Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes

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Summary
Plant development is characterized by precise control of gene regulation, leading to the correct spatial and temporal tissue patterning. We have characterized the Arabidopsis jabb-a-ID (jba-ID) mutant, which displays multiple enlarged shoot meristems, radialized leaves, reduced gynoecia and vascular defects. The jba-ID meristem phenotypes require WUSCHEL (WUS) activity, and correlate with a dramatic increase in WUS expression levels. We demonstrate that the jba-ID phenotypes are caused by over-expression of miR166g, and require the activity of the RNase III helicase DCL1. miR166g over-expression in jba-ID plants affects the transcripts of several class III homeodomain-leucine zipper (AtHD-ZIP) family target genes. The expression of PHABULOSA (PHB), PHAVOLUTA (PHV) and CORONA (CNA) is significantly reduced in a jba-ID background, while REVOLUTA (REV) expression is elevated and ATHB8 is unchanged. In addition, we show that miR166 has a dynamic expression pattern in wild-type and jba-ID embryos. Our analysis demonstrates an indirect role for miRNAs in controlling meristem formation via regulation of WUS expression, and reveals complex regulation of the class III AtHD-ZIP gene family.

Key words: microRNA, Arabidopsis thaliana, shoot apical meristem, polarity, HD-ZIP

Introduction
Eukaryotic development requires the precise spatial and temporal expression of regulatory genes that pattern tissues and control cell fate. It is becoming increasingly clear that small RNA molecules are important participants in these processes, providing sequence specificity for targeted regulation of key developmental factors at the post-transcriptional level. microRNAs (miRNAs) are 21-24 nucleotide single-stranded RNA molecules found in both plants and animals that can down-regulate gene expression by pairing to the messages of protein-coding genes (Bartel, 2004). They are produced from larger precursor transcripts of ~70-300 nucleotides in length that contain a characteristic hairpin secondary structure. In plants, mature miRNA sequences are processed from the double-stranded hairpin region of the precursor molecules by DICER-LIKE1 (Schauer et al., 2002), a ribonuclease III helicase protein that acts as a dsRNA-specific endonuclease (Park et al., 2002; Reinhart et al., 2002). The novel protein HEN1 and the nuclear dsRNA-binding protein HYL1 are also required for plant miRNA accumulation (Han et al., 2004; Park et al., 2002; Vazquez et al., 2004).

miRNAs appear to regulate gene expression by binding to complementary sequences in the mRNA transcripts produced by their target genes (Bartel, 2004). While animal miRNAs tend to have several mismatches with their target mRNA sequences, plant miRNAs are characterized by their near-perfect complementarity with their targets (Rhoades et al., 2002). Interaction of the miRNA with its target mRNA sequence is recognized by an RNA-induced silencing complex (RISC), leading either to specific cleavage of the mRNA via RISC endonuclease activity, or to translational repression (reviewed by Bartel, 2004). The RISC has been purified from fly and human cells (Hammond et al., 2000; Martinez et al., 2002), and in both cases contains a member of the ARGONAUTE (AGO) family of PPD proteins (Cerutti et al., 2000). Arabidopsis plants with reduced AGO1 gene activity accumulate miRNAs that are normally targeted for miRNA-mediated cleavage, confirming a role for AGO1 in the miRNA regulatory pathway (Vaucheret et al., 2004).

Mutants that lack DCL1 activity are embryo lethal (Schauer et al., 2002), revealing that miRNA metabolism is essential for normal plant development. Similarly, plants carrying weaker dcl1 alleles survive embryogenesis but display a wide spectrum of developmental defects (Schauer et al., 2002), while hen1 and hyl1 null mutants show reduced miRNA levels and morphological phenotypes that overlap with those of weak dcl1 alleles (Han et al., 2004; Park et al., 2002; Vazquez et al., 2004). ago1 null mutants are viable but have pleiotropic developmental phenotypes (Bohmert et al., 1998), while ago1 hypomorphic mutants exhibit morphological defects similar to those of dcl1, hen1 and hyl1 mutants (Vaucheret et al., 2004).

Consistent with the demonstration that activity of the miRNA pathway is important for plant development, it has been observed that a large fraction of the predicted target transcripts of plant miRNAs encode members of transcription
factor families (Rhoades et al., 2002). Many of these transcription factors have defined or predicted roles in developmental patterning, phase transition and/or cell fate control (Bartel, 2004). A direct role for miRNAs in regulating different aspects of Arabidopsis development has been experimentally demonstrated in a number of cases (Achard et al., 2004; Aukerman and Sakai, 2003; Baker et al., 2005; Chen, 2004; Emery et al., 2003; Mallory et al., 2004; Palatnik et al., 2004; Aukerman and Sakai, 2003; Baker et al., 2005; Chen, 2004; Emery et al., 2003; Mallory et al., 2004; Palatnik et al., 2004; Zhong and Ye, 2004).

Among the Arabidopsis developmental regulators targeted by miRNAs are five members of the class III homeodomain-leucine zipper (HD-ZIP) family of transcription factors (Sessa et al., 1998), REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CNA) and ATHB8. Loss-of-function phb, phv, cna and athb8 mutants are aphenotypic (Baima et al., 2001; Prigge et al., 2005), but rev mutants form defective lateral and floral meristems and develop aberrant stem vasculature (Otsuga et al., 2001; Talbert et al., 1995). rev phb phv triple mutants fail to establish a shoot apical meristem and produce abaxialized cotyledons, indicating that these three genes play overlapping roles in regulating SAM formation, leaf polarity and radial patterning (Emery et al., 2003; Prigge et al., 2005). PHB, PHV and CNA have overlapping functions in regulating meristem size, lateral organ polarity and vascular development that are distinct from REV (McConnell and Barton, 1998; McConnell et al., 2001; Prigge et al., 2005). ATHB8 has been proposed to play a role in vascular development (Baima et al., 1995; Baima et al., 2001), and acts redundantly with CNA to promote post-embryonic meristem activity (Prigge et al., 2005).

The five class III HD-ZIP gene transcripts are targeted by miRNAs from the miR165/166 group. Two MiR165 genes and seven MiR166 genes are encoded in the Arabidopsis genome, and the mature miR165 and miR166 sequences differ from one another by a single nucleotide (Reinhart et al., 2002). In wheat germ extracts, miR165/166 guides the RISC to efficiently cleave wild-type PHV mRNA (Tang et al., 2003) and CNA/ATHB15 mRNA (Kim et al., 2005). Increased expression of miR166a in an activation-tagged line causes a reduction in PHB, PHV and CNA transcript levels, leading to an expansion of xylem tissue and the interfascicular region, indicative of accelerated vascular cell differentiation (Kim et al., 2005). Stem fasciation and SAM enlargement are also reported in the miR166a over-expression line. Conversely, phv, phb and rev gain-of-function alleles that alter the miR165/166 complementary site are resistant to mRNA cleavage mediated by miRNA165/166 (Emery et al., 2003; Tang et al., 2003; Mallory et al., 2004; Zhong and Ye, 2004), and they confer specific patterning phenotypes as a result of ectopic expression of their target gene products (Emery et al., 2003; McConnell and Barton, 1998; McConnell et al., 2001; Zhong and Ye, 2004).

Using an activation tagging approach, we demonstrate that miR166g causes de novo SAM formation and disrupts the morphogenesis of leaves, vascular bundles and gynoecia when over-expressed in Arabidopsis. We show that jba-1D meristem cells express much higher than normal levels of WUS mRNA, and that WUS activity is required to obtain the jba-1D meristem phenotype. We find that miR166 is expressed in a dynamic pattern in developing wild-type and jba-1D embryos, being largely complementary to its HD-ZIP target transcripts in early stages but coincident with them in later stages. Over-expression of miR166g causes reduced accumulation of PHB, PHV and CNA transcripts in jba-1D seedlings and inflorescence apices. Increased accumulation of REV transcripts is also detected, but is not sufficient to account for the meristem defects. We propose that down-regulation of PHB, PHV and CNA mRNAs in jba-1D plants leads to up-regulation of WUS transcription in the SAM organizing center, which results in splitting and fasciation of the primary shoot apex.

Materials and methods

Plant growth and genetic analysis

Plants were grown in a 1:1:1 mixture of perlite:vermiculite:top soil under continuous cool-white fluorescent lights (120 μmol/min/second) at 22°C. Double mutants with dcl1-9, wus-1 and rev-6 were generated by crossing jba-1D/+ plants to dcl1-9/+ or wus-1/+ or rev-6 plants, and identified by PCR genotyping the F1 and F2 progeny. Control crosses to Col were performed with both dcl1-9 and wus-1, which originated from the Ler ecotype. The plasmid for recapitulating the jba phenotype was generated by amplifying from jba-1D genomic DNA a 1254 base pair fragment spanning from the 35S enhancers in the T-DNA right border to 129 base pairs downstream of the MIR166 locus. The amplified fragment was cloned into pBIN binary vector. Plant transformation was performed using the floral dip method (Clough and Bent, 1998).

Microscopy

Scanning electron microscopy was performed as described previously (Bowman et al., 1989) using a Hitachi 4700 scanning electron microscope with digital imaging capability. Confocal scanning electron microscopy analysis was performed as described (Running et al., 1995) using a LSM Zeiss 510 confocal microscope.

Histology

Tissues were fixed, embedded in Technovit 7100 resin, sectioned at 5 μm thick and stained in Toluidine Blue solution as described previously (Smith and Hake, 2003).

GUS staining

The GUS staining reaction and subsequent tissue embedding and sectioning were performed as described previously (Sieburth and Meyerowitz, 1997) using 2 mM 5-bromo-4-chloro-3-indolyl-β-D glucuronide (X-GLUC; Bioworld, Dublin, OH, USA).

In situ hybridization

Tissue fixation and in situ hybridization were performed as described previously (Jackson, 1992). Probes for in situ hybridization were transcribed using the digoxigenin labeling mix (Roche). For the REV probe, the full-length cDNA was used as a template. For the CNA and PHB probes, the nucleotide regions 1230-2511 and 1285-2554, respectively, relative to the ATG, were used as templates. For the miR166 probe, four concatamers of sense or antisense sequences were synthesized as oligonucleotides and cloned into pBS-SK. Hybridization with the miR166 probe was carried out at 42°C overnight. After hybridization, the slides were washed four times at 40°C for 10 minutes each.

Real-time and RT-PCR

Aerial parts of 28-day Col and jba-1D homozygous plants were used for real-time PCR analysis. cDNA synthesis was carried out in a 20 μl reaction using 1 μg DNase I-treated total RNA by the reverse transcription system (Promega). The cDNA reaction mixture was diluted 1:10 using DNase-, RNase-free Milli-Q water and 1 μl was taken for real-time amplifications. Amplifications were carried out in
The most dramatic jba-1D/+ phenotype was extreme fasciation of the flowering shoot apical meristem (inflorescence meristem), which caused the stem to grow as a wide strap-like structure rather than as a point (Fig. 1B). The second phenotype was a reduced or filamentous gynoecium, which rendered the hemizygous plants less fertile than their wild-type siblings (Fig. 1G). The third phenotype was epinastic leaf morphology (Fig. 1K). Homozygous jba-1D plants had more dramatic leaf phenotypes, producing severely downward curling and radialized leaves (Fig. 11,LM). jba-1D plants also displayed more extreme inflorescence meristem and flower phenotypes than jba-1D/+ plants, and were completely sterile (Fig. 1C,H). Thus the developmental phenotypes appeared to be dose-dependent – a single copy of this dominant mutation was sufficient to confer the phenotypes, but two copies made the phenotypes more severe. Pollen dehiscence was also reduced in jba hemizygous and homozygous plants, but all other aspects of development appeared to be unaffected by the mutation.

Examination of jba-1D plants revealed that at the transition to flowering, when the jba SAM phenotype becomes visible to
the naked eye, both jba-1D/+ and jba-1D plants had extremely enlarged primary inflorescence meristems (Fig. 2). In jba-1D/+ plants, the inflorescence meristems were both taller and wider than wild-type meristems, but the structural organization of the meristems was intact (Fig. 2A,B). In jba-1D plants, the primary reproductive shoot apex consisted of multiple, independently organized SAMs (Fig. 2C). Large numbers of floral meristems were initiated from each of the jba-1D inflorescence meristems, which continued to accumulate excess cells such that the shoot apices were ultimately visible to the naked eye (Fig. 1E). We interpret this phenotype as a hyper-activation of SAM activity that becomes progressively more severe during reproductive development. In contrast, the size of jba-1D floral meristems was only slightly increased relative to wild-type floral meristems, and floral organ number was relatively normal except for carpel number, which was reduced in jba-1D/+ flowers and essentially absent in jba-1D flowers (see Fig. S1 in supplementary material).

To determine when the jba SAMs first began to enlarge relative to wild-type SAMs, we assessed whether the vegetative meristems of jba-1D seedlings were distinguishable from those of wild-type seedlings. Sections through 11-day-old wild-type, jba-1D/+ and jba-1D seedlings revealed that the mutants already had enlarged vegetative SAMs that were both wider and taller than wild-type SAMs (Fig. 2D-F). In fact, jba-1D SAMs were measurably larger than wild-type SAMs by the time the embryos reached maturity. jba-1D mutant embryonic meristems were 36.6±1.1 μm wide and 5.4±1.2 μm tall (n=12), while Col embryonic meristems were 28.8±1.6 μm wide and 5.3±1.1 μm tall (n=9). The cells in jba-1D embryonic SAMs were approximately the same size as cells in wild-type SAMs, indicating that the mutant SAMs consisted of more cells than

![Fig. 2. jba-1D shoot apical meristem phenotypes.](image)

- **A** Wild-type inflorescence meristem at the transition to flowering.
- **B** jba-1D/+ inflorescence meristem that is both taller and wider than normal.
- **C** jba-1D inflorescence meristem that has split into multiple independent meristems (*), each generating floral meristem primordia on its flanks.
- **D** Wild-type seedling SAM after 11 days of vegetative growth.
- **E** Enlarged jba-1D seedling SAM.
- **F** Mean width and height of Col (n=12) and jba-1D (n=11) SAMs from 11-day-old seedlings.
- **G** Confocal micrograph of a wild-type mature embryo SAM.
- **H** Confocal micrograph of an enlarged jba-1D mature embryo SAM.
- **I** GUS expression from a pSTM::GUS reporter construct in the peripheral region (arrows) of a wild-type seedling SAM.
- **J** pSTM::GUS expression in a jba-1D seedling delimits two well-defined meristems, while the leaf primordium developing between them is radialized (arrowhead). Scale bars: 80 μm (A-E); 20 μm (G-H).
those of the wild type rather than larger cells. The organization of the jba-1D embryonic and vegetative meristems was not disturbed.

We confirmed the jba-1D SAM splitting phenomenon by using pSTM::GUS as a marker for the meristem boundary. This construct drives GUS expression at the SAM periphery, between the central region of the SAM and the developing lateral organ primordia (Fig. 2I). We found that after 11 days of vegetative growth, some jba-1D shoot apices consisted of two well-defined meristems rather than one single primary meristem. Each meristem was marked by pSTM::GUS expression at the periphery (Fig. 2I). Multiple meristems were never observed in jba-1D embryos, indicating that this phenomenon occurs de novo during post-embryonic development.

Cell fate in the SAM is controlled via a regulatory pathway involving the WUSCHEL (WUS) and CLAVATA (CLV) gene products (reviewed by Carles and Fletcher, 2003). The WUS gene is expressed in the central, interior region of the SAM, and encodes a homeodomain transcription factor that confers stem cell fate on the overlying cell population (Mayer et al., 1998; Schoof et al., 2000). CLV3 is expressed in the stem cells, and encodes a small, secreted polypeptide that limits the size of the WUS expression domain (Brand et al., 2000; Fletcher et al., 1999; Rojo et al., 2002). Using promoter-GUS constructs we determined that the WUS expression domain (Fig. 3A-D) and the CLV3 expression domain (Fig. 3E-F) enlarged coordinately in jba-1D SAMs, indicating that the fasciated meristem phenotype of jba-1D plants is due to the presence of many excess stem cells. In addition, we found that the distribution of pWUS::GUS expression was not uniform, and in some cases we observed two adjacent foci of WUS expression within the primary meristem (Fig. 3D). The appearance of such foci of WUS expression in jba-1D plants can explain the observed meristem phenotype, where independent domains of WUS expression lead to the formation of discrete, ectopic meristems. In serial longitudinal sections through entire 11-day old jba-1D seedlings, no WUS expression was detected outside of the shoot apical meristem.

In our pWUS::GUS experiments, we noted that GUS expression was detected in jba-1D meristems after a shorter incubation time than wild-type Col meristems (compare Fig. 3B and C), suggesting that cells in jba-1D SAMs might express higher levels of WUS mRNA than those in wild-type SAMs. To test this hypothesis we performed real-time quantitative RT-PCR (qRT-PCR) on aerial parts of 28-day-old wild-type and jba-1D plants using primers directed against CLV3 and WUS. We found that, while the relative level of CLV3 transcription was unchanged between wild-type and jba-1D plants, the level of WUS transcription was ~12-fold higher in jba-1D plants compared to wild-type plants (Fig. 3G). We next determined whether WUS function was sufficient to produce the jba meristem phenotypes by generating double mutants between jba-1D plants and plants carrying the wus-1 null allele. wus-1 jba-1D double mutant meristems were indistinguishable from wus-1 mutant meristems (see Fig. S2 in supplementary material), indicating that WUS activity is absolutely required to obtain the jba SAM phenotypes. Taken together, our evidence indicates that the presence of elevated levels of WUS transcription in jba-1D shoot apical meristems is sufficient to account for the fasciation and de novo meristem formation phenotypes.

We further examined the jba-1D fasciation phenotype by analyzing the stem vascular patterning. In wild-type Arabidopsis plants, the vascular tissue in the stem is present as a ring of approximately five to eight separate bundles (Fig. 4A).
The vascular bundles are collateral, with xylem positioned close to the center of the stem and phloem in more peripheral positions (Turner and Sieburth, 2001). Cross sections of the inflorescence stem revealed three types of alterations in the vascular patterning of jba-1D plants. First, jba-1D stems had increased numbers of discrete vascular bundles in peripheral positions along the stem (Fig. 4C). In the most extremely fasciated jba-1D stems, as many as 29 individual bundles could be observed. Second, jba-1D mutant stems displayed defects in the organization of the vascular cell types within these bundles. The wild-type collateral pattern of xylem toward the inside and phloem toward the outside (Fig. 4B) was disrupted in jba-1D stems by the appearance of ectopic xylem elements close to the periphery (Fig. 4D,F). This suggests a defect in the jba-1D plants in the production, reception or interpretation of positional signals that pattern the vasculature. Third, jba-1D mutants formed extra bundles abnormally located in the center of the stem (Fig. 4C). These bundles exhibited an amphivasal arrangement, with xylem surrounding phloem (Fig. 4E), and might represent veins from the multiply splitting meristems and/or from the radial leaves that protrude from the shoot apex. These results reveal that the jba-1D mutation affects the number, positioning and polarity of stem vascular bundles.

The striking phenotype of radial leaf formation between two or more adjacent meristems (see Fig. 2J and Fig. S1M in the supplementary material) in jba-1D plants led us to analyze the morphology and vascular patterning of these leaves. Examination of the radIALIZED leaves using light and scanning electron microscopy indicated that the leaves had adaxial features, such as trichomes and jigsaw-shaped cells, around the entire circumference (data not shown). The interior morphology of the radial rosette leaves also showed radial symmetry, with the cells resembling adaxial palisade mesophyll (Fig. 4G). However, the single vascular bundle within each radial leaf still exhibited polarity, with xylem on one side and phloem on the other side (Fig. 4H). In contrast, adaxialized radial leaves from other dominant leaf polarity mutants such as phb-1D exhibit radialized vascular bundles, with xylem surrounding phloem (McConnell and Barton, 1998). One interpretation of these data is that the developing jba-1D leaf may receive a normal adaxial polarizing signal from the SAM from which it is derived as well as weaker signals for adaxialization from adjacent meristems, leading to partial but incomplete radialization.

Since jba-1D plants show very similar shoot meristem and vascular phenotypes to those of the REV gain-of-function mutants avb1 and rev-10D (Emery et al., 2003; Zhong and Ye, 2004), we tested the contribution of REV to the jba-1D phenotype by crossing jba-1D plants to previously described rev-6 mutant plants (Otsuga et al., 2001). The REV locus and the jba-1D T-DNA insertion site are tightly linked on chromosome 5 with an estimated recombination frequency of 3.2% (~5 cM). From 250 F2 plants that were selected on plates for the jba-1D T-DNA insertion, we were able to identify three jba-1D/+ rev/rev plants. Analysis of these plants showed that the rev-6 mutation does not suppress the jba-1D/+ stem fasciation phenotype (see Fig. S2 in supplementary material). This result shows that wild-type REV function is not essential for the stem fasciation of jba plants. In contrast, radial leaves are not observed in the jba-1D/+ rev/rev plants, indicating that REV does play a role in conditioning the jba leaf phenotypes.

Fig. 4. Defective jba-1D inflorescence stem vasculature and lateral organ polarity. (A) Cross section through a wild-type stem. (B) Higher magnification of the boxed region in A. The vascular bundle consists of xylem (xy) located on the inner side of the bundle and phloem (ph) located on the outer side of the bundle. (C) Cross section through a jba-1D stem showing additional vascular bundles at the periphery and ectopic vascular bundles in the center of the stem. (D,F) Higher magnification of the boxed region in C and the boxed region in D, respectively. Xylem elements can be detected ectopically on the outer side of the bundle. (E) Higher magnification of the vascular bundles located at the center of the jba-1D stem (smaller box in C). The vascular bundles are radialized, with xylem cells (arrowheads) surrounding phloem cells. (G,H) Cross section through a radialized jba-1D leaf. Higher magnification the vascular bundles (H) shows a polarity in the vascular bundle, with phloem cells at one side of the bundle and xylem cells protruding to the other side. Scale bars: 500 μm (A,C); 50 μm (B,D,E,H,F); 100 μm (G).
**Over-expression of miR166g causes the jba-1D phenotypes**

The *jba-1D* T-DNA insertion falls in an intergenic region on chromosome 5 (Weigel et al., 2000). We determined that the T-DNA element was inserted 1890 base pairs (bp) downstream of At5g63710 and 1861 bp upstream of At5g63720 (Fig. 5A). Using RT-PCR we found that the At5g63720 gene is over-expressed in *jba-1D* mutant plants. However, transgenic *Arabidopsis* plants over-expressing At5g63720 under the control of the CaMV 35S promoter displayed a wild-type phenotype, indicating that this gene does not cause the *jba* phenotypes. Searching for other potential genes in the region we found a known microRNA locus, MIR166g, located 394 bp downstream of the T-DNA insertion site (Fig. 5A). The resulting T1 and T2 transformants were indistinguishable from *jba-1D/+* and *jba-1D* plants, demonstrating that over-expression of miR166g is sufficient to cause all of the *jba* phenotypes. We also performed a cross of *jba-1D/+* to plants carrying a hypomorphic allele of DCL1, which is required for miRNA biogenesis (Kurihara and Watanabe, 2004). Among the *jba-1D/+ dcl1-9/+* F1 progeny of the cross, approximately 50% displayed a completely wild-type phenotype, while the other 50% had slightly enlarged stems but were otherwise normal (see Fig. S2 in supplementary material). This result confirms that miRNA processing is required to obtain the *jba* phenotypes, and in addition, it reveals that one copy of the DCL1 enzyme is not enough to produce sufficient levels of miR166g to generate a phenotype.

The over-expression of miR166 in *jba-1D* plants was confirmed by hybridizing a low-molecular mass RNA blot with a 21-nucleotide probe complementary to the mature miR166 sequence (Fig. 5C). In *jba-1D* seedlings and inflorescences, the level of the 21-nucleotide miR166 was increased compared with wild type. In seedlings, measurement of the level of expression shows that *jba-1D* plants homozygous for the T-DNA insertion generated a higher level of miR166 transcripts than hemizygous *jba-1D/+* plants, accounting for the dose dependence of the *jba* phenotypes. miR166 is also expressed at much higher levels in both wild-type and *jba-1D* plants than in *jba-1D/+. This result confirms that miRNA processing is required to obtain the *jba* phenotypes, and in addition, it reveals that one copy of the DCL1 enzyme is not enough to produce sufficient levels of miR166g to generate a phenotype.

**Embryo expression of miR166 and an HD-ZIP target gene**

To investigate the role played by miR166g in regulating *Arabidopsis* morphogenesis, we determined the tissue distribution of the mature miR166 sequence in wild-type and *jba-1D/+* plants using in situ hybridization. Because the miR166g-mediated *jba-1D* phenotype was detectable in mature embryos, we focused our analysis on the embryonic stage of development. The miR166 expression pattern was identical in developing wild-type (Fig. 6A-E) and *jba-1D/+* (Fig. 6G-K) embryos, confirming that the 35S enhancer elements present in *jba-1D* up-regulate the affected gene in its normal expression pattern.
domain rather than inducing ectopic expression. The first stage at which we could reliably detect miR166 transcripts was the late globular stage, where initial expression is restricted to the periphery of the hypocotyl and the tips of the initiating cotyledons (arrows). During the heart and torpedo stages, miR166 expression expands to include the abaxial region and the distal tips of the cotyledons. In mature embryos, miR166 expression becomes localized to the SAM, the adaxial region of the cotyledons and the provascular tissues. Control hybridization with a miR166 sense probe, at the torpedo stage. REV expression in wild-type embryos. REV is expressed in the central apical region of late globular stage embryos. During the heart stage the REV expression expands to the adaxial region of the cotyledons and the central provascular tissues of the hypocotyl. REV expression in early and late torpedo stages is similar to the heart stage. In mature embryos, REV is localized to the SAM, the adaxial regions of the cotyledons and the central provascular tissues. Scale bars: 500 μm.

The expression patterns of the HD-Zip III target genes in Arabidopsis embryos have been well characterized (Emery et al., 2003; McConnell et al., 2001; Prigge et al., 2005). To compare the distribution of miR166 transcripts with those of its targets, we analyzed REV (Fig. 6M-Q) as its expression pattern encompasses all the others. During the late globular stage, miR166 expression was restricted to the periphery of the hypocotyl and the tips of the initiating cotyledons, while REV was expressed in the apical, central region of the embryo (Fig. 6M). Thus the initial REV expression pattern is reciprocal to that of miR166. During the heart and torpedo stages, REV expression expanded to the adaxial region of the cotyledons, the presumptive SAM and the central provascular tissues of the hypocotyl (Fig. 6N,O,P). At these stages the REV domain is reciprocal to miR166 in the hypocotyl and abaxial region of the cotyledons, but overlaps it in the adaxial region. These data suggest a possible role for miR166 in clearing REV transcripts.
from the peripheral regions of the developing embryo. In mature embryos, REV was localized to the SAM, the adaxial regions of the cotyledons and the central provascular tissues (Fig. 6Q). At this stage the expression of REV was coincident with that of miR166, suggesting that the effect of miR166 expression in mature embryos may be to modulate the mRNA transcript levels of REV.

We further investigated the effects of miR166g over-expression on the transcription levels of its target genes using semi-quantitative RT-PCR. The expression levels of PHB, PHV and CNA were significantly reduced in jba-1D seedlings compared to wild-type seedlings (Fig. 7). Decreased PHB and CNA transcript levels were also observed in jba-1D IFMs, while PHV transcript levels were slightly reduced. These results are consistent with the model that miR166g can direct cleavage and degradation of its target mRNAs. However, there were no differences in ATHB8 transcript levels in jba-1D seedlings or IFMs, while REV transcript levels were elevated in jba-1D plants compared to wild-type plants (Fig. 7). Thus over-expression of miR166g leads to differential effects on the overall transcript levels of its AtHD-ZIP target genes.

Finally, to analyze whether the increase in REV transcript levels and the decrease in CNA and PHB transcript levels in jba-1D plants is due to alteration in their tissue distribution, we performed an in situ hybridization analysis on seedlings using REV, CNA and PHB probes. The REV expression pattern in wild-type (Fig. 8A) and jba-1D (Fig. 8B) seedlings was similar. In both backgrounds, the REV mRNA accumulated in the adaxial regions of the leaf primordia, in the vascular tissues and in the central zone of the SAM. These results show that the tissue distribution of REV mRNA is not altered in jba-1D seedlings. The CNA expression in wild-type seedlings was restricted to the adaxial region of developing leaves, to the vascular tissues and to the central, interior region of the SAM (Fig. 8C). In jba-1D seedlings, the CNA expression pattern changed significantly (Fig. 8D). CNA transcripts accumulated at a very low level in the adaxial region of initiating leaf primordia and in the adaxial region of developing leaves (data not shown), but no signal was detected in the vascular tissues or in the center of the SAM. The PHB expression pattern was also altered in jba-1D seedlings. Wild-type seedlings express PHB in the adaxial region of the leaf primordia, in the vascular tissues and in the SAM, mostly in the overlying layers (Fig. 8E). In jba-1D seedlings, PHB was expressed at only a very low level in the adaxial region of initiating leaf primordia on the flanks of the SAM and in the vascular tissues, but no transcripts are detected in the SAM itself. These results are
consistent with the RT-PCR data and demonstrate that overexpression of miR166g has differential effects on the transcript levels and tissue distributions of its AtHD-ZIP target genes.

Discussion

A key role for miRNAs in plant development is inferred from the phenotypes of dcl1 and ago1 mutants, and from the overexpression or mutation of a number of miRNA loci. Here, we show that overexpression of miR166g in jba-1D mutants causes morphological defects in shoot apical meristems, stem vasculature, rosette leaves and gynoecia. Our experiments reveal a dynamic embryonic expression pattern of miR166, and a complex regulatory pathway controlling class III HD-ZIP gene transcription during development. They also identify an indirect role for miR166g in controlling WUS transcription and shoot meristem activity via regulation of class III HD-ZIP gene expression.

Over-expression of miR166g causes severe developmental defects

Several independent lines of evidence demonstrate that the jba-1D phenotypes are caused by over-expression of miR166g. First, miR166 transcripts are elevated in jba-1D seedlings and inflorescences, and this increase in transcript level occurs in a dose-dependent manner that corresponds with the increase in phenotype severity. Second, a 543 bp genomic region from the miR166 locus, but lacking coding sequences for any of the surrounding genes, is sufficient to recapitulate all aspects of the jba phenotype when introduced into wild-type Col plants. Third, DCL1 function is required to obtain the jba-1D phenotype when introduced into wild-type Col plants. Third, DCL1 function is required to obtain the jba-1D phenotype when introduced into wild-type Col plants. Third, DCL1 function is required to obtain the jba-1D phenotype when introduced into wild-type Col plants. Third, DCL1 function is required to obtain the jba-1D phenotype when introduced into wild-type Col plants. Finally, miRNA expression levels of the targets of miR166g, the five members of the class III HD-ZIP family of transcription factors, are altered in jba-1D plants.

Over-expression of miR166g in jba-1D plants causes specific developmental defects. The earliest detectable phenotype is an increase in jba-1D mature embryo SAM size, indicating that the regulatory activity of miR166 is already required during embryogenesis. The jba-1D SAM continues to enlarge throughout the vegetative and inflorescence phases, culminating in the splitting of the primary shoot apex into multiple independent SAMs and the fasciation of the inflorescence meristems. Meristem enlargement in jba-1D plants occurs through the coordinate expansion of CLV3-expressing cells in the overlying layers of the meristem and WUS-expressing cells in the interior, and requires wild-type WUS activity. The fasciated meristems of clv mutants also display coordinate expansion of CLV3-expressing and WUS-expressing cells, and are WUS dependent (Brand et al., 2000; Schoof et al., 2000). However, the fasciated meristems of clv plants and those of jba-1D plants are generated by different molecular mechanisms. The CLV pathway restricts the size of the WUS-expressing cell population by preventing its expansion laterally and upward into the L2 cell layer (Brand et al., 2000; Schoof et al., 2000). In contrast, miR166 appears to regulate meristem size by indirectly controlling the amount of WUS transcription within the organizing center itself. Thus miR166 and the CLV pathway function in parallel to regulate the level of WUS transcription and the number of WUS-expressing cells in vegetative and inflorescence meristems, respectively.

The jba-1D mutation also causes the adaxialization of rosette leaves and vascular bundles in the stem, and a reduction in gynoecium tissue. Given that PHB and PHV confer adaxial identity (Emery et al., 2003; McConnell and Barton, 1998), the adaxialization of jba-1D leaves seems counterintuitive. However, experiments have shown that a SAM-derived signal(s) is important for specifying adaxial leaf identity (Snow and Snow, 1959; Sussex, 1954; Waites and Hudson, 1995), and thus initiating jba-1D leaves may receive an excess of adaxializing signal(s) emanating from multiple SAMS. Increased accumulation of transcripts from REV, which plays a redundant role with PHB and PHV in conferring adaxial fate (Emery et al., 2003), may also contribute to conditioning this phenotype, as increased meristem size per se is not sufficient to cause leaf adaxialization (Clark et al., 1993). Similarly, the decrease in PHB, PHV and CNA transcript levels observed in jba-1D plants is unlikely to be the sole cause of the reduced gynoecium phenotype, as phb phv cna mutants form extra carpels instead of fewer carpels (Prigge et al., 2005). jba-1D floral meristems are the same size as wild-type meristems, and thus the gynoecium defect is probably not due to premature floral meristem termination. jba-1D gynoecia consist of fewer cell types than normal and occasionally lack vasculature (data not shown), suggesting instead a possible defect in gynoecium patterning and/or polarity. Further work will be required to unravel the precise roles of miR166 and the class III HD-ZIP genes in gynoecium development.

miR166 expression and regulation of the class III HD-ZIP genes

Our experiments show that over-expression of miR166g in activation-tagged jba-1D plants causes significant changes in the mRNA accumulation of their target class III HD-ZIP family members. We find that the overall transcription levels of the HD-ZIP genes are affected in jba-1D seedlings and inflorescences, but that the five target genes do not respond to miR166g over-expression in the same way. ATHB8 transcript levels are unaffected by miR166g over-expression, suggesting that ATHB8 may be targeted by other members of the miR165/166 group, such as miR166a (Kim et al., 2005). PHB, PHV and CNA are all down-regulated in jba-1D seedlings and inflorescence meristems, while REV transcription is elevated. Since REV shows a similar expression pattern in wild-type and jba-1-D seedlings (Fig. 8) and inflorescence meristems (data not shown), an explanation for the unexpected up-regulation of REV may be that REV is a target of negative regulation by PHB, PHV and/or CNA. This theory is consistent with previous observations that CNA and ATHB8 partially suppress the rev and rev phv lateral and floral meristem defects, indicating an antagonistic relationship between them (Prigge et al., 2005).

Interestingly, we found that miR166g has a dynamic expression pattern in developing wild-type and jba-1D embryos. During the early stages of embryogenesis, miR166 accumulates predominantly in the peripheral regions of the hypocotyls and throughout the developing cotyledons. The hypocotyl expression pattern of miR166 is reciprocal to those of its five target genes, which are all expressed in overlapping patterns but are largely restricted to the central cells (Emery et al., 2003; Prigge et al., 2005). These data suggest that during

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early embryogenesis miR166 acts to clear the transcripts of its
target gene(s) from the periphery of the developing hypocotyl.
In contrast, the expression patterns of miR166 and REV, PHB,
PHV and CNA overlap in the developing cotyledons, indicating
that the presence of the miRNA does not cause complete
turnover of its target transcripts in these tissues. In mature
embryos the miR166 expression pattern alters dramatically,
becoming confined to the SAM, the adaxial side of the
cotyledons, and the vasculature. At this stage of development
the expression pattern of miR166 is coincident with that of its
targets [REV in the SAM; REV, PHB and PHV in the
cotyledons and all five genes in the vasculature (Prigge et al.,
2005)] suggesting that a major effect of miR166 expression at
this stage may be to modulate the mRNA transcript levels of
the class III HD-ZIP genes. The Arabidopsis class III HD-ZIP
genes may thus represent examples of so-called miRNA
‘tuning targets’, messages for which miRNA regulation adjusts
the protein output in a fashion that permits customized
expression in different cell types yet a more uniform
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genes may thus represent examples of so-called miRNA
‘tuning targets’, messages for which miRNA regulation adjusts
the protein output in a fashion that permits customized
expression in different cell types yet a more uniform
expression level within each cell type (Bartel and Chen, 2004).
Alternatively, or additionally, the overlap of the miRNA and
and target mRNA expression patterns may suggest that the HD-ZIP
genes are at this stage of development either targeted for
translation repression or for methylation of their coding
regions, as has been previously shown for the PHB and PHV
loci (Bao et al., 2004).

**Regulation of shoot apical meristem activity by class III HD-ZIP genes**

The most dramatic phenotype caused by over-expression of
miR166g is the extensive SAM enlargement and stem
fasciation. Previous work has implicated members of the class
III HD-ZIP family in meristem regulation. rev phb phv mutants
lack a functional embryonic SAM (Emery et al., 2003), indicating
that REV plays a redundant role with PHB and PHV in
SAM establishment. rev mutants have reduced lateral and
floral meristem activity (Otsuga et al., 2001; Talbert et al.,
1995), indicating that REV also promotes post-embryonic
meristem initiation and function. REV gain-of-function
mutations avb1 and rev-10D lead to SAM enlargement,
deinfecte leaf polarity and altered vascular patterning (Emery et al.,
2003; Zhong and Ye, 2004). These phenotypes resemble
those observed in jba-1D plants, which accumulate higher than
normal levels of REV transcripts. However, eliminating REV
activity in a jba-1D background did not significantly attenuate
the jba shoot meristem phenotypes. This result indicates that
REV plays at most a minor role in conditioning the SAM
enlargement and stem fasciation defects caused by over-
expression of miR166g.

The shoot apical meristem phenotypes of jba-1D plants are
also very similar to those of phb phv cna mutants (Prigge et al.,
2005). Like jba-1D mutants, phb phv cna mutants produce
enlarged SAMs, fasciated meristems and internal vascular
bundles in the stem. The redundant role of PHB, PHV and CNA
in meristem regulation is independent of REV (Prigge et al.,
2005), as is the role of miR166g (see Fig. S2 in supplementary
material). Although cna mutations have no discernable effect
on their own they enhance the stem cell accumulation defects of
clv null mutant meristems, suggesting that CNA acts in
parallel with the CLV pathway to regulate shoot apical
meristem size (Green et al., 2005).

Our data confirm that proper regulation of class III HD-ZIP
gene activity is a critical feature of normal shoot apical
meristem function. We propose that in wild-type plants, PHB,
PHV and CNA restrict SAM activity by down-regulating WUS
transcription. During the late globular and heart stages of
embryogenesis, PHB, PHV and CNA are all expressed in apical
cells of the presumptive SAM (Prigge et al., 2005), in a pattern
coincident with that of WUS (Mayer et al., 1998). After
germination, PHB and CNA continue to be expressed in the
meristem (Fig. 8) (McConnell et al., 2001), the CNA expression
domain remaining coincident with that of WUS. Thus the transient expression of PHB, PHV and CNA early in
embryogenesis, and the persistent expression of PHB and CNA
in post-embryonic development, may be required to modulate
the level of WUS transcription, leading to the maintenance of
a stem cell population of the appropriate size. In jba-1D SAMs,
over-expression of miR166g would cause a reduction in the
level of PHB, PHV and CNA mRNAs and a resultant elevation
of WUS transcription. Abnormally high levels of WUS activity
could then promote excess stem cell accumulation and the
eventual establishment of new stem cell foci, leading to SAM
enlargement and ultimately to the splitting and fasciation of the
jba-1D shoot apical meristem. We also find that this activity of
PHB, PHV and CNA in regulating SAM size is largely
independent of REV, consistent with data from analysis of HD-
ZIP triple and quadruple mutant plants (Prigge et al., 2005).

Nine MIR165/166 loci are present in the Arabidopsis
genome, each of which has the potential to regulate any or all
of its five class III HD-ZIP gene targets either by clearing the
transcripts from specific cells or by regulating the level of
transcript accumulation. To account for the observed
expression of the HD-ZIP genes in combinatorial tissue- and
stage-specific patterns, it seems reasonable to expect that the
nine MIR loci will also have dynamic and differential
transcription profiles. Further examination of the regulatory
interactions between the various MIR165/166 family members,
their class III HD-ZIP target genes, and meristem maintenance
factors will reveal additional insights into the complex
interplay between polar lateral organs and the shoot apical
meristem from which they derive.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/16/3657/DC1

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