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Keep a distance to be different: axillary buds initiating at a distance from the shoot apical meristem are crucial for the perennial life style of *Arabis alpina*

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Summary

- In many seed plants, perennialism is achieved through axillary buds and side shoots that remain vegetative. This work aimed to analyse the pattern of axillary bud (AB) formation in the perennial model plant *Arabis alpina* and to study the role of the *LATERAL SUPPRESSOR* (*AaLAS)* gene.
- This study combines stereomicroscopic analysis with RNA sequencing to monitor the correlation between patterns of AB formation and gene expression. The role of *AaLAS* was studied using an RNAi approach.
- During vegetative development, ABs initiate at a distance from the SAM, whereas after floral induction, they initiate adjacent to the SAM. Dormant buds are established before the onset of vernalization. Transcript profiles of ABs initiated at a distance differed from those in the SAM, whereas those of buds initiated in close proximity were similar. Knock-down of *AaLAS* leads to the loss of dormant buds and vegetative side shoots, strongly compromising the perennial life cycle.
- AB formation is regulated differently during vegetative and reproductive development. New meristems that possess different gene expression profiles from those in the SAM are established at a distance from the SAM. *AaLAS* is essential for the perennial life cycle by modulating the establishment of dormant buds and vegetative side shoots.

Key words: *Arabis alpina*, axillary bud, axillary meristem, *LATERAL SUPPRESSOR*, perennial plant.

Introduction

Seed plants can be categorized into two major groups with respect to their life style: annual plants initiate flowering in all active shoot meristems in a single reproductive cycle (monocarpy) and die, whereas perennial plants live for many years and usually flower repeatedly (polycarpy) during their lifespan (Amasino, 2009). Flowering in perennial plants is restricted to a limited number of shoots, whereas other shoots/buds are maintained in a vegetative stage to continue the life cycle. Such vegetative shoots/buds also ensure the survival of the plant following damage, for example after herbivory, frost or fire.

During postembryonic development, the shoot apical meristem (SAM) regularly produces new phytomers, which consist of a leaf, an internode and an axillary bud (Lyndon, 2012). The initiation of AMs is differentially regulated during different phases of plant development. In the vegetative phase, *Arabidopsis thaliana* (*A. thaliana)* initiates AMs in an acropetal sequence at a distance from the SAM (Stirnberg *et al.*, 1999). After the onset of flowering, AMs initiate with an even distribution (Stirnberg *et al.*, 1999) or basipetally (Hempel & Feldman, 1994) in close proximity to the SAM. AM initiation is postulated to occur according to one of two conflicting hypotheses. The *de-novo* hypothesis states that AMs develop from completely or partially differentiated cells of the leaf base (McConnell & Barton, 1998). By contrast, the *detached meristem* hypothesis postulates that AMs develop from meristematic cells that are segregated from the SAM at the time of leaf initiation and never lose their meristematic potential (Steeves & Sussex, 1989). Recent results support the latter view (Wang & Jiao, 2018). For instance, in *A. thaliana*, continuous expression of the meristem marker *SHOOTMERISTEMLESS* (*STM*) in the leaf axil suggests developmental continuity with the SAM (Long & Barton, 2000; Shi *et al.*, 2016). To maintain their meristematic potential, AM progenitor cells are kept in a low-auxin environment (Q. Wang *et al.*, 2014; Y. Wang *et al.*, 2014) and divide infrequently (Rossmann *et al.*, 2015; Burian *et al.*, 2016).

The formation of AMs is modulated by a set of transcriptional regulators that includes *LATERAL SUPPRESSOR* (*Ls/LAS*) (Schumacher *et al.*, 1999; Greb *et al.*, 2003), *CUP-SHAPED COTYLEDON* (*CUC*) (Hibara *et al.*, 2006; Raman *et al.*, 2008) and *REGULATOR OF AXILLARY MERISTEMS* (*RAX*) (Keller *et al.*, 2006; Müller *et al.*, 2006). *LATERAL SUPPRESSOR* encodes a GRAS (GIBBERELLIN-ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA), SCARECROW (SCR)) protein, whose regulatory role in AM formation is conserved among tomato (Schumacher *et al.*, 1999), *A. thaliana* (Greb *et al.*, 2003) and many other species (Li *et al.*,

2003; Mizzotti *et al.*, 2017). In *A. thaliana* and tomato, *LAS* transcripts accumulate in a band-shaped domain at the adaxial side of young leaf primordia and loss of *LAS*/*Ls* function compromises AM formation during vegetative development (Schumacher *et al.*, 1999; Greb *et al.*, 2003; Rossmann *et al.*, 2015). The orthologues of *LAS* in rice (*MOC1*) and snapdragon (*ERA*) regulate AM formation during both vegetative and reproductive development (Li *et al.*, 2003; Mizzotti *et al.*, 2017).

After initiation, AMs produce a few leaf primordia that develop into an axillary bud (AB), which can either immediately grow into a side shoot or remain dormant, depending on its position on the plant, the developmental phase or environmental conditions (Grbić & Bleecker, 2000). Three types of bud dormancy have been described based on the signal that inhibits growth: paradormancy, ecodormancy and endodormancy (Lang, 1987). Paradormancy is imposed by an endogenous signal (e.g. apical dominance), ecodormancy is caused by environmental effects on bud activity, such as frost, which inhibits cell division and cell elongation, and endodormancy occurs mostly in perennial plants and is induced by signals within the bud. One mechanism that controls AB outgrowth is apical dominance (Cline, 1997). Removal of the shoot tip leads to axillary bud outgrowth, which can be reversed by application of auxin to the decapitated stump (Thimann & Skoog, 1933). It was hypothesized that auxin synthesized in young expanding leaves inhibits bud outgrowth; however, it is unclear how auxin exerts its effect. *BRANCHED 1* (*BRC1*) is a key regulator of shoot branching that integrates endogenous signals such as hormone signalling as well as environmental factors, including planting density (Aguilar-Martínez *et al.*, 2007; Wang *et al.*, 2019). *BRC1* encodes a TB1 CYCLOIDEA PCF (TCP) transcription factor and promotes bud growth arrest locally (Doebley et al., 1995; Doebley et al., 1997).

The developmental fate of axillary buds is modulated by many factors, including plant age and environmental cues, which influence a network of regulatory molecules. In *Arabis alpina* (*A. alpina*), the level of miR156 plays an important role in the age-dependent flowering response (Bergonzi *et al.*, 2013; Park *et al.*, 2017), which is mediated via *AaSPL15* (Hyun *et al.*, 2019). During vernalization, the transient repression of *PERPETUAL FLOWERING 1* (*PEP1*), the orthologue of *A. thaliana FLOWERING LOCUS C,* promotes flowering and the increase in *PEP1* transcription represses flowering following the return to warm temperatures (Wang *et al.*, 2009). The duration of cold treatment modulates the flowering response by determining the time window of *PEP1* repression (Lazaro *et al.*, 2018).

In this work, *A. alpina* was used as a model to study the pattern of AM initiation during different phases of perennial plant development. Microscopic analysis revealed the absence of AM initiation near the vegetative SAM and that dormant buds form before the onset of vernalization. The knock-down of *AaLAS* led to the loss of dormant buds and vegetative branches, culminating in premature senescence after one reproductive cycle.

Materials and Methods

Plant material and growth condition

All experiments were performed using *A. alpina* Pajares accession (Wang *et al.*, 2009). *A. alpina* seeds were stratified on wet filter paper for 4 days at 4°C in the dark and were then transferred to soil in the greenhouse with temperatures ranging from 20°C during the day to 18°C during the night in long days (LD, 16 h : 8 h, light : dark). Cold treatments were performed at 4°C either in short days (SD, 8 h : 16h, light : dark) to mimic natural winter conditions, or in LD conditions (16 h : 8 h, light : dark). Plants were then transferred back to greenhouse (LD, 20°C) until flowering. For prolonged vegetative development, plants were grown for 17 weeks in a Bronson growth chamber (LD) with a day-time temperature of 24°C and a night-time temperature of 18°C. To study AM initiation during the juvenile phase, plants were grown for two weeks in LD, followed by 15 weeks in cold (SD) and were moved back into the greenhouse (LD, 20°C).

Seeds of *A. thaliana* Col-0, *axr1-3* and *axr1-12* were obtained from the Nottingham Arabidopsis stock centre. To score the apical inhibition zone (AIZ), plants were grown in growth chambers (Mobylux GroBanks, CLF Plant Climatics, Germany) in SD conditions with a day-time temperature of 22°C and a night-time temperature of 18°C. The initiation of AMs in *A. thaliana* was monitored from three weeks after germination until the SAM transitioned to an inflorescence meristem. The AM initiation patterns in *A. alpina* and *A. thaliana* were characterised using a stereomicroscope to monitor the presence/absence of an AM/axillary bud in each leaf axil.

RNA *in-situ* hybridization

Sample preparation and *in-situ* hybridizations were performed as described by Coen *et al.* (1990) with slight modifications. 1-cm shoot apices were fixed with 4% paraformaldehyde fixative plus 0.03% Tween-20. Plant material was dehydrated without NaCl. Fixed material was embedded in Paraplast+ (Kendall) using an ASP300 tissue processor (Leica). Probes were not hydrolyzed.

Eight-µm-thin sections were hybridized according to Coen *et al.* (1990). For the synthesis of antisense probes from PCR products, the T7 promoter was included within the reverse primer. Primer sequences are listed in Table S1. *In vitro* transcription was performed with T7 RNA polymerase (Roche) and DIG RNA Labeling Mix (Roche) was used for RNA labelling with digoxigenin-UTP. For *AaSTM* antisense probe, nucleotides +1 to +1,158 relative to the ATG start codon (complete CDS) were synthesized from cDNA using primers U004 and U005 and were cloned into pCR BluntII TOPO vector (Thermo Fisher Scientific) in antisense orientation relative to the T7 promoter. Linearized plasmid was used for probe synthesis with T7 RNA polymerase. For the *AaLAS* antisense probe, nucleotides +498 to +1,335 relative to the ATG start codon were synthesized by PCR using primers AaFP2 and U009, and genomic DNA as a template.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on a DSM 940 (Zeiss) scanning electron microscope. Plant material was fixed overnight in 4% glutaraldehyde followed by dehydration with up to 100% ethanol. Samples were critical-point lyophilized and subsequently sputtered with a gold layer under vacuum.

Isolation of the *AaLAS* gene

The *A. alpina LAS* (*AaLAS*) sequence was obtained from RNA-seq analysis (GEO series GSE140316, BioProject PRJNA580332). Synteny analysis was performed using GEvo synteny analysis program.

Cloning and transformation

Knock-down constructs (*35S:AaLAS dsRNAi*) were created using Gateway cloning. A fragment of *LAS* from +1 to +660 bp was amplified from genomic DNA using primers U006 and U007 with attB1 and attB2 overhangs and was cloned into pDONOR201 containing the kanamycin resistance gene. The entry clone was cloned into the destination vector pJawohl-8-RNAi (GenBank accession number AF408413) harbouring the ampicillin resistance gene. *Agrobacterium*-mediated transformation of *A. alpina* was performed using the floral dip method according to Clough and Bent (1998). To select transgenic plants, T_1 seedlings were sprayed two or three times with 250 mg L^{-1} glufosinate (BASTA®, Hoechst). The presence of the transgene was verified by PCR using

the primers U006 and JaZP-Wrky-rev2. Single-copy transgenic lines were selected by qPCR by comparing the copy number of *AaLAS* with the single-copy *AaSPL15* gene. Primers are listed in Table S1.

Bud harvest and RNA-seq analysis

Young buds with a single leaf primordium and their corresponding shoot apical meristem including one leaf primordium were harvested using a BRAUN Sterican disposable cannula of either 0.60 × 25 mm or 0.45 × 25 mm under a Leica MZ-16FA stereomicroscope. Harvested tissue was immediately transferred to a pre-cooled (with liquid nitrogen) 2-mL collection tube. At stage1, the newly initiated ABs were harvested from L5 or L6 leaf axils of four-week-old plants; stage2_ABs were harvested from L15 or L16 leaf axils of eight-week-old plants; stage3_ABs were harvested from L24 or 25 leaf axils of plants that experienced four weeks of cold treatment and stage4_ABs were harvested from L33 or L34 leaf axils of plants that experienced seven weeks of cold treatment. Three biological replicates were prepared for each sample, except for stage1_AB (two replicates). In total, 120–150 buds and SAMs were harvested per replicate. Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich), following protocol A and was subjected to on-column DNase digestion. Details on library preparation and analysis on the RNA-seq data are provided in methods S1. The data are available at the NCBI database under BioProject ID PRJNA580332 with GEO series GSE140316.

RT-qPCR

Shoot apices including leaf primordia P1 to P4 (about 20 apices per sample) were harvested using a Leica MZ-16FA stereomicroscope. Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden). The RNA was digested using recombinant DNase I (Roche Applied Science) in the presence of Protector RNAse Inhibitor (Roche Applied Science). The RevertAid ™ H minus First-strand cDNA synthesis kit (Fermentas) was used for first-strand cDNA synthesis using total RNA, according to the manufacturer's manual. Approximately 500–1,000 ng total RNA was used for both expression analysis of *AaLAS* and validation of differentially expressed genes. The cDNA was diluted 1:5 and used for a 10-µL qRT-PCR reaction. Quantitative real-time PCR (qRT-PCR) was performed on a CFX 384 Touch Real-Time PCR Detection System (BIO-RAD) using SYBR Green PCR Master Mix (Applied Biosystems). Three technical replicates and two biological

replicates were analysed for each sample. Data were analysed using the standard curve method (Applied Biosystems, User Bulletin # 2, 2001). Primers used for DEGS validation of *PEP1*, *PEP2*, *AaSPL9*, *AaSPL15*,*AaLFY*, *AaTFL1* and *AaAP1* were described previously (Wang *et al.*, 2011; Bergonzi *et al.*, 2013; Lazaro *et al.*, 2018). Relative quantification was performed using internal standard curves and was corrected against a reference gene, *AaPP2A* (Bergonzi *et al.*, 2013; Hyun *et al.*, 2019). For list of primers used see table S1.

Results

During vegetative development axillary meristems initiate at a distance from the SAM

To identify when during development and where along the shoot axis new axillary meristems (AMs) initiate, we monitored axillary bud (AB) formation in the *A. alpina* accession Pajares at different developmental stages using a stereomicroscope. Eight weeks after germination in long days (LD), leaf axils L1–L7 developed side shoots (Fig. 1a, e), leaf axils L8–L13 contained fully developed axillary buds (Fig. 1b, e) and leaf axils L14–L23 harboured developing ABs or meristematic bulges (Fig. 1c, e). Notably, leaf axils L24–L32, which included the axils of the youngest leaves or leaf primordia in close proximity to the SAM, were devoid of ABs or any other morphological structures (Fig. 1d, e). Hereafter, these nodes will be termed the apical inhibition zone (AIZ). To test whether these empty leaf axils had already initiated an AM, we used *SHOOT MERISTEMLESS* (*STM)* as a molecular marker (Long and Barton (2000). Sections through eight-week-old vegetative shoot apices revealed *AaSTM* transcript accumulation in the SAM interprimordium regions (Fig. 1f, g) and the vasculature (Fig. 1h). Adjacent to leaf primordium 12, *AaSTM* mRNA accumulated at the adaxial centre of the leaf axil marking the youngest initiating AM (Fig. 1i). The observation that 10–11 leaf axils close to the vegetative SAM did not accumulate *AaSTM* mRNA suggests that AMs had not initiated in the AIZ.

Dormant buds initiate before the onset of vernalization

In Pajares, flowering is independent of day length, but is dependent on the length of the cold period that induces vernalization (Wang *et al.*, 2009; Wang *et al.*, 2011). To monitor the pattern of axillary bud formation in a complete reproductive cycle, plants were exposed to 12 weeks of cold

and phenotyped at four-week intervals. After four weeks, leaf axils L24–L32, which were empty before the cold treatment, had developed axillary buds (Fig. 2a, S1). In the same period, only few new leaf primordia were formed. As a result, the AIZ was reduced along an acropetal sequence. After eight weeks of cold treatment, the SAM of the primary shoot showed the characteristics of an inflorescence meristem and all leaf axils, including those initiated during the cold period (L33–L40), had developed axillary buds (Fig. 2b, S1). This indicates that the developmental transition from vegetative to reproductive development leads to AM initiation in close proximity to the SAM and consequent loss of the AIZ. After a 12-week cold period, plants were cultivated in the greenhouse (LD conditions) until flowering. Three weeks after transfer to the greenhouse, each AB/side shoot was inspected in detail to determine its developmental fate and degree of outgrowth. This analysis identified five distinct zones along the primary shoot axis (Fig. 2c, S2), which were named according to previous reports (Clarke *et al.*, 1999; Lazaro *et al.*, 2018). These observations lead to two important conclusions. Firstly, buds that remain dormant were formed before the onset of cold treatment. Secondly, flower buds initiate after the onset of cold treatment, either on the main shoot or on axillary shoots established during the early phase of main shoot development.

To ascertain whether the cold treatment is essential for the initiation of AMs close to the SAM, wild type plants were grown continuously in LD without cold treatment. After eight weeks, these plants showed the described pattern of ABs with an AIZ near the SAM (Fig. 3a). After 12 and 17 weeks in LD without cold, plants continued to produce ABs at a distance from the SAM following an acropetal gradient (Fig. 3b, c). Notably, the number of empty leaf axils increased in these plants (Fig. 3f). These experiments suggest that a cold period or cold-induced flowering is required to promote AM formation close to the SAM.

To discriminate a potential effect of cold treatment on AM formation from vernalization, we used juvenile plants that were not yet competent to flower (Wang *et al.*, 2011; Bergonzi *et al.*, 2013). Pajares plants were grown for two weeks in LD, followed by 15 weeks of cold treatment and were then returned to LD. Before the start of the cold period, these plants produced ABs in leaf axils L1–L4, but the young axils (L5–L11) near the SAM were empty (Fig. 3d). After 15 weeks of cold treatment, these juvenile plants that were not flowering maintained a set of leaf axils

near the SAM that were devoid of AMs (Fig.3e), which indicates that whereas cold *per se* is not sufficient, flowering is critical for the initiation of AMs adjacent to the SAM.

To compare the pattern of AM formation in perennial *A. alpina* with that in annual *A. thaliana*, we grew the Columbia accession in short-day (SD) conditions. Similar to *A. alpina*, an AIZ was also established during *A. thaliana* vegetative development and was filled in the reproductive phase (Fig. S3). Coudert et al. (2015) reported that an AIZ also forms in gametophores of *Physcomitrella patens*, the size of which changed after application of auxin. To analyse the potential role of auxin in AIZ formation in *A. thaliana*, we compared AB formation from three weeks after germination until the shoot apical meristem underwent transition to an inflorescence meristem (SD) in the auxin-resistant mutants *axr1-3* and *axr1-12* and wild type plants. At early and late vegetative development, *axr1-3* and *axr1-12* showed a significant reduction in the AIZ, whereas no difference was detected at the transition to reproductive development (Fig. S4). This indicates that auxin does potentially regulate the AIZ.

Transcript profiles of shoot apical meristems at different stages of the perennial life cycle

Based on the different patterns of AM initiation at different developmental stages, we hypothesized that a correlation exists between the fate of the SAM and that of initiating AMs. To test this, we first captured changes in the transcript profiles of *A. alpina* SAMs during different stages of the perennial life history. This information was then used as a reference for comparisons with newly initiated AMs. Terminal shoot meristems that included the youngest leaf primordium were harvested from vegetative plants 4 weeks (stage1_SAM) and 8 weeks (stage2_SAM) after germination, as well as from plants grown vegetatively for 8 weeks and cold-treated for 4 weeks (stage3_SAM) or 7 weeks (stage4_SAM) (Fig. 4a) to induce flowering. A gene was considered to be differentially expressed if its RPKM values were different at two developmental time points ($\geq$2 fold, p-value ≤ 0.05) (Table S2-S4). A comparison of the vegetative SAMs at stages 1 and 2 revealed very limited changes in transcript profiles, and gene ontology (GO) analysis showed enrichment for processes related to transcriptional regulation and metabolism (Fig. 4b and Table S5). By contrast, the strongest SAM reprogramming occurred between stages 2 and 3, which corresponded to the transition from vegetative to reproductive development (Fig. 4b).

Accordingly, an enrichment in GO terms related to reproductive phase transition and hormone-mediated signalling pathways was detected (Table S5). Genes involved in responses to auxin, ethylene and jasmonic acid were strongly downregulated (Table S5). In stage4_SAM transcript profiles, GO terms related to reproductive phase transition were enriched, indicating an increased commitment to flower. For example, the flowering repressors *PERPETUAL FLOWERING1* (*PEP1*) (Wang *et al.*, 2009) and *TERMINAL FLOWER 1* (*TFL-1*) (Alvarez *et al.*, 1992; Wang *et al.*, 2011) were downregulated (Fig. 4c, S5a, c and Table S5).

The RNA-seq analysis also identified many genes that were expressed at high levels or exclusively in SAMs that experienced cold. For example, genes that promote SAM transition from the vegetative to the reproductive phase (Table S5), such as *AGAMOUS-LIKE* (Ma *et al.*, 1991) and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL9 and SPL15*) genes (Schwarz *et al.*, 2008), were enriched in the SAMs of plants that experienced a cold period of at least four weeks (Fig. 4d, S5e, f). In addition, genes that modulate the competence to flower, such as *LEAFY* (*LFY*) (Weigel *et al.*, 1992), were expressed in the SAMs of plants treated with cold for four weeks and seven weeks (Fig. 4e, S5h). Transcripts of floral organ identity genes, including *APETALA 1* (*AP1*) (Irish & Sussex, 1990) and *SEPALLATA 1* (*SEP1*) (Pelaz *et al.*, 2000) accumulated exclusively in the SAMs of plants that experienced seven weeks of cold treatment (Fig. 4f, S5g). Taken together, vegetative SAMs showed the most similar transcript profiles and their developmental progression was reflected by changes in transcriptional regulation and metabolism. After the beginning of the cold period, changes in transcript profiles correlated primarily with reproductive development and responses to plant hormones, indicating a progression towards flowering.

Transcript profiles of axillary meristems formed during vegetative development differ from those of the SAM

To compare the transcript profiles of young ABs with those of the SAM, we harvested newly initiated ABs including their youngest primordium, from the same plants used to collect SAMs (Fig. 5a) and characterized their transcript patterns. Similar to the SAM, the smallest changes in transcript profiles were observed in the vegetative phase between stage1 and stage2_ABs (Fig. 5b

and Table S6). These included genes involved in hormonal regulation, such as an increase in the response to abscisic acid (Table S7). After the start of the cold treatment, transcript patterns showed profound changes (stage3_ABs) in comparison to those in the vegetative phase (Fig. 5b and Table S8). Although transcript levels of the flowering repressor *PEP1* were downregulated, commitment to flowering had not yet occurred, as indicated by the high expression of *TFL1* (Fig. 5c, S6a, c). Finally, stage4_ABs showed the most pronounced transcriptional reprogramming in comparison to that during vegetative development (Fig. 5b, Table S7 and S9). Genes involved in reproductive development, such as *SPL9*, *SPL15*, *LFY* and *API*, were upregulated and transcript levels of the negative regulators of flowering *TFL1*, *MAF1* and *MAF3* were reduced or remained low (*PEP1*; Fig. 5c, e, S6). Furthermore, genes involved in regulating AB dormancy, such as *BRANCHED 1* (*BRC1*) and *BRANCHED 2* (*BRC2*) (Aguilar-Martínez *et al.*, 2007), were downregulated in stage4_ABs (Fig. 5d, S6b). Together, these results suggest that ABs harvested soon after initiation at different stages of SAM development exhibit considerable differences in transcript profiles, which are gradually enhanced during the transition from vegetative development to flowering.

To analyse similarities between the transcript patterns of young ABs and SAMs, we performed hierarchical clustering analysis. This divided SAMs collected at different developmental stages into two separate clusters, with one representing vegetative SAMs (stage1_SAM and stage2_SAM) and another including the SAMs of cold-treated plants (stage3_SAM and stage4_SAM; Fig. 6a). This clustering was also supported by principal component analysis (Fig. 6b). Notably, all young ABs that initiated at a distance from the SAM (stage1_AB to stage3_AB) clustered separately from the SAM collected at the same time points. By contrast, young buds that initiated in close proximity to the SAM in stage 4 leaf axils grouped together with the SAM, indicating that AMs that initiate close to the SAM follow a similar developmental program to that of the SAM.

The role of *LATERAL SUPPRESSOR* in *A. alpina* shoot development

Because buds that initiated at a distance to the SAM, in particular dormant buds (V2) and V3 buds, are fundamentally important to the perennial life cycle of *A. alpina* (Wang *et al.*, 2009), we

tested whether the loss of these buds interferes with the perennial life cycle. Several transcription factors, including LAS (Greb *et al.*, 2003) and REGULATOR OF AXILLARY MERISTEMS (Keller *et al.*, 2006; Müller *et al.*, 2006), specifically affect AM formation during vegetative development, but it is unknown how they affect the ability of *A. alpina* plants to flower repeatedly and survive for many years. We firstly studied the role of *LAS*, because its function is evolutionarily well-conserved. Analysis of *A. thaliana LAS* (*AtLAS*) and *A. alpina LAS (AaLAS*) genomic environments revealed extended colinearity and the encoded proteins share 85% amino acid identity, suggesting that both genes are orthologous (Fig. S7). *AaLAS* mRNA was found to accumulate in a band-shaped domain at the boundary between young leaf primordia and the stem (Fig. 7a, b). *AaLAS* expression was first detected in the axils of P1 primordia and gradually decreased in those of older primordia until AMs became visible in the axils of P12 primordia. The *AaLAS* expression pattern in the shoot apex shows strong similarities with that of *AtLAS* mRNA in *A. thaliana* (Greb *et al.*, 2003).

To understand the biological role of *AaLAS* in *A. alpina* development, we generated knock-down lines using RNAi. Nine independent lines (*35S:AaLAS dsRNAi*) were generated, three of which contained a single copy T-DNA insertion and were analysed further. The *AaLAS* transcript level was significantly reduced in these transgenic lines (Fig. S8i). We compared the pattern of AB formation among these lines and the wild type at different developmental stages. In the vegetative phase, wild type plants developed ABs in most leaf axils (L1–L17, Fig. 7c, j) except for those of the AIZ (L18–L30, Fig. 7j). Among the *35S:AaLAS dsRNAi* plants, AB initiation was strongly inhibited in lines 1 and 3 (Fig. 7d, e S9a), but was only slightly compromised in line 2 (Fig. S8a, f, S9a). Eight weeks after germination, line 1 had not developed any ABs (Fig. 7j), whereas line 3 produced ABs in a few leaf axils (Fig. 7j). Scanning electron microscopy (SEM) analysis confirmed the empty leaf axils in knock-down lines 1 and 3 (Fig. 7i, S8d). In line 2, AB formation was frequently delayed in comparison to in wild type plants (compare Fig 7h with Fig. S8e).

After four weeks of cold treatment, line 1 plants had not produced an AB, whereas those of line 3 maintained a few branches in the V1 zone (Fig. S10a, S9b). At this stage, line 2 showed a mild defect, but otherwise the pattern of ABs along the shoot axis was similar to that of wild type plants (Fig. S9b, S10a). After eight weeks in the cold, wild type plants developed ABs in most

leaf axils, including the AIZ (Fig. 7k). A similar pattern was observed in knock-down line 2 (Fig. S8g, S9c). However, most leaf axils of line 1 plants remained empty, although a few that were empty before the floral transition initiated ABs (Fig. 7k. S9c). In line 3, ABs developed exclusively in the axils close to the apex (L26–L37), whereas those of L20 to L25 remained empty (Fig. 7k, S9c). Taken together, these results suggest that *AaLAS* is required in *A. alpina* to promote AM initiation during vegetative development and also during the initial phase of reproductive development.

Because knock-down of *AaLAS* function affected bud development before and during the cold period, we asked whether *AaLAS* knock-down interferes with the perennial life cycle of *A. alpina*. We addressed this by studying the flowering pattern and subsequent development of greenhouse-grown plants after an extended cold period of 15 weeks. After three weeks, line 1 plants did not develop any ABs or branches in the V1, V1a and V2 zones (Fig. 8b, e) and the number of V3 branches was significantly reduced in comparison to in wild type plants (Fig. S10b). Furthermore, some of these plants developed only few flowers in the I zone (Fig. 8b). Line 3 plants revealed an intermediate defect, exhibiting a reduced number of V1, V1a, V2 and V3 buds/branches (Fig. 8c, f), whereas plants of the weak knock-down line 2 showed all zones identified in wild type plants, with few empty leaf axils in each zone (Fig. 8a, d, S8b, h).

The absence of vegetative buds and shoots in the V2 and V3 zone of line 1 plants raised the question whether these plants could continue growth in a subsequent reproductive cycle. To address this, we followed the further development of a selected number of plants. Four months after cold treatment, plants of wild type, line 2 and line 3 developed seeds and initiated senescence only in the I zone of the main stem and in the flowering side shoots of the V1 zone (Fig. 8g, i, S11a, b). However, 13% of line 1 plants, which initiated buds only in the I zone, completely senescenced after seed set (Fig. 8h, S11c). The remaining 87% of line 1 plants continued growth through V3 branches, although they lacked V1, V1a and V2 buds/branches (Fig. S11d). Lazaro *et al.* (2018) reported that the fate of V3 branches is affected by the length of cold treatment. An extended cold treatment of 24 weeks caused 46% of line 1 plants to completely senescence after seed set, whereas all wild type plants developed further (Fig. 8k). This suggests that loss of *AaLAS* function interferes with the perennial life style of *A. alpina*, reducing its life span in extreme cases to one reproductive cycle.

Discussion

Axillary meristem initiation at a distance from the SAM correlates with the establishment of new transcript profiles

During *A. alpina* vegetative development, AMs initiate acropetally at a distance from the SAM and establish an AIZ (Fig. 1d). Similarly, vegetative *A. thaliana* plants lack AMs in the leaf axils adjacent to the SAM (Stirnberg *et al.*, 1999; Greb *et al.*, 2003). In the vegetative phase of the monocot rice (*Oryza sativa*), the earliest AM formation was observed in axils of P2/P3 primordia (Oikawa & Kyozuka, 2009), indicating that AMs initiate closer to the SAM than in the dicots studied here. After floral transition, the AIZ was reduced acropetally, leading to AM formation close to the SAM (Fig. 2b). This is similar to development in *A. thaliana* and other annual species, in which new meristems initiate close to the SAM during reproductive development (Hempel & Feldman, 1994; Stirnberg *et al.*, 1999).

The inhibition of AM initiation close to the SAM in vegetatively growing *A. alpina* plants suggests that the primary SAM inhibits AM initiation. Reports have postulated that a repressor is produced in the shoot apex and creates a decreasing basipetal concentration gradient (Reinhardt & Kuhlemeier, 2002; Greb *et al.*, 2003). Burian *et al.* (2016) showed that ablation of the *A. thaliana* SAM promotes the formation of a new meristem nearby, supporting the presence of an inhibitory signal from the SAM. Due to its role in apical dominance (Thimann & Skoog, 1933), auxin is a candidate repressor of AM initiation. However, evidence for this hypothesis is conflicting. Stirnberg *et al.* (1999) reported that auxin produced at the shoot apex in the auxin-resistant *A. thaliana axr1-12* mutant affects bud outgrowth, but not the timing of AM initiation. In this study, the AIZ of *axr1-3* and *axr1-12* was significantly reduced (Fig. S4), supporting the view that auxin is an inhibitory signal from the shoot apex. Alternatively, the requirement for an auxin minimum in leaf axils (Q. Wang *et al.*, 2014; Y. Wang *et al.*, 2014) may be reduced in these mutants. Gametophores of *Physcomitrella patens* also form an AIZ (Coudert *et al.*, 2015) and application of auxin extends the AIZ size, whereas a reduced auxin level in the auxin biosynthesis mutant *Ppshi2-1* results in a reduced AIZ. Although angiosperm shoots and moss gametophores are not equivalent structures, these results support the hypothesis that auxin is involved in mechanisms that establish or maintain a zone without ABs during development. However, we cannot exclude

the possibility that other compounds are involved in a signalling mechanism that inhibits the formation of AMs in the vegetative phase.

The detached meristem model (Grbić & Bleecker, 2000; Greb *et al.*, 2003; Q. Wang *et al.*, 2014; Y. Wang *et al.*, 2014) has been proposed to explain how new meristems are established at a distance from the primary SAM. For example, live-cell imaging of leaf axils in *A. thaliana* and tomato revealed that pools of cells expressing the meristem marker *STM* are maintained in leaf axils, the progenitors of which develop into AMs (Shi *et al.*, 2016). The maintenance of AM progenitor cells in leaf axils requires very specific conditions, such as a low auxin environment (Q. Wang *et al.*, 2014; Y. Wang *et al.*, 2014) and a low rate of cell divisions (Burian *et al.*, 2016), to prevent premature differentiation and the accumulation of deleterious mutations in these cells (Wang & Jiao, 2018). However, one important question is whether the distance from the SAM affects the fate of AMs. The transcript profiles in young ABs initiated during reproductive development close to the SAM are highly similar to those of the SAM. For example, *AaLFY*, *AaAP1*, *AaAP3*, *AaSEP4* and *AaPI,* which play pivotal roles in floral organ identity specification (Irish & Sussex, 1990; Weigel *et al.*, 1992; Pelaz *et al.*, 2000), are highly expressed only in young ABs initiated during the reproductive phase and in the SAM. By contrast, ABs that initiate at a distance from the SAM were characterized by transcript profiles that were highly dissimilar from those of the SAM. For example, *AaBRC1* transcripts accumulated highly in ABs initiated at a distance from the SAM, but not in the SAM or in buds formed in close proximity to the SAM. *BRC1* was reported to promote growth arrest and its downregulation causes bud outgrowth (Aguilar-Martínez *et al.*, 2007). These and other comparative transcript profiles between ABs and the SAM illustrate a general theme: New meristems (AMs) with different gene expression profiles from that in the SAM are readily established at a distance from the SAM, whereas such AMs are not initiated in close proximity to the SAM.

Knock-down of *AaLAS* compromises the perennial life cycle

In annual *A. thaliana*, knock-out of *LAS* leads to the absence of ABs in rosette leaf axils, resulting in a consequent reduction in the number of flowering shoots (Greb *et al.*, 2003), which limits

reproductive success. In perennial *A. alpina*, knock-down of the orthologous *LAS* gene not only reduces the number of vegetative V3 shoots, but it eliminates V1 side shoots and the dormant bud zone (V2). The perennial life cycle necessitates the maintenance of a reservoir of vegetative buds/side shoots for the subsequent season (Thomas *et al.*, 2000). As a consequence, 13% of *AaLAS* knock-down plants that developed buds only in the I zone underwent complete senescence after seed set and this fraction increased to 46% when plants were exposed to a prolonged cold treatment (Fig. 8j, k). A similar branching phenotype was observed in *A. alpina* mutants that harbouring a *PEP1* loss-of-function allele, which lacked the dormant bud zone and V3 side shoots, but developed V1 side shoots (Wang *et al.*, 2009). By contrast to the extreme *AaLAS* knock-down plants, *pep1-1* mutants flower perpetually and can survive for many years in the greenhouse, because they continue growth from V1 side shoots, which are absent in *AaLAS* knock-down plants. However, under experimental garden conditions, the mortality rate of *pep1-1* mutants was much higher than that of the corresponding Pajares wild type (Hughes *et al.*, 2019). Although the consequences of *LAS* loss-of-function between annual *A. thaliana* and perennial *A. alpina* differ, the initial defects in AM formation are very similar. In both species, a reduction or a complete loss of *LAS* function compromises AM initiation at a distance to the SAM. The developmental output differs, because the AMs that initiate in perennial *A. alpina* in this phase develop into dormant buds or vegetative side shoots, whereas in *A. thaliana*, all AMs finally probably transition to reproductive development. Furthermore, the reduction in the number of vegetative V3 shoots suggests that *AaLAS* is also required in the early phase of reproductive development after entering the cold period. This was not observed in *A. thaliana las-4* mutants, where AM formation in cauline leaf axils was not usually compromised. Similarly, in perennial tomato, the orthologous *ls* loss-of-function mutant usually forms two axillary shoots below each inflorescence, which continue sympodial shoot development (Schumacher *et al.*, 1999). We hypothesize that in this case, the sympodial meristem and the AM initiate close to the SAM of the main shoot and therefore are not affected by loss of *Ls* function. The AMs that initiate close to the SAM do not require *Ls*/*LAS*/*AaLAS* function. It is currently unclear whether an alternative mechanism assumes the role of *Ls*/*LAS*/*AaLAS* during this developmental period. The specific absence of AMs in cauline leaf axils in the *rsb1* mutant indicates that *AGL6* might play an important role in such a mechanism in *A. thaliana* (Huang *et al.*, 2012).

In annual plants, two types of senescence have been observed: During vegetative development, individual leaves senesce occasionally, but after seed set, senescence affects the whole plant and leads to plant death. By contrast, perennial plants senesce at the cellular or organ level, but whole-plant senescence is a rare event (Munné-Bosch, 2008). For example, senescence in *A. alpina* is usually restricted to shoots that have flowered (Wang *et al.*, 2009). In this study, up to 46% of the plants of the strong *AaLAS* knock-down line completely senesced upon prolonged cold treatment. To explain this, the continuation of the life cycle in perennial plants is dependent on the population of indeterminate vegetative meristems that can continue growth. On the contrary, determinate floral meristems usually initiate a set of reproductive structures and die. Plants of *AaLAS* knock-down lines that showed an extreme phenotype, contained floral buds that developed into flowers, but did not form V1 side shoots, V2 buds or V3 side shoots, resulting in the complete absence of vegetative buds/side shoots. In Arabidopsis, it has been shown that the presence of active ABs strongly affects longevity (Noodén & Penney, 2001; Guo & Gan, 2011). We speculate that the absence of vegetative buds can be sensed by *A. alpina* plants, which leads to the initiation of whole plant senescence. Tomato plants from which all vegetative meristems have been removed, initiate ectopic meristems on leaves (Rossmann *et al.*, 2015), which is dependent on the activity of *Ls*, an orthologue of *AaLAS*. The extreme *AaLAS* knock-down plants studied here showed no ectopic shoot formation in other parts of the plant body. This might be due to the absence of appropriate populations of pluripotent cells in other plant tissues, which in turn, might depend on the lack of *AaLAS* activity. Taken together, these observations indicate that *A. alpina* plants sense the presence of vegetative buds within the plant body and can initiate a whole-plant senescence programme if vegetative buds are absent. At first glance, whole-plant senescence in perennial *A. alpina* is similar to that in annual *A. thaliana*.

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Author contributions

U.P. and K.T. designed the research; U.P. performed research; U.P. and K.T. analysed the data; U.P. and K.T. wrote the manuscript.

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Supporting Information

The following Supporting Information is available for this article:

Fig. S1 Box plot representing the percentage of empty leaf axils in *A. alpina* at different developmental stages.

Fig. S2 Zonation and developmental fate of axillary buds along the shoot axis of *A. alpina* after completing one reproductive cycle.

Fig. S3 Axillary bud formation in *A. thaliana*

Fig. S4 Axillary bud formation in *axr1* mutants of *A. thaliana.*

Fig. S5 qRT-PCR of a subset of differentially expressed genes measured in shoot apical meristem harvested at different developmental stages.

Fig. S6 qRT-PCR of a subset of differentially expressed genes measured in axillary buds harvested at different developmental stages.

Fig. S7 Synteny analysis of *AaLAS* and *AtLAS.*

Fig. S8 Phenotypic characterization of *35S:AaLAS dsRNAi* line 2.

Fig. S9 Box plot representing the percentage of empty leaf axils in *A. alpina* wild type and *35S:AaLAS dsRNAi* plants.

Fig. S10 Knock-down of *AaLAS* affect axillary meristem initiation during cold treatment.

Fig. S11 Senescence pattern of wild type and *35S:AaLAS dsRNAi* line 1.

Table S1 List of primer used in this work.

Table S2 DEGs used for GO terms analysis of Stage1_SAM vs Stage2_SAM.

Table S3 DEGs used for GO terms analysis of Stage2_SAM vs Stage3_SAM.

Table S4 DEGs used for GO terms analysis of Stage3_SAM vs Stage4_SAM.

Table S5 GO term analysis of differentially expressed genes in comparisons between SAMs at successive developmental stages.

Table S6 DEGs used for GO terms analysis of Stage1_AB vs Stage2_AB.

Table S7 GO term analysis of differentially expressed genes in comparisons between young axillary bud at successive developmental stages.

Table S8 DEGs used for GO terms analysis of Stage2_AB vs Stage3_AB.

Table S9 DEGs used for GO terms analysis of Stage3_AB vs Stage4_AB.

Methods S1 RNA-seq analysis

Figure Legends:

Figure 1 Axillary bud initiation during *Arabis alpina* vegetative development.

(a) Side shoots dissected from the axils of L1 to L7 and one of the cotyledon leaf axils (left to right) of an eight-week-old plant. (b) SEM micrograph of a fully developed axillary bud with leaf primordia. (c) SEM micrograph of an emerging axillary meristem in a leaf axil. (d) SEM micrograph of an empty leaf axil. (e) Schematic representation of the pattern of axillary bud development at the vegetative stage. Each column represents a single plant and each square box represents a single leaf axil. Green squares indicate the presence of an axillary bud/side shoot and yellow squares indicate the absence of an axillary bud. Plants were grown in the greenhouse in long days for eight weeks; n = 15. (f) to (i) Transverse sections of eight-week-old shoot apices hybridized with an *AaSTM* antisense probe. An * indicates the shoot apical meristem and P indicates young leaf primordia numbered from the youngest (P1) to the oldest. (h) The arrow points to the broad expression of *AaSTM* in the vasculature. (i) Focused accumulation of *AaSTM* mRNA at the leaf axil of P12 marks the initiation of an axillary meristem. Scale bar: 100 μm (b, c, f, g, h and i) and 200 μm in (d).

Figure 2. Pattern of axillary buds, side shoots and flowers at different stages of *Arabis alpina* development.

(a and b) Schematic representation of the pattern of axillary bud initiation during transition to flowering. Each column represents a single plant and each square box represents a single leaf axil. Green squares indicate the presence of an axillary bud/side shoot and yellow squares indicate the absence of an axillary bud. The squares below the horizontal black line indicate the buds initiated before the onset of cold treatment and squares above indicate those that initiated during cold treatment. (c) The flowering pattern of side shoots after experiencing 12 weeks of cold treatment in long days (LD) and three weeks in LD in the greenhouse. Each square box in (c) indicates the fate of the side shoot/axillary bud in that leaf axil. Pink squares represent the main inflorescence with open flowers, pale green squares represent side shoots at the transition phase, green squares

represent dormant vegetative buds, purple squares represent side shoots with open flowers, pale-purple squares represent side shoots at the inflorescence stage and yellow squares represent empty leaf axils. Plants were grown for eight weeks in LD, cold-treated for 12 weeks in LD and then moved back to the greenhouse in LD until flowering. (a) n = 15, (b) n = 14 and (c) n = 15.

Figure 3. Transition to flowering is important to initiate axillary meristems adjacent to the main meristem in *Arabis alpina*.

Pattern of axillary bud development in wild type plants grown without cold treatment for eight weeks (a), 12 weeks (b) and 17 weeks (c). n = 10. (d and e) Pattern of axillary bud initiation in juvenile plants before (two-week-old plants) and after cold treatment (15 weeks of cold in short days (SD) and then grown in the greenhouse in long days (LD) for four weeks). n = 10 in (d) and n = 12 in (e). Each column represents a single plant and each square box represents a single leaf axil. Green squares indicate the presence of an axillary bud/side shoot, blue indicate accessory bud and yellow squares indicate the absence of an axillary bud. "v" indicates bifurcation of the main meristem. (f) Box plot showing the percentage of empty leaf axils near the shoot apical meristem in plants grown for 8, 12 or 17 weeks in LD. The first and third quartile of the box plot was separated by the median. Whiskers extend to data points that are less than 1.5 × IQR (inter-quartile range) away from first/third quartile. Data for two biological replicates were combined for the analysis. Double asterisks denotes significant difference between eight weeks and plants in 12 and 17 weeks in LD calculated using student t-test (two tail assuming equal variance and $P < 0.01$). n = 20.

Figure 4. Gene expression profiles of *Arabis alpina* shoot apical meristems (SAMs) at different developmental stages.

(a) Schematic representation of *A. alpina* plants at four different developmental stages. The red arrow at the tip of the plant marks the SAM, stereoscope images show shoot apices. The black arc and circle indicate regions harvested for RNA extraction. (b) Area-proportion Venn diagram based on the total number of significant differentially expressed genes (DEGs) in each pairwise

comparison and common genes between comparisons. (c–f) Changes in transcript profiles of genes involved in the developmental switch of the SAM from vegetative to inflorescence development: Negative regulators of flowering in (c), genes that promote transition to flowering in (d), floral meristem identity genes in (e) and floral organ identity genes in (f). The X-axis represents SAMs harvested at different developmental stages; the Y- axis represents the mean RPKM values of genes. The presented genes showed significant alterations in transcript accumulation: FDR corrected *p*-value ≤ 0.05 and fold change ≥ 2 or ≤ -2.

Figure 5. Transcriptome profiling of *Arabis alpina* axillary buds at different developmental stages.

(a) Schematic representation of *A. alpina* plants at four different developmental stages: red bulges on the leaf represent axillary buds, stereoscope images show axillary buds, the black arc indicates harvested region. (b) Area-proportion Venn diagram based on the total number of significant differentially expressed genes (DEGs) in each pairwise comparison. (c–e) Dynamics of transcript accumulation in axillary buds at different developmental stages. The X-axis represents different stages of plant development; the Y-axis represents mean RPKM values. The presented genes showed significant alterations in transcript accumulation: FDR corrected *p*-value ≤ 0.05 and fold change ≥ 2 or ≤ -2.

Figure 6. Comparison of transcript profiles between young axillary buds and the shoot apical meristem (SAM) at different developmental stages of *Arabis alpina*.

(a) Hierarchical clustering of transcripts and samples harvested at different time points. Each column corresponds to a developmental stage and each row represents a transcript. Transcripts are clustered based on the similarity of their expression profiles. Samples are clustered by the similarity of their transcript expression pattern. The heat map was created using Z-score normalized expression values for the transcripts. A gradient colour code was used to represent expression levels: yellow indicates a high transcript level and blue indicates a low transcript level. (b) Principal component analysis plot (2D) using the Z-score normalized expression values of transcripts. The variation between the young buds initiated at a distance from the SAM is shown

by a separation in PC1 that explains 26.9% of the total variation. The variation due to the developmental stage or exposure to cold treatment between the young bud and SAM is shown by a separation in PC2 that explains 15.4% of the total variation.

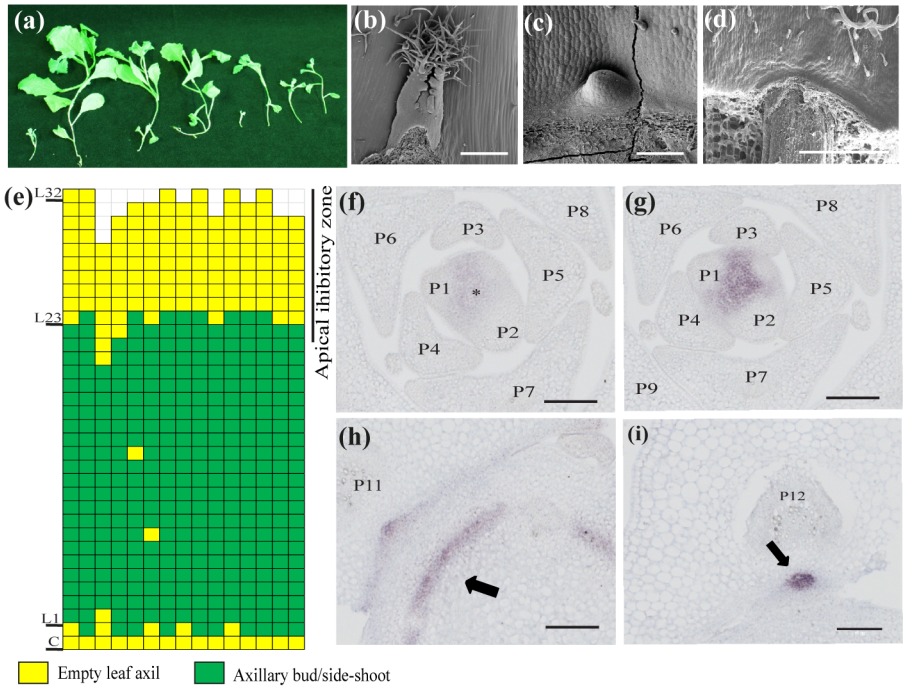
Figure 7. *LATERAL SUPPRESSOR* (*AaLAS*) modulates axillary bud formation in *Arabis alpina*.

(a and b) Transverse sections through eight-week-old shoot apices hybridized with an *AaLAS* antisense probe. An * indicates the shoot apex and P indicates young leaf primordia numbered from the youngest (P1) to the oldest. Scale bar: 100 µm. (c–e) Eight-week-old plants of *35S:AaLAS dsRNAi* line 1 (d) and line 3 (e) in comparison to wild type accession Pajares (c). Scale bar: 1 cm. (f and g) Close-up of leaf axils of the wild type and line 1. The orange arrows indicate the presence and absence of side shoot in wild type (f) and in line 1 (g), respectively. (h and i) SEM micrographs of a leaf axils (L10) of wild type (h) and line 1 (i). Scale bar: 200 µm. (j and k) Schematic representations of axillary bud development in wild type, *35S:AaLAS dsRNAi* line 1 and line 3. (j) Patterns of axillary bud development at the vegetative stage (eight-week-old plants). n = 12 (WT), n = 12 (line 1) and n = 10 (line 3). (k) Patterns of axillary bud development after transition to flowering (eight-week-old plants followed by eight weeks of cold treatment). n = 10 (WT), n = 10 (line 1) and n = 12 (line 3). Each column represents a single plant and each square box represents a single leaf axil. Green squares indicate the presence of an axillary bud/side shoot, blue indicate accessory bud and yellow squares indicate the absence of an axillary bud. The square boxes below the horizontal black line indicate buds initiated before the onset of cold treatment and square boxes above indicate buds initiated during cold treatment.

Figure 8. Knock-down of *AaLAS* affects perennial life style in *Arabis alpina*.

(a–c) Schematic representations (left) and cartoons (right) of patterns of flower and side shoot development in wild type, *35S:AaLAS dsRNAi* line 1 and line 3. Each column represents a single plant and each square box indicates the fate of the side shoot/axillary bud in that leaf axil. Pink squares represent the main inflorescence with open flowers, pale green squares represent side shoots at the transition phase, green squares represent dormant vegetative buds, purple squares

represent side shoots with open flowers, pale-purple squares represent side shoots and yellow squares represent empty leaf axils. n = 10 (WT), n = 12 (line 1) and n = 10 (line 3). (d–f) Pictures of flowering wild type, *35S:AaLAS dsRNAi* line 1 and line 3 plants. Plants were grown in long days (LD) for eight weeks, followed by 15 weeks of cold treatment and then in LD until flowering. Scale bar: 1 cm. (g–i) Pictures of senescing plants: wild type (g), *35S:AaLAS dsRNAi* line 1 (h) and line 3 (i) plants. Scale bar: 1cm. (j) Bar graph shows the percentage of senesced plants in wild type, *35S:AaLAS dsRNAi* line 1, line 2 and line 3. Data from both biological replicates were pooled before the analysis. n = 22. (k) The bar graph represents the percentage of plants that senescenced for wild type and *35S:AaLAS dsRNAi* line 1 after prolonged cold treatment. Data from both biological replicates were pooled before the analysis. *n* = 17 (WT) and n = 27 (line 1). Plants were grown in LD for eight weeks, followed by a cold treatment of 15 weeks (j) or 24 weeks (k), and were then moved back to LD to flower. The senescence pattern was recorded after four months. Error bars indicate ± SE.



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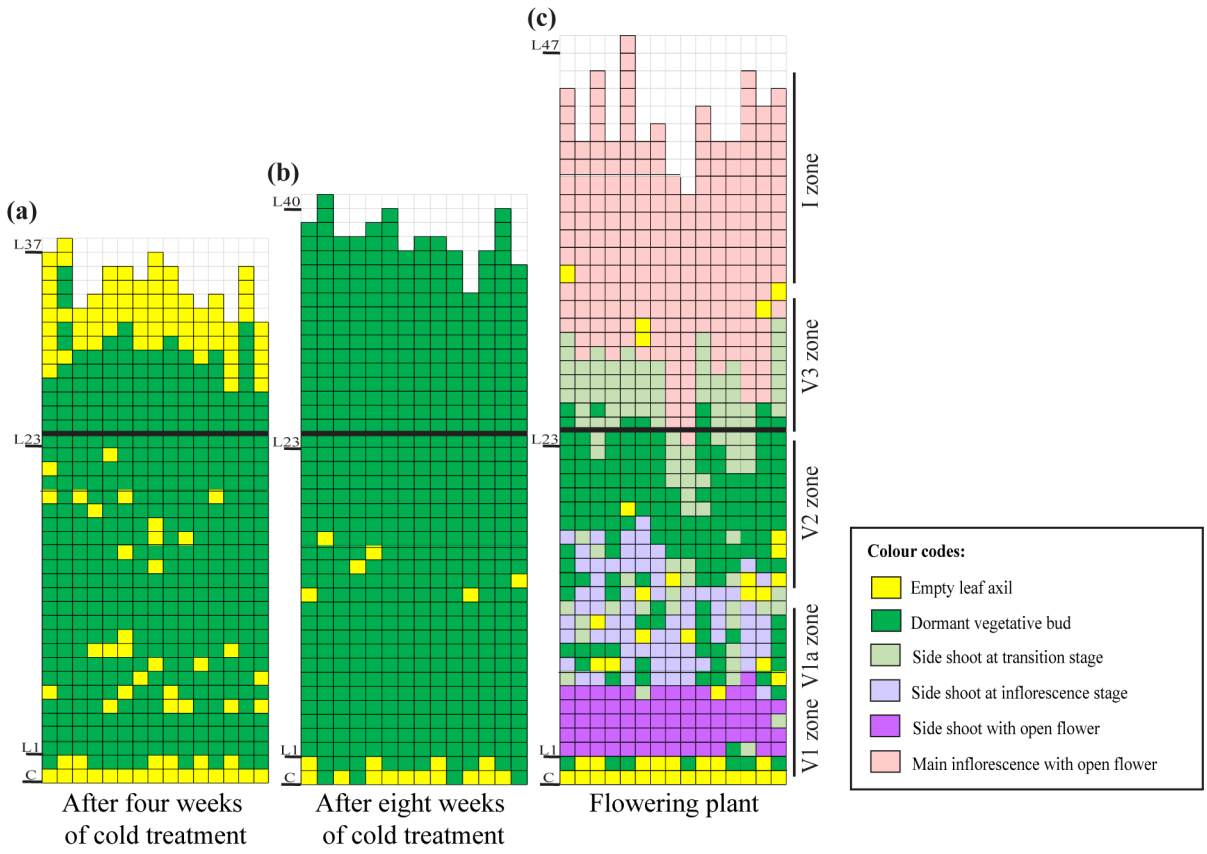

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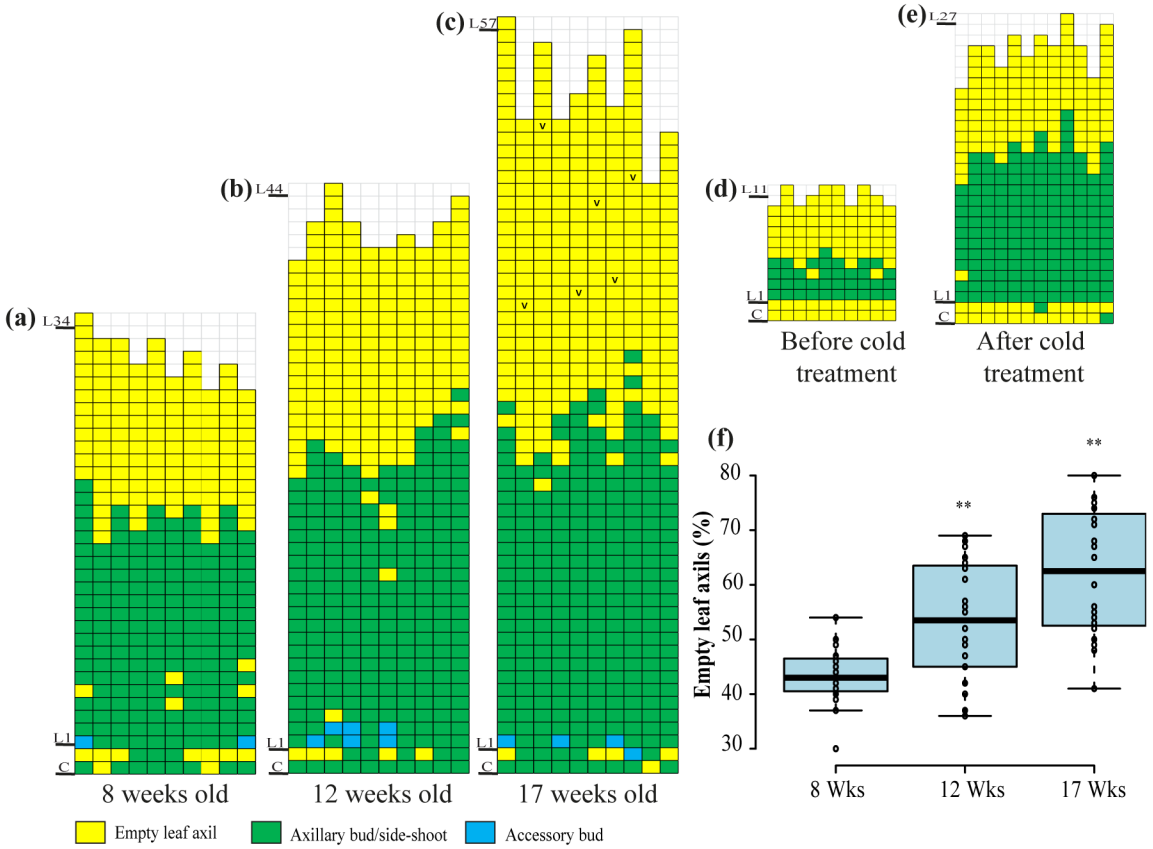

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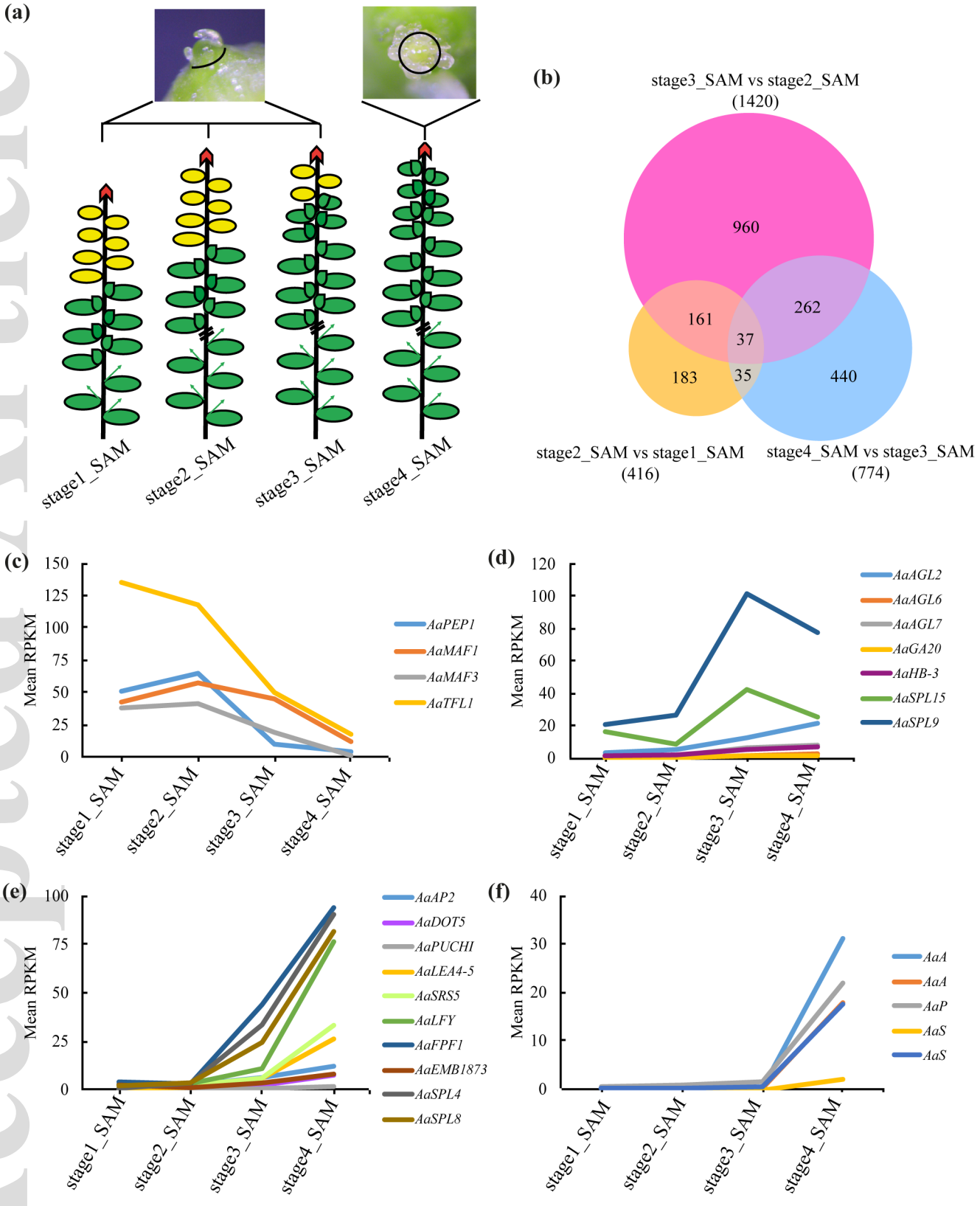

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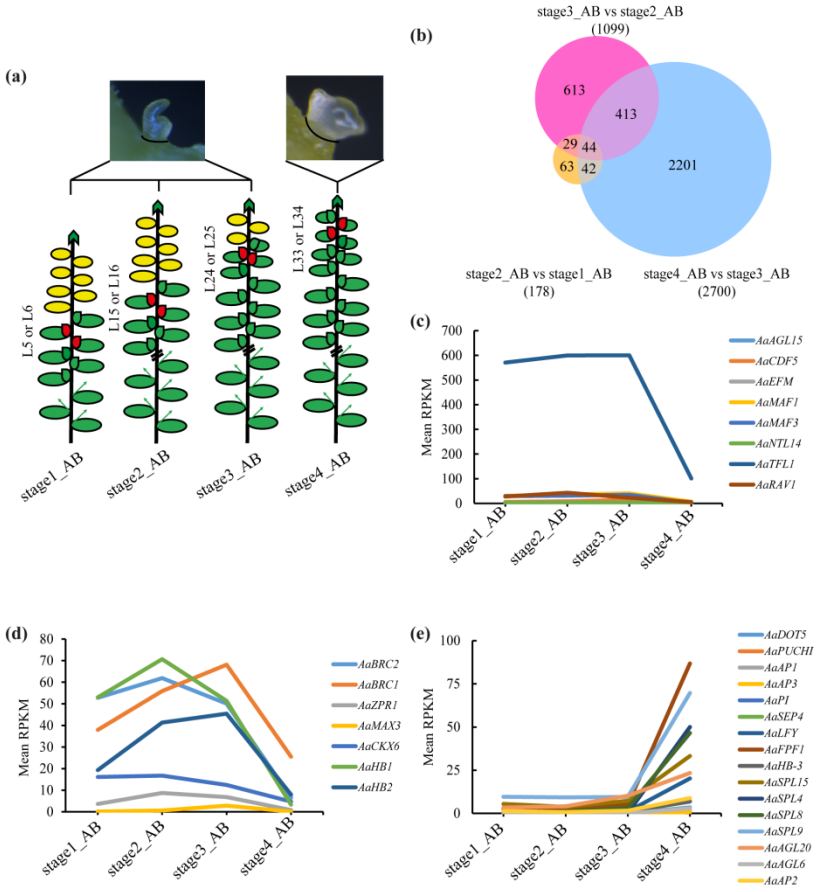

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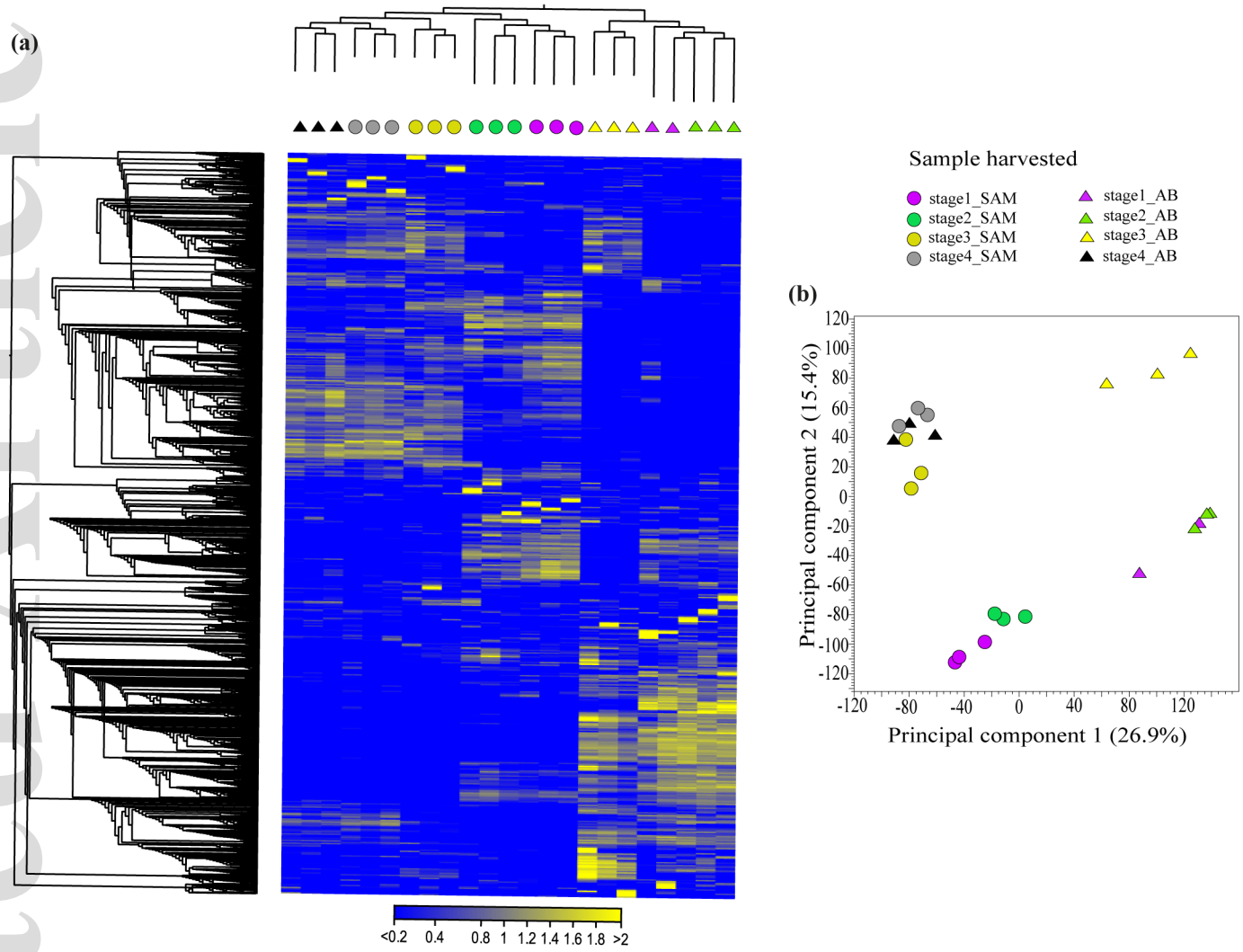

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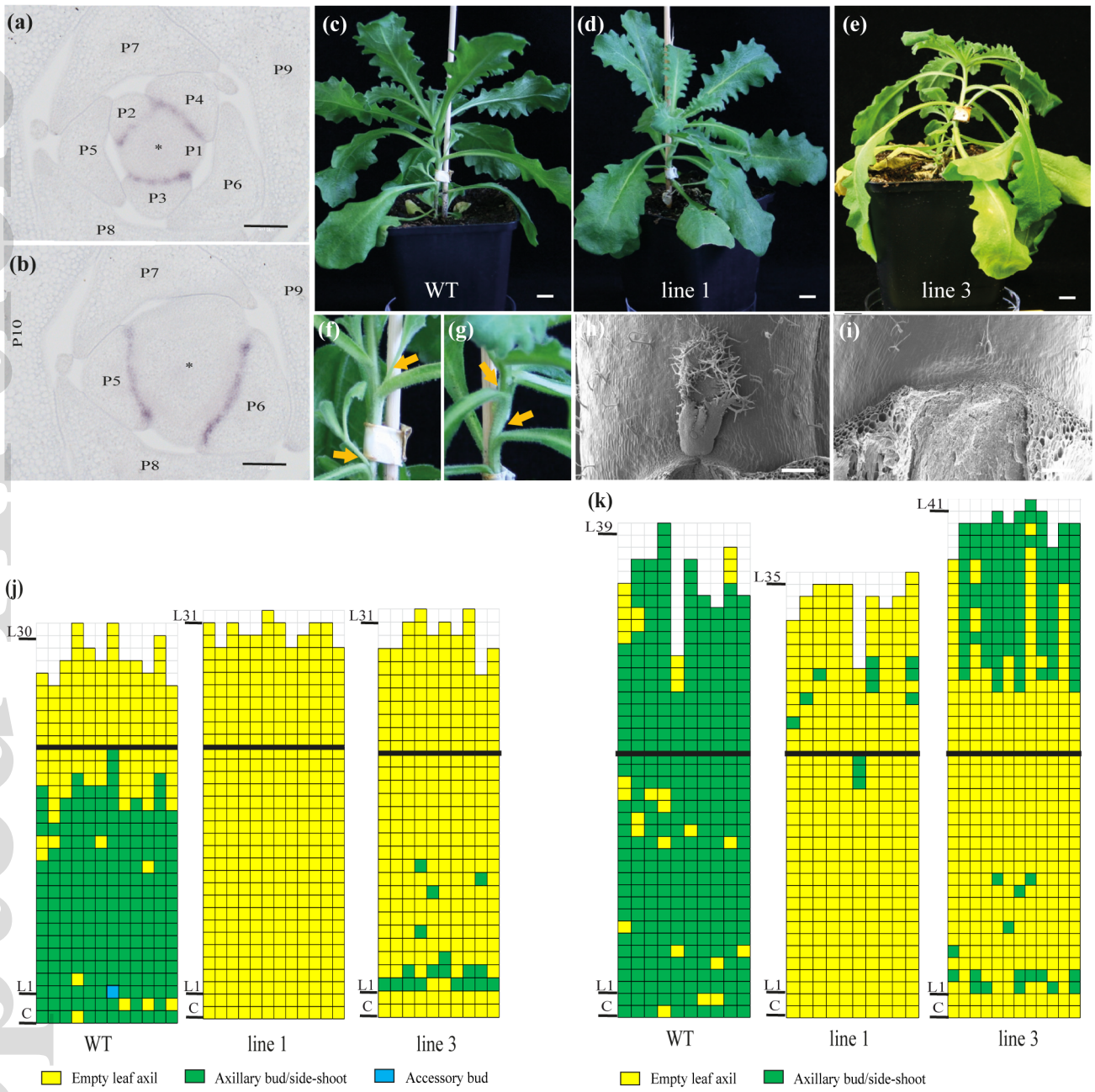

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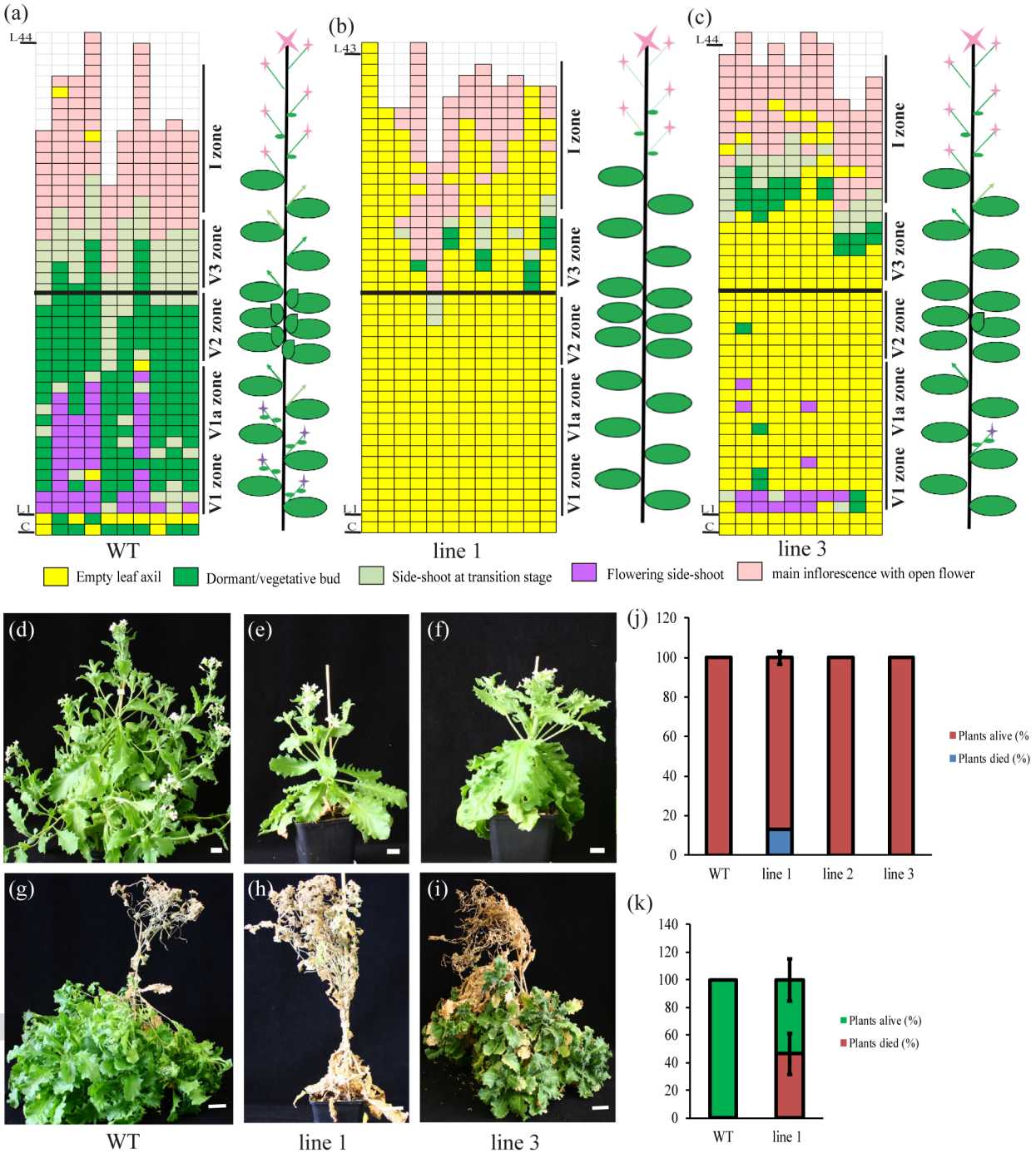

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