LETTERS

PEP1 regulates perennial flowering in *Arabis alpina*

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Annual plants complete their life cycle in one year and initiate flowering only once, whereas perennials live for many years and flower repeatedly. How perennials undergo repeated cycles of vegetative growth and flowering that are synchronized to the changing seasons has not been extensively studied[1]. Flowering is best understood in annual *Arabidopsis thaliana*[2,3], but many closely related species, such as *Arabis alpina*[4,5], are perennials. We identified the *A. alpina* mutant *perpetual flowering 1* (*pep1*), and showed that *PEP1* contributes to three perennial traits. It limits the duration of flowering, facilitating a return to vegetative development, prevents some branches from undergoing the floral transition allowing polycarpic growth habit, and confers a flowering response to winter temperatures that restricts flowering to spring. Here we show that *PEP1* is the orthologue of the *A. thaliana* gene *FLOWERING LOCUS C* (*FLC*). The FLC transcription factor inhibits flowering until *A. thaliana* is exposed to winter temperatures[6,7], which trigger chromatin modifications that stably repress *FLC* transcription[8–11]. In contrast, *PEP1* is only transiently repressed by low temperatures, causing repeated seasonal cycles of repression and activation of *PEP1* transcription that allow it to carry out functions characteristic of the cyclical life history of perennials. The patterns of chromatin modifications at *FLC* and *PEP1* differ correlating with their distinct expression patterns. Thus we describe a critical mechanism by which flowering regulation differs between related perennial and annual species, and propose that differences in chromatin regulation contribute to this variation.

Perennial plants repeatedly cycle between vegetative and reproductive development. In temperate climates these cycles are synchronized to the changing seasons, for example, by restricting flowering to spring and summer[12] or by arresting growth in the autumn[13]. Annual and perennial plants also show differences in the behaviour of shoot meristems—groups of undifferentiated cells from which all aerial tissues are derived. In annual plants all shoot meristems initiate reproductive development at similar times, a behaviour called monocarpy. In contrast, perennials are polycarpic and maintain vegetative growth after flowering, which allows them to flower and set seed many times during their lifetime. Vegetative growth is maintained either by conserving some meristems in the vegetative state after flower initiation[14] or by reverting back to vegetative development after flowering[15].

Control of the floral transition is best understood in the annual monocarpic model species *Arabidopsis thaliana*[2,3], a member of the *Brassicaceae*. Many other *Brassicaceae* species are polycarpic perennials, and therefore the molecular mechanisms underlying the difference between monocarpic and polycarpic plants can be approached by comparing *A. thaliana* with its close relatives. Similar comparative approaches were recently used to study the development of compound leaves[16] and to analyse the basis of heavy metal tolerance[17]. The arctic-alpine perennial *Arabis alpina* is a member of the *Brassicaceae*[4,5] that has favourable characteristics for use as a model perennial species, such as

being diploid and self-fertile, with a relatively small genome, and being susceptible to transformation by *Agrobacterium tumefaciens*. Here we describe a molecular-genetic analysis of the perennial traits seasonal flowering and polycarpy in *A. alpina*. We demonstrate that the *A. alpina* gene *PERPETUAL FLOWERING 1* (*PEP1*) encodes a MADS-box transcription factor that mechanistically links polycarpy and seasonal flowering. *PEP1* is the orthologue of the *A. thaliana* gene *FLOWERING LOCUS C* (*FLC*) and it is differently regulated in *A. alpina*, allowing the repeated response to seasonal cycles observed in perennial plants. This distinct pattern of regulation is related to species-specific differences in histone modifications at *PEP1*. These data provide insight into the mechanisms underlying evolution of life history in plants.

To facilitate a genetic study of flowering in *A. alpina* we first characterized the perennial growth habit and responses to environmental signals. The accession Pajares did not flower when grown continuously under long days (16 h light) or exposed to short days (8 h light) (Fig. 1a, Supplementary Fig. 1a and Methods). However, these plants flowered when exposed to low temperatures for several weeks (Fig. 1b), a treatment called vernalization. Although the flowering response to vernalization was independent of day length (Supplementary Fig. 1a), it was influenced by the duration of exposure to cold. Twelve weeks vernalization was sufficient to saturate the flowering response, causing all plants to produce fully developed inflorescences, whereas vernalization for shorter periods resulted in inflorescences that seemed to revert to vegetative growth (Fig. 1b, e and Supplementary Fig. 1b). Therefore, *Arabis alpina* Pajares plants only flower if exposed to prolonged vernalization treatments, and this response is independent of day length.

To understand the polycarpic behaviour of *A. alpina*, the fates of apical meristems of the main shoot and axillary shoots (branches) were followed before and after vernalization. The apices of the main shoot and axillary shoots formed before cold treatment produced flower buds during vernalization (Fig. 1c and Supplementary Figs 2 and 3a–d). Mature flowers emerged when plants were subsequently moved back to normal growth temperatures. Vegetative shoots developed from meristems that were not present or were still at an early developmental stage at the onset of vernalization, contributing to the polycarpic growth habit of the plant (Supplementary Fig. 3e, f). These shoots continued growing vegetatively until plants were again exposed to vernalization, inducing another round of flowering (Fig. 1d–h). Therefore, perenniality in this species involves maintenance of vegetative development after flowering, and the requirement for vernalization to induce flowering during each annual cycle.

To study the molecular mechanisms controlling flowering of *A. alpina*, mutants showing an impaired vernalization response were identified. From a total of 25,000 M_2 seedlings, two mutants were isolated that lacked obligate vernalization requirement. The most extreme early flowering mutant was called *perpetual flowering 1* (*pep1*). This mutant flowered with approximately 25 leaves under long
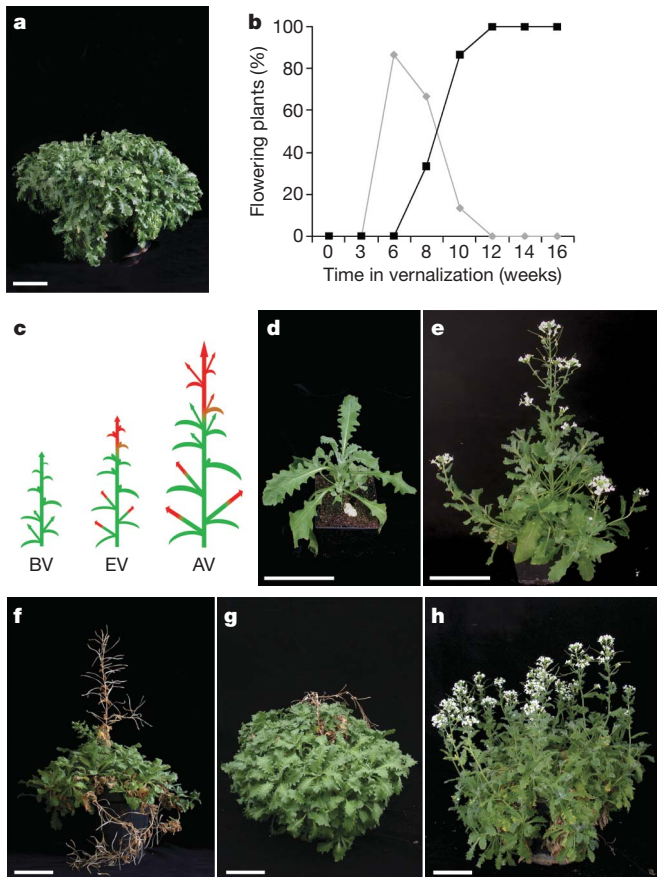
Figure 1 | *Arabis alpina* is a polycarpic perennial that requires vernalization each year to flower. a, An *A. alpina* (accession Pajares) plant grown for 3 and a half years without vernalization. The plant has not flowered.
b, Quantification of the duration of vernalization required for flowering of the Pajares accession. Plants were grown for 8 weeks in long days, vernalized for different times and subsequently grown in long days. Shown in grey are plants that responded partially to vernalization by producing a few flowers but also producing vegetative characteristics on the inflorescence (see also Supplementary Fig. 1b). Shown in black are plants that completely responded to vernalization and produced fully developed inflorescences. Twelve plants were scored at each time point. **c,** Diagram showing differential response of shoots to vernalization in *A. alpina*. The main shoot and axillary shoots are vegetative (green) before vernalization and become reproductive (red) by the end of the vernalization treatment. Axillary shoots that arise during or after vernalization remain vegetative (green). BV, before vernalization, plants grown for 8 weeks in long days; EV, end of a 12-week vernalization treatment under short days at 4 °C; AV, after vernalization, plants grown under long days. **d–h,** *Arabis alpina* does not die after flowering. The reproductive part of each shoot sets seed and senesces whereas vegetative shoots maintain vegetative growth indefinitely until exposed to another round of vernalization. Pictures from the same *A. alpina* plant were taken at successive stages of its life cycle: after 8 weeks in long days (**d**), vernalized for 12 weeks and then transferred to long days for 3 weeks (**e**), after 14 weeks in long days (**f**), after 25 weeks in long days (**g**), and after a new round of 12 weeks vernalization and then again for 4 weeks in long days (**h**). Scale bars, 10 cm.

days without exposure to vernalization, whereas wild-type plants never flowered under these conditions (Fig. 2a, b). In addition, the *pep1* mutant flowered continuously for at least 12 months (Supplementary Fig. 4a). To compare the duration of the flowering season of the *pep1* mutant and wild-type plants, both genotypes were vernalized and the duration of flowering was measured. Reproductive shoots in wild-type plants completed flowering 14 weeks after return to warm temperatures under long days, whereas under the same conditions *pep1* plants flowered for a further 5 months until the experiment was terminated (Fig. 2c, d). Flowering of wild-type plants was restricted to apical meristems of shoots that were present before vernalization, whereas the *pep1* mutant continued flowering from
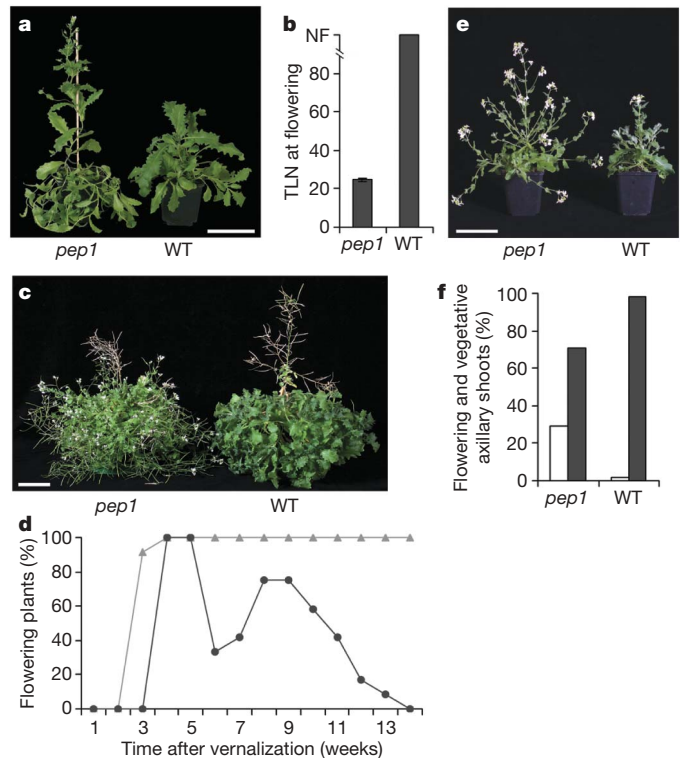

Figure 2 | *PEP1* restricts the flowering phase and enhances polycarpy of *A. alpina*. a, The *pep1* mutant flowers without vernalization. **b,** Flowering time of *pep1* plants compared to wild type (WT) without vernalization. NF, no flowering; TLN, total leaf number. **c,** *pep1* mutants show longer periods of flowering than wild-type plants. Plants were grown for 14 weeks under long days after 12 weeks vernalization. **d,** Duration of the flowering phase of *pep1* (grey) and wild-type plants (dark grey) after vernalization. **e,** More axillary branches undergo flowering in *pep1* plants than in wild-type as shown by plants grown for 3 weeks under long days after 12 weeks vernalization. **f,** Percentage of vegetative and flowering axillary shoots produced by *pep1* and wild-type plants. Plants were scored when the first open flower was present. Flowering shoots (white) and vegetative shoots (dark grey) are shown. In **c–f**, plants were grown under long days for 5 weeks before being vernalized for 12 weeks. $n = 12$ (**b, d, f**); scale bars, 10 cm.

secondary axillary shoots (Supplementary Fig. 4b). Moreover, the *pep1* mutant showed altered polycarpic behaviour. More shoots were induced to flower in the *pep1* mutant because axillary shoots that emerged during or after vernalization flowered in the *pep1* mutant but not in the wild-type plants (Fig. 2e, f and Supplementary Fig. 4c, d). Our data demonstrate that *PEP1* acts at different stages in the perennial life cycle. PEP1 prevents flowering before vernalization, whereas after vernalization it acts to restrict the duration of flowering and contributes to polycarpy by blocking flowering of axillary shoots. These three functions all involve the repression of the floral transition and are likely to be mechanistically related.

The reduced requirement for vernalization in the *pep1* mutant suggested that *PEP1* may be an orthologue of an *A. thaliana* gene conferring a vernalization requirement. To test this idea we isolated the *A. alpina* orthologue of *FLOWERING LOCUS C* (*FLC*), which encodes a MADS-box transcription factor that has an important role in establishing vernalization requirement in *A. thaliana*[6,7] and probably other annual *Brassicaceae* species[18,19]. *AaFLC* is present in a genomic region showing microsynteny with the *A. thaliana* chromosomal region containing *FLC* (Fig. 3a and Supplementary Table 1), and encodes a protein more closely related to FLC than to any other *A. thaliana* protein (Supplementary Fig. 5a). The function of AaFLC was tested by expressing the *AaFLC* complementary DNA from the *CaMV35S* promoter in the accession Columbia-0, which expresses the endogenous *FLC* gene at low levels[6,7]. The transgenic plants were late
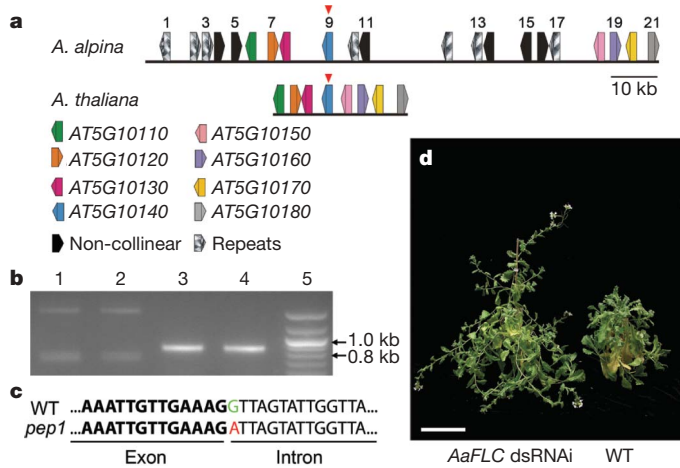
Figure 3 | PEP1 is the Arabis alpina orthologue of A. thaliana FLC.
a, Conserved synteny between the *Arabis alpina* (top panel) and *A. thaliana* (bottom panel) chromosomal regions containing *AaFLC* and *Arabidopsis FLC*, respectively. Vertical arrowheads show *AaFLC* and *Arabidopsis FLC* in each panel. Horizontal arrowheads illustrate genes and their orientation. Coloured arrowheads show syntenic genes, whereas black and striped arrowheads show the non-collinear genes and repeats, respectively. Genes (1–21) on the BAC containing the *AaFLC* gene are annotated in Supplementary Table 1. GenBank accession number FJ543377.
b, Accumulation of the full-length *AaFLC* transcript is impaired in *pep1* mutant plants. Amplified products from cDNA derived from *pep1* apices (lane 1), *pep1* leaves (lane 2), wild-type apices (lane 3) and wild-type leaves (lane 4). Lane 5 contains a 100-base-pair (bp) standard (NEB). Primers were designed to amplify by PCR the full-length coding sequence of *AaFLC* (819 bp). *AaFLC* cDNA in the *pep1* mutant and wild-type plant were sequenced and the detected splice variants are shown in Supplementary Fig. 6. In the *pep1* mutant, splice variants that were larger and smaller than the fully spliced *AaFLC* ORF were detected, accounting for the differently sized cDNAs detected in lanes 1 and 2. **c,** Mutation in the *AaFLC* gene in the *pep1* mutant. The G-to-A mutation at the splice donor site of exon 3 is indicated in green (wild type, WT) and red (*pep1*). **d,** Transgenic *35S:AaFLC* double-stranded (ds)RNAi *A. alpina* Pajares plants flower without vernalization. See also Supplementary Figs 5 and 8. Scale bar, 10 cm.

flowering (Supplementary Fig. 5b, c), demonstrating that AaFLC can act as a floral repressor in *A. thaliana* as previously shown for FLC[6,7].

To test the relationship between *AaFLC* and *PEP1*, the expression of *AaFLC* messenger RNA was compared in *pep1* and wild-type plants. Full-length *AaFLC* mRNA was below the level of detection in the *pep1* mutant (Fig. 3b and Supplementary Fig. 6), and by sequencing the *AaFLC* genomic region a mutation (G-to-A) was identified at the 5′ splice junction of the third intron (Fig. 3c). In the *pep1* mutant *AaFLC* mRNAs of different sizes were detected (Fig. 3b), and therefore *AaFLC* cDNAs made from *pep1* RNA were sequenced. None of the 14 cDNAs tested contained the full *AaFLC* open reading frame (ORF; Supplementary Fig. 6). In contrast, the cDNA containing the full ORF was by far the most abundant form detected in wild-type *A. alpina* plants (Fig. 3b and Supplementary Fig. 6). These data support the idea that the mutation present in *AaFLC* in the *pep1* mutant impairs production of the properly spliced mRNA.

We further tested whether reduced *AaFLC* activity was the cause of the *pep1* mutant phenotype. First, genetic linkage between *pep1* and the mutation in *AaFLC* was tested in an F[2] population constructed by backcrossing the *pep1* mutant to wild type. Under continuous long days, the ratio of flowering to non-flowering F[2] plants was approximately 1:3 (Supplementary Fig. 7). Genotypic data demonstrated that all of the flowering plants were homozygous for the mutation in *AaFLC*, and that all non-flowering plants were either heterozygous or homozygous for the wild-type allele (Supplementary Fig. 7). This experiment demonstrated that *pep1* is genetically linked to *AaFLC*. Second, transgenic plants, in which the level of *AaFLC* mRNA was reduced by RNA interference (RNAi), were generated. These plants

showed the characteristic phenotypes of *pep1* mutant plants. They flowered without vernalization, showed a longer duration of flowering and produced more flowering branches compared to the wild type (Fig. 3d and Supplementary Figs 5d, e and 8). These experiments further indicated that the *pep1* mutant phenotype is caused by impaired *AaFLC* activity and therefore *AaFLC* will be referred to as *PEP1*.

In *A. thaliana*, *FLC* transcription is repressed during vernalization, and after return to normal growth temperatures *FLC* repression is maintained[6–8,10]. The behaviour of *pep1* mutants suggested that *PEP1* functions before and after vernalization. To determine the *PEP1* expression pattern in *A. alpina*, apices and leaves of wild-type plants were analysed before, during and after vernalization using quantitative PCR with reverse transcription (qRT–PCR). During vernalization, *PEP1* transcript levels decreased markedly in shoot apices and leaves, and reached much lower levels at the end of the low temperature treatment, as observed in *A. thaliana*. However, after return to normal growth temperatures, *PEP1* mRNA levels increased in all tissues tested (Fig. 4a, b). To determine the spatial expression patterns of *PEP1*, RNA *in situ* hybridization experiments were performed. Before vernalization, *PEP1* mRNA was strongly detected in the shoot apical meristem, the adjacent axillary meristems and young leaves (Fig. 4c). At the end of vernalization, *PEP1* mRNA could not be detected in the flower buds at the shoot apical meristem, and was only weakly expressed in the primordia of newly formed vegetative axillary shoots (Fig. 4d, f). In agreement with the qRT–PCR results, *PEP1* transcript levels were restored after vernalization in both flowering and vegetative meristems (Fig. 4e, g). Therefore, in contrast to *FLC* in *A. thaliana*, after vernalization *PEP1* mRNA is restored to similar levels to those present before vernalization.

In *A. thaliana* stable repression of *FLC* by vernalization correlates with modification of histone H3 (refs 8, 9), and particularly with the addition of three methyl groups to the lysine residue at position 27 (H3K27me3)[11,20–22]. During vernalization the level of this histone modification increases close to the transcription start site of *FLC*, and in growing tissues it persists after return to normal growth temperatures, spreading across the gene and correlating with stable transcriptional repression[11]. In *A. alpina* the level of H3K27me3 also increased at *PEP1* during vernalization, but its levels decreased again after vernalization (Fig. 4h). Therefore, in contrast to *FLC* in *A. thaliana*, in *A. alpina* the H3K27me3 mark accumulated at *PEP1* during vernalization but did not persist after vernalization.

Thus, temporal changes in *PEP1* expression contribute to the perennial life history of *A. alpina*. Repression of *PEP1* transcription during vernalization and reactivation after return to warm temperatures correlate with unstable modifications of H3K27me3 at the *PEP1* locus. This observation is in contrast to *FLC* expression patterns in growing tissues of *A. thaliana*, in which *FLC* expression is stably repressed by vernalization and the H3K27me3 chromatin mark persists at the locus after vernalization[10,11,20–22]. Annual plants such as *A. thaliana* only flower once in their lifetime, and therefore to maximise seed production all growing shoot meristems undergo the transition to flowering when the environmental conditions are optimal. Thus, stable repression of the floral repressor *FLC* by vernalization is consistent with the monocarpic life strategy of *A. thaliana*. Mutations in a component of the polycomb-group protein complex required to produce the H3K27me3 mark prevent stable repression of *FLC* by vernalization in *Arabidopsis*, indicating that this mark is required for epigenetic silencing of *FLC*[10,20]. Moreover, *Arabidopsis* accessions that show variation in the duration of vernalization required to saturate the flowering response show differences in the accumulation of the H3K27me3 mark at the *FLC* locus during vernalization[23]. However, a reduction in the level of the mark after vernalization, as we observed in apices of fully vernalized *A. alpina* plants, has not been observed in growing tissues of *A. thaliana*, indicating that this aspect of *FLC/PEP1* regulation differs between species. Nevertheless, in *A. thaliana* the H3K27me3 mark at *FLC* does not persist in cells of fully expanded leaves that are
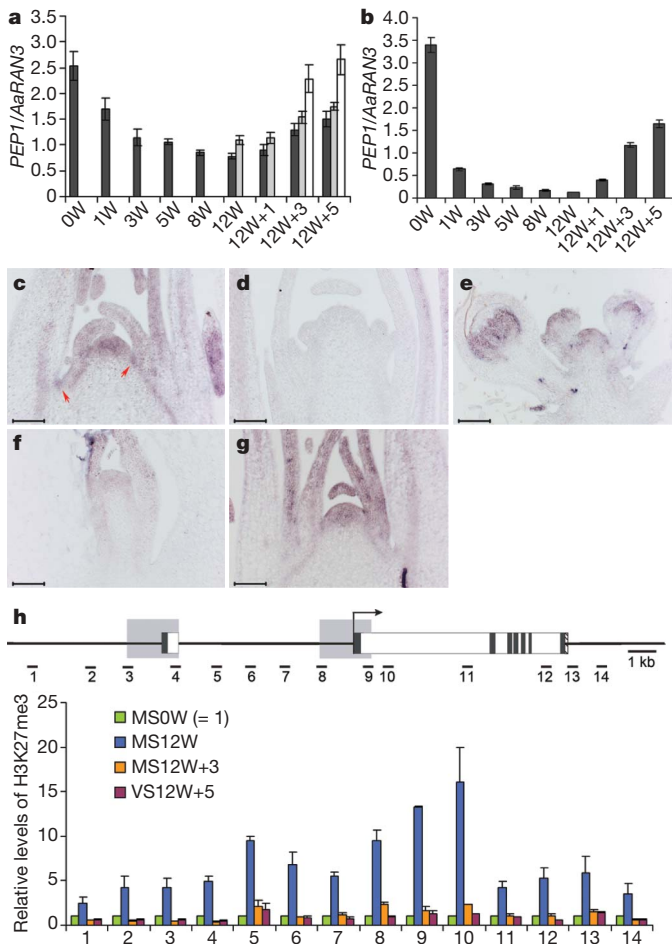
Figure 4 | Repression of *PEP1* expression by vernalization is unstable and correlated with changes in histone methylation. a, b, *PEP1* mRNA levels fall in apices (**a**) and leaves (**b**) during vernalization treatment (1 to 12 weeks (W)), and are restored after vernalization (12W + 1 to 5 weeks). RNA levels were measured by qRT–PCR. In **a**, dark grey bars represent main-shoot apices. Grey bars represent apices from axillary shoots produced before vernalization. *PEP1* expression in these axillary shoots was only measured at 12W, 12W + 1, 12W + 3 and 12W + 5 time points. White bars represent apices from axillary shoots produced after vernalization. In **a** and **b** the means of three technical replicates are shown; error bars indicate s.d. **c–e,** *In situ* hybridization of *PEP1* mRNA in the main-shoot apex after 8 weeks growth in long days before vernalization (**c**), followed by 12 weeks vernalization (**d**), and after a further 4 weeks in long days (**e**). Arrows highlight axillary meristems that will develop into vegetative axillary shoots during or after vernalization. **f, g,** *In situ* hybridization of *PEP1* mRNA in vegetative axillary shoots at the end of 12-weeks vernalization (**f**) and after a further 4 weeks in long days (**g**). Scale bars, 100 nm. **h,** The *AaFLC* locus showing the regions amplified by PCR after ChIP (top). Boxes represent exons (dark grey), introns (white), 3′ untranslated region (UTR; striped) and a 2-kb duplicated region present at the locus (grey). H3K27me3 levels determined by ChIP of 14 regions on the *AaFLC* locus (bottom). All data points were divided by the H3K27me3 level before vernalization (8 weeks in long days) to provide relative values. The level before vernalization is therefore arbitrarily assigned a value of 1.0. Main-shoot apices of *A. alpina* plants were grown for 8 weeks in long days (MS0W), after 12 weeks vernalization (MS12W), and followed by 3 weeks in long days (MS12W + 3). Apices from vegetative axillary shoots were grown for 5 weeks in long days after vernalization (VS12W + 5). The data presented are the means of two technical replicates, error bars denote s.e.m. Similar patterns of modification were obtained in a second set of biological replicates.

no longer undergoing mitosis, indicating that in these tissues the mechanism required for persistence of this mark at *FLC* is not active[11].

The instability of *PEP1* repression in *A. alpina* allows PEP1 to block flowering of all meristems that did not undergo flowering during the

preceding vernalization treatment, and therefore to take on roles in the perennial life cycle that are not required in the annual life cycle of *A. thaliana*. However, although differential regulation of *PEP1* enhances perennial characters and confers seasonality, *pep1* mutants are still long-lived and behave as perennial plants. This indicates that the differential regulation of *PEP1* and *FLC* is not the only difference between *A. thaliana* and *A. alpina* that is responsible for perennialism. Furthermore, although our data demonstrate an important role for PEP1 as an inhibitor of flowering in the perennial cycle, the presence of extra components, including floral promoters that increase in expression before each flowering cycle, cannot be excluded. Nevertheless, our analysis of *PEP1* provides insight into the different mechanisms underlying seasonal flowering in perennial and annual plants consistent with their life history, and how gene functions involved in the control of seasonal flowering can be incorporated into perennial-specific traits such as polycarpy. The distinction between annual and perennial life histories has arisen independently many times in the flowering plants[24,25], and in the *Brassicaceae* both life histories occur in several genera[26], suggesting that they diversified independently. Furthermore, not all perennials flower in response to vernalization and FLC is not a universal regulator of vernalization requirement[27], supporting the idea that different mechanisms must regulate the perennial growth habit in other groups of plants. Species-specific traits can evolve through alterations in the expression patterns of regulatory genes[28]. The differences in histone modifications at *FLC* and *PEP1* in *A. thaliana* and *A. alpina*, respectively, suggest that differences in chromatin regulation may be one of the mechanisms by which these alterations in gene expression patterns occur, thereby allowing diversification of rapidly evolving traits such as life history characters.

METHODS SUMMARY

Plant material. *Arabis alpina* L. accession Pajares was collected in the Cordillera Cantábrica mountain system in Spain (42°59′32′′ N, 5°45′32′′ W; 1,400 m altitude) and self-fertilized for six generations by single-seed descent. *Arabidopsis thaliana* experiments were performed using the Columbia-0 accession.

Ethyl methanesulphonate mutagenesis. *Arabis alpina* seeds were mutagenized with 0.35% ethyl methanesulphonate (Sigma) for 8–9 h. Twenty-five-thousand M_2 seedlings (2,500 M_1 families) were screened in long days at a 20 °C controlled-environment glasshouse.

BAC analysis and sequencing. Two positive BAC clones were identified by screening an *A. alpina* BAC library made from accession Pajares (R. Castillo, unpublished data) using *A. thaliana FLC* as a probe without the sequence encoding the MADS box. The DNA sequence of a positively hybridizing BAC was determined using the Sanger method, assembled and annotated manually.

Plant transformation. Plasmid constructs were generated by cloning PCR-amplified fragments into GATEWAY compatible binary vectors and introduced into *Agrobacterium* strain GV3101 (pMP90RK). Transgenic *A. thaliana* and *A. alpina* were generated by the floral-dip method[29].

Gene expression analysis. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) from expanded leaves, main-shoot apices and axillary-shoot apices grown before or after vernalization. cDNA synthesis and qRT–PCR were performed as previously described[30].

***In situ* hybridization.** *In situ* hybridization was performed on apices of the main shoot and of axillary shoots, as described previously[30].

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was carried out using apices of the main shoots (MS) of *A. alpina* plants grown for 8 weeks in long days (MS0W), then vernalized for 12 weeks (MS12W), and finally grown for 3 weeks in long days (MS12W + 3). Apices of axillary vegetative shoots grown during and after vernalization were collected at 5 weeks in long days (VS12W + 5). Chromatin samples were immunoprecipitated with anti-H3K27me3 (Upstate). Results were presented as a ratio of vernalized/non-vernalized samples and are the mean of two technical replicates.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

1. Battey, N. H. & Tooke, F. Molecular control and variation in the floral transition. *Curr. Opin. Plant Biol.* **5,** 62–68 (2002).

2. Turck, F., Fornara, F. & Coupland, G. Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annu. Rev. Plant Biol.* **59**, 573–594 (2008).
3. Baurle, I. & Dean, C. The timing of developmental transitions in plants. *Cell* **125**, 655–664 (2006).
4. Ansell, S. W., Grundmann, M., Russell, S. J., Schneider, H. & Vogel, J. C. Genetic discontinuity, breeding-system change and population history of *Arabis alpina* in the Italian Peninsula and adjacent Alps. *Mol. Ecol.* **17**, 2245–2257 (2008).
5. Koch, M. A. *et al.* Three times out of Asia Minor: the phylogeography of *Arabis alpina* L. (*Brassicaceae*). *Mol. Ecol.* **15**, 825–839 (2006).
6. Michaels, S. D. & Amasino, R. M. *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949–956 (1999).
7. Sheldon, C. C. *et al.* The *FLF* MADS box gene: A repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11**, 445–458 (1999).
8. Bastow, R. *et al.* Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* **427**, 164–167 (2004).
9. Sung, S. & Amasino, R. M. Vernalization in *Arabidopsis* thaliana is mediated by the PHD finger protein VIN3. *Nature* **427**, 159–164 (2004).
10. Gendall, A. R., Levy, Y. Y., Wilson, A. & Dean, C. The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. *Cell* **107**, 525–535 (2001).
11. Finnegan, E. J. & Dennis, E. S. Vernalization-induced trimethylation of histone H3 lysine 27 at *FLC* is not maintained in mitotically quiescent cells. *Curr. Biol.* **17**, 1978–1983 (2007).
12. Battey, N. H. Aspects of seasonality. *J. Exp. Bot.* **51**, 1769–1780 (2000).
13. Bohlenius, H. *et al.* CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science* **312**, 1040–1043 (2006).
14. Foster, T., Johnston, R. & Seleznyova, A. A morphological and quantitative characterization of early floral development in apple (*Malus × domestica* Borkh.). *Ann. Bot. (Lond.)* **92**, 199–206 (2003).
15. Diomaiuto, J. Periodic flowering or continual flowering as a function of temperature in a perennial species: the Ravenelle wallflower (*Cheiranthus cheiri* L.). *Phytomorphology* **38**, 163–171 (1988).
16. Hay, A. & Tsiantis, M. The genetic basis for differences in leaf form between *Arabidopsis thaliana* and its wild relative *Cardamine hirsuta*. *Nature Genet.* **38**, 942–947 (2006).
17. Hanikenne, M. *et al.* Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of *HMA4*. *Nature* **453**, 391–395 (2008).
18. Schranz, M. E. *et al.* Characterization and effects of the replicated flowering time gene *FLC* in *Brassica rapa*. *Genetics* **162**, 1457–1468 (2002).
19. D'Aloia, M., Tocquin, P. & Perilleux, C. Vernalization-induced repression of *FLOWERING LOCUS C* stimulates flowering in *Sinapis alba* and enhances plant responsiveness to photoperiod. *New Phytol.* **178**, 755–765 (2008).
20. Schubert, D. *et al.* Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. *EMBO J.* **25**, 4638–4649 (2006).
21. Sung, S., Schmitz, R. J. & Amasino, R. M. A PHD finger protein involved in both the vernalization and photoperiod pathways in *Arabidopsis*. *Genes Dev.* **20**, 3244–3248 (2006).
22. Greb, T. *et al.* The PHD finger protein VRN5 functions in the epigenetic silencing of *Arabidopsis FLC*. *Curr. Biol.* **17**, 73–78 (2007).
23. Shindo, C., Lister, C., Crevillen, P., Nordborg, M. & Dean, C. Variation in the epigenetic silencing of *FLC* contributes to natural variation in *Arabidopsis* vernalization response. *Genes Dev.* **20**, 3079–3083 (2006).
24. Thomas, H., Thomas, H. M. & Ougham, H. Annuality, perenniality and cell death. *J. Exp. Bot.* **51**, 1781–1788 (2000).
25. Bena, G., Lejeune, B., Prosperi, J.-M. & Olivieri, I. Molecular phylogenetic approach for studying life-history evolution: the ambiguous example of the genus Medicago L. *Proc. R. Soc. Lond. B* **265**, 1141–1151 (1998).
26. Beilstein, M. A., Al-Shehbaz, I. A. & Kellogg, E. A. Brassicaceae phylogeny and trichome evolution. *Am. J. Bot.* **93**, 607–619 (2006).
27. Yan, L. *et al.* The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. *Science* **303**, 1640–1644 (2004).
28. Prud'homme, B., Gompel, N. & Carroll, S. B. Emerging principles of regulatory evolution. *Proc. Natl Acad. Sci. USA* **104**, 8605–8612 (2007).
29. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
30. Searle, I. *et al.* The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. *Genes Dev.* **20**, 898–912 (2006).

METHODS

Growth conditions and phenotypic characterization. Plants were grown in long-day growth cabinets (16 h light at 22 °C, and 8 h dark at 18 °C) or long-day climate-controlled glasshouses (16 h light at 20 °C), short-day cabinets (8 h light at 22 °C, and 16 h dark at 18 °C), and vernalized at 4 °C in short days (8 h light/16 h dark). To compare the duration of flowering between the *pep1, 35S:AaFLC* dsRNAi line and wild-type plants, both genotypes were first grown for 5 weeks in long days (growth cabinet), vernalized for 12 weeks and then transferred back to long days. Flowering time was measured by scoring the leaves on the main shoot of at least eight individuals. Node position on the main shoot was determined by the presence of a true leaf and numbered from the bottom to the top of each plant.

Characterization of *pep1* mutant. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). Full-length *AaFLC* cDNA was amplified from expanded leaves and apices using primers on 5′ UTR (5′-AAACACAAAAAA AGAGTGAGAATAG-3′) and 3′ UTR (5′-AGTCTCTCAGCCATAGAGAG-3′) (30 cycles). PCR products were cloned into a pCR2.1-TOPO vector and 14 clones were sequenced. Genomic DNA from *pep1* mutant was extracted using the DNeasy DNA kit (Qiagen), and the *AaFLC* gene was sequenced using primers spanning the whole locus (primer sequences available on request). F₂ progenies were genotyped for the mutation in *AaFLC* using primers 5′-TTTGCCCCTTAG TTTTGTGG-3′ and 5′-TACCCGGGAAGACTACATGC-3′ flanking the mutation, and subsequently sequencing using primer 5′-TTTGCCCCTTAGTT TTGTGG-3′.

BAC analysis and sequencing. A BAC library was constructed using the pIndigoBAC-5 vector from partially HindIII-digested total genomic DNA of the accession Pajares (R. Castillo, unpublished data). The BAC library was screened using *Arabidopsis FLC* as a probe (without the MADS-box coding sequence) and two positive BACs were identified. Plasmid DNA was extracted from 100 ml overnight culture using the Qiagen Plasmid Midi kit. Positively hybridizing BACs were analysed by fingerprinting and all corresponded to one locus. The DNA sequence of a positively hybridizing BAC was determined using the Sanger method[31] and assembled using PHRED[32,33], PHRAP and Consed[34]. Gene prediction from GenMark.hmm[35], FGeneSH[36] and GenomeThreader[37] using several plant expressed-sequence-tag databases were integrated manually using the APOLLO genome editor[38]. Manual functional annotation of all predicted genes was on the basis of their homology to *A. thaliana* and other plant species, as well as on the presence of conserved domains identified using the InterPro[39] database.

Plasmid constructs. To generate the *35S::AaFLC* construct, the full-length (ATG to TAG) coding sequence of *AaFLC* was PCR-amplified with primer pair X-*AaFLC5*-F, 5′-(attB1)GAGACGAAGCCATGGGTAG-3′, and X-*AaFLC3*-R, 5′-(attB2)GGCTTAATTGAGTAGTGGGAG-3′, and verified by sequencing after cloning into pDONR221 (Invitrogen). Through site-directed recombination it was subsequently cloned into a binary vector pLEELA, which is a derivative of pJawohl3 RNAi (GenBank accession AF404854) containing a GATEWAY cassette introduced into the HpaI site.

To make the AaFLC dsRNAi construct for transformation of *A. alpina*, a fragment including sequences from exon 4 to 3′ UTR was amplified from cDNA using primer pair *AaFLC*-RNAi-F1, 5′-(attB1)AAGCTCGTGGCA CCAAATGTC-3′, and *AaFLC*-RNAi-R1, 5′-(attB1)AGTCTCTCAGCCATAGA GAGT-3′. The sequence of the *AaFLC* RNAi fragment is between coordinates 364 and 763 in the *AaFLC/PEP1* cDNA sequence (GenBank Accession Number FJ755930). The fragment was recombined to generate a GATEWAY entry clone and subsequently recombined into the binary vector pJawohl8 (GenBank accession AF408413) to generate an intron-spliced hairpin construct.

Gene expression analysis. Total RNA was extracted from expanded leaves and shoot apices (approximately 1 mm in length), and an on-column DNase treatment (Qiagen) was performed to reduce any DNA contamination. RNA was analysed by qRT–PCR using a BioRad iQ5 apparatus and SYBR Green I detection. Three micrograms of total RNA was used for cDNA synthesis with oligo-dT15 as a primer. cDNA was diluted to a final volume of 180 μl, and 3.5 μl of diluted cDNA was used for PCR. An *A. alpina RAN3* gene was used as a control to normalize the amounts of cDNA. For testing *AaFLC/PEP1* expression with qRT–PCR, the primer pair PEP1-RT-F1, 5′-CTTGTCGTCTCCTCCTCTGG-3′, and PEP1-RT-R1, 5′-ACTACGGCGAGAGCAGTTTC-3′ were used. Primer pair AaRAN3-F2, 5′-CACAGGAAAAACCACATTCGT-3′, and AaRAN3-R2, 5′-CCATCCCTAAGACCACCAAAT-3′ were used to detect *AaRAN3* transcript abundance.

***In situ* hybridization.** Methods of digoxigenin labelling of mRNA probes, tissue preparation and *in situ* hybridization were performed as already described[30]. Template DNA used for probe synthesis to detect *AaFLC/PEP1* transcripts were PCR-amplified from cDNA using the primer pair T3-*PEP1*-F2, 5′-<u>ATTA ACCCTCACTAAAGGG</u>AAGCCAGATGGAGAAGAAGAC-3′, and T7-*PEP1*-R2, 5′-<u>TAATACGACTCACTATAGGG</u>AACAAGGGTACGAAGATCCA-3′. The underlined nucleotide sequence indicates either the T3 or the T7 RNA polymerase-binding sites.

Chromatin immunoprecipitation. ChIP were carried out as described[30]. The antibody anti-H3K27me3 (Upstate) was used for the immunoprecipitation and an anti-rat IgG (Sigma) was used as a negative control. Two independent immunoprecipitations were performed with the anti-H3K27me3 antibody as technical replicates, and average values were obtained. The ChIP DNA was subjected to 33–34 cycles of PCR (non-saturated conditions). Primers used for PCR were designed to avoid the duplicated region in the *AaFLC* locus represented in Fig. 4h (Supplementary Table 2). *RAN3* amplification was used to normalize DNA concentrations. The semi-quantification of the data was performed using the QIAxcel System (Qiagen) and the QX DNA size marker 50–800-bp (Qiagen). An appropriate dilution of the input amplified in similar conditions was used to compare the ChIP data. Results are presented as a ratio of vernalized/non-vernalized samples and are the means of two technical replicates. Similar patterns of modification were obtained in a second set of biological replicates.

Scanning electron microscopy. Plants of similar sizes were selected and marked on the main shoot just below node 15 (as in Supplementary Fig. 2). Apices from the main shoot was sampled to include leaves grown above node 15 at: 8 weeks in long days, after 12 weeks in vernalization and after 1 subsequent week in long days. Expanded leaves were removed from shoots apices and fixed in 2.5% glutaraldehyde in 0.002 M phosphate buffer (pH 7) and 0.01% Nonidet P-40. Samples were dehydrated in ethanol and critical-point dried in liquid CO₂. Leaf primordia were removed under a binocular microscope and samples were mounted on stubs, coated and subjected to high resolution Zeis Supra 55 VP FEG scanning electron microscopy with a Gatan Alto 2500 cryo system. The presence of flowers or flowering shoots and the identity of shoots in the axils of each leaf were recorded starting from the top of each sample until the lower leaf, which corresponded to the leaf in node 15. Each plant was scored at each developmental stage. At every node, the presence or absence of a shoot was recorded, shoots were excised and leaf primordia were removed under the binocular microscope and subjected to high resolution scanning electron microscopy.

Phylogenetic analysis. Protein–protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST) was performed using the deduced amino acid sequence from AaFLC. ClustalX was used to create multiple alignments[40], and a phylogenetic tree was generated by the neighbour-joining method with Splitstree[41]. Bootstrap analysis was performed to estimate nodal support on the basis of 1,000 replicates. The accession numbers were: FLC, NP_196576; MAF1, NP_177833; MAF2, Q9FPN7; MAF3, NP_201311; MAF4, NP_201312; MAF5, AAO65320; AlFLC, AAV51231; ThFLC, AAY34137; AsFLC, AAZ92553; ArFLC, AAZ92552; BnFLC1, AAK70215; BnFLC2, AAK70216; BnFLC3, AAK70217; BnFLC4, AAK70218; BnFLC5, AAK70219; BrFLC1, ABI29999; BrFLC2, ABO40820; BrFLC3, ABI30001; BoFLC1, CAJ77613; BoFLC3, AAP31677; BoFLC4, AAQ76273; SaFLC, ABP96967; RsFLC, AAP31676; and AGL42, NP_568952.

31. Sanger, F., Nicklen, S. & Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467 (1977).
32. Ewing, B., Hillier, L., Wendl, M. C. & Green, P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**, 175–185 (1998).
33. Ewing, B. & Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**, 186–194 (1998).
34. Gordon, D., Abajian, C. & Green, P. Consed: A graphical tool for sequence finishing. *Genome Res.* **8**, 195–202 (1998).
35. Lukashin, A. V. & Borodovsky, M. GeneMark.hmm: new solutions for gene finding. *Nucleic Acids Res.* **26**, 1107–1115 (1998).
36. Salamov, A. A. & Solovyev, V. V. Ab initio gene finding in *Drosophila* genomic DNA. *Genome Res.* **10**, 516–522 (2000).
37. Gremme, G., Brendel, V., Sparks, M. E. & Kurtz, S. Engineering a software tool for gene structure prediction in higher organisms. *Inf. Softw. Technol.* **47**, 965–978 (2005).
38. Lewis, S. E. *et al.* Apollo: a sequence annotation editor. *Genome Biol.* **3**, research0082 (2002).
39. Apweiler, R. *et al.* InterPro—an integrated documentation resource for protein families, domains and functional sites. *Bioinformatics* **16**, 1145–1150 (2000).
40. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882 (1997).
41. Huson, D. H. SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73 (1998).