PLANT SCIENCE

A regulatory circuit conferring varied flowering response to cold in annual and perennial plants

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The reproductive strategies of plants are highly variable. Short-lived annuals flower abundantly soon after germination, whereas longer-lived perennials postpone and spatially restrict flowering. We used CRISPR/Cas9 and interspecies gene transfer to understand divergence in reproductive patterns between annual and perennial crucifers. We show that in perennial *Arabis alpina*, flowering in response to winter cold depends on the floral integrator SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15), whose activity is limited to older shoots and branches during cold exposure. In annuals, this regulatory system is conserved, but cold-induced flowering occurs in young shoots, without requirement for SPL15, through the photoperiodic pathway when plants return to warm. By reconstructing the annual response in perennials, we conclude that characteristic patterns of reproduction in annuals and perennials are conferred through variation in dependency on distinct flowering pathways acting in parallel.

Floral induction from environmental cues synchronizes plant reproduction to changing seasons (*1*). However, the ability of an individual plant to respond to permissive environmental cues also depends on its developmental stage. This interaction between environmental and developmental processes varies among species, creating diversity in reproductive strategy. Monocarpic annuals are short-lived and flower in response to permissive seasonal cues soon after germination. By contrast, flowering of perennials is delayed, enabling sufficient resource accumulation to sustain their repeated life cycle (*2*). Moreover, perennials spatially restrict flowering, so that vegetative branches are retained after reproduction, implementing polycarpic life history. Perennial Brassicaceae species, including *Arabis alpina*, express two systems that limit reproduction in time and space. The first involves orthologs of the *Arabidopsis thaliana* MADS box transcription factor FLOWERING LOCUS C (FLC), called PERPETUAL FLOWERING 1 (PEP1) in *A. alpina* (*3–7*). In perennials, repression of *FLC* orthologs in winter cold enables flowering to occur through a process called vernalization, whereas their reactivation in warm restricts flowering. The second system, based on microRNA156 (miR156), acts in young meristems to prevent the flowering response to vernalization, but in older meristems, transcriptional repression of *MIR156* allows the vernalization response to proceed (*6, 8*). We show

that the PEP1 and miR156 systems are mechanistically linked in shoot meristems by means of the flowering promoting factor SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15). Furthermore, we demonstrate that the extent to which plants depend on this pathway differs between annuals and perennials, conferring variation in the age at which plants respond to vernalization.

In diverse plant lineages, miR156 levels gradually decrease as plants age (*9–11*). In *A. alpina*, neither age nor miR156 affects *PEP1* down-regulation during vernalization (*8, 12*), suggesting that age-related vernalization is determined downstream of *PEP1*. Genome-wide studies in *A. thaliana* and *A. alpina* showed that FLC and PEP1 bind to corresponding regions in the promoters of *SPL15* and *AaSPL15*, respectively (*13–15*). *SPL15* mRNA is targeted by miR156, and in *A. thaliana*, SPL15 promotes flowering under short days (*16, 17*). The binding of PEP1 to *AaSPL15* was confirmed by means of chromatin immunoprecipitation–quantitative polymerase chain reaction (fig. S1), and *pep1* mutants exhibited increased *AaSPL15* mRNA levels, demonstrating the relevance of PEP1 binding for *AaSPL15* transcription (fig. S1) and supporting the effect of FLC detected in *A. thaliana* and *Cardamine hirsuta* (*13, 18*). Moreover, when older (6 weeks old) plants were exposed to vernalization, *AaSPL15* mRNA increased at the shoot apical meristem (SAM), correlating with *PEP1* repression and flowering (Fig. 1, A and B, and fig. S1) (*3*). However, vernalization was insufficient to promote *AaSPL15* expression at the SAM of young plants (2 weeks old) that contain high miR156 levels (Fig. 1, C and D, and fig. S1) (*8*). Thus, *AaSPL15* potentially integrates cues derived from winter cold and age of the plant at the SAM of *A. alpina*.

To test whether *AaSPL15* is involved in vernalization, mutations that inactivated *AaSPL15* were induced in *A. alpina* accession Pajares (Paj) by using CRISPR/Cas9 (Fig. 1E and fig. S2) (*19*). Resequencing the genome of *Aaspl15* mutants, we detected no mutations at potential off-target sites of the single-guide RNA (sgRNA) (fig. S3 and table S1). The isolated *Aaspl15* mutants were less sensitive to vernalization compared with Paj. Most 5- and 6-week-old *Aaspl15* mutants failed to flower after vernalization, whereas wild-type Paj were fully responsive at both ages (Fig. 1, F and G). Around 40% of *Aaspl15* individuals did produce flowers after vernalization; however, their inflorescences rapidly reverted to producing leaves (Fig. 1, F to I), suggesting that the mutant meristem was not committed to flowering. Similarly, loss of AaSPL15 activity delayed the expression of floral meristem identity gene *AaLEAFY* (*AaLFY*) during vernalization and the flowering of *pep1* mutant in warm long days (fig. S4). The mutation cosegregated with the effect on vernalization response (fig. S5) and was complemented by a *wtAaSPL15-VN* transgene in which the wild-type *AaSPL15* locus was fused to the fluorescent protein VENUS (fig. S6). These analyses demonstrate that *AaSPL15* is required for the flowering response to vernalization in *A. alpina*.

Lower *AaSPL15* mRNA levels in young compared with older plants suggested that miR156 influences vernalization through *AaSPL15*. To test this idea, we generated Paj plants that carried *wtAaSPL15-VN* or *rAaSPL15-VN*, which expresses a miR156-resistant form of *AaSPL15* mRNA (fig. S6). Confocal microscopy revealed that 8-week-old *wtAaSPL15-VN* accumulated VENUS:AaSPL15 protein during vernalization at the SAM, but that 3-week-old plants did not (Fig. 2A and fig. S7). By contrast, in *rAaSPL15-VN* plants, VENUS:AaSPL15 accumulated at the SAM of plants of both ages during vernalization (Fig. 2A). Thus, the sensitivity to miR156 ensures that SPL15 only accumulates at the SAM of adult plants. Moreover, young *rAaSPL15-VN* flowered in response to vernalization, whereas young *wtAaSPL15-VN* did not (Fig. 2B and fig. S8), indicating that SPL15 expression is sufficient to trigger flowering of young plants during vernalization. Thus, the antagonistic regulation of miR156 and SPL15 expression during cold determines the age at which *A. alpina* can respond to vernalization.

The resistance of *SPL15* to miR156 also modified the flowering response of lateral branches (Fig. 2C). Axillary meristems of *A. alpina* attain competence to flower dependent on their age and independent of the SAM (*8, 20*), so that after vernalization, lateral branches remain vegetative or flower in a predictable spatial pattern (Fig. 2D) (*3, 21*). Accumulation of VENUS:AaSPL15 varied among axillary meristems of *wtAaSPL15-VN* exposed to vernalization. The fusion protein accumulated only in meristems of lateral shoots old enough to have produced several leaves before vernalization (Fig. 2E), correlating with flowering response of these branches (fig. S9). By contrast, *rAaSPL15-VN* accumulated VENUS:

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AaSPL15 similarly in all axillary meristems, irrespective of the age of the lateral shoot (Fig. 2E and fig. S9). Also, a higher proportion of axillary meristems transitioned to flowering in *rAaSPL15-VN* (Fig. 2F). In rice, the closest homolog of SPL15 determines tiller bud outgrowth (*22*, *23*), but comparison of bud dormancy in *rAaSPL15-VN* and *wtAaSPL15-VN* (Fig. 2F) plants suggested that in *A. alpina*, axillary bud outgrowth is controlled independently of AaSPL15. Thus, the miR156-SPL15 module contributes to polycarpic development of *A. alpina* by conferring competence to flower independently on individual meristems.

Whether SPL15 participates in the vernalization response of *A. thaliana* remains unclear because the annual plant responds to vernalization independently of age (*20*). We detected that *SPL15* in *A. thaliana* is regulated in a manner similar to that described above in *A. alpina*. Thus, in 2-week-old *FRI-Col*, *FLC* down-regulation during vernalization was insufficient for VENUS:SPL15 accumulation because of high miR156 levels (Fig. 3, A and B, and figs. S10 and S11). Nevertheless, vernalization promoted flowering of these young plants. However, the transition took place only after returning to warm long days (Fig. 3C and fig. S12), which possibly involves the photoperiodic *FLOWERING LOCUS T* (*FT*) pathway. Accordingly, knockout alleles of *FT* and its paralogue *TWIN SISTER OF FT* (*TSF*) delayed flowering of 2-week-old *FRI*-Col exposed to vernalization (Fig. 3D). By contrast, 6-week-old *FRI*-Col, which contains low miR156 levels, accumulated VENUS:SPL15 during vernalization (Fig. 3A and fig. S11). The adult plants transitioned to flowering in cold (Fig. 3C and fig. S12), and *FT* no longer influenced flowering under these conditions (Fig. 3D and fig. S12). These data suggest that SPL15 and FT represent two independent pathways to flowering, and *A. thaliana* does not display age-related vernalization because on exposure to long days after vernalization, FT bypasses requirement for *SPL15*. Supporting this conclusion, *spl15 ft tsf* triple mutants in Col flowered late compared with either *spl15* or *ft tsf* mutants (Fig. 3E).

This model suggests that flowering of *A. alpina* in response to vernalization only occurs through the SPL15-dependent pathway. In *A. thaliana*, FLC directly represses *FT* transcription (*24*). Similarly, PEP1 in *A. alpina* down-regulated *AaFT1* and *AaFT3* (figs. S13 and S14), which can promote flowering (fig. S15). However, *PEP1* is only repressed in cold, whereas *FLC* remains stably repressed after vernalization (*3*, *4*, *25*). Therefore, PEP1 may prevent flowering through *AaFTs* after vernalization. Accordingly, ectopic *AaFT1* expression promoted flowering in *A. alpina* without cold exposure and independent of *AaSPL15* expression (fig. S15). To test whether differences in *PEP1*/*FLC* regulation between annuals and perennials affected flowering responses to cold, we used material derived from an interspecies cross between *A. alpina* and its annual relative *Arabis montbretiana* (*26*). Similar to *FLC* in *A. thaliana*, its ortholog in *A. montbretiana* (*AmFLC*) is stably repressed after vernalization, and this species responds to vernalization soon after germination (*26*). Back-crossing the interspecies hybrids to *A. alpina*, we derived two introgression lines, *PEP1*_IL and *AmFLC*_IL, that possess comparable genomic structures except for the region containing *PEP1* or *AmFLC* (Fig. 4A), and neither flowered without vernalization (fig. S16). However, in the hybrid plants, *AmFLC* remains stably repressed after vernalization, whereas *PEP1* is reactivated (*26*). We found that 2-week-old *AmFLC*_IL flowered in response to vernalization, whereas 2-week-old *PEP1*_IL did not (Fig. 4B and fig. S16). Thus, the age-related repression of vernalization response in young plants was compromised in *AmFLC*_IL. That *AmFLC* conferred this difference was confirmed by introducing *AmFLC* locus into *A. alpina pep1* mutant via *Agrobacterium*-mediated transformation, which recapitulated the phenotype of *AmFLC*_IL (Fig. 4, B and C, and fig. S17). Young *AmFLC pep1* showed neither *AaSPL15* mRNA accumulation nor flower formation in cold (Fig. 4, D and E, and fig. S18) but, as observed in *A. thaliana*, flowered after vernalization in response to long days, probably through *AaFT* activation (Fig. 4F and fig. S17). Thus, the stable repression of *AmFLC* after vernalization reconstructs the annual flowering response to winter cold in perennial *A. alpina*.

We show here that the age-dependent response to vernalization in perennial *A. alpina* occurs through SPL15 (fig. S19). Reactivation of *PEP1* in warm ensures that the FT pathway does not promote flowering after vernalization (fig. S20).
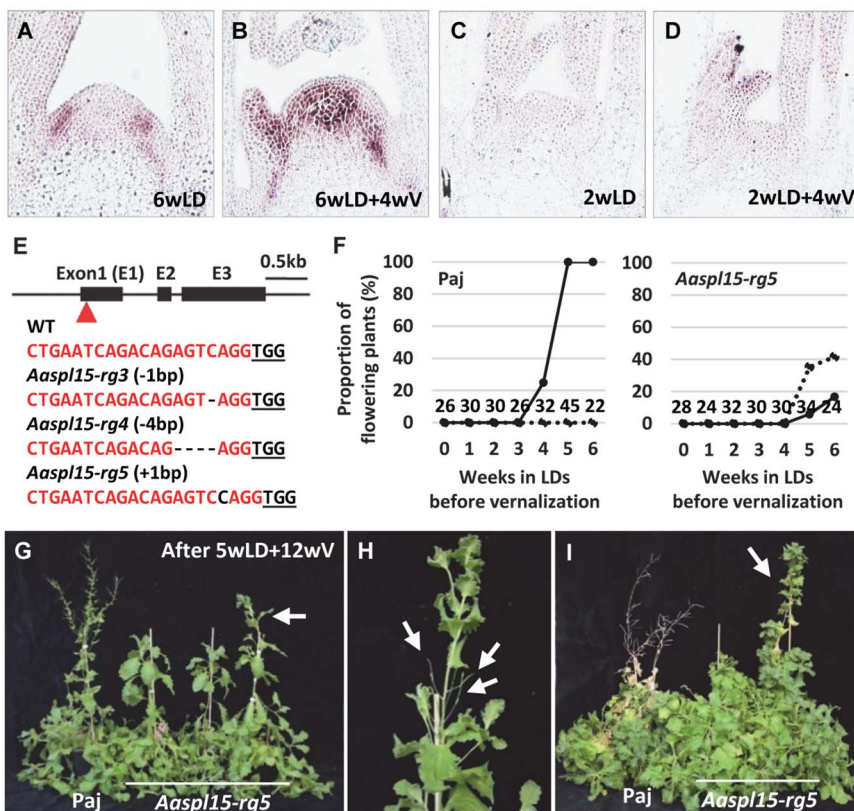

Fig. 1. AaSPL15 is a crucial component of the vernalization mechanism of *A. alpina*. (**A** to **D**) *AaSPL15* mRNA accumulation at shoot apices of *A. alpina* Paj before and after 4-week vernalization. Plants were grown under long days (LDs) for 6 or 2 weeks before vernalization. (**E**) *Aaspl15*-knockout alleles induced by use of CRISPR/Cas9. The arrowhead indicates the site targeted for mutagenesis. Twenty nucleotides homologous to the sgRNA and the PAM motif are marked with red letters and black underlined letters, respectively. (**F**) Vernalization response of Paj and *Aaspl15-rg5* at different ages. Solid and dashed lines indicate the proportion of plants flowering or reverted from flower formation back to vegetative growth, respectively. The number of analyzed plants are indicated. (**G** to **I**) Defects in the vernalization response of *Aaspl15* mutants. Arrows indicate [(G) and (I)] the plants that showed reversion and (H) siliques formed on the reverted shoot. Plants at (G) 1 month and (I) 4 months after vernalization treatment are presented.
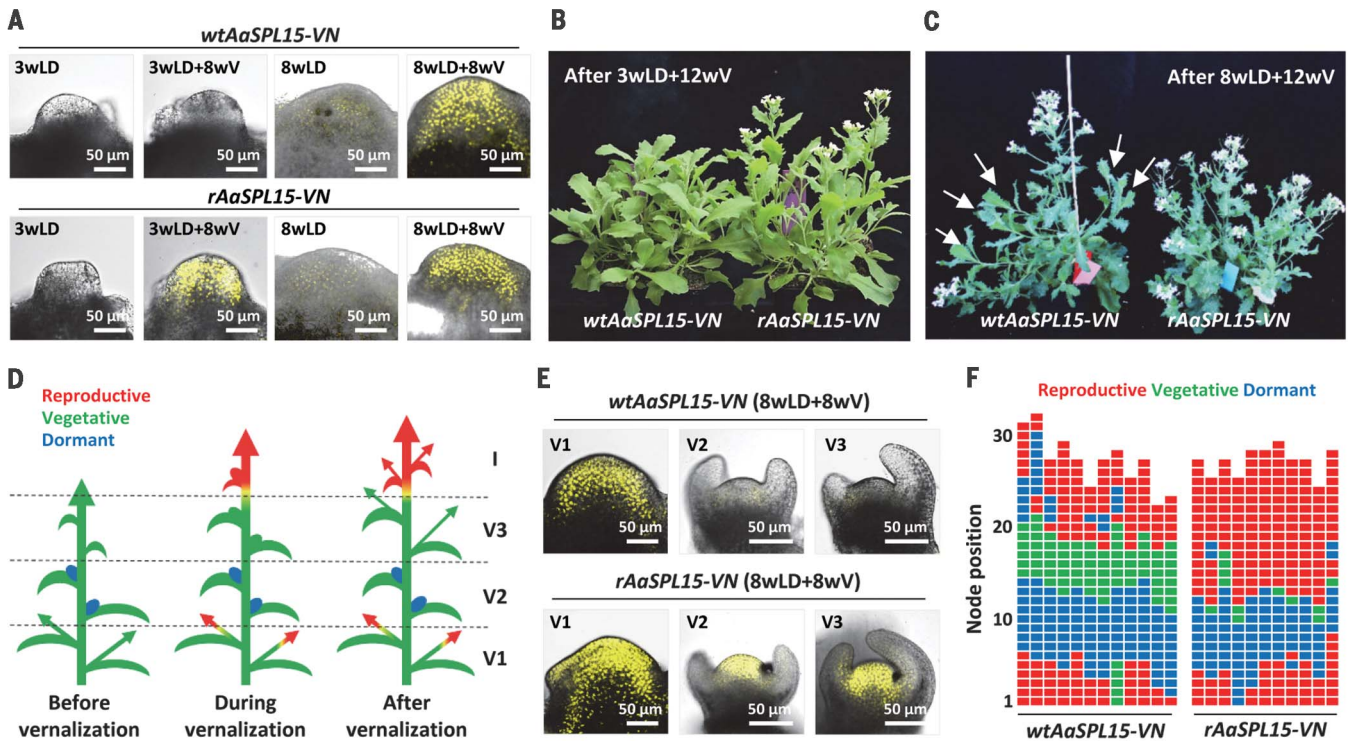
Fig. 2. *AaSPL15* **defines the competence of shoot meristems to flower in response to vernalization.** (**A**) VENUS:AaSPL15 protein accumulation before and during vernalization at main shoot apices of plants of different ages carrying the *wtAaSPL15-VN* or *rAaSPL15-VN* transgenes. (**B** and **C**) Earlier acquisition of sensitivity to vernalization in *rAaSPL15-VN*. Arrows indicate vegetative axillary branches formed after vernalization. (**D**) Schematic showing developmental fates of axillary meristems at different positions on the shoot of *A. alpina*. Arrows and ovals represent shoots and buds, respectively. (**E**) Accumulation of VENUS:AaSPL15 at axillary meristems of *wtAaSPL15-VN* and *rAaSPL15-VN* during vernalization. V1 to V3 indicate the positions on the shoot illustrated in (D). (**F**) Quantification of developmental fates of axillary meristems of *wtAaSPL15-VN* and *rAaSPL15-VN* at 6 weeks after exposure of 8-week-old plants to 12-week vernalization (8wLD+12wV). Each column depicts an individual plant.
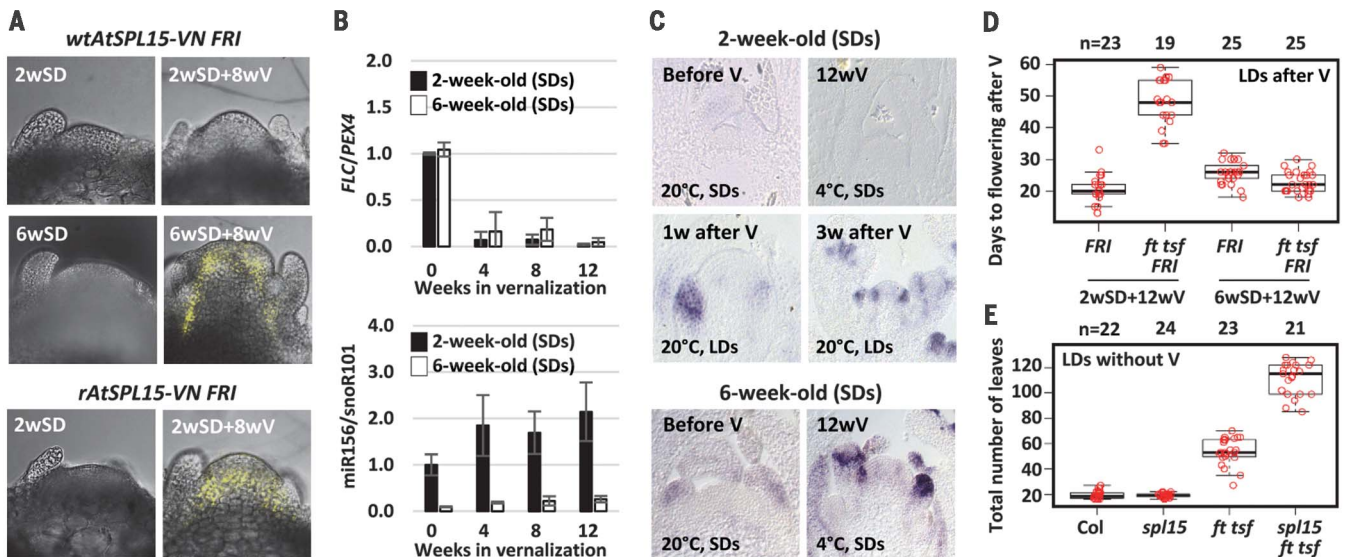
Fig. 3. FT bypasses the requirement for SPL15 in cold-induced flowering of young *A. thaliana*. (**A**) Accumulation of VENUS:SPL15 in *wtAtSPL15-VN FRI* and *rAtSPL15-VN FRI* before and during vernalization. (**B**) *FLC* mRNA and miR156 levels at the shoot apex of *FRI*-Col. The expression levels at 2 weeks old are set to 1.0 for normalization. The error bars indicate standard deviation of three biological replicates. (**C**) *LFY* mRNA accumulation in *FRI*-Col exposed to vernalization at 2wSD and 6wSD. (**D**) A lack of FT activity delays flowering of *FRI*-Col exposed to vernalization at 2wSD but not when exposed at 6wSD. (**E**) SPL15 and FT represent independent pathways to flowering on the basis of a synergistic genetic interaction under LDs. Flowering time is presented as (D) days to flowering after vernalization (V) and (E) total leaf number. In the box plots, the interquartile range box represents the middle 50% of the data. Whiskers extend 1.5 times the interquartile range and the center line shows the median.
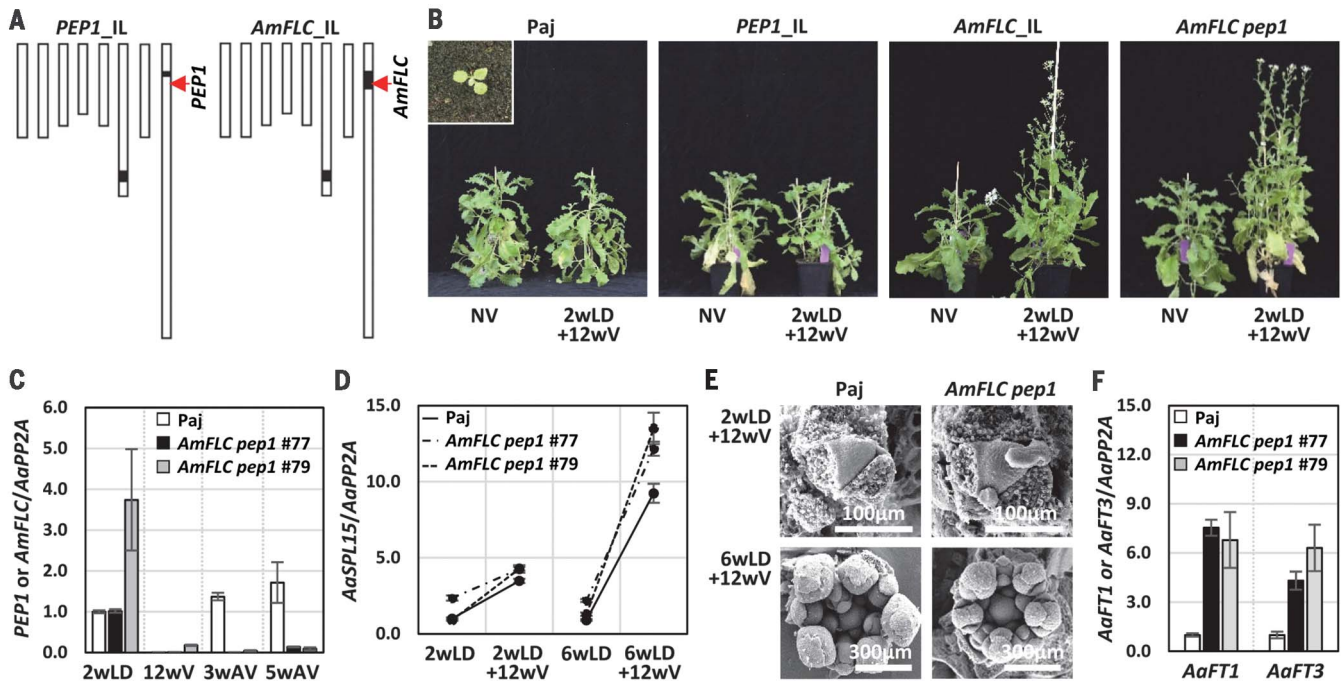
Fig. 4. Reconstruction of the annual response to age in perennial *A. alpina* by introducing *AmFLC*. (**A**) Schematic genome structure of *PEP1*_IL and *AmFLC*_IL. Black boxes indicate the introgressed *A. montbretiana* genomic segments. (**B**) Flowering response of Paj, *PEP1*_IL, *AmFLC*_IL, and *AmFLC pep1* exposed to cold as a 2-week-old seedling (inset). (**C** and **D**) *FLC* orthologs (C) and *AaSPL15* (D) mRNA levels at shoot apices of Paj and *AmFLC pep1*. AV, after vernalization. (**E**) Scanning electron microscopy of shoot apices of Paj and *AmFLC pep1* immediately after vernalization treatment. Neither Paj nor *AmFLC pep1* produced flowers during cold if exposed to vernalization as a young seedling (2wLD+12wV), but both did so if exposed to vernalization as an older plant (6wLD+12wV). (**F**) *AaFT1* and *AaFT3* mRNA levels in leaves of Paj and *AmFLC pep1* at 5wAV. The expression levels in [(C) and (D)] Paj at 2wLD and (F) Paj are set to 1.0 for normalization. Error bars indicate standard deviation of two biological replicates.

Despite the SPL15 pathway and its regulation being conserved, annual *A. thaliana* is not dependent on SPL15 because the stable repression of *FLC* allows young plants to flower through FT after vernalization. Redundancy between *SPL15* and *FT* may explain variation in severity of the *spl15* late-flowering phenotype (*16, 17*). Also, several Scandinavian accessions of *A. thaliana* require vernalization to flower and additionally carry hypomorphic *FT* alleles (*27*), suggesting that when exposed to short growing seasons, even annuals may benefit from reduced photoperiodic pathway activity and consequent increased dependency on SPL15. Thus, the ability to shift the balance between these pathways confers versatility in flowering physiology both within a species and during species divergence. These buffered signaling pathways enable flexible responses to shifting environments and diversification in life-history evolution (*28, 29*).

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Diversity in flowering regulation

Annual plants flower for one season and then die, whereas perennials can flower repeatedly year after year. Hyun *et al.* explain how different signaling pathways control such variation in flowering. The perennial pathway requires a floral integrator limited to older shoots. The annual pathway, on the other hand, allows a photoperiodic response to incite flowering on young shoots. Solutions to challenging environments may emerge through evolution as the balance between these regulatory systems shifts.

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