

Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*

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SUMMARY

Lateral root formation in plants involves the stimulation of mature pericycle cells to proliferate and redifferentiate to create a new organ. The simple organization of the root of *Arabidopsis thaliana* allows the development of lateral root primordia to be characterized histologically. We have divided the process of lateral root development into 8 stages defined by specific anatomical characteristics and cell divisions. To identify the cell types in the developing primordium we have generated a collection of marker lines that express β -glucuronidase in a tissue- or cell type-

specific manner in the root. Using these tools we have constructed a model describing the lineage of each cell type in the lateral root. These studies show that organization and cell differentiation in the lateral root primordia precede the appearance of a lateral root meristem, with differential gene expression apparent after the first set of divisions of the pericycle.

Key words: cell lineage, enhancer trapping, meristem, organogenesis, radial organization, patterning, *SCARECROW*

INTRODUCTION

One of the fundamental questions in developmental biology is how cells proliferate and organize to form discrete organs. Unlike animals, where organogenesis occurs primarily in the embryo, normal growth in plants involves both embryonic and postembryonic organogenesis. The primary shoot and root apical meristems, which are responsible for the growth of the primary shoot and root, form as part of the developing embryo. After germination, shoot buds are formed in the axils of leaf primordia as the shoot grows (Esau, 1965). These axillary shoot buds appear to be composed of undifferentiated cells that can be triggered to become vegetative meristems, which are then responsible for the lateral proliferation of the shoot system (Esau, 1965; Steeves and Sussex, 1972). In contrast, no secondary buds are laid down as the root grows. As a result, the root system must proliferate via meristems formed from non-meristematic tissues.

Histological studies have shown that, in angiosperms, lateral roots are derived from the pericycle layer deep within the parent root tissues. There is also a contribution from dividing endodermal cells in some monocots (McCully, 1975). The initiation of lateral roots occurs some distance away from the root apical meristem in the differentiation zone of the root, where the pericycle cells are not actively dividing (Esau, 1965). The mature pericycle cells, once stimulated, dedifferentiate and proliferate to form a lateral root primordium (LRP). The LRP grows through the overlying cell layers of the parent root and eventually breaks through the epidermis and emerges. At some point during LRP development an active lateral root apical meristem must form. Little information is available about the events involved in either the initiation or development of the LRP. The pericycle cells that become 'founder'

cells for the LRP are positioned at the xylem poles, but it is difficult to predict which pericycle cells along the length of the root will be recruited to the lateral root program (Charlton, 1991).

Development of the LRP involves several stages which may be regulated by distinct pathways. There is evidence that stimulated pericycle cells begin to lose their mature characteristics, or dedifferentiate, even before cell division begins (Foard et al., 1965; McCully, 1975). This suggests that changes in cell size and shape are separable from cell division in LRP formation. Furthermore, several mutants have recently been isolated in *Arabidopsis thaliana* that initiate LRP which fail to develop beyond a certain point, again indicating multiple phases in the development process (Celenza et al., 1995; Cheng et al., 1995). In support of this notion, other studies in *Arabidopsis* have shown that, at a certain stage, the LRP becomes competent to continue development when explanted to hormone-free media (Laskowski et al., 1995). Based on these findings, it has been suggested that LRP formation can be thought of as a two stage process: (1) stimulation of dedifferentiation and proliferation in the pericycle layer to form the LRP; (2) redifferentiation to form a lateral root meristem, which establishes and perpetuates the organization of the lateral root (Celenza et al., 1995; Cheng et al., 1995; Laskowski et al., 1995).

We are attempting to understand these complex developmental events in the model dicot *Arabidopsis*. The root of *Arabidopsis* provides an excellent system for the study of organ development. The arrangement of cells in both the primary and lateral roots is simple and predictable (Dolan et al., 1993). The outer four radial layers, each one cell thick, are (from the outside) the epidermis, cortex, endodermis and pericycle (Fig. 1A). The pericycle forms the outermost layer

of the stele, which also contains phloem, xylem, and stelar parenchyma cells (not shown). Each cell type forms vertical files of cells that can be traced to meristematic initials in the root apical meristem (Fig. 1B). The initials are responsible for maintaining the cellular organization of the root. Each time they divide they regenerate the initial and add one cell to the vertical cell file. There are four sets of meristematic initials in *Arabidopsis* roots: one that forms the epidermis and lateral root cap (Fig. 1B); one that forms columella root cap (not shown); one that produces both the cortex and endodermal layers (Fig. 1B) and one that produces the cells of the stele (not shown) (Dolan et al., 1993). In the primary root, these initials appear to be established during embryogenesis (Scheres et al., 1994). In contrast, the meristematic initials in the lateral roots must form postembryonically. In this paper we use the term root apical meristem to refer to these sets of meristematic initials and the actively dividing cells adjacent to them.

As a first step to understanding lateral root patterning and development, we have taken advantage of the simplicity of the *Arabidopsis* root structure to define anatomically a series of discrete developmental stages in LRP development. We have complemented these studies by generating a collection of marker lines that express β -glucuronidase (GUS) in a cell type-specific manner in each of the cells of the root. This allows the differentiation state of cells to be established based on molecular characteristics. Furthermore, the marker line expression patterns can be used to follow the fates of cells in the developing LRP. Our results show that the LRP forms through a highly ordered series of divisions that generates a structure with a radial organization similar to that of the mature root tip. The cells within the LRP become non-identical after the first periclinal division (Stage II) and begin to acquire characteristics of their mature cell fate during the early stages of LRP development. Based on these findings we propose a new model of the key stages in lateral root formation.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis seeds of ecotypes WS, NO-O and Columbia were sterilized and sown on nutrient agar medium (Murashige and Skoog) plates containing 4.5% sucrose. Plates were oriented vertically to ease the observation and removal of intact roots. Growth conditions were 18 hours light, 6 hours dark, 22°C.

Histology and histochemistry

1- to 3-week old seedlings were stained for GUS activity for up to 3 days in the following solution: 1× GUS buffer, 20% methanol, 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronidase). Addition of methanol greatly improves the specificity and reproducibility of staining (Kosugi et al., 1990). Staining solution was made fresh from a 10× buffer that was stored in darkness for no more than one week. 10× GUS buffer: 1 M Tris pH7.5 containing 29 mg/ml NaCl, 6.6 mg/ml $K_3Fe(CN)_6$.

For observation of whole mounts, both stained and unstained roots were transferred to small Petri dishes containing 0.24 N HCl in 20% methanol and incubated on a 57°C heat block for 15 minutes. This solution was replaced with 7% NaOH, 7% hydroxylamine-HCl in 60% ethanol for 15 minutes at room temperature.

(The hydroxylamine can be omitted with no decrease in effective clearing). Roots were then rehydrated for 5 minutes each in 40%, 20% and 10% ethanol, and infiltrated for 15 minutes in 5% ethanol, 25% glycerol. Roots were mounted in 50% glycerol on glass microscope slides.

For transverse sections, samples were fixed and embedded in Historesin as described by DiLaurenzio et al. (1996).

All samples were observed using Nomarski optics on a Leitz Laborlux S microscope. Photographs were taken using a Leitz MPS52 camera, and images were scanned into Adobe Photoshop 3.0 to create figures. In some cases the intensity of the blue color was enhanced.

Enhancer trap lines

Plant Cloning Vector (PCV) (Koncz et al., 1994) contains a *Bam*HI site immediately adjacent to the Right Border sequences. The β -glucuronidase (*uidA*) coding region fused to the TATA region (-46 to +8) of the Cauliflower Mosaic Virus (CaMV) 35S promoter were introduced into this site (Benfey et al., 1990). 300 transgenic lines were generated by root transformation of WS and NO-O ecotypes as described by Marton and Browse (1991), and 4 independent lines from each transformant were screened for GUS activity in the root.

Cortex and epidermis marker lines were provided by Drs J. Harada and W. Rerie, respectively. Construction of these lines has been described (Dietrich et al., 1992; Masucci et al., 1996).

RESULTS

Developmental stages of lateral root formation

Roots of *Arabidopsis* seedlings were cleared and observed in whole mounts in order to define an index of LRP development. The process has been divided into 8 stages (Stage I-VII and Emergence), shown in Fig. 2A-K.

Stage I. The first evidence of LRP initiation is the appearance of closely spaced cell walls in the pericycle layer in perpendicular orientation to the root axis (arrows, Fig. 2A). An increased frequency of anticlinal divisions is clearly seen as compared to the pericycle cells at the opposite side of the stele. In the longitudinal plane, approximately 8-10 'short' pericycle cells are formed, which enlarge in a radial direction.

Stage II. A periclinal division occurs that divides the LRP into two layers (outer layer (OL) and inner layer (IL)) (Fig. 2B). Not all the small pericycle-derived LRP cells appear to participate in this division; typically the most peripheral cells do not divide. Hence, as the OL and IL cells expand radially the domed shape of the LRP begins to appear.

Stage III. The OL divides periclinaly, generating a three layer primordium comprising OL1, OL2 and IL (Fig. 2C). Again, some peripheral cells do not divide, creating outer regions that are one and two cell layers thick. This further emphasizes the domed shape of the LRP.

Stage IV. The IL divides periclinaly, creating a total of four cell layers (OL1, OL2, IL1, IL2) (Fig. 2D). At this stage the LRP has penetrated the parent endodermal layer.

Stage V. A central cell in OL1 and OL2 divides anticlinaly to form four small cuboidal cells (Fig. 2E, arrow; cells 5 and 6 in Fig. 2F). The cells adjacent to these two cells in the OL1 and OL2 also divide (arrows, Fig. 2F), creating an outer layer (OL1) that contains 10-12 cells (Fig. 2F). In addition, cells in IL2 enlarge radially and divide (short arrow, Fig. 2F), pushing

the overlying layers up and apparently compressing the cells in IL1 and OL2. The LRP at this stage is midway through the parent cortex.

Stage VI. This stage is characterized by several events which appear to occur at approximately the same time. (1) Cells of OL2 undergo a periclinal division, creating a new internal layer (Fig. 2G, arrows). These layers are designated OL2a and OL2b. (2) The four central cells of OL1 divide periclinally (Fig. 2H, arrow). (This division is particularly useful in identifying the median longitudinal plane in the enlarging LRP.) After this division, OL1 at the tip of the primordium is considered to be the inner of these two layers. At this point there are a total of twelve cells in OL1, four in the middle that have undergone the periclinal division and four on either side (Fig. 2G,H). The LRP has passed through the parent cortex layer and has penetrated the epidermis. Note that the cells at the core of the LRP, apparently derived from IL2, have a distinct elongated shape characteristic of vascular elements (Fig. 2H, arrowhead).

The Stage VI LRP begins to resemble the mature root tip (compare with Fig. 1B), containing 3 layers that could correspond to epidermis, cortex, endodermis surrounding a core of presumptive stelar tissue, and a potential root cap at the tip of the LRP.

Stage VII. As the primordium enlarges it becomes more difficult to characterize the divisions, particularly in the internal layers. It appears that many of the cells of the LRP continue to undergo anticlinal divisions. In the OL1, this results in 8-10 cells on either side of 8-10 central cells (Fig. 2I). We refer to this as the 8-8-8 cell pattern. The LRP appears to be just about to emerge from the parent root.

Emergence occurs primarily through cell expansion

The cell number in the OL1 of the LRP at Stage VII is nearly invariant and therefore useful for monitoring subsequent cell divisions during emergence. As shown in Fig. 2J,K, the LRP

clearly increases in overall length as it emerges from the parent root. Occasionally the basal OL1 cells appear to divide anticlinally, and the number of small cells near the LRP tip may increase by 2 or 4. However, the increase in the length of the LRP appears to be largely due to enlargement of the basal cells. This enlargement can be seen by comparing the OL1 cells in Fig. 2I,J,K, and L. Therefore, it seems that the LRP emerges from the parent root primarily by expansion of the preexisting cells rather than by cell division.

Functional meristem formation

After emergence the basal cells in the OL1 continue to expand, but in addition the cell number begins to increase, with new smaller cells appearing near the apex (Fig. 2L). Hence, the peripheral regions of the OL1 still contain 8-10 greatly elongated cells, while the number of cells close to the central region of the LRP tip increases. Thus, it appears that the root is growing via divisions in cells at the apex, suggestive of a functional lateral root apical meristem. We refer to the organ at this stage as a lateral root (LR) rather than an LRP, to indicate that at this point meristematic initials are contributing to the increase in root length.

Generation of a collection of root cell type-specific marker lines

There are few visible characteristics that allow the cell types of the LRP to be distinguished from each other at early developmental stages. The position of cells in the LRP is suggestive of their fate. However, cell type-specific markers are needed to confirm these predictions. In addition, histology cannot address when cells in the LRP begin to be distinct from one another and when they begin to acquire mature characteristics – in other words, distinguish between cell proliferation and differentiation. To address these issues we have created a collection of marker lines that each express GUS activity in a different cell-type. These lines should allow identification of differentiated cells even if they are morphologically similar.

An enhancer trapping cassette was created by fusing the GUS coding sequence to the TATA region (−46 to +8) of the 35S promoter from CaMV. This minimal promoter does not confer detectable GUS expression. However, its presence allows other upstream elements to direct GUS expression in a developmental and/or cell-specific manner (Benfey et al., 1990). The use of a minimal promoter instead of a promoterless construct allows GUS expression to occur even if the enhancer trap cassette inserts at a distance from the enhancer element. Furthermore, since the insert does not have to be within the structural gene we do not necessarily expect mutations to be generated in the enhancer trap lines. The minimal promoter:GUS construct was cloned immediately adjacent to the Right Border of PCV (Koncz et al., 1994 and Methods), and introduced into *Arabidopsis* via *Agrobacterium*-mediated root transformation (see Materials and Methods). 300 independent lines were generated and stained for GUS activity in the root. From these lines we assembled a collection of useful markers.

Mature stained root tips of each marker line are shown in Fig. 3. Optical sections of cleared whole mounts were used, as

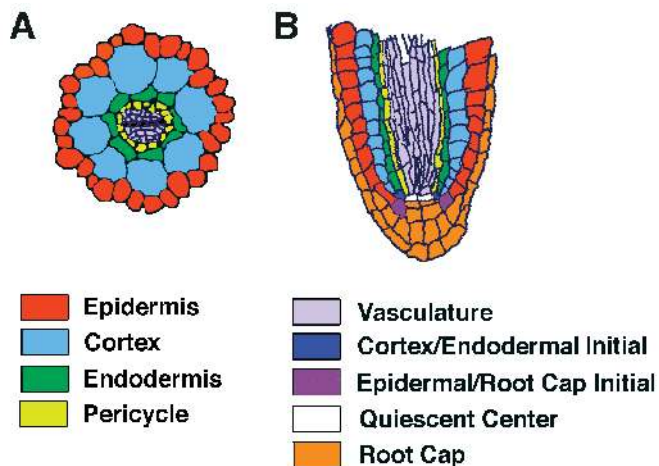


Fig. 1. The primary root of *Arabidopsis*. (A) Schematic of a transverse section was made by tracing cells from an image of sectioned material. (B) Schematic of a longitudinal section was made by tracing cells from a Nomarski image of a whole mount. Cell types are color coded as defined in the figure. Similar schematics were used by DiLaurenzio et al. (1996).

they are particularly useful in identifying the nature of staining patterns in three dimensions, especially in the proximity of the meristematic initials which are difficult to visualize in longitudinal sections. Transverse sections were also made to confirm the boundaries of the stained tissues. All of the lines discussed in this paper were generated through enhancer trapping, as described above, except for CorAx92 (Dietrich et al., 1992) and EpiGL2 (Masucci et al., 1996), which are transgenic plants that contain cell type-specific cis-elements fused to the GUS coding region.

The following lines most clearly define each cell type:

Ste05 expresses GUS in the stele, including the pericycle layer, throughout primary and lateral roots (Fig. 3A). At the root tip, staining becomes difficult to detect in the elongation and meristematic zones; therefore, it is likely that only differentiated stele cells express GUS activity. Expression is also seen in the vasculature of aerial parts of the plant. A transverse section is shown in 3B.

End195 expresses in the endodermis of primary and lateral roots (Fig. 3C). Staining can be seen most clearly in cells in the meristematic zone of the root, although overstaining also reveals expression in more mature cells. Staining towards the root tip includes the first endodermal daughter cell of the cortex/endodermal initial, and may also include the initial itself (asterisk). Expression is also seen at the base of young leaves and in the stipules. A transverse section is shown in Fig. 3D.

End199 expresses in the endodermis of primary and lateral roots, again most clearly in cells in the meristematic zone (Fig. 3E). Unlike End195, staining in End199 appears to include both the cortex/endodermal initial (asterisk) and, in younger roots, the cells of the quiescent center. There sometimes also appears to be lower level expression throughout the stele. Expression is also observed in young leaf primordia. A transverse section is shown in Fig. 3F.

CorAx92. This line was generated by fusing the 5' and 3' sequences from a cortex-specific gene isolated from oilseed rape to the GUS coding sequence (Dietrich et al., 1992). Root expression is strongest in the meristematic zone and is limited to the cortex layer, extending to but not including the cortex/endodermal initial (asterisk) (Fig. 3G). Staining is also apparent in the petioles and leaf blades of expanded leaves. A transverse section is shown in Fig. 3H.

EpiGL2. This line was generated by fusing the *GL2* promoter to the GUS coding sequence (Masucci et al., 1996). Expression is seen in the non-hair forming epidermal cells (atrachoblasts), resulting in a striped pattern in the epidermal layer (Masucci et al., 1996; Fig. 3I). Staining is strongest in the meristematic zone, including the first daughter cell of the epidermal/lateral root cap initial and perhaps the initial (asterisk) as well. Staining is also seen in the trichomes, leaf primordia, and the epidermis of the hypocotyl and leaf petioles. A transverse section is shown in 3J.

CRC219 shows expression in the columella root cap only (Fig. 3K).

LRC244 shows expression in the lateral root cap only (Fig. 3L).

Using marker lines to understand LRP organization

The marker lines described above allow the identification of

specific tissues and cell types in mature roots. The appearance of staining in specific cells in the LRP of a marker line indicates that these cells display a gene expression pattern characteristic of a mature cell type. Hence, it is likely that these cells represent the progenitors of that tissue. (The developmental stage at which staining is first apparent in the LRP differs for each marker. This does not necessarily reflect the onset of cell differentiation in the LRP, which may occur earlier, but only the onset of detectable expression of one cell type-specific gene.) We have analyzed staining patterns in the LRP of the marker lines in the context of our histological studies to create a lineage map of each cell type in the lateral root.

Epidermis

The epidermal marker line EpiGL2 stains 2-4 cells in the OL1 of the LRP at Stage VI (Fig. 4A). Staining is not continuous around the perimeter of the LRP, but excludes the 4 cells at the center that have undergone a periclinal division. At later stages, the staining pattern is clearly limited to the peripheral cells in OL1 (Fig. 4B,C).

Cortex

Cortex marker line CorAx92 stains 2-4 cells in the OL2 at Stage VII (Fig. 4D; it is difficult to distinguish OL2a and OL2b

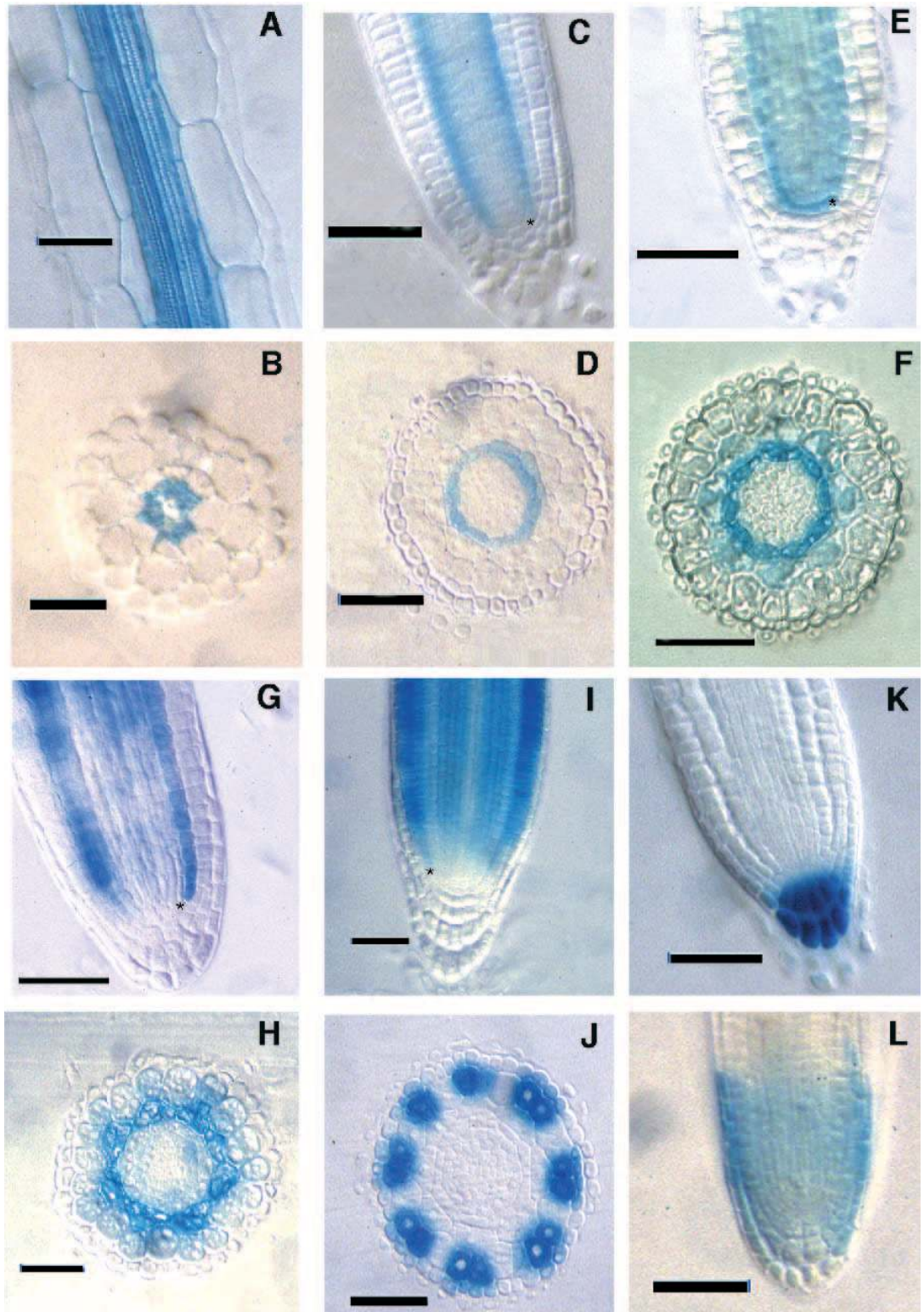
Fig. 2. Stages of lateral root primordium development. Shown are Nomarski images of cleared whole mounts of 2- to 6-week old roots. (A) Stage I. Arrows point to new cell walls indicating anticlinal divisions in the pericycle. (B) Stage II. A periclinal division has divided the LRP into two layers, outer layer (OL) and inner layer (IL). (C) Stage III. A periclinal division in OL has created a total of three layers. (D) Stage IV. A periclinal division in IL creates a fourth layer. (E) Stage Va. Arrow indicates a single anticlinal division in the center cells of OL1 and OL2. (F) Stage Vb. Arrows indicate two additional anticlinal divisions in OL1 and OL2. The short arrow indicates the region in which cells of the IL2 undergo expansion and division, distorting the shape of IL1 and OL2. Cells in the outermost layer are numbered to indicate the constant organization at this stage. (G) Stage Via. A periclinal division in all but the center cells of OL2 creates a new tier of cells (arrows). The two tiers are designated OL2a and OL2b. (H) Stage Vlb. Four central cells in OL1 divide periclinaly to create another new tier at the tip of the primordium (arrow). Numbering shows that there are now four cells in OL1 on either side of four divided central cells. The OL1 is considered to be the inner of these two layers of divided central cells. The arrowhead points to the elongated cells reminiscent of vascular elements. The events shown in G and H may not necessarily occur sequentially; they are shown in two panels for clarity. (I) Stage VII. All the cells in the OL1 have undergone anticlinal divisions, as evidenced by the cell shape and the increased number of cells in this layer. This gives the characteristic 8-8-8 cell pattern, as indicated by cell numbering. In the central region of the tip of the primordium, numbers are on top of the relevant cells. (J,K) Emerging LRP. Note that the cells in the OL1 are enlarged. The number of cells near the apex has increased slightly, potentially indicating the first divisions of meristematic initials. (L) Fully emerged LR. Note the gross increase in size that has occurred in the basal cells of the OL1. Cell numbering indicates that there are now more cells in this layer near the root tip, consistent with the presence of an active lateral root apical meristem. Bar, 50 μ m.

in this image). At emergence, staining appears to extend throughout the majority of the OL2a (Fig. 4E,F). As in the epidermal marker line, staining does not include the cells in the center of the layer.

Endodermis

Endodermal marker line End195 initially stains 2-4 cells of the OL2 at Stage V (Fig. 4G). After OL2 undergoes a periclinal division staining persists most strongly in cells of the OL2b,

Fig. 3. GUS expression patterns in mature root tips of the marker lines. Shown are Nomarski images of mature root tips from tissue or cell type-specific marker lines stained for GUS activity. A,C,E,G,I,K and L are cleared whole mounts. There is always some difficulty in observing staining patterns in whole mounts, as in some cases it is necessary to focus through strongly stained layers to visualize internal layers. Therefore, transverse sections are also shown (B,D,F,H,J). In some cases the sections are near the root tip; therefore, one or more layers of lateral root cap cells can be seen external to the epidermal layer (D,F,H,J). (A) Ste05. Staining is in the vasculature in a mature region of the root. There is no staining at the root tip (not shown). (B) Transverse section of mature region of Ste05. (C) End195. Staining is specific to the endodermis, and may extend to the cortex/endodermal initial (asterisk). (D) Transverse section of End195 near the root tip. The section is slightly oblique, such that cells from two layers of endodermis can be seen on the right side of the image. (E) End199. Staining is strongest in the endodermis, including the cortex/endodermal initial (asterisk) and quiescent center. There appears to be fainter staining in the stele. (F) Transverse section of End199 near the root tip. A similar image appears in DiLaurenzio et al., 1996. (G) CorAx92. Staining is specific to the cortex, extending up to but not including the cortex/endodermal initial (asterisk). (H) Transverse section of CorAx92 near the root tip. (I) EpiGL2:GUS. Staining is in the atrichoblasts, a subset of epidermal cells. Staining extends up to and may include the epidermal/lateral root cap initial (asterisk). (J) Transverse section of EpiGL2:GUS near the root tip. (K) CRC219. Columella root cap staining. (L) LRC244. Lateral root cap staining. Bar, 50 μ m.



excluding the cells in the center of the layer (Fig. 4H,I) The single cell at the tip of the root primordium that abuts the OL2a and OL2b layers also appears to stain.

Stele

Stele marker line Ste05 stains in the center of the base region of the emergent LRP (Fig. 4J).

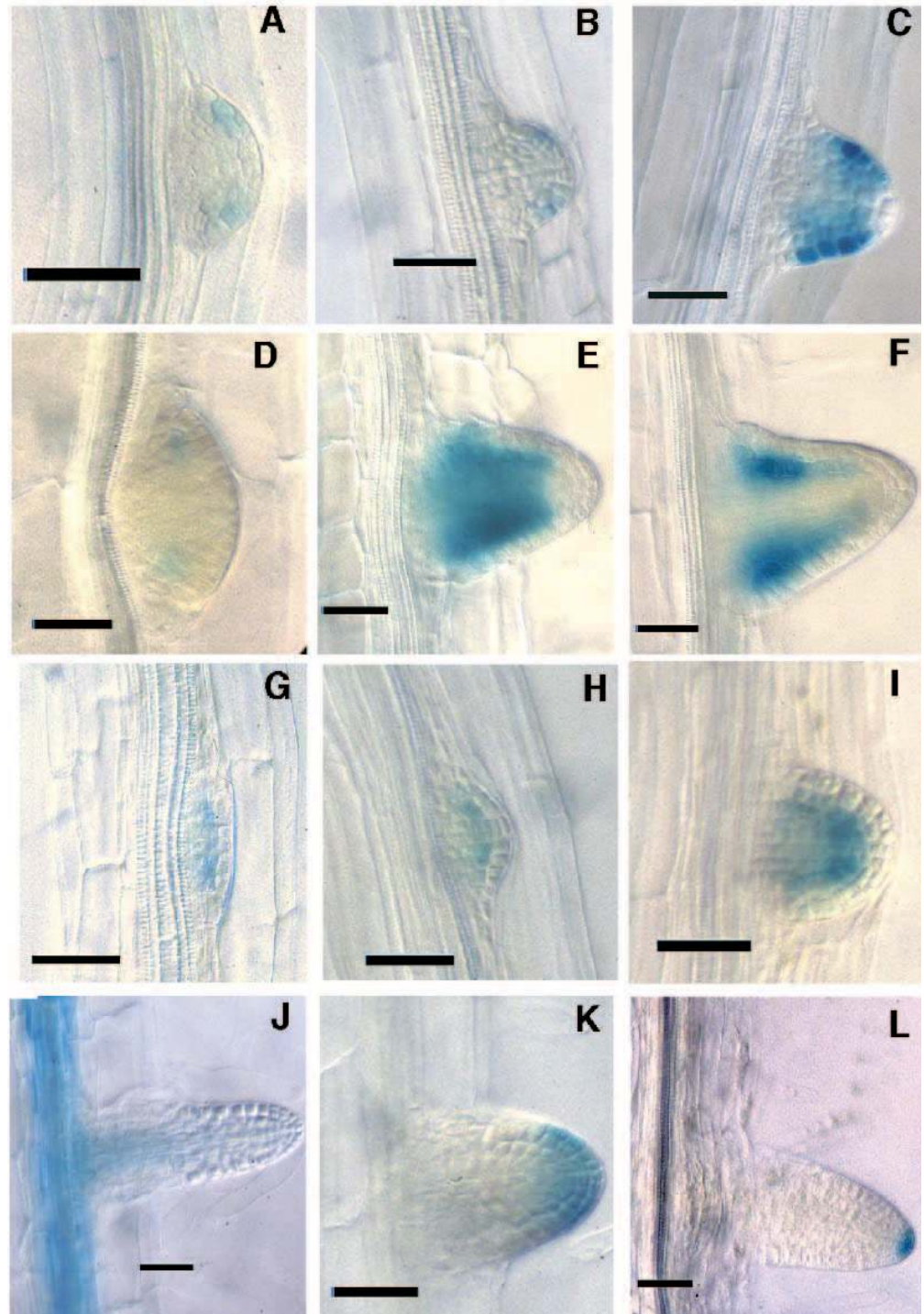
Cap

Lateral root cap marker line LRC244 stains a dome of cells in

the outermost layer at the tip of the emergent LRP (Fig. 4K). The stained region does not include the cells stained in the epidermal marker line. As the LRP develops into an LR, a small number of cells at the very center of the tip are excluded from staining in LRC244 (not shown). These unstained cells correspond to the cells that stain in the columella root cap marker line, CRC219 (Fig. 4L), suggesting that the two regions of the root cap may become distinct at this point.

These results support the prediction that by Stage VI the LRP has a radial organization similar to that of the mature root

Fig. 4. GUS expression in developing lateral root primordia in the marker lines. Shown are Nomarski images of lateral root primordia from tissue- or cell type-specific marker lines stained for GUS activity. (A-C) EpiGL2. (A,B) Stage VI and VII. Staining appears in OL1 cells adjacent to the central four, divided cells. (C) Emergence. Staining is strong in 4 enlarged cells on either side of the divided central cells. (D-F) CorAx92. (D) Stage VII. Staining first appears in two OL2 cells. (E) Emergence. Staining is seen in a file of cells in the OL2. The region at the tip of the primordium does not stain. (F) At this stage it is clear that staining is limited to OL2a, the outer tier of cells formed after the periclinal division in OL2 at Stage VI. (G-I) End195. (G) Stage V. Staining is seen specifically in two cells in the OL2 at either side of 2-4 central cells. (H) Stage VI. Staining is in four cells of the OL2, two on either side of two central, unstained cells. It is likely that the two stained cells derive from the single stained cells in Stage V. (I) Stage VII. Staining includes a file of cells. The shape and position of these cells confirms that they are in the OL2b layer, the inner tier formed after the periclinal division of OL2 at Stage VI (compare to CorAx92). The single cell that abuts OL2 and OL2b also appears to stain. (J) Ste05 postemergence. Staining is seen in the vasculature of the primary root extending into the core of the emergent LRP. (K) LRC244 at emergence. Staining is strongest throughout the domed outermost layer of the LRP, surrounding the other cells at the LRP tip. (L) CRC219 postemergence. Staining is apparent in a small number of cells at the very tip of the primordium. Bar, 50 μ m.



tip. Cell layers appear histologically to be organized into a root cap and a single layer of epidermis, cortex and endodermis surrounding a central stele. Cell type-specific markers for each of these cell types are expressed in the predicted layer. The expression patterns of GUS in epidermis, cortex and endodermal marker lines are summarized in a schematic form in Fig. 5. Correlating these expression patterns with the histologically defined stages of development (Fig. 2) allows the lineage of each cell type to be traced back through the earliest stages of LRP formation (see Discussion).

Onset of differentiation in the lateral root primordium

The marker lines described in the previous section, EpiGL2, CorAx92 and End195, provide evidence that cells of the LRP are non-identical at early developmental stages, since adjacent cells are showing differential gene expression. Two additional marker lines show differential staining at even earlier stages of LRP development. One of these, LRB10 (lateral root base), does not express GUS in the primary root tip at all (not shown). Staining is apparent in all cells of the LRP at Stage I (Fig. 6A), and Stage II (not shown). However, by Stage IV only the cells at the periphery of the LRP are still expressing GUS (Fig. 6B). As the LRP develops, these cells continue to stain, although less intensely, resulting in a ring of GUS-expressing cells at the base of the LRP (Fig. 6C-E) and of the LR (not shown).

Another line, End199, presents a different early expression pattern. Staining is first apparent at Stage II in only the three central cells of the OL (Fig. 6F). As the LRP reaches Stage V the staining remains strongest in the central 4 cells of OL2 (Fig. 6G,H). By Stage VII staining also includes the newly formed OL2b (Fig. 6I), and staining in both this layer and the central cells persists beyond emergence (Fig. 6J).

The GUS expression patterns of LRB10 and End199 are summarized schematically in Fig. 7. These expression patterns clearly demonstrate non-identity between LRP cells at very early stages, Stage IV in the case of LRB10 and within the OL at Stage II in End199. Staining in postemergent lateral roots indicates that the cells at the circumference of the LR in LRB10 (not shown) and the central cells in the End199 root (Figs 6J, 3E) continue to show GUS activity at maturity. This suggests that at the early stages of LRP development, cells are not only becoming non-identical but are also gaining attributes that reflect their differentiated cell fates. These observations suggest a very early onset of differentiation in the cells of the LRP.

DISCUSSION

Organization and cell lineages in lateral root primordia

Arabidopsis roots have a simplicity of structure that makes it possible to define developmental stages in lateral root primordia by histological observations. The LRP develops through a consistent series of divisions, in which a multilayered structure is created and then subdivided to produce all of the cell layers present in a mature root. The ordered nature of these divisions suggests that there is no stage at which pericycle cells are proliferating in an unorganized manner.

At later developmental stages the organization of the LRP

resembles that of the primary root tip, such that the identity of many of the cells can be tentatively assigned. The creation of tissue- and cell type-specific marker lines makes it possible to confirm the identity of these cells. The origin of each cell type can then be inferred by tracing its lineage back through histologically defined stages. Together, our histological studies and marker line staining patterns lead to predictions of the lineage of each cell type in the developing LRP. Although these lineages need to be confirmed by dynamic cell-fate analysis, we can formulate a working model, as shown in Fig. 8. In this model, the epidermis is derived from the OL at Stage II, and from the OL1 after the subsequent periclinal division. Characteristic anticlinal divisions in this layer are denoted with arrows (Va and b, Fig. 8). Cortex and endodermis are both derived from the Stage II OL, and from OL2 after the Stage III division of OL. Cortex and endodermis become distinct from each other via a periclinal division at Stage VI (arrow, VIb in Fig. 8). Note that at this and all subsequent stages the putative endodermal and cortical layers abut a single cell at the tip of the primordium which is in a position similar to that of the cortex/endodermal initial in the mature apical root meristem (shown in dark blue at Stage VIb; compare to Fig. 1B). The cells of the pericycle and stele appear to derive from IL in Stage II. The pericycle probably arises from IL1 after the periclinal division of IL at Stage IV, while the other stele cells arise from proliferation in IL2. The IL2 cells enlarge (arrowhead, Va in Fig. 8) and undergo extensive divisions and take on an elongated shape (VIa and b, Fig. 8) characteristic of vascular elements. The root cap is defined at Stage VI (arrow, VIa in Fig. 8) via a periclinal division in OL1.

The cluster of cells at the tip of the LRP which do not express GUS activity in the epidermal, cortex or endodermal marker lines (white in Fig. 8, Stages VI and VII) are situated to form the quiescent center and potentially contribute to the initials of the new apical meristem. Of all of our marker lines, only End199 stains in these cells. In fact, End199 staining in the putative precursors of these cells at Stage II is the earliest observed instance of differential expression within the LRP. Fortuitously, we discovered that the GUS cassette in End199 is situated approximately 1 kb upstream of the start of translation of the *SCARECROW* (*SCR*) gene (DiLaurenzio et al., 1996). In situ RNA analyses indicate that the GUS patterns in End199 accurately reflect expression of the *SCR* gene (Helariutta and Benfey, unpublished). Mutants in the *SCR* gene are completely lacking one of the radial layers between the epidermis and pericycle in both primary and lateral roots, apparently because the periclinal division of the cortex/endodermal initial that forms the two cell files does not take place (Scheres et al., 1995; DiLaurenzio et al., 1996). The expression of *SCR* in the central cells of the OL at Stage II is consistent with a regulatory role for *SCR* in radial organization, and suggests that these central cells may play an important role in organization of the LRP at very early stages.

Cell differentiation

Even the earliest stages of LRP development are characterized by highly consistent periclinal and anticlinal divisions. The LRP can therefore be thought of as an organized structure from initiation onwards. However, this does not necessarily indicate

that the cells within that structure have unique identities or that they have differentiated into specific cell types. The marker lines can provide information about the onset of cell differentiation as GUS expression in a subset of LRP cells indicates that different molecular events are occurring in these cells as compared to their neighbors.

As noted above, the earliest observed differential expression is in End199 at Stage II. Expression is in the central cells of OL, indicating not only that the two layers of cells in the Stage II LRP are non-identical, but also that cells have different identities within a single layer. Early differential staining is also seen in LRB10 at Stages IV and in End195 at Stage V. Although in some cases we do not know the identity of the genes whose expression are reflected in the marker lines, it is clear that the same genes are specifically regulated in the mature root. Therefore, it is likely that the onset of staining indicates that cells are beginning to acquire mature characteristics. Based on these observations, it appears that differentiation of LRP cells begins in the earliest stages of LRP formation.

Early activity of the LR apical meristem

It is clear that both organization and cell differentiation in the LRP occur very early in development, and therefore precede the formation of a lateral root apical meristem. However, in a mature root, it is divisions of initials in the meristem that maintain the organization of the root by generating ordered files of cells of each type. Therefore, at some time in lateral root formation a meristem must be established to fulfill this function. We define a functional meristem as a group of cells which includes a set of stem cell-like initials, cells that divide to regenerate themselves and produce new cells of a particular cell type. There are also some stereotypical patterns of division that are hallmarks of the initials of the mature root apical meristem. For example, the epidermal/lateral root cap initial undergoes a periclinal division to generate both epidermal cells and cells of the lateral root cap. The cortex/enodermal initial undergoes a periclinal division to generate cortex and endodermal cell files.

Before Stage V, it is relatively easy to observe the cell divisions in the LRP, and it appears that there are no particular cells that serve as initials. However, at Stage VI divisions become more complex and difficult to follow. Furthermore, between Stage VI and Stage VII the number of small cells at the tip of the primordium in the OL1 doubles from 4-8, and the cell number in OL2 appears to undergo a similar increase. It is also at Stages VI and VII that files of cells of specific cell types can be distinguished in the marker lines. Since many cells of the LRP are actively dividing at Stages V-VII, the definitive question is whether there are any cells that can be identified as initials during these stages.

In the absence of specific markers for initials, it is difficult to resolve this question. For the epidermal, cortical and endodermal marker lines, staining is first observed in a small number of cells (2-4 cells in a median longitudinal section). It is possible that these cells will be the first to function as initials, giving rise to all the cells that will show GUS staining at subsequent stages and maintaining themselves in a meristematic state. Even if this is the case, the putative 'initials' in the early LRP do not resemble the initials in the mature root tip. In the

OL1, the first cells that stain in the epidermal marker line, EpiGL2, are not derived from a cell that generated both the epidermis and lateral root cap, nor do they appear to undergo a periclinal division to generate these two tissues. Nevertheless, these cells could form a 'temporary meristem', and generate the first files of differentiated cells in the LRP at Stages VI and VII. Hence, radial patterning would be established via divisions in an apical meristem. An alternative model is that the files of cells that stain in the marker lines differentiate in response to positional cues, and are derived from anticlinal and periclinal divisions throughout the LRP. In this scenario, no active meristem is established until later (see below). This is analogous to embryonic root development, in which radial organization precedes active meristem formation (Scheres et al., 1994).

A recent study has identified an important stage in early LRP development. Laskowski et al. (1995) defined a developmental point after which an excised LRP could continue to develop in hormone-free media. This functional assay therefore defined the stage at which the LRP is autonomous. Although it is difficult to compare our stages with those described in this paper, it appears that the autonomous LRP are somewhere between Stages III and V. Based on the data of Laskowski et al. (1995) it is clear that the LRP is sufficiently organized at this stage to proceed with development of an LR if isolated from the parent root. However, this capability does not necessarily mean that a meristem has been established, but only that the isolated LRP can direct the appropriate set of cell divisions to eventually generate a meristem.

Apical meristem activity during and after LRP emergence

The number of cells in the OL of the developing LRP changes only slightly, if at all, from Stage VII until after emergence. If there is already a functional meristem during this stage, it is not highly active. As the LRP increases in length the basal-most cells become grossly enlarged. Hence, emergence of the LRP from the parent root and the initial growth of the LR occur primarily by cell expansion. This is consistent with studies in *Vicia faba* that used [³H]thymidine labeling to show that LRP emergence was accompanied by a period of low mitotic activity, which increased again post-emergence (McCully, 1975). The period of low mitotic activity during emergence is suggestive of growth through expansion of existing cells. Together, these findings support the idea that divisions in meristematic initials are not the primary cause of elongation during LRP emergence from the primary root. After this point, the number of cells in the outer layer near the tip begins to increase dramatically while basal cell numbers remain constant, strongly indicating the activation of a meristem that directs growth from this stage onward.

The idea that emergence of the LRP is not dependent on an active apical meristem is consistent with the findings of Cheng et al. (1995), who have isolated a mutant, *rml-1*, that initiates LRPs which emerge from the parent root and then cease to develop. If these mutants are unable to form an active root apical meristem, as suggested by the authors, they would be expected to arrest at just this developmental stage, with

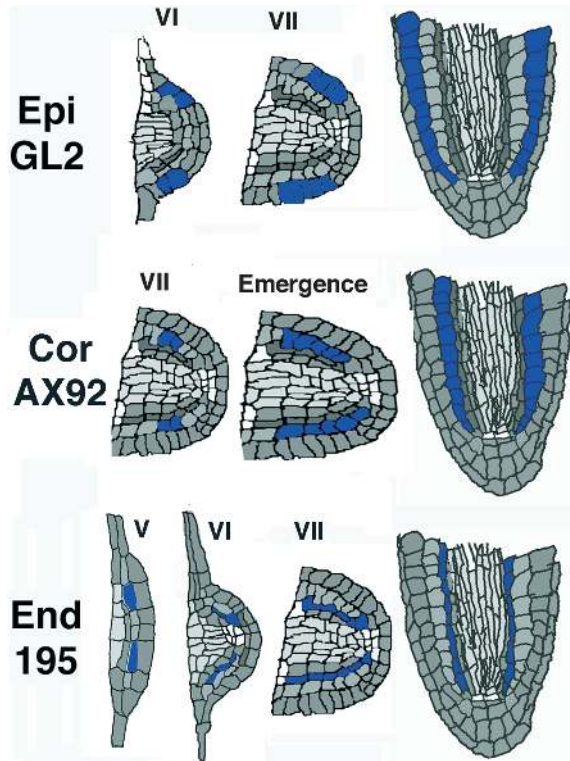


Fig. 5. Schematic representation of staining in EpiGL2, CorAx92 and End195. These diagrams correspond to the images in Fig. 4 with some additional stages. The diagrams at the far right show staining patterns in a mature root tip.

emergence being driven by cell expansion. The same interpretation might be applied to the *alf-3* mutant isolated by Celenza et al. (1995). It would be interesting to determine if the lateral roots of *rml-1* and *alf-3* arrest at the 8-8-8 cell pattern seen from Stage VII until root meristem activity commences. (*rml-1* lateral roots reportedly arrest with 17 cells in the epidermal cell file, whereas we predict 12-14 cells per file, potentially coinciding with their observations). It is possible that all the events up to and including LRP emergence have to proceed correctly for a root apical meristem to be formed and become

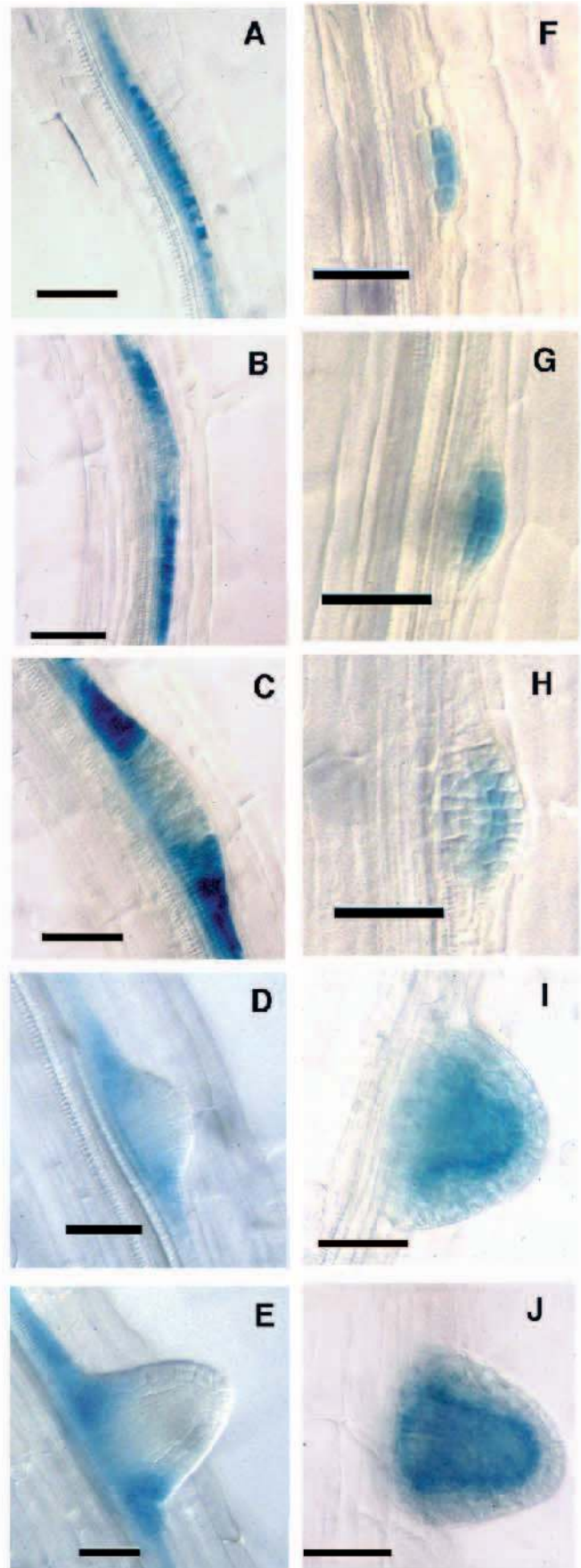


Fig. 6. GUS expression in lateral root primordia of LRB10 and End199. Shown are Nomarski images of developing LRP. These two lines show differential GUS expression at very early stages of LRP development. (A-E) LRB10. (A) Stage I. Staining is ubiquitous throughout the early primordium. (B) Stage IV. Staining begins to be restricted to the periphery of the LRP. (C) Stage V. Restriction of staining to the peripheral cells is clearer at this stage. (D) Stage VI. Staining becomes limited to a ring of cells at the base of the LRP. (E) Emergence. Staining is fainter than at earlier stages, but remains as a clear ring around the base of the emerged root. (F-J) End199. (F) Stage II. Staining is restricted to three cells in the center of the OL. (G) Stage III. Staining is strongest in the central cells of OL1 and OL2. (H) Stage V. Staining has become restricted to the central cells of OL2. There may also be weaker staining in other regions of the LRP. (I) Stage VII. Staining is now not only in the central cells but also in the OL2b layer. (J) Emergence. Staining persists in a pattern similar to that seen in mature lateral roots of End199, extending through the OL2b layer and the central cells near the tip of the primordium. Bar, 50 μ m.

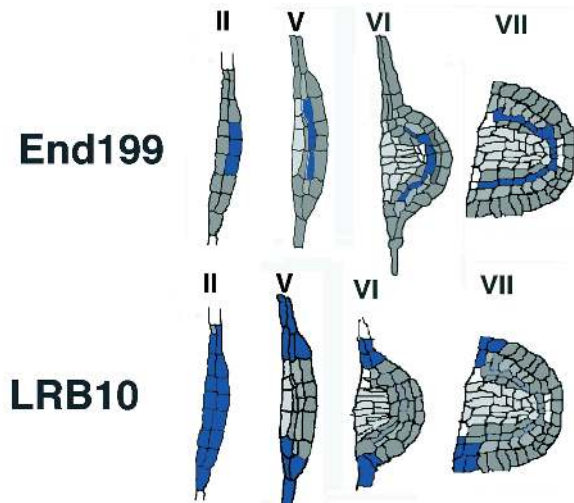


Fig. 7. Schematic representation of the GUS expression patterns in LRB10 and End199. The diagrams correspond to the images shown in Fig. 6.

active, and that a defect in any part of the LRP development process would result in a *rml-1/alf-3*-like phenotype.

Key stages in lateral root formation

Taken together with previous work, our studies suggest that lateral root formation can be divided into the following major stages:

- (1) Stimulation and dedifferentiation of pericycle cells.
- (2) Ordered cell divisions and cell differentiation to generate a highly organized LRP, which may include a group of cells that function as an apical meristem.
- (3) Emergence via cell expansion.
- (4) Activation of the lateral root meristem to allow continued growth of the organized lateral root.

Lateral root development may recapitulate embryonic root formation

It is interesting to compare the development of the LRP with the development of the embryonic root. In both cases, a highly organized structure with differentiated cells is established first and meristematic initials then set aside (Scheres, 1994; this paper). Germination could be regarded as analogous to LRP emergence. In both cases, once organization is established there is a period during which growth is driven primarily by cell expansion (Cheng et al., 1995; this paper). After this period, growth occurs via divisions in the new root meristem.

The notion that embryonic and lateral root organization are established via the same molecular mechanism is supported by radial organization mutants such as *scarecrow*, *short-root* and *fass* (Scheres et al., 1995) and the meristem activation mutant *rml-1*, (Cheng et al., 1995) in which a similar defect is seen in both primary and lateral roots. However, the *alf-3* and *alf-4* mutant phenotypes appear only in the lateral root (Celenza et al., 1995), suggesting that there are unshared mechanisms. Studies are in progress to define the embryonic expression patterns of the marker lines to further compare the embryonic and postembryonic developmental processes. Preliminary data indicate that many genes expressed during early LRP devel-

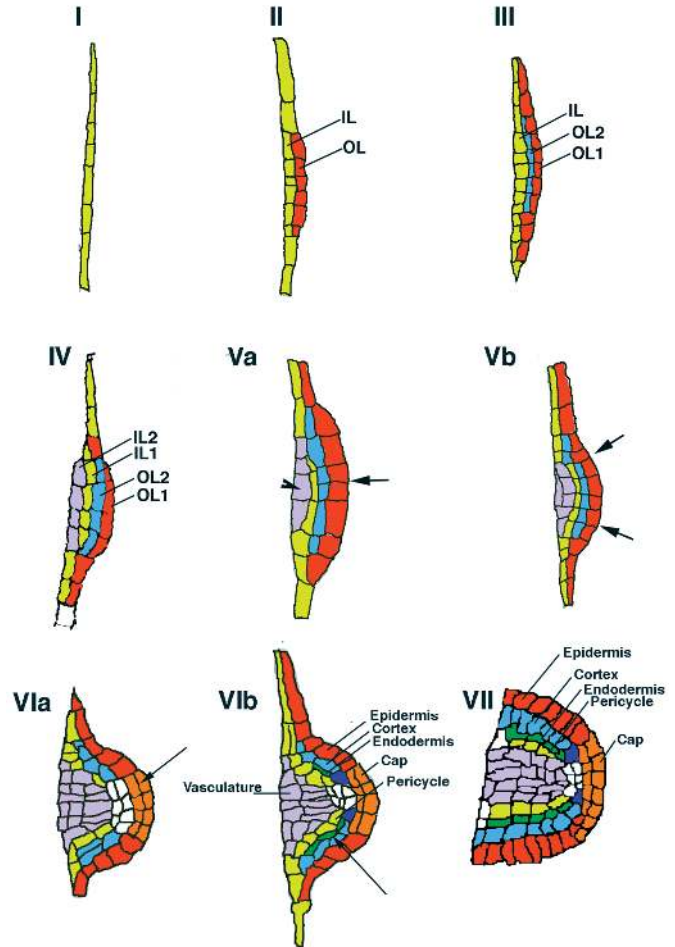


Fig. 8. Model of LRP development in *Arabidopsis*. Stages are indicated above each diagram. Images were created by tracing cell outlines from Fig. 2. Color coding shows the putative derivation of each tissue from Stage I through Stage VII, based on the information from the histological studies and the marker lines. Colors correspond to the same cell-types as in Fig. 1. Note that by Stage VI6 all the radial pattern elements of the primary root are present in the LRP. The cluster of white cells near the LRP tip at Stages VI and VII cannot be clearly identified, but the position and lack of staining of these cells in differentiated cell-specific marker lines suggest that they develop into initials and quiescent center. White cells at the base of the LRP could not be identified. See text for further details.

opment are also expressed during embryonic root development. If this should indeed prove to be the case, lateral roots would provide an easily observed, accessible model for embryonic development of roots as well as a system for the study of postembryonic organogenesis.

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