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# Embryo patterning genes and reinforcement cues determine cell fate in the *Arabidopsis thaliana* root

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*The majority of plant organs arise from groups of continuously dividing cells, the meristems. Little is known about mechanisms of cell specification in meristems. Within the* Arabidopsis *root meristem, the fate of every cell can be predicted accurately, and the origin of these cells during the formation of the embryonic root primordium is known. Laser ablations reveal that, despite the regularity in cell lineage, position remains important to reinforce cell specification. Genetic analysis has revealed that many genes involved in the specification of the main cell types in the root act early, during embryogenesis, and an important question is whether the same or other genes are involved in the reinforcement of specification. Sub-specification of cell types, as exemplified by epidermal root hair cell specification, involves two pathways, one of which may act to reinforce earlier patterning events mediated by the other.*

**Key words:** *Arabidopsis* / cell specification / embryogenesis / meristem / root



DURING PLANT organogenesis, a specific spatial arrangement of differentiated cell types is laid down. Plants are unique in that this is not limited to embryogenesis but perpetuated throughout the life cycle.[1] Within the embryo, a simple pattern of cell types is formed in the radial dimension, whereas different organ primordia are laid down in the apical–basal dimension[2] (Figure 1). The primary shoot and root meristems at the apical and basal ends of the embryo consist of a subset of cells within the shoot and root primordia that remain capable of continued development. Upon germination, these two meristems constitute the local regions where new cells are added to the plant body to elaborate on the preexisting cellular pattern of the embryo. Furthermore, the meristems can give rise to secondary meristems that re-iterate the activity of the primary meristem.

Recent work on the small weed *Arabidopsis thaliana* is beginning to shed light on the mechanisms of pattern formation within meristems. Floral meristem development and the regulation of shoot meristem size are areas investigated successfully by molecular genetic approaches.[3-6] Noteworthy, shoot apical meristems produce organ primordia as lateral appendages, which only subsequently give rise to differentiated cell types. Research on the shoot and floral meristems focuses mainly on the definition of the meristem as a whole, the allocation of cell groups to primordia, and the establishment of identity of the primordia.

The underground root meristem lacks the complexity of lateral appendage formation. Its ontogeny during embryogenesis can be described with a resolution at the cellular level. The root meristem resides at the distal end of a single organ and the fate of each of its cells can be predicted from their position. The predictable destiny of root meristem cells allows two different approaches to study cell fate determination. Firstly, the transparent roots allow in-vivo manipulations of cells with known fate within the meristem and analysis of the effects. Secondly, mutations that interfere with cell type specification within the root meristem allow straightforward interpretation of phenotypes. Below I will discuss how these studies are beginning to provide clues to the mechanisms by which cells become specified within the root meristem, and how the post-embryonic pattern of cell types relates to genes involved in embryonic pattern formation.

## Structure and ontogeny of the *Arabidopsis* root meristem

Plant roots have a simple tissue organization, in which outer rings of epidermal and cortical cells surround a central vascular bundle. *Arabidopsis* roots are a paragon of this regularity. They contain a surprisingly constant number and arrangement of cells in cross-section (Figure 2a). In longitudinal view, files of each cell type terminate in so-called initial cells (Figure

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2b). A small set of initials for all tissues surround four quiescent cells.[7] Quiescent centre and initials together are termed the promeristem, the minimal construction centre of the root.[8] All cells of the promeristem are laid down in their typical cellular organization during embryogenesis, and exhibit their characteristic division pattern from the heart stage of embryogenesis onward. Cells that leave the meristem progressively differentiate into the various mature cell types as predicted by their position.

Both anatomical and clonal analysis have been performed to describe how the primary root meristem is laid down during embryo development. Clonal analysis exploits blue sectors which arise by transposon excision from the *uidA* (*GUS*) marker gene in transgenic plants. Large sectors mark the progeny of a single embryonic cell. Analysis of the end points and width of these sectors has allowed the deduction of a complete fate map for the *Arabidopsis* root.[9,10] The root meristem arises from daughters of both the basal and apical cell which are separated at the first zygotic division: the quiescent centre and columella root cap arise from the hypophyseal cell that is, in turn, derived from the basal cell, while the proximal initials arise from the apical cell (Figure 1). Apparently, the daughters of the hypophyseal cell come to cooperate with the proximal initials to give rise to the functionally integrated root meristem.

The separation of the main tissue layers: protoderm, ground tissue and procambium, also occurs early during embryogenesis (Figure 1). These divisions, like the first zygotic division, form clonal boundaries that separate different cell types. The root meristem initials that continue to produce protoderm-, ground tissue- and procambium-derived cells are set apart from these separate layers at the heart stage. In conclusion, the main tissues of the *Arabidopsis* root, both in the radial and in the apical–basal dimension, arise from progenitor cells that are separated at an early stage of embryogenesis.

Secondary root meristems are formed after embryogenesis, and hence arise in a different developmental context. The ontogeny of lateral root primordia, which originate from the pericycle of the primary

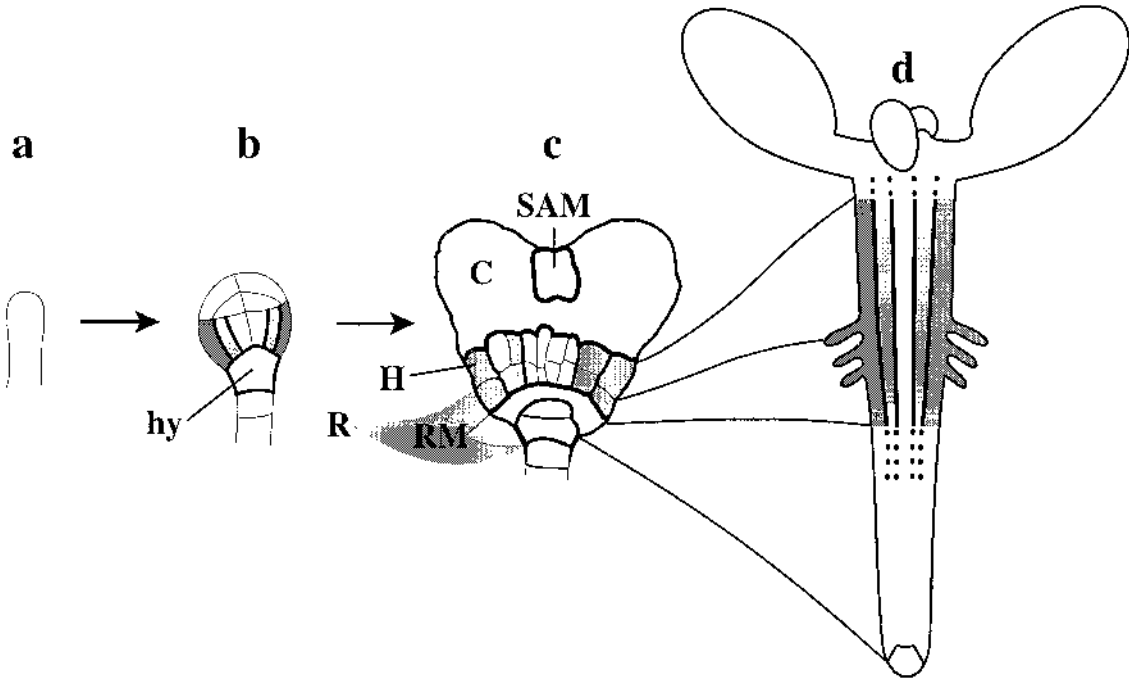


**Figure 1.** Origin of organs and main tissues in the *Arabidopsis* embryo. (a) zygote; (b) 32-cell-stage embryo; (c) early heart stage embryo; (d) seedling. hy, hypophyseal cell; SAM, shoot apical meristem; C, cotyledons; H, hypocotyl; R, root; RM, root meristem. Tissues indicated by shading the middle region of the embryo/seedling: dark grey, protoderm (origin of epidermis and lateral root cap); light grey: ground meristem (origin of cortical parenchyma and endodermis); white, procambium (origin of pericycle and vascular cell types).

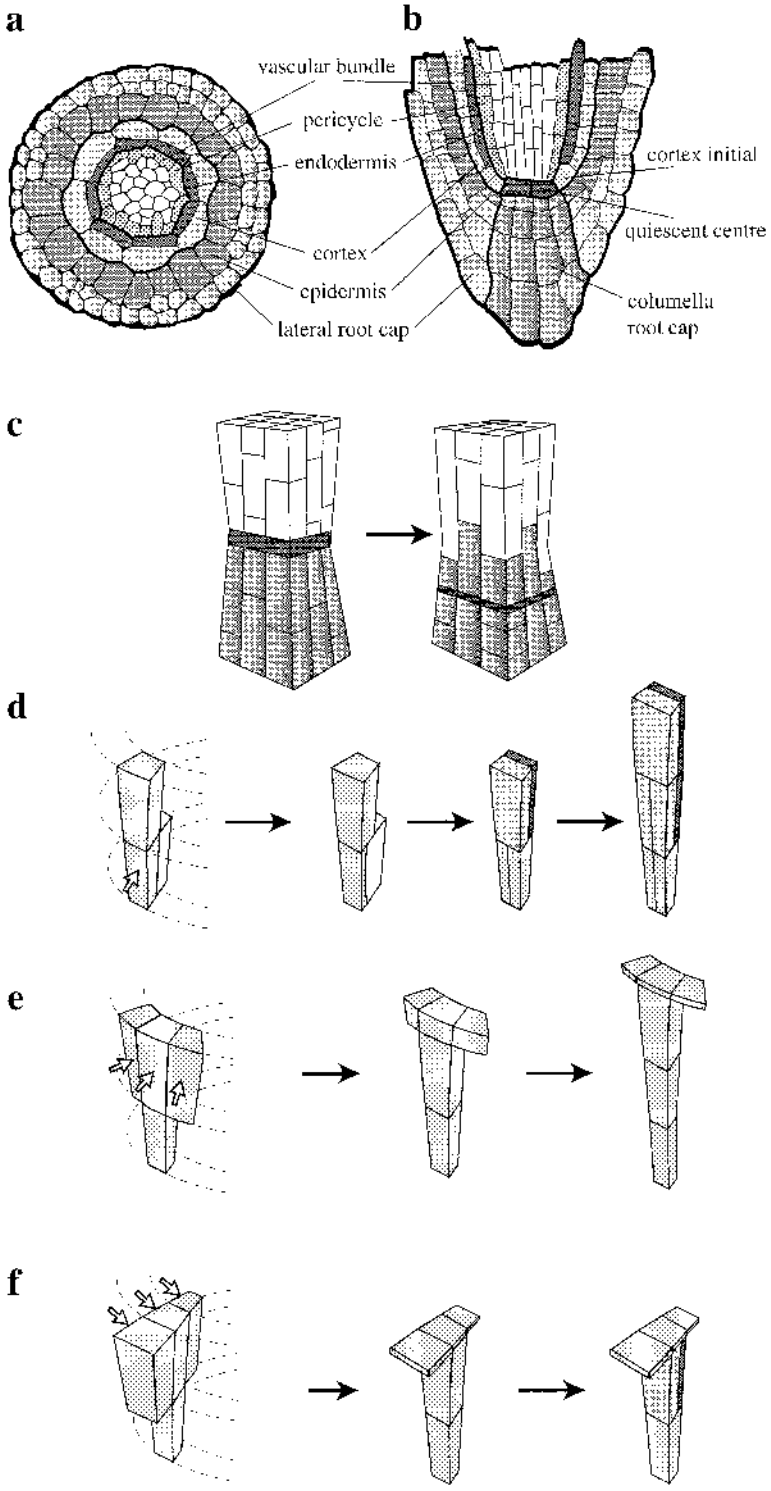


**Figure 2.** Lineage and flexibility in the *Arabidopsis* root. Cell types in the seedling root in (a) transverse and (b) longitudinal view. (c) Effect of quiescent centre ablation. Vascular initial daughters move distally and switch fate. (d) Effect of cortical initial ablation. Pericycle cell daughters move outward and switch fate. (e) Effect of ablation of cortical initial daughter cells (arrows). The initial that is deprived from cortical contacts does not divide asymmetrically. (f) Ablation of other cells contacting the cortex initial does not disturb the asymmetric division of the initial.

root, has been analysed using a variety of tissue-specific enhancer trap lines as markers. Also in lateral roots the major cell types appear to be specified at very early stages of primordium development, well in advance of the occurrence of a root apical meristem.[11] Clonal analysis on lateral roots has not been reported yet. However, based on the regularity of their ontogeny it seems likely that early divisions separate cell types and act as clonal boundaries as in the primary root.

## Flexibility of cell fate

The strong correlation between cell type and embryonic lineage in the *Arabidopsis* meristem could be explained in terms of lineage-dependent development after early and transient instructions on cell fate during embryonic pattern formation. On the other hand, positional signalling might continuously determine cell fate. Such signals would have to act at a single-cell resolution, since many layers in the root meristem comprise only one cell. Being superimposed on an essentially invariant pattern of cell divisions, such a position-dependency would go undetected. The evidence for positional information in the shoot meristem obtained by clonal and chimera analysis and the capacity of roots to regenerate missing cell types form strong precedents to propose a role for signals in the root meristem.[12-14] The clear role of cell-to-cell signalling in the development of the nematode *C. elegans*, which has an invariant cell lineage, also demonstrates that invariant cell lineage does not downplay the importance of position in developmental decisions.[15]

To investigate positional signalling in the *Arabidopsis* root, laser ablation experiments have been performed.[16] Note that cells continuously divide within the root meristem. Therefore, laser ablations can demonstrate the existence of positional signals, but not whether mitotic activity is essential for the competence to respond to these signals. Upon ablation of quiescent centre cells, cells originating from the vascular bundle were displaced toward the root tip. Promoter–marker gene fusions showed that these displaced cells now displayed columella-specific gene expression (Figure 2c). Therefore, the clonal boundary set by the first zygotic division does not restrict the developmental potential of the resulting daughter cells. However, the occurrence of vascular- and columella-marker gene expression in vascular initials and in columella initials demonstrates that initial cells show at least some specification towards particular cell fates.

Upon ablation of cortical initial cells, cells derived from the pericycle but now within the cortical cell layer were capable of switching fate and formed both endodermis and cortex (Figure 2d). Hence the early embryonic divisions in the radial plane, like the first zygotic division in the apical–basal plane, are not instrumental in restricting developmental potential. Since vascular initials and also cortical initials (see later) display tissue-specific gene expression, these cells are, despite their developmental flexibility, specified to some extent, as discussed above for vascular versus root cap cell fates.

In descriptions of animal development, the covert commitment that precedes overt differentiation has been divided into two steps, which are operationally defined by the way in which cells develop in ectopic sites.[17] Cells that develop autonomously in a neutral environment, but switch when subjected to different signals, are named ‘specified’. Cells that do not switch fate when subjected to different positional signals are called ‘determined’. The laser ablations summarized above demonstrate that root meristem initial cells are not determined. The tissue-specific gene expression patterns that persist into initial cells, on the other hand, indicate that cell specification has already taken place. Transplantation of plant cells to a neutral environment would be the experiment to test whether the term ‘specification’ is correct in view of the definition stated above. An analogous experiment is to create a neutral environment within the root meristem. In this vein, cortical initial cells have been deprived from contacts with more mature cells by ablation of daughter cells[16] (Figure 2e). The ablated cells stayed in between the initial cell and more mature cells. The result was that cortex initial cells no longer performed their specific asymmetric division pattern. The disturbance occurred only if the contact with the more mature cortex layer was disrupted, and not if other overlying cells were ablated (Figure 2f). This implies that cortex initial cells cannot differentiate autonomously, and they need reinforcement signals from more mature cortex cells. Hence, although gene expression patterns show that meristem cells are specified to some extent, they appear to continuously require signals. The emerging picture of the root meristem is one in which all cells, including the meristem initials, are at least partly specified but need reinforcement cues from more mature tissue.

These reinforcement cues are able to correct the pattern of cell specification when cells are displaced from one tissue layer to another, either by damage or by occasional aberrant cell divisions.

Until what stage do root cells remain competent to switch fate when they are forced into another position? Analysis of root hair development has given clues to this question. The epidermis in the *Arabidopsis* root is composed of hair- and non-hair cells. Normally, hair cells are located exclusively over a radial cortical cell wall.[7] Hair cells have a more dense cytoplasm as compared to non-hair cells already at a very short distance from the epidermal initial cell, which indicates that they are at least partially specified within the meristem. Analysis of rare, spontaneous longitudinal divisions in the epidermis, giving rise to recognizable 'T-clones', has revealed that clones as small as two cells in the longitudinal dimension can be found, of which one of the cell files has switched fate according to its new position relative to the underlying cortex.[18] This clone size shows that even cells which are about to leave the meristem can switch fate. Thus, several lines of evidence suggest that many if not all meristematic cells remain competent to switch fate. This raises two questions: when are cells stably determined, and what is the source of the positional signals that are present?

When are cells determined? Formally, determination takes place when a differentiated cell loses its competence for interpreting positional signals. Within the root, this may translate to the observation that some differentiated cells become cytoplasmically uncoupled.[19] Hence, differentiated cells would be withdrawn from the population of cells that can respond to patterning information (Figure 3a). However, it is also possible that short-range positional information is generated by a spatially restricted set of root cells. This would be consistent with another model, in which fully differentiated cells are never fully determined, but no longer have access to positional information (Figure 3b).

The chemical nature and the source of positional signals are at present a matter of speculation. If it is assumed that differentiated cells can no longer respond to signals because they are uncoupled (cf. Figure 3a), then it becomes unlikely that these differentiated cells can act as a source of these signals. On the other hand, if this information resides in more mature, but not yet fully differentiated cells within or close to the root meristem, this would be compatible with both models in Figure 3.

## Towards molecules: mutational analysis of root development

Identification of genes that are involved in generating, perceiving and responding to the signals that allocate cells to tissues in the root is a major strategy to unravel the molecular details of this process. Below, the characteristics of a few genes that are required for the formation of specific radial, apical–basal and circumferential elements of the cellular pattern of the root are highlighted.

### *Radial organization*

A number of mutants have been described that alter the radial organization of the root.[20,21] Given the allele frequencies of the identified loci, probably many of these genes are as yet unidentified. This can be attributed to several reasons, among which is the possibility that mutations that severely disrupt radial patterning in the embryo are embryo-lethal. Two mutants, *shortroot* (*shr*) and *scarecrow* (*scr*), are affected in the specification of the separate cell layers of cortical parenchyma and endodermis from the ground tissue (Figures 1, 2b). These layer-specific phenotypes persist in the hypocotyl, and the mutants have an embryonic phenotype. Therefore the corresponding genes appear to be necessary to organize

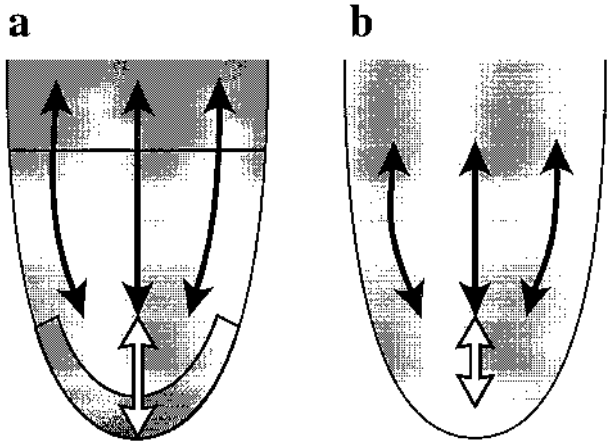


**Figure 3.** Mechanisms for stable cell differentiation. (a) Cell fate signals are ubiquitous but differentiating cells (dark shading) become unresponsive to signals, for example by eliminating cytoplasmic contacts. (b) Short-range cell fate signals arise from specific groups of cells within the meristem. Differentiating cells grow out of the signalling range. Closed arrows: signals for radial pattern elements (e.g. cortical cell types). Open arrows: signals for distal pattern elements (e.g. root cap).

the radial pattern of the complete seedling axis from embryogenesis onward.

Genetic evidence suggests that the *shr* and *scr* mutants disturb two different aspects of the organization of the cortical cell layers. Firstly, the *shr* mutant lacks an endodermis and the mutant cell layer has cortical parenchyma attributes, whereas *scr* mutants have a mutant cell layer that has attributes of both endodermis and cortical parenchyma.[22] Secondly, double mutants of *shr* and a *fass* allele that produces multiple cortical layers do not restore an endodermis, while *scr,fass* double mutants give rise to an inner cell layer with endodermal attributes surrounded by multiple cortical layers without endodermal attributes.[21] These data suggest that the *SCR* gene product is involved in the asymmetric cell division that leads to the separation of the two ground tissue layers. The *SHR* gene product, in this scenario, is a cell specification factor for the endodermis. This outcome suggests that pattern formation in plants is the result of a balanced interplay between genes specifically involved in certain asymmetric divisions, and genes involved in cell specification.

The *SCR* gene has been cloned and it contains a basic domain reminiscent to that in bZIP transcription factors.[22] While this result does not yet lead to an understanding of how this gene is related to mechanisms for specific cell division, the expression pattern of the gene, visualized both by in-situ hybridization and an enhancer trap located 1 kb upstream from the gene, confirms the notion that the gene product is active during embryogenesis in the ground tissue layer. The gene appears to be expressed in the cortical initial and in the endodermal daughters. The enhancer trap expression indicates that the restriction to endodermis is due to restriction of transcription, and not to mRNA partitioning. Interestingly, the enhancer trap is also expressed very early and at the site of the prospective ground tissue in lateral root primordia.[11] This is consistent with the observation that *scr* and other radial mutants display their characteristic phenotypes in lateral roots and in roots derived from callus. Therefore, embryonic gene activities necessary for the correct radial organization of roots are employed again when secondary roots are formed.

Based on these results and those from laser ablation experiments, it becomes attractive to envisage the root meristem as a group of dividing cells that is competent to react to layer-specific signals from more mature cells. These signals, in turn, depend on the correct activity of radial pattern formation genes that are first active during embryogenesis. However, cells in the distal region of the root, containing the quiescent centre and the root cap, are programmed differently since they do not form elements of the radial tissue pattern. Yet the laser ablation experiments show that cell specification within this region also depends on positional information. In the next section, genetic data will be presented that suggest the great importance of this distal region in establishing a root meristem.

### *Apical–basal organization*

The root and root meristem are elements of the apical–basal embryonic pattern. Clonal analyses showed that the boundary between the root and the hypocotyl does correlate, but not with cellular precision, to early embryonic divisions.[9] This indicates that root and hypocotyl fate are only separated at late stages of embryo development, and thus that root and hypocotyl fate are connected during early pattern formation. A few *Arabidopsis* genes have been described which are required for the formation of hypocotyl and root. The effects of mutations in the *MONOPTEROS* (*MP*) gene have been analysed in detail.[23] This gene is required for the formation of both root and hypocotyl in the embryonic context, but not for secondary root formation. The *mp* phenotype further illustrates the intimate relation between embryonic root and hypocotyl specification. Analysis of the embryonic and post-embryonic defects in *mp* alleles suggests a specific role of the *MP* gene in cell axialization of provascular cells in the early embryo and in post-embryonic organs.[24] In *mp* embryos, a lack of axialization of provascular cells apparently interferes with root/hypocotyl specification.

The fate map of the *Arabidopsis* seedling shows that the majority of the primary root cells arise from the root meristem initials which are first defined at the late heart stage of embryogenesis. However, a small region covered with root hairs that connects root and hypocotyl originates from an adjacent region in the embryo and is referred to as the ‘embryonic root’. A genetic screen for mutant seedlings that contain the embryonic root, but lack an organized promeristem has identified a series of independent mutations in the *HOBBIT* (*HBT*) locus (Willemsen *et al*, in preparation). Seedlings homozygous for strong *hbt* alleles display no root meristem activity. All seedlings homozygous for *hbt* alleles have abnormal root meristem anatomy. Mutants homozygous for strong *hbt* alleles contain no differentiated columella root cap, based

on the absence of starch granules. *hbt* mutants show abormalities in division pattern within the hypophyseal cell from the preglobular stage of embryogenesis onward. These aberrations are restricted to the hypophyseal cell at early stages of embryogenesis. At late stages of embryogenesis and in the seedling, the proximal initials, most notably the epidermal initials that should form a lateral root cap, can also become abnormal (cf Figure 1, 2b; Willemsen *et al*, in preparation). This mutant phenotype suggests three functions of the *HBT* gene: (i) specifying hypophyseal cell descendants; (ii) triggering of mitotic activity in the proximal meristem; and (iii) proper formation of a lateral root cap. The second and third function may be a downstream result of the presence of a correctly programmed hypophyseal cell. In summary, *hbt* mutants are defective in the following four distal pattern elements of the root: the columella root cap, the quiescent centre and the lateral root cap (cell types), and the proximal meristem (a collection of cell types distinguished by their mitotic activity).

The root phenotype in *hbt* mutants is not confined to the embryo. Adventitious roots from *hbt* mutants, generated from the hypocotyl of seedlings or via tissue culture, have the characteristic mutant phenotype and arrest development. Therefore the *HBT* gene, unlike the *MP* gene (but reminiscent of the radial patterning genes *SCR* and *SHR*) is not only required for embryonic root formation but also for root formation in all developmental contexts (Willemsen *et al*, in preparation; cf refs 21, 23, 24).

### *Circumferential organization*

Upon specification of the main tissues in the root, they are further sub-specified to give rise to specialized cell types. This aspect of pattern formation is particularly amenable to analysis in the epidermal cell layer. The strikingly consistent patterning of hair- and non-hair epidermal cell files in the *Arabidopsis* root has allowed identification of mutations that disrupt the circumferential pattern within the epidermis. A surprising insight into root hair patterning was obtained when the effect of trichome (leaf/stem hair) mutants[25] on root hair formation was investigated. *transparent testa glabra* (*ttg*) mutants, which have no trichomes, were found to possess ectopic root hairs and, accordingly, densely cytoplasmatic precursor cells in the root meristem.[26] Overexpression of the maize *R* gene (a myc transcription factor homologue) can complement the *ttg* mutation and it therefore may be a homologue of the *ttg* gene. It is of note that *R* overexpression leads to excess trichomes as well as to hairless root epidermal cells.[26] Furthermore, mutants in the *GLABRA2* (*GL2*) homeobox gene which have partly specified, greatly reduced trichomes, have ectopic root hair cells. In contrast to the ectopic hair files in *ttg* mutants, the *gl2* ectopic hair cells are partly specified as non-hair cells because the corresponding cell files are non-vacuolated in the meristematic region.[27] Thus, the *GL2* gene appears to serve a subfunction of the *TTG* gene in a specification of trichomes as well as in the specification of the root hairless cell type. Based on in-situ hybridization and reporter gene fusions, the *GL2* gene is preferentially expressed in the differentiating hairless cell, with a distal expression boundary close to the epidermal initials in the root meristem, and proximally extending well into the elongation zone.[27] Thus, at least two transcription factors required for trichome formation are also involved in hair cell patterning in the root. The surprise is that this transcription factor module specifies hair formation in the shoot whereas it specifies the hairless cell type in the root. It will be interesting to find out whether the *GL2* gene is first expressed during embryogenesis, which would imply that the pattern of hair- and non hair-cells is first laid down in the embryo.

The phytohormone ethylene has been implicated as a positive regulator of hair cell development. First, addition of the ethylene precursor ACC leads to ectopic hairs.[28] Second, blocking either synthesis or the perception of ethylene with AVG or $Ag^+$ leads to hairless cells.[28,29] Third, plants, which are mutant for the *RAF* homologue *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), a negative regulator of ethylene signal transduction,[30] have ectopic hairs.[10] An attractive proposition is that, in normal development, ACC or ethylene reaches epidermal cells through the clefts in the cortical cells at the sites of the radial walls.[28] There it would mediate inactivation of *CTR1* (possible involving a receptor of the *ETR1* family[30]) to trigger root hair formation. *root hair development6* (*rhd6*) mutants have more hairless cells and this phenotype can be rescued by the addition of ACC or ethylene. The *RDH6* gene may be a downstream effector of hair outgrowth in this pathway.[29,31]

What is the relation between the negative (trichome-transcription-factor) and the positive (ethylene-related) pathway to root hair patterning? Treatment of *ttg* and *gl2* mutants with the ethylene blocker AVG, or the construction of double mutants with the (possibly non-null) *rdh6* mutant leads to a reduction but not elimination of root hairs.[31] Hence the

ethylene pathway could be downstream and negatively regulated by *TTG* and *GL2*. Two observations form circumstantial evidence that there is a prepattern of *TTG, GL2* expression with which the hormone pathway interacts. First, the densely cytoplasmic root hair precursor cells are still present in *ctr1* and *rdh6* mutants. Second, the hairless-cell-specific expression pattern of a *pGL2-GUS* function is neither affected by these two mutants nor by the addition of AVG and ACC. These observations are suggestive of a ubiquitous phytohormone-controlled reinforcement signal that is modulated by the *TTG/GL2* prepattern and only involved in one aspect of differentiation: the execution of hair outgrowth (Figure 4, all arrows). However, it cannot be excluded at this point that the ethylene pathway is a spatially restricted patterning activity itself (Figure 4, solid arrow).

## Future prospects

Laser ablation experiments indicate that the determination of cell fates in the *Arabidopsis* root meristem involve the occurrence of 'top-down' signalling, whereby more mature cells provide reinforcement cues to the daughters of initial cells. However, initial cells are not developmentally naive, because they express certain tissue-specific marker genes. Analysis of mutants defective in cell specification in the radial and apical–basal dimension has defined genes that act early during embryogenesis. Taken together, these observations suggest that embryonic pattern formation — and its replay during lateral root formation — sets the stage for cell specification. The information that is first generated there is perpetuated and continuously available to the meristem, enabling dislocated cells to adjust to the existing cellular pattern. Such a mechanism may have evolved to ensure correct pattern formation even when inappropriate cell divisions or damage occurs within the meristem. With the first genes involved in embryonic cell specification at hand, the search for the molecular mechanisms behind this remarkably flexible mode of development can now begin. One of the first questions that arises is whether the same genes that set up the pattern of cell types are involved in the reinforcement process. Continued investigation of the interplay between transcription factors and phytohormone perception pathways may soon elucidate whether specification and reinforcement pathways provide patterning information independently during sub-specification of epidermal cell types.

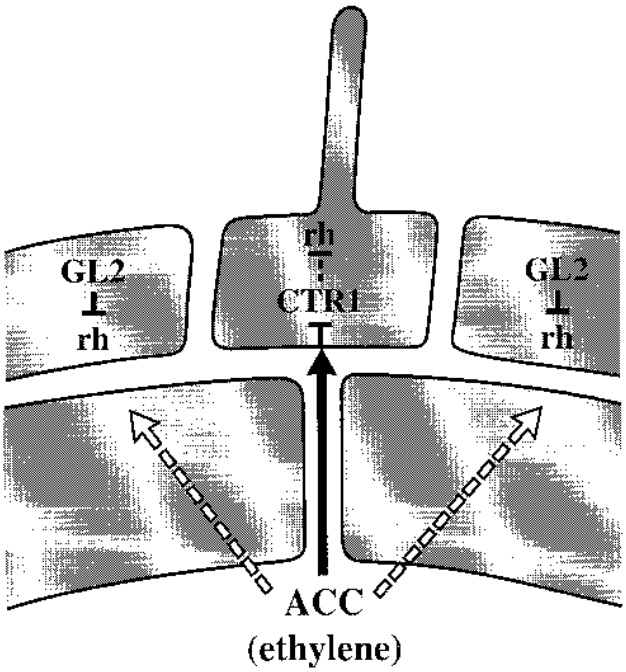


**Figure 4.** Root epidermal hair cell patterning. The *GL2* and *ACC* synthase activities are shown in the regions where they are localized. Arrows: movement of ACC or ethylene as a positive signal for root hair development that releases the *CTR1*-mediated inhibition of root hair formation. This signal may either be present ubiquitously (all arrows), or as an independent spatially restricted patterning clue (black, solid arrow only).

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