Time-lapse imaging

Cells were stably transfected with pBOS–histone-H2B–GFP[15] and analysed on a TE200 inverted microscope (Nikon) with a ×40 objective enclosed in a temperature- and carbon-dioxide-controlled incubator (Neve). Images were acquired with a CCD camera (Princeton) at intervals ranging from 1 to 10 min and analysed by the MetaMorph software program (Molecular Devices).

Fluorescent *in situ* hybridization

Methods for FISH analysis with chromosome-specific centromeric probes and quantitative analysis of chromosome loss rates have been described[16]. A pan-centromeric FISH probe (IDbright Pan-Centromeric, ID Labs) was used in accordance with the manufacturer's instructions. Multicolour FISH with centromeric probes for chromosomes 7, 17, 18 and X was also done on interphase nuclei once cells had gone through 25 generations after clone isolation. To prepare metaphase spreads, we treated cells with 0.1 $\mu g\,ml^{-1}$ colcemid (KaryoMax, Invitrogen) for 1 h and processed them by standard methods. Karyotyping was done by standard G-banding procedures.

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Correspondence and requests for materials should be addressed to C.L. (lengauer@jhmi.edu).

Spatially restricted microRNA directs leaf polarity through ARGONAUTE1

Catherine A. Kidner & Robert A. Martienssen

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA

Gene regulation by RNA interference requires the functions of the PAZ domain protein Argonaute. In plants, mutations in *ARGONAUTE1* (*AGO1*) are associated with distinctive developmental defects that suggest a role for microRNA (miRNA) in organ polarity. Potential targets of miRNA regulation are the homeodomain/leucine zipper genes *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*)[1]. These genes are expressed in a polar fashion in leaf primordia and are required for adaxial cell fate[2,3]. Here we show that a 21-nucleotide miRNA that directs cleavage of *PHB*/*PHV* messenger RNA accumulates first in the embryonic meristem, and then in the abaxial domain of the developing leaf. miRNA distribution is disrupted by mutations in AGO1, indicating that AGO1 affects the regulation of miRNA. In addition, interactions between homeodomain/leucine zipper genes and an allelic series of *ago1* indicate that miRNA acts as a signal to specify leaf polarity.

Surgical isolation of early leaf primordia results in outgrowth of 'centric' or radial organs, suggesting that a meristem-derived signal directs adjacent regions of the leaf primordia to adopt an adaxial fate, and directs the regions of the leaf primordia furthest from the meristem to become abaxial[4]. In *Arabidopsis*, mutants in *AGO1* (ref. 5) and the related gene *PINHEAD*/*ZWILLE* (*PNH*/*ZLL*)[6,7] have overlapping defects in meristem maintenance and leaf polarity consistent with a defect in this signalling process, and the two genes are partially redundant[6]. However, whereas *ago1* leaves are adaxialized (see below), *pnh* leaves are weakly abaxialized[6].

Argonaute genes are defined by carboxy-terminal PAZ and PIWI domains, and are widespread in eukaryotes[8]. In *Drosophila*, mutations in *sting*/*aubergine* and *dAGO1* have defects in germline development[9], and Sting/Aubergine is required for translation of *oskar* in the polar granules[10]. Argonaute genes are required for post-transcriptional gene silencing and RNA interference (RNAi)[8,11], and AGO1-like proteins are part of the RISC (RNA interference silencing complex), which targets mRNA for degradation using miRNA as a guide[12,13]. miRNAs from *Arabidopsis* match several transcription factor genes, potentially accounting for *ago1* and *pnh/zll* phenotypes[1,14].

We isolated an allelic series of *AGO1* to investigate its role in polarity (Fig. 1). The strong alleles *ago1-9* and *ago1-10* are frame-shift truncations of 384 and 404 amino acids respectively, deleting the PAZ and PIWI domains. Vegetative organs are strap-like or radial[5,6], with radial organs (*ago1-10*) or reduced flowers (*ago1-9*) in the inflorescence (Fig. 1), suggesting a complete loss of adaxial/abaxial polarity. Ectopic ovules are found occasionally (*ago1-9*) or frequently (*ago1-11* and *ago1-12*) on the outside (abaxial) carpel wall, usually near the base (Fig. 1c). *ago1-11* and *ago1-12* are both weak alleles that retain the PAZ domain. *ago1-11* has an A-to-T conversion at the splice acceptor site of the fourteenth intron, leading to mis-splicing and exon skipping. The first pair of leaves are sometimes radial or trumpet-shaped but the remainder are flattened. Trichomes appear on the abaxial rather than the adaxial side of early leaves, suggesting an inversion of epidermal polarity in this weak allele (Fig. 1d). *ago1-12* has a C-to-T transition at nucleotide 2,414, resulting in a leucine substitution for an absolutely conserved histidine residue in the PIWI domain. *ago1-12* leaves are almost always trumpet-shaped with glossy dark-green tissue (adaxial) outside and reflective epidermis (abaxial) inside the

bell (Fig. 1b). This phenotype resembles the dominant mutant *Phabulosa1-D*[3].

Enhancer-trap reporter ET2689 is inserted in an intergenic region upstream of *At3g28925* and is expressed on the proximal abaxial side of leaves. In *ago1-10*, expression is lost from the leaves and appears ectopically in the apical hook (Fig. 2a, b). ET3964 is expressed on the adaxial side of leaves and flowers, as well as in axils, and is inserted in the 5′UTR of *At1g67260*, a TCP gene[15]. Other members of the TCP gene family are regulated by the JAW miRNA and control growth[16]. In *ago1-10*, ET3964 is ectopically expressed and upregulated (Fig. 2c,d), suggesting that *At1g67260* is also regulated by RNAi. *FILAMENTOUSFLOWER* (*FIL*) and *KANADI1* (*KAN1*) are expressed abaxially in wild-type organs and regulate polarity[17–20].

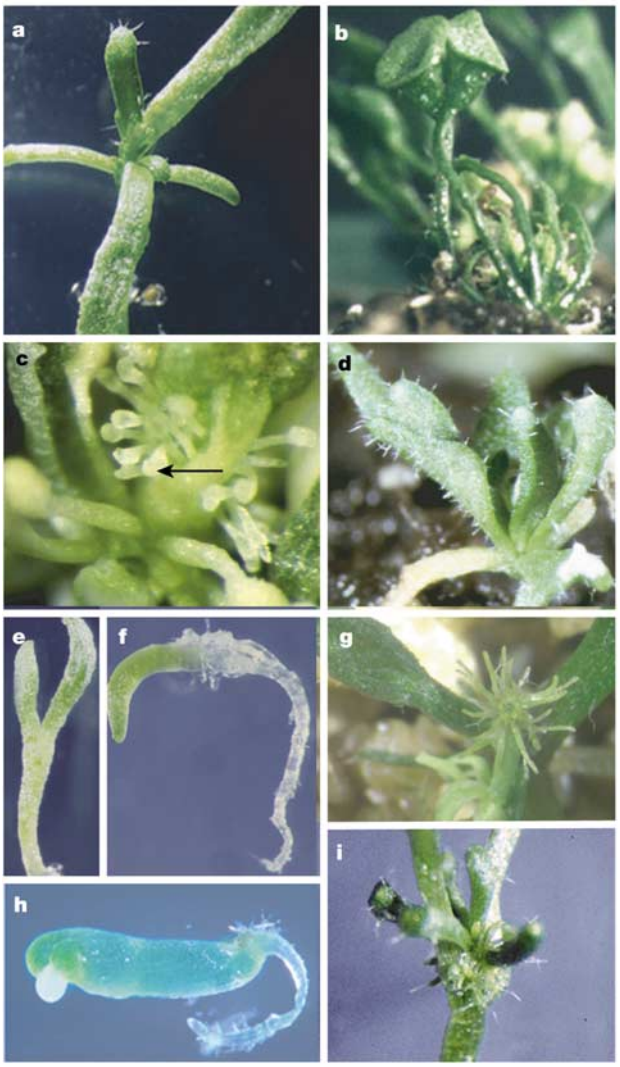

Figure 1 Mutations in *AGO1* are adaxialized and mutations in *REV* are enhanced by *ago1* and *dcl1-9*. **a**–**d**, Six-week-old plants. **a**, *ago1-10* (*ago1-9* has a very similar phenotype); **b**, *ago1-12*; **c**, **d**, *ago1-11*. **c**, In wild-type carpels, ovules are formed only on the adaxial surface; however, in *ago1-11*, ovules emerge from the abaxial surface as well. **d**, First or second leaves of *ago1-11*. At this stage, wild-type leaves have trichomes on the adaxial side only, and *ago1-11* has trichomes abaxially. **e**, *ago1-11*/*ago1-11*,*dcl1-9*/*dcl1-9* seedlings lack meristem function and organ polarity. **f**, *ago1-10*/*rev-6* double mutants lack a shoot apical meristem and cotyledons are reduced or absent. **g**, *dcl1-9*/*rev-6* mutants have narrow, epinastic leaves and form filamentous organs in the place of flowers. **h**, *Phb1-D*/*Phb1-D*,*ago1-10*/*ago1-10* seedlings lack meristem function and have no, or only rudimentary, organs. **i**, *Phb1-D*/+,*ago1-12*/+ seedlings have an enhanced polar defect, resembling *Phb1-D* homozygotes.

FIL is expressed at a much lower level in *ago1-9* but *KAN1* is expressed at normal levels (Fig. 2e).

The closely related homeodomain/leucine zipper (HD-ZIP III) genes *PHB* and *PHV* convey leaf polarity[2]. *PHB* and *PHV* are located in segmental genome duplications[21] and are probably redundant. Loss-of-function mutants in *PHB* and *PHV* have no phenotype[22], although the closely related gene *REVOLUTA* (*REV*) is required for axillary meristem development and vascular patterning[23], and the *phb*,*phv*,*rev* triple mutant has severe loss of polarity and of meristem function[22]. *PHB* negatively regulates *FIL*, and both *REV* and *PHV* are negatively regulated by *KAN1* and *KAN2* (refs. 17,20). In early embryos *PHB* and *REV* are expressed in the shoot apical meristem (SAM), in adaxial domains of developing cotyledons, and in vasculature[23,2]. PHB expression is lost from the SAM of torpedo-stage embryos but is regained late in embryogenesis, extending in 'rays' to young primordia after germination, and becoming progressively restricted to the adaxial domain and the vasculature[2]. *REV* expression forms an inverted-cup pattern within the SAM. In organ primordia *REV* is restricted to the vasculature and to a broader adaxial domain than *PHB*. The combined expression pattern of both genes overlaps with that of *PNH*/*ZLL*. *AGO1* is expressed uniformly in embryos, SAMs and developing primordia[6].

Two miRNAs from *Arabidopsis*, miR165 and miR166 (ref. 24), match *PHB*, *PHV* and *REV* mRNA, and direct their cleavage[22,25]. The 19-base-pair (bp) miRNA match is specifically disrupted in each of

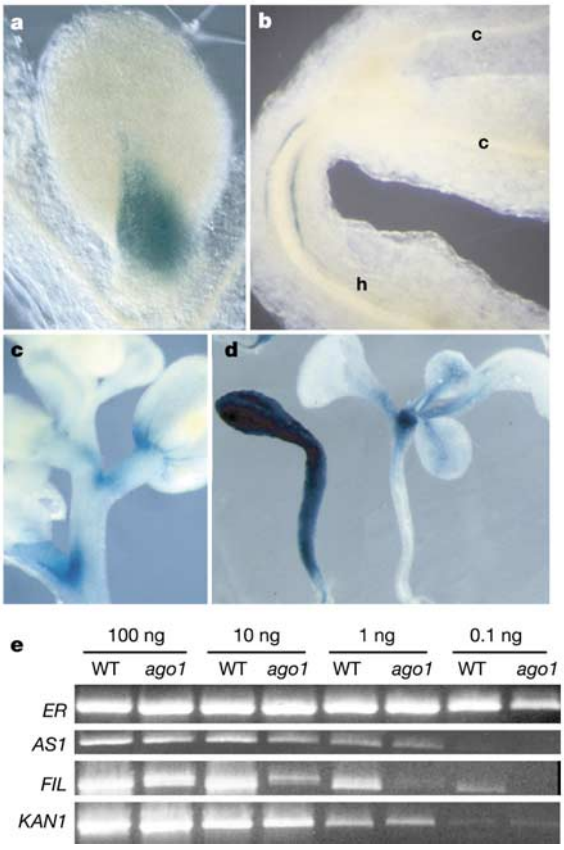

Figure 2 Reporter gene expression in *ago1-10* demonstrates adaxialization. **a**, ET2689 is expressed on the abaxial proximal side of the leaf. **b**, In *ago1-10*, no expression is seen in the leaves but two stripes of expression appear at the apical hook (c, cotyledon; h, hypocotyl). **c**, **d**, ET3964 is expressed on the adaxial side of the leaf and cotyledon axil (**c** and right side of **d**). In *ago1-10* it is overexpressed (left side of **d**). **e**, *FIL* is expressed at lower levels in *ago1-10*. A dilution series of total RNA from the wild type and *ago1-10* has consistently lower amplification by RT–PCR when using primers specific for *FIL* but not for *ERECTA* (*ER*), *ASYMMETRIC LEAVES1* (*AS1*) or *KANADI1* (*KAN1*).

nine independent dominant mutations in *PHB* and *PHV* that have strongly adaxialized organs and form trumpet-shaped leaves[2]. The dominant mutations in PHB lead to ectopic expression[2], and transcripts of the dominant mutants cannot be cleaved *in vitro*[25].

Both *ago1-12* and *Phb-1D* have trumpet-shaped leaves with adaxial tissue on the outside. These similarities indicated that the *ago1* phenotype might be due to failure to downregulate *PHB* and *PHV* mRNA. Consistent with this view, the *Phb1-D* gain-of-function phenotype was enhanced by *ago1*, presumably by upregulation of *PHV* due to loss of miRNA regulation (Fig. 1h, i). However, loss-of-function mutants in the *PHB* homologue *REV* were also strongly enhanced (Fig. 1f). In *ago1-10*;*rev-6* double mutants, SAMs and cotyledons fail to develop at the heart stage, although root growth is unaffected (Fig. 1f). We explored this further by examining *rev-6* in combination with *dcl1-9*. *DCL1* encodes a homologue of the double-stranded RNase DICER and fails to accumulate miRNA[24]. *ago1-10*;*dcl1-9* double mutants are lethal at the embryo stage (data not shown) and *ago1-11*;*dcl1-9* double mutants lose meristem function and organ polarity (Fig. 1e). This confirms that the RNAi pathway is required for polarity. *rev-6*;*dcl1-9* double mutants had filamentous flowers resembling *rev*;*fil* (ref. 26), and *FIL* was severely downregulated in *rev-6*;*dcl1-9* inflorescences (Fig. 1g and Supplementary Information). This loss of abaxial fate would be expected from the loss of miRNAs regulating *PHB* and *PHV* but is only revealed in the absence of *REV*.

ago1 and *dcl1* have different effects on the accumulation of miRNA[27], perhaps accounting for their different interactions with *rev*. We examined this further by *in situ* hybridization using the miRNA precursor as a probe (Fig. 3). Consistent with northern blot analysis[27], hybridization was only detected with probes that included the miRNA (Fig. 3a and Supplementary Information). In bent cotyledon-stage wild-type embryos, the miRNA accumulates in the vasculature and in the SAM (Supplementary Information), but in germinating seedlings, the miRNA accumulates specifically in the abaxial domain of developing leaf primordia (Fig. 3a). Weaker hybridization can be detected in the SAM (Fig. 3a). This pattern thus limits miRNA-directed cleavage of *PHB*/*PHV*/*REV* to the abaxial side of young primordia, and is largely complementary to the expression patterns of these genes[2]. miR165 was not detected in P0, although *Phb1-D* is ectopically expressed in incipient primordia. It is possible that low levels could not be detected in these cells.

To determine whether miRNA accumulation influenced leaf polarity through this mechanism, we examined PHB expression. In the wild type, *PHB* is expressed on the adaxial side of the leaf, as previously reported[2] (Fig. 3c). In *ago1-10*, *PHB* is ectopically expressed throughout leaf primordia, accounting for adaxialization of the leaf (Fig. 3d). However, miR165 is also ectopically expressed in the adaxial domain of *ago1*, and is lost from the meristem (Fig. 3c). The ectopic accumulation of miRNA in *ago1* may be due to failure to enter the RNAi pathway. Recently, the PAZ domain has been shown to bind miRNA and may be important in miRNA maturation and incorporation into the RISC[28].

The pattern of accumulation of miR165 accounts for the different roles of PNH/ZLL and AGO1 in leaf polarity, namely that *ago1* is adaxialized whereas *pnh*/*zll* is weakly abaxialized. *PNH*/*ZLL* is expressed in the adaxial domain[6], where it could not direct *PHB*/*PHV* cleavage because of the absence of miRNA (Fig. 3f). However, PNH/ZLL could direct cleavage in *ago1*, as miR165 is mis-localized to the adaxial domain. Reduction of adaxial *PHB* in *ago1* would enhance loss-of-function mutants in *REV*. Conversely, *pnh-2* would have no effect, and is indeed epistatic to *rev-6* (data not shown). PNH/ZLL and AGO1 may even positively regulate *PHB*/*PHV*/*REV* in the absence of miRNA, by analogy with *aubergine* and *oskar* in *Drosophila*[10]. Thus differential accumulation of miRNA accounts for differences between *pnh* and *ago1*, and their interactions with *rev*.

Sussex[4] proposed that a signal passed between the meristem and leaf primordia to convey organ polarity. Information also flows from the leaf to the meristem, as loss of organ polarity can result in meristem arrest[2,3,17]. In tobacco, disruption of RNA trafficking using viral movement proteins leads to radial leaves that may be at least partially adaxialized, indicating a mobile RNA signal[29]. Our results indicate that miR165 and miR166, or their precursors, contribute to this polarizing signal: miRNA initially accumulates in the meristem, becoming localized to the abaxial domain during leaf development. This process requires AGO1, indicating that miRNA localization is, at least in part, under post-transcriptional control. The loss of

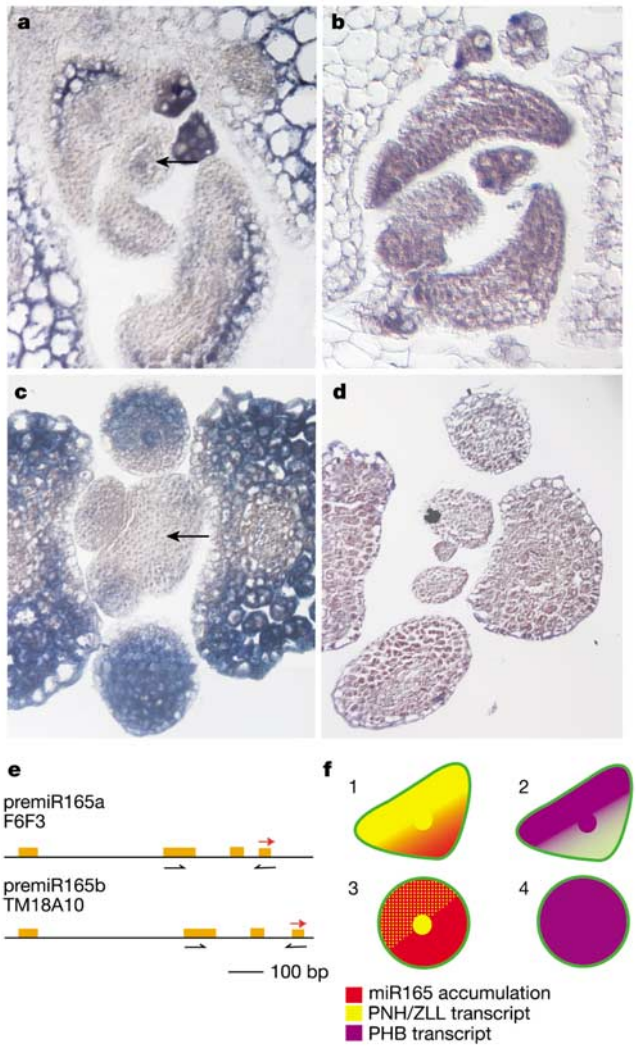

Figure 3 Transcripts from miR165 accumulate in the embryonic meristem and on the abaxial side of the leaf. **a**, In the wild type, miR165 accumulates on the abaxial side of 7-day-old leaves at stages P1, P2 and P3 and also weakly in the meristem (arrow). **b**, *PHB* accumulates on the adaxial side of the leaf primordia in the wild type. **c**, In *ago1-10*, miRNA expression extends adaxially and is at a higher level than in the wild type. It is not observed in the meristem (arrow). **d**, In 7-day-old *ago1-10*, *PHB* is expressed throughout the leaf primordia. **e**, premiR165a and premR165b loci. Orange boxes indicate areas of conserved sequence. Red arrows indicate the position of the miRNA and black arrows the primers used to generate the antisense probe. **f**, Accumulation of transcripts in the P2 leaf of the wild type (1, 2) and *ago1-10* (3, 4). In the wild type, miR165 (red) is expressed abaxially. This leads to accumulation of *PHB* transcript (purple) on the adaxial side of the primordia only (2). In *ago1-10*, miR165 (red) accumulates ectopically on the adaxial side of the primordial (3), resulting in co-localization with *PNH*/*ZLL* (yellow stipple). Loss of miRNA-directed regulation in *ago1-10* results in ectopic accumulation of *PHB* transcript (4) and an adaxialized phenotype.

abaxial cell fate in *ago1* indicates that miRNA regulation is required for leaf polarity. Other factors may guide the polarizing signal, but this remains to be determined. □

Methods

Growth conditions and plant material

All mutant and transgenic lines were analysed in the Landsberg *erecta* background, unless otherwise noted. *ago1-10* and *ago1-11* arose as point mutants in our gene-trap populations. *ago1-12* was kindly provided by S. Poethig, *dcl1-9* by S. Jacobsen, *rev-6* by L. Comai, and *pnh-2* by K. Barton. Gene-trap and enhancer-trap lines were generated as previously described[30].

Seedlings were grown on germination medium containing 1 × MS salts (Gibco), 1% sucrose and 0.5% phytagel (Sigma). Soil-grown plants were grown in Metromix 200 (Scotts) supplemented with 14-14-14 Osmocote (Scotts) at a rate of 2.65 kg m^{-3} and Marathon systemic insecticide (Olympic) at a rate of 0.88 kg m^{-3}. Plants were grown at 22 °C in 24 h light (200 microeinsteins m^{-2} s^{-1}). *dcl1-9*/+,ET2689/+,ET3964/+ seedlings were selected on germination medium containing kanamycin (50 μg ml^{-1}) and 0.7% agar instead of phytogel.

Histochemical localization of GUS activity

GUS staining was carried out using a substrate solution containing 100 mM sodium phosphate pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.5 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 100 μg ml^{-1} chloramphenicol, and 2 mM each of potassium ferricyanide and potassium ferrocyanide.

Molecular biology

DNA and RNA extraction and manipulation were carried out using standard methods. Polymerase chain reaction with reverse transcription (RT–PCR) was carried out using total RNA from 21-day-old plants and the Qiagen Onestep kit. Products were sequenced or probed with complementary DNAs to confirm their identity: for *FILAMENTOUS FLOWER* (*FIL*) the oligonucleotide primers were 5′-GTTTGTTCACCGGACCACTT-3′ forward and 5′-TTCTTGGCAGCAGCACTAAA-3′ reverse; for *KANADI1* (*KAN1*) the primers were 5′-GGAGGAGGAGACGTGGATCA-3′ forward and 5′-ACCTGCAAATGGCTCTTCAC-3′ reverse; for premiR165 the primers were 5′-CCCATCATTCCCTCAT CATAA-3′ forward and 5′-AAGCCTGGTCCGACGATAC-3′ reverse; for *PHB*-3′ the primers were 5′-GCCAAGGCATCTATGTTGCT-3′ forward and 5′-AATGTGAAAACCGGTGAAGC-3′ reverse.

In situ hybridizations were performed using a protocol from J. Long (www.its.caltech.edu/~plantlab/protocols/insitu.pdf), using embryos provided by K. Barton and 7-day-old wild-type and *ago1-10* seedlings. premiR165 RT–PCR and PHB-3′ products were cloned into pCR2.0 to make RNA probes. *STM* was used as a positive control and a sense transcript from the premiR165b locus that did not include the miRNA sequence was used as a negative control.

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Correspondence and requests for materials should be addressed to R.A.M. (martiens@cshl.org).

microRNA-mediated repression of *rolled leaf1* specifies maize leaf polarity

Michelle T. Juarez[1,2], Jonathan S. Kui[1,3], Julie Thomas[1], Bradley A. Heller[1] & Marja C. P. Timmermans[1]

[1]*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA*
[2]*Graduate Program in Genetics, State University of New York, Stony Brook, New York 11794, USA*
[3]*The Watson School of Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA*

In both animals and plants, many developmentally important regulatory genes have complementary microRNAs (miRNAs), which suggests that these miRNAs constitute a class of developmental signalling molecules[1]. Leaves of higher plants exhibit a varying degree of asymmetry along the adaxial/abaxial (upper/lower) axis. This asymmetry is specified through the polarized expression of class III homeodomain/leucine zipper (*HD-ZIPIII*) genes[2–4]. In *Arabidopsis*, three such genes, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), are expressed throughout the incipient leaf, but become adaxially localized after primordium emergence. Downregulation of the *HD-ZIPIII* genes allows expression of the *KANADI* and *YABBY* genes, which specify abaxial fate[5–8]. *PHB*, *PHV* and *REV* transcripts contain a complementary site for miRNA165 and miRNA166, which can direct their cleavage *in vitro*[9–11]. Here we show that miRNA166 constitutes a highly conserved polarizing signal whose