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FLOWERING NEWSLETTER REVIEW

Regulation of flowering time by the miR156-mediated age pathway

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Abstract

Precise flowering time is critical to reproductive success. In response to diverse exogenous and endogenous cues including age, hormones, photoperiod, and temperature, the floral transition is controlled by a complex regulatory network, which involves extensive crosstalks, feedback, or feedforward loops between the components within flowering time pathways. The newly identified age pathway, which is controlled by microRNA156 (miR156) and its target *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) transcription factors, ensures plants flower under non-inductive conditions. In this review, I summarize the recent advance in understanding of the age pathway, focusing on the regulatory basis of the developmental decline in miR156 level by age and the molecular mechanism by which the age pathway is integrated into other flowering time pathways.

Key words: Age, microRNA, flowering time.

Introduction

The aerial lateral organs of a plant are derived from the shoot apical meristem (SAM), a population of pluripotent stem cells at the shoot apex that are formed during embryonic development. After seed germination, organ primordia are continuously formed on the flanks of the SAM. Based on the identity of the lateral organs, the post-embryonic development of a plant can be divided into vegetative and reproductive phases. The SAM produces leaves during the vegetative phase, whereas it gives rise to flowers in the reproductive phase (Poethig, 2003). Vegetative phase can be further divided into juvenile and adult phases. Adult phase differs from juvenile phase in terms of reproductive competence and morphological differences such as leaf epidermal cell differentiation and leaf complexity (Huijser and Schmid, 2011; Poethig, 2013).

The floral transition, namely the switch from vegetative to reproductive phase, is coordinately controlled by multiple genetic pathways in response to various developmental and environmental cues (reviewed in Andres and Coupland, 2012; Bäurle and Dean, 2006; Srikanth and Schmid, 2011). The past two decades have seen fundamental advances in our understanding of the molecular mechanism underlying floral transition. Studies of the annual model *Arabidopsis thaliana* identified five flowering time pathways, known as age, autonomous, gibberellin (GA), photoperiod, and vernalization (Amasino and Michaels, 2010). A central aspect of our knowledge of flowering time regulation is that multiple floral inductive cues are integrated into a set of flowering time integrator genes, including MADS-box genes such as *APETALA 1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), and a plant-specific transcription factor *LEAFY* (*LFY*) (Amasino and Michaels, 2010; Lee and Lee, 2010; Srikanth and Schmid, 2011). In this Review, I begin with a brief description of the five flowering time pathways in *A. thaliana*. Then I turn to a discussion of the molecular basis of the age pathway and how age cues are integrated into other flowering inductive cues.

Flowering behaviour in *A. thaliana* can be divided into two types, winter annual and rapid cycling, based on their requirement for a prolonged exposure to low temperature, a treatment called vernalization (reviewed in Heo and Sung,

2011; Song *et al.*, 2012; Song *et al.*, 2013). Winter annual types are late flowering and such a late-flowering phenotype can be eliminated by vernalization. Genetic studies have revealed that *FLOWERING LOCUS C* (*FLC*), a MADS-box gene, acts as the master regulator in vernalization pathway. Before vernalization, *FRIGIDA* (*FRI*), a gene of unknown biochemical function, activates *FLC* (Choi *et al.*, 2011). FLC delays flowering through repressing *FT* in the leaves and *SOC1* at shoot apex (Searle *et al.*, 2006).Transcription of *FLC* rapidly decreases in response to vernalization. It has been demonstrated that the repression of *FLC* by cold is regulated by complex mechanisms involving long-non-coding RNAs (lncRNAs), histone modification and higher order chromatin assembly (Crevillen *et al.*, 2013; Rosa *et al.*, 2013; Song *et al.*, 2012; Sun *et al.*, 2013; Zografos and Sung, 2012). In contrast to winter annual, rapid-cycling accessions are early flowering in the absence of vernalization, which is often due to naturally occurring mutations in *FRI* (Johanson *et al.*, 2000).

A. thaliana is a long day plant, in which the onset of flowering is accelerated when the length of daylight is prolonged compared with darkness. Molecular and genetic analyses demonstrate that the seasonal changes in day length are measured by *CONSTANS* (*CO*), which encodes a zinc finger and CCT-domain-containing transcription factor (Putterill *et al.*, 1995). *co* mutants show delayed flowering in long days but not in short days (Putterill *et al.*, 1995). Classical physiological experiments reveal that the floral transition in response to day length involves a systemic signal formed in the leaves that induces floral transition at the SAM. Consistent with this notion, *CO* is mainly expressed in leaf vascular tissues (An *et al.*, 2004). *CO* expression is regulated by light at both the transcriptional and post-transcriptional level. In short days, the expression of *CO* peaks after dusk, so that CO protein is subjected to COP1-mediated degradation (Jang *et al.*, 2008; Liu *et al.*, 2008; Valverde *et al.*, 2004; Yanovsky and Kay, 2002). In contrast, *CO* expression coincides with light in long days, which leads to stabilization of CO. The accumulation of CO leads to the activation of *FT*, which encodes a putative phosphatidylethanolamine-binding protein. With the help of an endoplasmic reticulum (ER) membrane protein, FT-INTERACTING PROTEIN1 (FTIP1), FT proteins move from the leaves to the shoot apex (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Liu *et al.*, 2012; Mathieu *et al.*, 2007). In the SAM, FT, by binding with the transcription factor FD, activates the expression of *LFY* and MADS-box genes, such as *AP1* and *SOC1*, and thereby induces flowering (Abe *et al.*, 2005; Kobayashi and Weigel, 2007; Wigge *et al.*, 2005).

In addition to photoperoid and vernalization, GAs also play a critical role in flowering time. The GA biosynthetic mutant, *ga1*, never flowers under non-inductive short day conditions (Wilson *et al.*, 1992). GA signalling transduction is mediated by ubiquitin–proteasome degradation (reviewed in Harberd, 2003; Schwechheimer and Willige, 2009). By binding to GA, GIBBERELLIN INSENSITIVE DWARF1 (GID1), a nuclear-localized GA receptor, promotes the degradation of the transcriptional repressors called DELLAs. The *Arabidopsis* genome encodes five DELLA genes, namely *REPRESSOR OF GA1-3* (*RGA*), *GA INSENSITIVE* (*GAI*), *RGA-LIKE 1* (*RGL1*), *RGL2*, and *RGL3* (Murase *et al.*, 2008). The DELLA motif, which is 17 amino acid residues long and located in the amino-terminal of DELLAs, is essential for the degradation of DELLA proteins by the proteasome (Dill *et al.*, 2001). The negative role of GA on flowering time is mediated by DELLAs such as GAI and RGA. The *gai* or *rga-Δ17* mutant, which carries the deletion of the DELLA motif, is insensitive to GA-induced proteolysis and delays flowering (Dill *et al.*, 2001; Peng *et al.*, 1997). It has been shown that GAI represses flowering through *SOC1* because the expression of *SOC1* is induced by GA treatment but reduced in the *gai* mutant (Moon *et al.*, 2003). Recent studies identified two GATA-type transcription factors, GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and GNL (GNC-LIKE) as new components in the GA pathway (Richter *et al.*, 2010). Expression analyses indicate that *GNC* and *GNL* act downstream of DELLAs and promote flowering through activating *SOC1* (Richter *et al.*, 2013).

The autonomous pathway is constituted by a group of genes that promote flowering by suppressing *FLC*. FCA, FPA, and FLOWERING LOCUS K (FLK) contain an RNA-binding domain (Lim *et al.*, 2004; Macknight *et al.*, 1997; Mockler *et al.*, 2004; Schomburg *et al.*, 2001), whereas *FY* and *FLOWERING LOCUS D* (*FLD*) encode a protein homologous to the yeast poly-adenylation factor Pfs2p and a dimethylated histone H3 at lysine 4 (H3K4me2) demethylase, respectively (He *et al.*, 2003; Simpson *et al.*, 2003). It has been shown that FCA, interacting with a component of the CPSF complex, targets CstF-dependent 3' processing to the proximal site on *FLC* antisense transcripts. With the help of FPA, FY, and FLD, this targeted processing triggers localized histone demethylase activity and results in reduced *FLC* sense transcription (Liu *et al.*, 2010; Manzano *et al.*, 2009). Because the regulatory basis of these genes is largely unknown, the biological relevance of autonomous pathway remains unclear.

In summary, the identities and actions of the components in flowering time pathways reveal that photoperiodic pathway acts as a positive regulator of flowering, whereas other pathways promote flowering through alleviating flowering repressors. The fact that flowering eventually occurs in the photoperiodic mutants indicates that there is another flower-promoting pathway that ensures plants flower under non-inductive conditions.

miR156–SPL defines the age pathway

microRNAs (miRNAs) are 21–24 nt long, small noncoding RNAs widely distributed in animals and plants (Bartel, 2009). It has been shown that plant miRNAs regulate gene expression through transcript cleavage (Llave *et al.*, 2002; Reinhart *et al.*, 2002) and translational inhibition (Brodersen *et al.*, 2008; Chen, 2004; Li *et al.*, 2013).

miR156 is one of the most evolutionally conserved miRNAs in plants. The targets of miR156 encode a family of transcription factors, called SQUAMOSA PROMOTER BINDING LIKEs (SPLs) (Cardon *et al.*, 1999; Rhoades *et al.*, 2002). In the *Arabidopsis* genome, there are 11 *SPLs* targeted by miR156. Based on the size of encoded proteins, these *SPL* genes can be divided into two major groups, represented by *SPL3* (*SPL3*, *SPL4*, and *SPL5*) and *SPL9* (*SPL2*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13*, *SPL13-like*, and *SPL15*) (Xing *et al.*, 2010). SPL3, SPL4, and SPL5 are much smaller than the other gene products, with the DNA-binding domain making up most of the protein. In addition, the miR156-binding site is located in the 3'UTR of *SPL3* (also *SPL4* and *SPL5*) and miR156 regulates *SPL3* expression through transcript cleavage as well as translational inhibition (Gandikota *et al.*, 2007).

Expression of miR156 is temporally regulated. Mature miR156 is highly abundant in seedlings and decreases with time (Wang *et al.*, 2009a; Wu *et al.*, 2009; Wu and Poethig, 2006). This expression pattern is observed not only in *A. thaliana*, but also in other species including *Arabis alpina*, *Cardamine flexuosa*, maize, poplar, rice, and tomato (Bergonzi *et al.*, 2013; Chuck *et al.*, 2007; Wang *et al.*, 2011a; Xie *et al.*, 2012; Yoshikawa *et al.*, 2013; Zhou *et al.*, 2013). The developmental decline in miR156 is partially mediated by sugars, the products of photosynthesis (Proveniers, 2013; Yang *et al.*, 2011; Yang *et al.*, 2013; Yu *et al.*, 2013). Exogenous sugar treatment results in a rapid decease in miR156 expression. The repression of miR156 by sugar occurs at both transcriptional and post-transcriptional levels. Consistent with these findings, the *A. thaliana chlorina1* (*ch1*) mutant, which has impaired photosynthesis, accumulates higher level of miR156 than wild type. Similarly, defoliation delays juvenile-to-adult phase transition with a concomitant rise in miR156 level (Yang *et al.*, 2011).

The importance of miR156 in flowering is inferred from the observation that overexpression of miR156 delays flowering (Jung *et al.*, 2011b; Schwab *et al.*, 2005; Schwarz *et al.*, 2008; Wu and Poethig, 2006; Yamaguchi and Abe, 2012; Zhou and Wang, 2013). Notably, the effect of miR156 overexpression on flowering is much pronounced under non-inductive short day conditions, together with the fact that miR156 expression is regulated by age, indicating that miR156 acts as an endogenous flowering cue. In agreement with this finding, overexpression of *SPL3* results in an early flowering phenotype irrespective of photoperiodic length (Wang *et al.*, 2009a; Wu and Poethig, 2006; Yamaguchi *et al.*, 2009). In contrast, the effect of *SPL9* on flowering time is ambiguous (Wang *et al.*, 2009a). Despite the fact that *SPL9* overexpression lines flower nearly at the same time as wild type, the floral transition of *SPL9* overexpression lines is clearly accelerated when the flowering time is measured by the number of leaves produced when the plants start to flower. These contradictory results can be explained by the negative role of *SPL9* on leaf initiation rate (Wang *et al.*, 2008). Indeed, overexpression of miR156 under a shoot apex specific promoter delays flowering without affecting leaf initiation rate (Wang *et al.*, 2009a). Therefore, these results suggest an antagonistic effect between growth rate and flowering time, which prevents plants from precocious flowering.

The role of miR156 in flowering seems widely conserved among angiosperms. Overexpression of miR156 caused late flowering phenotype in many species including *A. alpina*, *C. flexuosa*, maize, potato, and rice (Bergonzi *et al.*, 2013; Bhogale *et al.*, 2014; Chuck *et al.*, 2011; Eviatar-Ribak *et al.*, 2013; Xie *et al.*, 2006; Zhou *et al.*, 2013).

Integration of age and photoperiodic pathways

Genetic studies have placed the age pathway in parallel with photoperiodic pathway. Overexpression of miR156 in an *ft* background results in a severely delay in flowering (Wang *et al.*, 2009a). In the extreme case, flowering never occurs under short day conditions. These results indicate that the miR156-mediated age pathway ensures plants flower in the absence of exogenous inductive cues.

Recent efforts have provided insight into how the miR156–SPL module regulates flowering in *A. thaliana*. In the juvenile phase, the levels of miR156-targeted *SPL* genes are low because of high amount of miR156. As plants age, the amount of miR156 is decreased, resulting in an increase in miR156-targeted *SPL* level. *SPL3* and *SPL9* promote flowering in leaves and shoot apex through two distinct mechanisms (Figure 1). In the shoot apex, *SPL3* and *SPL9* induce flowering through activating MADS-box genes, including *AP1*, *LFY*, *FUL*, and *SOC1* (Wang *et al.*, 2009a; Yamaguchi *et al.*,

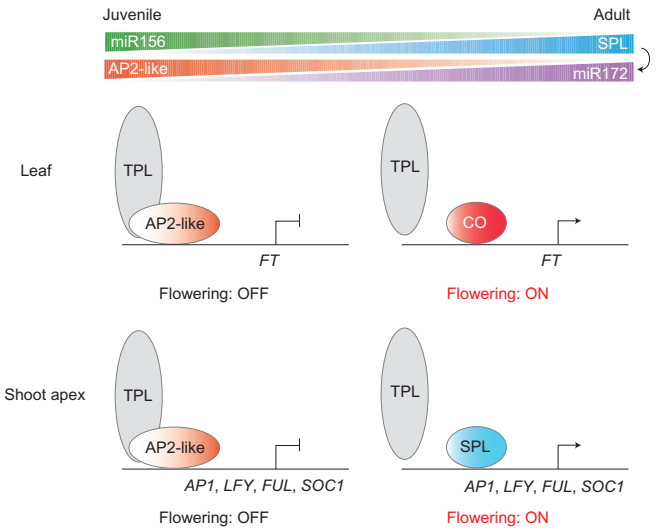

Fig. 1. miR156-mediated age pathway. miR156 level is high in juvenile phase but significantly reduced in adult phase. As a result, the level of miR156-targeted *SPLs* rises, which leads to activation of miR172 and thereby reduction in the levels of miR172-targeted *AP2-like* genes (*AP2*, *TOE1*, *TOE2*, *SMZ*, and *SNZ*). In the juvenile phase, miR172-targeted AP2-like proteins, with the help of TPL, repress flowering through *FT* in leaves, and flower-promoting genes (*AP1*, *FUL*, *LFY*, and *SOC1*) in the shoot apex. In the adult phase, CO activates *FT* expression in leaves and miR156-targeted *SPLs* induce flowering through activating the expression of flower-promoting genes in the shoot apex.

2009). Forced expression of *SPL3* or *SPL9* under the shoot apex specific promoter leads to early flowering phenotype under both long day and short day conditions (Wang *et al.*, 2009a).

In leaves, SPL9 activates another miRNA, miR172, by direct binding to and transcriptional activation of *MIR172b* (Wu *et al.*, 2009). miR172 targets a family of AP2-like transcription factors, including *AP2*, SCHLAFMUTZE (*SMZ*), SCHNARCHZAPFEN (*SNZ*), *TARGET OF EAT1* (*TOE1*), *TOE2*, and *TOE3* (Aukerman and Sakai, 2003; Wu *et al.*, 2009). All these miR172-targeted AP2-like transcription factors act as flowering repressors. Overexpression of miR172 causes an extremely early flowering phenotype under both short days and long days (Jung *et al.*, 2007; Yant *et al.*, 2010), whereas the increased level of *TOE1* results in late flowering (Mathieu *et al.*, 2009). Chromatin immunoprecipitation sequencing (ChIP-SEQ) analyses reveal that TOE1 and AP2 not only inhibit *FT* expression in leaves, but also repress many other flowering time regulators acting downstream of *FT* in the shoot apex (Mathieu *et al.*, 2009). The repression of these genes by TOE1 is mediated by TOPLESS (TPL), a transcriptional co-repressor (Causier *et al.*, 2012; Long *et al.*, 2006). Intriguingly, AP2 also negatively regulates miR172 and positively regulates miR156, suggesting a miR156–miR172 feedback loop in fine-tuning the flowering response (Yant *et al.*, 2010).

Previous studies have suggested that miR156-targeted *SPL* genes act downstream of photoperiodic pathway because up-regulation of *SPL3* and *SPL9* is readily detectable within 3 days after transfer of vegetative plants from short days to inductive long days, and this induction is much reduced in *co* or *ft* mutants (Schmid *et al.*, 2003). In agreement with this finding, *SPL3* has been shown to be directly regulated by SOC1 (Jung *et al.*, 2011a) and *SPL4* expression is reduced in a *soc1 ful* double mutant (Torti *et al.*, 2012) (Figure 2). Furthermore, mutations in two BELL1-like homeobox genes, *PENNYWISE* (*PNY*) and *POUND-FOOLISH* (*PNF*), impair the photoperiodic induction of *SPL3*, *SPL4*, and *SPL5* (Lal *et al.*, 2011).

Another layer of crosstalk between age and photoperiodic pathways comes from the regulation of miR172 by photoperiodic length in leaves (Figure 2). GIGANTEA (GI) positively regulates *CO* transcription (Fowler *et al.*, 1999; Jung *et al.*, 2007; Park *et al.*, 1999). miR172 abundance is substantially reduced in a *gi* mutant (Jung *et al.*, 2007). It is suggested that GI regulates miR172 at the miRNA processing level because the level of primary transcript of *MIR172* is not accordingly reduced but elevated in a *gi* mutant.

Crosstalk between age and gibberellin pathways

Under non-inductive short day conditions, age and GA pathways play the predominant roles in flowering. DELLA represses flowering in both leaves and the shoot apex. Forced expression of GA-insensitive *RGA* or GA catabolic

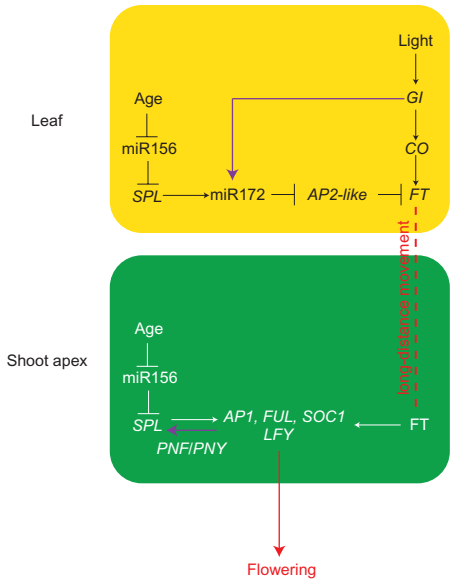

Fig. 2. Integration of age and photoperiodic pathways. The integration of age and photoperiodic pathways take place at two levels (purple arrow lines). First, miR172 abundance is regulated by photoperiod via GI-mediated miRNA processing. Second, miR156-targeted SPLs acts not only in parallel with, but also downstream of photoperiodic pathway. The level of miR156-targeted *SPLs* is rapidly induced when *A. thaliana* plants are shifted from short days to long days. This action seems to be mediated by two MADS-box genes, *SOC1* and *FUL*.

genes under leaf or shoot apex specific promoters results in a late flowering phenotype (Galvao *et al.*, 2012; Porri *et al.*, 2012; Yu *et al.*, 2012). Interestingly, GA treatment does not markedly accelerate flowering in an miR156 overexpression line, indicating that GA promotes flowering partially through the miR156–SPL module. In light of this finding, Yu *et al.* (2012) revealed that GA and age pathways are integrated through a physical interaction between DELLAs (RGA, GAI, RGL1, RGA2 and RGL3) and miR156-targeted SPL9-like proteins (SPL2, SPL9, SPL10 and SPL11). The binding of RGA to SPL9 interferes with SPL9 transcriptional activities on *MIR172b*, *SOC1*, and *FUL*. As a result, DELLA delays flowering by reducing *FT* expression through repressing miR172 in leaves, whereas it inhibits floral transition by repressing *SOC1* and *FUL* in the shoot apex (Figure 3).

Integration of age and vernalization pathways

In *A. thaliana*, plants become competent to vernalization after germination. However, recent studies indicate that age regulates the timing of sensitivity in response to vernalization in *A. alpina* and *C. flexuosa*, two polycarpic perennials closely related to *A. thaliana*. Independently, Wang *et al*. and Zhou *et al*. revealed that young *A. alpina* and *C. flexuosa* are insensitive to cold treatment (Wang *et al.*, 2011b; Zhou *et al.*, 2013). This flowering behaviour is mediated by the levels of miR156 and miR172 (Bergonzi *et al.*, 2013; Zhou *et al.*, 2013). Overexpression of miR156 prevents flowering in response to

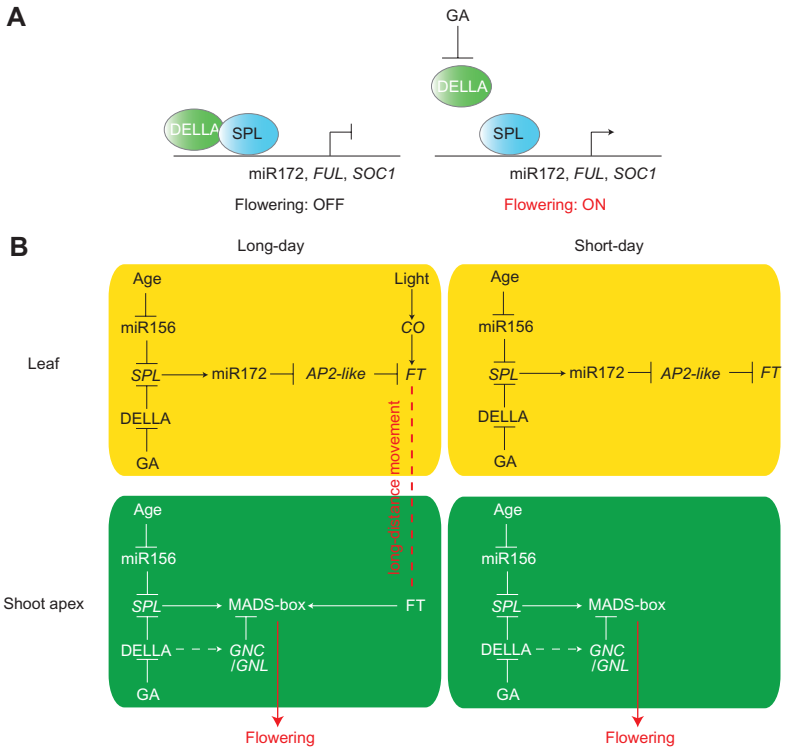

Fig. 3. Integration of Age and GA pathways. (A) DELLA acts a repressor of miR156-targeted SPL. The binding of DELLA with SPL inhibits the transcriptional activation of SPL targets, such as miR172, *FUL*, and *SOC1*. (B) DELLA represses flowering in two distinct mechanisms. The binding of DELLA with miR156-targeted SPLs compromises the activation of miR172 in leaves, and the activation of MADS-box genes (*SOC1* and *FUL*) in the shoot apex. In addition, it has been shown that DELLAs indirectly repress *SOC1* through two GATA transcription factors, GNC and GNL.

vernalization, whereas the reduced activity of miR156 or *PERPETUAL FLOWERING2* (*PEP2*, an miR172-targeted *AP2-like* gene) results in an accelerated acquisition of floral competence in response to vernalization. In addition, *A. alpina TERMINAL FLOWER1* (Aa*TFL1*) was found to block flowering of young *A. alpina* plants exposed to vernalization (Wang *et al.*, 2011b). The integration of age and vernalization pathways thus offers an advantage for the perennial growth habit by ensuring that plants do not flower until they develop axillary vegetative shoots and sufficient biomass.

Although the role of miR156 and miR172 in setting a threshold for the sensitivity in response to vernalization is conserved between *A. alpina* and *C. flexuosa*, the underlying molecular mechanism differs in the following two aspects (Fig. 4). First, *C. flexuosa FLC* expression is not reduced when miR172-targeted *AP2* group genes are suppressed by miR172 overexpression, whereas *PEP1*, the *A. alpina FLC* orthologue (Wang *et al.*, 2009b), is decreased in the *pep2* mutant (Fig. 4). Second, the expression of flowering activator miR172 is coupled with the flowering repressor miR156 in *A. thaliana*, maize, rice, and poplar. In *C. flexuosa*, miR172 is similarly linked to miR156, whereas it seems that *A. alpina* is an exception from this rule (Fig. 4). Interestingly, although the level of miR172 is not increased during vegetative phase, a rise in miR172 abundance is observed in developing floral primordia, which leads to alleviate the flowering repressive effect of miR172-targeted AP2-like proteins (Bergonzi *et al.*, 2013).

The above two differences reflect different strategies in the two perennial species. In *A. alpina*, because PEP2 positively regulates *FLC*, miR172-targeted *AP2*-like genes have to be uncoupled from miR156 and its SPL targets. Otherwise, the age-dependent increase in miR172 will cause a loss of *FLC* activity and thus promote flowering. Conversely, in *C. flexuosa*, because miR172 has remained under the control of miR156–SPL module, *FLC* has to be uncoupled from miR172-targeted *AP2*-like genes (Fig. 4).

Future directions

The past 20 years have witnessed a great increase in our knowledge of the basic molecular mechanisms of flowering. Most remarkably, functional genetic studies in *A. thaliana* and rice have identified signalling pathways that act as master regulators of floral transition and that are conserved in monocots and dicots. Growing evidence suggests that the integration of each floral inductive cue varies in different species. As described above, although both vernalization and age pathways operate in *A. thaliana*, this species does not have a pronounced age-dependent vernalization response. Thus, a major challenge in the future will be to understand how the flowering pathways are differentially regulated and integrated in different species.

The identification of sugar as an upstream regulator of miR156 suggests that sugar may play an important role in flowering. Consistently, trehalose-6-phosphate (T6P), a disaccharide molecule, was recently revealed as a new

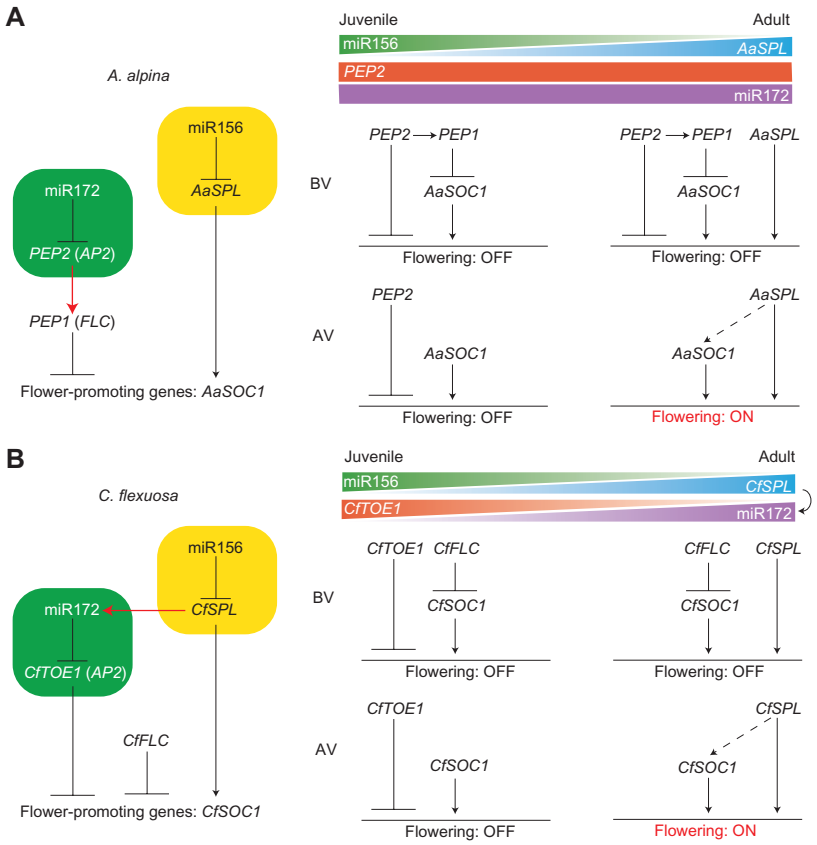

Fig. 4. Integration of age and vernalization pathways. (A) In *A. alpina*, miR172 is not regulated by an miR156-mediated age pathway. PEP2 (an *A. alpina* AP2) activates the expression of *PEP1*, an *FLC* orthologue in *A. alpina* (red arrow line). Note that miR172 level is not changed with age but arises in developing floral primordia. The dashed arrow line indicates an indirect activation of *SOC1* by miR156-targeted AaSPL. (B) In *C. flexuosa*, the miR156–SPL module is directly connected to the miR172–AP2 module (red arrow line). The expression of *CfFLC* is not regulated by *CfTOE1* (a *C. flexuosa* miR172-targeted AP2-like gene).

regulator of flowering (van Dijken *et al.*, 2004; Wahl *et al.*, 2013). Therefore, another challenge in future is to explore the means by which carbohydrate or energetic status regulates flowering.

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