

# Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156

Li Yang, Susan R. Conway and R. Scott Poethig\*

## SUMMARY

Vegetative phase change in *Arabidopsis* is regulated by miR156, a microRNA that promotes the expression of the juvenile phase and represses the expression of the adult phase. miR156 is expressed at a very high level early in shoot development and then decreases, leading to the onset of the adult phase. To determine the source of the factors that regulate vegetative phase change, we examined the effect of root and leaf ablation on the timing of this transition. Ablation of the root system or cotyledons had no effect on the timing of vegetative phase change, but ablation of leaf primordia delayed this transition in a miR156-dependent fashion. This treatment produced an increase in the overall abundance of miR156, which was attributable to an increase in the transcription of some, but not all, of the miR156 genes in *Arabidopsis*, and decreased the expression of SPL genes regulated by miR156. miR156 levels were also elevated by leaf ablation in *Nicotiana benthamiana* and in rejuvenating shoot apices of maize cultured in vitro. We conclude that vegetative phase change is initiated by a signal(s) produced by leaf primordia, which acts by repressing the transcription of specific members of the miR156 gene family.

**KEY WORDS:** Developmental timing, Heteroblasty, Heterochrony, miR156, Phase change, Shoot development, *Arabidopsis*

## INTRODUCTION

The shoot apex of a plant produces different types of leaves, buds and internodes at different times in development. This phenomenon is termed heteroblasty and is the result of number of overlapping processes (Allsopp, 1967; Goebel, 1900; Wareing, 1959). A major contributor to heteroblasty is the phenomenon of phase change – the transition between juvenile, adult and reproductive stages of shoot development (Poethig, 2003). The molecular mechanism of the transition to reproductive development (floral induction) is now well understood (Amasino, 2010), but molecules involved in the juvenile-to-adult transition (vegetative phase change) have only recently been identified. Among the most important of these is the microRNA (miRNA) miR156. In *Arabidopsis* (Wu et al., 2009; Wu and Poethig, 2006) and maize (Chuck et al., 2007) miR156 is highly expressed early in shoot development and declines dramatically during the juvenile-to-adult transition. Constitutive expression of miR156 prolongs the expression of the juvenile phase, whereas a reduction in miR156 activity accelerates vegetative phase change, demonstrating that miR156 is a key regulator of this transition (Chuck et al., 2007; Wu et al., 2009; Wu and Poethig, 2006). miR156 promotes juvenile development by repressing members of the SBP/SPL family of transcription factors (Chuck et al., 2007; Gandikota et al., 2007; Schwab et al., 2005; Schwarz et al., 2008; Wu and Poethig, 2006), the targets of which include the miRNA miR172b (Wu et al., 2009) and the transcription factors LFY, AP1, FUL, SOC1 and AGL42 (Wang et al., 2009; Yamaguchi et al., 2009).

Although much has been learned about the genetic pathways underlying vegetative phase change, the source and the identity of the signals that initiate this transition are unknown. Experiments in

a number of woody species (Doorenbos, 1954; Frank and Renner, 1956; Greenwood et al., 2010; McDaniel, 1980; Schwabe and Al-Doori, 1973) suggest that the root system is a source of a juvenilizing factor, whereas defoliation experiments implicate leaves as the source of an adult-promoting factor (Ashby, 1948; Njoku, 1956b). Other studies have suggested that the overall size (Day et al., 2002; Greenwood et al., 2010) and growth rate (Borchert, 1964) of the shoot are key factors in this process. However, the different growth habits of the species used in these studies, as well as the fact that different traits were used as evidence of vegetative phase change in the different species, make it difficult to compare the results of these studies. Many traits change during shoot development and it can be difficult to distinguish traits that are components of the vegetative phase change program from those that are regulated by pathways involved in floral induction or some other aspect of shoot physiology. Although the presence or absence of reproductive structures (flowers or cones) is commonly used to distinguish juvenile and adult shoots (Jones, 1999), these phases of shoot development were originally defined on the basis of vegetative morphology, not reproductive competence (Goebel, 1900). In fact, the relationship between the vegetative morphology of the shoot and its reproductive behavior is still poorly defined; many species flower long after entering an adult vegetative phase, and plants that flower in a juvenile vegetative phase are well known (Wiltshire et al., 1991; Zimmerman et al., 1985). Vegetative morphology can also be misleading. Leaf shape is commonly used to distinguish juvenile versus adult identity, but light intensity can sometimes affect leaf shape in ways that resemble the effect of vegetative phase change (Njoku, 1956a) without necessarily operating via this mechanism (Jones, 1995). The discovery that miR156 regulates vegetative phase change in both dicots and monocots (Chuck et al., 2007; Wu and Poethig, 2006) provides a solution to this problem. Given the central role of miR156 in vegetative phase change, it is now possible to identify key regulators of this process by identifying the factors that control the expression of this miRNA.

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To identify the source of the factors that regulate the timing of vegetative phase change and the mechanism by which these factors operate, we examined the effect of root, cotyledon and leaf ablation on shoot morphology and the expression of miR156 in *Arabidopsis*, maize and *Nicotiana benthamiana*. Our results indicate that vegetative phase change is mediated by a factor(s) produced by leaf primordia, and that this factor acts by repressing the expression of miR156.

## MATERIALS AND METHODS

### Genetic stocks and growth conditions

All the *Arabidopsis* genetic stocks used were in a Columbia background. The enhancer trap E1735 was generated in our laboratory using a GAL4-UAS::GFP vector provided by Jim Haseloff (Haseloff, 1999). The UAS::DTA line (Laplaze et al., 2005) was also obtained from Jim Haseloff. This transgene was then transferred from C24 into Columbia by five rounds of backcrossing. An enhancer trap line that expresses GFP in hydathodes (E325) was used to examine the effect of defoliation on hydathode number. Seeds were sown on Fafard #2 soil and left at 4°C for 2 days prior to being transferred to a growth chamber. Plant age was measured from the time seeds were transferred to the growth chamber. With the exception of the root ablation experiments, which were conducted under long day conditions (16 hours light:8 hours dark, 125  $\mu\text{mol}/\text{m}^2/\text{second}$ , 23°C), all experiments were conducted with plants grown in short days (10 hours light:14 hours dark, 23°C), under a 3:1 combination of cool white (F032/841/Eco, Sylvania) and wide spectrum (Gro Lite WS, Interlectric) fluorescent lights, at a light intensity of 300  $\mu\text{mol}/\text{m}^2/\text{second}$ .

### Transgenic plants

We first generated the p3300-Gateway-GUS+ plasmid. For this purpose, the GUSplus cassette in pCambia 3301-GUSplus was amplified using the forward primer 5'-(*Bam*HI)-GGATCCATGGTAGATCTGAGG-GTAAATTTCTAGTTTTCTCC-3' and the reverse primer 5'-(*Sac*I)-GAGCTCCACTGATAGTTAATCCCGATCTAGTAAACATAG-3'. This PCR product was cloned into the pCambia 3300 vector using the *Sac*I and *Bam*HI restriction sites. The Gateway cassette was amplified from pEarleyGate 202 using the forward primer 5'-GCGAAGCTTAATT-AAGCGCGGCGCGCCGGACACGCTCGAGATCACAAG-3' and the reverse primer 5'-GCCTAGGCACCACTTGTACAAG-3'. The pCambia 3300-GUSplus plasmid was cut with *Bam*HI and the overhangs were blunted using Klenow DNA polymerase. The Gateway PCR product was then cloned into this plasmid, generating p3300-Gateway-GUS+.

To generate pSPL9:SPL9-GUS+ and pSPL9:rSPL9-GUS+, a 5.2 kb fragment containing the *SPL9* promoter and coding region was amplified and cloned upstream of GUS+ in p3300-Gateway-GUS+ using the primers described in Table S1 in the supplementary material. The miR156-resistant *rSPL9* gene was produced by introducing silent mutations into the PCR primers that were used to amplify this gene. To generate pSPL3:rSPL3-GUS+, a 3.4 kb fragment containing the *SPL3* promoter and coding region without the 3' UTR (which contains the miR156 target site) was amplified and introduced into p3300-Gateway-GUS+. To generate pSPL3:GUS+-SPL3, the GUS+ coding region was inserted into a 3.9 kb fragment containing the *SPL3* genomic region, between the *SPL3* promoter and its start codon using PCR fusion. The whole sequence was amplified and cloned into p3300 between the *Eco*RI and *Bam*HI sites. The PCR primers used for these experiments are listed in Table S1 in the supplementary material.

### Defoliation

The first two leaves of 8- to 12-day-old *Arabidopsis* seedlings grown in short days were removed using forceps. At the first time point, these leaves were ~1 mm long. A small wound on leaf 1 or 2 was made at the same time on control plants. Gene expression was analyzed in shoots (excluding cotyledons and the first two leaves) harvested 3 days after defoliation. Cotyledon(s) or the first leaf of *Nicotiana benthamiana* plants grown in long days were removed when plants were 2 weeks old. Shoot apices with leaves less than 1 cm long were harvested 3 days after manipulation. Shoot apices from 3-week-old maize seedlings were dissected and cultured as described (Orkiszewski and Poethig, 2000).

### RNA blots

RNA blots were processed as described previously (Wu and Poethig, 2006). Briefly, total RNA was isolated using Trizol (Invitrogen), purified with RNeasy (Qiagen) and treated with RNase-free DNase (Qiagen). Quantitative (q) RT-PCR was performed using SuperScript II reverse transcriptase and Power SYBR Green PCR Master Mix (Applied Biosystems), and normalized using *ACTIN 2* (At3g18780) as a standard. The primers used for qRT-PCR are described in Table S1 in the supplementary material.

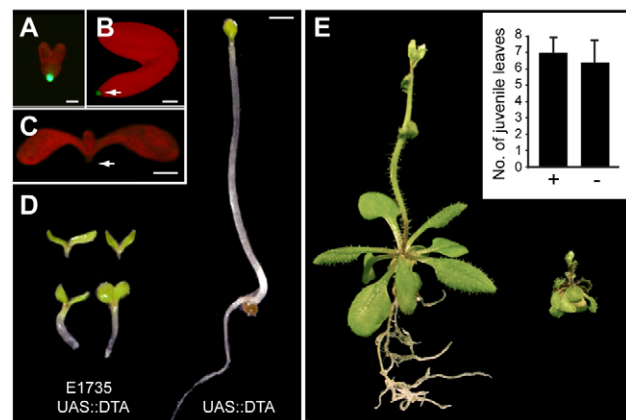
### GUS staining

The fifth leaf primordium was harvested from untreated and defoliated plants 6 days after defoliation and stained using the protocol described by Senecoff et al. (Senecoff et al., 1996). The incubation time for the rSPL3-GUS and rSPL9-GUS reporters was reduced to 1 hour to compensate for the high level of GUS activity in these lines.

## RESULTS AND DISCUSSION

### Roots are not required for vegetative phase change

To investigate the role of the root system in vegetative phase change in *Arabidopsis*, we generated rootless plants using a transgenic approach. For this purpose, we took advantage of an enhancer trap line (E1735) that expresses the yeast transcriptional activator GAL4 in the quiescent cells of the embryonic and postembryonic root apical meristem, starting at the heart stage of embryogenesis (Fig. 1A,B) (<http://enhancertraps.bio.upenn.edu>). E1735 was crossed to a line hemizygous for a transgenic construct in which the alpha chain of diphtheria toxin (DTA) is fused to the GAL4 promoter (UAS::DTA). The F1 progeny from this cross segregated phenotypically normal plants, as well seedlings displaying varying degrees of hypocotyl and root formation (Fig. 1C,D). The most severely affected seedlings had a very short hypocotyl and no visible root system; these plants failed to express the GFP reporter present in E1735, indicating that they completely lacked root cells (Fig. 1C,D). On MS



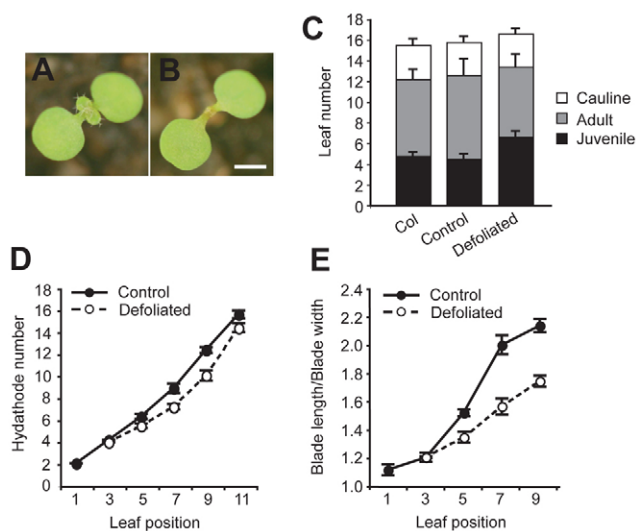
**Fig. 1. Roots are not required for vegetative phase change.**

(A,B) Heart stage (A) and bent cotyledon stage (B) E1735 *Arabidopsis* embryos expressing GFP in the root apical meristem (arrow). (C) Rootless E1735/+ UAS::DTA/+ seedling. This seedling does not express GFP in the position of the root tip (arrowhead). (D) UAS::DTA/+ seedling (right) and the rootless progeny from a cross of this line to E1735 (left). (E) Rosette morphology and the average number of leaves without abaxial trichomes (juvenile leaves) of sibling E1735/+ (left, +) and E1735/+ UAS::DTA/+ rootless (right, -) plants.  $n=10$ ;  $P>0.05$ ; error bars indicate mean + s.d. Scale bars: 20  $\mu\text{m}$  in A,B; 1 mm in C; 2 mm in D.

medium supplemented with 1% sucrose these rootless plants produced abnormally small, but viable, rosettes, and eventually flowered (Fig. 1E). In *Arabidopsis*, juvenile leaves lack trichomes on the abaxial surface of the leaf blade, whereas adult leaves possess abaxial trichomes (Chien and Sussex, 1996; Telfer et al., 1997). Despite their difference in size, there was no significant difference between the number of leaves lacking abaxial trichomes (juvenile leaves) in plants with or without roots (Fig. 1E). We conclude that the root system does not play a significant role in vegetative phase change in *Arabidopsis*.

### Leaf ablation delays phase change by increasing the expression of miR156

We then examined whether cotyledons or leaves regulate vegetative phase change by removing these organs at various times after germination. Removing cotyledons from 7-day-old seedlings delayed their growth significantly and produced a slight delay in the production of abaxial trichomes, but later treatments had no significant effect on either growth rate or abaxial trichome production (see Fig. S1 in the supplementary material). By contrast, ablating the first two leaf primordia 8 to 12 days after germination had long-lasting effects on both growth and vegetative phase change (Fig. 2A,B; see Fig. S1 in the supplementary material). Defoliated plants displayed a transient increase in the rate of leaf initiation (see Fig. S2 in the supplementary material) and exhibited a marked delay in the expression of several phase-specific leaf traits. Abaxial trichome production was delayed by one or two plastochrons (Fig. 2C) and the hydathode number and the length-to-width ratio of the lamina increased more gradually in defoliated plants than in controls (Fig. 2D,E).



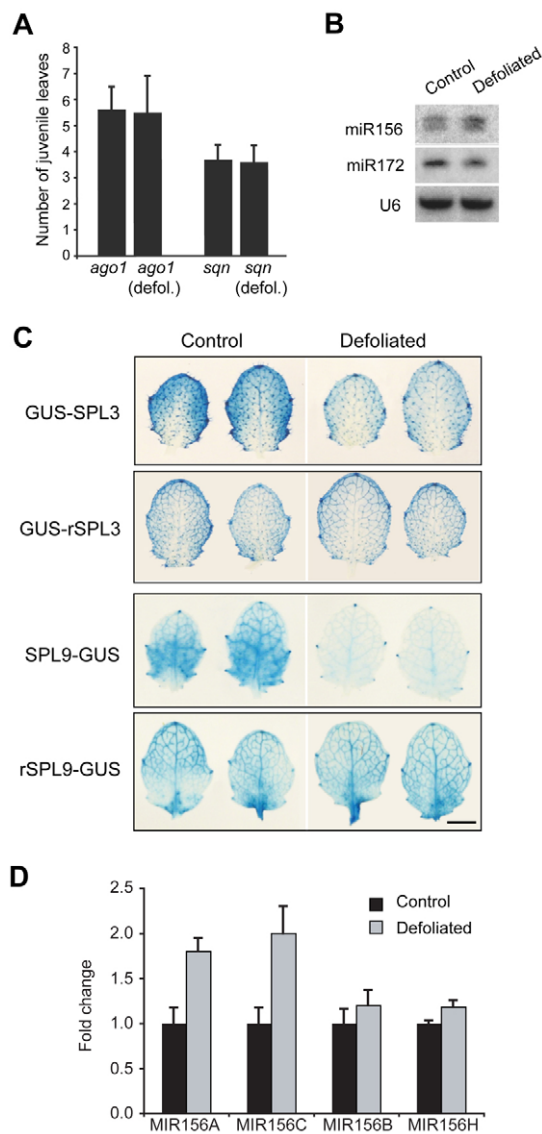
**Fig. 2. Defoliation delays phase change.** (A,B) Eight-day old *Arabidopsis* seedling before (left) and after (right) defoliation. Scale bar: 2 mm. (C) Defoliated plants produce significantly more leaves lacking abaxial trichomes (juvenile leaves) than untreated (Col) and wounded control plants.  $n=20$ ;  $P<0.001$  (Student's *t*-test); error bars indicate mean + s.d. (D,E) The average number of hydathodes (D) and the length:width ratio of successive leaves (E) in wounded control and defoliated plants. Defoliated plants have a slower rate of increase in hydathode number and have rounder leaves than control plants. D,  $n=10$ ; E,  $n=7$ ; error bars indicate mean  $\pm$  s.e.m.

The number of leaf primordia present at the time of cotyledon or leaf ablation was determined by dissecting a matched set of plants expressing LFY::GUS, a reporter that is expressed in young leaf primordia. This analysis revealed that the first transition leaf (leaf 5 or 6) was produced 7 or 8 days after germination, and was therefore present on many, if not most, of the seedlings used for leaf ablation (see Fig. S2 in the supplementary material). The fact that leaf ablation was capable of changing the morphology of this pre-existing leaf indicates that the loss of leaf primordia has a rapid effect on the identity of the shoot apex, and implies that leaves 1 and 2 are either a source or a sink of a phase change signal before, or shortly after, the stage at which they were ablated, i.e. at a length of  $\sim 1$  mm.

We tested the hypothesis that defoliation acts by affecting the expression or activity of miR156 by examining the effect of this treatment on two mutants, *sqn-1* and *ago1-45*, that have reduced miR156 activity (Smith et al., 2009). Although these mutations also affect the activity of other miRNAs, their effect on phase change is completely attributable to a defect in miR156 (Smith et al., 2009). Leaf ablation had no effect on the timing of abaxial trichome production in either mutant (Fig. 3A), indicating that miR156 is required for the effect of leaf ablation on vegetative phase change. We then examined the effect of leaf ablation on the expression of miR156 and three transcripts repressed by miR156: miR172, *SPL3* and *SPL9*. miR156 is present at high levels early in shoot development and declines during vegetative phase change, whereas miR172, *SPL3* and *SPL9* have the opposite expression pattern (Aukerman and Sakai, 2003; Jung et al., 2007; Wang et al., 2009; Wu et al., 2009; Wu and Poethig, 2006). Consistent with their juvenilized phenotype, defoliated plants had higher levels of miR156 and lower levels of miR172 than wounded controls (Fig. 3B).

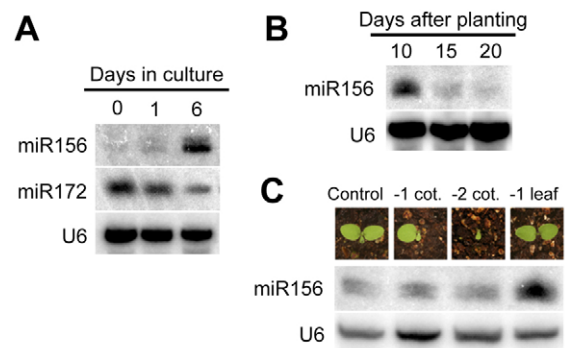
To examine the effect of defoliation on the expression of *SPL3* and *SPL9* we took advantage of reporter lines containing miR156-sensitive or miR156-resistant genomic constructs of these genes fused to GUS. GUS activity was assayed in the fifth leaf primordium of plants expressing wild-type reporters for *SPL3* and *SPL9* (GUS-*SPL3*, *SPL9*-GUS) and in the third (r*SPL9*-GUS) or fifth (GUS-r*SPL3*) leaf primordium of plants expressing miR156-resistant reporters for these genes; the third leaf primordium was examined in the case of r*SPL9*-GUS because these plants grow more slowly than those expressing *SPL9*-GUS. Leaf ablation decreased GUS expression in plants expressing the miR156-sensitive transgenes, but had no effect on the expression of the miR156-insensitive reporters (Fig. 3C). This result demonstrates that the decrease in the expression of the miR156-sensitive reporters in defoliated plants is mediated by miR156, and is consistent with the increased level of miR156 in these plants (Fig. 3B).

miR156 is encoded by eight genes in *Arabidopsis*. We used quantitative RT-PCR to measure the abundance of the primary transcripts of four of these genes (*MIR156A*, *MIR156B*, *MIR156C* and *MIR156H*) to determine whether the increase in miR156 in defoliated plants is mediated at a transcriptional or post-transcriptional level. Defoliation increased the expression of the primary transcripts of *MIR156A* and *MIR156C*  $\sim 2$ -fold, but had no effect on the expression of *MIR156B* and *MIR156H* (Fig. 3D), suggesting that only some miR156 genes play a role in vegetative phase change. This result also indicates that defoliation acts by increasing the transcription of a subset of miR156 genes, rather than by increasing the rate of miRNA processing. If defoliation increased the level of mature miR156 transcripts by enhancing the



**Fig. 3. The effect of defoliation on vegetative phase change is mediated by miR156.** (A) Defoliation has no significant effect on the number of leaves without abaxial trichomes in *Arabidopsis ago1-45* and *sqn-1* mutants.  $n=13$ ;  $P>0.05$ ; error bars indicate mean + s.d. (B) RNA blot of 17-day-old plants. miR156 is elevated and miR172 is reduced in defoliated plants compared with wounded controls. U6 was used as a loading control. (C) GUS activity in leaf primordia of transgenic plants expressing miR156-sensitive (GUS-SPL3, SPL9-GUS) and miR156-resistant (GUS-rSPL3, rSPL9-GUS) reporters for *SPL3* and *SPL9*. Defoliation reduces expression of the miR156-sensitive reporter but not of the miR156-resistant reporter. miR156-insensitive primordia were stained for a shorter time than miR156-sensitive primordia to compensate for their higher level of GUS activity. All leaves are shown at the same magnification. Scale bar: 1 mm. (D) Quantitative RT-PCR of the primary transcripts of miR156 genes in control and defoliated plants.  $n=3$  technical replicates; error bars indicate mean + s.d. Samples were normalized to *ACTIN 2*.

processing of the primary transcripts it would be expected to produce a decrease, not an increase, in the abundance of the primary transcripts. Furthermore, factors that affect miR156 processing would be expected to affect all miR156 transcripts, not just a subset.



**Fig. 4. The effect of defoliation on miR156 levels in maize and *Nicotiana benthamiana*.** (A) miR156 expression increases and miR172 expression decreases in shoot apices of adult maize plants growing in culture. (B) miR156 expression decreases between 10 and 15 days after planting in *N. benthamiana* seedlings. (C) The expression of miR156 in *N. benthamiana* seedlings is unaffected by cotyledon ablation, but increases in response to defoliation. -1 cot., one cotyledon ablated; -2 cot., two cotyledons ablated; -1 leaf, first leaf ablated. U6 was used as a loading control.

### Defoliation induces miR156 expression in maize and *Nicotiana benthamiana*

Culturing shoot apices from adult maize plants after removing most leaves and leaf primordia causes either complete or partial rejuvenation of the shoot apex, depending on the number of leaf primordia remaining on the explant (Irish and Karlen, 1998; Orkiszewski and Poethig, 2000). An analysis of the effect of this treatment on the expression of miR156 and miR172 revealed that miR156 was slightly elevated in 1-day-old explants and much more so in 6-day-old explants, whereas miR172 had the opposite expression pattern (Fig. 4A). This increase in miR156 is consistent with the observation that *SPL* gene expression decreases in explanted maize apices (Strable et al., 2008), and is likely to be the basis for the rejuvenated phenotype of cultured shoot apices.

In *N. benthamiana*, miR156 expression decreases between 10 and 15 days after planting (Fig. 4B). Removal of one or two cotyledons from 14-day-old plants had no effect on the level of miR156, confirming that the expression of this miRNA is insensitive to wounding and suggesting that embryonic factors play little or no role in vegetative phase change. By contrast, removal of a single leaf primordium produced a significant increase in miR156 expression (Fig. 4C). Along with the results obtained in maize, this observation strongly suggests that the function of leaf primordia in vegetative phase change is evolutionarily conserved.

### Conclusions

Our results indicate that vegetative phase change is regulated by a factor produced by leaf primordia that acts by repressing the expression of miR156. This discovery suggests several ways in which the timing of vegetative phase change may be regulated. The most obvious possibility is that vegetative phase change is initiated by an increase in the level of this factor that results from an increase in leaf number, i.e. by a leaf-counting mechanism. However, this hypothesis is inconsistent with previous studies indicating that the timing of vegetative phase change is regulated independently of leaf number (Hamada et al., 2000; Telfer et al., 1997). Other possibilities are that vegetative phase change is regulated by an increase in the production of this inducer by individual leaf primordia, as occurs in the case of FT during floral

induction (Bohlenius et al., 2006; Kardailsky et al., 1999; Kobayashi et al., 1999), or by an increase in the sensitivity of the shoot apex to this factor. In this latter scenario, the factor would function in a permissive rather than in an instructive role. Whatever the case might be, the evidence that vegetative phase change is mediated by a factor produced by leaf primordia is an important advance in our understanding of the mechanism of this transition and will aid in the identification of this key developmental regulator.

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#### Competing interests statement

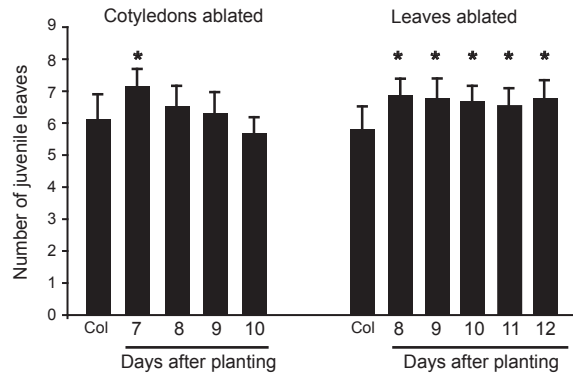
The authors declare no competing financial interests.

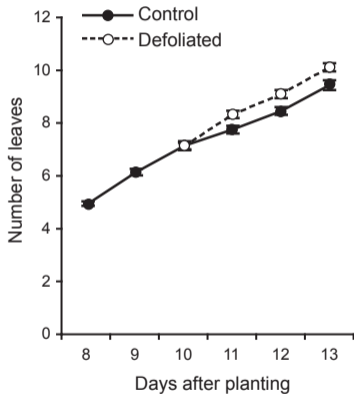
#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058578/-/DC1>

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**Table S1. PCR primers**

Primer	Sequence (5' to 3')
SPL9-cacc-F	CACCATCACATTTGAGCGTCCAAAGATATGGTCTCTGC
SPL9-R	GAGAGACCAGTTGGTATGGTGAGAAG
SPL9r-1	AGAGAGTAGTGACAGTGCGCAGTTTGAGTCGCCAATTCCTC
SPL9r-2	TGCGCACTGTCTACTCTCTAATCCACATCAACCACATGA
GUSp-SPL3-R1	CATGGACCTGATAGCGGCGTTGCTAGCGTCTTGTAGCCGA
GUSp-SPL3-F1	ATGGTAGATCTGAGGGTAAATTTCTAGTTTTTCTCCTTCA
GUSP-SPL3-R3	TTTACCCTCAGATCTACCATCTGCAAATTCAACTCTCTC
GUSP-SPL3-F2	AACGCCGCTATCAGGTCCATGATGAGTATGAGAAGAAGCAA
SPL3-G1	CCGGAATTCCTGTAAAGATAATTGTG
SPL3-G2	GGCCGGATCCGATTAGTCTTCCAATC
qMIR156A-F	CATCTTGTAGATCTCTGAAGTTGGACT
qMIR156A-R	GAGATTGAGACATAGAGAACGAAGACA
q156B-F2	GAAGAGGGAGAGATGGTGATTG
q156B-R2	GCAGAGATAGGCAACTGACAG
qMIR156C-F	AAGAGAAACGCATAGAACTGACAG
qMIR156C-R	GGGACCGAATCGGAGCCGGAATCTGAC
qMIR156H-F	GAAAGAGAGCACAACCTGGGATTAGC
qMIR156H-R	CGCAATGATGGTGGCAGAAGGAAAGAG