miR156-independent repression of the ageing pathway by longevity-promoting AHL proteins in Arabidopsis

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Summary

- Plants age by developmental phase changes. In Arabidopsis, the juvenile to adult vegetative phase change (VPC) is marked by clear heteroblastic changes in leaves. VPC and the subsequent vegetative to reproductive phase change are promoted by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors and repressed by miR156/157 targeting SPL transcripts.
- By genetic, phenotypic, and gene expression analyses, we studied the role of the longevity-promoting AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15) and family members in SPL-driven plant ageing.
- Arabidopsis ahl loss-of-function mutants showed accelerated VPC and flowering, whereas AHL15 overexpression delayed these phase changes. Expression analysis and tissue-specific AHL15 overexpression revealed that AHL15 affects VPC and flowering time directly through its expression in the shoot apical meristem and young leaves, and that AHL15 represses SPL2/9/13/15 gene expression in a miR156/157-independent manner. The juvenile traits of spl loss-of-function mutants appeared to depend on enhanced expression of the AHL15 gene, whereas SPL activity prevented vegetative growth from axillary meristem by repressing AHL15 expression.
- Our results place AHL15 and close family members together with SPLs in a reciprocal regulatory feedback loop that modulates VPC, flowering time, and axillary meristem development in response to both internal and external signals.

Introduction

Plant development progresses through several distinct developmental phases, starting with embryogenesis and followed successively by the juvenile vegetative, adult vegetative, reproductive, and gametophytic phases. In the juvenile vegetative phase, plants are generally not competent to flower, and flowering requires the transition from juvenile to adult vegetative development, which is referred to as vegetative phase change (VPC). Depending on the species, VPC may be marked by morphological changes, such as increased internode length, adventitious root production, and changes in leaf size and shape and trichome distribution, resulting in the presence of both juvenile and adult organs on a plant, a phenomenon referred to as heteroblasty (Huijser & Schmid, 2011). In Arabidopsis thaliana (Arabidopsis), leaf heteroblasty provides a clear indicator of VPC. Juvenile leaves have smooth margins, are rounder (length : width), and lack abaxial trichomes, whereas adult leaves have serrated margins, are more elongated (length : width ratio) and have abaxial trichomes (Telfer et al., 1997).

Compared with the adult-to-reproductive phase transition (or reproductive phase change), which is one of the key traits in crops, much less is known about the molecular mechanisms that mediate VPC. However, research in Arabidopsis has demonstrated that microRNAs (miRNAs) miR156, miR157, and miR172, are major regulators of VPC in Arabidopsis and other plant species (Poethig, 2013; Teotia & Tang, 2015). During the Arabidopsis life cycle the gradual decrease in miR156/miR157 expression results in increased expression of the SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) miR156/miR157 target genes. SPL transcription factors, in turn, promote the adult developmental programme at the shoot apical meristem (SAM), resulting in the transition from juvenile to adult leaf production and eventually from vegetative to reproductive development (Wu et al., 2009; He et al., 2018). The gradual decrease of miR156/miR157 expression during shoot maturation is accompanied by an SPL-induced increase in miR172 expression. miR172 promotes the development of trichomes on the abaxial side of leaves by repressing the expression of the APETALA2-LIKE (AP2-like) transcription factors TARGET OF EARLY ACTIVATION TAGGED1 (TOE1) and TOE2 (Wu et al.,
In eukaryotes, a wide range of DNA binding proteins have been identified that bind to AT-rich sequences in the minor groove of DNA by a small AT-hook domain (Reeves, 2010). These AT-hook proteins are considered as chromatin architectural factors involved in a diverse array of crucial cellular processes, including cell growth, cell differentiation, cell transformation, cell proliferation, cell death, and DNA replication and repair, by regulating chromatin remodelling and gene transcription (Reeves, 2010; Sgarra et al., 2010; Ozturk et al., 2014). The Arabidopsis genome encodes 29 AT-HOOK MOTIF NUCLEAR-LOCALIZED (AHL) proteins that contain either one or two AT-hook domains and a Plant and Prokaryote Conserved (PPC) domain (Fujimoto et al., 2004; Matsushita et al., 2007; Street et al., 2008; Zhao et al., 2013). These AHL proteins have been shown to be implicated in several aspects of plant growth and development, including hypocotyl growth (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013), root vascular tissue differentiation (Zhou et al., 2013), flower development (Ng et al., 2009), and flowering time (Street et al., 2008; Xiao et al., 2009; Yun et al., 2012; Xu et al., 2013). Based on mutant and protein–protein interaction studies, the AHL family members have been proposed to bind AT-rich DNA regions as hetero-multimeric complexes that recruit other transcription factors through their interacting PPC domains (Zhao et al., 2013). In addition, AHL proteins have been shown to repress transcription of several key developmental regulatory genes, possibly through modulation of the epigenetic code in the vicinity of their DNA binding regions (Lim et al., 2007; Ng et al., 2009; Yun et al., 2012). Some evidence has been obtained that AHL proteins function by altering the organization of chromatin structure (Lim et al., 2007; Ng et al., 2009; Yun et al., 2012; Xu et al., 2013). However, since this plant-specific class of nuclear proteins has only been studied more recently, their exact mode of action is still elusive.

Recently, we have shown that the AHL15 gene and other members of the AHL gene family are important for embryogenesis (Karami et al., 2021) and that they promote plant longevity by delaying axillary meristem (AML) maturation (Karami et al., 2020). In view of the seemingly antagonistic effect of AHL15 and its family members on the plant ageing pathway, we here study their possible role in the regulation of VPC and flowering time. Our analyses in Arabidopsis and Nicotiana tabacum (tobacco) showed that AHL15 overexpression prolongs the juvenile vegetative phase and delays flowering, whereas ahl15 loss of function results in precocious appearance of adult vegetative traits. A more detailed analysis indicated that AHL15 delays developmental phase changes by repressing SPL gene expression in an miRNA-independent manner. We further show that, in turn, AHL15 expression is repressed through feedback regulation by the SPLs.

Materials and Methods

Plant material and growth conditions and phenotype analysis

All Arabidopsis mutant and transgenic lines used in this study are in the Columbia (Col-0) background. The ahl15, ahl19, p35S:AHL15, p35S:AHL15-GR, pAHL15:GUS, pAHL15:AHL15, and pAHL15: AHL15ΔG, p35S:amiAHL20 and ahl15+/+ pAHL15:AHL15ΔG, pAHL15:AHL15ΔG, and ahl15+/ pAHL15:AHL15-GUS plant lines have been described previously (Karami et al., 2020, 2021). The sp9 spl15, p35S:miR156, p35S:MIM156, pSPL9:rSPL9-GR, pSPL2:rSPL2-GUS, pSPL9:rSPL9-GUS, pSPL10:rSPL10-GUS, pSPL11:rSPL11-GUS, pSPL13:rSPL13-GUS, pSPL15:rSPL15- GUS, mirR156a/b, mirR156a/b/d miR157a/c, and sp29/10/11/13/15 plant lines were obtained from the Nottingham Arabidopsis Stock Centre. The reporter lines pmiR156A:GUS, pmiR156B:GUS, and pmiR156C:GUS have been described previously (Yu et al., 2015). Plant lines and F1, F2, or F3 plants from crosses were PCR genotyped using primers described in the Supporting Information Table S1. Seeds were directly sown on soil in pots and grown at 21°C, 65% relative humidity, and a 16 h (long day, LD) or 8 h (short day, SD) photoperiod. One exception: SD was 10 h for the top panel of Fig. S3 (see later). To score for aerial rosette leaf production by AMs, wild-type, mutant, or transgenic plants were transferred to larger pots about 2 wk after flowering. Nicotiana tabacum cv SR1 Petit Havana (tobacco) wild-type or p35S:AHL15-GR plants (Karami et al., 2020) were grown in medium-sized pots at 25°C, 70% relative humidity, and a 16 h photoperiod. For dexamethasone (DEX; Sigma-Aldrich) treatment, Arabidopsis and tobacco plants were sprayed with 20 and 30 μM DEX, respectively. Leaf size (leaf length, and leaf width) was measured directly using a ruler.

The number of juvenile leaves (without abaxial trichomes) was scored once they appeared using a stereomicroscope. For imaging of leaf shape, fully expanded leaves were removed, attached to cardboard with double-sided tape, flattened, and photographed with a Nikon D5300 camera (Nikon Corp., Tokyo, Japan). Leaf images were optimized and changed into black-and-white images and assembled using Adobe ILLUSTRATOR CC 2017 (Adobe Inc., San Jose, CA, USA). Potted plants were photographed with a Nikon D5300 camera. All measurements were statistically analysed and plotted into graphs in GRAPHPAD PRISM 8 (GraphPad Software Inc., San Diego, CA, USA).

Plasmid construction and transgenic Arabidopsis lines

To generate the constructs pFD:AHL15 and pANT:AHL15, 3 kb regions upstream of the ATG initiation codon of the FD (At4G35900) and ANT (At4G37750) genes were amplified from Col-0 genomic DNA using the forward and reverse PCR primers indicated in Table S1. The resulting fragments were first inserted into pDONR207 by BP reaction, and subsequently cloned upstream of the genomic fragment containing the AHL15 transcribed region in destination vector pGW-AHL15 (Karami et al., 2021) by LR reaction. To generate pFD:miR156, first pGW-miR156 was constructed by replacing the AHL15 fragment.
Histochemical staining and microscopy

Histochemical staining of plant tissues for β-glucuronidase (GUS) activity was performed using 1 mg l⁻¹ X-gluc (R0852; Thermo Fischer Scientific, Waltham, MA, USA) as described previously (Anandalakshmi et al., 1998). Tissues were stained for 4 h at 37°C, followed by chlorophyll extraction and rehydration by incubation for 10 min in a graded ethanol series (75%, 50%, and 25%). GUS-stained tissues were observed and photographed using a Leica MZ12 microscope (Leica Geosystems AG, Heerbrugg, Switzerland) equipped with a Leica DC500 camera.

The tissue-specific GUS-staining intensity was quantified as mean grey values by analysing images of independent samples capturing the same region of interest using ImageJ, as previously described (Béziat et al., 2017).

Quantitative real-time PCR analysis

RNA was isolated from the shoot apex and a few young leaves close to the shoot apex (Fig. S1) or from the rosette base nodes using the RNaseasy® kit (Qiagen). First-strand complementary DNA was synthesized using the RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed on three biological replicates along with three technical replicates using the SYBR® green dye premixed master-mix (Thermo Fisher Scientific) in a C1000 Touch® thermal cycler (Bio-Rad, Hercules, CA, USA). Ct values were obtained using Bio-Rad CFX Manager 3.1. The relative expression level of genes was calculated according to the 2⁻ΔΔCt method (Livak & Schmittgen, 2001). The β-TUBULIN-6 and ACTIN2 genes were used as reference. Since similar results were obtained for these two genes in all qPCR experiments, expression was normalized using the β-TUBULIN-6 gene. The miRNAs’ abundance was quantified using 1 μg of RNA in a reverse transcription reaction by using the stem–loop method with an miRNA-specific forward primer. The miRNA levels were normalized using snoR101 as internal control. The data were analysed and plotted into graphs in GraphPad PRISM 8. Three biological replicates were performed, with three technical replicates each. The primers used are described in Table S1.

Results

AHL genes delay vegetative phase change and flowering time

When studying plants overexpressing AHL15 (p35S:AHL15), we noticed that the leaf blade length:width ratio was significantly reduced compared with that of wild-type plants (Fig. 1a–e). These initial observations and more detailed analysis showed that p35S:AHL15 plants had a significantly increased number of leaves without abaxial trichomes, both under SD and LD conditions (Fig. 1d), indicating AHL15 overexpression delayed VPC in Arabidopsis. By contrast, abl15 loss-of-function mutants developed leaves with a slightly increased blade length:width ratio and a reduced number of leaves without abaxial trichomes (Fig. 1a–d). abl15 mutant plants complemented with a pAHL15:AHL15 genomic clone again showed wild-type leaf development (Fig. 1a–d), indicating that the mutant phenotypes were caused by abl15 loss of function. AHL15 is part of a large gene family in Arabidopsis, where it clusters together with two close homologues: AHL19 and AHL20 (Zhao et al., 2013; Karami et al., 2021). In line with the previously reported high degree of functional redundancy between AHL genes (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013; Karami et al., 2021), abl15 abl19 p35S:amiRAHL20 triple-mutant plants showed a stronger increase in the leaf blade length:width ratio compared with the abl15 single or abl15 abl19 double mutants. However, the reduction in number of leaves without abaxial trichomes was comparable to that of abl15 or abl15 single or abl15 abl19 double-mutant plants under both SD and LD conditions (Fig. 1a–d).

Previously, we and others have shown that it is possible to overcome the functional redundancy among AHL genes by expression of a dominant negative mutant version of an AHL protein. Such a mutant version can be obtained either by deleting the conserved GRFEIL motif in the PPC domain (ΔG) or by generating a C-terminal fusion with GUS reporter protein (Zhao et al., 2013; Karami et al., 2020, 2021). Plants homozygous for a transgenic locus expressing AHL15-ΔG under control of the AHL15 promoter in the wild-type background mostly showed wild-type development, except for a decreased number of leaves without abaxial trichomes under LD conditions (Fig. 1c,d). By contrast, pAHL15:AHL15-GUS plants showed an increase in the leaf blade length:width ratio and a decrease in the number of leaves without abaxial trichomes under, respectively, LD and SD conditions (Fig. 1c,d). Introduction of the pAHL15:AHL15-ΔG or pAHL15:AHL15-GUS loci into the abl15 mutant background by reciprocal crosses resulted in wild-type F1 plants. F2 plants homozygous for both abl15 loss of function and the transgenic locus could not be obtained because of embryo lethality (Karami et al., 2020). However, selected abl15/+ pAHL15:AHL15-ΔG or abl15/+/pAHL15:AHL15-GUS F2 plants, heterozygous for abl15 loss of function and homozygous for the transgenic locus, showed a strong increase in leaf blade length:width ratio and also a significant reduction in leaves without abaxial trichomes, indicative of early VPC (Figs 2a–d, S2a–d). Since Arabidopsis plants require VPC before they can shift to the reproductive phase (Huijser & Schmid, 2011), we also monitored the flowering time of our mutant lines, as quantified by the number of rosette leaves developed until flowering. The single or combined abl15 and abl19 loss-of-function mutations did not significantly affect flowering time, whereas abl15 abl19 p35S:amiRAHL20 triple-mutant plants showed a significant reduction in flowering time both under LD and SD conditions (Fig. S3a, b). By contrast, p35S:AHL15 plants showed a significant delay of

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New Phytologist (2022) 235: 2424–2438
www.newphytologist.com
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AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15) and family members delay vegetative phase change in Arabidopsis thaliana. (a) The rosette phenotype of 5-wk-old wild-type (Col-0), p35S:AHL15, ah15, ah15 ahl19 p35S:amiRAHL20, or ah15/+ pAHL15:AHL15-ΔG plants grown under short day (SD) conditions. Bar, 1 cm. (b) Shape of the successive rosette leaves of 7-wk-old wild-type, p35S:AHL15, ah15 ah19 p35S:amiRAHL20, or ah15/+ pAHL15:AHL15-ΔG plants grown under SD conditions. (c) The length : width ratio of the second, fifth, and seventh leaves of 7-wk-old wild-type, p35S:AHL15, ah15 ah19 p35S:amiRAHL20, or ah15/+ pAHL15:AHL15-ΔG plants grown under SD conditions. (d) The juvenile leaf number (leaves without abaxial trichomes) in wild-type, p35S:AHL15, ah15, ah15 pAHL15:AHL15, ah15 ah19, ah15 ah19 ah15 ah19 p35S:amiRAHL20, pAHL15:AHL15-ΔG, and ah15/+ pAHL15:AHL15-ΔG plants grown under SD (up) and long day (down) conditions. (c, d) A coloured dot indicates the individual measurement per plant (n = 15 biologically independent plants per line), the horizontal line and the number below this line indicate the mean, and error bars indicate the SEM. Different letters indicate statistically significant differences (P < 0.01) as determined by one-way ANOVA with Tukey’s honest significant difference post hoc test.
flowering under both SD and LD conditions (Fig. S3a,b). Moreover, previously, we already showed that \textit{ahl15+} p\textit{AHL15}: \textit{AHL15}ΔG plants, but not p\textit{AHL15}: \textit{AHL15}ΔG plants, flowered significantly earlier under both LD and SD conditions compared with wild-type plants (see also Fig. S3a). Here, we also observed a strong reduction in flowering time for both p\textit{AHL15}: \textit{AHL15}-GUS and \textit{ahl15+} p\textit{AHL15}: \textit{AHL15}-GUS plants (Fig. S2e). Together, these results indicate that \textit{AHL} genes act redundantly
to delay VPC and, most likely as a result, also the reproductive phase change. Clearly, the AHL15-GUS fusion has a stronger dominant negative effect on overcoming this redundancy than AHL15-ΔG does.

We also analysed the effect of AHL15 overexpression on the same developmental phase changes in tobacco, a plant species from a different family, using available p35S:AHL15-GR tobacco lines (Karami et al., 2020). Previous analysis has shown that juvenile leaves in tobacco are rounder (lower length : width ratio) and smaller, show a reduced venation pattern, and are more pale green than adult leaves (Feng et al., 2016). In contrast to DEX-treated wild-type plants or mock-treated p35S:AHL15-GR tobacco plants, which showed wild-type leaf development with three round juvenile leaves preceding the production of the typically larger and longer adult leaves (Feng et al., 2016), DEX-treated p35S:AHL15-GR plants formed many small leaves with juvenile features (round, pale green/yellow, reduced venation pattern; Fig. S4a). By spraying these plants once per week with DEX, the SAM continued to produce small leaves, and plants could be kept in the vegetative state for more than a year, resulting in highly branched and bushy plants that did not flower (Fig. S4b,c). By contrast, DEX-treated wild-type plants and p35S:AHL15-GR control plants that were not treated with DEX developed normally and flowered after 2 months (not shown). This result indicates that AHL15 overexpression can also strongly delay, if not prevent, VPC and flowering in a non-Brassicaceae plant species, such as tobacco.

AHL15 delays vegetative phase change through its expression in the shoot apical meristem and in leaf primordia

To further understand the role of AHL genes in these developmental switches, we analysed the expression dynamics of AHL15 and its close homologues AHL19 and AHL20 during VPC. Reverse transcription qPCR experiments showed that expression of all three AHL genes in the shoot apex and young leaves of 3-wk-old seedlings was reduced compared to 1-wk-old seedlings grown under short day (SD) conditions (Fig. 2a). The reduced AHL15/19/20 expression in week 3 is in line with the timing of VPC, which occurs at 17–20 d after germination (DAG) in Arabidopsis ecotype Col-0 grown under SD conditions (Xu et al., 2016). Similarly, analysis of pAHL15:GUS reporter lines showed that AHL15 was expressed throughout the shoot at 7 DAG (week 1), that its expression declined in the SAM at 12 DAG (week 2), and that at 17 DAG (week 3) its expression was off in the SAM and severely reduced in the young leaves (Fig. 2b, c). The gene expression dynamics of AHL15 and its close homologues supports their role in maintaining vegetative juvenile traits and thus suppressing VPC.

Previous studies have shown that VPC is regulated by both internal factors at the SAM (Fouracre & Poethig, 2019) and lateral organ-derived signals (Yang et al., 2011, 2013; Yu et al., 2013). During VPC, AHL15 remains expressed in expanded lateral organs, but its expression is reduced in the SAM and newly formed organs, suggesting that its expression in these tissues is most relevant for its function. To confirm this, we expressed AHL15 under the control of the FD (pFD) and AINTEGUMENTA (pANT) promoters, which are predominantly active in the shoot apex (Yamaguchi et al., 2016; Fouracre & Poethig, 2019). Expression of AHL15 under these promoters significantly delayed VPC, as indicated by the reduced length : width ratio of the leaves and increased number of leaves lacking abaxial trichomes (Fig. 2d–g), and also delayed flowering (Fig. 5a,b) under both SD and LD conditions. Combining both pFD:AHL15 and pANT:AHL15 constructs in one plant line led to a further delay of VPC (Fig. 2d–g) and flowering (Fig. 5a,b). This additive effect can be explained by the slightly different but overlapping activities of the selected promoters (Yamaguchi et al., 2016; Fouracre & Poethig, 2019), causing enhancement of AHL15 expression in the SAM and in young leaf primordia (Fig. S6). Our results indicate that AHL15 expression in these tissues is sufficient to delay VPC and flowering (Fig. 2b).

AHL proteins repress SPL gene expression in an miR156/miR157-independent manner

In Arabidopsis, VPC is mediated by a gradual decrease in miR156/miR157 expression, which increases the expression of the SPL genes that are targets of these miRNAs. SPL genes, in turn, promote the adult developmental programme in the shoot apex, resulting in the production of adult instead of juvenile...
leaves and eventually in the initiation of flowering (Wu et al., 2009). One possible mode of action of AHL proteins might be that they suppress SPL expression by enhancing the miR156/miR157 pathway. A qPCR comparison of the expression of SPL genes (SPL2, SPL9, SPL10, SPL11, SPL13, and SPL15) known to promote VPC (Xu et al., 2016) showed that the transcript levels of SPL2, SPL9, SPL13, and SPL15 were significantly upregulated in shoots of 10-d-old or 3-wk-old ahl15 ahl19 p35S:MockDEX p35S:AHL15-GR pSPL2:rSPL2-GUS p35S:AHL15-GR pSPL9:rSPL9-GUS p35S:AHL15-GR pSPL10:rSPL10-GUS p35S:AHL15-GR pSPL11:rSPL11-GUS p35S:AHL15-GR pSPL13:rSPL13-GUS p35S:AHL15-GR pSPL15:rSPL15-GUS p35S:AHL15 p35S:MIM156 p35S:MIM1562

(a) Long day
(b) Short day
(c) Short day
(d) Short day
(e) p35S:AHL15-GR pSPL2:rSPL2-GUS p35S:AHL15-GR pSPL9:rSPL9-GUS p35S:AHL15-GR pSPL10:rSPL10-GUS p35S:AHL15-GR pSPL11:rSPL11-GUS p35S:AHL15-GR pSPL13:rSPL13-GUS p35S:AHL15-GR pSPL15:rSPL15-GUS

(f) (g) (h) (i)
amiRAH2.0 triple mutant and abhl15/+ pAHL15: AHL15-ΔG plants compared with wild-type seedlings under, respectively, LD or SD conditions (Fig. 3a–c). Under these conditions plantlets developed four and five leaves, respectively, which were juvenile based on their morphology. In the abhl15 abhl19 double mutant, only SPL9 and SPL13 showed enhanced expression. These results suggest that AHL proteins, namely AHL15, -19, and -20, repress the expression of SPL2, SPL9, SPL13, and SPL15 during VPC.

Not all SPL genes tested were simultaneously upregulated in the abhl15/+ pAHL15: AHL15-ΔG mutant background, and this made us wonder whether AHRs repress SPL expression independent of the miR156/miR157 pathway. Indeed, the pmiri156a/GUS, pmiri156b:GUS, and pmiri156d:GUS reporters (Yu et al., 2015) did not show a major change in expression upon AHR15 activation by DEX treatment in the p35S:AHL15-GR background (Fig. S7). Also, qPCR analysis showed that miR156/157 levels were not significantly different in 2- or 3-week-old wild-type or abhl15+/ pAHL15: AHL15-ΔG seedlings grown under juvenile-phase-prolonging SD conditions (Fig. 3d). In addition, when testing the expression of six miR156/miR157-insensitive pSPL: rSPL-GUS reporters (rSPL2, rSPL9, rSPL10, rSPL11, rSPL13, and rSPL15) (Xu et al., 2016) in the p35S:AHL15-GR background, the expression of rSPL2, rSPL9, rSPL13, and rSPL15 appeared to be downregulated by DEX treatment in the shoot apex, petioles, and leaves (Fig. 3e,f).

In plants overexpressing a target mimic of miR156 (p35S: MIM156), SPL gene expression is enhanced (Franco-Zorrilla et al., 2007), resulting in the accelerated appearance of adult leaves (Fig. 3g,h) and early flowering (Fig. S8a,b). AHR15 overexpression negated the precocious appearance of adult vegetative traits (Fig. 3g,h) and early flowering (Fig. S8) of p35S:MIM156 plants, bringing these traits back to or close to wild-type levels, whereas the MIM156 expression level was not affected by the p35S:AHL15 construct (Fig. S9).

In the reverse experiment, where the p35S:AHL15 construct was introduced into the p35S:miR156 background having low SPL levels, we observed a remarkably additive effect on both the vegetative and the reproductive phase transition (Fig. S10a,b). qPCR analysis showed that AHR15 and miR156 overexpression levels were maintained in the p35S:AHL15 p35S:miR156 background (Fig. S11a,b). Together, these results indicate that AHR15 and family members suppress the expression of specific SPL genes involved in VPC in an miR156/miR157-independent manner.

SPLs promote the vegetative phase change in part by repressing AHR15 expression

During VPC in 2-week-old Arabidopsis seedlings, increasing SPL levels (Wang et al., 2009) coincided with downregulation of AHR15, AHR19, and AHR20 (Fig. 2a–c). This led us to hypothesize that the SPL transcription factors are mediating downregulation of AHR gene expression. Based on this hypothesis, we expected AHR expression to be downregulated in Arabidopsis lines with elevated SPL levels (pSPL:rSPL-GUS lines, p35S:MIM156, and mirR156a/c/d mirR157a/c) and to be upregulated in Arabidopsis lines with reduced SPL levels (p35S: miR156 or SPL loss-of-function mutants). qPCR analysis indeed showed that the expression of AHR15 and AHR20 was significantly reduced in 10-day-old Arabidopsis seedlings of lines with elevated SPL levels compared with wild-type seedlings (Fig. 4a). In line with this result, expression of the pAHL15:GUS reporter was reduced in 1-week-old seedlings overexpressing MIM156 (Figs 4b, S12a), or in DEX- compared with mock-treated pSPL:rSPL9-GR seedlings (Fig. 4c). By contrast, expression of the pAHL15:GUS reporter was enhanced in 2-week-old seedlings by miR156 overexpression (Figs 4d, S12b). qPCR analysis also showed enhanced expression of AHR15 and AHR20 in 2-week-old p35S:miR156, sp9p15, and sp29/10/11/13/15 seedlings compared with wild-type seedlings (Fig. 4c). Based on our hypothesis, the delayed VPC and flowering phenotypes of plants with reduced SPL levels (e.g. the sp29/10/11/13/15 mutants and p35S:miR156) would be dependent on the elevated expression of functional AHR genes. The abhl15 loss-of-function
mutation in the \textit{ahl15} \textit{spl9} \textit{spl15} triple mutant indeed partially rescued the delayed VPC phenotypes of the \textit{spl9} \textit{spl15} double mutant. The length:width ratio of \textit{ahl15} \textit{spl9} \textit{spl15} leaves was almost restored to wild-type levels, and VPC of \textit{ahl15} \textit{spl9} \textit{spl15} plants was significantly accelerated compared with that of \textit{spl9} \textit{spl15} double-mutant plants, both in SD and in LD conditions.
SQUAMOSA PROMOTOR BINDING PROTEIN-LIKEs (SPLs) promote vegetative phase change by repressing AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15) and AHL20 expression in Arabidopsis thaliana. (a) Relative expression level of AHL15, AHL19, or AHL20 in the shoot apex and young leaves of 10-d-old wild-type (Col-0), pSPL2:rSPL2-GUS, pSPL9:rSPL9-GUS, pSPL10:rSPL10-GUS, pSPL11:rSPL11-GUS, pSPL13:rSPL13-GUS, pSPL15:rSPL15-GUS, p35S:MIM156, or mirR156a/b/d mirR157a/c seedlings grown under long day (LD) conditions. (b–d) Histochromic staining for (left) and quantification of (right) β-glucuronidase (GUS) activity in the shoot apical meristem and leaf primordia of (b) 8-d-old pAHL15:GUS or p35S:MIM156 pAHL15:GUS seedlings, (c) 10-d-old water (mock) or 20 μM dexamethasone (DEX)-treated pAHL15:GUS p35S:SPL9-GR seedlings, or (d) 15-d-old pAHL15:GUS or pAHL15:GUS p35S:mir156 seedlings grown under LD conditions. Bar, 1 mm. Dots in (b–d) indicate the values of 10 biological replicates per plant line, horizontal line and the number below this line indicate the mean, and error bars indicate the SEM. *, P < 0.01, indicates a significant difference with wild-type background as determined by a two-sided Student’s t-test.

Our previous results have shown that AHL15 does play a central role in the developmental phase identity of AMs; that is, the vegetative to reproductive phase change (Karami et al., 2020). Overexpression of AHL15 (p35S:AHL15 or pMYB:AHL15) repressed this developmental switch, leading to prolonged vegetative activity of AMs and resulting in the formation of aerial rosette leaves from inflorescence nodes (Fig. S15a,b). Such aerial rosette leaves are not formed on wild-type Arabidopsis (Col-0) plants grown under LD conditions (Fig. S15a,b), but they can be induced by growing plants under SD conditions or by mutating the SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) and FRUITFULL genes encoding transcription factors that suppress AHL15 expression (Karami et al., 2020). Previously, we have demonstrated that the aerial rosette phenotype is dependent on a functional AHL15 gene (Karami et al., 2020). Interestingly, the AMs in the axis of cauline leaves of spl9 spl15 mutant or p35S:miR156 (or pFD:miR156) overexpression plants also produced aerial rosette leaves (Fig. S15a,b), revealing an as yet unidentified role for SPLs in promoting the vegetative to reproductive transition of AMs. Introduction of spl9 spl15 or pFD:miR156 in the abh15 loss-of-function mutant background strongly reduced the aerial rosette leaf phenotype of both mutant lines (Fig. 5a,b), supporting our previous conclusion that AHL15 function is important for this phenotype. As aerial rosette leaves are reproducibly absent in wild-type and abh15 plants when grown under the same LD conditions (Fig. 5a), we did not take them along in this analysis. In line with the role of the SPL transcription factors as AHL repressors, AHL15 expression was significantly increased in spl9 spl15 and p35S:miR156 AMs compared with wild-type AMs (Fig. 5c,d). Based on these results, we concluded that AHL15 acts downstream of the SPL genes and that repression of AHL15 by SPLs in wild-type plants promotes the reproductive identity of AMs, whereas under conditions where SPL activity is reduced (e.g. in p35S:miR156 or spl9 spl15 plants), elevated AHL15 expression enhances the vegetative activity of AMs, resulting in the formation of aerial rosette leaves.

Discussion

Plants during their lifetime progress through distinct consecutive developmental phases. What drives and regulates the transition from one developmental phase to another is a longstanding...
fundamental question in plant developmental biology. In Arabidopsis and several other plants, VPC and the reproductive phase change (flowering) have been shown to be driven by the ageing pathway. In this pathway, the SPL transcription factors promote phase transitions, and miR156 and miR157 repress these transitions by targeting the SPL transcripts (Poethig, 2013; Teotia & Tang, 2015). Previously, we have shown that Arabidopsis AHL15 and its paralogues enhance plant longevity by delaying maturation of AMs (Karami et al., 2020). Here, we show that AHL15 and its close homologue AHL20, and possibly also AHL19, interfere with the ageing pathway by repressing SPL2/9/13/15 gene expression in an miRNA-independent manner and that, in turn, AHL15 and AHL20 expression is repressed through feedback regulation by the SPLs. In addition, we show that this reciprocal negative feedback loop between AHL15/19/20 and SPL2/9/13/15 genes not only regulates VPC and flowering
but also AM maturation, and thus controls plant longevity and life history.

Although the reproductive phase change is agronomically most important, VPC also has a strong impact on plant fitness and biomass, not in the least because it determines the balance between vegetative growth and reproductive development (Demura & Ye, 2010). Although the timing of VPC can be influenced by environmental factors such as photoperiod, light intensity, and temperature, this developmental switch is mainly regulated by endogenous genetic components (Poethig, 2013). Studies in Arabidopsis have revealed that the gradual decline in the expression of miR156/miR157 increases the abundance of the SPL transcription factors, which promote VPC (Poethig, 2013; Teotia & Tang, 2015). The change in leaf morphology during VPC is accompanied by a reduced expression of AHL15 and close homologues in the SAM and young leaves, which is in line with our model that AHL genes are repressed by SPLs (Fig. 6).

Recently, it has been reported that these internal factors at the SAM maintain the juvenile phase during early shoot development, but that the SAM plays a relatively minor role in the regulation of leaf identity at later stages of VPC (Fouracre & Poethig, 2019). Our observations that abl1 loss-of-function plants (abl1+ pAHL15:AHL15-ΔG) immediately form adult leaves and that p35S:AHL15 seedlings form multiple juvenile leaves confirm that AHLs belong to the internal factors in the shoot apex that maintain the juvenile phase in early shoot development. VPC was also strongly delayed when we overexpressed AHL15 specifically in the SAM and young leaf primordia using the FD and ANT promoters, suggesting that AHL might also regulate VPC through lateral organ-derived signals (Yang et al., 2011, 2013; Yu et al., 2013).

Our analysis showed that AHL15 represses the expression of SPL genes in an miR156/miR157-independent manner. Previous studies have shown that the abundance of miR156 strongly declines (c. 90%) at the shoot apex of Arabidopsis seedlings within a 2 wk period, whereas the RNA level for most SPL genes remains low or increases only slightly during this period (Xu et al., 2016). Here, we show that the SPL2, SPL9, SPL13, and SPL15 expression levels are significantly higher at the shoot apex of abl15 loss-of-function than in wild-type plants, whereas the miR156/miR157 levels did not differ between wild-type or abl15 loss-of-function mutant plants. This indicates that the maintained repression of SPL expression during early plant development is mediated by AHL15 independent of miR156/157. How AHL15 represses SPL genes remains unknown. AHL15 might directly bind to the SPL loci. However, preliminary yeast one-hybrid assays did not provide an indication for direct binding of AHL15 to the SPL promoter regions (data not shown). Recently, we have shown that AHL15 overexpression suppresses the biosynthesis of the plant hormone gibberellin (GA; Karami et al., 2020). Since DELLA proteins, the degradation targets of GA signalling, have been shown to repress SPL expression (Yu et al., 2012), AHL15 may repress SPLs indirectly by reducing GA biosynthesis and thus stabilizing the DELLA proteins (Fig. 6).

Previously, it has been shown that SPLs promote trichome development on the abaxial side of leaves partially by repressing the AP2-like transcription factors TOE1 and TOE2 (Wu et al., 2009; Wang et al., 2019; Xu et al., 2019). How SPLs promote
the other adult leaf traits, such as leaf elongation and leaf serration, was not known until now. In this study, we show that the promotion of adult traits, including leaf elongation and trichomes on the abaxial side of leaves, is in part mediated by the repression of AHL gene expression by SPLs.

We have recently published that AHL15 globally reorganizes the chromatin configuration (Karami et al., 2021). Therefore, AHL15 may control the ageing pathway by inducing changes in higher-order chromatin organization that lead to repression of SPL genes, GA3 biosynthesis, and other pathways. In animals, the contribution of higher-order chromatin organization to ageing processes has been well documented (Moraes, 2014; Chandra & Kirschner, 2016). However, the actual involvement of higher-order chromatin organization in the juvenile-to-adult transition in plants remains to be investigated.

In conclusion, based on our findings, we propose a model in which both miR156/157 and AHL15/20 proteins independently slow down plant ageing by repressing SPL2/9/13/15 expression: miR156/157 by targeting the SPL mRNAs (Wang et al., 2009; Wu et al., 2009; He et al., 2018) and AHL15/19/20 possibly by binding directly to SPL loci or indirectly through the DELLA proteins by downregulating biosynthesis of the ageing-promoting GA (Yu et al., 2012; Karami et al., 2020) (Fig. 6). In turn, SPLs reciprocally repress AHL expression possibly by binding directly to the AGL upstream regions, or indirectly by promoting the expression of SOC1, a negative regulator of the AGL15 gene (Wang et al., 2009; Karami et al., 2020) (Fig. 6). The floral integrator SOC1 (Huijser & Schmid, 2011) connects AHL15 to the photoperiodic pathway, which explains why, under SD conditions, AGL15 expression in the Arabidopsis SAM and AMs is enhanced, leading to delayed VPC and the production of aerial rosette leaves. Our findings place AHL15 and its close homologue AGL20, and possibly also AGL19, together with SPLs in a reciprocal regulatory feedback loop that allows modulation of the ageing pathway in plants by both internal (e.g. miRNA156/157) and external (e.g. photoperiod) signals.

Acknowledgements
We are grateful to Scott Poethig for providing different SPL and miR156 related lines, and to Nam-Hai Chua for providing the pmmiR156-GUS reporter lines. We thank Kim Boutilier and Lena Maas for performing yeast one hybrid experiments, and Ward de Winter, Jan Vink, Nick Surtel, and Mariel Lavrijsen for their technical support. OK was in part supported by subsidies from Generade and from the Building Blocks of Life research programme (no. 737.016.013, to RO), which is (partly) financed by the Dutch Research Council (NWO).

Author contributions
AR, OK and RO conceived the project, designed the experiments, and analysed and interpreted the results. AR performed most of the experiments. SB and OK contributed to some of the experiments. OK and RO supervised the project. AR, OK and RO wrote the manuscript. All authors read and commented on versions of the manuscript.

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Data availability
The raw experimental data and the plant lines described are available upon request. The Arabidopsis AGI locus identifier for each gene described is provided in the Table S1.

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Supporting Information

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

Fig. S1 Preparation of shoot apex and young leaf samples from young Arabidopsis thaliana plants for RNA isolation.

Fig. S2 Expression of a dominant negative AHL15-GUS fusion protein leads to precocious development of adult traits and early flowering in Arabidopsis thaliana.

Fig. S3 Arabidopsis thaliana AHL15 and close homologues redundantly regulate flowering time.

Fig. S4 Extreme delay of vegetative phase change and flowering by heterologous expression of Arabidopsis thaliana AHL15 in Nicotiana tabacum.

Fig. S5 Shoot apical meristem- or young leaf-specific AHL15 overexpression delays flowering time in Arabidopsis thaliana.

Fig. S6 Tissue-specific AHL15 overexpression in Arabidopsis thaliana.

Fig. S7 AHL15 does not affect the expression of miR156A, -B, or -D in Arabidopsis thaliana.

Fig. S8 AHL15 and SPLs antagonistically control flowering time in Arabidopsis thaliana.

Fig. S9 Overexpression of the mimic miR156 (p35S:MIM156) is not altered by p35S:AHL15 in Arabidopsis thaliana.

Fig. S10 AHL15 and SPLs synergistically control vegetative phase change and flowering time in Arabidopsis thaliana.

Fig. S11 AHL15 and miR156 are overexpressed in Arabidopsis thaliana p35S:miR156 p35S:AHL15 plants.
Fig. S12 Overexpression of the mimic miR156 (p35S:MIM156) or miR156 (p35S:miR156) in the Arabidopsis thaliana pAHL15:GUS background.

Fig. S13 The rescue of miR156 overexpression phenotypes by abhl5 loss of function in Arabidopsis thaliana is most likely caused by silencing of the p35S:miR156 construct.

Fig. S14 Delay of flowering by spl loss of function in Arabidopsis thaliana is largely AHL15 independent.

Fig. S15 Aerial rosette leaves in Arabidopsis thaliana by reduced SPL expression or AHL15 overexpression.

Table S1 Gene IDs and primers used for cloning, genotyping, and quantitative PCR.

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