The Plant Cell, Vol. 14, 3029–3042, December 2002, www.plantcell.org © 2002 American Society of Plant Biologists

Regulation of Axis Determinacy by the Arabidopsis *PINHEAD* Gene

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Plants produce proximal-distal growth axes with two types of growth potential: they can be indeterminate, in which case growth continues indefinitely, or they can be determinate, in which case growth is limited to the production of a single organ or a discrete set of organs. The indeterminate shoot axes of Arabidopsis *pinhead/zwille* mutants frequently are transformed to a determinate state. *PINHEAD* (*PNH*) is expressed in the central domain of the developing plant: the provascular tissue, the shoot apical meristem, and the adaxial (upper) sides of lateral organ primordia. Here, we show that ectopic expression of *PNH* on the abaxial (lower) sides of lateral organs results in upward curling of leaf blades. This phenotype correlates with a loss of cell number coordination between the two surfaces of the blade, indicating that ectopic PNH can cause changes in cell division rates. More strikingly, moving *PNH* expression from the central to the peripheral domain of the embryo causes transformation of the determinate cotyledon axis to an indeterminate state. We propose that growth axes are specified as determinate versus indeterminate in a *PNH*-mediated step. Our results add to a growing body of evidence that radial positional information is important in meristem formation. These results also indicate that genes regulating cell division and axis determinacy are likely to be among PNH targets.

INTRODUCTION

Numerous proximal-distal growth axes are found in shoot systems of angiosperm plants (Figure 1A). These include that of the main shoot, each lateral branch, each cotyledon, leaf, floral organ, and so on. Perpendicular to each proximal-distal axis is the radial axis, which runs in the central to peripheral direction (for cylindrical structures) or in the adaxial to abaxial direction (for planar structures; Figure 1B). Proximal-distal axes of plants can exhibit either indeterminate growth, resulting in the formation of an unlimited number of organs, or determinate growth, resulting in the formation of a single organ or a discrete set of organs. In flowering plants, the terms "determinate" and "indeterminate" frequently are applied to inflorescence architectural features characterized by terminal or nonterminal flowers, respectively. Here, we use the terms in their broader developmental sense to indicate growth potential of any proximal-distal axis (not just the inflorescence).

A key requirement of indeterminate growth potential for a shoot axis is the presence of a shoot apical meristem (SAM) at the distal end. The SAM is equipped specifically to give rise to organ primordia on its lateral flanks while maintaining a reservoir of self-replacing totipotent cells at its center. Several mutants have been isolated or engineered that lack or have a nonfunctional SAM. These include *shoot meristemless* (*stm*; Barton and Poethig, 1993), *cup-shaped cotyledon1* and *-2* double mutants (*cuc*; Aida et al., 1997), *wuschel* (*wus*; Laux et al., 1996), *pinhead/zwille* (*pnh*; McConnell and Barton, 1995; Moussian et al., 1998), and plants overexpressing *YABBY3*, *FILAMENTOUS FLOWER* (*FIL*; Siegfried et al., 1999), or *KANADI* (*KAN*; Eshed et al., 2001; Kerstetter et al., 2001). The corresponding genes act at several levels in the hierarchy that controls SAM development and function.

At the highest level, genes are responsible for establishing pattern. For instance, the SAM arises from the central, apical region of the globular embryo. Therefore, failure to elaborate central and/or apical identities would be expected to result in failure to form a SAM. Perhaps the best evidence for this is the fact that when the *KAN* gene (a gene normally expressed in a peripheral, or abaxial, domain of the embryo) is expressed throughout the entire embryo, the result is a lack of central, or adaxial, fates, including central vascular cylinder cells and a SAM (Eshed et al., 2001; Kerstetter et al., 2001). Alterations in organ polarity also play a role in postembryonic meristem formation. As evidence of this, adaxialized *phabulosa* dominant mutants form extra axillary meristems on the undersides of their leaves (McConnell and

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.005132.

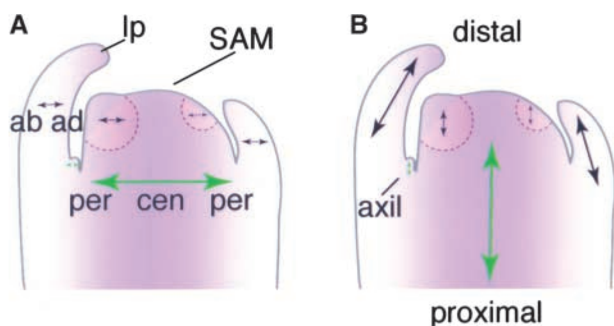

Figure 1. Axes of the Arabidopsis Shoot.

In these cartoon representations of a longitudinal section through a vegetative shoot apex, purple represents central/adaxial positional information and arrows represent axes.

(A) Central-peripheral and adaxial-abaxial organization. The SAM is organized into central and peripheral zones. It produces new leaf primordia sequentially from its periphery. These leaf primordia have adaxial and abaxial zones that correspond to positions in the central-peripheral dimension of the meristem. ab, abaxial; ad, adaxial; cen, central; lp, leaf primordium; per, peripheral.

(B) Proximal-distal axes of the growing shoot. Arrows represent axes of growth in the proximal-distal (to the root-shoot junction) dimension. Through the action of the SAM, the main shoot axis (large green arrow), which is indeterminate, gives rise to the new growth axes of the leaf primordia (black arrows), which are determinate. Note that although the leaf will eventually be oriented nearly perpendicular to the long axis of the plant, its orientation is parallel during its primordial stages. New indeterminate axes (small green arrow) arise in the axils of the leaves with the production of an axillary meristem.

Barton, 1998), whereas the likely abaxialized *revoluta* mutants fail to form axillary meristems (Talbert et al., 1995; Otsuga et al., 2001).

The *CUC* gene also seems to act at the level of pattern formation, specifically in directing the transition from radial symmetry to bilateral symmetry. *cuc* mutants are largely radially symmetrical, with a single, cup-shaped cotyledon and no SAM (Aida et al., 1997).

Downstream of such pattern formation functions are genes that dictate cell fates within the embryo or the SAM. For instance, the *STM* gene product (a homeodomain-containing protein) is required for cells to act as meristem cells. In the absence of *STM*, these cells adopt fully differentiated fates according to their positions (Barton and Poethig, 1993; Long et al., 1996).

Finally, genes required for cell division and its regulation are essential for SAM function. Such genes may act globally (Cockcroft et al., 2000; De Veylder et al., 2001), whereas the action of others may be more specific to the meristem. The *CLAVATA* (*CLV*) and *WUS* genes define a pathway that has as its principal role the regulation of growth of stem cells within the meristem. In the absence of the CLV1 receptor or its ligand, the *CLV3* gene product, these stem cells grow excessively (Clark et al., 1993, 1995, 1997; Fletcher et al., 1999), whereas in the absence of *WUS*, a homeodomain-containing protein, they cease dividing prematurely (Laux et al., 1996; Mayer et al., 1998).

Similar to *KAN* overexpressors and *cuc*, *stm*, and *wus* mutants, *pnh* mutants frequently lack a functional SAM. Although a SAM is formed during embryogenesis in *pnh* mutants, the indeterminate shoot axis acts like a determinate axis; these mutants produce only one organ from the first SAM. *pnh* mutants also exhibit a defect in axillary meristem formation, which is the mechanism by which plants create new indeterminate growth axes after embryogenesis. In addition, *pnh* mutants make shorter siliques and narrower leaves and petals than wild-type plants and show abnormalities in floral organ number (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999). (*pnh* mutants almost always go on to flower and set seed as a result of the formation of buds at the cotyledon bases.)

Collectively, the *PNH* expression domain comprises the central and adaxial cells of developing tissues. *PNH* mRNA is found at moderate levels throughout the SAM and in the adaxial domains of organ primordia (Lynn et al., 1999). This is consistent with the defects in meristem and organ development. *PNH* mRNA is found at higher levels in the precursors to the vasculature, especially the phloem precursors (Moussian et al., 1998; Lynn et al., 1999). Despite this fact, defects in vascular development have not been seen in *pnh* mutants (Moussian et al., 1998; Lynn et al., 1999).

Recently, Nishimura et al. (2002) described transgenic rice plants expressing an RNA antisense to a rice *PNH* homolog (*OsPNH1*). Similar to *pnh* mutants, these plants had abnormal meristems with reduced KNOX gene expression. In addition, the plants showed significant abnormalities in leaf development with altered vascular arrangement, suggesting that *OsPNH1* may play a more significant role in the rice leaf than *PNH* does in the Arabidopsis leaf.

PNH is a member of a small gene family in Arabidopsis whose founding member is the *ARGONAUTE* (*AGO*) gene (Bohmert et al., 1998). Genetic analysis has shown that *PNH* and *AGO*, which, in contrast to *PNH*, is expressed ubiquitously, are partially redundant genes (Lynn et al., 1999). Mutations in *AGO* confer a meristem determinacy defect similar to that of *PNH* mutations (Lynn et al., 1999). However, *ago* mutants have many additional defects, including an inability to silence genes post-transcriptionally (Fagard et al., 2000). In other species, several genes similar to *AGO*/*PNH* have been described. These genes are involved in processes such as post-transcriptional gene silencing (Tabara et al., 1999; Catalanotto et al., 2000), translational regulation (Wilson et al., 1996; Zou et al., 1998; Grishok et al., 2001), and germline stem cell development (Cox et al., 1998; Reinke et al., 2000), implicating this gene family in the regulation of developmental processes, perhaps through the post-transcriptional regulation of target genes.

The *Caenorhabditis elegans agl-1* and *agl-2* genes encode *AGO*/*PNH*-like products required for the accumulation

of short RNAs that negatively regulate the translation of genes involved in developmental timing (Grishok et al., 2001). A physical association between such short RNAs and *PNH/AGO*-like gene products has been seen in rabbit reticulocytes, in which the *AGO/PNH*-like gene product eIF2C2 has been shown to exist in 15S ribonucleoproteins that also include a helicase and micro-RNAs (Mourelatos et al., 2002).

The loss-of-function *pnh* phenotype is consistent with a role for the *PNH* gene at several levels in the hierarchy of developmental events in the shoot. It may be involved in pattern formation and/or in the more limited roles of cell division control or the promotion of the undifferentiated state. To better understand the level at which *PNH* functions in the shoot, we investigated *PNH* using gain-of-function analysis. Our results support a role for *PNH* at several levels in the developmental hierarchy of shoot development. Expanding the expression of *PNH* outside of its normal domain resulted in a cell division control defect that manifested as upward curling of leaf margins. A more dramatic result was found when *PNH* was moved from its normal domain to a more peripheral domain: such mutants show a conversion of the determinate cotyledon axis to an indeterminate state. Together with the loss-of-function phenotype, this result suggests that *PNH* regulates the indeterminacy of the proximal-distal shoot axes. Furthermore, this finding highlights the importance of radial positional information in the overall architectural development of the plant body and shows that *PNH* acts largely cell nonautonomously.

RESULTS

Placing *PNH* under the Control of the 5′ Region of the *FIL* Gene Causes Its Abaxial Expression

In wild-type plants, high levels of *PNH* mRNA are observed in developing vascular tissue (Moussian et al., 1998; Lynn et al., 1999), and low levels are seen throughout the SAM and on the adaxial, but not abaxial, sides of lateral organ primordia (Figures 2A to 2D) (Lynn et al., 1999). In effect, *PNH* is expressed throughout a central domain of the developing regions of the plant body. The expression of *PNH* was expanded by constructing a *FIL*::*PNH* transgene in which the 5′ sequences of the *FIL* gene were fused to a *PNH* cDNA (Figure 3). The *FIL* gene is expressed on the abaxial sides of developing lateral organ primordia (Sawa et al., 1999; Siegfried et al., 1999), in an apparently complementary fashion to *PNH*. *FIL* expression in leaves is rapidly limited to marginal domains and disappears as the leaf differentiates (Siegfried et al., 1999).

To verify the new expression domain, in situ hybridization experiments were performed on *PNH; FIL*::*PNH* transgenic plants using a *PNH* antisense probe. In addition to the wild-type expression domain, transformants expressed *PNH* on the abaxial sides of cotyledons and near the margins on the abaxial sides of true leaves at levels resembling those on the adaxial sides (Figures 2E to 2H, arrows). To assess which part of the expanded *PNH* domain was derived from the *FIL*::*PNH* transgene, in situ hybridization using an antisense β-glucuronidase (*GUS*) probe was performed on *FIL*::*GUS* transgenic plants (Figure 3). These experiments confirmed that the *FIL* sequences used in this study drive expression in the abaxial cells of lateral organs (Figures 2I to 2L), which are the same cells in which *FIL* mRNA was detected under our conditions (data not shown). Note that GUS RNA persisted in medial abaxial portions of the leaf primordia longer than either endogenous FIL RNA (Siegfried et al., 1999) or PNH RNA expressed from the FIL promoter. This is most likely the result of the increased stability of the GUS RNA relative to the PNH and FIL RNAs.

Abaxial Expression of *PNH* Causes Upward Curling of Lateral Organs

Of 21 independent *PNH; FIL*::*PNH* lines examined, 2 gave rise to plants resembling the wild type, 3 gave rise to plants resembling *pnh* mutants, and 16 gave rise to plants with curled leaves, described below. Lines appearing wild type likely resulted from transgene silencing, whereas those resembling *pnh* mutants likely resulted from cosuppression of the endogenous *PNH* gene.

As a negative control, 130 independently derived lines expressing the *FIL*::*PNH-FRAMESHIFT* (*FS*) transgene were examined (Figure 3). This transgene is identical to *FIL*::*PNH* except for a single nucleotide deletion in the coding sequence that creates a premature stop codon. When this sequence was expressed in *Escherichia coli*, a truncated protein was produced (data not shown). Thirty-three lines resembled *pnh* mutants and likely resulted from the cosuppression of *PNH*. The remaining 97 lines resembled the wild type. Thus, ectopic expression of the wild-type *PNH* gene product is required for the curling phenotype.

The *PNH; FIL*::*PNH* plants exhibited upwardly curled blades in rosette leaves (Figures 4A to 4C), cauline leaves (Figures 4I to 4L), petals, and sepals (Figures 4M and 4N), giving them an involute appearance compared with the wild type. Other lateral organs, such as cotyledons, stamens, and carpels, were normal.

Blade curling results from a differential between the amount of growth on the adaxial and abaxial sides of the leaf blade. This growth can be caused by cell expansion, cell division, or both. To investigate the mechanism of blade curling in *PNH; FIL*::*PNH* plants, the ratios of adaxial to abaxial epidermal cell numbers were compared in curled and wild-type rosette leaves. We expected that if abnormal cell expansion caused curling, the ratio of adaxial to abaxial cells would be the same in *PNH; FIL*::*PNH* and wild-type blades; if abnormal cell division rates caused curling, we expected this ratio to change. Cells from the youngest curled

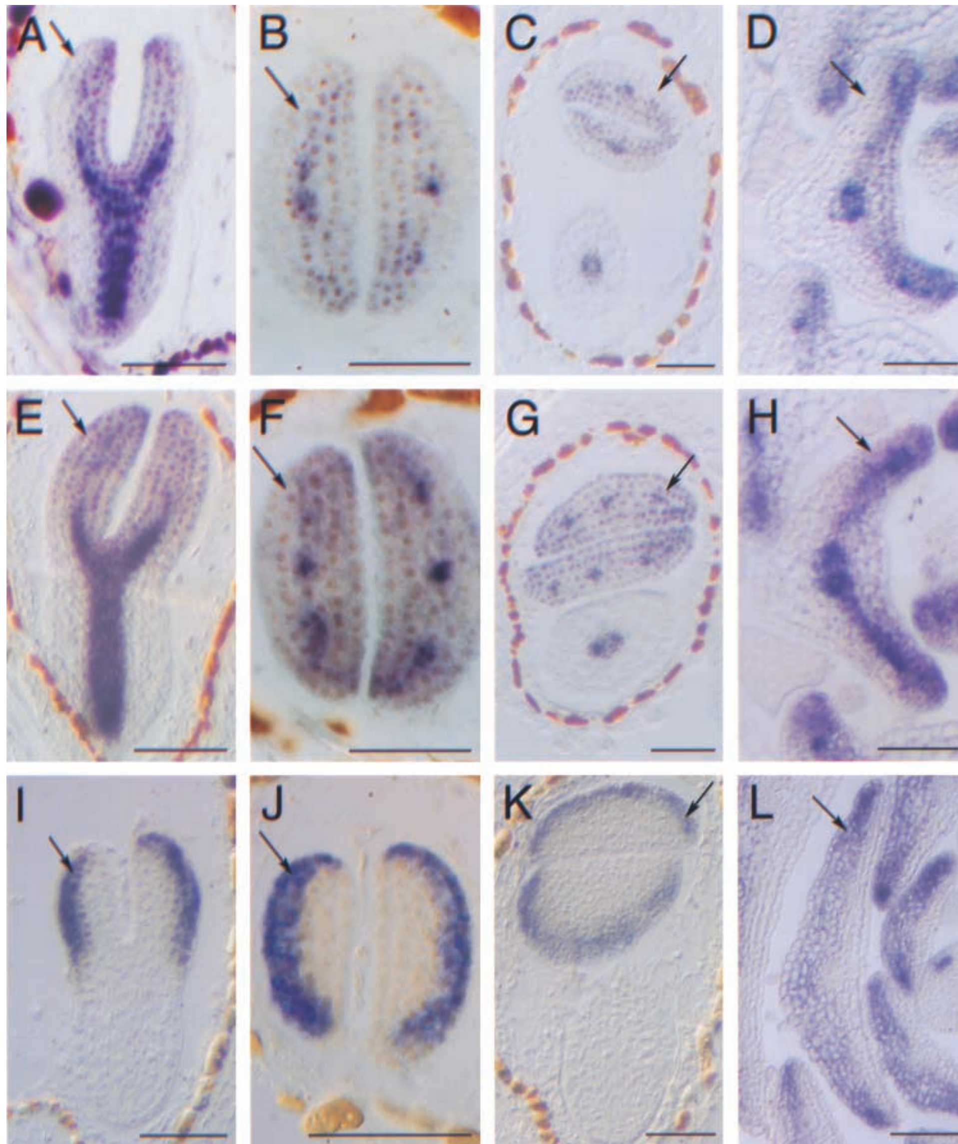

Figure 2. Expression Analysis of Transgenic Plants.

(A) to **(D)** *PNH* antisense RNA probe on wild-type plants. *PNH* RNA is undetectable in abaxial cells of cotyledon (**[A]** to **[C]**) and leaf primordia **(D)** (arrows).

(E) to **(H)** *PNH* antisense RNA probe on *PNH; FIL*::*PNH* transgenic plants. *PNH; FIL*::*PNH* plants express *PNH* mRNA in the wild-type domain and in abaxial cotyledon primordia cells (**[E]** to **[G]**) and in abaxial cells near the margin in leaf primordia **(H)** (arrows).

(I) to **(L)** *GUS* antisense probe on *FIL*::*GUS* transgenic plants. The activity of the *FIL* 5′ regulatory sequences used in this analysis is limited to the abaxial cotyledon cells in the embryo (**[I]** to **[K]**) and to the abaxial cells in the leaf primordia **(L)** (arrows).

Bars = 50 μm.

PNH; FIL::*PNH* leaves (plastochron 8 [P8] to P10) and similarly staged wild-type leaves were counted using transverse sections of shoot apices (Figures 4E to 4H). The ratio of adaxial to abaxial epidermal blade cells changed from an average of 1.01 ± 0.04 (SE) with a range of 0.9 to 1.11 in wild-type blades to 0.63 ± 0.0095 (SE) with a range of 0.60 to 0.66 in curled *PNH; FIL*::*PNH* blades (n = 5 blades for each genotype). Thus, ectopically expressing *PNH* in the abaxial leaf domain either causes an excess of cell divisions in this domain or represses cell division on the opposite, adaxial, side of the leaf.

The latter possibility seems less likely because plants ex-

pressing *PNH* in both leaf domains from the constitutive 35S promoter of *Cauliflower mosaic virus* did not show a similar degree of leaf curling. Plants carrying the *35S*::*PNH* transgene had round leaf blades with gently ruffled surfaces (Figure 4O). Occasionally, the margins of the leaves curled up, but not to the same extent seen with *FIL*::*PNH*. In addition, the leaves frequently were held upright, rather than lying flat against the soil. Sepals and petals resembled those of *PNH; FIL*::*PNH* flowers, and carpels often were bumpy in appearance (data not shown). Finally, the leaves of *PNH; FIL*::*PNH* plants seemed to be the same width as wild-type leaves rather than narrower, as might have been expected if the transgene repressed cell division.

The involute morphology affected leaves from the early stages of blade outgrowth, at approximately the P8 or P9 stage, with the P1 leaf being defined as the youngest primordium that is morphologically distinguishable on the flanks of the SAM. The curling phenotype was seen until just before full expansion of the leaf. At this point, the blades corrected the curling until they nearly resembled wild-type blades (Figures 4I to 4L). This correction likely is permitted by a cessation of ectopic *PNH* expression in maturing leaves, because plants expressing a *FIL*::*GUS* transgene showed *GUS* activity in young but not maturing leaves (Figure 4D).

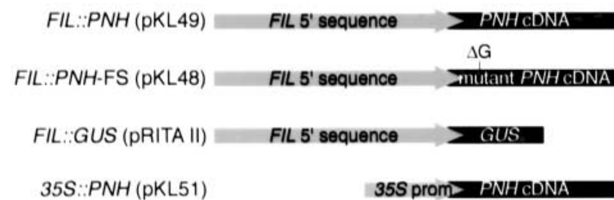

Figure 3. Transgenes.

The 5′ regulatory sequences of the *FIL* gene were fused to a *PNH* cDNA, a mutant *PNH* cDNA, or the marker gene *GUS*. The 35S promoter of *Cauliflower mosaic virus* was fused to a *PNH* cDNA. See Methods for construct details.

pnh; FIL::*PNH* Plants Make Double Cotyledons

We next investigated the effects of moving *PNH* expression from its natural domain to a new domain. To achieve this, the *FIL*::*PNH* transgene was introduced into *pnh-2* mutant plants. The resulting *pnh; FIL*::*PNH* plants should have a domain of wild-type *PNH* expression that mimics the pattern of *GUS* expression in *FIL*::*GUS* plants (Figures 2I to 2L). However, it was not possible to directly test the wild-type *PNH* expression domain because *pnh-2* mutants produced stable mutant transcripts. Therefore, mutant transcripts detected in in situ hybridization were indistinguishable from wild-type transcripts. However, we tested *FIL* expression in *pnh; FIL*::*PNH* plants and found that it was expressed normally (data not shown).

When the *FIL*::*PNH* transgene was expressed in a *pnh* mutant background, none of the *pnh* defects (SAM and axillary meristem formation, floral organ morphology, and number) was rescued. The inability of abaxial *PNH* to rescue the mutant phenotype shows that normal localization of the *PNH* transcript is critical. Surprisingly, unlike *PNH; FIL*::*PNH* leaves, *pnh; FIL*::*PNH* leaves did not curl. Thus, expression of *PNH* in its normal domain is essential for the transgene to cause leaf curling.

pnh; FIL::*PNH* individuals germinated and grew much more slowly than wild-type individuals. In addition, a novel phenotype was seen in *pnh; FIL*::*PNH* cotyledons. The novel phenotype consisted of cotyledon primordia splitting at various points in development to form fused or unfused “double” cotyledons (Figure 5). This phenotype segregated in four of four independent lines transformed with the *FIL*::*PNH* transgene (many other T1 individuals died before setting seed). This phenotype also was seen when the transgene was transformed into wild-type plants and crossed into a *pnh* background. When the *FIL*::*PNH-FS* negative control construct was transformed into homozygous *pnh-2* plants, none of the 96 T1 generation plants had the novel phenotype. Furthermore, none of the hygromycin-resistant T2 progeny from 11 of 11 independent lines examined showed the novel cotyledon phenotype (Table 1). These data verify that the novel phenotype is conferred by the ectopic expression of PNH.

We classified double cotyledons according to degree of separation. For example, cotyledons in class A were completely normal, and those in class F were separated completely into two cotyledons (Figure 5G). Eighty-seven percent of hygromycin-resistant seedlings ($n = 530$ seedlings) showed full or partial doubling of at least one cotyledon (classes B to F). Of the double cotyledons, 80.8% were fused (classes B to E); the remainder were completely separate (class F) ($n = 604$ half-seedlings).

The cotyledons of each seedling were arranged in two opposite groups, just as a wild-type seedling arranges its two cotyledons. For example, individuals with three separate cotyledons (one class-A and one class-F cotyledon) usually held two smaller cotyledons in close proximity, opposite to a larger third cotyledon. We observed no cotyledons that split into unequal parts, nor did we observe multiple sinuses.

To observe the early stages of cotyledon doubling, *pnh; FIL*::*PNH* embryos were examined. Early and late heart-stage *pnh; FIL*::*PNH* embryos were very wide at the top, and their cotyledons were abnormally splayed compared with the wild type (Figures 6A, 6B, 6E, and 6F). By the early torpedo stage, evidence of cotyledon doubling emerged as a flattening (Figure 6G) or indentation (Figure 6I) of the distal tip of the cotyledon primordium compared with the wild type (Figure 6D).

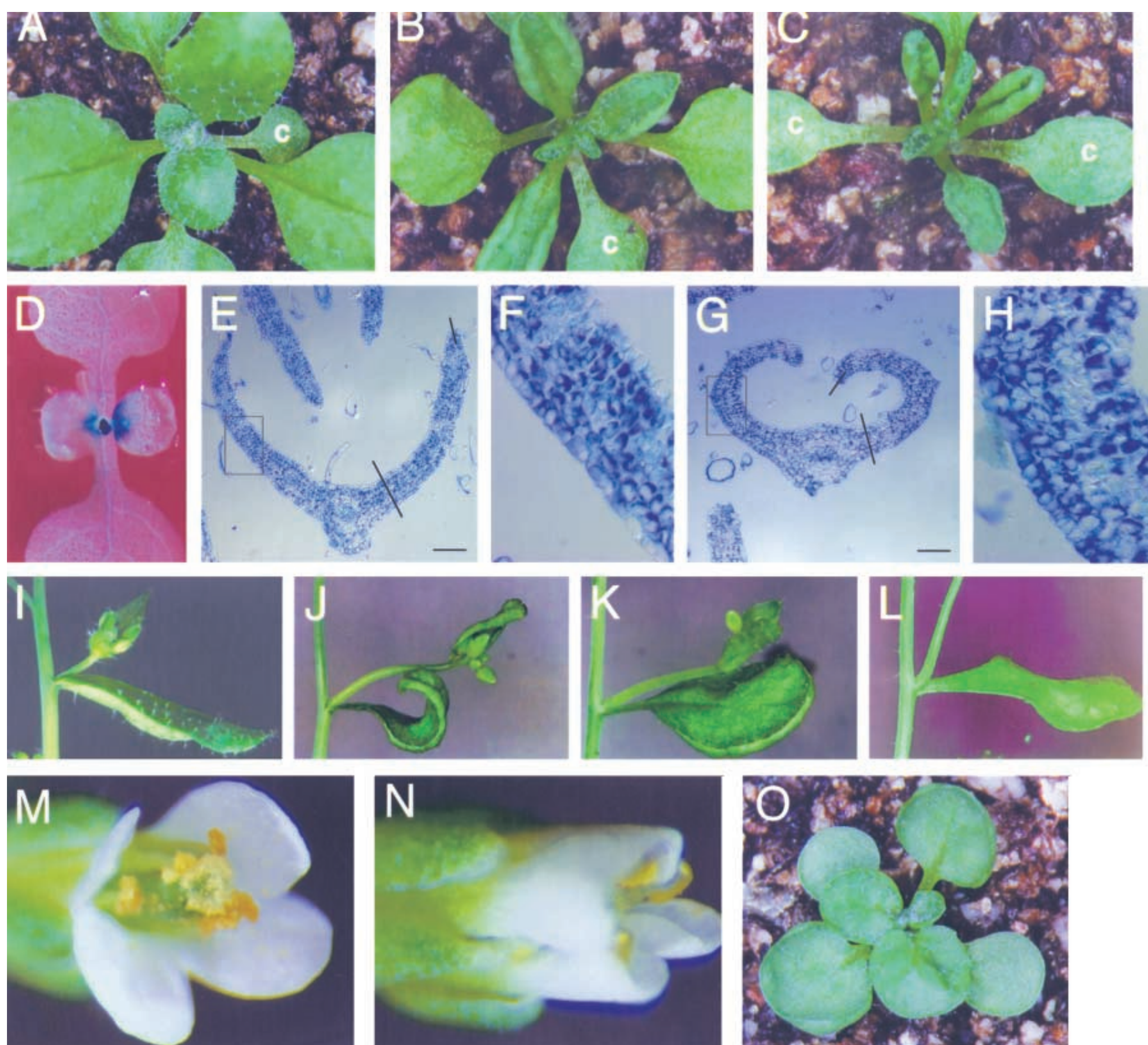

Figure 4. Phenotype of *PNH; FIL*::*PNH* Transformants.

(A) to **(C)** Rosettes from wild-type **(A)** and mildly **(B)** and severely **(C)** affected *PNH; FIL*::*PNH* transgenic plants. *PNH; FIL*::*PNH* rosette leaves are involute, whereas wild-type rosette leaves are slightly revolute. c, cotyledon.

(D) *FIL*::*GUS* plant stained for GUS activity. Young leaves express GUS on their abaxial side, but expanding leaves lose *GUS* expression in a basipetal fashion.

(E) and **(G)** Toluidine blue–stained paraffin sections of wild-type **(E)** and *PNH; FIL*::*PNH* **(G)** leaves. The surface at top is adaxial. Lines show the locations of blade/midrib and adaxial/abaxial cutoffs. Boxes show regions magnified in **(F)** and **(H)**. Bars = 50 μm.

(F) and **(H)** Higher magnification of leaves in **(E)** and **(G)**, respectively. The epidermal cell sizes are approximately the same in both leaves.

(I) to **(L)** Cauline leaves from wild-type **(I)** and *PNH; FIL*::*PNH* transgenic **(J)** to **(L)** plants. The leaves in **(J)**, **(K)**, and **(L)** are the youngest, middle, and oldest cauline leaves, respectively, from the same plant. Young leaves are curled up at the margins but uncurl as they mature.

(M) and **(N)** Flowers from wild-type **(M)** and *PNH; FIL*::*PNH* transgenic **(N)** plants. Sepals and petals of transgenic plants are involute; however, stamens and carpels are unaffected. Overall floral organ morphology and number are as in the wild type.

(O) *35S*::*PNH* rosette with ruffled leaves.

Together, these observations suggest that each *pnh; FIL*::*PNH* embryo originally initiated two cotyledon primordia, as does the wild type, but many of these primordia proceeded to initiate two additional cotyledon primordia. This is consistent with our further observations of two morphological features of cotyledons: vascular patterning and hydathode formation. Wild-type cotyledons exhibited a stereotypical pattern of vascularization, which was similar in *pnh* mutants (Figures 6J and 6K). Although the patterning of the vasculature was aberrant in continuity and the placement of branch

points with respect to the wild type, the overall pattern was duplicated in double cotyledons rather than widened to service both sides (Figures 6L and 6M). The hydathode is a specialized structure for guttation found at the margins of leaves and cotyledons (Esau, 1977). In both wild-type and *pnh* plants, there was only one hydathode per cotyledon, as demonstrated by staining plants carrying a hydathode::*GUS* marker gene (Figure 6N and data not shown). Double cotyledons had two foci of stain, indicating that this structure had been duplicated (Figures 6O and 6P). We conclude that double cotyledons result from duplication of the cotyledon initiation process before vascular and hydathode patterning.

pnh; FIL::*PNH* Double Cotyledons Produce Ectopic Meristems

In the wild type, the SAM develops between the two cotyledon axes. Similarly, ectopic growths formed between the two halves of 33.0% of fused double cotyledons (classes B to E; $n = 488$ half-seedlings) (Figure 5G). Ectopic growths were found only along the fusion zone, at the distal tip, petiole, blade, or a combination of these sites (Figures 7A to 7D). A greater incidence of ectopic growth formation was seen in cotyledons that had two distinct lobes (classes C to E) than in those with only an indentation (class B). On separate (class F) cotyledons, the fusion zone was in virtually the same location as the normal site of adventitious SAM formation in *pnh* seedlings (Figures 7E and 7F); thus, these two types of SAMs could not be distinguished, and ectopic growth formation was not scored for class-F cotyledons. No ectopic growths were observed on normal (class A) cotyledons.

Ectopic growths fell into two groups: differentiated outgrowths, which were composed of large differentiated cells, and SAMs, which were defined by the production of at least one leaf-like structure such as a regular *pnh* SAM would produce. In 2-week-old seedlings, 56.2% of ectopic growths were SAMs ($n = 73$ half-seedlings), whereas in 3-week-old seedlings, 90.7% of ectopic growths were SAMs ($n = 108$ half-seedlings). Combined with the observation that SAMs often emerged from differentiated outgrowths, these data suggest that differentiated outgrowths typically lead to SAM formation. Ectopic SAMs occasionally were observed to produce full rosettes and inflorescences; ectopic root growth never was observed.

Consistent with the formation of ectopic SAMs on double cotyledons, the activity of the *STM* promoter was seen in double cotyledons. An *STM*::*GUS* transgene that confers GUS activity in the SAM of wild-type seedlings (McConnell and Barton, 1998) drove the expression of GUS activity in the double cotyledon fusion zones of *pnh; FIL*::*PNH* plants (data not shown). These data indicate that ectopic meristem formation likely occurs through the ectopic activation of meristem genes such as *STM*.

pnh FIL::*PNH* Cotyledon Primordia Ectopically Express Genes Required for SAM Initiation in the Embryo

Because the *CUC1* and *CUC2* genes are required for SAM formation and organ separation and are expressed in the region where separation occurs (Aida et al., 1997, 1999), we reasoned that these genes might be involved in the separation of *pnh; FIL*::*PNH* cotyledon primordia into double cotyledons. If this were true, we would expect *CUC1* and *CUC2* to be expressed at the distal tip of *pnh; FIL*::*PNH* cotyledon primordia before doubling. Early in development, the expression of *CUC2* in *pnh; FIL*::*PNH* embryos was confined to the same region as in the wild type (Figure 8A). However, by the late heart stage, when cotyledon primordia are easily distinguishable, expression of this gene also was evident at the distal tip of some cotyledon primordia (Figure 8B). At later stages of embryogenesis, staining was seen in a higher proportion of cotyledon primordia (data not shown).

Similarly, *STM* was expressed only in its normal domain at the early and middle stages of embryogenesis, but it was expressed in some cotyledon primordia by the bent-cotyledon stage (Figures 8C and 8D). Unlike *CUC2* expression, *STM* expression in cotyledon primordia was detected only after they were morphologically distinguishable as double cotyledons. The *CUC2* and *STM* genes normally are expressed only at the shoot apex. That they were expressed in the cotyledon primordia in our experiments reveals that these cotyledon primordia had activated at least part of the program normally reserved for the indeterminate axis that forms during embryogenesis.

DISCUSSION

Through the analysis of ectopic expression phenotypes, we have gained a broader understanding of the role that *PNH* plays in plant development. Ectopic *PNH* activity is able to influence development at what appears to be a late stage in the developmental hierarchy—cell division—and also at a very early stage in the developmental hierarchy—the specification of a growth axis as indeterminate versus determinate. This finding is consistent with the highly pleiotropic nature of the *pnh* loss-of-function phenotype (Lynn et al., 1999).

The ability of ectopic *PNH* to create such substantial changes in the development of the plant when expressed ectopically suggests a regulatory role for *PNH*. The *PNH* gene is involved in the transformation of a normally determinate growth axis into an indeterminate growth axis.

Role of *PNH* in Cell Division Regulation and Leaf Polarity

In the wild type, the ectopic expression of *PNH* in the abaxial domains simply adds a new region to the normal *PNH*

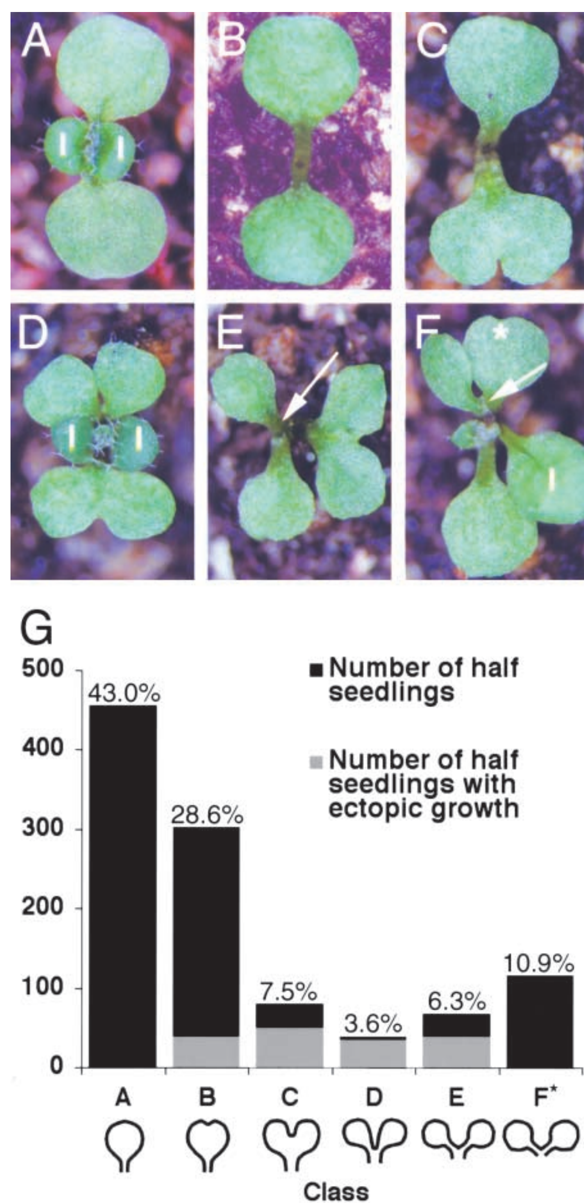

Figure 5. Phenotypes of *pnh-2; FIL*::*PNH* Transformants.

(A) Wild-type seedling showing normal cotyledon shape. l, leaf.
(B) *pnh-2* seedling with normal cotyledon morphology.
(C) to **(F)** *pnh-2; FIL*::*PNH* seedlings displaying the variable cotyledon phenotypes. Note the ectopic meristem formation (arrows). The double cotyledon in **(F)** has indentations in the two new cotyledons, as if a second round of doubling was initiated (star).
(G) Frequency of *pnh-2; FIL*::*PNH* cotyledon phenotypes and ectopic growth formation. Two- or 3-week-old seedlings from three independent transformed lines were examined, with similar results for each experiment except as discussed in the text. Cotyledon phenotypes were divided into six classes as follows: A, normal; B, indentation with most of blade fused (bottom cotyledon in **[C]**); C, notched with most of blade separate (cotyledon at right in **[E]**); D, blades completely separate but petiole fused (top cotyledon in **[D]**); E, petiole partially separate (top cotyledon in **[F]**); F, completely separate petiole and blade (cotyledon at left in **[E]**). Percentages refer to percent of total half-seedlings examined with that phenotype. * Cotyledons in class F could not be scored for ectopic growth formation (see text).

Table 1. Cotyledon Phenotypes in Control Plants

Phenotype	Number of *pnh-2* Plants	Number of *pnh-2; FIL*::*PNH-FS* Plants
Two cotyledons	617	965
Fused cotyledons	3 (class B or C)[a]	0
Three cotyledons	6	9
Four cotyledons	0	1
Total	626	975

[a] Classes are described in Figure 6.

expression pattern. These new abaxial regions of *PNH* expression grow more than the corresponding adaxial regions, resulting in upward curling of the leaf margins. The change in growth appears to be attributable to cell division, rather than expansion. Abaxial overgrowth could be caused by an increase in abaxial cell division or by a decrease in adaxial cell division. We favor a model in which *PNH* promotes abaxial cell division in *PNH; FIL*::*PNH* leaves for two reasons. First, in plants that express *PNH* constitutively from the viral 35S promoter, both surfaces of the leaf appear to grow excessively, resulting in large ruffled leaf blades that are held upright. Second, the repression of adaxial cell division by *PNH* in *PNH; FIL*::*PNH* plants seems less likely because it would entail a cell-nonautonomous effect of abaxial *PNH* on adaxial cells, which already express *PNH* from the natural promoter.

The adaxial nature of *PNH* expression in lateral organs coupled with the abaxialized phenotype seen in some *pnh* organs (Lynn et al., 1999) might indicate that *PNH* plays a role in the polar development of the leaf. Indeed, there is some evidence that the axillary bud is an adaxial character (McConnell and Barton, 1998) that fails to develop in *pnh* plants (Lynn et al., 1999). Also, *pnh ago*/+ mutant petals show some indication of alterations in polarity. However, *PNH; FIL*::*PNH* plants did not show any obvious alterations in polar leaf development: axillary meristems, trichomes, and other polar leaf characters were normal, and *PHABULOSA* and *FIL* were expressed in the adaxial and abaxial cells, respectively, just as in the wild type (data not shown). Thus, in the leaf, ectopic *PNH* function is sufficient to promote only a specific subset of adaxial traits: growth and meristem formation.

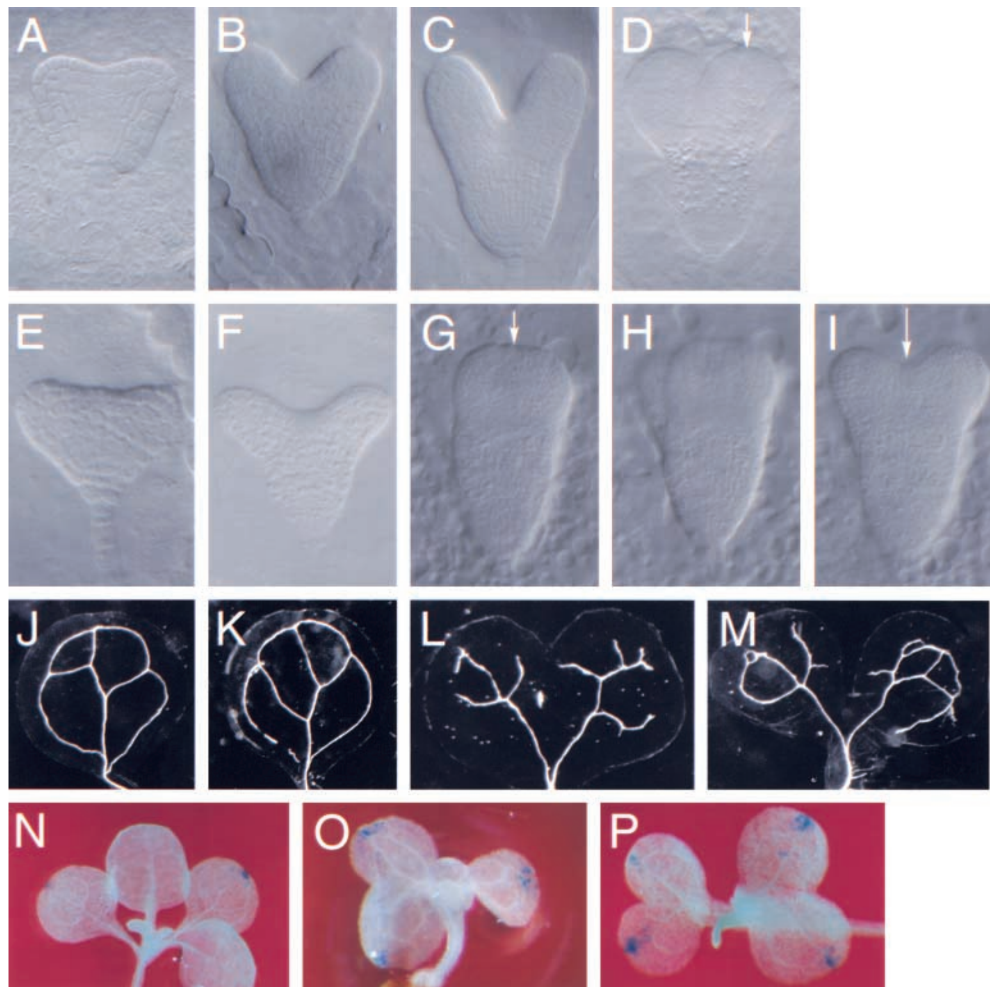

Figure 6. Morphological Analysis of *pnh-2; FIL*::*PNH* Cotyledons.

(A) to **(I)** Cleared embryos from wild type (**[A]** to **[D]**) or *pnh-2; FIL*::*PNH* (**[E]** to **[I]**) plants at the early heart stage (**[A]** and **[E]**), late heart stage (**[B]** and **[F]**), and early torpedo stage (**[C]**, **[D]**, and **[G]** to **[I]**). Torpedo-stage embryos are depicted in serial optical sections of the same embryo. Note the rounded distal tip of the wild-type cotyledon (arrow in **[D]**) compared with the flattened (arrow in **[G]**) or indented (arrow in **[I]**) shape of the *pnh; FIL*::*PNH* cotyledons.

(J) to **(M)** Cleared cotyledons from wild-type **(J)**, *pnh* **(K)**, or *pnh-2; FIL*::*PNH* (**[L]** and **[M]**) seedlings.

(N) to **(P)** GUS-stained seedlings expressing the hydathode::GUS marker in the wild-type **(N)** or *pnh-2; FIL*::*PNH* (**[O]** and **[P]**) genetic background.

PNH Is Necessary and Sufficient for the Indeterminacy of Shoot Axes

In addition to expanding the *PNH* expression domain, we also moved it to a new location. This was done by expressing the *FIL*::*PNH* transgene in *pnh* mutants. In this situation, *pnh* transcript was not expressed in its normal domain—a central region of the embryo—but rather was expressed in the cotyledon primordia. The result of altering the pattern of *PNH* expression in this way is that the cotyledons split into two new cotyledons and, in the most extreme cases, were transformed from determinate to indeterminate growth axes. In these extreme cases, each cotyledon primordium seems to recapitulate the development of the shoot pole of the globular embryo.

The development of the indeterminate *pnh; FIL*::*PNH* cotyledon axis is similar, both molecularly and morphologically, to the development of the indeterminate wild-type embryonic

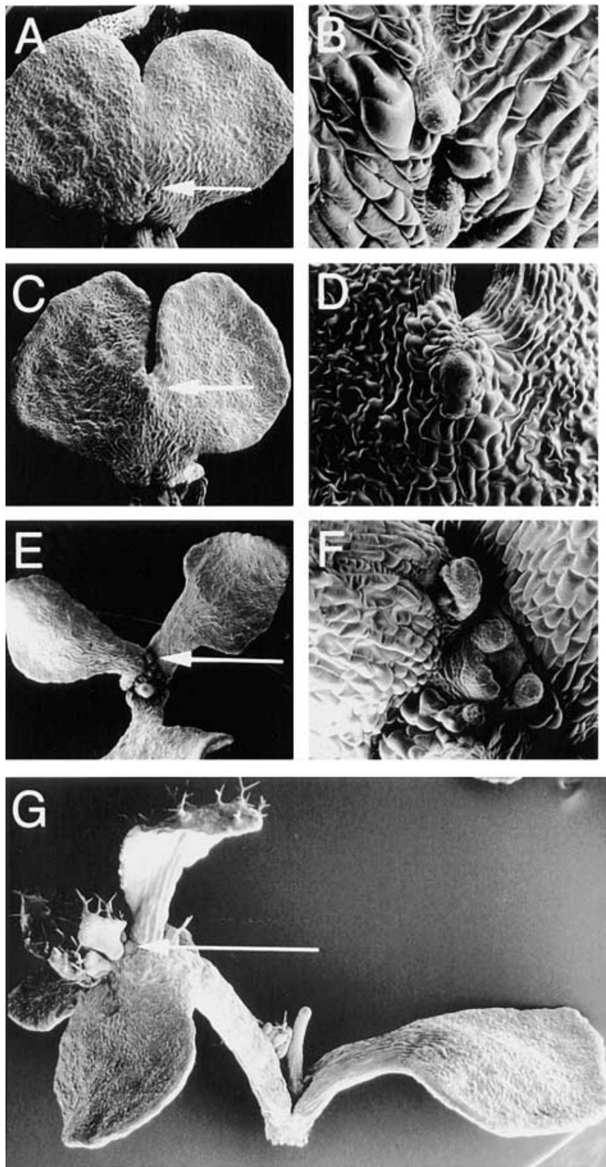

Figure 7. Ectopic Meristem Formation in *pnh-2; FIL*::*PNH* cotyledons.

(A) to **(F)** Scanning electron micrographs of 18-day-old *pnh-2; FIL*::*PNH* seedlings forming ectopic meristems on cotyledons on the blade (**[A]** and **[B]**) or distal tip of the fusion zone (**[C]** to **[F]**). Arrows in **(A)**, **(C)**, and **(E)** indicate areas magnified in **(B)**, **(D)**, and **(F)**, respectively.

(G) A typical *pnh; FIL*::*PNH* seedling at a later stage (∼23 days old). The arrow shows an ectopic meristem developing at the end of one of the cotyledons.

axis (Figure 9). In the wild-type embryo, the formation of the embryonic axis occurs as growth slows at the distal tip at the late globular stage, allowing two outgrowths to form the two cotyledon primordia on either side. This process is mediated by the *CUC* genes, which are expressed at the site where growth will slow at the globular stage of embryogenesis (Aida et al., 1997, 1999; Takada et al., 2001). In this region of slowed growth, a SAM forms in a process that requires the *STM* gene (Barton and Poethig, 1993; Long et al., 1996). This axis is indeterminate; growth will continue from this SAM, and a continuous stream of new organs will arise from this axis.

In a similar manner, growth slows at the distal end of *pnh; FIL*::*PNH* cotyledon primordia, allowing the outgrowth of two lobes. Expression of the *CUC2* gene occurs at this site before the formation of an indentation. Then, a SAM is formed in the region of slowed growth. The *STM* gene is expressed here as it is in the indeterminate axis of the wild type. This axis also is capable of indeterminate growth, as shown by rosettes and inflorescences that can develop from *pnh; FIL*::*PNH* cotyledons.

The following model explains the conversion of the determinate cotyledon axis to an indeterminate axis in *pnh; FIL*::*PNH* plants. In the wild type (Figure 9C), *PNH* expression marks the central domain of the main indeterminate axis of the embryo. At the distal end of this axis is the SAM. Two new axes are specified in the cotyledon primordia; these are determinate organ axes and do not have a SAM at the distal end. In addition, the expression domain of *PNH* in these axes includes central and adaxial regions. In *pnh* embryos (Figure 9D), PNH activity is absent or reduced and the indeterminate axis behaves like a determinate axis, with only one organ at the shoot apex. In *pnh; FIL*::*PNH* embryos (Figure 9E), *PNH* activity is decreased or absent in its normal domain, again causing the indeterminate axis to behave like a determinate axis. However, PNH activity has been added back to the cotyledon cells, and the cotyledon primordia take on the role of the indeterminate axis in these individuals. Thus, PNH appears to be necessary and sufficient for the indeterminacy of proximal-distal shoot axes. Although the FIL promoter directs expression in the abaxial cotyledon domain throughout most of cotyledon development, this promoter may be active throughout the cotyledon primordia in their earliest stages of development (Siegfried et al., 1999). Thus, we do not know whether axis indeterminacy is caused by the expression of *PNH* throughout the early cotyledon primordia or by *PNH* expression limited to the abaxial cotyledon domain.

It is interesting that the *PNH; FIL*::*PNH* plants do not show the double cotyledon and ectopic SAM phenotypes (Figure 9F). Perhaps the main indeterminate axis, which functions normally in *PNH; FIL*::*PNH* plants, has an inhibitory effect on other axes, preventing them from becoming indeterminate. A somewhat related possibility is that a contiguous block of *PNH*-expressing cells can contribute to the formation of only one indeterminate axis.

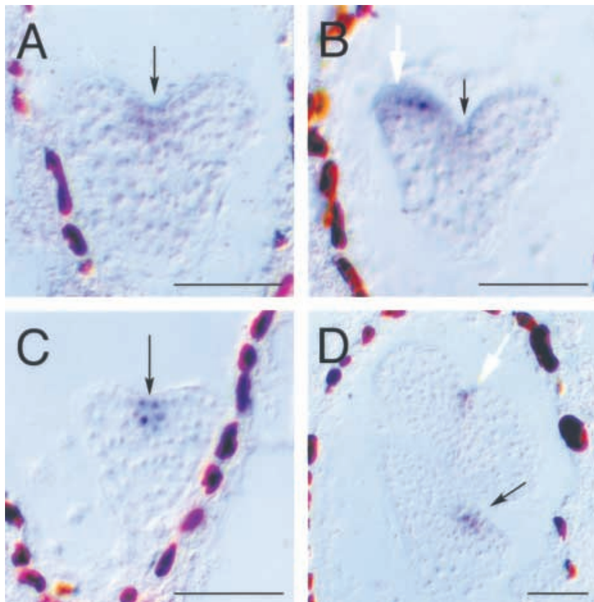

Figure 8. Gene Expression in *pnh; FIL*::*PNH* Embryos.

(A) and **(B)** In situ hybridization using a *CUC2* antisense probe on *pnh; FIL*::*PNH* embryos expressing *CUC2* in the normal domain **(A)** or ectopically in the cotyledon primordium **(B)**. Cotyledon stain was visible in multiple sections.

(C) and **(D)** In situ hybridization using an antisense *STM* probe on *pnh; FIL*::*PNH* embryos. *STM* is expressed in the normal domain early **(C)** but also is expressed in the cotyledon primordium by the bent-cotyledon stage, when cotyledon doubling is clearly distinguishable **(D)**.

Black arrows indicate the presumptive SAM, and white arrows indicate the presumed location of ectopic meristem formation. Bars = 50 μm.

These experiments underscore a growing body of evidence that indicates that radial pattern in the embryo and in the shoot is important in promoting SAM formation. Gene expression patterns show that the apical half of the embryo is divided into a central and a peripheral portion before a meristem is developed (Long and Barton, 1998). Mutants that lack a SAM, such as the *stm* and *wus* mutants, have normal adaxial/abaxial organ polarity (Barton and Poethig, 1993; Laux et al., 1996), indicating that the development of a meristem is not necessary for the proper development of organ polarity. On the other hand, alterations in polarity have profound effects on the ability of either the main SAM or axillary meristems to form (McConnell and Barton, 1998; Eshed et al., 1999, 2001; Kerstetter et al., 2001; McConnell et al., 2001). Together, these data indicate that radial polarity—that is, central (or adaxial) and peripheral (or abaxial) domains of the embryo (or leaf axil)—are established first and that the SAM is dependent on spatial cues in these distinct domains for its formation.

PNH Acts Cell Nonautonomously

Our results show that expressing *PNH* ectopically has consequences for both leaf and cotyledon primordia. Thus, *PNH* acts either cell nonautonomously or at least over a

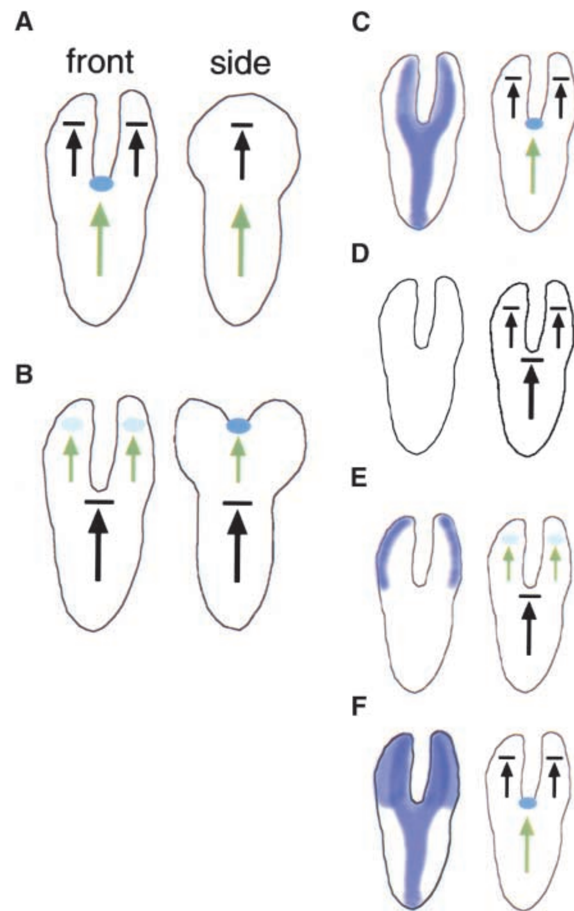

Figure 9. Models.

(A) and **(B)** Cartoons of the front and side views of wild-type **(A)** and *pnh; FIL*::*PNH* **(B)** embryos. Black arrows indicate determinate axes, and green arrows indicate indeterminate axes. SAM precursors are indicated with blue ovals. Compare the side view of the cotyledon in **(B)** with the front view of the embryo in **(A)**.

(C) to **(F)** Cartoons of embryos depicting the *PNH* expression domain in purple (left) and the behavior of the growth axes (right). Genotypes depicted are wild type **(C)**, *pnh* **(D)**, *pnh; FIL*::*PNH* **(E)**, and *PNH; FIL*::*PNH* **(F)**.

short range, emphasizing the importance of the spatially restricted *PNH* domain.

The new regions of *PNH* expression are regions in which *AGO* is expressed: *PNH* is expressed in a central domain of the developing embryo and shoot and in the presumptive vasculature, whereas the redundantly acting *AGO* gene is expressed throughout the embryo and the shoot apex. From this finding, we conclude either that the *PNH* and *AGO* gene products have overlapping but distinct functions or that the absolute levels of *PNH/AGO* function are important in development. These are testable hypotheses that have implications for the sets of genes targeted for regulation by the *PNH/AGO* genes: *PNH* and *AGO* may associate with overlapping sets of micro-RNAs, and this causes them to have different but overlapping sets of target genes. Alternatively, the two genes may have identical targets, but high levels of *PNH/AGO* activity are important for adaxially expressed targets, whereas lower levels are required for abaxially expressed targets.

The notion of distinct roles for *PNH* and *AGO* is consistent with the finding that *AGO*, but not *PNH*, is required for post-transcriptional gene silencing in Arabidopsis (Morel et al., 2002). However, it also is possible that post-transcriptional gene silencing simply requires a certain level of *PNH/AGO* activity and that this level is decreased more in *ago* mutants (because *AGO* is expressed ubiquitously and *PNH* is not) than in *pnh* mutants. To determine the extent of overlap between the functions of these gene products will require additional experiments.

Implications for the Molecular Function of *PNH*

In other systems and in plants, *PNH/AGO* activity is associated with the regulation of translation and/or the degradation of mRNAs (Fagard et al., 2000; Grishok et al., 2001). Therefore, it is very likely that *PNH/AGO* will mediate their effects at a post-transcriptional level with the specificity of their targets determined by the exact micro-RNAs the *PNH* and *AGO* gene products interact with. This study predicts that some of these targets will be mRNAs whose products regulate the cell cycle and mRNAs whose products regulate indeterminate axis formation. Identifying these targets and the mechanism by which they are regulated by *PNH* and *AGO* activity should tell us a great deal about these very basic aspects of plant development.

METHODS

Growth Conditions

Arabidopsis thaliana plants were grown at 24°C under continuous cool-white fluorescent light in Metromix 200 (Sierra, Grace, Milpitas, CA) unless noted otherwise. Sterile medium was Murashige and Skoog (1962) medium (Sigma, St. Louis, MO) with 2% Suc. Where indicated, hygromycin was added to a concentration of 40 μg/mL and carbenicillin to 100 μg/mL.

Histology

For in situ hybridization, specimens were processed as described at http://www.wisc.edu/genetics/CATG/barton/protocols.html, with color substrate incubations of 4 to 18 h. Riboprobes were synthesized from pKL3 for *PNH* (Lynn et al., 1999), pMACUC2 for *CUC2* (Aida et al., 1999), pmeriHB1 for *STM* (Long et al., 1996), pJM1 for *PHABULOSA* (McConnell et al., 2001), pY1-Y for *FIL* (Siegfried et al., 1999), and pGUS for β-glucuronidase (*GUS*) (J. Long, unpublished data). For embryo visualization, developing seeds were cleared in Hoyer's solution (100 g of chloral hydrate and 5 mL of glycerol in 30 mL of water). For leaf cell analysis, 6-μm wax sections were stained for 15 min in 0.025% toluidine blue and then deparaffinized. For scanning electron microscopy, specimens were fixed overnight in 2% glutaraldehyde, dehydrated through a graded ethanol series, critical point dried in liquid CO_2, sputter coated with gold, and visualized in a Hitachi S570 scanning electron microscope at 5 kV (Tokyo, Japan).

GUS Staining

For β-glucuronidase activity detection, samples were incubated overnight at 37°C in GUS assay buffer (100 mM phosphate buffer, pH 7, 0.1% Triton X-100, 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 5 mM FeCN, and 10 mM EDTA) and then cleared in 70% ethanol. The hydathode::GUS marker line was a fortuitous consequence of an altered STM promoter::GUS transformation. The transgene does not show expression in the STM domain.

Constructs and Plant Transformation

FIL 5′ sequences were 3971 bp of genomic DNA from 4036 to 65 bp upstream of the ATG of the *FIL* gene. *PNH* coding sequences were 3014 bp of a *PNH* cDNA, including 65 bp of the 5′ untranslated region. *PNH-FS* coding sequences have a single nucleotide deletion in codon 302, which causes a frameshift and introduces a premature stop codon in the *PNH* coding sequence. pKL49, pKL48, and pKL51 were constructed in pCAMBIA 1300 (www.cambia.org.au/main/r_et_quickpick.htm), which confers hygromycin resistance, sequenced, and transformed into *Agrobacterium tumefaciens* strain GV3101. Landsberg *erecta* or *pnh-2* flowering plants were transformed by floral dip (Clough and Bent, 1998). Seeds resulting from self-pollination of transformed plants were sterilized in bleach with 1% Tween-20 and plated to sterile medium with hygromycin and carbenicillin to select transformants (T1 generation). pRITA II transgenic seeds were a gift from John Bowman (University of California, Davis, CA).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We thank John Bowman, John Emery, Yuval Eshed, and Rita Khodosh for sharing the *FIL* promoter as well as seeds from pRITA II transgenic lines. We are grateful for helpful comments on the manuscript from Pablo Jenik. This work was funded by National Science Foundation Grant IBN-0080853 to M.K.B. and a National Science Foundation predoctoral fellowship to K.L.N.

Received June 6, 2002; accepted September 17, 2002.

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