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The GATA Factor *HANABA TARANU* Is Required to Position the Proembryo Boundary in the Early *Arabidopsis* Embryo

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SUMMARY

Division of the *Arabidopsis* zygote defines two fundamentally different developmental domains, the proembryo and suspensor. The resulting boundary separates domain-specific gene expression, and a signal originating from the proembryo instructs the suspensor to generate the root stem cell niche. While root induction is known to require the phytohormone auxin and the Auxin Response Factor *MONOPTEROS*, it has remained largely elusive how the two domains involved in this process are initially specified. Here, we show that the GATA factor *HANABA TARANU* (*HAN*) is required to position the inductive proembryo boundary. Mutations in *HAN* cause a coordinated apical shift of gene expression patterns, revealing that *HAN* regulates transcription in the basal proembryo. Key auxin transporters are affected as early as the 8 cell stage, resulting in apical redistribution of auxin. Remarkably, *han* embryos eventually organize a root independent of *MONOPTEROS* and the suspensor around a new boundary marked by the auxin maximum.

INTRODUCTION

Among the strategies that multicellular life has evolved to answer the problem of pattern formation, inductive signaling across discrete domains within developing tissues emerges as a common theme. In animals, such domains can take the form of compartments, lineage-restricted regions defined by the expression of regulatory "selector" genes (Mann and Carroll, 2002). At compartment boundaries, specialized signaling takes place to coordinate development. For example, expression of *engrailed* (*en*) specifies the posterior compartment of the wing imaginal disc in the *Drosophila* larva (Morata and Lawrence, 1975). Downstream of *en*, Hedgehog signals to bordering cells of the anterior compartment, which respond by producing a narrow band of Decapentaplegic morphogen expression (Irvine and Rauskolb, 2001; Blair, 2003). Boundary-dependent signaling is critical for correct patterning of the wing.

Although in higher plants, cell migration is prohibited by cell walls and lineage typically plays a minor role in cell specification, evidence for active developmental boundaries does exist. Discrete lateral shoot and floral organs are separated by regions of cells with reduced proliferation that signal back to the meristem from which they were derived (Waites et al., 1998). Outgrowth of laminar plant organs such as the leaf blade requires juxtaposition of adaxial and abaxial cellular domains, each with a unique profile of gene expression (Bowman et al., 2002).

In the plant embryo, a key lineage and fate border is established with the asymmetric division of the zygote. While the smaller apical daughter goes on to form the roughly spherical proembryo, the basal cell undergoes a series of transverse divisions to form a filamentous, largely extraembryonic support structure known as the suspensor (Figure 1). The seminal decision between embryonic and extraembryonic fates requires signaling by the *SSP* Pelle/IRAK-like kinase gene and the *YDA* mitogen-activated protein kinase (MAPK) kinase kinase, which act after fertilization to promote suspensor development in the basal lineage (Lukowitz et al., 2004; Bayer et al., 2009). With the third round of divisions in the apical lineage (eight-cell stage), the proembryo is divided into an upper tier that will produce most of the embryonic leaves as well as the shoot meristem, and a lower tier that will derive the hypocotyl in addition to the proximal root meristem. Differences between these anatomically defined regions are supported by the combinatorial expression of multiple *WUSCHEL-LIKE HOMEOBOX* (*WOX*) genes, which uniquely mark four domains along the main axis of the embryo, including upper and lower tier; however, phenotypic analysis of multiple *wox* mutants reveals a complicated interdependence of *WOX* genes, making it difficult to pinpoint specific functions for each *WOX* expression domain (Haecker et al., 2004; Breuninger et al., 2008). By the globular stage, HD-ZIP III homeodomain genes required for normal shoot development become restricted to the upper tier of globular stage embryos by a micro-RNA-dependent pathway. Loss of miRNA-mediated repression completely arrests the patterning process, suggesting that an early separation of shoot and root domains is crucial (Grigg et al., 2009). Also at the globular stage, the lower tier cells of the proembryo initiate an important inductive event which

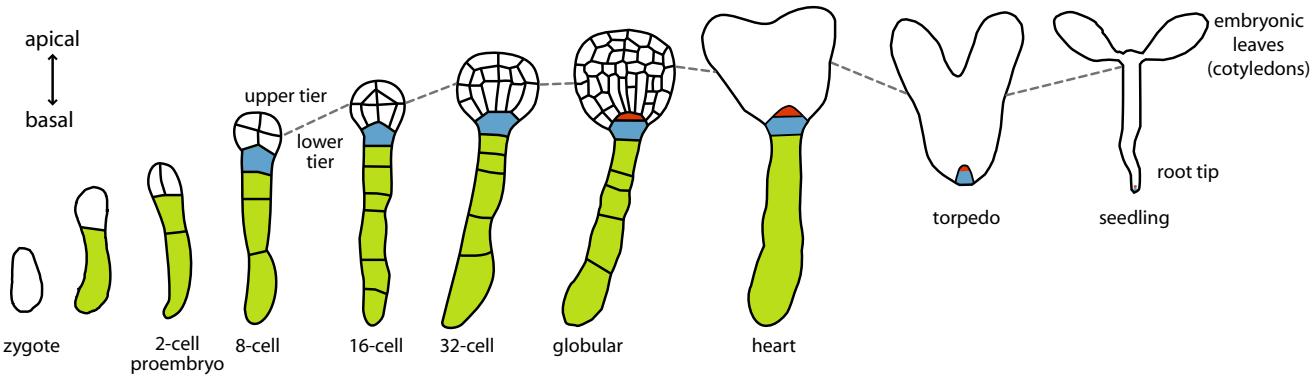

Figure 1. Key Events in *Arabidopsis* Embryonic Development

Division of the zygote gives rise to a distinct apical proembryonic lineage (white) and basal suspensor lineage (colored). The hypophysis, or uppermost suspensor cell (blue), divides at the globular stage to give rise to the QC (red), which is the site of the incipient root meristem. The proembryonic lineage is divided into an upper tier and lower tier at the 8 cell stage, with derivatives in later stages approximately indicated by dotted lines.

recruits the hypophysis, or uppermost cell of the suspensor, to form the quiescent center (QC), or stem cell niche of the future root meristem.

Root initiation is linked to the formation of a primary axis and requires the plant hormone auxin. Loss of the Auxin Response Factor (ARF) *MONOPTEROS* (*MP*), or a gain-of-function allele of its repressor, *BODENLOS* (*BDL*), results in embryos that lack basal axis elements including the root (Berleth and Jurgens, 1993; Hamann et al., 1999). Both factors are only expressed in the proembryo and act nonautonomously to specify division of the hypophysis (Hardtke and Berleth, 1998; Hamann et al., 2002; Weijers et al., 2006). Similarly, abrogation of polar auxin flux by chemical inhibition or multiple mutations in the *PIN-FORMED* (*PIN*) auxin efflux carrier family can result in apolar embryos (Friml et al., 2003). Indeed, PIN7 is one of the earliest known basal lineage markers, and its polar localization in apical suspensor membranes is thought to direct auxin to the preglobular proembryo. In contrast, PIN1 protein specifically accumulates in the young proembryo and is initially distributed relatively evenly along all internal cell walls (Friml et al., 2003). Only at the early globular stage does PIN1 acquire a basal membrane polarization in the central domain of the proembryo and PIN7 localization switch to basal membranes within the suspensor. This new configuration establishes apical-to-basal auxin flux and produces a robust auxin maximum in the hypophysis which immediately precedes, and is required for, its asymmetric division to form the progenitor of the QC (Friml et al., 2003). Both *mp* and *bdl* mutants lack a detectable auxin maximum at the base of the embryo. Failure to rescue these mutants by exogenous auxin application implies that a second, auxin-independent signal is required for hypophysis specification (Weijers et al., 2006). The MP target TMO7, a predicted transcription factor that moves from the proembryo into the suspensor, has recently been proposed as a component of this signal (Schlereth et al., 2010).

Here, we report that mutations in the GATA transcription factor *HANABA TARANU* (*HAN*) have a very early and specific effect on transcription in the proembryonic lower tier. A number of transcripts, including those coding for the auxin transport apparatus, exhibit a coordinated apical shift in the proembryo, thereby juxtaposing embryonic and suspensor domains at a new position between the upper and lower tier. Remarkably, a hypophysis-independent root can form at this juxtaposition in older *han* embryos, lending support for the concept of a proembryo boundary: a developmental border separating suspensor and embryonic fates across which the root is induced. We propose that *HAN* is required to position the proembryo boundary in *Arabidopsis*.

RESULTS

Lack of *HAN* Expression in the Early Embryo Coincides with Predominantly Basal Defects in Patterning

In a microscope-based screen for abnormal early embryo morphology (Lukowitz et al., 2004), we isolated four allelic mutations with a novel and severe effect on embryonic patterning (Figure 2; see Figure S2, available online). Aberrations are apparent by the 16 cell stage, when the tangential divisions that give rise to the protoderm in lower-tier cells frequently fail, and misaligned, oblique divisions are commonly observed in the upper tier (Figures 2A and 2B). Subsequently, the strongest phenotypes develop at the basal pole: From the 16 cell stage, lower-tier derivatives begin to resemble suspensor cells in that they divide less frequently and vacuolate, such that the basal pole of *han* embryos is eventually populated by fewer, abnormally large cells (Figures 2C and 2D). In addition, suspensor cells cease to divide (Figure 2G; Landsberg *erecta* [*Ler*] versus *han-2* or *han-16*, student's t test, $p < 0.0001$), and the hypophysis arrests its development without ever producing the progenitor cell of the QC (*han-16*, 1 division in 104 embryos; Figures 2C–2F). Neither elongated provascular cells nor an incipient root meristem can be recognized by anatomical criteria, suggesting that axialization and root initiation are disrupted. Eventually, however, at a time when their wild-type siblings have reached the late heart or torpedo stage, elongated provascular cells appear in the center of *han* proembryos and a root meristem forms at their base (Figure 2F; see below).

Positional cloning led to the discovery of the *HAN* locus, encoding a GATA factor with a single Zn-finger domain and a short second domain shared by only two other related genes in *Arabidopsis* (Figure 2H) (Reyes et al., 2004). In addition to its

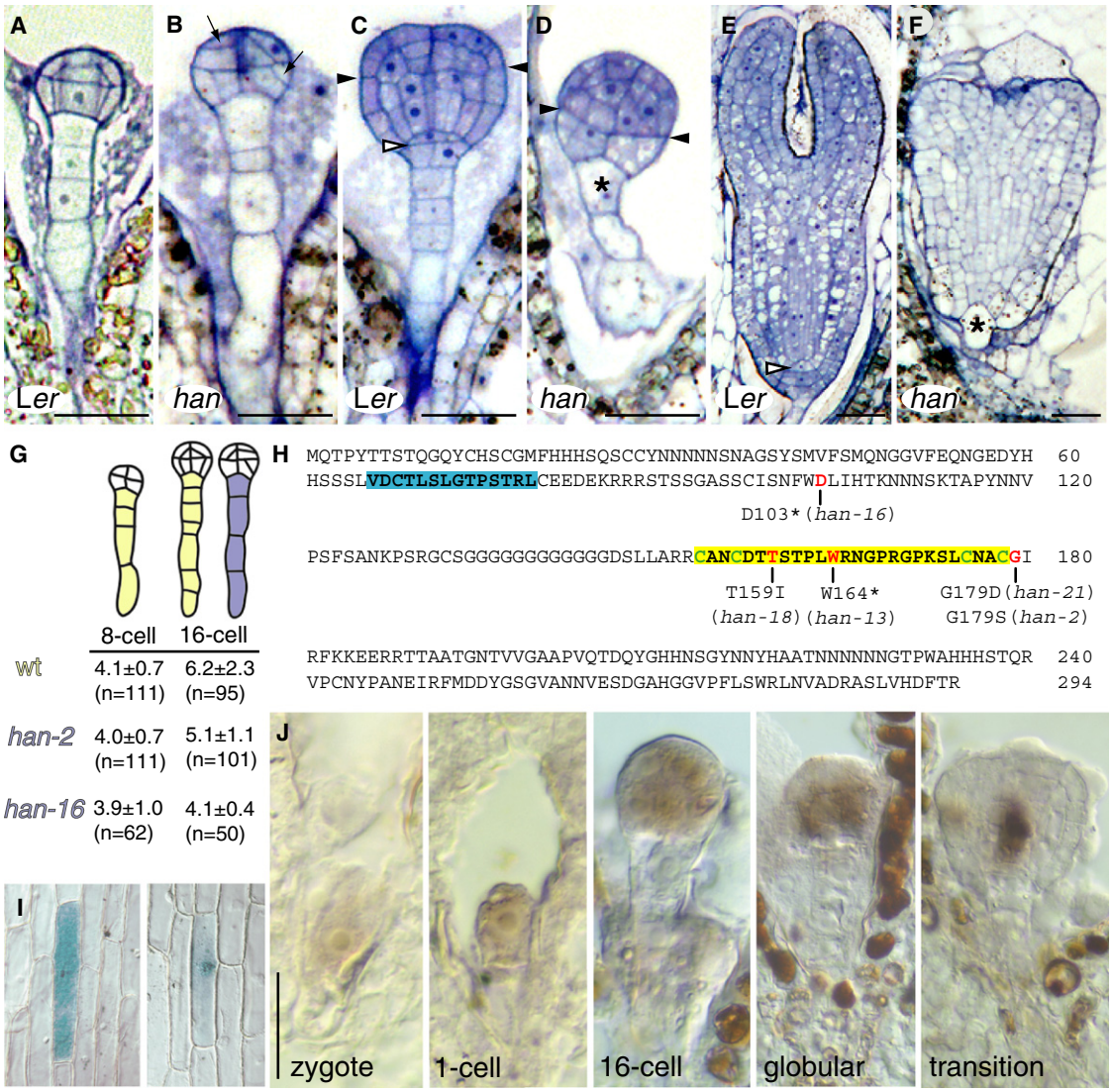

Figure 2. HAN Is Expressed in the Proembryo and Causes Predominantly Basal Defects When Mutated

(A–G) The *han* mutant phenotype, wild-type (A, C, and E), and *han-16* mutant (B, D, and F) embryos at 16 cell proembryo (A and B), globular (C and D), and torpedo (E and F) stages. Arrows mark aberrant division planes at the 16 cell stage. The hypophysis, or uppermost suspensor cell, divides to form the QC in wild-type (open arrowheads) but not in *han* (asterisks) embryos. Cells from the 8 cell stage lower-tier lineage (below filled arrowheads) are fewer and larger in the mutant. (G) Reduced suspensor cell numbers in *han-2* and *han-16* embryos at the 16 cell stage compared with wild type (±SEM).

(H) HAN protein sequence highlighting conserved region shared with two HAN family members (blue), single Zinc-finger domain (yellow) showing cysteines that form disulfide bridges (green), and altered residues (red) in mutants from this work as well as *han-2* (Zhao et al., 2004).

(I) 35S:*HAN*:*GUS* (right panel) protein fusion accumulates in the nucleus of onion epidermal cells, unlike a broadly localized 35S:*GUS* control (left panel).

(J) RNA in situ hybridization reveals *HAN* expression in the zygote that confines to the apical cell lineage with the first division and is then progressively restricted to the provasculature beginning at the globular stage. Scale bars, 20 μm. See also Figure S2.

role in the embryo, *HAN* is also required in the inflorescence: mutant plants display an aberrant phyllotaxis and do not form a complete set of floral organs. These traits were the basis for the independent isolation and naming of the gene by Zhao and colleagues (2004). *han* plants are almost completely sterile, only occasionally producing seeds, which are smaller than wild type (data not shown). Notably, crossing wild-type pollen into plants homozygous for a strong *han* allele results in embryos indistinguishable from wild type, ruling out a maternal effect on embryogenesis (data not shown).

All four of the *han* alleles discovered in this work are associated with similar defects as the putative null allele *han-16*, which gives rise to a truncated protein missing the Zn-finger domain. One allele, *han-21*, results from a nonconservative (Gly to Asp) mutation flanking the Zn-finger domain (Figure 2H). A conservative mutation in the same residue (Gly to Ser) gives rise to the weaker *han-2* phenotype, which can be maintained as a homozygote with reduced fertility (Zhao et al., 2004). Transformation of a genomic DNA fragment encompassing the entire *HAN* locus is sufficient to complement the defects

associated with *han* mutations (see Experimental Procedures for details).

In support of a predicted function in gene transcription, a HAN-β-glucuronidase (GUS) fusion protein localizes to the nucleus when transiently expressed in epidermal cells (Figure 2I). Using RNA in situ hybridization, we found that *HAN* expression initiates in the zygote and is subsequently confined to the apical lineage (Figure 2J). Expression progressively restricts to the provasculature beginning at the midglobular stage. The early expression of *HAN* gave the first indication that it may function prior to when anatomical differences can be discerned and is consistent with its role as a regulator of early embryonic development.

Mutations in *HAN* Result in an Apical Shift in the Fate Map of the Globular Embryo

We used a panel of genes expressed in well-defined spatial domains to better understand the dynamics of patterning events in the *han* mutant background. Given the striking suspensor and hypophysis phenotypes of *han*, we first sought to determine the expression of basal markers in the mutant. Expression of *SUCROSE TRANSPORTER3* (*SUC3*) is confined to the suspensor cells until it begins to mark the root cap of advanced wild-type embryos (Meyer et al., 2004). Indeed, we found that p*SUC3*:*GFP* undergoes a clear apical shift into the *han* proembryo as early as the 16 cell stage (Figure 3A). The homeobox gene *WOX5* is first expressed in the hypophysis of the globular embryo and is subsequently confined to the QC. In the postembryonic root meristem, it functions in the QC to maintain the adjacent stem cells of the columella root cap (Sarkar et al., 2007). In *han* embryos, p*WOX5*:*GFP* expression shifts apically, broadens significantly, and appears more variable. Ectopic expression is found in the proembryo (Figure 3B) and occasionally in cells derived from the upper tier (data not shown). We also assayed expression of *SHR*, a transcription factor transcribed in the stele, which is required for QC function and specification of the ground tissue (Nakajima et al., 2001). *SHR* expression initiates upon hypophysis division in the provascular cells that directly abut the QC progenitor in wild type (Figures 3C and 3D). In *han*, we detect a consistent loss or severe reduction of p*SHR*:*GFP* expression in cells next to the hypophysis. Collectively, the changes in lower-tier gene expression—ectopic expression of suspensor-specific p*SUC3*:*GFP* and p*WOX5*:*GFP* and loss of p*SHR*:*GFP*—are consistent with the anatomical resemblance of these cells in the mutant to suspensor cells.

We also assayed gene expression in the apical proembryo, which exhibits some early, albeit less severe, cell division defects. The stem cell promoting factor *WUSCHEL* (*WUS*) begins to mark the shoot meristem organizing center at the apex of the proembryo as early as the 16 cell stage (Mayer et al., 1998). As assayed by in situ hybridization, *WUS* expression is largely unaffected in the *han* background at early stages (Figures 3E and 3F). Disrupted protodermal divisions are one of the earliest visual hallmarks of the *han* phenotype, therefore we examined the expression of *MERISTEM LAYER1* (*ML1*) and the p*ML1*:*H2B-YFP* reporter, which are initially expressed in all cells of the proembryo but restrict to the protoderm by the midglobular stage in wild-type embryos (Figures 3G–I; Lu et al., 1996; Takada and Jürgens, 2007). Neither *ML1* RNA nor reporter expression are significantly altered in early or late *han* embryos.

Interestingly, transcription of at least one basally expressed gene does not shift in *han* mutants (Figure 3J). The *PLETHORA1* (*PLT1*) transcription factor is required redundantly for embryonic root formation and postembryonic root meristem function (Aida et al., 2004; Galinha et al., 2007). Early p*PLT1*:*CFP* reporter expression does not change substantially in *han* embryos, in agreement with the fact that its wild-type expression domain already spans both the suspensor and the lower tier.

In summary, we found no evidence that loss of *HAN* eliminates specific cell or tissue types, such as protoderm. Rather, it leads to a coordinated apical shift in the embryonic fate map. The cells of the lower tier acquire a number of suspensor characteristics at the level of both anatomy and gene expression, suggesting that the boundary between suspensor and proembryo is affected.

Altered Auxin Flux Defines a New Border between DR5rev:*GFP*-Expressing and Nonexpressing Cells in the *han* Mutant

Since the suspensor is thought to direct auxin toward the proembryo, we reasoned that an apical shift of suspensor fates may affect the distribution of auxin in the *han* mutants. Auxin perception maxima can be visualized using the DR5rev:*GFP* reporter of auxin-dependent transcriptional activation. In wild-type globular embryos, strong expression of DR5rev:*GFP* is found in the hypophysis and, occasionally, adjacent cells of the suspensor (Friml et al., 2003). In contrast, we found that *han* mutant embryos exhibit a robust apical shift of DR5rev:*GFP* reporter expression into the proembryo, with a clear demarcation along the border that separates the upper and lower tiers (Figures 4A and 4B).

To trace the origin of this novel, ectopic auxin perception maximum, we examined the expression of two key components of the auxin transport machinery, the efflux carriers PIN1 and PIN7, using whole-mount immunolocalization. These two auxin transporters show mutually exclusive expression domains in the preglobular embryo, with PIN7 accumulating in the suspensor and PIN1 in the proembryo. Establishment of apical-to-basal auxin flux in the proembryo precedes hypophysis specification and is brought about by a combination of PIN7 protein switching from apical to basal membranes in suspensor cells and PIN1 protein switching from nonpolar to basal localization in the provascular cells of the proembryo (Friml et al., 2003). In *han* mutants, both PIN7 and PIN1 exhibit a dramatic apical shift as early as the 8 cell stage, giving rise to two mutually exclusive domains within the proembryo that follow the upper-tier lower-tier demarcation (Figures 4C and 4D). This misregulation occurs at the level of transcription, as confirmed by PIN7 RNA in situ hybridization (Figure 4E). Moreover, PIN1 polar localization to basal cell membranes appears compromised or delayed in *han* (Figure 4C), implying that apical-to-basal auxin flux is generally diminished. The resulting backup may explain why the outgrowth of cotyledons is delayed and their separation is often incomplete in *han* embryos (Figure 2F).

These results imply that in normal development, *HAN* functions as an activator of *PIN1* expression. To test whether *HAN* expression is sufficient to drive *PIN1*, we quantified transcript in postembryonic roots bearing the constitutive overexpressor 35S:*HAN*:*GR* (Zhao et al., 2004) and found increased *PIN1* in the presence of dexamethasone induction (7.6-fold increase ± 2.3 SEM; student's t test, $p = 0.055$). Likewise, protoplasts

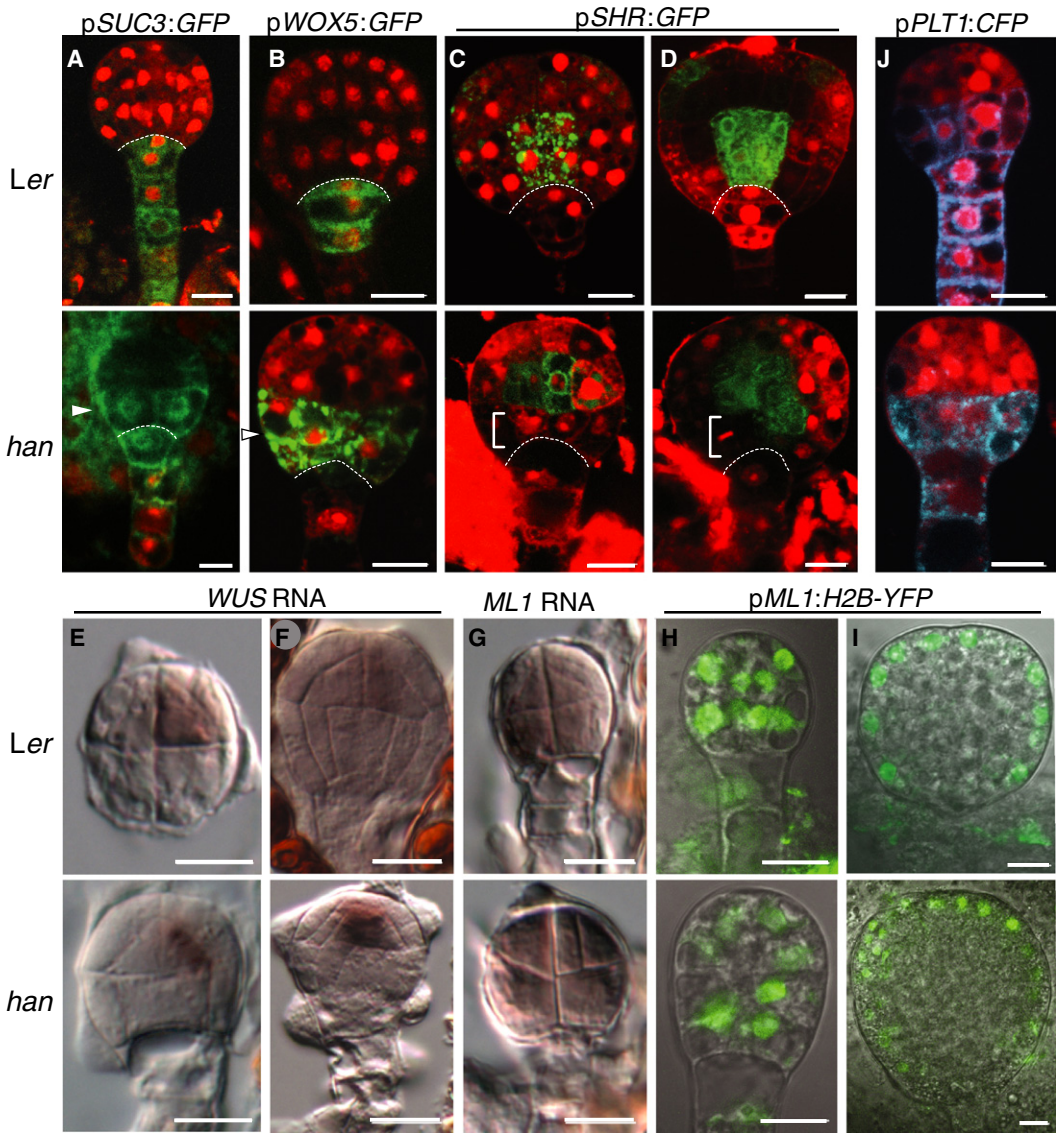

Figure 3. Gene Expression Shifts Apically in *han* Mutant Embryos

(A) Ectopic expression of p*SUC3*:*GFP* is detected in the lower tier of *han* proembryos. (B) p*WOX5*:*GFP* expression is lost in the hypophysis and appears ectopically in *han* proembryos. (C and D) p*SHR*:*GFP* expression is lost from lower-tier cells (bracket) of *han* globular (C) and transition stage (D) embryos. (E and F) *WUS* expression is unchanged in 16 cell (E) or globular (F) *han* embryos as assayed by RNA in situ hybridization. (G) *ML1* expression is unchanged in *han*, both at early stages when *ML1* is ubiquitous (G and H), and at later stages when it restricts to the outer layer (I), by RNA in situ and the p*ML1*:*H2B-YFP* reporter. (J) p*PLT1*:*CFP*, normally expressed in both suspensor and lower tier, does not change significantly in *han*. Apical is up for all images. Dotted line highlights the apical cell wall of the uppermost suspensor cell. Arrowheads indicate regions of ectopic expression. Red stain is propidium iodide (A–D and J). Scale bar, 10 μm.

derived from postembryonic roots and transformed with a 35S:*HAN*-bearing plasmid show increased *PIN1* levels (4.0-fold ± 0.2 SEM; student's t test, p = 0.007) compared with empty vector (Bargmann and Birnbaum, 2009).

The altered pattern of *PIN* gene expression in *han* confirms that a very clear and precise shift of proembryo and suspensor fates occurs as early as the 8 cell stage, with a significant effect on auxin distribution. As auxin signaling is known to inform root development in both the embryonic and postembryonic context, we next asked whether genes required for organizing the root meristem become expressed coincidentally with the ectopic auxin maximum in the *han* mutant.

Delayed Organization of an Ectopic Root Meristem in the Center of *han* Embryos

In wild type, the embryonic root is initiated by an inductive event at the globular stage, when an auxin-dependent signal originating from the proembryo instructs the uppermost suspensor cell, the hypophysis, to produce the lens-shaped progenitor cell of the root QC. This division is not observed in *han*. Nevertheless, and unlike rootless mutants deficient in auxin signaling, 90% of *han* mutants (*han-16*, n = 137) eventually recover, managing to establish an embryonic axis (as defined by elongated provascular cells; Figure 2F) and a relatively normal root meristem (Figures 5A and 5B).

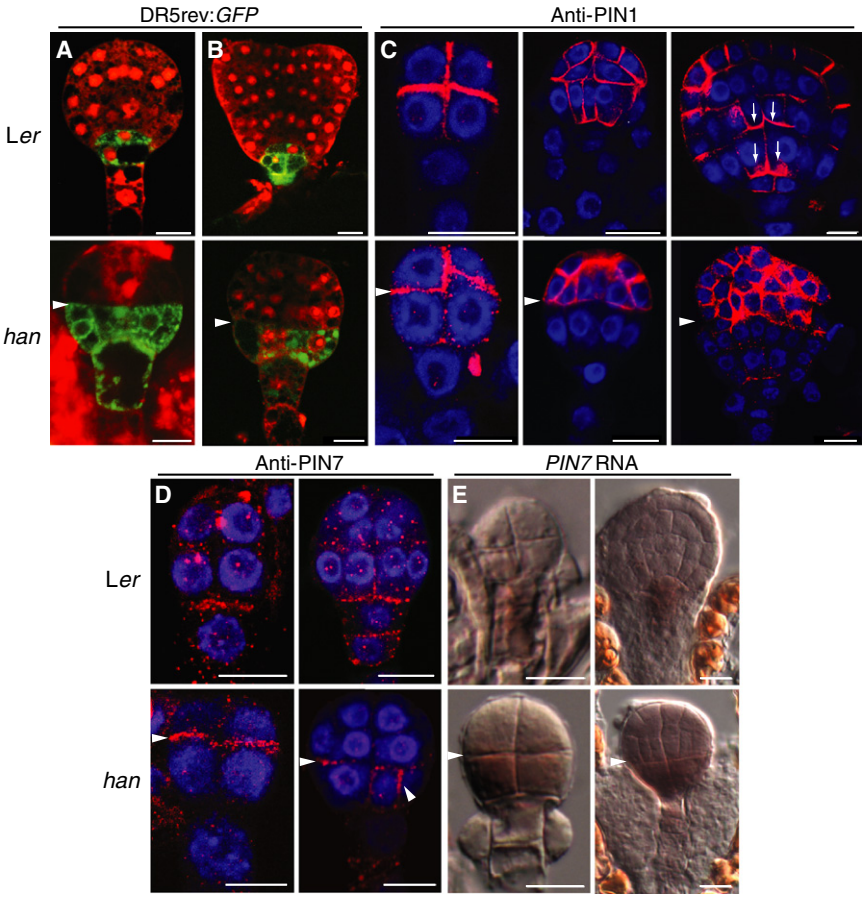

Figure 4. PIN Mislocalization Shifts the Auxin Perception Maximum from the Hypophysis to the Lower Tier in *han* Mutants

(A and B) DR5rev:*GFP* expression in globular (A) and heart-stage (B) embryos expands into the lower tier in the *han* background (arrowhead, apical boundary of ectopic DR5rev:*GFP* expression).

(C) Whole-mount immunolocalization of PIN1 protein. Arrows indicate the switch to basal subcellular localization at the globular stage in wild type, which does not occur in *han* mutants. PIN1 is not detected in the lower half of the embryo (below solid arrowhead).

(D) PIN7 whole-mount immunolocalization. PIN7 is detected in the wild-type suspensor, but shows stronger expression in the lower tier of *han* proembryos (arrowheads, ectopic expression).

(E) In situ hybridization confirms PIN7 misexpression at the RNA level (arrowheads, apical limit of ectopic expression).

Red stain is propidium iodide (A and B); blue nuclear stain is DAPI (C and D). Scale bar, 10 μm.

Maintenance of the QC is thought to depend on three coincident factors: a local auxin maximum, and coexpression of the PLT family and SHR/SCARECROW (SCR) transcription factors (Di Laurenzio et al., 1996; Nakajima et al., 2001; Aida et al., 2004; Scheres, 2007). Since expression of *SHR* is shifted apically in *han* embryos, there is no or only minimal overlap with the *PLT1* expression domain at the globular stage when QC formation is normally initiated (compare Figures 3C and 3J). However, expression of *SCR* and *PLT1* begins to overlap in the lower tier of *han* embryos by the time their wild-type siblings have reached the late heart stage (Figures 5C–5E). As in wild type, the overlap corresponds to a position just below the boundary deliniated by the DR5rev:*GFP* expression domain and is eventually marked by a tightly restricted focus of *WOX5* transcription (Figure 5F). Meristematic fate is further confirmed by the expression of *PIN4*—normally confined to QC and initials (Sundaresan et al., 1995; Friml et al., 2002)—below the line that distinguishes upper and lower tier (Figure 5G).

The *han* embryo is therefore able to produce a root independent of the hypophysis by coordinating root meristem gene activity at a later stage of development. Taken together, the creation of a new boundary between suspensor and proembryo gene expression domains and the associated shift of root induction to a position roughly below this juxtaposition argues for the existence of a developmental "proembryo boundary", the position of which is normally maintained by *HAN*.

Formation of the Ectopic Root Is Independent of *MP*

To clarify the relationship between *HAN*- and auxin-dependent gene regulation, we examined the interactions between *han* and *mp* mutations. *MP* encodes a key positive regulator of auxin-dependent transcription in early development and is required in the provascular cells of the proembryo for inducing the asymmetric division of the hypophysis. Like *han*, the *mp* mutant hypophysis never produces a QC, but unlike *han*, the mutant embryo fails to recover a root later in embryogenesis (Berleth and Jurgens, 1993). Both *MP* and its repressor *BDL* share a very similar expression pattern with *HAN* (Hardtke and Berleth, 1998; Hamann et al., 2002). By in situ hybridization, *MP* expression is unaffected in the *han* mutant (Figures 6A and 6B).

Surprisingly, *han* is epistatic over *mp* in the embryo. Prior to the early globular stage, when *mp* embryos are essentially indistinguishable from wild type, only normal and *han* phenotypes, but no novel phenotypes, were observed in the progeny of *han-21*/+ *mp-T370*/+ plants. After the early globular stage, 25% (n = 733) were indistinguishable from *han* embryos, while only 17% resembled *mp* embryos, indicating that double mutants resemble *han* embryos by anatomy. To monitor root formation in the double mutants, we analyzed GUS expression in the progeny of plants that were heterozygous for *han-16* and *mp-T370* as well as homozygous for the *pin4-LENNY* allele, harboring a Ds GUS insertion downstream of the promoter (Sundaresan et al., 1995; Friml et al., 2002; n = 714). Expression of *PIN4* is dependent on auxin signaling and was absent in all *mp*-resembling embryos. In contrast, all *han*-resembling embryos from these parents were also GUS positive, indicating that *han mp* double mutants retain the ability to express *PIN4* and, by extension, respond to auxin. Consistent with this conclusion, double-mutant seedlings, identified using PCR-based

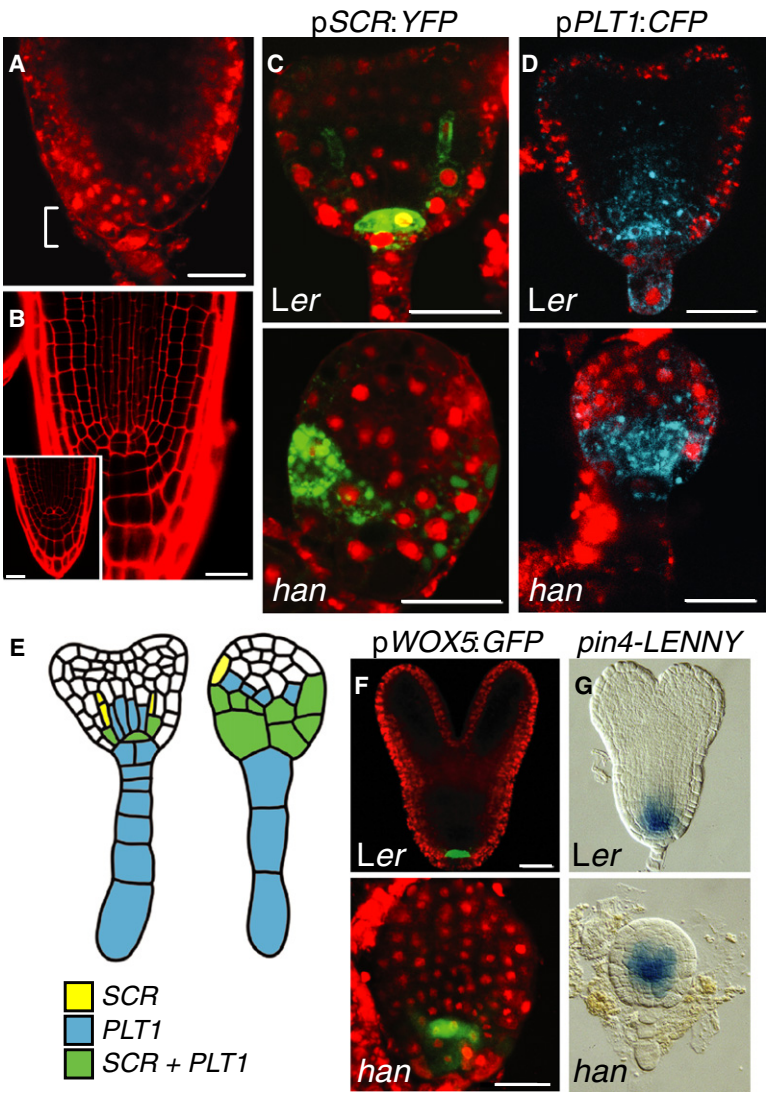

Figure 5. Root Meristem Gene Expression Organizes Near the Center of Advanced *han* Mutant Embryos, which Are Able to Generate a Functional Root

(A) Lower-tier derived cells are eventually pushed to the edge of the *han* root (bracket).

(B) Postembryonic *han-2* roots are similar to wild type in organization (inset, wt).

(C–E) Genes required for root meristem function, as marked by p*SCR*:*GFP* (*C*) and p*PLT1*:*CFP* (*D*), overlap in older *han* embryos.

(E) Schematic of overlapping expression in wild-type (left) and *han* (right) heart-stage embryos.

(F and G) Confirmation of root meristem specification by a restricted focus of p*WOX5*:*GFP* (F) and *pin4-LENNY* GUS promoter trap (G) expression in *han*. Red nuclear stain is propidium iodide. Scale bar, 20 μm.

markers for the *mp-T370* and *han-21* mutations, had a functional root meristem. Double-mutant seedlings went on to produce adults with characteristic *mp* defects such as crinkled leaves and pin-like inflorescences (Figures 6C–6F; Przemeck et al., 1996). These results argue against a direct effect of *HAN* on auxin signaling and reveal that *han* embryos can perceive auxin in a *MP*-independent fashion, likely involving other auxin response factors expressed in the proembryo.

DISCUSSION

Nearly all of the earliest divisions in the *Arabidopsis* embryo appear to be decisive for cell fate. As new spatial domains are created by cell division, they become marked by distinct transcriptional signatures, exemplified by the expression of *WOX* family genes in dynamic and specific patterns (Haecker et al., 2004). Here, we show that the GATA-factor *HAN* contributes to this process by directing gene expression in the lower tier of the developing embryo as soon as this domain arises. As a consequence, loss of *HAN* profoundly alters the spatial layout of auxin transport and auxin responses, and perturbs the developmental boundary between proembryo and suspensor across which the root is initiated.

Coordinated Regulation of Expression Domains at the Base of the Embryo

Similar to the selector genes of animals, *HAN* appears to specify a lineage-defined domain—the lower tier of the proembryo—by regulating the expression of an entire suite of transcripts within the domain. By anatomical criteria, mutants develop without noticeable defects prior to the 16 cell stage, forming a filamentous suspensor and a spherical embryo with an upper and lower tier. However, *han* mutants already show dramatic differences in gene expression from wild type by the 8 cell stage, with the domains of most genes undergoing a coordinated apical shift. *SUC3* and *PIN7* transcripts, normally only detected in the suspensor, are found ectopically in the lower tier of the proembryo, while *WOX5* expression disappears from the hypophysis and expands even further into the apex. Reciprocally, *SHR* and *PIN1* expression is lost from the lower tier of *han* embryos but remains undisturbed in the upper tier. These results indicate an accumulation of suspensor attributes in the lower tier. In support of this, the lower tier appears structurally similar to the extra-embryonic suspensor, with large and highly vacuolated cells, low cell division rates, and little or no apparent contribution to the mature embryo.

HAN transcripts accumulate throughout the proembryo, yet gene expression and development is affected much more dramatically in the lower tier than in the upper tier of mutants. Conceivably, redundant functions—perhaps one of the two *HAN-LIKE* genes (Zhao et al., 2004)—may mitigate the loss of *HAN* in the upper tier, or *HAN* may require a domain-specific cofactor. Alternatively, the upper and lower tier of the early embryo could already possess fundamentally different properties, related to their eventual fates as shoot and root (Grigg et al., 2009; Smith and Long, 2010), that would make them respond differently to *HAN*.

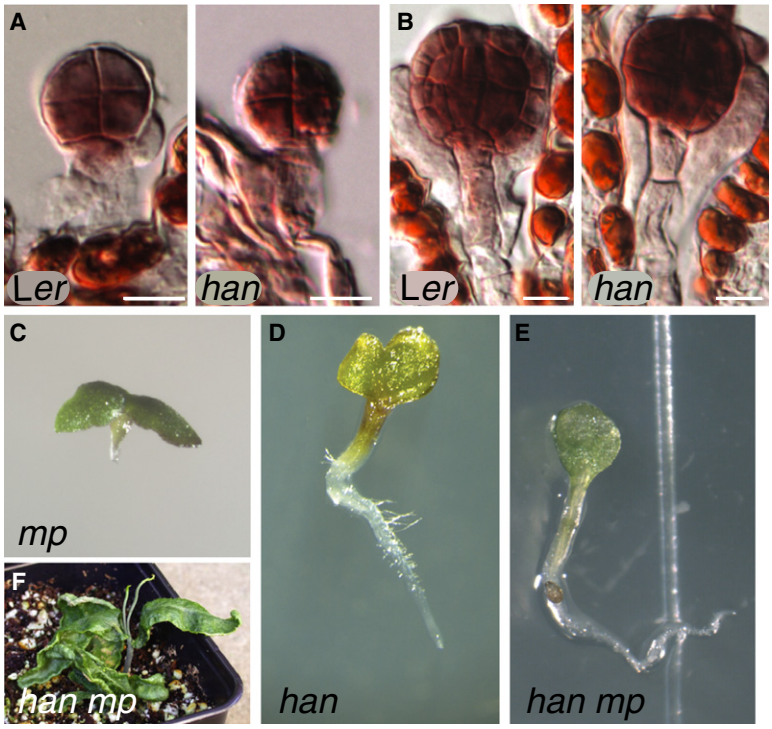

Figure 6. *han* Suppresses the *mp* Rootless Phenotype
(A and B) *MP* expression is unaltered in 8 cell (A) or globular stage (B) *han* embryos by RNA in situ.
(C) *mp* seedling lack a primary root.
(D and E) *han* and *mp han* double-mutant seedlings displaying primary roots.
(F) Example of a *mp han* double-mutant plant grown on soil from seed and displaying a *mp* adult phenotype.
Scale bar, 10 μm.

A Mutant with Altered Auxin Distribution

Localized auxin flux and signaling informs most of what we know about the embryonic patterning process, including axis and root formation (see Introduction). In some respects, loss of *HAN* and disruption of auxin transport or signaling are associated with similar effects: Like auxin signaling and transport mutants, *han* embryos fail to axialize early or to initiate a root through hypophysis division. Yet, and in striking contrast to auxin mutants, these processes are not permanently blocked but typically recover, resulting in the initiation of an embryonic root later and at a more apical position. As would be expected from this ability of *han* embryos, auxin perception remains essentially intact and is thus unlikely to be affected directly by *HAN* activity. Instead, analysis of the DR5rev:*GFP* reporter reveals an inability to drain the lower tier of auxin at the globular stage—an effect that can be attributed to an altered configuration of PIN efflux carriers from the 8 cell stage on. The coordinated shift of *PIN1* and *PIN7* expression is the earliest defect we uncovered in mutant embryos, suggesting that these genes may be targets of *HAN*. In support of this view, induced expression of *HAN* in postembryonic roots or root protoplasts is sufficient to activate *PIN1* transcription.

Given the fundamental role of auxin in organizing the plant body, the mechanisms controlling expression and polar localization of PIN transporters on a cellular as well as organismal level are of obvious significance. Although key details remain elusive, evidence for both context-dependent and self-organizing modes of regulation have been described (Reinhardt et al., 2003; Jönsson et al., 2006; Smith et al., 2006; Xu et al., 2006; Sena et al., 2009; Sauer et al., 2006). Auxin-dependent positive feedback likely orchestrates PIN1 expression in the shoot meristem to create auxin maxima at the sites of lateral organ initiation (Reinhardt et al., 2003). Tissue-specific factors, such as the serine/threonine kinase *PINOID* (*PID*) and the protein phosphatase *PP2A*, appear to regulate polar relocalization of PIN1 to the cellular apex in the developing cotyledons (Friml et al., 2004; Michniewicz et al., 2007). Our results reveal *HAN* as a domain-specific regulator of *PIN* gene expression before the globular stage and indicate that cell identity plays an important role in establishing the framework of auxin transport in the early embryo.

Regulation of the Proembryo Boundary and Root Induction

Targeted expression studies show that hypophysis division, and thus root initiation, requires *MP* activity specifically in the central cells of the early globular proembryo, implying a mechanism of inductive signaling from these cells to the suspensor. *MP* activity mediates apical-to-basal auxin flux toward the hypophysis and, consequently, formation of the local auxin maximum associated with root formation. However, auxin does not seem to be the only signal in this process, as accumulation of exogenous auxin in the suspensor of *mp* mutants does not trigger root formation (Weijers et al., 2006). Indeed, recent work has pinpointed TMO7, a mobile transcription factor activated by *MP*, as a likely second signal (Schlereth et al., 2010).

Loss of *HAN* disrupts induction of the hypophysis. Furthermore, the shift in expression of molecular markers delineating the proembryo domain corresponds to the site of root initiation later in development. These observations lend strong support to the concept of an instructive proembryo boundary that supercedes lineage and is positioned at the preglobular stage by a *HAN*-dependent molecular mechanism (Figure 7). Do the same signals as in wild type induce root induction across this boundary in *han*?

A distal auxin maximum has long been known to be a prerequisite for root development, and our results indicate that auxin does accumulate below the shifted proembryo boundary. On the other hand, root formation in *han* is not dependent on *MP* activity, and it is unclear whether other auxin response factors, such as *NPH4*, which is expressed in the apical embryo (Hardtke et al., 2004), can substitute for *MP* to generate the second inductive signal in the mutants. This model would require that lower-tier cells in *han* retain a hypophysis-like ability to respond to the second signal. Alternatively, the auxin maximum may reorganize root meristem gene expression independently, as proposed for root regeneration (Sena et al., 2009). Ectopic auxin accumulation in the embryonic apex, caused by dominant

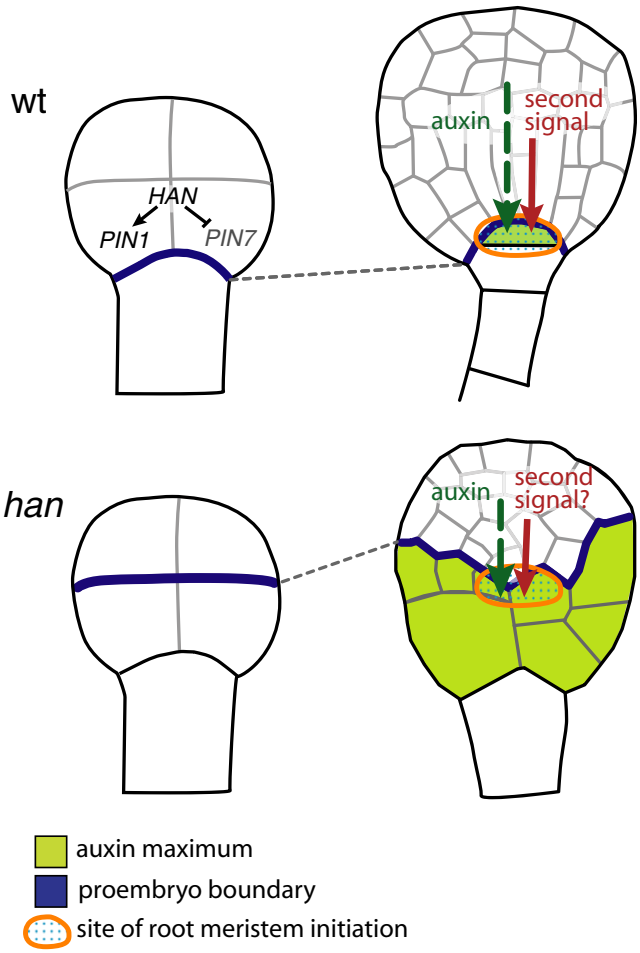

Figure 7. Model of HAN Function

As early as the 8 cell stage (left), *HAN* activity promotes embryo-specific (including *PIN1*) and represses suspensor-specific (including *PIN7*) gene expression in the lower tier of the proembryo. These two expression domains are shifted to the border between upper tier and lower tier in the mutant. By the globular stage (right), PIN1 and PIN7 proteins polarize to basal membranes in the wild type, producing an auxin maximum in the hypophysis required for root initiation. In *han*, PIN1 is slow to polarize and ectopic PIN7 remains expressed throughout the lower tier. This altered configuration results in an ectopic auxin maximum that directs hypophysis-independent and *MP*-independent root initiation near the shifted proembryo boundary. A second, auxin-independent signal normally required for root induction may or may not be maintained at the more apical position in *han* mutants.

negative *ara7* mutations that inhibit PIN polarization, has been shown to induce root formation at the tips of cotyledon primordia (Dhonukshe et al., 2008). According to this view, the effect of *han* mutations on root induction may largely be due to the coordinated apical shift of *PIN* gene expression. Consequently, removing *PIN* function should modify the effect of *han* mutations on the embryo. No such effect has been observed in *han pin1* and *han pin7* double mutants, which essentially develop like *han* and show similar expression of the auxin reporter *DR5rev:GFP* (T.N. and W.L., unpublished data). This result may not be entirely surprising, as *PIN* genes are known to form a robust network, where the loss of one or more genes can be compensated by other family members (Friml et al., 2003; Blilou et al., 2005; Vieten et al., 2005). But it implies that more sophisticated approaches will have to be developed for directly testing the role of auxin in signaling across the proembryo boundary.

EXPERIMENTAL PROCEDURES

Plant Growth, Genetic Strains, and Genotyping

Plants were grown under constant illumination (65 μmol/m^2/s) at 21°C and 60% relative humidity on commercial potting mix (RediEarth, Sun Gro Horticulture) containing systemic insecticide (Marathon 1% G, Olympic Horticultural Products) and slow-release fertilizer (19/12/6 Osmocote, Scotts Miracle-Gro Co.).

The mutant alleles *han-13*, *han-16*, *han-18*, and *han-21* were induced by ethyl methanesulfonate treatment of L*er* seed and recovered from a microscope-based screen for abnormal embryo morphology (Lukowitz et al., 2004). Other mutants and reporter lines originated as follows: *DR5rev-GFP*, Col accession (Friml et al., 2003); *han-2*, L*er* accession (Zhao et al., 2004); *ML1:H2B-YFP*, Col accession, gift of A. Roeder and E. Meyerowitz (Caltech); *mp-T370*, L*er* accession (Hardtke and Berleth, 1998); *PIN4:GUS*, L*er* accession (Friml et al., 2002); *pPLT1:CFP*, L*er* accession (Galinha et al., 2007); *pSCR:GFP*, L*er* accession (Wysocka-Diller et al., 2000); *pSHR:GFP*, L*er* accession (Helariutta et al., 2000); *pWOX5:GFP*, Col accession (Blilou et al., 2005); *SUC3:GFP*, C24 accession (Meyer et al., 2004). Codominant PCR-based markers for *han-16*, *han-21*, and *mp-T370* enabled detection of mutant plants independently of their phenotype (see Supplemental Information for primer sequences).

Positional Cloning

The *han-21* mutation was mapped to a ~1 cM interval on the lower arm of chromosome III, flanked by the PCR-based markers *B9* and *MUR1* (kindly communicated by S. Turner and C. Somerville, and I. Wilson and S. Somerville, respectively) (mapping markers are listed in Supplemental Information). Twenty-five recombination events within this interval were collected from a mapping population of about 1200 F2 plants as described (Lukowitz et al., 2000). Fine mapping positioned *HAN* on a ~22 kb fragment contained in the bacterial artificial chromosome (BAC) clone F18B3, defined by the markers *M011*, *M015*, and *M016* (seven, four, and one recombination events, respectively) on one side and the markers *M021*, *M017*, *M018* (two recombination events), and *M008* (three recombination events) on the other. This DNA segment contains three protein-coding genes as well as a tRNA gene (At3g50870 to At3g50900); sequencing revealed that all *han* alleles harbor point mutations in At3g50870.

Molecular Complementation

A 9.1 kbp EcoRI-SalI fragment spanning the entire *HAN* locus was subcloned from BAC F18B3 into a modified pBIN T-DNA vector containing a glufosinate resistance gene (kindly provided by S. Cutler and C. Somerville).

Transient Expression in Onion Cells

A full-length HAN cDNA clone was isolated from an inflorescence library, amplified using primer pair HAN-ATG/TAA (see Supplemental Information) and placed in-frame at the C terminus of a GUS open reading frame under the control of the viral 35S promoter (derived from pCambia1201) (McElroy et al., 1995) using an introduced BamHI restriction site. A particle gun (Biorad PDS1000) was used to transform the plasmid into onion epidermal cells. Onion peels were incubated on Murashige and Skoog (MS) plates for 24 hr and GUS activity assayed by incubating for 2 to 6 hr in staining solution (1 mM X-glucuronic acid [Rose Scientific], 100 mM potassium phosphate [pH 7], 10 mM EDTA, 0.5 mM each potassium ferri- and ferrocyanide).

Histology and Light Microscopy

Whole-mount immature seeds were dissected from siliques, transferred to a modified Hoyer's solution (70% chloral hydrate, 4% glycerol, 5% gum arabic), and examined whole mount using differential interference contrast (DIC) optics (Leica DMRB). Mutant embryos were staged by comparison to their wild-type siblings from the same silique. In case this was not possible, approximate stages were determined on the basis of overall size and cell

number. Histological sections were prepared from fixed immature seed embedded in Spurr's resin as described (Lukowitz et al., 2004).

RNA in Situ Hybridization

Siliques were fixed overnight at 4°C (4% formaldehyde in phosphate-buffered saline) and processed for sectioning and hybridization as described (Long et al., 1996) with minor modifications. Please see the Supplemental Information for RNA probe preparation.

Imaging of Cell Fate Markers

Reporter crosses to the strong *han-16* allele were used for marker visualization. Except in a few cases, the resulting lines were propagated as *han* heterozygotes because of sterility, and segregating mutant embryos were identified by phenotype. Deviations from wild-type expression patterns only occurred in homozygous mutant embryos, which segregated in a 3:1 ratio from heterozygous parents and were strictly correlated with mutant morphology. Markers were also introduced into the weaker *han-2* allele, where they could be visualized in the progeny of homozygous mutant plants. Expression in early embryos (1–16 cell stage, when the anatomy of *han* mutants is indistinguishable from wild type) was typically assayed in this background.

For imaging fluorescent markers, immature seeds were dissected from siliques into a drop of 5%–15% glycerol and gently squeezed under a coverslip until embryos were released. Subsequent visualization under a Zeiss LSM 510 or LSM 5 Pascal confocal was carried out within 1–2 hr of seed rupture. Propidium iodide (PI) at 10 mg/l was used as counterstain. Fluorophores were excited using the 488 nm (GFP, YFP, PI) or 457 nm (CFP) line of an argon ion laser and fluorescence imaged using standard filter sets. *PIN4:GUS* was assayed by staining unfixed embryos for 2–6 hr in GUS staining buffer, as described above.

Whole-Mount Immunolocalization

Immunolocalization was carried out on whole-mounted embryos as described (Friml et al., 2003) using antibody dilutions of 1:1000 (PIN1) and1:50 (PIN7) in combination with Cy3-conjugated secondary antibody (Jackson Immunoresearch, Westgrove, PA). 4′,6-Diamidino-2-phenylindole (DAPI) was added at 1 mg/l as a nuclear stain. Stained embryos were imaged with a Leica TCS SP2 with 405 nm diode laser or Zeiss LSM 510 Meta fitted with a Chameleon XR tunable laser for UV excitement of DAPI.

Quantitation of PIN1 Transcript

Root tissue was collected from 8 days post germination (dpg) vertically grown 35S:*HAN*:*GR* seedlings (kind gift of Eliot Meyerowitz; Zhao et al., 2004) that were induced for 7 hr on 10 μM dexamethasone or ethanol mock treatment in triplicate. Whole-root protoplasts were obtained from vertically grown 11 dpg seedling using the method of Bargmann and Birnbaum (2009). Approximately 1 million protoplasts were transfected for 18 hr with either 50 μg of pBEACON_35S:*HAN cDNA* or control pMON999_35S:RFP, cell sorted for transformed (red) cells, and collected in RTL buffer (QIAGEN, Santa Clarita, CA) in triplicate. RNA was extracted using the QIAGEN RNeasy Micro procedure, with a grinding and Qiashredder step for root tissue, and reverse transcription performed using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA). To quantify transcript levels, PIN1rt-F and PIN1rt-R primers were used and compared against *ACTIN2* control, using ACT2rt-F and ACT2rt-R primers (Pfaffl, 2001) (see Supplemental Information).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2010.06.004.

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