Short title: *PEP1* regulates inflorescence fate

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Extended vernalization regulates inflorescence fate in *Arabis alpina* by stably silencing *PERPETUAL FLOWERING 1*

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One sentence summary: *PERPETUAL FLOWERING 1* has a dual role regulating meristem fate after cold; it prevents flowering of new axillary branches and antagonizes inflorescence development.

Footnotes:

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ABSTRACT

The alpine perennial *Arabis alpina* initiates flower buds during prolonged exposure to cold. In the accession Pajares we demonstrate that the length of vernalization influences flowering time and inflorescence fate, but does not affect the axillary branches that maintain vegetative growth. The expression of floral organ identity genes gradually increases in the main shoot apex during vernalization, correlating with an increase in floral commitment. In northern *Arabidopsis thaliana* accessions, the length of vernalization modulates the stable silencing of the floral repressor *FLOWERING LOCUS C* (*FLC*). We demonstrate that expression of *PERPETUAL FLOWERING 1* (*PEP1*), the orthologue of *FLC* in *A. alpina*, is similarly influenced by the duration of the exposure to cold. Extended vernalization results in stable silencing of *PEP1* in the inflorescence. In contrast, insufficient vernalization leads to *PEP1* reactivation after cold treatment, which correlates with delayed flowering and the appearance of floral reversion phenotypes such as bracts and vegetative inflorescence branches. Floral reversion is reduced in the *pep1-1* mutant, suggesting that *PEP1* regulates the fate of the inflorescence after vernalization. The effect of vernalization duration on stable silencing of *PEP1* is specific to meristems that initiate flowering during cold treatment. Extended vernalization fails to silence *PEP1* in young seedlings and axillary branches that arise from buds initiated during cold treatment, which remain vegetative. We conclude that the duration of vernalization in *A. alpina* differentially regulates *PEP1* in the inflorescence and axillary branches. *PEP1* has a dual role regulating meristem fate; it prevents meristems from flowering and antagonizes inflorescence development after vernalization.

Keywords: *Arabis alpina*, vernalization, perennial, inflorescence, *PERPETUAL FLOWERING 1*, *FLOWERING LOCUS C*, preformation, floral reversion

INTRODUCTION

Perennials live for many years, and most of them are capable of undergoing several reproductive events during their lifetime. In contrast to annual species, which complete their life cycle after seed set, perennials keep some meristems in a vegetative state to maintain growth from one year to the next. Therefore, the orchestration of developmental and seasonal cues that promote or repress flowering contributes to the perennial growth habit. Many perennials initiate flowering several months or years before anthesis. This growth pattern provides developmental flexibility, which is essential for the perennial life cycle and advantageous in arctic and alpine environments where spring and summer seasons are short (Diggle, 1997; Meloche and Diggle, 2001; Battey and Tooke, 2002). Preformation of flower buds can facilitate rapid anthesis after snowmelt and influences plant responses to environmental cues (Billings and Mooney, 1968; Aydelotte and Diggle, 1997).

We used the alpine perennial Brassicaceae species *Arabis alpina* as a model to study the effect of prolonged exposure to winter chilling temperatures on flowering and perennial traits. In nature *A. alpina* flowers soon after snowmelt (Torang et al., 2015), suggesting that, similar to other alpine species, the induction of flowering and anthesis might be uncoupled. *A. alpina* accessions such as Pajares have an obligate vernalization requirement for flowering, although other accessions do not require vernalization (Wang et al., 2009; Albani et al., 2012). Pajares plants flower in response to vernalization only if they are grown for at least five weeks in long days before cold treatment, suggesting that *A. alpina* has a juvenile phase, during which it is not competent to flower (Wang et al., 2011; Bergonzi et al., 2013). Under controlled environmental conditions, adult Pajares plants initiate flowering during vernalization, and this transition is marked by an increase in the expression of the floral meristem identity gene *AaLEAFY* (*AaLFY*), which becomes detectable after five weeks of cold treatment (Wang et al., 2009). However, seven additional weeks of vernalization are required for the formation of flower buds in the main shoot apex, suggesting that vernalization in *A. alpina* might also regulate other processes downstream of flowering induction (Wang et al., 2009). Vernalization fails to trigger flowering in axillary branches that arise from buds initiated during cold treatment (Wang et al., 2009; Park et al., 2017). This asynchronous development of different meristems within the same plant contributes to the perennial growth habit (Albani and Coupland, 2010; Bergonzi and Albani, 2011; Wang et al., 2011; Bergonzi et al., 2013; Park et al., 2017).

In *A. alpina*, flowering in response to vernalization is regulated by *PERPETUAL FLOWERING 1* (*PEP1*), the orthologue of *FLOWERING LOCUS C* (*FLC*) in the annual model species *Arabidopsis thaliana* (Michaels and Amasino, 1999; Sheldon et al., 2000; Wang et al., 2009). The *pep1* mutants and accessions with non-functional alleles of *PEP1* flower without vernalization (Wang et al., 2009; Albani

et al., 2012; Nördstrom et al., 2013). Furthermore, as shown for *FLC*, *A. alpina PEP1* expression is downregulated during vernalization. However, one key difference between the two species is the timing of flower initiation in relation to *FLC*/*PEP1* temporal expression patterns. In *A. thaliana*, flowering is initiated after vernalization; therefore, stable repression of *FLC* is essential for flowering to occur. In *A. alpina*, flowering is initiated during vernalization, when *PEP1* mRNA levels are low. After vernalization, high *PEP1* expression levels ensure the continuation of vegetative growth by repressing flowering in the axillary branches that arise from buds initiated during cold treatment (Wang et al., 2009). Thus, *PEP1* provides a second layer of regulation to define the fate of these axillary branches after returning to warm temperatures, in addition to the age-dependent flowering pathway. This role of *PEP1* in maintaining vegetative growth is also supported by the phenotype of *pep1-1*, in which all axillary branches flower (Wang et al., 2009). *PEP1* expression pattern resembles that of *FLC* in extreme northern *A. thaliana* accessions, in which insufficient vernalization fails to stably silence *FLC* (Shindo et al., 2006; Li et al., 2014; Duncan et al., 2015). Interestingly, both in *A. alpina* and in *A. thaliana*, *PEP1* and *FLC* expression are substantially reduced after six weeks of vernalization (Shindo et al., 2006; Wang et al., 2009). However, the *A. alpina* accession Pajares and northern *A. thaliana* accessions require longer cold exposure periods to accelerate flowering. In *A. thaliana*, this requirement is related to the initial accumulation of the H3K27me3 repressive mark on the *FLC* locus, which occurs in autumn (Coustham et al., 2012; Duncan et al., 2015). In *A. alpina*, H3K27me3 levels on *PEP1* correlate negatively with *PEP1* expression in both flowering and vegetative branches. Interestingly, plants still flower although *PEP1* expression is reactivated in flower buds after 12 weeks of vernalization (Wang et al., 2009; Castaings et al., 2014; Kiefer et al., 2017).

In *A. thaliana*, the floral integrators *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) are directly repressed by *FLC* in the leaves and shoot apex, respectively (Helliwell et al., 2006; Searle et al., 2006; Deng et al., 2011). *SOC1* is the earliest molecular marker upregulated in the shoot apical meristem at floral transition and, together with *FRUITFUL* (*FUL*), it plays a redundant role in the maintenance of the inflorescence meristem (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000; Melzer et al., 2008). *soc1* mutants, combined with mutations in *FUL*, fail to maintain the determinacy of the inflorescence, which reverts to vegetative development and produces aerial rosettes (Melzer et al., 2008). Interestingly, *soc1 ful* double mutants show perennial traits and an extended life cycle, suggesting that lack of inflorescence determinacy might contribute to perennial growth (Melzer et al., 2008). After floral transition, *FT* is expressed in the inflorescence in an *FLC*-dependent manner and is required to maintain floral commitment in *A. thaliana* (Liu et al., 2014; Müller-Xing et al., 2014). Under short photoperiods, *ft* mutants produce inflorescences with reverted phenotypes containing vegetative branches with rosette-like cauline leaves (Melzer et al., 2008; Liu

et al., 2014). These results support the hypothesis that flowering time and floral commitment might be regulated by the same genes.

In *A. thaliana*, inflorescences show two clear zones (I1 and I2) marked by the development of different organs. The I1 zone consists of flowering inflorescence branches, and the I2 zone consists of solitary flowers. The branches in the inflorescence are produced by the basipetal activation of buds on the axils of leaf primordia initiated before floral induction (Hempel and Feldman, 1994). At the same time, flowers in the I2 zone differentiate acropetally, marking floral commitment (Hempel and Feldman, 1994). *LFY* and the MADS-box transcription factor *APETALA 1* (*AP1*) play an instrumental role in this process. Mutations in these meristem identity genes result in the extension of the I1 zone over the I2 zone as flowers are transformed into inflorescence branches (Irish and Sussex, 1990; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Weigel and Nilsson, 1995).

In contrast to *A. thaliana*, where flower primordia acquire a floral fate very rapidly, in perennials continuous exposure to flower-inducing stimuli is required to prevent floral reversion where the flower itself loses floral identity and has vegetative or inflorescence features (Battey and Lyndon, 1990; Hempel et al., 1997; Battey and Tooke, 2002). Here we show that insufficient vernalization results in floral reversion phenotypes and late flowering in *A. alpina*, probably because floral commitment was not achieved during cold. Floral reversion phenotypes increase with *PEP1* reactivation after the return to warm temperatures, whereas extended vernalization silences *PEP1* in the shoot apex and enhances the reproductive potential of the inflorescence. The achievement of a floral fate during vernalization is important for meristem behavior after the plants return to warm temperatures and to maintain stable silencing of *PEP1*. In contrast, in young plants and axillary vegetative branches, which do not initiate flowering during the cold period, the duration of vernalization has no influence on *PEP1* expression after vernalization. Overall, our results show that *PEP1* expression is regulated by the duration of vernalization depending on whether meristems acquire floral or vegetative identity during the cold treatment.

RESULTS

The duration of vernalization determines inflorescence fate but has a marginal effect on axillary branches initiated during vernalization

A. alpina flower buds are initiated during vernalization. After vernalization, the main shoot apex gives rise to an inflorescence stem whereas axillary branches follow different fates depending on whether they initiated before or during cold treatment (Fig. 1A; Wang et al., 2009; Park et al., 2017). The axillary branches initiated before vernalization flower after the return to warm temperatures, similar

to the main shoot apex (V1 in Fig. 1A). In contrast, the axillary branches located right below the inflorescence arise from buds initiated during vernalization and remain vegetative afterward (V3 in Fig. 1A; Wang et al., 2009; Park et al., 2017). To test the effect of the vernalization duration on flowering and bud fate (inflorescence and axillary) we subjected plants to different vernalization periods. Plants were grown for eight weeks in long-day greenhouse conditions and vernalized for 8, 12, 15, 18, 21 or 24 weeks. Treatments were synchronized so that all plants returned to greenhouse conditions on the same day. A longer vernalization period accelerated flowering after the return to warm temperatures (Fig. 1, B and D). Plants vernalized for 24 weeks flowered within two weeks after being transferred to the greenhouse, whereas plants vernalized for eight weeks required an additional three weeks in long days to flower. Similarly, plants exposed to longer vernalization periods bolted earlier than plants vernalized for shorter periods (Fig. 1, C and D). The length of the inflorescence was also influenced by the duration of vernalization, with prolonged vernalization resulting in longer inflorescence stems (Fig. 1C). These results suggest that extended vernalization accelerates bolting and flowering after the return to warm temperatures and that the duration of vernalization determines the final length of the inflorescence.

Plants vernalized for 12 weeks or longer produced an inflorescence showing the typical I1 and I2 zonation described in *A. thaliana.* Interestingly, I1 inflorescence branches either flowered or stayed vegetative (Fig. 1D). The total number of inflorescence branches in the I1 zone varied according to the duration of vernalization (Supplemental Fig. S1). In addition, the ratio between flowering and vegetative I1 inflorescence branches depended on the duration of vernalization (Fig. 1E). Extended vernalization resulted in an increased frequency of I1 branches that produced flowers and, therefore, a reduction in the number of I1 inflorescence branches that remained vegetative (Fig. 1E). Nevertheless, the commitment of I1 inflorescence branches to flowering showed a similar pattern of growth as in *A. thaliana* (Hempel and Feldman, 1994). Inflorescence branches committed basipetally to floral identity according to the duration of vernalization (Fig. 2A and Supplemental Fig. S2). Similarly, the length of the I1 branches increased with vernalization duration and showed a basipetal pattern of growth with higher branches being longer than lower branches (Fig. 2B). In plants vernalized for eight weeks, inflorescences were abnormal and occasionally single flowers reverted to inflorescence branches (Supplemental Fig. S3; Wang et al., 2009). Inflorescences remained vegetative even after plants were grown under long days (LDs) for five months, and showed delayed senescence (Supplemental Fig. S3). A prominent feature of short vernalization periods was the presence of bracts in the axils of solitary flowers in the I2 zone, which is also a phenotype associated with floral reversion (Müller-Xing et al., 2014). Plants vernalized for 21 weeks or longer did not develop bracts, suggesting that prolonged vernalization reduced the vegetativeness of the inflorescence (Fig. 1F). Consequently, the number of siliques produced in the inflorescences increased according to the

vernalization period. In general, plants vernalized for less than 15 weeks produced a reduced number of siliques compared to plants exposed to longer vernalization (Supplemental Fig. S4). Overall, our results suggest that at least 15 weeks of vernalization are required for inflorescences to reach their reproductive potential.

In contrast to what was observed in the main stem, the duration of vernalization had a minor influence on the fate of the axillary branches initiated during vernalization. In some plants, only after 24 weeks of vernalization did the V3 upper branch commit to reproductive development (Fig. 2A and Supplemental Fig. S2). Taken together, these results show that the duration of vernalization influences the architecture and the fate of the inflorescence but has a marginal effect on those axillary buds initiated during cold treatment.

Floral commitment after extended vernalization correlates with changes in the expression of meristem identity genes

The floral reverting phenotypes observed after short vernalization indicated that floral commitment had not been achieved during cold treatment. We measured the expression of *SOC1*, *FUL*, *LFY*, *AP1*, and *TFL1* orthologues in apices of three- and six-week-old plants after different vernalization periods. Three-week-old plants of the *A. alpina* accession Pajares are not competent to flower in response to cold and remain vegetative during and after vernalization, whereas six-week-old plants initiate flowering during vernalization (Wang et al., 2011; Bergonzi et al., 2013; Park et al., 2017). Apices of three-week-old plants are composed of the vegetative shoot apical meristem and leaf primordia. Apices of six-week-old plants are composed of I1, I2, and V3 initials as well as leaf primordia. *AaSOC1* mRNA levels increased similarly in all apices regardless of whether they were reproductive or not at the end of the vernalization treatment (Fig. 3A; Wang et al., 2011). In contrast, *AaFUL*, *AaLFY*, *AaAP1,* and *AaTFL1* were differentially expressed between flowering and vegetative apices. *AaFUL*, *AaLFY*, and *AaAP1* showed higher expression in flowering apices after prolonged vernalization, whereas *AaTFL1* mRNA levels were gradually reduced specifically in flowering apices (Fig. 3, C, E, G and I). Interestingly, *AaAP1* expression was barely detectable after eight weeks of cold treatment, suggesting that floral commitment was not achieved.

In V3 axillary branches the expression of *AaSOC1*, *AaFUL*, *AaLFY*, and *AaAP1* was very low, while *AaTFL1* expression was high after all cold treatment periods (Fig. 3, B, D, F, H, and J). Our results suggest that floral commitment in *A. alpina* is marked by the accumulation of *AaFUL*, *AaLFY*, and *AaAP1* mRNA in the shoot apical meristem at the end of vernalization.

Extended vernalization prevents reactivation of *PEP1* mRNA in meristems that achieved flowering identity during vernalization

The effect of different vernalization periods on flowering in *A. alpina* resembled those of northern *A. thaliana* accessions such as Lov-1, in which longer vernalization is required to stably silence *FLC* (Shindo et al., 2006; Finnegan and Dennis, 2007; Angel et al., 2011; Li et al., 2014; Duncan et al., 2015). To check whether the effect of extended vernalization on flowering may be related to the stable silencing of *PEP1*, we measured *PEP1* mRNA levels in apices of plants exposed to 18 weeks of cold. In the main shoot apex, *PEP1* expression was downregulated during vernalization and remained low after plants returned to warm temperatures (Fig. 4A). This result is in contrast to previous studies, in which *A. alpina* plants vernalized for 12 weeks showed a reactivation of *PEP1* mRNA in the main shoot apex after the return to warm temperatures (Wang et al., 2009; Castaings et al., 2014; Kiefer et al., 2017).

To determine whether differences in growth conditions may explain this discrepancy, we compared *PEP1* mRNA levels after short (eight weeks) and extended (18 and 24 weeks) vernalization. As expected, eight weeks of chilling temperatures were sufficient to ensure full repression of *PEP1*, such that at the end of all vernalization treatments tested, *PEP1* mRNA levels in the main shoot apex were low (Fig. 4A and B; Wang et al., 2009). In agreement with our previous observations, the duration of the vernalization affected the silencing of *PEP1* (Fig. 4A and B). Vernalization treatments of 18 and 24 weeks achieved stable silencing of *PEP1*, whereas after eight weeks of vernalization *PEP1* mRNA levels increased ten-fold in the shoot apex (Fig. 4A and B). These data are in agreement with previous results showing the influence of vernalization length on the expression of *FLC* in *A. thaliana* and *FLC* orthologues in other Brassicaceae species (Shindo et al., 2006; D'Aloia et al., 2008; Irwin et al., 2016). However, in *A. alpina* the upregulation of *PEP1* after eight weeks of vernalization correlated with the appearance of a vegetative-like inflorescence, suggesting that differential *PEP1* silencing after different vernalization periods might contribute to floral reversion (Supplemental Fig. S3).

To check whether extended vernalization also silences *PEP1* in the axillary branches that continue vegetative growth (V3 in Fig.1A), we measured *PEP1* mRNA levels in the apices of the V3 branches after vernalization. All plants showed high levels of *PEP1* transcript five weeks after they were returned to warm temperatures, indicating that the duration of vernalization did not affect *PEP1* mRNA levels in V3 axillary branches (Fig. 4A and C). These results suggest that the role of *PEP1* in maintaining vegetative development after flowering is not affected by the length of vernalization, although 24 weeks of vernalization seem to slightly reduce the percentage of V3 branches that remain vegetative (Fig. 2B and Supplemental Fig. S2). The differential silencing of *PEP1* in the main shoot apex compared to axillary branches might be due to their identity (flowering or vegetative) at the end of vernalization. To check this hypothesis, we measured *PEP1* mRNA levels in main shoot apices of three-week-old seedlings after different cold treatment periods. *PEP1* expression was

reactivated after vernalization in the shoot apical meristem of three-week-old plants regardless of the duration of the cold treatment (Fig. 4D). These results suggest that *PEP1* silencing after vernalization is regulated differently in meristems that either achieved floral identity or remained vegetative at the end of vernalization.

Previous studies in *A. thaliana* suggested that *FT*, which is a target of FLC, is involved in memory of floral commitment (Melzer et al., 2008; Liu et al., 2014; Müller-Xing et al., 2014). Therefore, we compared the expression of the *A. alpina FT* homolog, *AaFT1* (Adrian et al., 2010), in shoot apices after different periods of vernalization. At the end of vernalization, *AaFT1* expression was low in all treatments tested (Fig. 5A). When plants were transferred to long days, *AaFT1* mRNA was detectable after different cold treatments in flowering plants, but not in apices of three-week-old vernalized plants or V3 branches (Fig. 5). These results suggest that *AaFT1* is upregulated specifically in the apices of flowering plants after vernalization and follows an opposite expression pattern to *PEP1*.

PEP1 antagonizes the commitment of the inflorescence to reproductive development after vernalization

The fact that floral reversion phenotypes after short vernalization correlated with the reactivation of *PEP1* mRNA in the shoot apex hinted that *PEP1* might regulate the fate of the inflorescence after vernalization. We further explored this by scoring flowering and inflorescence traits in the *pep1-1* mutant after different cold treatment periods. To ensure that *pep1-1* plants were not induced to flower before prolonged exposure to cold, we vernalized younger plants that were still competent to flower. Five-week-old Pajares and *pep1-1* mutant plants were subjected to different vernalization periods (8, 12, 15, 18, or 21 weeks). Vernalization treatments were synchronized so that all plants returned to greenhouse conditions on the same day. *pep1-1* plants flowered earlier than the wild-type irrespective of the length of vernalization but responded to different cold treatment periods (Fig. 6A and Supplemental Fig. S5). Moreover, the inflorescence stem was shorter in *pep1-1* than in wild-type plants, except in those subjected to eight weeks of vernalization (Supplemental Fig. S5). Taken together, these data show that *PEP1* represses flowering and inflorescence outgrowth in *A. alpina*.

Interestingly, floral reversion phenotypes were greatly reduced in *pep1-1*, especially after eight weeks of vernalization, while wild-type plants showed more enhanced floral reversion (Fig. 6, B and C). The number of bracts subtending solitary flowers in the I2 zone was reduced in *pep1-1* compared to the wild-type for almost all vernalization lengths applied (Fig. 6B). However, the number of siliques in the I2 zone was similar between Pajares and *pep1-1* suggesting that *PEP1* does not influence the reproductive potential of the inflorescence in the I2 (Fig. 6E). The most significant phenotype in *pep1-1* compared to the wild-type was observed in the I1 zone. None of the inflorescence branches

in *pep1-1* remained vegetative regardless of the duration of vernalization (Fig. 6C). In addition, the number of siliques in these inflorescence branches was very similar for all vernalization durations, suggesting that *PEP1* influences the silique number in the I1 zone (Fig. 6D). Nevertheless, after extended vernalization wild-type plants developed more siliques in the I1 zone than *pep1-1* plants, suggesting that additional genes contribute to the reproductive potential of the inflorescence in the I1 zone.

PEP1 plays a role in defining the reproductive potential of I1 zone; therefore, we tested whether this is correlated with differential expression of *PEP1* among I1 branches. We compared *PEP1* expression levels in I1 branches in wild-type plants vernalized for 12 weeks, in which the lower branches in the inflorescence maintained vegetative development while the higher branches flowered (Fig. 2A and Supplemental Fig. S6A). As expected, *PEP1* mRNA levels were high in vegetative branches compared to flowering I1 branches (Supplemental Fig. S6B). Similar to *PEP1, AaTFL1* mRNA levels were high in vegetative branches compared to flowering I1 branches (Supplemental Fig. S6D). *AaFUL* and *AaAP1* showed the opposite pattern, being less expressed in the vegetative branches compared to flowering branches (Supplemental Fig. S6, C, and E). These results indicate that *PEP1*, *AaTFL1*, *AaFUL*, and *AaAP1* expression correlates with inflorescence branch fate in the I1 zone. Overall, our data suggest that *PEP1* antagonizes the commitment of the inflorescence to reproductive development after vernalization specifically by influencing the fate of the I1 branches.

DISCUSSION

Extended vernalization ensures floral commitment by stably silencing *PEP1*

Understanding the role of winter chilling temperatures in flowering is important for most species, regardless of whether they follow an annual, biennial or perennial life strategy. However, low temperatures can regulate different processes depending on the plant's life strategy. In winter annual and biennial species, cold winters are required to initiate flowering in the spring. Perennial species in temperate or alpine environments initiate flower buds several months or years before flowering; thus, cold winters are important to enhance bud break and achieve uniform flower emergence in the spring (Atkinson et al., 2013).

The quantitative effect of vernalization on flowering has been reported in several Brassicaceae species such as *Sinapis alba* and *A. thaliana* accessions from northern latitudes, in which extended cold exposure is required to accelerate flowering (Shindo et al., 2006; D'Aloia et al., 2008; Li et al., 2014; Duncan et al., 2015). In this study, we investigated whether the duration of vernalization has an effect on flowering in the alpine perennial species *A. alpina.* Similar to *A. thaliana*, longer vernalization gradually accelerates flowering in Pajares plants (Fig. 1). The length of vernalization

334 determines whether *PEP1* is successfully silenced after the return to warm temperatures, an effect
335 also described for *FLC* in *A. thaliana* (Fig 7; Shindo et al., 2006). Thus, *A. alpina* Pajares plants
336 accelerate flowering in response to vernalization in a similar way to northern *A. thaliana* accessions.
337 In both species, after insufficient vernalization, *FLC* and *PEP1* are not stably silenced, and flowering is
338 delayed (Fig. 7; Shindo et al., 2006; Duncan et al., 2015). *pep1-1* mutant plants still respond to
339 different cold treatment periods, suggesting that additional genes might regulate flowering in
340 response to vernalization (Fig. 6). *FLC*-independent mechanisms in the vernalization pathway have
341 also been reported in *A. thaliana* as *flc* null mutants also respond to vernalization (Michaels and
342 Amasino, 2001; Schönrock et al., 2006). The orthologue of *TERMINAL FLOWER 1* (*AaTFL1*) in *A. alpina*
343 was also previously shown to regulate the duration of vernalization required for flowering (Wang et
344 al., 2011). *TFL1* regulates flowering time and inflorescence architecture in *A. thaliana*, as *tfl1* mutants
345 flower early and develop inflorescences which terminate prematurely after producing a few flower
346 buds (Ratcliffe et al., 1998). Silencing of *AaTFL1* in *A. alpina* does not abolish the vernalization
347 requirement to flower but allows the plants to respond to shorter cold treatment periods, such that
348 they flower if exposed to only five weeks of vernalization (Wang et al., 2011). These data suggest that
349 genes regulating the duration of vernalization required for flowering also play a role in inflorescence
350 determinacy in *A. alpina*.

351 In this study, we show that the duration of vernalization determines inflorescence fate by regulating
352 *PEP1* stable silencing (Fig. 7 and Supplemental Fig. S6). This role of vernalization through *FLC* has not
353 been demonstrated in *A. thaliana*. The most interesting difference in the role and regulation of
354 vernalization in *A. alpina* is the fact that flower buds are initiated during prolonged exposure to cold,
355 and not after cold as described in *A. thaliana* (Wang et al., 2009; Wang et al., 2011). During
356 vernalization, new axillary buds are also initiated in the axils of the leaves below the inflorescence
357 meristem. Branches that arise from these axillary buds (V3 in Fig. 1A) maintain vegetative growth the
358 following season and, therefore, sustain the perennial growth habit of *A. alpina*. We have previously
359 shown that *PEP1* represses flowering in these V3 axillary branches (Wang et al., 2009). Here, we
360 tested the effect of the duration of vernalization on *PEP1* expression in the apices of V3 branches.
361 Extended vernalization has a marginal effect on the fate of V3 branches, which show high *PEP1*
362 mRNA levels irrespective of the duration of vernalization (Fig. 7). These results suggest that the
363 length of vernalization affects *PEP1* silencing specifically in the main shoot apical meristem, but not
364 in the axillary branches that arise from buds initiated during the cold treatment. In V3 branches, it is
365 likely that the high expression of miR156, a microRNA that promotes juvenility and represses
366 flowering, ensures that these shoots remain vegetative (Park et al., 2017). The downregulation of
367 miR156 accumulation is slowed down by vernalization but after extended vernalization miR156 levels
368 are reduced (Bergonzi et al., 2013), which may result in the marginal commitment of V3 branches.

The duration of vernalization also increases the reproductive potential of the inflorescence, since inflorescence branches commit basipetally to flowering. After insufficient vernalization, the lower inflorescence branches remain vegetative.

Extended vernalization ensures floral commitment during cold treatment

Flower bud preformation is common among alpine species, as it facilitates acceleration of anthesis and successful seed set in arctic-alpine environments. We demonstrate here that flower initiation during vernalization is associated with the upregulation of *AaSOC1*, *AaFUL*, *AaLFY,* and *AaAP1* and the downregulation of *AaTFL1* in the shoot apex (Fig. 7). Among these genes, *AP1* is considered a marker of floral commitment in *A. thaliana* (Mandel and Yanofsky, 1995; Hempel et al., 1997; Liljegren et al., 1999; Wagner et al., 1999). Thus, the upregulation of *AaAP1* in the shoot apical meristem probably indicates an increase in the number of floral meristems formed in Pajares during vernalization. The extreme floral reversion phenotypes observed and the absence of detectable *AaAP1* expression in the shoot apex after eight weeks of vernalization highlight that, in *A. alpina*, floral commitment has to be achieved in the cold. In *A. thaliana*, the MADS-box transcription factors *SOC1* and *FUL* act redundantly to regulate the maintenance of the inflorescence meristem (Melzer et al., 2008). Our results show that *AaFUL* levels in the shoot apex at the end of vernalization correlate with flowering initiation. In contrast, *AaSOC1* expression increases irrespective of whether the shoot meristem initiates flowering or remains vegetative during vernalization (Fig. 7). Acceleration of flowering after extended vernalization also correlates with the repression of *AaTFL1* in adult plants that will flower after the cold treatment. These results suggest that the mechanisms regulating commitment to flowering in *A. thaliana* are partially conserved in *A. alpina*.

Our data may also give insights into the effects of global warming in arctic-alpine species at the molecular level. Similar to the insufficiently vernalized Pajares plants, which have reduced seed set, ecological studies in the alpine shrub *Salix herbacea* have demonstrated that early spring snowmelt results in a decrease in plant reproductive output (Supplemental Fig. S3; Wheeler et al., 2016). Plants vernalized for longer than 12 weeks show a typical inflorescence architecture divided into the I1 zone with inflorescence branches and the I2 zone with solitary flowers. However, plants vernalized for 12 or 15 weeks show subtle reverting phenotypes, such as bracts in the I2 zone (Fig. 1). In *A. thaliana*, the floral primordium emerges as a cryptic bract, but bracts do not outgrow due to the development of the associated floral meristem (Chandler, 2012). Thus, bracts in *A. thaliana* only appear in mutants with compromised floral meristem identity (Mandel et al., 1992; Weigel et al., 1992; Chandler, 2012; Müller-Xing et al., 2014). In *A. alpina*, the number of bracts in the I2 zone was reduced after longer vernalization periods, following an opposite pattern to the expression of floral identity genes in the

inflorescence meristem during cold treatment. These results indicate that extended vernalization in *A. alpina* ensures floral development during cold treatment, and favors the development of the floral meristem over its subtending bract after the return to warm temperatures. In addition, longer vernalization leads to a higher percentage of flowering I1 inflorescence branches (Fig. 1). In plants vernalized for 12 weeks the higher branches in the inflorescence commit to reproductive development, whereas the lower inflorescence branches remain vegetative. Differences in floral identity within the same inflorescence also correlate with higher expression of *AaAP1* and *AaFUL* in flowering I1 branches compared to vegetative I1 branches (Supplemental Fig. S6). This spatial pattern of commitment to flowering within the inflorescence can also give insights into the sequence of developmental events in the inflorescence meristem. Similarly to *A. thaliana*, inflorescence branches in *A. alpina* commit basipetally to floral identity (Fig. 2; Hempel and Feldman, 1994). The length of inflorescence branches also shows a basipetal pattern, with higher branches being longer than lower branches. These results suggest that flowering in *A. alpina* is a continuous process, and after flowering induction, prolonged periods of cold temperatures are required for the development of the floral meristem. Longer vernalization leads to the formation of more flower buds during cold treatment and higher expression of floral organ identity markers such as *AaAP1* (Fig. 7). Thus, preformation of flower buds during cold treatment facilitates the acceleration of flowering after the return to warm temperatures. A similar requirement for saturating floral-promoting inputs before the return to warm temperatures has been reported in the cold-induced perennials *Boronia megastigma* and *Hypocalymma angustifolium* (Day et al., 1994).

Reactivation of *PEP1* after insufficient vernalization antagonizes inflorescence fate, likely by repressing *AaFT1*

Reverted inflorescences have previously been observed in other perennial Brassicaceae species, such as *Arabidopsis halleri* growing in nature (Aikawa et al., 2010). Interestingly, the presence of floral reversion phenotypes in *A. halleri* population coincided with a seasonal upregulation of *AhgFLC* (Aikawa et al., 2010). In northern *A. thaliana* accessions, despite the upregulation of *FLC* after insufficient vernalization, floral reversion has not been demonstrated. However, constitutive expression of *FLC* in Columbia results in plants that flower late and have aerial rosettes in the inflorescence (Wang et al., 2007). In addition, the *A. thaliana* accession Sy-0 has been reported to produce aerial rosettes, which are partially caused by high *FLC* expression and are abolished with vernalization (Grbic and Bleecker, 1996; Poduska et al., 2003; Wang et al., 2007). In *A. alpina*, we show that the upregulation of *PEP1* in the inflorescence after vernalization might counteract the commitment to flowering. Besides, *AaFT1* mRNA levels in flowering apices gradually increase after

vernalization (Fig. 7; Müller-Xing et al., 2014). *FT* is a direct target of FLC and antagonizes floral reversion in *A. thaliana* (Helliwell et al., 2006; Searle et al., 2006; Liu et al., 2014; Müller-Xing et al., 2014). In *A. alpina*, PEP1 binds directly to *AaFT1* chromatin (Mateos et al., 2017), in accordance with our results demonstrating that *AaFT1* follows the opposite expression pattern to *PEP1* in the shoot apex after vernalization (Fig. 7). In plants that initiate flowering during cold treatment, extended vernalization stably silences *PEP1,* and *AaFT1* is upregulated. However, in young seedlings that do not initiate flowering during cold treatment, extended vernalization fails to silence *PEP1* and *AaFT1* expression is low. These results lead us to speculate that *PEP1* might antagonize inflorescence fate by repressing *AaFT1* after vernalization.

Feedback mechanisms that prevent *PEP1* reactivation in meristems that achieved floral identity at the end of vernalization might also exist. FLC and PEP1 shares targets with AP1 and another MADS-box protein, SEPALLATA 3 (SEP3), which also promotes floral organ identity (Kaufmann et al., 2009; Kaufmann et al., 2010; Deng et al., 2011; Mateos et al., 2017). Our results also contribute to the understanding of how flower initiation and maintenance is achieved in perennials, as the expression of *FLC*- and *FT-like* genes has been associated with bud break in aspen, apple, and pear trees (Böhlenius et al., 2006; Ito et al., 2016; Kumar et al., 2017). Altogether, our results highlight the importance of floral repressors such as *PEP1/FLC*, which are expressed both in vegetative and inflorescence meristems, in modulating the commitment to flowering in different types of shoots.

CONCLUSION

Our study demonstrates that extended vernalization in *A. alpina* enhances flowering and reduces floral reversion by ensuring flower bud development before exposure to warm temperatures. Seasonal changes in floral promoters or repressors might regulate the outgrowth of the initiated flower buds. We also show that the floral repressor *PEP1* regulates the final stages of flowering by antagonizing floral commitment. Extended vernalization silences *PEP1* in the inflorescence to accelerate flowering, but does not affect *PEP1* upregulation in the vegetative branches ensuring the return to vegetative development after flowering.

MATERIALS AND METHODS

Plant material, growth conditions, and phenotyping

The *Arabis alpina* Pajares accession and the *pep1-1* mutant were used for physiological analysis and expression studies. The accession Pajares was collected in the Cordillera Cantábrica mountains in Spain at 1,400 meters altitude (42°59´32´´ N, 5°45´32´´ W) and *pep1-1* is an EMS derived mutant in the Pajares background (Wang et al., 2009). To score flowering and inflorescence traits, plants were grown in an greenhouse under long day conditions (16 h light and 8 h dark) under temperatures

ranging from 20°C during the day to 18°C during the night prior to vernalization. All vernalization treatments were performed at 4°C in short day conditions (8 h light and 16 h dark) and experiments were synchronized in such a way that plants vernalized for different periods were moved back to LD greenhouse (16 h light and 8 h dark) on the same day.

For the characterization of flowering and inflorescence traits in Pajares after different periods of vernalization (eight, 12, 15, 18, 21 and 24 weeks), plants were grown for eight weeks before they were vernalized. For the characterization of flowering and inflorescence traits in Pajares *versus pep1-1*, plants were grown for five weeks in LD greenhouse conditions to ensure that *pep1-1* did not initiate flowering before vernalization. Flowering time was measured by recording the date in which the first flower opened. Length of the Pajares inflorescence stem was measured at eight weeks after vernalization or when the last flower in the inflorescence opened. In Pajares, the inflorescence is well defined and is always above a zone of vegetative branches which senesce after flowering. In *pep1-1*, the inflorescence is not well defined as the vegetative zone is missing. To indicate the beginning of the inflorescence in *pep1-1*, the last leaf was marked before plants entered vernalization. Inflorescence traits were measured at 8 and 14 weeks after vernalization, or when the last flower in the inflorescence opened. The number of siliques was scored eight weeks after vernalization to avoid loss of siliques or when the last flower in the inflorescence opened. The number of siliques was measured as a proxy for yield in I1 and I2 zones separately. All experiments were performed with at least 12 plants.

Gene Expression Analysis

To follow the expression patterns of *PEP1* in the main shoot apex, Pajares plants were grown for three or six weeks in long days before being vernalized for different periods (8, 18 and 24 weeks). All treatments were synchronized so that the plants were transferred to the greenhouse on the same day. Main shoot apices of three- and six-week-old vernalized plants were harvested at the end of the different periods of vernalization, and one, three, and five weeks after vernalization. From six-week-old-vernalized plants, axillary vegetative apices (V3 in Fig. 1A) were harvested five weeks after vernalization. Apices of inflorescence branches of plants vernalized for 12 weeks which had either flowering or vegetative identity were harvested eight weeks after vernalization. An average of 15 apices were pooled in each sample. Apices from the main shoot apex were harvested at the same time from 15 different plants. Five seedlings per pot were grown in the case of three-week-old plants, and individual plants were grown in each pot in the case of six-week-old plants. Samples from apices of V3 axillary branches were harvested from 10 six-week-old plants, two V3 axillary branches per plant. Samples from I1 inflorescence branches were harvested from 10 six-week-old plants grown

in individual pots. Two pools were harvested simultaneously, a pool of apices in Node 1 of the Inflorescence and a pool of apices in Node 7.

Total plant RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and a DNase treatment was performed with Ambion DNAfree-kit DNase treatment and removal (Invitrogen) to reduce any DNA contamination. Total RNA (1.5 µg) was used to synthesize cDNA through reverse transcription with SuperScript II Reverse Transcriptase (Invitrogen) and oligo dT (18) as a primer. Two microliters of a cDNA dilution (1:5) was used as the template for each quantitative PCR. The RT-qPCR was performed using a CFX96 Real-Time System (Bio-Rad) and the iQ SYBR Green Supermix detection system. Each data point was derived from two independent replicates in three-week-old plants or three independent replicates in six-week-old plants and is shown as mean ± s.d.m. *AaRAN3* and *AaPP2A* were used for expression data normalization.

Primers used for RT-qPCR for *PEP1*, *AaSOC1*, *AaLFY*, *AaTFL1*, *AaRAN3,* and *AaPP2A* were described previously (Wang et al., 2009; Wang et al., 2011; Bergonzi et al., 2013). Primers for *AaAP1* are as follows: AaAP1_F: ATGAGAGGTACTCTTACGCCGA and AaAP1_R: GTCATCTCCAAGATAATGCCTC. Primers for *AaFUL* are as follows: AaFUL_F: GGATACTTGAACGCTATGATCG and AaFUL_R: TCAACGAATCAAGATCTTCCCC*.* Primers for *AaFT1* are as follows AaFT1_F: GATCTAAGGCCTTCTCAAGTCCAA and AaFT1_R: CTGTCGGAACAATATCAGCACGATA (Wang, 2007).

Statistical analysis

Statistical analyses were performed using the R software. Data distribution for time to flower emergence, number of inflorescence branches, total number of siliques and number of siliques in I1 and I2 were checked with a Shapiro-Wilk test of normality. The Kruskal–Wallis test was employed as an omnibus test to detect significant differences as data were not normally distributed. This was followed by a posthoc test for pairwise multiple comparisons using the Mann–Whitney U test. The Type I error rate (α) was set at 0.05, and the Bonferroni p-value adjustments method was used. For the *pep1-1* physiological analysis, we conducted multiple pairwise Bonferroni tests (α = 0.05) to detect significant differences between Pajares and *pep1-1*. Here, a nonparametric test could not be conducted due to ties created during rank assignment.

To detect significant differences in gene expression we controlled for a false discovery rate of 0.05 when conducting multiple pairwise comparisons by using Benjamini-Hochberg-corrected p-values or Hochberg-GT2.

Treatments with significant differences are represented with letters or asterisks.

Accession numbers:

Sequence data used in this article can be found in the GenBank/EMBL databases under the following accession numbers: cDNA of *PEP1* (FJ755930), coding sequence of *AaLFY* (JF436956), coding sequence of *AaSOC1* (JF436957), *AaAP1* (AALP_AA2G117200), coding sequence of *AaTFL1* (JF436953), *AaFUL* (Aa_G837900), *AaFT1* (Aa_G437270).

SUPPLEMENTARY MATERIALS

Supplemental Figure S1. The duration of vernalization influences the number of branches in the inflorescence.

Supplemental Figure S2. Inflorescence branches in *A. alpina* commit basipetally to floral identity.

Supplemental Figure S3. The length of vernalization determines inflorescence reversion and senescence.

Supplemental Figure S4. Extended vernalization increases the number of siliques in the inflorescence.

Supplemental Figure S5. The *pep1* mutant flowers earlier than Pajares but also responds to different periods of vernalization.

Supplemental Figure S6. *PEP1* antagonizes the commitment of the inflorescence to flowering.

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FIGURE LEGENDS

Figure 1. The length of vernalization determines inflorescence outgrowth and floral reversion in *A. alpina*. (A) Schematic representation of a flowering *A. alpina* plant after vernalization. Axillary branches have different fates according to their position on the plant: (V1) axillary branches that flower and partially senesce, (V2) dormant axillary buds, and (V3) axillary vegetative branches. The inflorescence consists of the (I1) zone with inflorescence branches and the (I2) zone with solitary flowers. The I1 branches can be distinguished from the V3 branches by the bolting of the inflorescence stem. (B) Flowering time of Pajares plants vernalized for 8, 12, 15, 18, 21 or 24 weeks. (C) Length of the inflorescence in Pajares plants vernalized for 8, 12, 15, 18, 21 or 24 weeks measured eight weeks after vernalization. (D) Picture of *A. alpina* Pajares exposed to 8, 12, 15, 18, 21 or 24 weeks of vernalization (8w, 12w, 15w, 18w, 21w or 24w) and subsequently grown for three weeks in

an LD greenhouse. Bar = 10 cm. (E) Percentages of flowering and vegetative inflorescence branches in the I1 zone after 8, 12, 15, 18, 21 and 24 weeks of vernalization followed by eight weeks in a LD greenhouse. Close-up pictures of flowering (top) and vegetative (bottom) I1 inflorescence branches. Bar = 0,5 cm. (F) Number of bracts in the I2 zone of plants exposed to 8, 12, 15, 18, 21, and 24 weeks of vernalization followed by 14 weeks in a LD greenhouse. Close-up picture of the bracts in the inflorescence of plants exposed to 12 weeks of vernalization followed by 14 weeks in a LD greenhouse. Bar = 3 cm. Arrows indicate two bracts as an example. The error bars represent the s.d.m; n = 9-12. Letters indicate significant differences determined by omnibus Kruskal-Wallis test followed by pairwise multiple comparison using Mann-Whitney U test (α-value of 0.05). No letters indicate no significant differences.

Figure 2. The duration of vernalization determines the commitment to floral identity of inflorescence and V3 branches in a basipetal pattern. (A) Ratio of flowering branches after different periods of vernalization (8, 12, 15, 18, 21, and 24 weeks). Nodes are numbered from the bottom of the inflorescence. Positive values represent the I1 inflorescence zone. Negative values represent the V3 zone. The basis of the inflorescence bolted stem is indicated by zero (0). (B) Mean length of inflorescence branches at consecutive nodes along the inflorescence in plants vernalized for 12 or 24 weeks followed by eight weeks in a LD greenhouse. The error bars represent the s.d.m; n = 11-12.

Figure 3. The transcripts of floral meristem identity genes accumulate in the apices of six-week-old plants that initiate flowering during cold treatment. (A, C, E, G and I) Expression level of *AaSOC1, AaFUL, AaTFL1, AaLFY* and *AaAP1* in the shoot apical meristem of three- and six-week-old Pajares plants at the end of different periods of vernalization. Three- or six-week-old plants were vernalized for 8, 18, or 24 weeks. Apices were harvested at the end of the vernalization treatments. (B, D, F, H, and J) Expression level of *AaSOC1, AaFUL, AaTFL1, AaLFY,* and *AaAP1* in V3 axillary branches. Six-week-old plants, vernalized for 8, 18, or 24 weeks and transferred to a LD greenhouse for five weeks. The data are the means of two or three biological replicates, and error bars represent the s.d.m. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05). Graphs with no letters indicate no significant differences.

Figure 4. Extended vernalization silences *PEP1* in the inflorescence. (A) *PEP1* expression in the main shoot apex of six-week-old plants before (0), during (1, 3, 5, 8, 12, and 18 weeks) and after 18 weeks of vernalization. After vernalization, main shoot apices were harvested one, two, three and four weeks after the plants were transferred back to a LD greenhouse. *PEP1* expression was also measured in the apices of axillary V3 branches after vernalization. (B) *PEP1* expression at the end of vernalization in the apices of plants vernalized for 8, 18, and 24 weeks (0 indicates expression at the

end of vernalization) and after vernalization (one, three and five weeks in a LD greenhouse). (C) *PEP1* expression in the apices of axillary V3 branches vernalized for 8, 18, or 24 weeks and transferred back to a LD greenhouse for five weeks. (D) *PEP1* expression in the main shoot apex of three-week-old seedlings at the end of 8, 18, or 24 weeks of vernalization (0 indicates expression at the end of vernalization) and after five weeks in a LD greenhouse. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05) for A and D and Hochberg-GT2 for B. Graphs with no letters indicate no significant differences. All data are the means of two or three biological replicates, and error bars represent the s.d.m.

Figure 5. *AaFT1* transcript gradually accumulates in the apices of six-week-old plants after different cold treatment periods. (A) *AaFT1* expression level in the shoot apical meristem of three- and six-week-old Pajares plants. Plants were vernalized for 8, 18, or 24 weeks (8w, 18w, and 24w) and apices were harvested at the end of each vernalization treatment (0 indicates expression at the end of vernalization) and five weeks after vernalization. (B) *AaFT1* expression level in the apices of V3 axillary branches vernalized for 8, 18, or 24 weeks and transferred back to a LD greenhouse for five weeks. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05). Graphs with no letters indicate no significant differences. The data are the means of two or three biological replicates, and error bars represent the s.d.m.

Figure 6. Floral reversion phenotypes are reduced in the *pep1* mutant. Phenotypes were measured in wild-type Pajares (WT) and *pep1-1* plants exposed to 8, 12, 15, 18, and 21 weeks of vernalization. (A) Time to flower emergence; (B) Number of bracts; (C) Percentage of flowering I1 inflorescence branches (FB); (D) Number of siliques in the I1 branches; (E) Number of siliques in the I2 inflorescence zone. All measurements in (B-E) were performed at the end of flowering. The error bars represent the s.d.m; n = 9-12. Asterisks indicate significant differences between wild-type and the *pep1-1* mutant at each time point determined by multiple pairwise Bonferroni tests (α-value of 0.05).

Figure 7. Schematic representation of a model for the genetic regulation of flowering and vegetative branches in a fully vernalized *A. alpina* plant. Flowering is initiated during vernalization in the apical meristem of the main shoot and in the V1 axillary branches (orange circle). V3 vegetative branches arise from buds below the inflorescence initiated during cold treatment (green circle). The expression of *PEP1* and *AaTFL1* is downregulated in the main shoot apical meristem, whereas the expression of *AaSOC1*, *AaFUL*, *AaLFY,* and *AaAP1* is upregulated (triangles). After the return to LD greenhouse conditions, the I1 and I2 zones of the inflorescence develop and the V1 branches flower.

PEP1 remains stably silenced in the shoot apex, and *AaFT1* expression is upregulated (triangle). The length of vernalization influences the expression levels of meristem identity genes during vernalization and of *PEP1* and *AaFT1* after vernalization. In contrast, the V3 branches show high *PEP1* and *AaTFL1* expression levels irrespective of the duration of vernalization.

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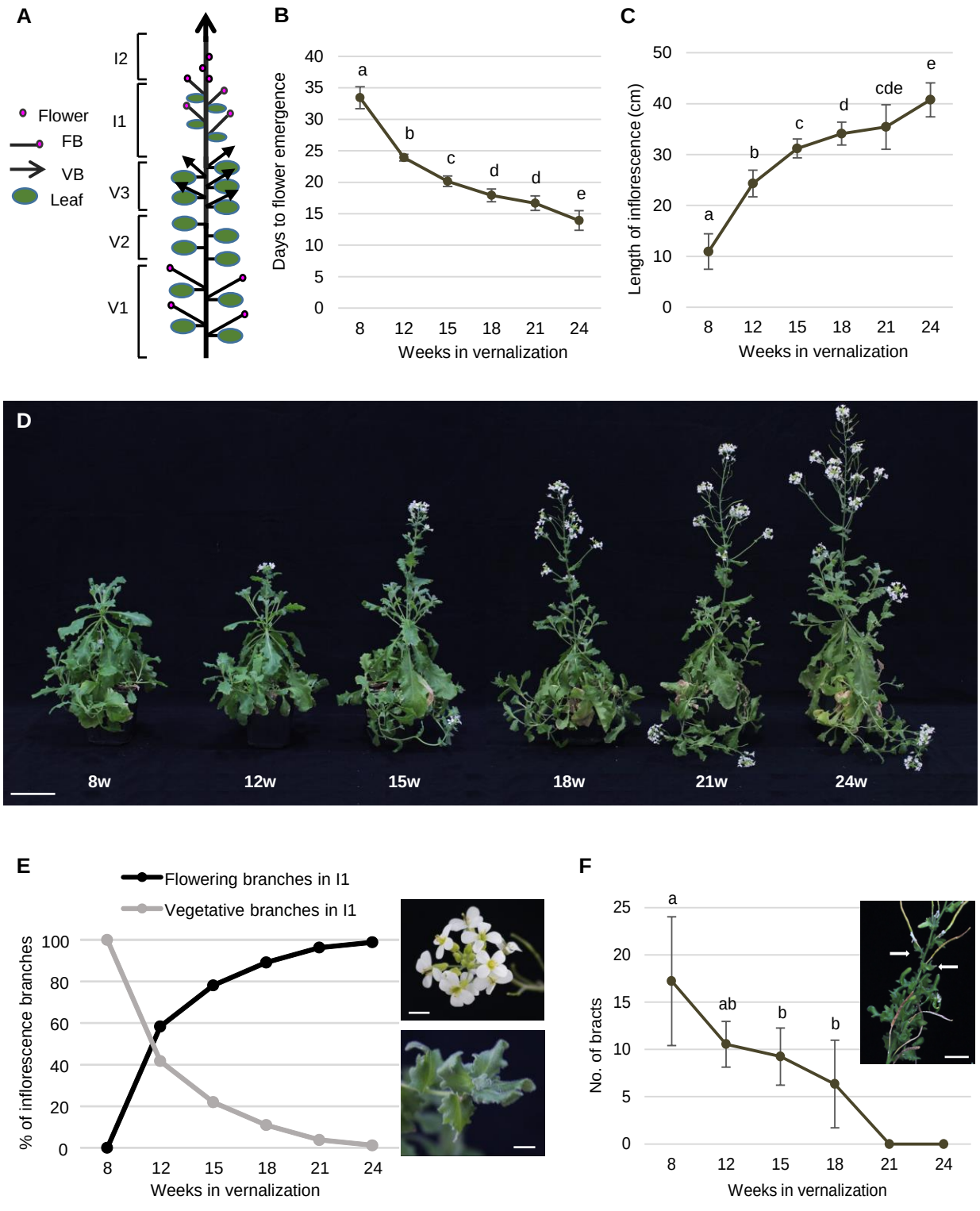

Figure 1. The length of vernalization determines inflorescence outgrowth and floral reversion in *A. alpina*. (A) Schematic representation of a flowering *A. alpina* plant after vernalization. Axillary branches have different fates according to their position on the plant: (V1) axillary branches that flower and partially senesce, (V2) dormant axillary buds, and (V3) axillary vegetative branches. The inflorescence consists of the (I1) zone with inflorescence branches and the (I2) zone with solitary flowers. The I1 branches can be distinguished from the V3 branches by the bolting of the inflorescence stem. (B) Flowering time of Pajares plants vernalized for 8, 12, 15, 18, 21 or 24 weeks. (C) Length of the inflorescence in Pajares plants vernalized for 8, 12, 15, 18, 21 or 24 weeks measured eight weeks after vernalization. (D) Picture of *A. alpina* Pajares exposed to 8, 12, 15, 18, 21 or 24 weeks of vernalization (8w, 12w, 15w, 18w, 21w or 24w) and subsequently grown for three weeks in an LD greenhouse. Bar = 10 cm. (E) Percentages of flowering and vegetative inflorescence branches in the I1 zone after 8, 12, 15, 18, 21 and 24 weeks of vernalization followed by eight weeks in a LD greenhouse. Close-up pictures of flowering (top) and vegetative (bottom) I1 inflorescence branches. Bar = 0,5 cm. (F) Number of bracts in the I2 zone of plants exposed to 8, 12, 15, 18, 21, and 24 weeks of vernalization followed by 14 weeks in a LD greenhouse. Close-up picture of the bracts in the inflorescence of plants exposed to 12 weeks of vernalization followed by 14 weeks in a LD greenhouse. Bar = 3 cm. Arrows indicate two bracts as an example. The error bars represent the s.d.m; n = 9-12. Letters indicate significant differences determined by omnibus Kruskal-Wallis test followed by pairwise multiple comparison using Mann-Whitney U test (α-value of 0.05). No letters indicate no significant differences.

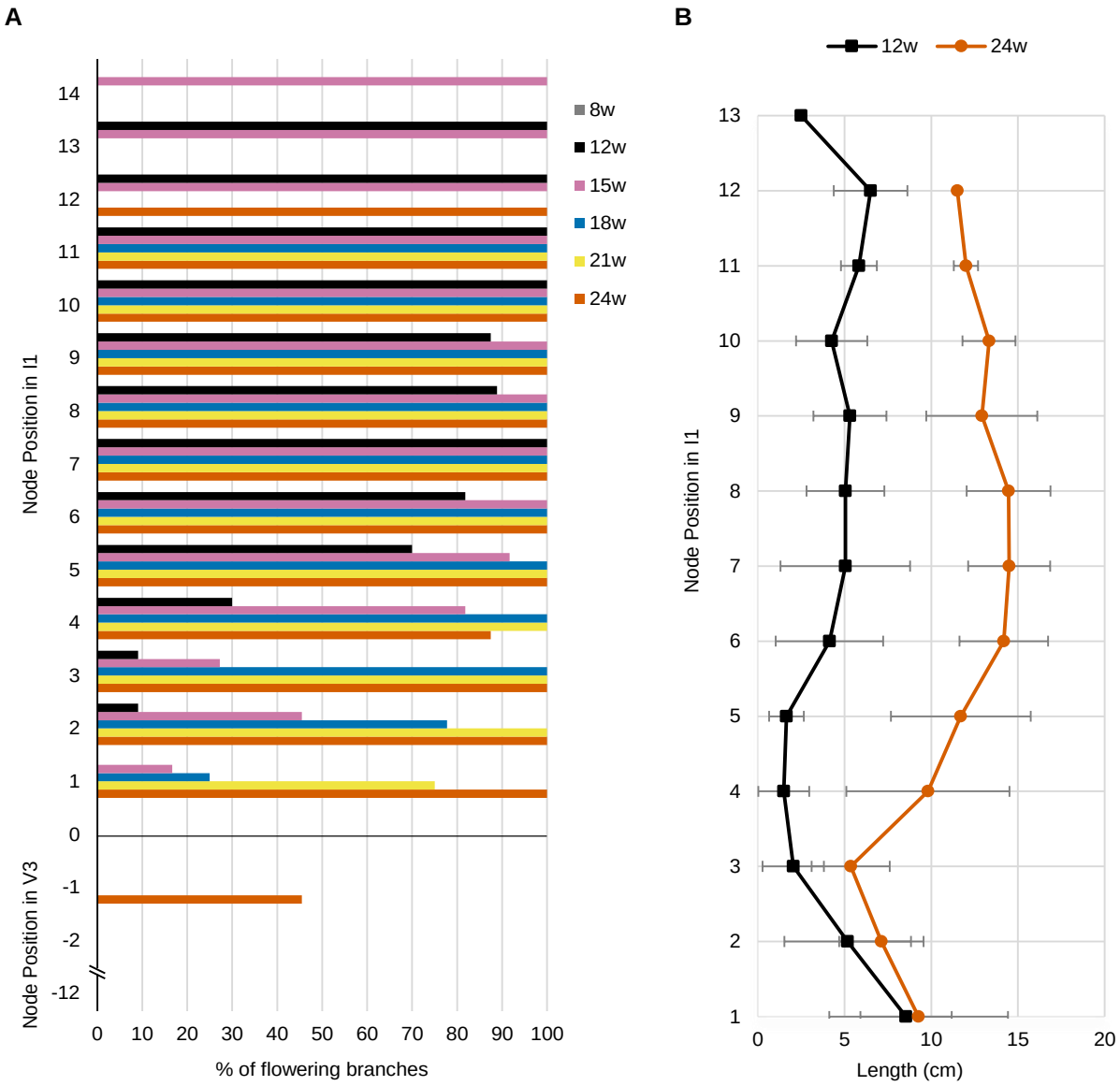

Figure 2 . The duration of vernalization determines the commitment to floral identity of inflorescence and V3 branches in a basipetal pattern. (A) Ratio of flowering branches after different periods of vernalization (8, 12, 15, 18, 21, and 24 weeks). Nodes are numbered from the bottom of the inflorescence. Positive values represent the I1 inflorescence zone. Negative values represent the V3 zone. The basis of the inflorescence bolted stem is indicated by zero (0). (B) Mean length of inflorescence branches at consecutive nodes along the inflorescence in plants vernalized for 12 or 24 weeks followed by eight weeks in a LD greenhouse. The error bars represent the s.d.m; n = 11-12.

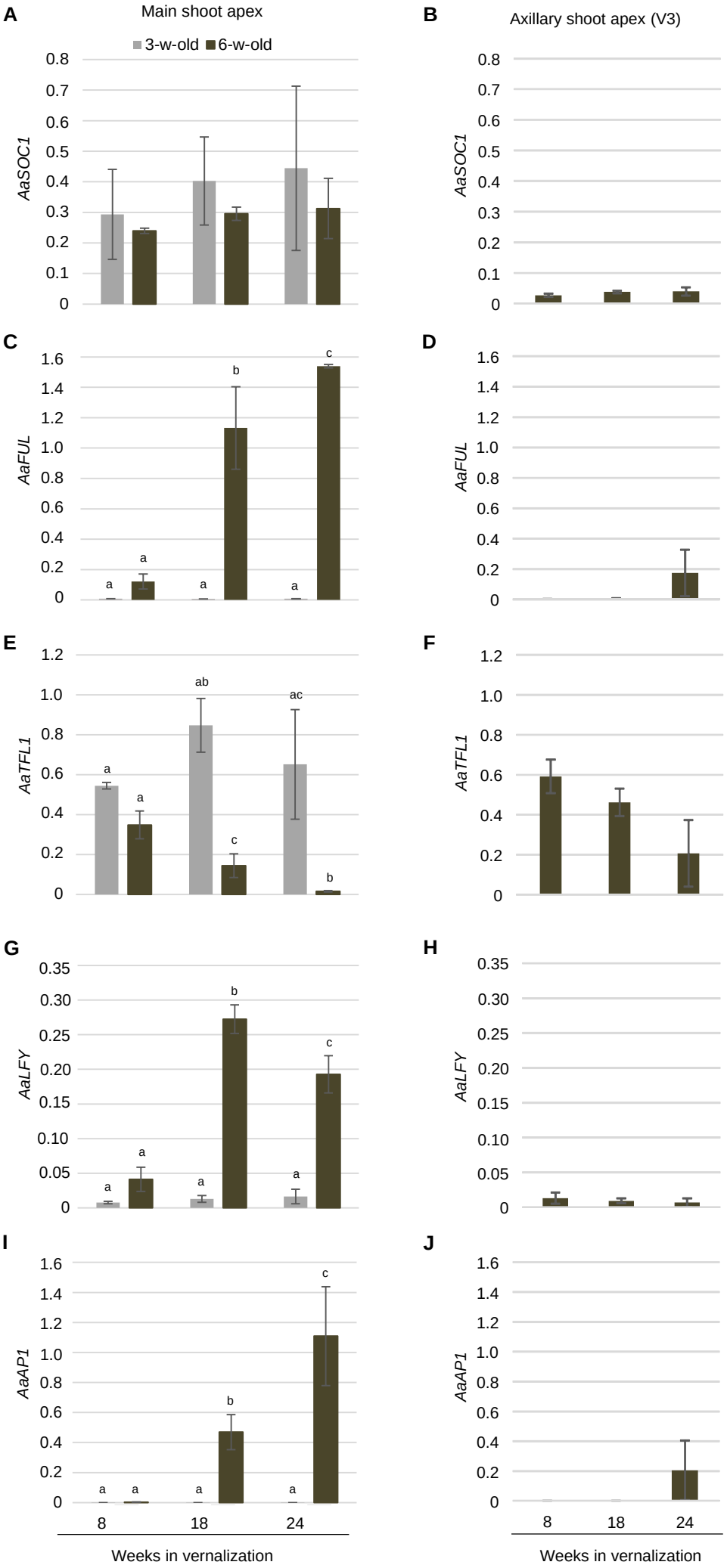

Figure 3. The transcripts of floral meristem identity genes accumulate in the apices of six-week-old plants that initiate flowering during cold treatment. (A, C, E, G and I) Expression level of *AaSOC1, AaFUL, AaTFL1, AaLFY* and *AaAP1* in the shoot apical meristem of three- and six-week-old Pajares plants at the end of different periods of vernalization. Three- or six-week-old plants were vernalized for 8, 18, or 24 weeks. Apices were harvested at the end of the vernalization treatments. (B, D, F, H, and J) Expression level of *AaSOC1, AaFUL, AaTFL1, AaLFY,* and *AaAP1* in V3 axillary branches. Six-week-old plants, vernalized for 8, 18, or 24 weeks and transferred to a LD greenhouse for five weeks. The data are the means of two or three biological replicates, and error bars represent the s.d.m. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05). Graphs with no letters indicate no significant differences.

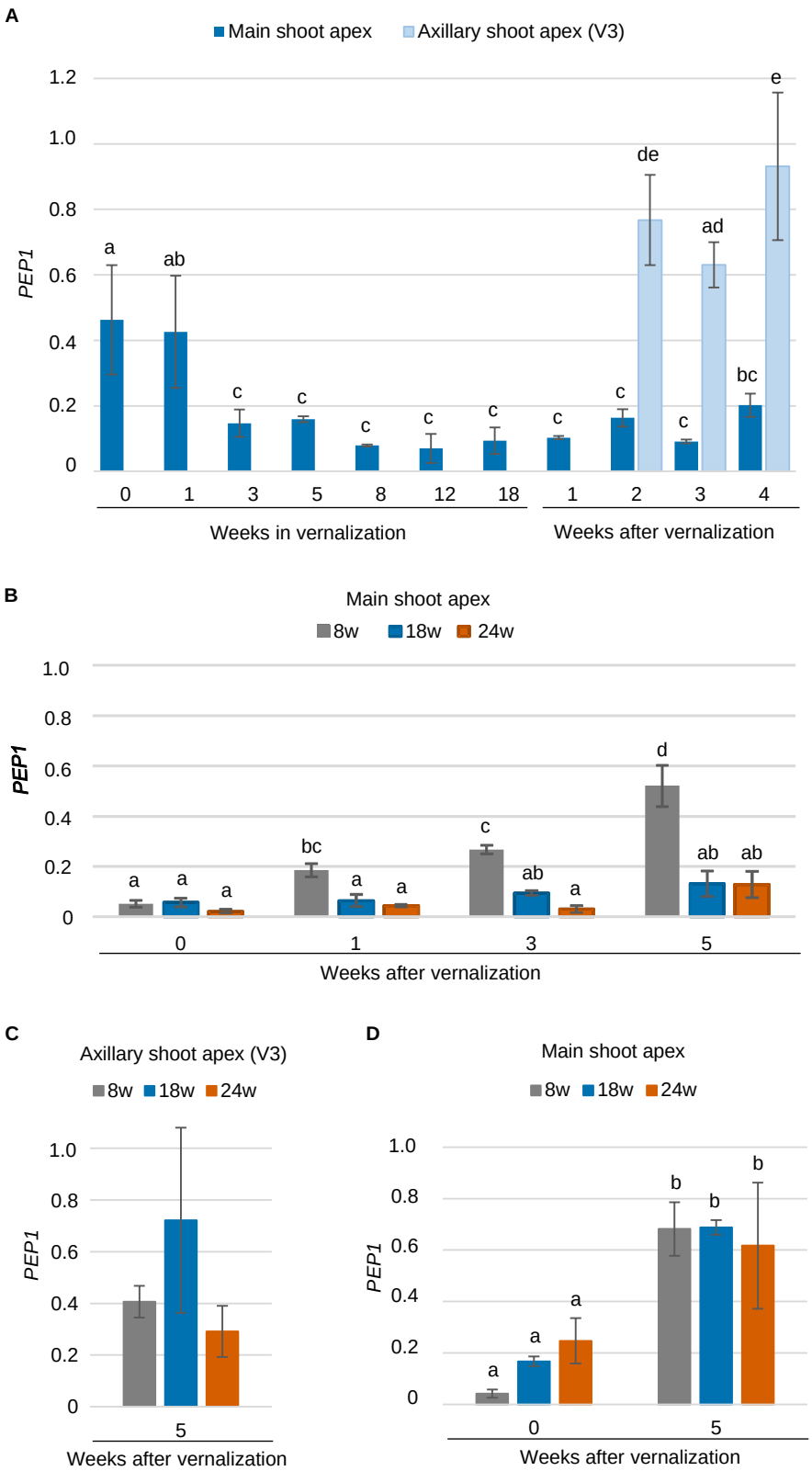

Figure 4. Extended vernalization silences *PEP1* in the inflorescence. (A) *PEP1* expression in the main shoot apex of six-week-old plants before (0), during (1, 3, 5, 8, 12, and 18 weeks) and after 18 weeks of vernalization. After vernalization, main shoot apices were harvested one, two, three and four weeks after the plants were transferred back to a LD greenhouse. *PEP1* expression was also measured in the apices of axillary V3 branches after vernalization. (B) *PEP1* expression at the end of vernalization in the apices of plants vernalized for 8, 18, and 24 weeks (0 indicates expression at the end of vernalization) and after vernalization (one, three and five weeks in a LD greenhouse). (C) *PEP1* expression in the apices of axillary V3 branches vernalized for 8, 18, or 24 weeks and transferred back to a LD greenhouse for five weeks. (D) *PEP1* expression in the main shoot apex of three-week-old seedlings at the end of 8, 18, or 24 weeks of vernalization (0 indicates expression at the end of vernalization) and after five weeks in a LD greenhouse. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05) for A and D and Hochberg-GT2 for B. Graphs with no letters indicate no significant differences. All data are the means of two or three biological replicates, and error bars represent the s.d.m.

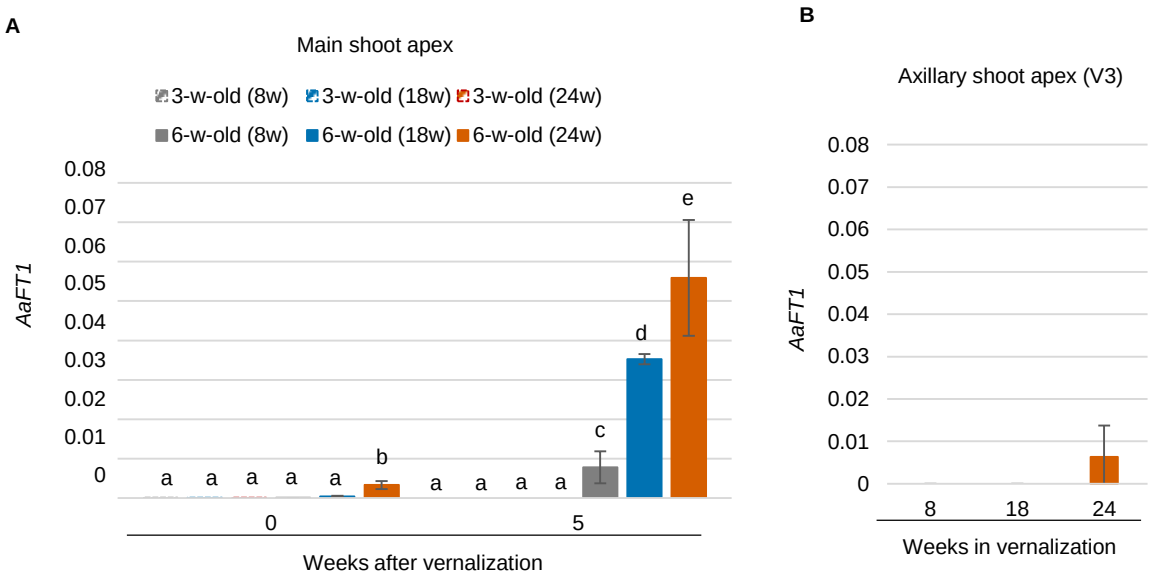

Figure 5. *AaFT1* transcript gradually accumulates in the apices of six-week-old plants after different cold treatment periods. (A) *AaFT1* expression level in the shoot apical meristem of three- and six-week-old Pajares plants. Plants were vernalized for 8, 18, or 24 weeks (8w, 18w, and 24w) and apices were harvested at the end of each vernalization treatment (0 indicates expression at the end of vernalization) and five weeks after vernalization. (B) *AaFT1* expression level in the apices of V3 axillary branches vernalized for 8, 18, or 24 weeks and transferred back to a LD greenhouse for five weeks. Letters above the columns indicate significant differences determined by multiple pairwise comparisons using Benjamini-Hochberg-corrected p-values (α-value of 0.05). Graphs with no letters indicate no significant differences. The data are the means of two or three biological replicates, and error bars represent the s.d.m.

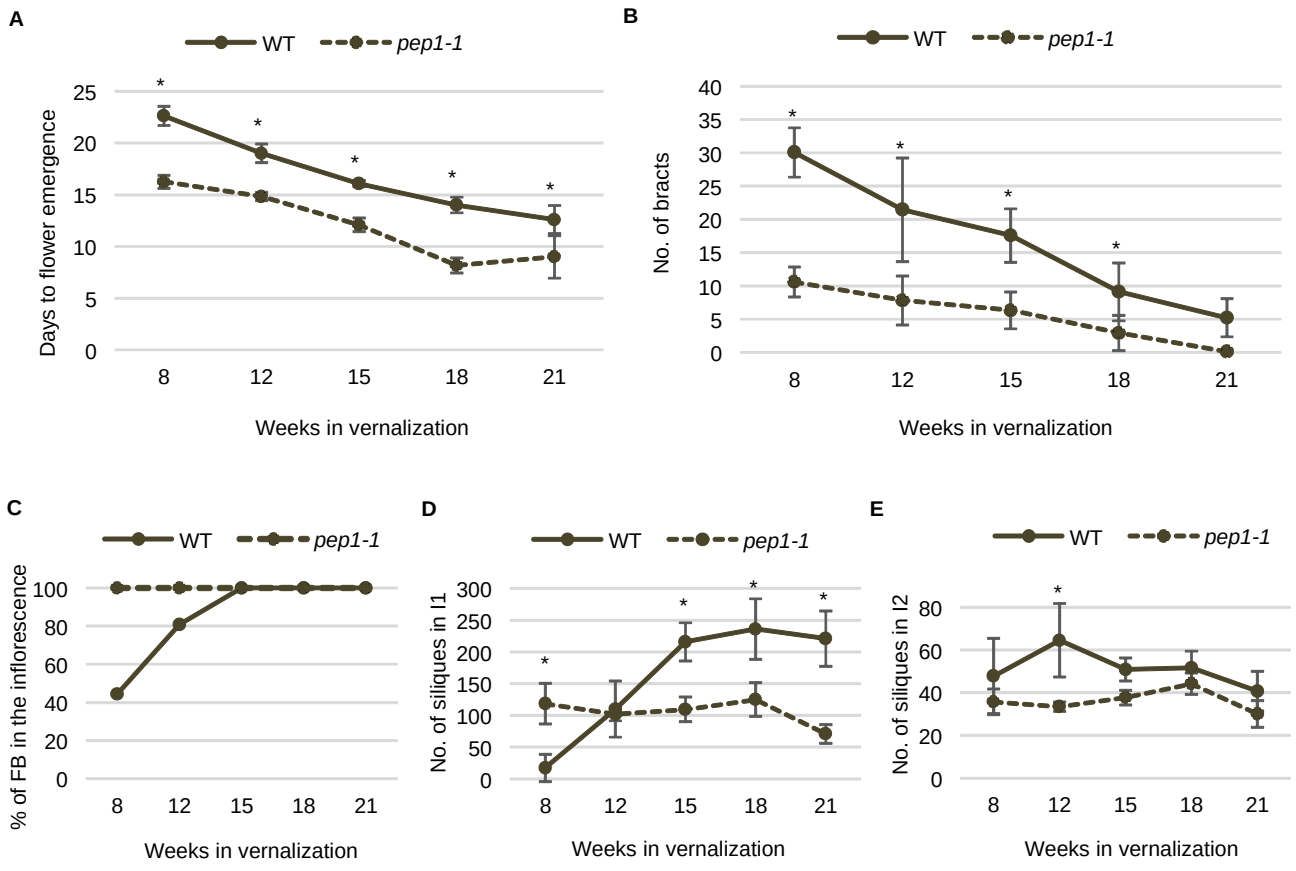

Figure 6. Floral reversion phenotypes are reduced in the *pep1* mutant. (A) Time to flower emergence in wild type Pajares (WT) and *pep1-1* plants exposed to eight, 12, 15, 18 and 21 weeks of vernalization measured as the number of days to the first open flower. (B) Number of bracts in the I2 of wild type and *pep1-1* plants exposed to eight, 12, 15, 18 and 21 weeks of vernalization. (C) Percentage of flowering I1 inflorescence branches (FB) in wild type and *pep1-1* exposed to eight, 12, 15,18, 21 and 24 weeks of vernalization (D) Number of siliques in the I1 branches of wild type and *pep1-1* after eight, 12, 15, 18, 21 and 24 weeks of vernalization. (E) Number of siliques in the I2 inflorescence zone of wild type and *pep1-1* stem after eight, 12, 15, 18, 21 and 24 weeks of vernalization. All measurements in (B-E) were performed at the end of flowering. The error bars represent the s.d.m; n = 9-12. Asterisks stand for significant differences between wild type and the *pep1-1* mutant at each time point determined by multiple pairwise Bonferroni tests (α-value of 0.05).

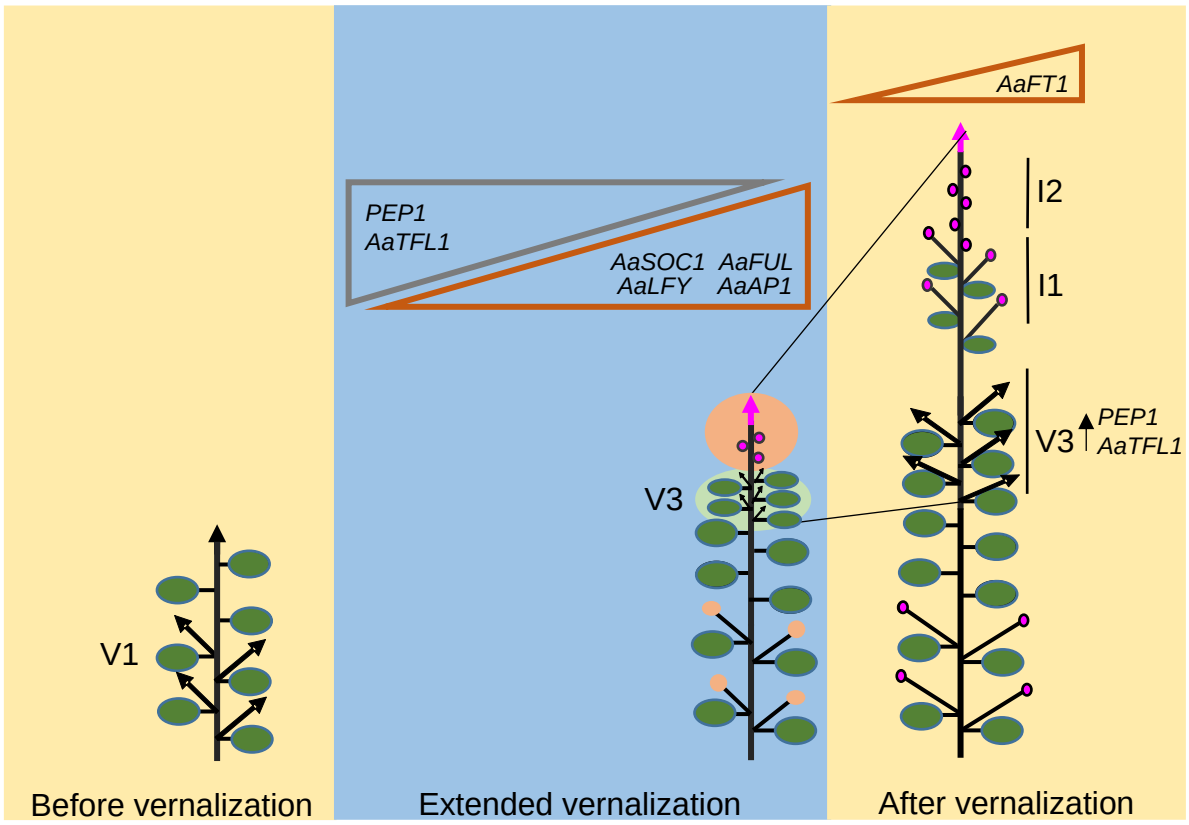

Figure 7. Schematic representation of a model for the genetic regulation of flowering and vegetative branches in a fully vernalized *A. alpina* plant. Flowering is initiated during vernalization in the apical meristem of the main shoot and in the V1 axillary branches (orange circle). V3 vegetative branches arise from buds below the inflorescence initiated during cold treatment (green circle). The expression of *PEP1* and *AaTFL1* is downregulated in the main shoot apical meristem, whereas the expression of *AaSOC1*, *AaFUL*, *AaLFY,* and *AaAP1* is upregulated (triangles). After the return to LD greenhouse conditions, the I1 and I2 zones of the inflorescence develop and the V1 branches flower. *PEP1* remains stably silenced in the shoot apex, and *AaFT1* expression is upregulated (triangle). The length of vernalization influences the expression levels of meristem identity genes during vernalization and of *PEP1* and *AaFT1* after vernalization. In contrast, the V3 branches show high *PEP1* and *AaTFL1* expression levels irrespective of the duration of vernalization .

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