Role for the shoot apical meristem in the specification of juvenile leaf identity in *Arabidopsis*

Jim P. Fouracre[a] and R. Scott Poethig[a,1]

[a]Biology Department, University of Pennsylvania, Philadelphia, PA 19104

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The extent to which the shoot apical meristem (SAM) controls developmental decisions, rather than interpreting them, is a longstanding issue in plant development. Previous work suggests that vegetative phase change is regulated by signals intrinsic and extrinsic to the SAM, but the relative importance of these signals for this process is unknown. We investigated this question by examining the effect of meristem-deficient mutations on vegetative phase change and on the expression of key regulators of this process, miR156 and its targets, SPL transcription factors. We found that the precocious phenotypes of meristem-deficient mutants are a consequence of reduced miR156 accumulation. Tissue-specific manipulation of miR156 levels revealed that the SAM functions as an essential pool of miR156 early in shoot development, but that its effect on leaf identity declines with age. We also found that *SPL* genes control meristem size by repressing *WUSCHEL* expression via a novel genetic pathway.

vegetative phase change | miR156 | WUSCHEL | leaf development | shoot apical meristem

As plants mature they transition through a number of distinct developmental phases. A transition from juvenile-to-adult vegetative growth, before the onset of reproductive development, has been recognized since the late 19th century (1). Depending on the species, this transition, known as vegetative phase change (VPC), may lead to changes in, among others, leaf size and shape, plastochron length, shoot physiology, adventitious root production, disease resistance, and reproductive competence (reviewed in ref. 2). In the model plant *Arabidopsis thaliana*, the switch to adult growth is associated with the production of large, spatulate, and serrated leaves that produce trichomes on their abaxial surface. Leaves with a juvenile identity are small, round, smooth, and lack abaxial trichomes.

The master regulator of VPC in *Arabidopsis* and all other studied flowering plants is the microRNA miR156 (2). The expression of miR156 declines temporally during shoot maturation, allowing the expression of its target genes in the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) family to increase (3–5). *Arabidopsis* has 10 *SPL* genes that are targeted by miR156 and that promote adult growth to varying degrees with *SPL9*, *SPL13*, and *SPL15* having the largest effect (6). miR156 and the partly redundant miR157 also exist in multigene families, with the loci *MIR156A*, *MIR156C*, *MIR157A*, and *MIR157C* being the most important for suppressing *SPL* activity during juvenile growth (7).

miR156 and *SPL9/13/15* are all expressed throughout the shoot apex in both the shoot apical meristem (SAM) and young leaf primordia (6). While there has been recent progress in elucidating the molecular mechanisms that control the temporal decline of miR156 expression (8–13), how expression of the miR156-*SPL* pathway and the process of VPC are coordinated across the shoot apex remains unknown. It has long been thought that the identity of a shoot is determined by the maturation state of the SAM (14, 15). A regulatory role for the SAM is further supported by the phenotypes of plants that have reduced meristems and that immediately produce leaves with adult traits, such as *wuschel* (*wus*) and *paused* (*psd*) mutants (16, 17). However, leaf ablation studies have shown that signaling from existing juvenile leaves promotes the subsequent production of adult leaves (18–20). These results sit in apparent conflict: if juvenile leaves are required to initiate adult growth, how do *wus* and *psd* immediately produce adult leaves? We aimed to resolve this question by analyzing the effects of perturbations to the meristem on the miR156-*SPL* pathway. We demonstrate that the *wus* phenotype is a consequence of reduced miR156 expression and that expression of miR156 within the SAM, both in *wus* and developmentally normal meristems, affects leaf identity. We further show that expression of *WUS* is regulated by feedback from *SPL* genes.

Results

The Precocious Formation of Adult Leaves in SAM-Defective Plants Is Not Attributable to a Delay in Leaf Initiation. Mutations in the homeobox gene, *WUS*, and the tRNA export receptor, *PSD*, interfere with development of the SAM, delay leaf emergence, and exhibit precocious vegetative development; the first two leaves produced by these mutants prematurely display adult traits (Fig. 1 *A*–*C*) (16, 17). To determine if the effect of these mutations on leaf identity is attributable to their effect on the SAM, we examined the phenotype of two other meristem-deficient mutants: *shoot meristemless* (*stm-1*) and the triple mutant *arabidopsis thaliana homeobox 1*; *pennywise*; *pound-foolish* (*ath*; *pny*; *pnf*) (21). Like *wus* and *psd*, the first two leaves of these mutants resembled leaves produced at later plastochrons in developmentally normal plants (Fig. 1 *A*–*C*).

Significance

Developmental transitions during shoot development in plants are regulated by factors originating outside and within the shoot apical meristem (SAM). The best-known example of this is the vegetative-to-reproductive transition, which is initiated by a leaf-derived signal that transforms the vegetative SAM into a developmentally stable inflorescence meristem. Although the juvenile-to-adult vegetative transition (vegetative phase change) is also thought to be regulated by factors exogenous and internal to the SAM, how this process is coordinated spatially remains unknown. Here we demonstrate that the SAM specifies leaf identity early in development, but that leaves become more important determinants of shoot identity as the shoot ages. We also reveal a role for the plant aging pathway in the regulation of meristem size.

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[1]To whom correspondence should be addressed. Email: spoethig@sas.upenn.edu.

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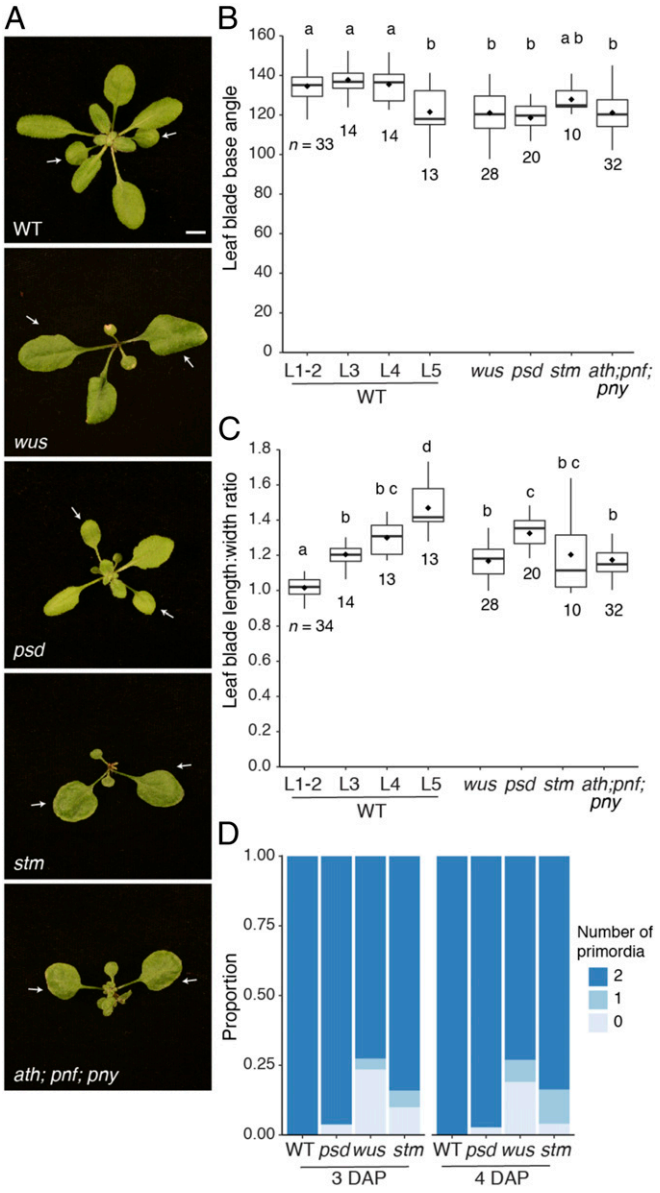

Fig. 1. The precocious phenotype of meristem-defective mutants is not caused by a delay in leaf initiation. (*A*) The phenotype of WT Columbia and mutant plants at 23 DAP in LD conditions. White arrows point to leaves 1 and 2. (Scale bar, 5 mm.) (*B* and *C*) The angle of the leaf base (*B*) and the length:width ratio of the leaf blade (*C*) in leaves 1–5 of WT plants and in leaves 1 and 2 of meristem-defective mutants. Boxes display the interquartile range (IQR) (boxes), median (lines), and values beyond 1.5* IQR (whiskers); mean values are marked by a solid diamond (◆). Significantly distinct groups were determined by one-way ANOVA with post hoc Tukey multiple comparison test (letters indicate statistically distinct groups; $P < 0.05$). Sample sizes are shown on the graphs. (*D*) Distribution of leaf primordia number identified by staining of a *LFY::GUS* reporter in respective meristem-defective mutants (*wus* and *stm* were segregating 25% mutant plants; *psd* was homozygous for the mutation).

It has been suggested that the first-formed leaves of *psd* and *wus* have an adult identity because leaf initiation does not occur in these mutants until after the shoot has transitioned to the adult phase (16, 22). This hypothesis implies that the timing of VPC is regulated independently of leaf production and is inconsistent with the evidence that leaves promote VPC. To resolve this inconsistency, we re-examined the effect of *psd*, *wus*, and *stm* on leaf initiation, using lines containing a *LFY::GUS* reporter that is expressed in leaf primordia; this reporter makes it possible to visualize young leaf primordia immediately after seed germination (*SI Appendix*, Fig. S1*A*). Three days after planting (DAP), 100% (20/20) of Col *LFY::GUS* plants and 96% (25/26) of *psd LFY::GUS* mutants had two leaf primordia, and a similar number of plants had two leaf primordia at 4 DAP (Fig. 1*D*). These primordia were slightly smaller in *psd* than in wild type (WT), consistent with previous studies indicating that *psd* delays leaf initiation (22). However, this result demonstrates that *psd* initiates leaves before VPC (which occurs between plastochron 5 and 6), implying that its effect on leaf identity cannot be attributed to this delay in leaf initiation. Because *stm* and *wus* cannot be maintained in homozygous condition, their effect on leaf initiation was determined by staining populations segregating 25% for these mutations. At 3 DAP, 84% of the seedlings segregating for *stm* had two leaf primordia, 6% had one primordium, and 10% had no visible primordia; by 4 DAP, the number of plants with at least one leaf primordium had increased to 96%. Given that 25% of these seedlings were homozygous for *stm*, this result demonstrates that a majority of *stm* mutants initiate at least one leaf during the juvenile phase. Similar results were obtained with *wus*. At 3 DAP, 72% of the seedlings segregating for *wus* had two leaf primordia, 4% had one leaf primordium, and 24% had no visible leaf primordia. At 4 DAP, 73% of these seedlings had two leaf primordia, 8% had one leaf primordium, and 19% had no leaf primordia. Thus, a significant number of *wus* seedlings initiate leaves during the juvenile phase. These results confirm previous studies indicating that *psd*, *stm*, and *wus* delay leaf initiation, but demonstrate that this delay does not account for the adult identity of the leaves that they produce.

To determine if the precocious phenotype of these mutants might be due to a delay in leaf expansion, we examined the relationship between the timing of leaf emergence and leaf identity in *wus*. We found that the first leaf of *wus* mutants reached a length of ≥0.5 mm at different times after planting (between 6 and 12 DAP), but that there was no qualitative difference in the final shape of leaves that emerged at different time points (*SI Appendix*, Fig. S1 *B* and *C*). These results suggest that the effect of *psd*, *stm*, and *wus* on leaf identity is not due to a delay in either leaf initiation or leaf growth.

Precocious Adult Leaves Exhibit Elevated SPL Activity. An alternative possibility is that meristem-defective mutations affect the activity of genes that regulate VPC. To test this hypothesis, we examined the effect of *wus* and *psd* on the abundance of miR156—the master regulator of VPC—and one of its direct targets, *SPL9*. Analyses were carried out on 10-d-old shoot apices bearing leaf primordia ≤1 mm and on 1- to 2-mm primordia of leaves 1 and 2; these samples were harvested from plants grown in short days to eliminate differences in gene expression resulting from the potential effect of these mutations on floral induction (the *wus* and *psd* mutant phenotypes persist in SD conditions). miR156 was reduced and *SPL9* was elevated in both the shoot apices and leaf primordia of *wus* and *psd* compared with WT seedlings (Fig. 2 *A* and *B*). *wus* had no effect on miR156 expression in cotyledons (*SI Appendix*, Fig. S2). We then examined the effect of *wus* on the abundance of SPL proteins by crossing miR156-sensitive (*sSPL9-GUS*, *sSPL13-GUS*) and miR156-resistant (*rSPL9-GUS*, *rSPL13-GUS*) reporters into *wus* (Fig. 2*C*). Fluorescence assays of proteins using 4-methylumbelliferyl ß-D-glucuronide (MUG) extracted from LP1 and -2 revealed that sSPL9-GUS was expressed 3× higher and sSPL13-GUS was expressed 5.2× higher in *wus* than in WT primordia (Fig. 2*D*). rSPL9-GUS and rSPL13-GUS were also more highly expressed in *wus* than in WT, although to a much smaller extent than the miR156-sensitive reporters. These data suggest that the precocious vegetative phenotype of *psd* and *wus* is primarily attributable to a decrease in the abundance of miR156, although transcriptional up-regulation of *SPL* genes may also contribute to this phenotype.

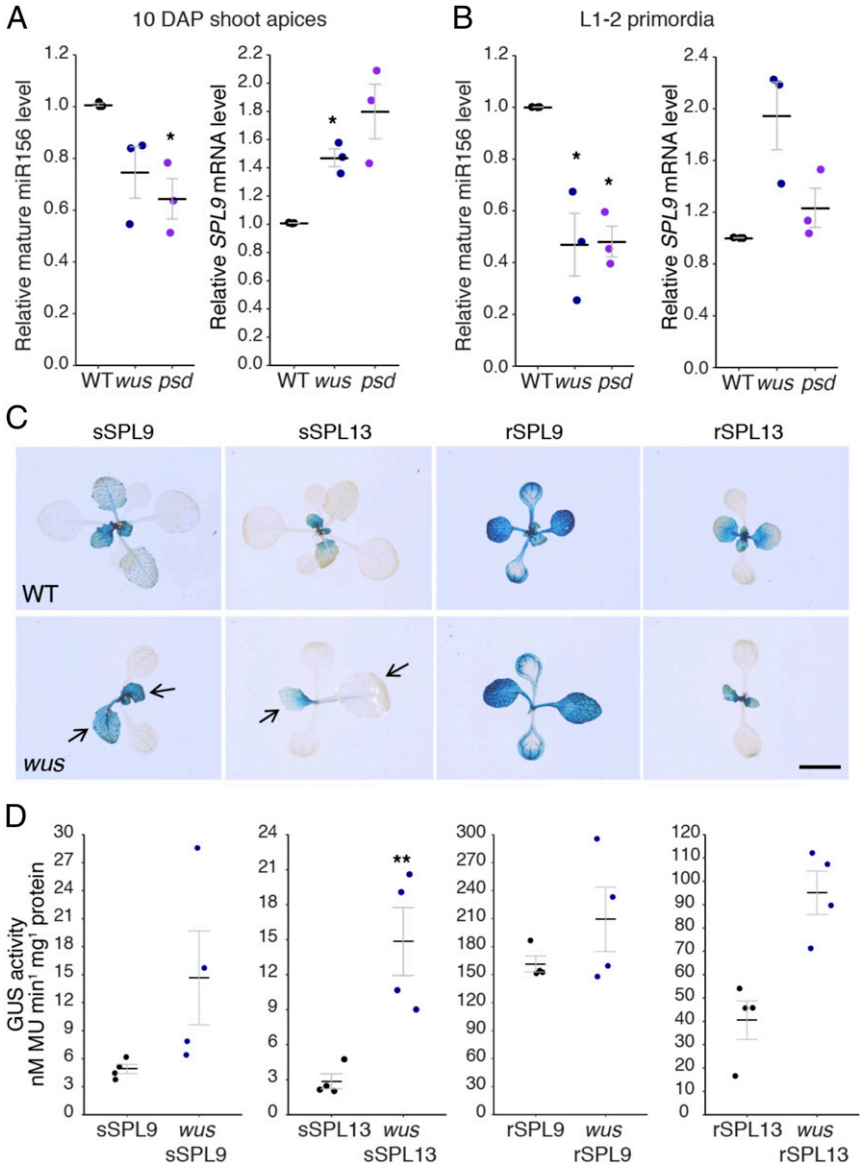

Fig. 2. *wus* and *psd* have reduced levels of miR156 and elevated levels of *SPL9*. (*A* and *B*) miR156 miRNA and *SPL9* mRNA levels in 10-DAP shoot apices with leaf primordia ≥1 mm removed (*A*) and in isolated 1- to 2-mm primordia of leaves 1 and 2 (*B*) in SD conditions. Relative levels were quantified by RT-qPCR, normalized to snoR101 (for miR156) or *ACT2* (for *SPL9*) as internal control genes and expressed as a ratio of expression to WT plants. Each data point represents a biological replicate and is the average of three technical replicates. Black bars represent the mean and gray bars the SEM. Significant differences to WT were determined by two-tailed *t*-test (*$P < 0.05$). (*C*) GUS staining of miRNA-sensitive and miR156-resistant SPL-GUS reporter constructs at 21 DAP in SD conditions. Black arrows point to precocious accumulation of SPL proteins in leaves 1 and 2. (Scale bar, 5 mm.) (*D*) MUG assays of lines shown in *C*. Protein was extracted from 1- to 2-mm primordia of leaves 1 and 2 in SD conditions. Each data point represents a biological replicate. Significant differences from WT were determined by two-tailed *t* test of log-transformed data (**$P < 0.01$).

Elevated miR156 Expression Suppresses the Leaf Phenotype of *wus*. If the effect of *wus* on leaf identity is attributable to a decrease in the level of miR156, overexpression of miR156 should correct this phenotype. To test this prediction, we expressed miR156 constitutively and in localized regions of the shoot apex in *wus* mutants. Constitutive high expression of *MIR156A* under the regulation of the CaMV 35S promoter largely suppressed the adult leaf phenotype of *wus*; *wus*; *35S::MIR156A* leaves more closely resembled the juvenilized leaves of *35S::MIR156A* plants than the adult-like leaves of *wus* mutants (Fig. 3 *A* and *B*). *35S::MIR156A* also partially suppressed the effect of *wus* on leaf number (Fig. 3*A*). Expressing *MIR156A* in *wus* using the *AINTEGUMENTA* (*ANT*) promoter—which is transcribed across the shoot apex but predominantly in incipient and young leaf primordia (23, 24) (*SI Appendix*, Fig. S3*A*)—produced a phenotype similar to that of *35S::MIR156A*. Expressing miR156 in the central domain of the SAM using the *WUS* promoter (*WUS::MIR156A*) also partly rescued the effect of *wus* on leaf shape and leaf production (Fig. 3). Heterologous gene expression driven by the *WUS* promoter has previously been shown to be confined to the meristem in a *wus* background (25). These results therefore suggest that the precocious phenotype of *wus* plants is due to a reduction in the abundance of miR156 and demonstrate that expressing miR156 specifically within the SAM can partially compensate for the effect of *wus* on shoot development.

SAM-Derived miR156 Regulates Leaf Identity. The similarity between the morphological and molecular phenotypes of plants

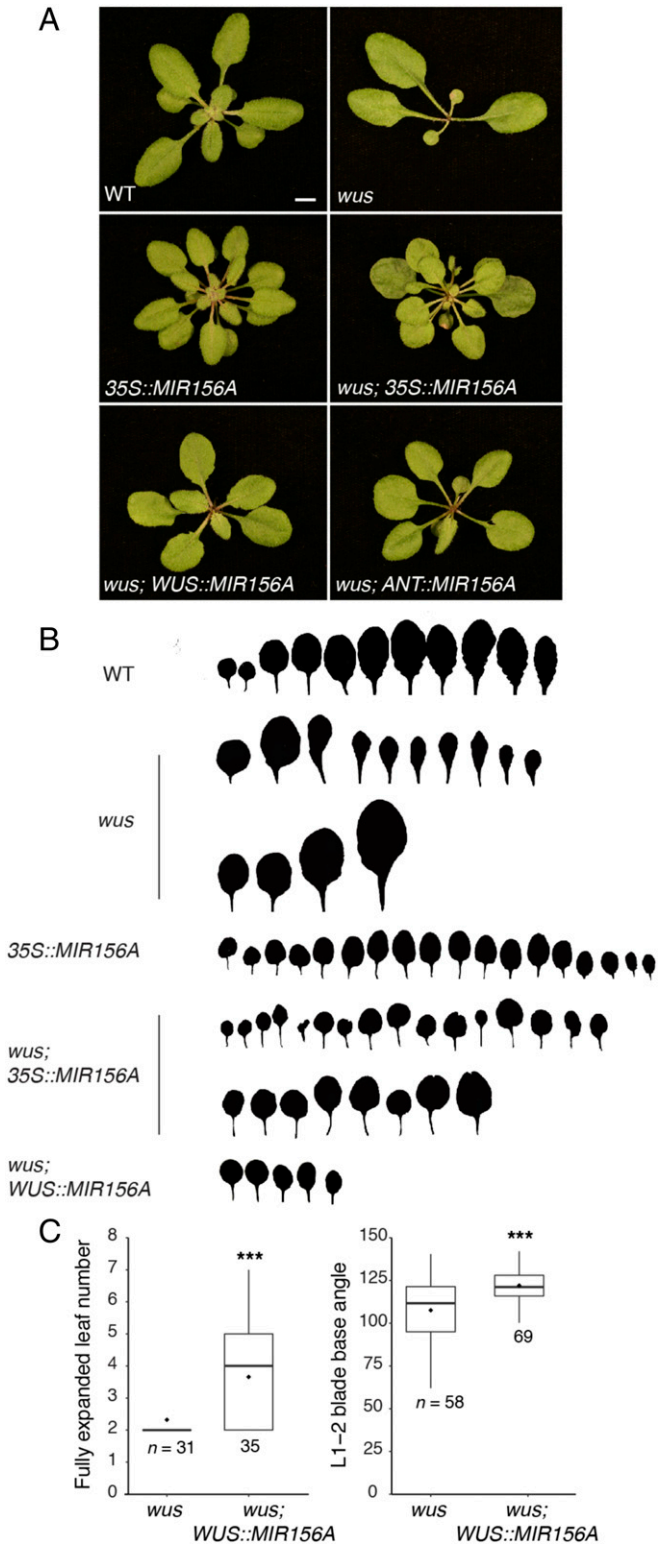

Fig. 3. Enhanced miR156 expression suppresses the *wus* leaf phenotype. (*A*) Photographs were taken at 21 DAP in LD. (Scale bar, 5 mm.) (*B*) Heteroblasty of lines shown in *A*; two examples of *wus* and *wus*; *35S::MIR156A* are shown to indicate the range of phenotypes observed. (*C*) Boxplots showing the effects of *WUS::MIR156A* on leaf formation at 24 DAP and the angle of blade bases in leaves 1 and 2 in a *wus* background. Only fully expanded leaves without polarity defects were counted. Boxes display the IQR (boxes), median (lines), and values beyond 1.5* IQR (whiskers); mean values are marked by a solid diamond (◆). Significant differences between the two lines were determined by two-tailed *t* test (***$P < 0.001$); sample sizes are indictated on the graphs.

with defective SAMs, and the ability of *WUS::MIR156A* to partially rescue the effect of *wus* on leaf shape, suggest that miR156 (or a downstream target) produced by the SAM acts noncell-autonomously to regulate leaf identity. To test this hypothesis, we manipulated the abundance of miR156 in different domains of WT and miR156-deficient shoot apices by expressing *MIR156A* or the miR156/157 target site mimic *MIM156* [which reduces miR156/157 activity (26)] in transgenic plants using promoter sequences from *ANT* (27), *FLOWERING LOCUS D* (*FD*) (28, 29), *STM* (30), and *WUS* (31). Promoter::GUS fusions confirmed that the *ANT* sequence drives expression throughout the entire shoot apex; the *FD* sequence drives expression in the SAM and leaf primordia ≤250 μM in size; the *STM* sequence drives expression in the peripheral region of the SAM but not in leaf primordia; and the *WUS* sequence drives expression in the central region of the SAM (*SI Appendix*, Fig. S3*A*). Transgene expression was confirmed by RT-PCR (*SI Appendix*, Fig. S3*B*).

To determine if miR156 produced by the SAM is capable of rescuing the phenotype of plants deficient for miR156/157, we fused the promoter sequences mentioned above to a *MIR156A* genomic sequence and introduced these constructs into a *mir156a mir156c mir157a mir157c* quadruple mutant (*mir156/157* qm) (7). *WUS::MIR156A* partly suppressed the leaf shape (Fig. 4 *A*, *K*, and *L*) and abaxial trichome phenotype (Fig. 4*B*) of the *mir156/157* qm, and significantly accelerated the emergence of the first two leaves (Fig. 4*E*), but had no effect on leaf number or bolting time (Fig. 4 *C* and *D*). *STM::MIR156A* had a stronger effect on abaxial trichome production, leaf shape, and the rate of leaf initiation and significantly increased the number of rosette leaves, but also had no effect on bolting time. *FD::MIR156A* nearly completely corrected the vegetative phenotype of the *mir156/157* qm (Fig. 4 *A*–*E* and *SI Appendix*, Fig. S3*C*) in long days (LD) and delayed VPC and flowering relative to WT plants in noninductive SD conditions (*SI Appendix*, Fig. S4). *SPL* expression is reduced in SD (32), which probably accounts for the stronger effect of *FD::MIR156A* under these conditions. The observations that meristematic expression of miR156 had the strongest effect on the morphology of early leaves (Fig. 4*K* and *SI Appendix*, Fig. S3*C*), was only able to delay trichome formation from leaf 2 to leaf 3 (in the case of *WUS::MIR156A* and *STM::MIR156A*) (Fig. 4*B*), and accelerated the emergence of leaves 1–2 but not later leaves (Fig. 4*E*) suggest that the regulatory capacity of SAM-derived miR156 is highest during early development—i.e., during the initiation of rosette leaves 1–3—but declines with age. In this regard, the observation that the continued expression of miR156 in leaf primordia under the *ANT* promoter maintained plants in the juvenile phase for a prolonged period (Fig. 4 *A*–*C*) supports the conclusion that leaves are a more important source of miR156 than the SAM throughout most of shoot development.

To determine if miR156 produced by the SAM is necessary for juvenile leaf identity, we also characterized the phenotype of WT plants transformed with constructs containing a miR156 target site mimic (*MIM156*) fused to the *WUS*, *STM*, *FD*, or *ANT* promoters. *WUS::MIM156* and *STM::MIM156* accelerated abaxial trichome production and reduced rosette leaf number but did not affect the rate of leaf initiation (Fig. 4 *F*–*J*). Expression of *MIM156* from the *STM* promoter additionally affects the shape of leaf 3 but not leaves 1 or 2 (Fig. 4 *K* and *M*). *FD::MIM156* accelerated abaxial trichome production (Fig. 4*G*) and the production of leaves with adult morphology (*SI Appendix*, Fig. S3*C*) and also reduced leaf number (Fig. 4*H*); however, like *WUS::MIM156* and *STM::MIM156*, this construct did not affect the rate of leaf initiation (Fig. 4*J*). Some lines transformed with these constructs bolted slightly earlier than normal, but this effect was quite variable. These results demonstrate that miR156 produced by the SAM

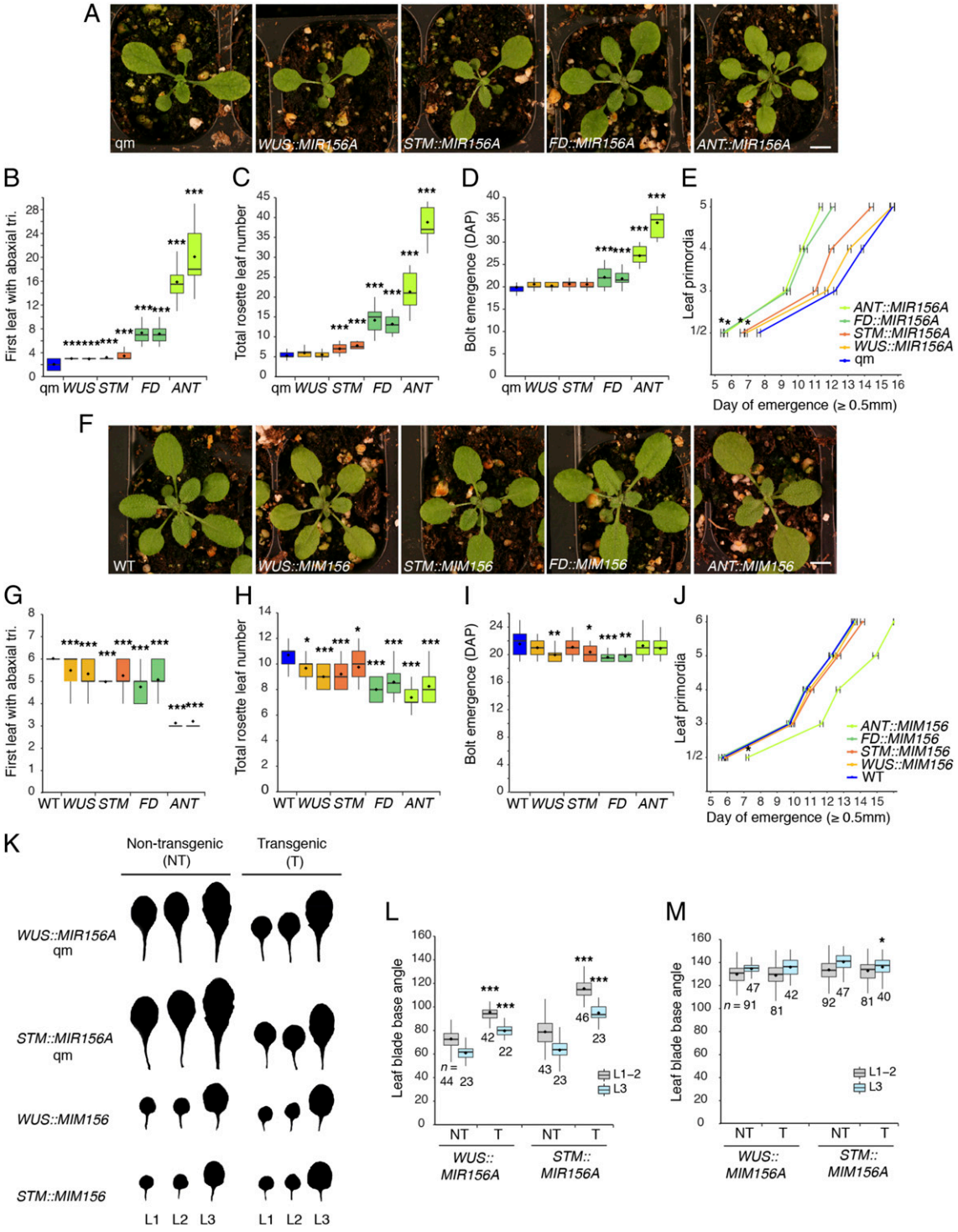

Fig. 4. Localized expression of *MIR156A* and *MIM156* in the SAM rescues loss of *mir156* function and leads to precocious phase change. (*A–E*) Expression of *MIR156A* in a *mir156ac mir157ac* qm background. (*F–J*) Expression of *MIM156* in a WT background. (*A* and *F*) Photographs taken at 18 DAP in LD conditions. (Scale bar, 5 mm.) (*B–D* and *G–I*) Gene names on the *x* axis describe the promoter used to drive expression. Two independent T3 lines are shown for each promoter. Significant differences from quadruple mutant or WT controls were determined by one-way ANOVA of log-transformed data with post hoc Dunnett's multiple comparison test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). $n = 31–48$ (*B* and *G*); $n = 12–24$ (*C*, *D*, *H*, and *I*). (*E* and *J*) Emergence was scored when leaves were visible in the rosette without manipulating other leaves. Single representative T3 lines are shown. Error bars represent the SEM; significant differences between the timing of the emergence of leaves 1 and 2 relative to the qm or WT controls were determined by one-way ANOVA of log-transformed data with post hoc Dunnett's multiple comparison test (*$P < 0.001$; $n \geq 21$). (*K*) Silhouettes of leaves 1–3 in segregating T2 sibling plants in which the denoted transgene was either homozygous or absent. Transgene homozygosity was evaluated based on the fluorescence strength of a seed-specific red fluorescent protein marker located on the transgene. (*L* and *M*) The leaf blade base angle of the plants shown in *K*. Significant differences from nontransgenic sibling controls were determined by a two-tailed *t* test (*$P < 0.05$; ***$P < 0.001$); sample sizes are indicated on the graph. Boxes in *B–D*, *G–I*, *L*, and *M* display the IQR (boxes), median (lines), and values beyond 1.5* IQR (whiskers); mean values are marked by a solid diamond (◆). All phenotypic analyses were carried out in LD conditions.

is both necessary and sufficient for the production of at least some aspects of juvenile leaf identity.

To determine if the ability of the *WUS::MIR156A* and *STM::MIR156A* constructs to correct the *mir156/157* qm phenotype is attributable to the mobility of miR156 (or a downstream component of the miR156-*SPL* pathway), we tested the effects of localized miR156 activity on the accumulation of a *SPL9::SPL9-GUS* reporter. We crossed a *SPL9::SPL9-GUS*; *mir156/157* qm line (7) to *WUS::MIR156A*; *mir156/157* qm and *STM::MIR156A*; *mir156/157* qm lines. The progeny of these crosses were thus hemizygous for *SPL9::SPL9-GUS* and hemizygous for either *WUS::MIR156A* or *STM::MIR156A* in a *mir156/157* qm background. To ensure that the *SPL9::SPL9-GUS* construct was actually capable of responding to *miR156*, the *SPL9::SPL9-GUS*; *mir156/157* qm line was crossed to Col and to *FD::MIR156A*; *mir156/157* qm and *ANT::MIR156A*; *mir156/157* qm plants. The progeny of the cross to Col were heterozygous for *mir156a mir156c mir157a mir157c* and hemizygous for *SPL9::SPL9-GUS* and were morphologically WT. Plants were harvested and stained for GUS activity 2 wk after planting when miR156/157 are relatively abundant (7).

SPL9::SPL9-GUS/+; *mir156/157* qm seedlings had GUS activity in the epidermis and mesophyll of leaf primordia, but had no obvious GUS activity in the SAM (Fig. 5). In contrast, *SPL9::SPL9-GUS*/+ *mir156/157* qm plants expressing *MIR156A* from either the *ANT* or *FD* promoters had no obvious GUS staining in both leaf primordia and the SAM. This result demonstrates that the *SPL9::SPL9-GUS* reporter is sensitive to miR156/157. Furthermore, the observation that *FD::MIR156A* is capable of repressing *SPL9::SPL9-GUS* expression outside the expression domain of the *FD* promoter suggests that miR156 is diffusible. This conclusion is further supported by the observation that expressing *MIR156A* under the regulation of the meristem-specific *STM* or *WUS* promoters strongly repressed *SPL9::SPL9-GUS* expression in very young leaf primordia and more weakly repressed *SPL9::SPL9-GUS* expression in older leaf primordia (Fig. 5 and *SI Appendix*, Fig. S5). This latter result suggests that miR156 diffuses from the SAM into leaf primordia. These results are consistent with the observation that *WUS::MIR156A* and *STM::MIR156A* are capable of partially rescuing the *mir156/157* qm phenotype (Fig. 4), and, further, they suggest that the suppression of *SPL* activity during early primordia development is sufficient to determine leaf identity.

The miR156-SPL Pathway Represses *WUS* Expression. *35S::MIR156A* and *WUS::MIR156A* significantly increase the number of leaves produced by *wus* mutants (Fig. 3), raising the possibility that *SPL* genes negatively regulate the size or function of the SAM. To test this hypothesis, we measured the size of the SAM in the *mir156/157* qm, in which *SPL* expression is strongly up-regulated (7), and in the *spl2/9/10/11/13/15* sextuple mutant (*spl* sxm), which has very low levels of SPL activity (6). The SAM of the *mir156a/157* qm was significantly narrower than WT, whereas the SAM of the *spl* sxm was significantly wider than WT (Fig. 6*A*). These results are consistent with the phenotype of plants expressing a miR156-resistant version of SPL9 (3) and suggest that *SPL* genes repress the growth of the SAM.

To determine if the expanded size of the SAM in the *spl* sxm is associated with enhanced *WUS* expression, we introduced a *WUS::GUS* reporter into this line. Three *spl* sxm; *WUS::GUS* lines were crossed to WT to generate plants heterozygous for these mutations and hemizygous for *WUS::GUS*. These phenotypically WT F1 plants were compared with *spl* sxm *WUS::GUS*/+ plants, which were identified in the T2 progeny of the original transgenic lines by the intermediate fluorescence intensity of the seed-specific *OLE1::OLE1-RFP* selection marker (33) on the *WUS::GUS* construct. Although the expression level of the *WUS::GUS* transgene varied between lines, GUS activity was consistently higher in *spl* sxm; *WUS::GUS*/+ than in *spl* sxm/+; *WUS::GUS*/+ plants (Fig. 6*B*). This result suggests that SPL transcription factors directly or indirectly repress the expression of *WUS*.

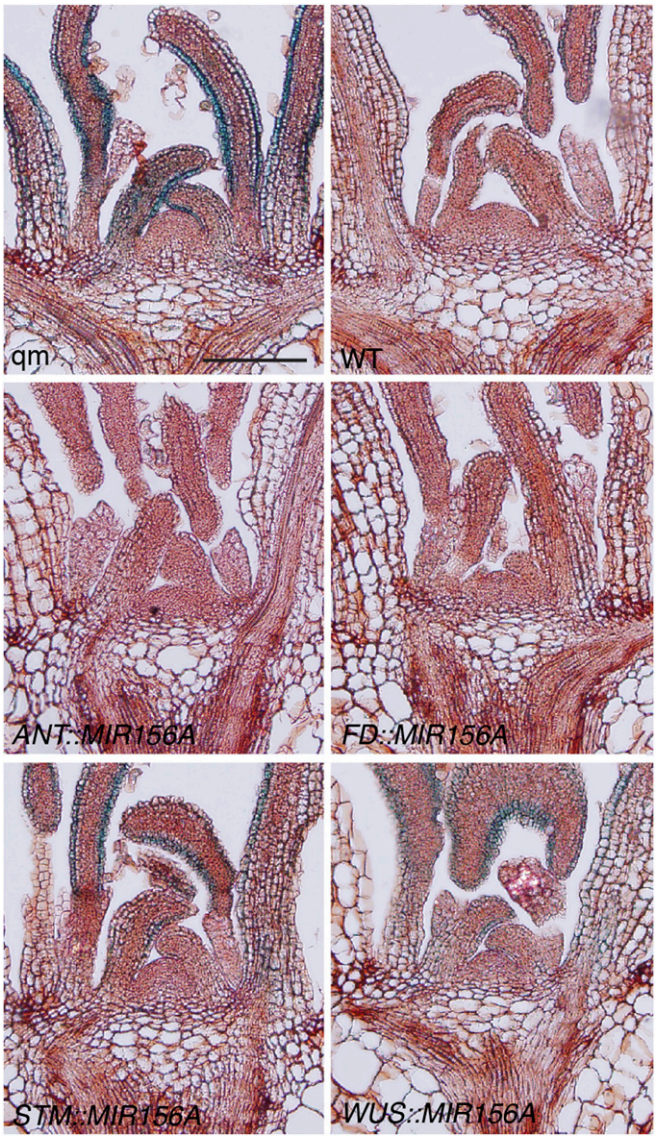

Fig. 5. The miR156 module acts noncell-autonomously across the shoot apex. GUS-stained sections of plants hemizygous for a *SPL9::SPL9-GUS* reporter construct. All plants except for WT are homozygous for the qm combination *mir156a miR156c mir157a miR157c*; WT is heterozygous for these mutations. Plants labeled *ANT::MIR156A*, *FD::MIR156A*, *STM::MIR156A*, and *WUS::MIR156A* are hemizygous for these transgenes. (Scale bar, 100 μm.)

To obtain additional evidence for this conclusion, we examined *WUS* expression in the shoot apices of WT and *spl* sxm plants using RT-qPCR. Consistent with the expression of the *WUS::GUS* reporter, *WUS* transcripts were significantly more abundant in the *spl* sxm mutant than in WT (Fig. 6*C*). WUS positively regulates the expression of *CLAVATA3* (*CLV3*), which feeds back via its receptor, CLV1, to repress *WUS* expression (27, 34). To determine if SPL transcription factors (TFs) repress *WUS* expression via this pathway, we first examined the effect of the *spl* sxm on *CLV3* expression. We found that the *spl* sxm had significantly elevated levels of *CLV3* transcripts (Fig. 6*C*), which is inconsistent with the hypothesis that SPL TFs repress *WUS* expression by promoting the expression of *CLV3*. We then tested

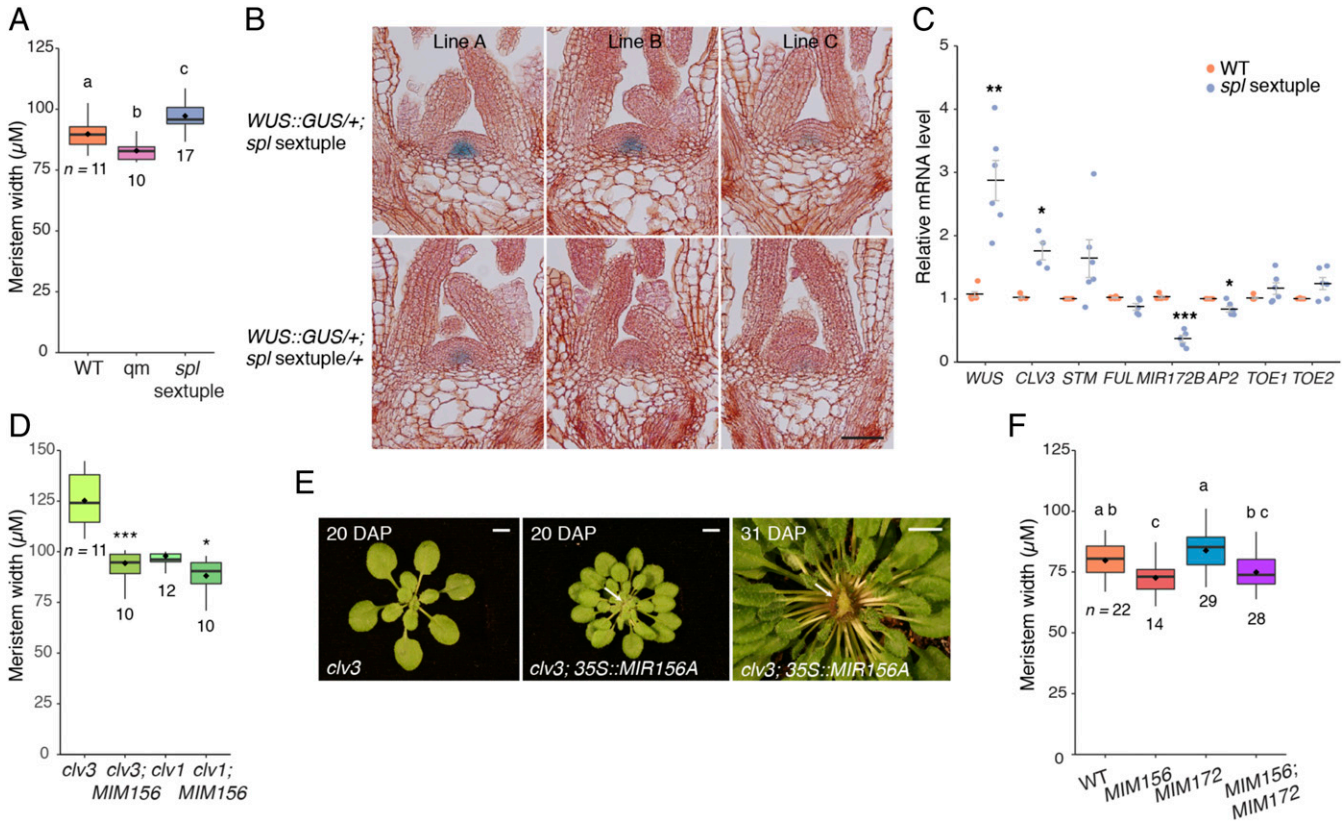

Fig. 6. *SPL* genes repress *WUS* expression and reduce meristem size. (*A*, *D*, and *F*) Statistically distinct genotypes were identified by one-way ANOVA with post hoc Tukey multiple comparison test (letters indicate statistically distinct groups; $P < 0.05$) (*A* and *F*) or pairwise two-tailed *t* tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) (*D*). Boxes display the IQR (boxes), median (lines), and values beyond 1.5* IQR (whiskers); mean values are marked by a solid diamond (◆). (*B*) Histological sections of three independent *WUS::GUS* lines. (Scale bar, 50 μm.) (*C*) mRNA levels in shoot apices with leaf primorida larger than 0.5 mm removed. Relative levels were quantified by RT-qPCR, normalized to *ACT2* as an internal control gene, and expressed as a ratio to the level in WT plants. Each data point represents a biological replicate and is the average of three technical replicates. Black bars represent the mean and gray bars the SEM. Significant differences between *spl* sextuple and WT plants were determined by two-tailed *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (*E*) White arrows indicate visibly fasicated mersitems (young leaves have been removed from the 31-DAP plant to more clearly show meristem size). (Scale bars, 5 mm.) All analyses were carried out on 2-wk-old plants in SD.

if *CLV3* and *CLV1* are required for the negative effect of *SPL* overexpression on meristem size. If SPL TFs repress *WUS* expression by promoting the expression of *CLV3* and/or *CLV1*, loss-of-function mutations in *CLV3* and *CLV1* should block the effect of *SPL* overexpression on meristem size. Specifically, the enlarged meristem phenotype of *clv3* and/or *clv1* should be epistatic to the small meristem phenotype of plants containing *35S::MIM156*, which increases SPL expression. Instead, we found that *35S::MIM156* significantly decreased the size of the SAM in both *clv1* and *clv3* (Fig. 6*D*), whereas *35S::MIR156A* dramatically enhanced the effect of these mutations on meristem size (Fig. 6*E*). These additive/synergistic interactions suggest that SPL TFs regulate *WUS* expression independently of the *CLV3-CLV1* pathway. The inconsistent change in *STM* expression in the *spl* sxm (Fig. 6*C*) suggests that the repressive effects of *SPL* genes on meristem size are also independent of *STM*.

An alternative possibility is that SPL TFs repress *WUS* expression through their effect on the expression of APETALA2-like (AP2-like) TFs. Several AP2-like TFs are repressed transcriptionally by the SPL-target *FRUITFULL* (*FUL*) and posttranscriptionally by the SPL target *MIR172B* (4, 35, 36). Because AP2-like TFs promote the expression of *WUS* (35, 37), a reasonable hypothesis is that SPL TFs repress *WUS* expression by promoting the transcription of *FUL* or *MIR172B*. To test this hypothesis, we examined how *FUL*, *MIR172B*, and three miR172-regulated *AP2* family members (*AP2*, *TOE1*, and *TOE2*) are expressed in the *spl* sxm. We saw no significant decrease in *FUL* expression whereas the primary transcript of *MIR172B* was significantly reduced in the shoot apices of *spl* sxm seedlings (Fig. 6*C*). We observed no increase in the transcript levels of *AP2*, *TOE1*, and *TOE2*. This latter result is consistent with previous studies showing that changes in the level of miR172 do not produce changes in the abundance of its target transcripts, possibly because miR172 regulates gene expression at a translational level (38, 39).

As a more direct test of the hypothesis SPL genes repress the growth of the SAM by repressing *AP2-like* gene expression, we measured the size of the SAM in *MIM156*, *MIM172*, and *MIM156*; *MIM172* plants (Fig. 6*F*). Consistent with the phenotype of the *mir156/157* qm mutant (Fig. 6*A*), *MIM156* had a significantly narrower SAM than WT plants. *MIM172* plants had wider meristems than WT, although this difference was not large enough to be statistically significant. The width of the SAM in *MIM156*; *MIM172* was not significantly different from *MIM156*. These results support the conclusion that miR156-regulated SPL TFs repress, and miR172-regulated AP2-like TFs promote, the development of the SAM. However, the phenotype of *MIM156*; *MIM172* is inconsistent with the hypothesis that SPL TFs regulate meristem size via the miR172-AP2 pathway. If this were true, *MIM172* should be epistatic to *MIM156*. Rather, these results suggest that either SPL TFs and AP2-like TFs operate independently to regulate SAM size or that AP2-like TFs operate upstream of SPL TFs in this process.

Discussion

The extent to which the SAM autonomously regulates shoot development—as opposed to simply responding to regulatory factors produced by organs and tissues outside the SAM—is a classic question in plant development. Classically, vegetative phase change was thought to result from changes in the developmental identity of the SAM; indeed, the apparent stability of the juvenile and adult phases provided some of the first evidence that cells in the SAM can become determined for specific developmental fates (14, 15, 40). It is also clear that the timing of vegetative phase change can be influenced by various environmental factors and by organs/tissues external to the SAM. In particular, the discovery that preexisting leaves promote the adult identity of later-formed leaves (18–20, 41, 42) raises the possibility that vegetative identity is specified primarily by signaling from leaves to the SAM and leaf primordia, rather than by the autonomous activity of the SAM. The results presented here suggest that the identity of newly formed leaves is regulated both by SAM and by preexisting leaves, but that the relative importance of these sources of information changes during shoot development (summarized as a model in Fig. 7).

Several observations suggest that the SAM promotes juvenile leaf identity during early shoot development, but plays a relatively minor role in the regulation of leaf identity at later stages of shoot development. The best evidence that the SAM is required for juvenile leaf identity early in shoot development is the observation that meristem-defective mutations, such as *wus*, *psd*, and *stm*, exhibit accelerated adult development. Previous studies have suggested that this phenotype is due to the effect of these mutations on leaf initiation (16, 22), but our results indicate that it is more likely attributable to their low level of miR156/157. In particular, we found that, in addition to having reduced levels of miR156, the effect of *wus* on leaf morphology can be suppressed by expressing miR156 in the SAM. Meristem-specific expression of miR156 also partially corrects the phenotype of the *mir156/157* qm, providing additional evidence that the SAM promotes juvenile leaf identity. However, meristem-specific miR156 expression did not have a major effect on the rate of initiation or on the morphology of later-formed leaves in the *mir156/157* qm. Even *FD::MIR156A*, which confers constitutive expression of miR156 in the SAM and young leaf primordia, did not completely correct the phenotype of the *mir156/157* qm. Indeed, despite a delay in the timing of VPC for *FD::MIR156A*, *WUS::MIR156A*, and *STM::MIR156A* plants, all these genetic lines still progressed to the adult phase, and they did so more quickly than *ANT::MIR156A* plants, in which miR156 is maintained in leaf primordia. Furthermore, reducing miR156/157 in the SAM of otherwise WT plants has only a minor effect on leaf identity. Together, these results suggest that the SAM specifies the identity of the first leaves produced by the shoot, but plays a much smaller role in determining the identity of subsequent leaves. Thus, how and where a plant makes developmental decisions shifts with age and shoot status. During initial growth the SAM is fundamental to maintaining a plant in the juvenile phase. However, as a plant produces leaves the control of development becomes more spatially diffuse, and the determination of shoot identity is coordinated by peripheral organs. Such a process has been well demonstrated in the case of floral induction, where the initiation of flowering is controlled by the leaf-derived protein FLOWERING LOCUS T (FT), but the specification of floral meristem identity is largely intrinsically regulated (28, 29).

One argument against this conclusion is that *wus* and *stm* have a much larger effect on VPC than the *WUS::MIM156* and *STM::MIM156* transgenes. However, this is readily explained by observation that *wus* and *stm* have significantly less miR156 than plants expressing *WUS::MIM156* and *STM::MIM156*. miR156 is broadly expressed throughout the SAM (3), so it is to be expected that mutations that delay the initiation and dramatically reduce the size of the SAM—such as *wus* and *stm*—have a more significant effect on the level of miR156 than *WUS::MIM156* and *STM::MIM156*, which are expressed in only a small number of cells in the SAM. Indeed, it is remarkable that *WUS::MIR156A* and *WUS::MIM156* actually affect leaf identity, given the very small number of cells in which these transgenes are expressed.

How does the SAM specify leaf identity during early development? One possibility is that cells acquire their identity in the

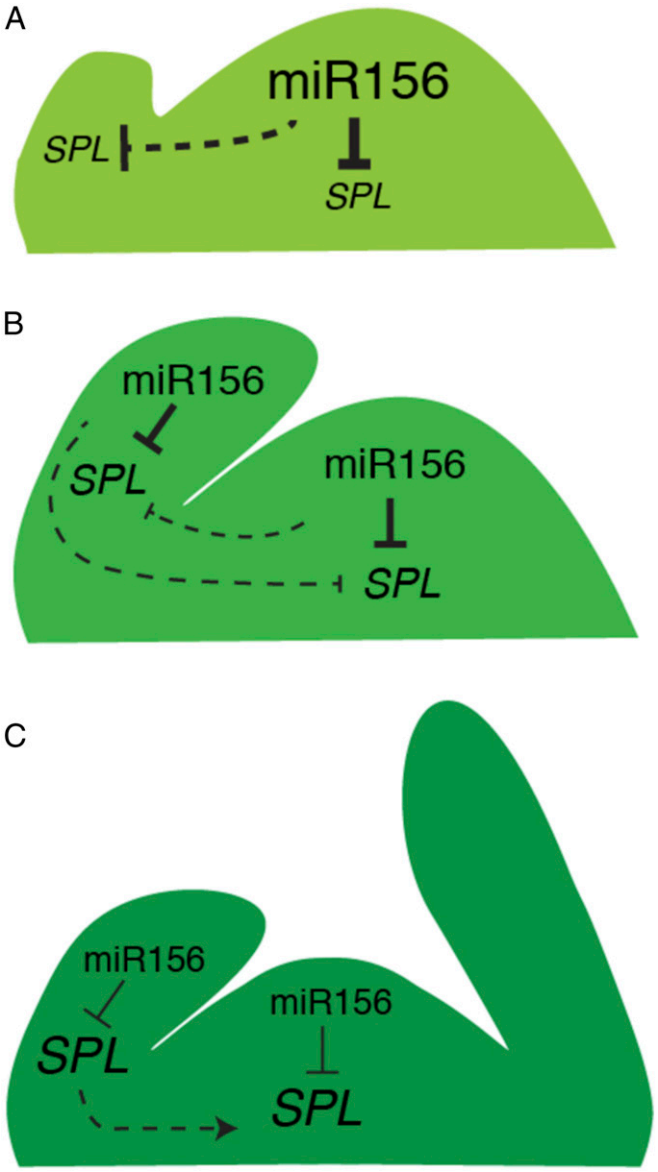

Fig. 7. Model for the coordination of miR156 regulation across the shoot apex during vegetative development. (*A*) During the first few plastochrons, miR156 produced by the SAM represses *SPL* expression in emerging leaves. (*B*) During subsequent stages of juvenile growth, *SPL* expression in leaves is repressed predominantly by miR156 produced by leaves; SAM-derived miR156 plays a much smaller role in the regulation of SPL gene expression at these stages. (*C*) A temporal decline in miR156 across the shoot apex leads to the derepression of *SPL* genes and vegetative phase change, promoted in part by SPL protein mobility (3). Dashed lines indicate movement of either transcript (miR156) or protein (SPL) from site of expression. Lettering and line thickness reflect relative levels of expression (lettering) and repressive interactions (line thickness).

SAM and retain this identity as they divide and differentiate into leaves. We think this is unlikely because experiments in maize have shown that the phase identity of a leaf is not determined until after leaf initiation (42). Furthermore, it would be surprising if the amount of miR156 acquired by cells in the SAM is sufficient to regulate gene expression throughout leaf development if the transcript pool of a cell decreases by 50% with each division. A more likely scenario is that miR156, or a miR156-dependent factor, diffuses from the SAM into leaves. This hypothesis is supported by our observation that the meristem-specific transgenes *WUS::MIR156A* and *STM::MIR156A* strongly repress *SPL* expression in young leaf primordia, but are less effective in older leaf primordia. It is also supported by previous studies indicating that miR156 acts noncell-autonomously in potato (43) and in maize (44, 45) and is consistent with a model in which early leaf development is regulated by mobile gradients of a number of small RNAs (46). In contrast, a recent study on miRNA mobility found that an artificial miRNA expressed within the *WUS* domain functions cell-autonomously in this domain (47). However, this study did not investigate the movement of endogenous miRNAs, and it is possible that miR156 mobility is regulated by factors specific to this miRNA. In any case, our observation that the more broadly expressed meristem-specific *STM::MIR156A* transgene is also capable of repressing SPL expression in leaves supports the hypothesis that the SAM promotes juvenile leaf identity because miR156 diffuses from the SAM into leaf primordia. miR156 is expressed in leaves and increases in abundance as leaves expand (7, 48). Diffusion of miR156 from preexisting leaves into the SAM and newly formed leaf primordia could explain why a reduction in the level of miR156 specifically within the SAM had relatively little effect on leaf identity. Although the movement of miR156 from the SAM into leaf primordia is the most parsimonious explanation of our results, it is also possible that a repressor of *SPL* activity, the expression of which is promoted by miR156, could diffuse from the SAM to leaf primordia. To unambiguously demonstrate that miR156 functions noncell-autonomously within the shoot apex, it will be necessary to develop methods for specifically sequestering this miRNA within the SAM.

SPL proteins regulate many aspects of shoot development. In addition to promoting adult leaf identity, SPL proteins repress branching (49, 50) and reduce the size of the SAM when they are overexpressed as a result of the loss of miR156 regulation (ref. 3; this report). Our results suggest that SPL proteins reduce meristem size by repressing *WUS* transcription independent of the *CLV3-CLV1* signaling pathway. One of the ways in which SPL proteins could do this is through their effect on the expression of miR172. SPL proteins promote the expression of miR172, which in turn represses a family of AP2-like transcription factors that promote *WUS* expression (37). However, our results indicate that the effects of SPL proteins on meristem size are largely independent of the miR172-AP2–like pathway. SPL proteins have also been shown to restrict cytokinin signaling via interference with type-B ARABIDOPSIS RESPONSE REGULATOR (ARR) protein activity (51). Given that type-B ARRs induce *WUS* expression (52), another possibility is that *SPL* genes restrict meristem size via this mechanism. The recent finding that *Arabidopsis* and soybean orthologs of SPL9 and WUS physically interact (53) suggests that SPL proteins could also regulate WUS activity posttranslationally. However, we observed that *wus* mutants expressing miR156 under the regulation of constitutive or meristem-specific promoter produce significantly more leaves than *wus*. This result suggests that WUS is not absolutely required for the function of SPLs in the SAM and raises the possibility that SPL proteins regulate meristem size or activity by both WUS-dependent and WUS-independent mechanisms.

SBP/SPL genes are present in algae and all land plants (54, 55) whereas miR156/157 did not evolve until plants colonized land (56–58). In the moss *Physcomitrella patens*, gametophytes with reduced SBP/SPL activity are more highly branched and initiate more leafy buds (59, 60). This suggests that one of the earliest functions of miR156/157 in land plant evolution was to promote apical growth and that this function has been conserved in angiosperms. The time course of miR156/SPL evolution, and the evidence that in flowering plants loss of miR156/157 causes leaves to adopt an adult identity, suggest that the adult phase is the default state of the shoot. We suspect that miR156 and miR157 evolved in response to environmental conditions that selected for more vigorous and prolonged shoot growth and that species-specific juvenile traits evolved later, as plants expanded into a variety of distinct habitats.

Materials and Methods

Col was used as the genetic background for all lines used in this work. Detailed descriptions of the methods used to generate transgenic plants and of the molecular analyses employed are presented in *SI Appendix*. Details of plant growth conditions and statistical tests are included in the relevant figure legends.

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