Beyond flowering time: diverse roles of an APETALA2-like transcription factor in shoot architecture and perennial traits

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Summary

- Polycarpic perennials maintain vegetative growth after flowering. PERPETUAL FLOWERING 1 (PEP1), the orthologue of FLOWERING LOCUS C (FLC) in Arabis alpina regulates flowering and contributes to polycarpy in a vernalisation-dependent pathway. pep1 mutants do not require vernalisation to flower and have reduced return to vegetative growth as all of their axillary branches become reproductive.
- To identify additional genes that regulate flowering and contribute to perennial traits we performed an enhancer screen of pep1. Using mapping-by-sequencing, we cloned a mutant (enhancer of pep1-055, eop055), performed transcriptome analysis and physiologically characterised the role it plays on perennial traits in an introgression line carrying the eop055 mutation and a functional PEP1 wild-type allele.
- eop055 flowers earlier than pep1 and carries a lesion in the A. alpina orthologue of the APETALA2 (AP2)-like gene, TARGET OF EAT2 (AaTOE2). AaTOE2 is a floral repressor and acts upstream of SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 5 (AaSPL5). In the wild-type background, which requires cold treatment to flower, AaTOE2 regulates the age-dependent response to vernalisation. In addition, AaTOE2 ensures the maintenance of vegetative growth by delaying axillary meristem initiation and repressing flowering of axillary buds before and during cold exposure.
- We conclude that AaTOE2 is instrumental in fine tuning different developmental traits in the perennial life cycle of A. alpina.

Introduction

Plants have evolved different life history strategies. Annuals are monocarpic and set seed and senesce after flowering. Most perennials are polycarpic, being able to maintain vegetative growth from axillary meristems (AMs) and restricting senescence only to the reproductive branches (Amasino, 2009; Albani & Coupland, 2010). The distinction between annuals and perennials is underlined by several traits. Typically, most perennials have a prolonged juvenile phase during which they are not competent to respond to flower inductive stimuli. The polycarpic behaviour of perennial plants is a result of the asynchronous and differential behaviour of AMs as some commit to reproductive development, while others develop into vegetative branches or arrest growth after producing a few leaves and forming a bud (Tan & Swain, 2006; Costes et al., 2014; Park et al., 2017; Vayssières et al., 2020). Thus, understanding the molecular mechanisms underlying the activity and fate of AMs can give insight into plant life history evolution.

Comparative studies between two Brassicaceae models, the annual Arabidopsis thaliana and the polycarpic perennial Arabis alpina have been successfully used as a tool to dissect at the molecular level the evolutionary changes driving the adoption of the annual or perennial life histories. These studies have demonstrated that several genes that regulate flowering time in Arabidopsis thaliana have similar and unique roles in A. alpina and, most importantly, that the A. alpina specific roles contribute to the perennial life cycle. One characteristic example is the MADS box transcription factor FLOWERING LOCUS C (FLC), a key floral repressor in the vernalisation pathway, in which prolonged cold exposure promotes flowering by stably silencing FLC mRNA levels in Arabidopsis thaliana (Michaels & Amasino, 1999; Sheldon et al., 2000). The FLC orthologue in A. alpina, PERPETUAL FLOWERING1 (PEP1) also regulates flowering in response to cold (Wang R. et al., 2009). pep1 mutants flower without cold exposure, whereas wild-type plants have an obligate vernalisation requirement to flower (Wang R. et al., 2009). The expression pattern of PEP1 differs from FLC in Arabidopsis thaliana so that PEP1 mRNA levels are upregulated in axillary branches after vernalisation to maintain vegetative development and sustain the polycarpic growth habit of A. alpina (Wang R. et al., 2009; Lázaro et al., 2018). Other flowering time
regulators that typically control flowering through the age pathway, such as the A. alpina orthologues of APETALA2 (PERPETUAL FLOWERING2, PEP2), SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE15 (AaSPL15) and TERMINAL FLOWER1 (AaTFL1) also have similar and unique roles in A. alpina (Wang et al., 2011; Bergonzi et al., 2013; Hyun et al., 2019; Lázaro et al., 2019). PEP2, AaTFL1 and AaSPL15 determine the age at which plants become competent to cold treatment, control the duration of cold exposure required for flowering and (similar to PEP1) ensure maintenance of vegetative development in some axillary branches (Wang R. et al., 2009, 2011; Bergonzi et al., 2013; Hyun et al., 2019; Lázaro et al., 2019). The age and the vernalisation pathways are also integrated in different ways. PEP1 binds to the AaSPL15 locus, whereas PEP2 acts upstream of PEP1 to ensure the upregulation of PEP1 mRNA levels in axillary branches after vernalisation (Hyun et al., 2019; Lázaro et al., 2019).

Similarly, microRNA156 (miR156) that targets most SPLs in the age pathway has similar and unique roles in A. alpina and in Arabidopsis thaliana. miR156 has similar expression patterns in both species and its accumulation is high in young seedlings and declines as plants get older (Wang J.W. et al., 2009a; Bergonzi et al., 2013). miR156 in A. alpina also regulates flowering in the age pathway, which is evident from A. alpina transgenics that constitutively express MIR156b and do not flower in response to vernalisation, whereas the ones with suppressed miR156 activity by target mimicry (35S:MIM156) respond to vernalisation at a younger age (Bergonzi et al., 2013). The miR156-SPL module also regulates other traits such as plastochron, branching and AM initiation (Wang et al., 2008; Wu et al., 2009; Tian et al., 2014; Jung et al., 2016; Gao et al., 2018). These additional roles of miR156 and some of its targets have been reported in Arabidopsis thaliana and other species but not in A. alpina (Bergonzi et al., 2013; Hyun et al., 2019). miR172 and its targets, AP2 and the AP2-like genes SCHLAFMUTZE (SMZ), SCHNARCHZAPFEN (SNZ), TARGET OF EAT1-3 (TOE1-3) are also placed in the age pathway (Aukerman & Sakai, 2003; Wu et al., 2009). In Arabidopsis thaliana, the accumulation of miR172 increases progressively as plants age and follow an opposite pattern to that of miR156 (Aukerman & Sakai, 2003; Wu et al., 2009). These complementary expression patterns of miR156 and miR172 are not observed in A. alpina, although one of the miR172 targets (A. alpina AP2/PEP2) regulates age-dependent flowering in response to vernalisation (Bergonzi et al., 2013; Lázaro et al., 2019).

Our experiments have previously demonstrated that the pep1-1 mutant still responds to different durations of vernalisation, suggesting that there are other repressors regulating flowering in parallel with PEP1 (Lázaro et al., 2018). Here we aimed at identifying such additional floral repressors and at characterising their contribution to the perennial life cycle. We performed an enhancer screen of pep1-1 and identified a mutant, enhancer of pep1 055 (eop055). We demonstrated that eop055 is a toe2 mutant in A. alpina. To characterise the role of AaTOE2 in the A. alpina life cycle and perennial traits, we introgressed the Aatoe2 mutation in the wild-type background that has an obligate vernalisation requirement to flower and an active PEP1 allele. We demonstrated that AaTOE2 regulates the age-dependent response to vernalisation and shoot architecture. We have previously shown that shoot architecture in A. alpina is organised in zones of differential bud activity and fate according to position on the plant named as V1 (zone of flowering axillary branches), V2 (zone of dormant buds) and V3 (zone of vegetative axillary branches) (Lázaro et al., 2018; Vayssières et al., 2020). Here we demonstrate that AaTOE2 contributes to shoot architecture by repressing flowering in axillary branches and determining the number of metamers in each zone. Many of these roles have been tailored to contribute to the perennial growth habit and have not been described for TOE2 in Arabidopsis thaliana.

**Materials and Methods**

**Plant material, growth conditions and phenotypic analysis**

The pep1-1 mutant does not require vernalisation to flower and was previously obtained after the mutagenesis of the A. alpina accession Pajares (Wang R. et al., 2009b). Here, c. 6000 pep1-1 seeds were mutagenised with 0.35% ethyl methanesulphonate (Sigma) for 8–9 h. In total, 18 000 M2 seedlings (1500 M1 families) were screened in under long day (LD, 16 h:8 h, light:dark) glasshouse and mutants that flowered earlier than pep1-1, named as enhancers of pep1 (eop), were selected. The phenotype of mutants was subsequently confirmed in the M3 generation. The eop055 mutant was crossed with wild-type Pajares, which requires vernalisation to flower, and an introgression line (Aatoe2_IL) was selected from the F2 segregating population that carried the single nucleotide polymorphisms (SNP) in AaTOE2 and lacked the SNP in PEP1 (responsible for the pep1-1 phenotype). Primers used for genotyping are listed in Supporting Information Table S1. The Arabidopsis thaliana toe2-1 mutant (SALK_065370) was obtained from the Nottingham Arabidopsis Stock Centre (NASC).

For most experiments, seeds were first stratified in darkness for 3–5 d at 4°C. Depending on the experiments, plants were grown in a long day (LD) or short day (SD) (8 h:16 h, light:dark) glasshouse with light intensity varying from 200–500 μmol m⁻² s⁻¹ and temperature at c. 22°C. Vernalisation experiments were performed in a SD growth chamber at 4°C and light intensity 14 μmol m⁻² s⁻¹.

Flowering time experiments with pep1-1, eop055 and Arabidopsis thaliana toe2-1/IL genotypes were performed by scoring total leaf number at flowering. Aatoe2_IL and Pajares plants were characterised for several traits:

1. Ability to flower without vernalisation, by scoring flowering in plants growing in a LD glasshouse for up to 22 wk.
2. Juvenile phase, by growing plants for 3 wk in a LD glasshouse, cold treating them for 12 wk and scoring flowering after plants were returned to LD glasshouse conditions.
3. Duration of vernalisation required for flowering, by exposing 8-wk-old plants grown in a LD glasshouse to 8, 12 and 18 wk of vernalisation and scoring flowering after they were returned to LD glasshouse conditions.
(4) Shoot architecture, by scoring bud activity and fate in every leaf node of flowering plants exposed to 12 wk of vernalisation and subsequently grown for 8 wk in a LD glasshouse.

(5) Leaf initiation rate, by monitoring total visible leaf number from 2–8 wk in a LD glasshouse.

(6) AM initiation, by monitoring the presence or absence of a bud or an AM in each leaf node under the stereomicroscope.

All experiments were performed with at least 12 plants. Pheno-
typic data were analysed using Student’s t-test.

Mapping-by-sequencing and fine mapping

A BC1F2 mapping population was created by backcrossing eop055 to pep1-1 and scored for flowering time in an LD glasshouse. From 450 eop055BC1F2 plants, 109 flowered with a similar number of leaves as eop055, suggesting that a single gene was segregating for flowering time. Genomic DNA was extracted from a pool of flower buds collected from 84 eop055BC1F2 early flowering plants. Flower buds from 48 pep1-1 plants were also harvested for DNA extraction. Genomic DNA samples were sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA, USA) at the Max Planck Genome Center Cologne (Germany) and yielded 203 042 704 reads for pep1-1 and 200 336 368 reads for eop055BC1F2. In total, 190 190 100 (93.67%) reads from pep1-1 and 194 532 678 (95.42%) reads from eop055BC1F2 were aligned to the A. alpina V5 reference genome using Bowtie (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome.

We applied SAMTOOLS and BCFTOOLS to identify SNPs between samples and the A. alpina reference genome (Li et al., 2009). SHOREMAP v.3.6 was used to extract the SNPs and to visualise the allele frequency (AF) for EMS-induced mutations in eop055BC1F2. In total, 190 190 100 (93.67%) reads from pep1-1 and 194 532 678 (95.42%) reads from eop055BC1F2 were aligned to the A. alpina V5 reference genome using Bowtie 2 (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome. We applied SAMTOOLS and BCFTOOLS to identify SNPs between samples and the A. alpina reference genome (Li et al., 2009). SHOREMAP v.3.6 was used to extract the SNPs and to visualise the allele frequency (AF) for EMS-induced mutations in eop055BC1F2. In total, 190 190 100 (93.67%) reads from pep1-1 and 194 532 678 (95.42%) reads from eop055BC1F2 were aligned to the A. alpina V5 reference genome using Bowtie 2 (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome. A. alpina V5 reference genome using Bowtie (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome. A. alpina V5 reference genome using Bowtie 2 (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome. A. alpina V5 reference genome using Bowtie 2 (Langmead & Salzberg, 2012; Jiao et al., 2017), resulting in an average coverage of 72.4 and 72.7 for the respective resequenced genome.

SNPs with a quality score lower than 40 were filtered out of the resequencing data. Mapping-by-sequencing data were analysed using Student’s t-test.

To fine map the EOP055 locus, 11 molecular markers were developed based on SNPs detected by the resequencing data to be polymorphic between eop055BC1F2 and pep1-1. For fine mapping, we screened 503 BC1F2 plants. Primers used for fine mapping are listed in Table S1. Plant transformation in A. alpina and Arabidopsis thaliana was performed using the Agrobacterium-mediated floral dip method (Clough & Bent, 1998).

RNA extraction and expression studies

For main shoot samples, apices from 12–50 plants per sample were combined for all experiments. For V3 bud samples, buds from leaf axils 21–26 were harvested under a stereomicroscope from 24–48 plants per sample. Total RNA was isolated from plant tissues using the miRNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to DNase treatment using DNA-free DNase (Ambion). For normal qRT-PCR reactions, 1 µg of total RNA was used as a template for the synthesis of cDNA by reverse transcription with SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT) (18) primer. For qRT-PCR of miRNA reactions, 200 ng of total RNA was used for reverse transcription using specific key primers. Quantitative PCRs to follow gene expression were carried out as described in Lázaro et al. (2019) and for miRNAs as described in Bergonzí et al. (2013). For each sample, three technical replicates and three biological replicates were analysed. Transcript accumulation of selected genes in A. alpina samples was normalised using the A. alpina PROTEIN PHOSPHATASE 2A (AaPPT2A) and RAN GTPASE 3 (AaRAN3) and in Arabidopsis thaliana samples using ACTIN. qPCRs for miR156 and miR172 were normalised using AatnoR101. Expression data were analysed using Student’s t-test. Primers used in this analysis are listed in Table S1.

RNA-seq

For transcriptome analysis we dissected main shoot apices and V3 buds from Aatoe2-1L and wild-type plants as described above in three biological replicates per sample. RNA was extracted using the miRNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to DNase treatment using DNA-free DNase (Ambion). PolyA enrichment, library preparation and sequencing were performed at the Max Planck Genome Center, Cologne (Germany). PolyA RNA was isolated from 1 µg of total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Frankfurt am Main, Germany) and used for library construction with NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Sequencing was performed on an Illumina HiSeq 3000 system with 1 × 150-bp single-read lengths.

Sequencing reads were mapped and aligned to the reference genome (A. alpina V5) using STAR software (Dobin et al., 2013). Raw read counts per gene were quantified with HTSEQ v.0.5.4p1. The differentially expressed genes with more than a two-fold change and an adjusted P-value below 0.05 were
Gene Ontology (GO) enrichment analysis was performed using the DAVID resource (Huang et al., 2008). DEGs with homologues in *Arabidopsis thaliana* were used as input data for GO analysis. GO terms with a Benjamini-Hochberg adjusted P-value lower than 0.05 were plotted using the R-based graphical tool BACA (Fortino et al., 2015).

**Results**

**EOP055** is the orthologue of the AP2-like gene TOE2 and represses flowering in *A. alpina* and *Arabidopsis thaliana*

To identify additional genes that regulate flowering and perennial traits in *A. alpina*, we used an enhancer screen in the *pep1-1* background. The enhancer of *pep1-1 O55* (*eop055*) mutant flowered earlier than *pep1-1* in LD and SD glasshouse conditions (Fig. 1a,b). We combined mapping-by-sequencing and classical map-based cloning to identify the causal mutation in *eop005* (Hartwig et al., 2012; Schneeberger, 2014; Andrés et al., 2015).

SHOREMAP analysis indicated that this causal mutation in *eop055* was located in a broad region spanning 40 Mb on the *A. alpina* chromosome 8 (Figs 1c, S1a; Table S2). We reduced the original mapping region (defined between markers M1 and M8) by screening 503 BC1F2 plants with markers designed based on SNPs from SHOREMAP. Fine mapping narrowed down the causal mutation to a 433 kb genomic region between markers M5 (41 140 691 bp) and M10 (41 574 114 bp). The detailed cloning approach is presented in Fig. S1. (d) Scheme of the *AaTOE2* locus (*Aa*G234670) showing the position of the mutation and the single nucleotide polymorphism (SNP) found in *eop055*. In (a) bar, 5 cm. In (b) n = 24-28. ** Indicates significant differences at P < 0.01 according to Student’s t-test. The boxes indicate the interquartile range (IQR), the horizontal line in the middle is the median, the vertical lines (whiskers) correspond to the maximum or minimum value within 1.5 × IQR and the dots are the sample values. In (d) black boxes indicate the exons and white boxes indicate the untranslated regions. Red lines indicate the AP2 domains and the orange line indicates the *miR172* binding site. G171D indicates the nonsynonymous amino acid substitution in the *eop055* mutant from glycine (G) to aspartic acid (D) at amino acid 171. TOE2, TARGET OF EAT 2; EOP055, ENCHANCER OF PEPI; PEPI, PERPETUAL FLOWERING 1.
contained a conserved miR172 binding site (Figs 1d, S3a). Overall, these results suggested that Aa_G234670 is the orthologue of TOE2.

The SNP in AaTOE2 (Aa_G234670) causes a nonsynonymous amino acid substitution from glycine (G) to aspartic acid (D) at amino acid 171 within the first AP2 domain (AP2-R1) (Fig. S3b; Table S2). To verify whether the SNP in AaTOE2 was responsible for the early flowering phenotype in eop055, we complemented the eop055 mutant using the wild-type 7.8 kb AaTOE2 genomic region spanning 4128 bp upstream of its translational start and 776 bp downstream of its translational stop. Three independent T3 homozygous lines with a single copy insertion were selected and showed delayed flowering compared with eop055 (Fig. 2a,b). We also overexpressed the wild-type AaTOE2 and mutated Aatoe2 allele in Arabidopsis thaliana toe2-1. AaTOE2 complemented the early flowering phenotype of the toe2-1 mutant, whereas Aatoe2 did not (Figs 2c,d, S4). These results suggested that the role of AaTOE2 in flowering is conserved in Arabidopsis thaliana and that the G171D amino acid substitution affects its function. Taken together, these results demonstrate that the eop055 mutant phenotype was caused by the loss of function of AaTOE2, which represses flowering in A. alpina and Arabidopsis thaliana.

In Arabidopsis thaliana, TOE proteins regulate flowering through the photoperiod pathway (Yant et al., 2009; Zhang et al., 2015). In A. alpina, however, the role of the photoperiod pathway in flowering is not very clear and wild-type plants initiate floral buds during cold treatment under SDs (Wang R. et al., 2009). Flowering in response to prolonged cold treatment depends on plant age. Wild-type plants have a juvenile phase of 5 wk so that only plants older than 5 wk are competent to flower in response to prolonged cold treatment (Wang et al., 2011). We found that transcript accumulation of AaTOE2 in wild-type plants was high in the main shoot apex of vegetative plants and is transiently silenced during cold exposure. However, this is true only for plants that can initiate flowering during cold (adult, 8-wk-old) and not for cold-treated young seedlings (juvenile, 3-wk-old) which remain vegetative (Fig. 3). Transcript accumulation of AaTOE2 in AMs (within the V3 zone), which also remain vegetative, is similar during and after cold exposure (Fig. 3a). These results suggested AaTOE2 is a floral repressor that is highly expressed in vegetative apices and is differentially regulated spatiotemporally.

![Figure 2](image-url)

**Fig. 2** AaTOE2 complements the early flowering phenotypes of the Arabis alpina eop055 and Arabidopsis thaliana toe2-1 mutants. (a, b) Complementation of eop055. (a) Flowering phenotypes of pep1-1, eop055 and three independent pAaTOE2:G:AaTOE2 eop055 transgenic lines grown in long days (LDs) for 57 d. (b) Total leaf number at flowering of pep1-1, eop055 and transgenic lines. (c, d) Complementation of Arabidopsis thaliana toe2-1 mutant. (c) Flowering phenotypes of Col, toe2-1 and transgenic lines constitutively expressing the wild-type allele (35S:Aatoe2) and mutated allele (35S:Aatoe2) of AaTOE2. The picture was taken 35 d after sowing in LDs. (d) Total leaf number at bolting of Col, toe2-1 and transgenic lines in LDs. AaTOE2 transcript accumulation in Arabidopsis thaliana transgenic lines is presented in Supporting Information Fig. S4. Bars: (a) 5 cm; (c) 2 cm. n = 12–15. ns, not significant. **Indicates significant differences compared with eop055 in (b) and toe2-1 in (d) at P < 0.01 according to Student’s t-test. The boxes and the horizontal line in the middle represent the interquartile range (IQR) and the median, respectively. The whiskers correspond to the maximum or minimum value within 1.5 × IQR, the dots indicate the sample values. TOE2, TARGET OF EAT 2.
Aatoe2_IL responds to cold treatment at a young age

To understand the function of AaTOE2 in A. alpina we introgressed the Aatoe2 allele into the wild-type background by crossing eop055 with Pajares and selecting an Aatoe2_IL homozygous for Aatoe2 which contained a functional PEP1. We have previously shown that AP2 in A. alpina (PEP2) regulates flowering and perennial traits through the age pathway and plays a role in the vernalisation pathway by enhancing PEP1 mRNA levels (Lázaro et al., 2019). The pep2-1 mutant does not require cold treatment to flower and has reduced PEP1 mRNA levels compared with the wild-type (Bergonzi et al., 2013). We checked whether AaTOE2 contributed to the obligate vernalisation requirement of A. alpina. We grew Aatoe2_IL plants in a LD glasshouse and tested whether they could flower without cold treatment. Similar to the wild-type, Aatoe2_IL plants did not flower in LDs. Similar to the wild-type, Aatoe2_IL plants did not flower in LDs. This result suggested that AaTOE2 does not regulate flowering through PEP1.

AP2-like genes regulate the juvenile to adult phase change and TOE2 mRNA levels in Arabidopsis thaliana decrease in older plants (Jung et al., 2007; Wang, 2014). We tested AaTOE2 transcript accumulation in the main shoot apex of plants growing for 2–8 wk in a LD glasshouse without cold treatment. AaTOE2 transcript accumulation did not decrease as plants became older and resembled the expression patterns of PEP2 published previously (Fig. S5a; Bergonzi et al., 2013). Transcript accumulation of PEP1 in apices was similar between wild-type and Aatoe2_IL plants at all ages tested confirming again that AaTOE2 does not
regulate flowering through PEP1 (Fig. S5i). Interestingly, PEP2 mRNA levels were slightly upregulated in Aatoe2_IL plants at most time points tested (Fig. S5j). AP2 proteins regulate flowering through several feedback mechanisms to upstream components in the age pathway (Yant et al., 2010). To test whether this is also the case in A. alpina, we also analysed the accumulation of miR156 and some of its target genes (AaSPL3/4/5, AaSPL9/15 and AaSPL10) (Figs 5a,b, S5b–f). miR156 accumulation was similar in both Aatoe2_IL and wild-type plants and reduced with plant age (Fig. 5a). Among all genes tested, only the accumulation of AaSPL5 transcripts differed in an age-dependent pattern between the Aatoe2_IL and wild-type (Figs 5b, S5b–f). AaSPL5 mRNA levels were higher in Aatoe2_IL and increased in the apices of older plants (Fig. 5b). In Arabidopsis thaliana, SPL3/4/5 transcript levels are upregulated in transgenic plants constitutively expressing 35S:MIR172b and are increased in double and triple mutants of AP2-like genes (Jung et al., 2011). To test whether miR172 accumulation was also coupled with the changes in AaSPL5, we compared miR172 levels between Aatoe2_IL and wild-type plants (Fig. 5c). The results we observed were opposite to what we would have expected based on studies in Arabidopsis thaliana and also in comparison with previous findings in A. alpina (Jung et al., 2011; Bergonzi et al., 2013). We found that miR172 accumulation increased with plant age and that in Aatoe2_IL it was lower compared with the wild-type (Fig. 5c). This result also correlated with the slight reduction in PEP2 mRNA levels in Aatoe2_IL apices (Fig. S5j). In Arabidopsis thaliana, SPL3/4/5 promote flowering by transcriptionally activating the floral meristem identity genes such as AP1, LFY and FUL in conjunction with the transcription factor FD (Yamaguchi et al., 2009; Wang J.W. et al., 2009a; Jung et al., 2016).

In Aatoe2_IL plants growing in a LD glasshouse, the expression of meristem identity genes did not differ compared with the wild-type (Fig. S5g,h). These results were in agreement with the fact that Aatoe2_IL plants do not flower without cold treatment.

The effect of the age pathway in A. alpina is obvious after cold treatment (Wang et al., 2011; Bergonzi et al., 2013; Hyun et al., 2019; Lázaro et al., 2019). To test whether AaTOE2 regulated the age-dependent response to vernalisation, we vernalised 3-wk-old wild-type and Aatoe2_IL seedlings for 12 wk and tested whether they flowered (Fig. 5d). Aatoe2_IL seedlings flowered in response to cold treatment, whereas wild-type plants did not, suggesting that AaTOE2 regulated the response to vernalisation in an age-dependent manner (Fig. 5d). This result is also in agreement with the age-dependent differences observed in the silencing of AaTOE2 mRNA levels by cold.

Overall, these results suggested that AaTOE2 regulates the age-dependent response to vernalisation and influences miR172 and AaSPL5 mRNA levels at the shoot apical meristem (SAM).

Aatoe2_IL has more flowering axillary branches compared to the wild-type

In A. alpina, all genes identified so far that regulate the age-dependent response to vernalisation also contribute to the duration of vernalisation required for flowering and determine the fate of...
AaTOE2 extends the duration of cold treatment required for flowering in adult Arabis alpina plants. (a) Days to flowering of wild-type (WT) and Aatoe2_IL plants exposed to different durations of cold treatment. Flowering time was measured in plants grown for 8 wk in long days (LDs), exposed to 8, 12 or 18 wk of cold treatment and subsequently returned to a LD glasshouse. Days to flowering were counted from the day that plants were transferred to LD glasshouse conditions after vernalisation. (b) Flowering phenotype of WT and Aatoe2_IL plants cold-treated for 8 wk, followed by 4 wk in LDs. The boxes indicate the interquartile range (IQR), the horizontal line in the middle is the median, the vertical lines (whiskers) correspond to the maximum or minimum value within 1.5 × IQR and the dots are the outliers. **Indicates significant differences between WT and Aatoe2_IL at each cold treatment at $P < 0.01$ according to Student’s t-test. Bar, 5 cm. n = 12. TOE2, TARGET OF EAT 2.

To understand how AaTOE2 regulates flowering in the main shoot and V3 axillary branches, we performed a transcriptome analysis. We exposed 8-wk-old Aatoe2_IL and wild-type plants to 4°C for 8 wk to induce flowering (Fig. S6), and dissected their SAM and V3 buds. Principal component analysis (PCA) analysis indicated that the variable explaining most of the variability (PC1) was tissue, which separated the SAM and V3 bud samples, whereas PC2 separated the samples according to their genotype (Fig. 8a). Most differentially expressed genes (DEGs) between genotypes were detected in V3 buds, with 422 genes being differentially expressed in V3 buds compared with 309 genes in the SAM (Fig. 8b; Table S3). In both tissues, GO analysis indicated enrichment in several biological processes, including response to various stresses and regulation of cellular, metabolic and developmental processes (Fig. S9). Several genes involved in stress responses were differentially expressed between Aatoe2_IL and the wild-type (Fig. 8c). In the SAM, many genes involved in the reproductive development were upregulated in the Aatoe2_IL (e.g. AP1, AGL5 and DYT1) (Fig. 8c; Table S3; Alejandra Mandel et al., 1992; Savidge et al., 1995; Zhang et al., 2006). This result suggested that Aatoe2_IL initiated flowering during cold treatment earlier than wild-type plants. Among the genes upregulated specifically in the V3 buds of Aatoe2_IL, we detected again SPLs (SPL3, 4, 5 and 10) and also several TCP genes (AaTCP4, 10, 24) known to be involved in leaf growth, flower development, flowering time and jasmonic acid (JA) metabolism (Fig. 8c; Table S3; Wu et al., 2009; Nag et al., 2009; Danisman et al., 2012; Xu et al., 2016; Jung et al., 2016; Lucero et al., 2017; Chang et al., 2018). In the V3 buds, miR172 accumulation was higher in Aatoe2_IL, whereas miR156 was lower compared with the wild-type (Fig. S10a). These results differed from the miR172 subapical axillary branches (Wang et al., 2011; Hyun et al., 2019; Lázaro et al., 2019). To test whether this also stands for AaTOE2, we exposed 8-wk-old Aatoe2_IL plants to 8, 12 or 18 wk of cold treatment and tested flowering time after transfer to an LD glasshouse. Aatoe2_IL plants flowered earlier than wild-type after different durations of cold treatment (Fig. 6a). The biggest effect was observed in plants cold-treated for 8 wk when wild-type plants did not flower but Aatoe2_IL plants did (Fig. 6a, b). This result suggested that AaTOE2 regulates the duration of vernalisation required for flowering in A. alpina. Interestingly, the shoot apical meristems of wild-type and Aatoe2_IL plants were both reproductive at the end of the 8 wk of cold treatment (Fig. S6), suggesting that AaTOE2 antagonises the commitment to flowering after vernalisation.

We also scored shoot architecture in plants exposed to 12 wk of vernalisation, as it was previously shown that this was sufficient to ensure the formation of zones with differential bud activity and fate (Lázaro et al., 2018; Vayssières et al., 2020). As previously published, in wild-type plants the axillary branches at lower leaf nodes (V1) flowered in response to vernalisation and differed from inflorescence branches (I) because they only senesced partially after flowering (Fig. 7b; Wang R. et al., 2009b; Lázaro et al., 2018; Vayssières et al., 2020). Wild-type A. alpina plants also maintained a zone of dormant axillary buds (V2) and vegetative axillary branches (V3), which are vital to maintain the inflorescence was not influenced in Aatoe2_IL plants (Figs 7c, S8).
expression patterns we obtained in the main shoot apex of vegetative plants in a LD glasshouse (Fig. 5c), although AaSPL5 mRNA levels followed a similar trend and were higher in both vegetative shoot apices and V3 buds of Aatoe2_IL plants (Figs 5, S10). V3 buds are formed during vernalisation (Ponraj & Theres, 2020) and therefore can commit to reproductive development if they acquire competence. To check whether V3 buds of Aatoe2_IL plants become reproductive during vernalisation, we cold-treated plants for 12 wk and looked at axillary buds in the V3 zone (Fig. S11). In wild-type plants, the axillary buds were vegetative whereas the ones from Aatoe2_IL plants had the characteristic dome shape of a flowering meristem (Fig. S11). These results suggested that flowering in V3 buds of Aatoe2_IL plants is initiated during vernalisation and that the increase of miR172 accumulation and AaSPL5 mRNA levels correlated with the initiation of flowering.

Taken together, these results suggested that AaTOE2 represses flowering in the SAM and V3 buds during vernalisation.

Initiation rate of AMs and leaves are affected in Aatoe2_IL

In Aatoe2_IL plants, the number of basal flowering axillary branches (corresponding to V1) was increased (Fig. 7b,c). As V1 axillary branches are formed before cold exposure, we compared...
the number of axillary branches between genotypes before vernalisation (Vayssières et al., 2020). Here, 8-wk-old Atoe2_IL plants had 12.3 ± 2.0 branches compared with the wild-type that had only 7.2 ± 1.2 branches (Figs 9a, S12b). Components of the age pathway regulate other traits, including leaf initiation rate (plastochron) and branching (Wang & Wang, 2015). The toe2 mutants do not have a clear plastochron phenotype and in 35S: MIR172b plants the plastochron is not affected (Jung et al., 2011). Nevertheless, in the triple mutant spL3/4/5 plastochron is reduced (Jung et al., 2016). We followed leaf initiation in plants grown for up to 8 wk in LDs, and by contrast to the branching phenotype, we detected a statistically significant reduction in the leaf initiation rate in Atoe2_IL plants compared with the wild-type (Fig. S12).

We also had a closer look at the branching phenotype in the Atoe2_IL by scoring the presence or absence of an AM/bud in each leaf node at different developmental stages (Fig. 9b–d). In wild-type plants, it has recently been reported that during vegetative development the leaf axils close to the SAM do not contain an AM (Ponraj & Theres, 2020). These leaf axils fill up during vernalisation when the SAM transitions to reproductive development (Ponraj & Theres, 2020). Atoe2_IL plants have a reduced number of empty leaf axils before and during vernalisation, suggesting that AM initiation is enhanced compared with the wild-type, which results in the increased number of basal axillary branches (Fig. 9e,f). Empty apical leaf axils in plants before vernalisation serve as sites where V3 buds will be formed during vernalisation (Ponraj & Theres, 2020), explaining the reduced number of V3 axillary branches in Atoe2_IL plants (Fig. 9e,g,h).

These results indicated that AaTOE2 regulates AM formation and shoot architecture in A. alpina.

Discussion

In perennials, regrowth from one year to the next is ensured by vegetative axillary or apical meristems. Therefore, perennial
Fig. 9 AaTOE2 enhances the number of apical leaf primordia without an axillary meristem in vegetative Arabis alpina plants. (a) Axillary branches in Aatoe2_IL and wild-type (WT) plants grown for 8 wk in a long day (LD) glasshouse. (b–d) Scanning electron microscopy (SEM) images of (b) an axillary bud, (c) an axillary meristem (AM) and (d) an empty leaf axil. (e) Schematic representation of axillary bud formation of WT and Aatoe2_IL plants grown for 8 wk in an LD glasshouse (8wLD), vernalised for 4 wk or 8 wk. Each column represents a single plant and each box within a column represents a single leaf axil numbered from the bottom to the top of each plant. Green indicates the presence of a branch, light green of an axillary bud/AM and beige of a leaf axil without an AM. (f–h) Number of (f) axillary branches, (g) axillary buds/AMs, (h) leaf axils without an AM in plants presented in (e). ** Indicates significant differences between WT and Aatoe2_IL plants at each time point at P < 0.01 according to Student's t-test. Bars: (a) 3 cm; (b–d) 100 μm. In (e) and (f) n = 12. Arrows indicate an axillary bud in (b), an axillary meristem in (c) and an empty leaf axil in (d). TOE2, TARGET OF EAT 2.
species usually show a complex shoot architecture that can consist of dormant buds and axillary vegetative and/or flowering branches (Costes et al., 2014; Varssier et al., 2020). This strategy of splitting resources towards vegetative and reproductive development affects yield (Bazzaz et al., 1987). Thus, although perennial crops are considered to be the future in sustainable agriculture, they could eventually have a reduced yearly yield compared with established annual crops (Cox et al., 2006). Understanding shoot architecture in perennials can provide us with tools to improve crop yield. In Arabidopsis thaliana, the flowering phenotype of mutants such as toe2 is not very strong due to functional redundancy with other AP2-like genes (Aukerman & Sakai, 2003; Jung et al., 2007; Zhai et al., 2015). We describe that in A. alpina, although it contains six AP2-like genes, the toe2 mutant has a strong early flowering time phenotype. Thus, we concluded that functional redundancy between AP2-like genes might be weaker in A. alpina. We also show that AaTOE2 contributed to the polycarpic growth habit by regulating the number and fate of axillary branches that will stay vegetative the following year. This role of AaTOE2 can affect the final yield as it modulates the balance between reproductive and vegetative development. AaTOE2 also controls the vernalisation response by regulating the age at which plants are sensitive to vernalisation as well as the duration of cold required for flowering. Hence, we concluded that AaTOE2 regulates different developmental traits that contribute to the perennial life cycle of A. alpina.

AaTOE2 regulates reproductive competence and the duration of cold treatment required for flowering in A. alpina

Wild-type Pajares plants flower only if exposed to cold treatment at an age older than 5 wk (Wang et al., 2011; Bergonzi et al., 2013). Similar to other species, in A. alpina miR156 and members of the SPL and AP2-subfamilies also regulate the competence to flower and determine the age at which plants are able to respond to vernalisation (Bergonzi et al., 2013; Teotia & Tang, 2015; Hyun et al., 2019; Lázaro et al., 2019). Transgenic A. alpina lines with reduced miR156 activity or overexpressing the miR156-resistant form of AaSPL15, flower when vernalised as young seedlings (grown for 2 or 3 wk before vernalisation) (Bergonzi et al., 2013; Hyun et al., 2019). We demonstrated that miR172 accumulation increased when A. alpina plants became older, resembling previous studies in Arabidopsis thaliana and other species, a mechanism regulated by SPL9, SPL10 and SPL3/4/5 (Aukerman & Sakai, 2003; Chuck et al., 2007; Wu et al., 2009; Xie et al., 2020). We also established a role for AaTOE2 in reproductive competence and placed AaSPL5 downstream of AaTOE2 (Fig. 10). In Arabidopsis thaliana SPL3/4/5 mRNA levels are upregulated in plants overexpressing miR172 and in toe1 toe2 double or toe1 toe2 smz triple mutants (Jung et al., 2011). Thus, it has been suggested that SPL3/4/5 are post-transcriptionally regulated by miR156 and transcriptionally by miR172 probably through the AP2-like genes (Jung et al., 2011). Our data indicated that in A. alpina there are parallel pathways to those described in Arabidopsis thaliana and feedback loops influence the expression of SPLs and miR172. However, by contrast with Arabidopsis thaliana, we detected that AaTOE2 in vegetative shoot apices positively regulates miR172 accumulation (Yant et al., 2010). SPL3/4/5 induced flowering by binding to the promoters of APETALA1, LEAFY, and FRUITFULL (Yamaguchi et al., 2009; Wang J.W. et al., 2009a; Jung et al., 2016). In our experiments, A. alpina plants do not initiate flowering without exposure to cold treatment. The upregulation of AaSPL5 mRNA in the SAM before cold exposure might be an indicator of enhanced sensitivity to flowering inductive stimuli. AaTOE2 also regulates the duration of cold treatment required to achieve floral

![Fig. 10 Schematic diagram showing the network of genes regulating flowering in the perennial Arabis alpina.](image-url)
commitment in cold, which is an important trait for alpine species adapted to environments with short growth seasons (Billings & Mooney, 1968; Aydelotte & Diggle, 1997; Wang R. et al., 2009b; Lázaro et al., 2018). Interestingly, all genes that regulate competence to flower in *A. alpina* also influence the duration of vernalisation required for flowering (Fig. 10). We have previously published that *A. alpina* mutants in floral repressors (e.g. AaTFFL1, PEP2) that regulate the age-dependent response to vernalisation also flower after a shorter duration of vernalisation, whereas mutants in floral promoters (e.g. AaSPL15) show strong floral reversion phenotypes after cold exposure (Wang et al., 2011; Bergonzi et al., 2013; Hyun et al., 2019). It is, therefore, very interesting to understand why and how these two traits are associated, especially because transcript accumulation of these genes does not differ between nonvernalised young and old seedlings (Wang et al., 2011; Bergonzi et al., 2013; Hyun et al., 2019). AP2 and AP2-like genes have been mainly studied for traits such as floral development, inflorescence and spikelet architecture (Greenwood et al., 2017; François et al., 2018; Gattolin et al., 2018; Harrop et al., 2019; Debernardi et al., 2020). Similarly, TFL1 and SPL15 have also been reported to play a role in inflorescence architecture in other species (Jiao et al., 2010; Jiang et al., 2013). For AaTOE2, we did not observe a role in inflorescence architecture. However, the fact that the development of floral buds in *A. alpina* occurs during vernalisation may explain the role of AaTOE2 in the duration of vernalisation required for flowering. Our transcriptome analysis also indicated that AaTOE2 may influence how plants modify developmental transitions due to environmental stresses, as previously suggested for other flowering time genes (Riboni et al., 2014; Kazan & Lyons, 2016).

AaTOE2 regulates shoot architecture by inhibiting AM initiation and by repressing flowering in AMs formed during cold treatment

Shoot architecture in *A. alpina* is organised in zones of consequent nodes that behave in a similar way and is determined by the differential response of AMs to cold treatment (Lázaro et al., 2018; Vayssières et al., 2020). We have previously shown that the maintenance of vegetative growth is determined after vernalisation by PEP1, whose mRNA levels are upregulated in the subapical axillary branches (V3 branches) to repress flowering (Wang R. et al., 2009b; Lázaro et al., 2018). AMs that will give rise to V3 axillary branches are formed during vernalisation in the axis of the leaf primordia close to the SAM (Ponraj & Theres, 2020). In the *Aatoe2* IL plants, V3 AMs initiate flowering during cold treatment, suggesting that the fate of these meristems is regulated during vernalisation by AaTOE2. The initiation of flowering in V3 buds was also correlated with a strong increase of miR172 levels, which differed to our results on the vegetative shoot apices. Thus, the network between different components in the age pathway may vary between vegetative and flowering apices. AaSPL5, probably, does not participate in this differential AaTOE2/miR172 feedback. The *A. alpina* AP2 (PEP2) regulates the fate of V3 branches by ensuring the upregulation of PEP1 mRNA levels after vernalisation (Lázaro et al., 2019). In this respect, AaTOE2 differs from PEP2 as it does not influence PEP1 expression levels. Nevertheless, AaTOE2 has an effect on flowering only in vernalised plants suggesting that PEP1 plays a prominent role repressing flowering in parallel to AaTOE2. PEP2 regulates flowering through the age and the vernalisation pathways (Lázaro et al., 2019). Thus, although AaTOE2 does not influence PEP1 mRNA levels, it could have overlapping functions with PEP2 through the age pathway.

AMs are initiated acropetally at a distance from the SAM and during vegetative development in *Arabidopsis thaliana* and *A. alpina* plants the leaf axes adjacent to the SAM lack an AM (Stirnberg et al., 1999; Reinhardt & Kuhlemeier, 2002; Greb et al., 2003; Ponraj & Theres, 2020). In *Arabidopsis thaliana*, the signal from the SAM that inhibits AM initiation may be auxin, as auxin resistant mutants (*axr1-3* and *axr1-12*) have a reduced number of leaf nodes without an AM (Ponraj & Theres, 2020). Empty leaf axes close to the SAM are filled with an AM soon after *A. alpina* plants are exposed to cold (Ponraj & Theres, 2020). Cold treatment also causes a reduction of endogenous IAA levels in *A. alpina* stems (Vayssières et al., 2020). These results suggested that in *A. alpina* similar mechanisms might contribute to the inhibition and release of growth in the subapical leaf axes. In this study, we observed that *Aatoe2* IL plants had an enhanced AM initiation rate and a reduced number of leaves without an AM. TOE2 or other AP2-like genes or even their regulator *mir172* have not been reported in *Arabidopsis thaliana* to play a role in AMs initiation and/or to have a branching phenotype (Mathieu et al., 2009; Wu et al., 2009; Yant et al., 2010; Zhai et al., 2015). Nevertheless, *miR156* and the SPLs contribute to branching and this role is also coupled with shortened plastochron lengths, which is again a trait that has not been reported for AP2-like genes and *mir172* (Wang & Li, 2008; Schwarz et al., 2008; Jung et al., 2011; Zhou et al., 2013). OsSPL14, which is the orthologue of the *Arabidopsis thaliana* SPL15, regulates tiller number in rice (Jiao et al., 2010; Luo et al., 2012). The SPLs in *Arabidopsis thaliana* (specifically SPL9 and SPL15) suppress LATERAL SUPPRESSOR (LAS) expression, which is a central regulator of AM initiation (Greb et al., 2003; Tian et al., 2014). The *spl9*-*splt5*-1 double mutants have accessory buds which is an indicator of LAS overexpression (Tian et al., 2014). We observed differences in the number of leaf axes dedicated to V1 and V3 zones in *Aatoe2* IL plants, which is a consequence of the enhanced AM initiation rate in vegetative plants before being exposed to cold treatment. *Aatoe2* IL plants had more basal V1 axillary branches and fewer branches dedicated to the V3 zone. This phenotype has not been reported in the *Aaspl15* mutant, suggesting that the role of AaTOE2 in AMs initiation is independent of AaSPL15 (Hyun et al., 2019).

Overall, we demonstrated that AaTOE2 regulates several developmental traits that contribute to the perennial life cycle of *A. alpina*. So far, the *miR156/SPL* module has been described as a regulatory hub due to its conservation in different species and the range of different phenotypes it influences (Wang, 2015;
Wang & Wang, 2015). We propose that AP2-like genes will be useful to breed for improved plant architecture and enhanced yield.

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Author contributions

YZ and MCA planned and designed the research, MCA did the mutagenesis screen, YZ performed most experiments for the mutagenesis screen, YZ and MCA wrote the manuscript. All authors read and commented on the manuscript.

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References

Research 15


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Cloning of the cop055 mutant.

Fig. S2 Phylogenetic relationship of the AP2-like family in Arabis alpina and Arabidopsis thaliana.

Fig. S3 Aa_G234670 is the Arabis alpina orthologue of TOE2.

Fig. S4 AaTOE2 is constitutively expressed in Arabidopsis thaliana toe2-1 complementation lines.

Fig. S5 Transcript accumulation of several flowering time regulators does not differ between Arabis alpina wild-type and Aatoe2-IL plants grown in continuous long day conditions.

Fig. S6 Scanning electron microscopy images of the main shoot apex from wild-type and Aatoe2-IL plants vernalised for 8 wk.

Fig. S7 V1 axillary branches of vernalised Arabis alpina wild-type and Aatoe2-IL plants after they were returned for 20 wk to a long day glasshouse.

Fig. S8 Inflorescence architecture of Arabis alpina is not influenced in Aatoe2-IL plants.

Fig. S9 Gene ontology enrichment analysis of differentially expressed genes detected between wild-type and Aatoe2-IL plants in two different tissues: the main shoot apical meristem and V3 axillary buds.

Fig. S10 Transcript accumulation of AaTOE2, AaSPL5, miR172 and miR156 in V3 buds of Arabis alpina wild-type and Aatoe2-IL plants.

Fig. S11 V3 buds in Arabis alpina Aatoe2-IL plants transitioned to flowering during vernalisation.

Fig. S12 Plastochron and number of axillary branches are increased in Arabis alpina Aatoe2-IL plants.

Table S1 Primers used in this study.

Table S2 List of single nucleotide polymorphism within coding sequence regions in chromosome 8 for the Arabis alpina cop055 mutant.

Table S3 DEGs between Aatoe2-IL and wild-type in the main shoot apical meristem and V3 buds.

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