturation of the shoot (Goebel, 1908). Application of exogenous sucrose reduced miR156 levels and specifically the transcription of *MIR156A* and *MIR156C*. Also sucrose and glucose levels were higher in older plants therefore showing an inverse relationship to miR156 and mutants exhibiting impaired rates of photosynthesis had higher levels of miR156 as well as delayed transition to adult vegetative phase (Yang et al., 2013; Yu et al., 2013). These and related experiments suggest that the higher levels of sugar, particularly sucrose but also glucose, in older plants contributes to the downregulation of *MIR156* gene transcription to accelerate transition to adult phase. However, it remains unclear from these experiments whether sugar also acts as the timer in the shoot meristem to regulate floral transition.

A further series of genetic experiments implicated another sugar, trehalose 6-phosphate (T6P), in repression of miR156 levels during flowering (Gomez et al., 2010; Wahl et al., 2013). Trehalose 6-phosphate is present in low concentrations in plant cells and is proposed to act as a signaling molecule rather than to have a function in primary metabolism (Lunn et al., 2006). Mutations impairing TREHALOSE 6 PHOSPHATE SYNTHASE (TPS) are embryo lethal (Gomez et al., 2010), but if this defect is complemented with a transgene active in embryos then the resulting plants are viable and late flowering (Gomez et al., 2010; Wahl et al., 2013). In these plants, miR156 levels were up to 8 times higher than in wild-type, suggesting that this is one of the causes of the late-flowering phenotype. Consistent with this interpretation, the *SPL3*, *SPL4* and *SPL5* genes were expressed at lower levels in *tps* mutants. These results suggest that T6P signaling might be important in regulating *MIR156* transcription as part of the sugar signaling pathway. However, in *tps* mutants other flowering-time genes were altered in expression, and as described above roles for SPL3, SPL4 and SPL5 in flowering-time control have not been established, so the mechanism and extent to which T6P controls flowering time through miR156 regulation still requires elucidation.

Other genetic systems controlling competence to flower

In addition to the miR156/SPL module, other genetic systems have been proposed to contribute to the age at which plants become sensitive to environmental cues that induce flowering. Notable among these are the TEMPRANILLO (TEM) transcription factors that repress the response to photoperiod in young *A. thaliana* plants. TEM1 and TEM2 are members of the RAV transcription factor family and contain two DNA binding domains related to those of AP2 and to B3 (Castillejo and Pelaz, 2008). TEM1 binds directly to the promoter of *FT* and to exons of genes encoding GA biosynthetic enzymes to repress their transcription and thereby delay floral induction (Castillejo and Pelaz, 2008; Osnato et al., 2012). Furthermore, *TEM1* mRNA abundance falls abruptly between 8 and 10 days after germination under long days (Castillejo and Pelaz, 2008). The timing of this reduction correlates with a strong increase in *FT* mRNA and to enhanced sensitivity of the plants to long photoperiods for floral induction (Castillejo and Pelaz, 2008; Sgamma et al., 2014). These results suggest that *TEM* genes act mainly in young plants to block the response to long days and that reduction in their expression contributes to acquisition of competence to flower in response to photoperiod. Furthermore, this system appears to be evolutionarily conserved because the mRNA of a *TEM* orthologue from *Antirrhinum majus* was also reduced in abundance at the time at which plants became sensitive to photoperiod to induce flowering (Sgamma et al., 2014). How the levels of *TEM1* and *TEM2* mRNA are reduced with age is unknown, but in *A. thaliana* exposure to long days reduces *TEM1* and *TEM2* mRNA levels (Osnato et al., 2012), suggesting that the repressive effects of these transcription factors on flowering may be regulated directly by environmental conditions rather than or as well as by endogenous mechanisms associated with aging of the plant.

Finally, the floral repressor TERMINAL FLOWER 1 (TFL1) extends the phase during which plants are insensitive to inductive cues. In perennial *A. alpina* TFL1 activity blocked activation of the floral meristem identity gene *LFY* during vernalization of young plants (Wang et al., 2011). Reduction of *TFL1* expression by RNA interference allowed *LFY* transcription and flowering to occur during vernalization of young plants, in a similar way as in *35S:MIM156* plants (Wang et al., 2011; Bergonzi et al., 2013). These data together with the work described above on miR156/SPL function in *A. alpina* suggest that repression of flowering by TFL1 is required to block flowering of young plants and that this can be overcome later through the action of SPL transcription factors. TFL1 is proposed to interact with the bZIP transcription factor FD and thereby repress transcription (Hanano and Goto, 2011), so it is possible that TFL1 and SPL transcription factors have common target genes and that whether flowering proceeds is determined by the relative abundance of each class of protein.

Variation in competence to flower among annuals and perennials

The acquisition of competence to flower is usually strongly delayed in perennials whereas annuals can flower rapidly after germination. This delay in perennials allows the plant to produce more biomass and axillary meristems prior to reproduction, and thereby likely increases the possibility of surviving flowering and reproducing the following year (Bergonzi and Albani, 2011). Annual and perennial life history can diverge rapidly during evolution, suggesting that the genetic system conferring competence to flower can also change relatively quickly (Bergonzi et al., 2013; Zhou et al., 2013). By contrast, the miR156/SPL system appears to be ancient and present in all flowering plants (Morea et al., 2016). In the Brassicaceae this discrepancy is proposed to be explained by increased dependency on the miR156/SPL system for flowering in perennials, whereas annuals evolve genetic mechanisms that bypass the requirement for SPLs during flowering. For example, in *A. thaliana* there is a strong requirement for SPL15 to promote flowering under non-inductive short days, whereas in long days this requirement is bypassed so that *spl15* mutants have a very mild phenotype under these conditions (Hyun et al., 2016). Therefore, the balance of quantitative activities of different flowering pathways can explain how the time taken to acquire competence is more important in determining flowering time of some species than others. Similarly, evolution in annuals of pathways that bypass the requirement for miR156/SPL to induce flowering can explain how the miR156/SPL module is present and similarly expressed in annuals and perennials but annuals do not show a strong requirement for acquisition of competence to flower. The recent progress in defining closely related annual and perennial experimental systems that differ in competence phenotypes provide a means of understanding how these bypass pathways evolve and how their activities vary quantitatively.

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Box 1

Interactions between SPL transcription factors and GAs

Several phenotypic and molecular observations suggest a close functional relationship between SPL transcription factors and GA signal transduction (Zhang et al., 2007; Porri et al., 2012; Yu et al., 2012; Yamaguchi et al., 2014; Hyun et al., 2016). The SPL transcription factors promote flowering under short days and juvenile to adult vegetative phase transition, two processes also regulated by GA.

At the level of transcription, SPL8 induces expression of genes involved in GA biosynthesis and signaling in the anther (Zhang et al., 2007), while depletion of GA from the shoot meristem delayed transcription of several *SPL* genes during floral transition (Porri et al., 2012).

GA signaling also directly affects the activity of SPL transcription factors at the post-translational level. The DELLA protein REPRESSOR OF GA1-3 (RGA), a negative regulator of GA signaling that is degraded in the presence of GA, interacts with SPL9 in heterologous assays performed in yeast and *Nicotiana benthamiana*. Also, reducing GA from plants with the biosynthetic inhibitor paclobutrazol, which leads to an increase in DELLA protein levels, reduced the ability of overexpressed SPL9 to activate its target genes *SOC1* and *MIR172b* (Yu et al., 2012). These data suggest that interaction of DELLA with SPL9 reduces the ability of the transcription factor to activate *SOC1* and *MIR172b* transcription. Another set of experiments showed that RGA is recruited to a similar position on the *AP1* promoter as SPL9, and this association was prevented when miR156 expression was induced from a transgene (Yamaguchi et al., 2014). Furthermore, simultaneous induction of expression of LFY and the DELLA protein RGA enhanced *AP1* transcription compared to induction of each transgene separately. This experiment suggests that reduction of GA in the floral primordium by enzymes whose transcription is activated by LFY promotes floral development by increasing the abundance of DELLA and the transcriptional activation activity of SPL9-DELLA. Taken together these results indicate that RGA interacts with SPL9 at promoters and depending on the context leads to reduced (*MIR172b* and *SOC1*) or increased transcription (*AP1*).

DELLA proteins also bind directly to SPL15 and both proteins associate with similar regions of target genes (Hyun et al., 2016). RGA association with SPL15 target genes is abolished in *spl15* mutants, indicating that SPL15 recruits DELLA proteins to DNA. Furthermore, the interaction of SPL15 with DELLA blocks the capacity of SPL15 to activate its *FUL* and *MIR172b* target genes (Hyun et al., 2016), as was proposed at *MIR172b* for SPL9-DELLA (Yu et al., 2012). The functional significance of this interaction was supported by genetic experiments in which GA was depleted from the meristem by overexpression of a GA catabolic enzyme and in these plants the interaction between SPL15 and DELLA protein RGA was enhanced while the ability of SPL15 to promote flowering was reduced (Hyun et al., 2016). Thus at least one of the routes by which DELLA delays flowering is by interacting with and repressing the activity of SPL15, which is required to promote flowering under short days.

Figure 1. Age-related responsiveness to vernalization controlled by the miR156/SPL and miR172/AP2-like modules in *Arabis alpina*, a perennial relative of *A. thaliana*. Top row. Young plants that have not achieved competence to flower are exposed to vernalization. In the shoot apex miR156 is broadly expressed across the SAM and leaves, and the abundance of miR156 remains high during vernalization. Flowering does not occur during vernalization. After vernalization, miR156 is downregulated and SPL encoding genes are expressed but flowering does not proceed until the plants are vernalized. The *AP2* orthologue *PEP2* is expressed at all stages throughout the plant and is shown here at the SAM. Bottom row. Older plants that have achieved competence are exposed to vernalization. The age-related downregulation of miR156 has occurred allowing expression of SPL encoding genes at the shoot apex. The level of miR172 is markedly increased at the SAM during vernalization presumably through the activity of SPLs. The increase of miR172 at the SAM inhibits the accumulation of the floral repressor PEP2 in the center of the meristem. Flowering is induced during vernalization and floral meristem identity genes such as *LFY* are expressed. In another perennial Brassicaceae species *Cardamine flexuosa*, the miR172 level was shown to be increased during growth of adult plants even before vernalization. The miR172/AP2 module plays a role also in the floral meristem formed on the flanks of the SAM to determine the identity of developing floral organs. Based on data from (Bergonzi et al., 2013; Zhou et al., 2013).

Figure 2. Spatially distinct roles of *SPL genes* in the *Arabidopsis* SAM. Left panel. Two closely-related genes *SPL9* and *SPL15* are expressed before floral induction in leaves and the SAM, respectively. *SPL9* is expressed in leaves where in adult plants after reduction in miR156 it participates in the accumulation of miR172 to promote the transition to adult leaf morphology. Middle panel. During floral induction under SDs, the accumulation of miR172 and mRNA of the floral activator *FUL* at the SAM requires the function of *SPL15*. During and after floral induction, *SPL9* mRNA appears on the flanks of the meristem and the protein activates the floral identity gene *AP1* in cooperation with DELLA and LFY. *SPL3, SPL4* and *SPL5* mRNAs are expressed at the shoot apex. Right panel. After floral induction. *SPL15* is expressed in the floral meristem and the inflorescence meristem. *SPL3*, *SPL4* and *SPL5* are expressed in specific patterns at the apex. See text for references.

Figure 3. Mechanism by which SPL15 activates its target genes *FUL* and *MIR172b* during floral induction under SDs. A. Activation of transcription requires SPL15 and the MADS box transcription factor SOC1. SOC1 recruits the histone H3 K27me3 demethylase REF6 allowing removal of repressive chromatin marks from the target gene. SPL15 interacts with the Mediator complex to promote transcription through RNA PolII. B. If GA levels are low at the meristem, DELLA protein levels are high. DELLA interacts with SPL15 at the target gene promoter, preventing interaction of SPL15 with the Mediator complex. No transcription occurs. C. In the absence of SPL15, SOC1 binds to the target gene and REF6 is recruited but no transcription occurs. The Mediator complex is not recruited to the target gene. D. In the absence of SOC1, SPL15 binds to the target gene but REF6 is not recruited. The repressive chromatin mark H3K27me3 remains on the gene and no transcription occurs. Data from (Hyun et al., 2016).

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