

## Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the embryonic axis

Ben Scheres<sup>1,\*</sup>, Laura Di Laurenzio<sup>2</sup>, Viola Willemsen<sup>1</sup>, Marie-Therès Hauser<sup>2</sup>, Kees Janmaat<sup>1</sup>, Peter Weisbeek<sup>1</sup> and Philip N. Benfey<sup>2</sup>

<sup>1</sup>Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

<sup>2</sup>Department of Biology, New York University, 1009 Main Building, Washington Square, New York NY 10003, USA

\*Author for correspondence

### SUMMARY

The primary root of *Arabidopsis thaliana* has a remarkably uniform cellular organisation. The fixed radial pattern of cell types in the mature root arises from proliferative divisions within the root meristem. The root meristem, in turn, is laid down during embryogenesis. We have analysed six mutations causing alterations in the radial organisation of the root. Embryonic phenotypes resulting from *wooden leg*, *gollum*, *pinocchio*, *scarecrow*, *shortroot* and *fass* mutations are described. While mutations in the *fass* gene affect morphogenesis of all cells, the five other mutations cause alterations in specific layers. *Wooden leg* and *gollum*

mutations interfere with the proper organisation of the vascular tissue. *Shortroot*, *scarecrow* and *pinocchio* affect the endodermis and cortex. The layer-specific phenotypes caused by all five mutations are also apparent in the hypocotyl. All these phenotypes originate from defects in the radial organisation of the embryonic axis. Secondary roots, which are formed post-embryonically, also display layer-specific phenotypes.

Key words: *Arabidopsis*, root meristem, radial pattern, embryogenesis, *shortroot*, *scarecrow*, *pinocchio*, *gollum*, *fass*

### INTRODUCTION

The continuous activity of meristems in which cells divide and produce new organs is a remarkable aspect of plant development. Primary meristems of angiosperm plants are laid down during embryogenesis. They contribute to post-embryonic development upon germination by producing organs and secondary meristems. Cells within the primary shoot meristem give rise to various organs such as leaves, the stem, and the different floral organs. In contrast, the primary root meristem elaborates a single organ, the primary root, with a stereotyped radial pattern of tissues. In *Arabidopsis thaliana* this radial pattern is of elegant simplicity. In the mature region of the root, single layers of epidermis, cortex, endodermis, and pericycle surround a small number of vascular cells (Dolan et al., 1993). Clonal analysis has revealed that the meristematic root can be traced back to 'initial cells' originating from a single tier of cells in the heart stage embryo (Scheres et al., 1994). The radial pattern of the mature *Arabidopsis* root correlates with the spatial arrangement of initials at the early torpedo stage of embryogenesis. This embryonic arrangement of initials results from early cell divisions that create the tissue layers of both root and hypocotyl (Scheres et al., 1994). Histological studies on several other species confirm the idea that cell divisions in the embryonic axis generally determine the radial organisation of the seedling root and hypocotyl (Von Guttenberg et al., 1955; Ramji, 1975; Kuras, 1980).

Experimental studies have pointed to the capacity of a region

within the root meristem itself to dictate pattern. Studies in which root apices have been cultured in vitro have been interpreted to demonstrate that pattern information resides in the root apex (Reinhard, 1954; Torrey, 1954). Excision experiments in *Zea mays* indicate that such information may be present in a subset of slowly dividing cells in the root apex termed the quiescent centre (Feldman and Torrey, 1976). However, these experiments do not determine conclusively where the information to establish the radial pattern of the root originates. The pattern information could be generated in the root apex with the aid of apex-specific gene products. Alternatively, radial pattern information could be generated in the embryo and this information would remain active throughout root development.

To distinguish between these two possibilities, we have identified mutations that cause radial pattern defects in the root. Our aim was to establish whether these defects are specific for roots and therefore provide evidence for the existence of a separate set of genes specifying radial organisation within the root meristem. Six mutant phenotypes are characterised in this study which have defects in pericycle, vascular tissue, endodermis, cortex and overall radial organisation of the root. We show that in all cases the defect in the radial pattern of the root is apparent in the embryonic axis and in both hypocotyl and root of the seedling. Therefore the majority of genetic information determining the cellular organisation of the root appears to be involved in the radial organisation of the embryo axis as a whole.

## MATERIALS AND METHODS

### Plant growth conditions and mutagenesis

For root and hypocotyl anatomy studies seeds were sterilized in 5% sodium hypochlorite for 10 minutes and imbibed for 2 days at 4°C in the dark in sterile water. Seedlings were grown on Petri plates containing 1× Murashige and Skoog salt mixture and 0.5 g/l 2(N-morpholino) ethanesulfonic acid, pH 5.8, in 0.8% Duchefa agar. Plates were incubated in a near-vertical position in a plant chamber at 22°C, 75% humidity with a 16 hours light/8 hours dark cycle.

For embryo anatomy studies, *A. thaliana* ecotype 'Columbia' was grown on soil until seed set became apparent using conditions as described above.

The *shortroot* (*shr*), *scarecrow* (*scr*) and *pinocchio* (*pic*) mutants were recovered as seedlings with slow-growing roots among T-DNA mutagenised ecotype WS seeds (Feldman, 1991; Benfey et al., 1993). The *wooden leg* (*wol*) mutant phenotype was recovered within bulk M2 offspring of EMS mutagenised seeds as a seedling with retarded root growth, and outcrossed to determine the segregation ratio. A single-family screening strategy was adapted to allow the recovery of seedling-lethal mutations affecting the radial pattern of root tissues. *A. thaliana* ecotype 'Columbia' dry seeds were mutagenised with 10 mM ethyl methane sulfonate (EMS) in water for 24 hours at 22°C. Single siliques were harvested from individual M1 lines. The *gollum* (*glm*) and *fass* (*fs*) mutant phenotypes were identified within M2 offspring. M3 progeny of the non-mutant siblings was re-tested for appearance and segregation ratio of the phenotype.

### Genetic analysis

Lines segregating double mutants were constructed as follows. F<sub>1</sub> plants carrying one *wol* and one *shr* allele were obtained by crossing parents homozygous for the single allele. Plants homozygous for *wol* were used as acceptor because *shr* homozygous plants have low seed set.

Plants homozygous for the *fs* allele used are sterile. For that reason F<sub>1</sub> plants carrying one *shr*, *scr* or *wol* allele together with the *fs*<sup>1213</sup> allele were constructed by using homozygous *shr*, *scr* or *wol* plants as acceptor and donor pollen from plants that were tested to be heterozygous at the *fs* locus.

For double mutant analysis F<sub>1</sub> families segregating both parental phenotypes were selected, numbered and photographed, and subsequently examined microscopically for the occurrence of novel phenotypes. In this way specific features of seedlings with novel microscopical phenotype could be recorded. Families from *shr* × *fs*/+ and *shr* × *wol* crosses segregated a novel phenotype. Seedlings of putative *shr fs* double mutants as determined by microscopy had the overall appearance of the *fs* parent. Six of 36 seedlings with *fs* appearance segregated the novel phenotype (lacked a casparian strip), consistent with an expected 1:3 ratio of double mutants in the *fs* phenotype class in the case of unlinked genes ( $\chi^2 = 1.3$ ). The seedling appearance of the putative *wol shr* double mutants resembled the *wol* single mutant. Five of 22 seedlings with *wol* single mutant appearance segregated the novel phenotype, consistent with the expected ratio of 1:3 double mutants for unlinked genes ( $\chi^2 = 0.06$ ).

In families segregating both parental phenotypes from *wol* × *fs* and *scr* × *fs* crosses no novel phenotype was detected. Because the phenotype of *fs* mutants is extremely pleiotropic while the *wol* and *scr* mutant phenotype is most prominent in roots, the *fs* overall seedling phenotype was assumed to be epistatic to the *wol* and *scr* seedling phenotype, as observed in the *shr* × *fs* cross. For the *wol* × *fs* cross 20 seedlings with *fs* appearance were analysed microscopically and none showed an additional phenotype. Hence the hypothesis that a novel double mutant phenotype is present in the *fs* phenotype class can be rejected ( $\chi^2$  at 1:3 expectation = 6.7). 10 seedlings with *wol* appearance were sectioned as a control and these revealed no novel phenotype. For the *scr* × *fs* cross 40 seedlings with *fs* appear-

ance were analysed microscopically and these revealed no new phenotype ( $\chi^2$  at 1:3 expectation = 13.3).

### Light microscopy

Dissected embryos of various stages as well as seedlings were fixed in 1% glutaraldehyde and 4% formaldehyde in 50 mM sodium phosphate buffer, pH 7.2, dehydrated and embedded in Technovit 7100 (Kulzer, Hereaus). For the transverse sections of embryos complete siliques were embedded in an oriented fashion. 3 µm sections from all specimens were made on a Reichert-Jung 1140 rotary microtome carrying a disposable Adamas steel knife. Embryo sections were stained with 2.5% Astra blue (Merck) dissolved in 0.5 M sodium phosphate buffer, pH 4.4, at 50°C for 8 minutes, mounted in DePeX (BDH) and photographed using a Zeiss Axioskop using Kodak Technical Pan film. Root and hypocotyl sections were stained with 0.05% toluidine blue-O (Merck) in water at room temperature for 1 minute, mounted in DePeX and photographed using a Zeiss Axioskop using Kodak Ektar 25 film.

Suberin staining on fresh sections was performed essentially as described by Brundrett et al (1988). Agarose embedded and hand sectioned tissue was incubated overnight in 0.1% (w/v) berberine hemisulphate (Sigma) at root temperature, rinsed with distilled H<sub>2</sub>O and incubated in 0.5% (w/v) aniline blue WS (Polysciences) for 90 minutes at room temperature. Agarose slices were rinsed as above, transferred to a 0.1% FeCl<sub>3</sub> in 50% (v/v) glycerin solution for 5-10 minutes, and mounted on microscope slides in the same solution. The sections were photographed using a Zeiss axioskop fluorescence microscope with excitation filter G 365 nm, chromatic beam splitter FT395 nm and barrier filter LP420, using Ilford XP2 film.

### Tissue culture experiments

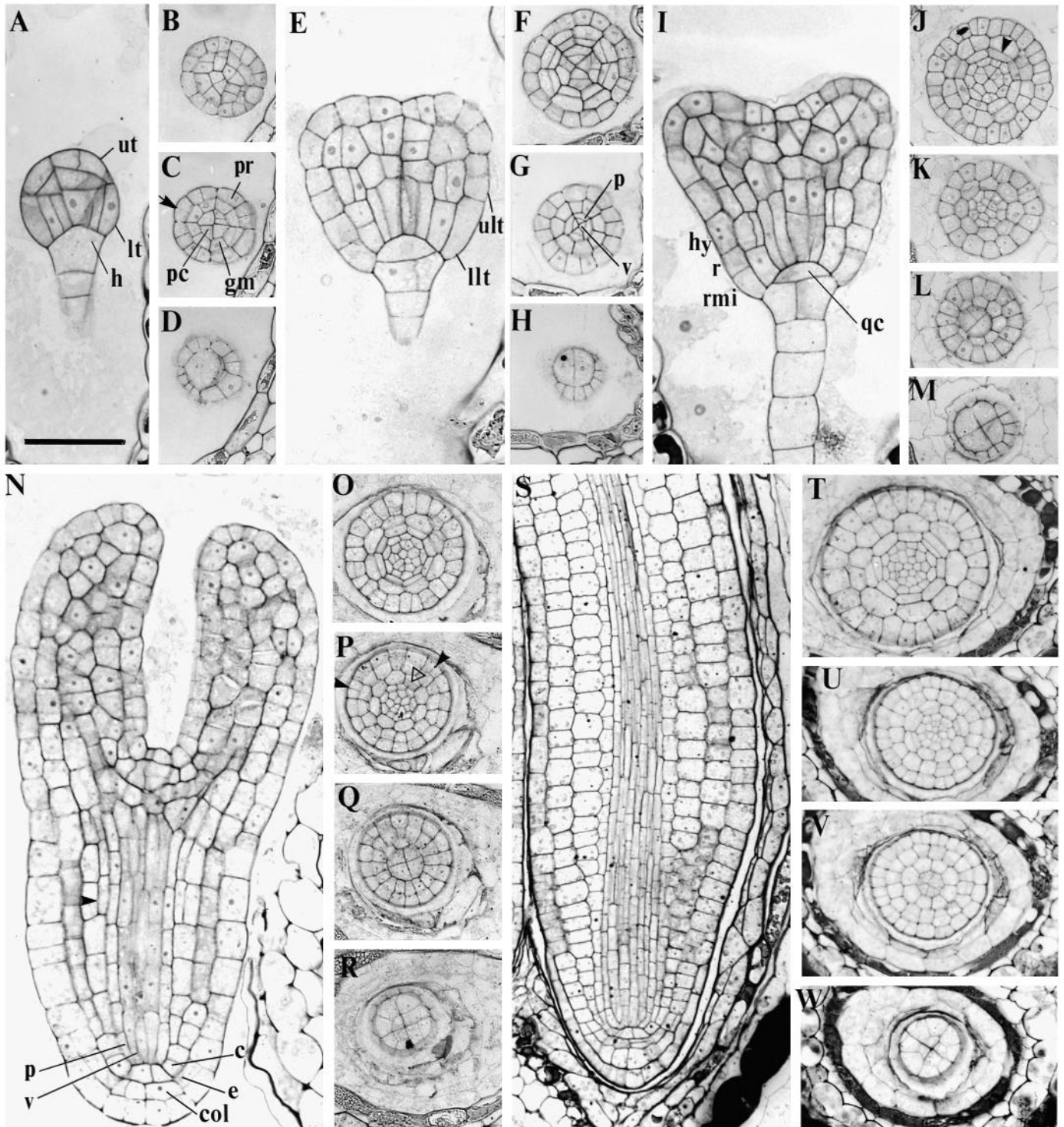
Explants from wild-type and *shr*, *scr*, *pic*, *wol* and *fs* mutant seedlings that were germinated on MS 4.5% agar plates were incubated for 48 hours in callus inducing medium (Valvekens et al., 1988) at room temperature, and subsequently placed on root inducing medium (MS basal salts (Sigma), 0.5 g/l MES (Sigma), 4.5% sucrose, 0.8% agar (BBL, Becton & Dickson), 1mg/l IAA (Sigma), 0.1 mg/l kinetin (Sigma), 0.2% IBA (Sigma), set to pH 5.9 with KOH). The explants were incubated for 7-14 days under the conditions described above. The radial tissue organisation of the regenerated roots was examined using fresh sections.

## RESULTS

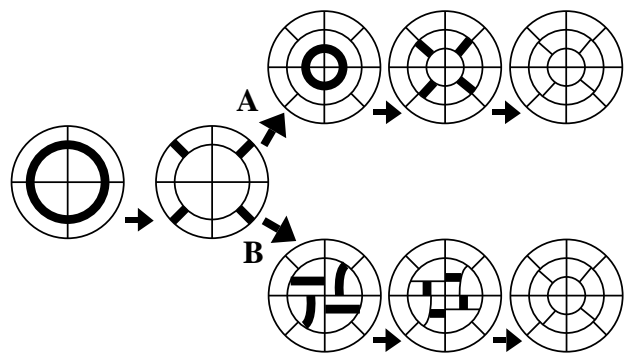
### Ontogeny of the radial organisation of the *Arabidopsis* embryonic axis

The radial organisation of the different tissues of wild-type *Arabidopsis* roots reflects the arrangement of initials in the lower cell tier of the embryonic axis at the torpedo stage of embryogenesis (Scheres et al., 1994). We investigated the cell divisions leading to the radial organisation of the wild-type embryonic axis to provide a framework for mutant analysis. Transverse sections at different levels of the axis in embryos of several developmental stages are shown in Fig. 1. Median longitudinal sections of embryos at corresponding developmental stages are included to facilitate the description.

At the early globular stage the *embryo proper* is split in an upper and a lower tier (Fig. 1A). In the lower tier, protoderm, ground meristem, and procambium are separated as the progenitors of the major tissue types in the embryonic axis by regular periclinal divisions (Fig. 1C). Anticlinal divisions have determined the number of ground meristem cells at eight and are at this stage elaborating the number of protoderm cells



**Fig. 1.** Radial organisation of the *Arabidopsis* embryo at successive stages. The depicted stages are defined according to Jürgens and Mayer (1994). (A) Median longitudinal (ML) section of globular embryo. (B-D) Transverse (TS) sections of globular embryo at ut, lt and h levels. (E) Triangular embryo, ML. (F-H) Triangular embryo, TS at ult, llt and h levels. (I) Early heart stage embryo, ML. (J-M) Early heart stage embryo, TS at hy, r, rmi, and h levels. (N) Mid-torpedo stage embryo, ML. (O-R) Mid-torpedo stage embryo, TS at hy, r, qc and col levels. (S) Mature embryo, ML. (T-W) Mature embryo, TS at hy, root proximal initials, qc and col regions. Sections were stained with astra blue. ut, upper tier; lt, lower tier; h, hypophysis; ult, upper subtier of lt; llt, lower subtier of lt; pr, protoderm; gm, ground meristem; pc, procambium; p, pericycle; v, vascular primordium; hy, prospective hypocotyl; r, prospective root; rmi, root meristem initials; qc, quiescent centre; e, epidermis; c, cortex; col, columella. Bar, 50 µm.



at sixteen (Fig. 1C, arrow shows recently deposited cell wall). At later stages this radial cell number of protoderm and ground meristem is preserved in the cells immediately adjoining the hypophyseal cell. This subtier harbours the progenitors of the root meristem initials (cf. Fig. 1G,L,Q,V). Therefore early anticlinal divisions largely determine the number of epidermal and cortical root meristem initials in the seedling root.

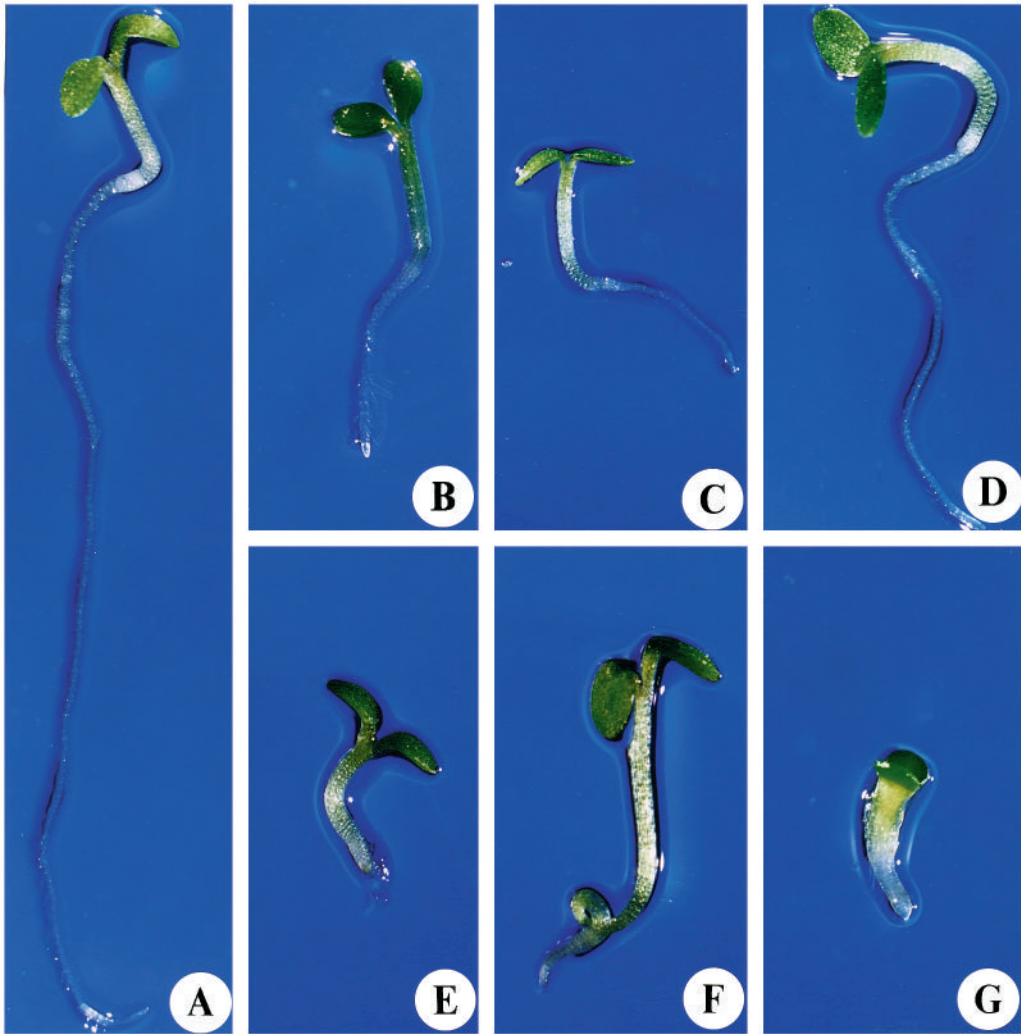
At the late globular/triangular stage the lower tier has split in two subtiers, *upper lower tier (ult)* and *low lower tier (llt)* (Fig. 1E). The *llt* region will produce the embryonic axis, forming root and hypocotyl (Scheres et al., 1994). The four procambium cells have performed a periclinal division to generate the future pericycle and the vascular primordium (Fig. 1F,G; p and v). In the *llt* region, regular periclinal divisions create four inner and four outer cells (Fig. 1G). In the *ult* region, cells within the procambium perform less regular tangential divisions (Fig. 1F).

At the early heart stage the three *llt* subtiers corresponding to the incipient hypocotyl, embryonic root and root meristem initials become separated (Fig. 1I; hy, r, rmi; Scheres et al., 1994). The number of cells in the pericycle increases to 8-9 by anticlinal divisions, approaching the number of initials counted in the seedling root. Periclinal divisions have doubled the ground meristem in the future hypocotyl region (Fig. 1J, arrowhead) and only in the outer layer additional anticlinal divisions occur.

**Fig. 2.** Schematic representation of division patterns in the embryonic axis. (A) Early division pattern with alternating periclinal and anticlinal divisions. (B) Late, less regular division pattern involving tangential divisions. A higher pressure in the inner cell mass normalises the orientation of new cell walls in B. Thick lines represent recent divisions.

Protoderm cells in the hypocotyl region divide anticlinally to approach the final epidermal cell number in the seedling hypocotyl.

The early torpedo stage embryo contains the root promeristem with all initials and derived cell types (Fig. 1N). Within the vascular primordium additional irregular cell divisions have occurred to establish the number of vascular initials present in the seedling root meristem (Fig. 1O,P). The epidermal initials have given rise to a layer of lateral root cap (Fig. 1N,P,Q). The prospective lateral root cap cells undergo a radial division which doubles their number (Fig. 1P, arrowheads). Characteristic periclinal divisions in the daughter cells of the cortex initials give rise to the endodermis (Fig. 1P, open triangle). The inner ground meristem layer in the hypocotyl



**Fig. 3.** Mutant seedling phenotype. Appearance of seedlings 7 days after germination on water agar. (A) Wild-type seedling. (B) *shr*; (C) *scr*; (D) *pic*; (E) *glm*; (F) *wol*; (G) *fs*. All seedlings photographed at the same magnification.



also undergoes periclinal divisions, resulting in a continuous endodermis layer in root as well as hypocotyl of the seedling (Fig. 1N, arrowhead). At the mature embryo stage the embryonic axis has elongated considerably by transverse divisions, which preserve the radial organisation of the torpedo stage embryo (Fig. 1S-W).

The radial arrangement of derivatives of the hypophyseal cell (Fig. 1A; h) is established by a sequence of divisions reminiscent of those described above for the *lt* region in the embryo proper. The prospective quiescent centre cells form a quadrant at the heart stage (Fig. 1M,Q). Within the columella cell tier a quadrant is formed by cell divisions which may be oriented similarly to the quiescent centre quadrant (as in Fig. 1R). However, a shift in orientation of vertical cell walls in the two tiers is frequently observed (data not shown). The elaboration of the final pattern of columella cells '4 surrounded by 8' is completed at the torpedo stage either by periclinal/anticlinal or by tangential divisions (Fig. 1R,W).

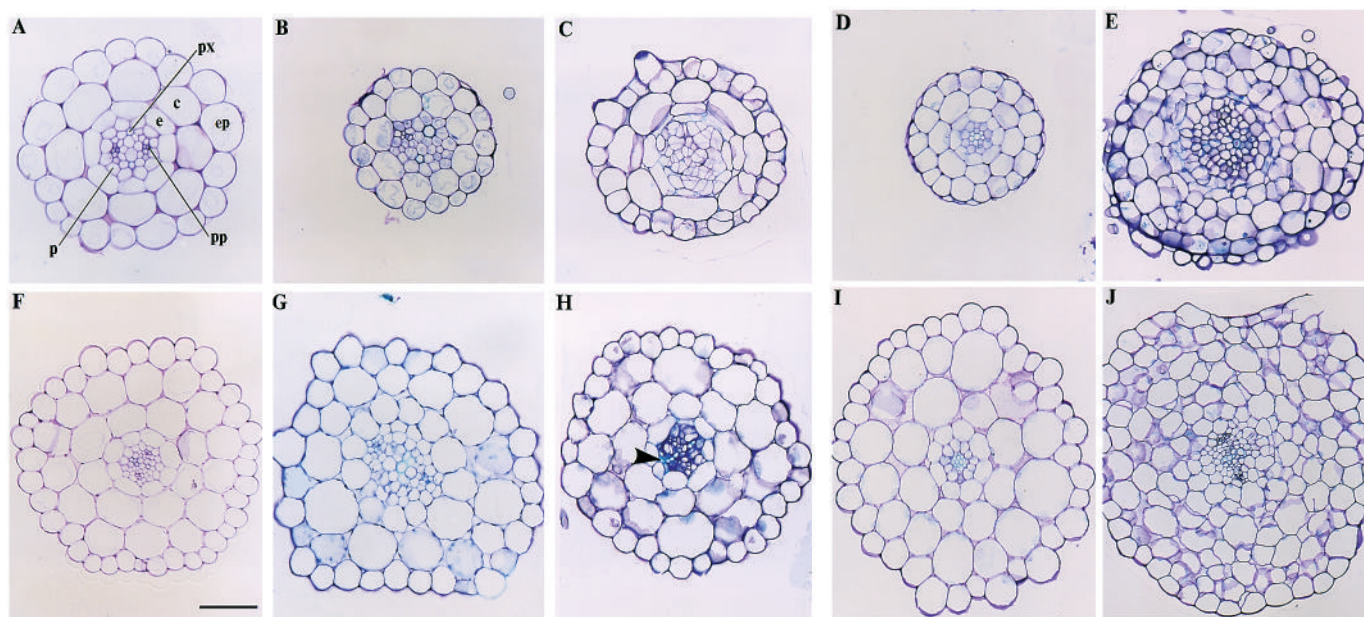
A summary of the observed division patterns is shown in Fig. 2. Periclinal cell divisions in centrally located cells of the embryo separate the different tissues. Anticlinal divisions subsequently determine the number of cells of each layer in a regular manner (Fig. 2A). As these divisions proceed in the stele region, the cells become smaller, and tangential divisions can be observed (Fig. 2B). We have no indications that tangential division patterns in the pericycle region lead to aberrations in the seedling pericycle layer. Therefore we conclude that, to a certain extent, forces acting on the young cell wall correct the radial organisation (Fig. 2B). Both the typical periclinal/anticlinal and tangential patterns of division occur in the clonally distinct basal subtiers of the hypophyseal cell that form the columella of the root. Hence the radial pattern of all root and hypocotyl cells in the mature embryo of *Arabidopsis* is the result of remarkably consistent cell

divisions exhibited throughout the different stages of embryo development.

### Mutations affecting radial organisation of root and hypocotyl

To investigate the nature of genetic controls exerted on radial pattern formation we performed a genetic screen for mutations affecting the radial organisation of root tissues. Slow root growth had been observed in the radial pattern mutant *shortroot* (*shr*), perhaps as a consequence of the lack of endodermal tissue (Benfey et al., 1993). The rationale of our screen was that more radial pattern defects would cause retardation of root growth. Seedlings from different batches of mutagenized seeds (see Materials and Methods) were preselected for retarded root growth and microscopically examined for defects in radial organisation. In this way the *scarecrow* (*scr*), *pinocchio* (*pic*), *gollum* (*glm*) and *wooden leg* (*wol*) mutants were identified. Fig. 3 displays the phenotype of seedlings homozygous for the different mutations. The major characteristic of all mutant seedlings is retarded root growth (Fig. 3A-F). In addition we have further characterised a mutant previously named *dikkiedap*, whose increased diameter immediately suggested alterations in radial pattern (Fig. 3G). Complementation analysis revealed this mutation to be an allele of the *fass* (*fs*) gene (R. Torres-Ruiz, personal communication; Mayer et al., 1991). All phenotypes segregated as if caused by single nuclear recessive mutations. Complementation and mapping data reveal that the *shr*, *scr*, *pic*, *wol* and *fs* mutations define different genes (data not shown).

The radial pattern defects found in mutant seedlings are visualised in the transverse sections depicted in Fig. 4. Wildtype primary roots in the differentiation zone contain single layers of epidermis, cortex, endodermis, and pericycle with constant cell numbers (Fig. 4A; Dolan et al., 1993).



**Fig. 4.** Transverse sections of mature root and hypocotyl regions from seedlings 7 days after germination. (A-E) Root; (F-J) hypocotyl. (A,F) Wild type; (B,G) *shr*; (C,H) *glm*; (D,I) *wol*; (E,J) *fs*. Sections stained with toluidine blue. Note that walls of xylem cells stain light blue. ep, epidermis; c, cortex; e, endodermis; p, pericycle; pp, protophloem; px, protoxylem. Arrowhead points to a xylem cell in the pericycle position. Bar, 50 µm.

Within these layers vascular cells are present containing two protoxylem and two protophloem elements. The wild-type hypocotyl has a similar organisation but contains an additional cortex cell layer (Fig. 4F).

The stereotyped tissue organisation is differently affected in the different mutants. Roots from *shr* seedlings lack the endodermal layer (Fig. 4B, Benfey et al., 1993). *scr* and *pic* roots also lack a ground tissue layer and transverse sections look similar to those of *shr* roots. Suberin staining indicated that the remaining cell layer in both *scr* and *pic* contains the casparian strip characteristic for endodermis cells. We concluded that the cortex layer is absent in these mutants. The hypocotyl in *shr*, *scr* and *pic* contains two irregular layers of cells in the normal location of the endodermis and two cortex layers (Fig. 1G, *shr* is shown).

Roots from *glm* seedlings lack the characteristic organisation of the vascular tissue and pericycle (Fig. 4C). The pericycle layer in the hypocotyl of *glm* seedlings is incomplete and the arrangement of xylem and phloem elements is atypical (Fig. 1H, arrow points to xylem cell in pericycle position). *Wol* seedlings contain roots with a vascular system in which fewer cells are present compared to wild type, and all these vascular cells differentiate into xylem elements (Fig. 4D). The lower part of the hypocotyl in *wol* seedlings shows the same deviation in vascular cell number and type observed in the root (Fig. 4I). The number of vascular cells increases in the upper part of the hypocotyl at the site just basal to the separation of the vascular bundles to the cotyledons. It is noteworthy that phloem vessels are present in this region.

Roots of *fs* seedlings show supernumerary cell numbers. This results in an enlargement of the vascular cylinder and multiple cortical cell layers (Fig. 4E). More epidermis and pericycle cells are present in *fs* roots but they still appear as a continuous layer. Suberin staining remains restricted to one single layer, indicating that also a single layer of endodermis is present (cf. Fig. 6C). The hypocotyl of *fs* seedlings contains, like the root, too many cells in all regions, leading to the expansion of the presumptive ground tissue towards five irregular layers in comparison with the two layers in wild type (Fig. 4J). It is interesting to note that the widening of the vascular bundle frequently permits the presence of more than two protoxylem poles. A detailed analysis of *fs* alleles has shown that the presence of more protoxylem strands is linked to the number of cotyledons in *fs* seedlings (Torres-Ruiz and Jürgens, 1994).

In summary, the phenotypes caused by six different mutations affecting different aspects of the radial organisation of the root are not specific to the part of the root formed by the root meristem but extend into the hypocotyl region.

### All mutants display alterations in radial organisation of the embryonic axis

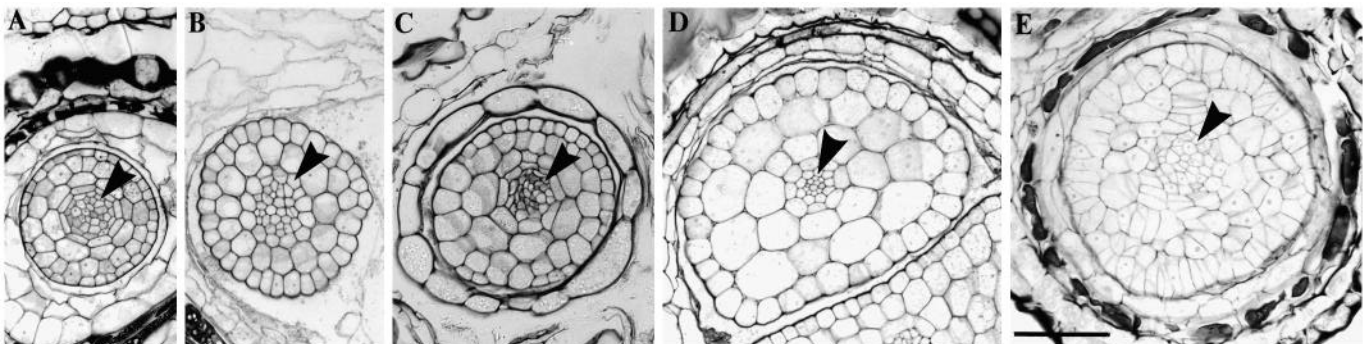
The occurrence of a radial pattern defect in the root as well as in the hypocotyl of seedlings homozygous for the various mutations strongly suggested a defect occurring during embryogenesis. To detect such a defect we sectioned embryos at different stages that were homozygous for the various mutations. Representative sections are depicted in Fig. 5.

*Shr*, *scr* and *pic* mutants have in common a lack of cell layers normally derived from the ground meristem. Embryos homozygous for the *shr*, *scr*, and *pic* mutation are first recognised as different from wild type at the early heart stage, when the periclinal division that doubles the ground meristem layer in the axis is absent. In sections of mid torped stage embryos, only one layer derived from the ground tissue is present (Fig. 5B). The periclinal division giving rise to the endodermis does not occur in the ground meristem of the prospective hypocotyl, nor in the descendants of the root cortex initial (cf. Dolan et al., 1993).

The radial pattern defects observed in the stele of *glm* and *wol* seedlings are also apparent in embryos. Stele cells of the mature *glm* embryo are disorganised compared to wild-type embryos (Fig. 5C). Both the root and the hypocotyl region in mature *wol* embryos show about 10 cells within the layer of cells resembling the pericycle layer in wild-type embryos. The number of cells within the pericycle of wild-type mature embryos is about 18 (Fig. 5D, cf. Fig. 1T). The division pattern elaborating all the other tissues before the heart stage of embryogenesis is normal (data not shown). Therefore the last divisions to complete the number of cells in the stele appear absent in *wol* embryos.

Embryos homozygous for *fs* display multiple misoriented cell divisions at essentially all stages of development (Torres-Ruiz and Jürgens, 1994; cf. Fig. 5E). While the orientation of these divisions remains predominantly anticlinal in the presumptive pericycle and epidermis, it is randomised in the ground meristem leading to the formation of additional layers (Fig. 5E).

The formation of a root primordium with the correct radial



**Fig. 5.** Radial organisation of mutant embryos. (A) Wild type, mature stage, root region; (B) *shr*, mid torped stage, hypocotyl region; (C) *glm*, mature, root; (D) *wol*, mature, hypocotyl; (E) *fs*, mature, first root section above putative quiescent centre region; Astra-blue staining. Arrowheads indicate position of the pericycle (putative in E). Bar, 50  $\mu$ m.

organisation is not restricted to embryogenesis. Lateral roots are newly formed along the primary root during post-embryonic development. Furthermore, the formation of root primordia can be induced in tissue culture. In order to assess whether the mutant phenotypes were specific for the developmental context of the embryo, we examined lateral roots emerging from pericycle cells of the primary root, and of roots induced from callus by treatment with auxins. Fresh sections of lateral roots as well as callus-induced roots from *shr*, *scr*, *pic*, *wol* and *fs* mutants revealed in all cases the mutant phenotype (data not shown). Seedlings homozygous for the *wol* mutation produced no lateral roots from the primary root. Lateral roots emerged only from the upper hypocotyl and these had an intermediate vascular phenotype (L. DiL. and P. B., unpublished data). From the observations on lateral and callus-induced roots we concluded that all mutations described here interfere with radial pattern formation during primary as well as secondary root development.

### Are the *shr*, *scr* and *wol* mutations primarily interfering with cell specification or morphogenesis?

Seedling and embryo analysis showed that the absence of specific cell types in *shr*, *scr* and *wol* seedlings is linked to the absence of particular divisions during embryogenesis. The absence of a cell type could be a consequence of a primary defect in cell division. Alternatively, these mutations could have a primary defect in cell specification during pattern formation, leading to the absence of later cell divisions. In *fs* mutants the frequency and orientation of cell divisions is altered, while pattern formation is unaffected as judged by the presence of all tissues in appropriate positions (Torres-Ruiz and Jürgens, 1994). Therefore we created double mutant combinations with *fs*. If the lack of particular cell divisions in *shr*, *scr*, and *wol* single mutants was counteracted by additional divisions in the *fs* background, then the question of whether cell specification defects would still occur could be addressed. Potential double mutants were identified as outlined in materials and methods.

Since *shr* mutants contain no endodermis the progeny of *shr* × *fs* crosses was analysed by suberin staining. Seedlings were found with the appearance of a *fs* single mutant in which the endodermis-specific casparian strip could not be detected in root and hypocotyl tissue, as shown in Fig. 6B. Nevertheless multiple layers derived from the ground meristem are present in such seedlings. In wild-type seedlings and single *fs* mutants, suberin staining visualises the endodermis (Fig. 6A,C). The *fs* mutant seedling appearance of the presumptive double mutant is consistent with the pleiotropic effect of mutant *fs* alleles on cell morphogenesis. We concluded that the block in periclinal divisions of the ground meristem, characteristic for embryos homozygous for *shr*, can be released by mutation of the *FS* gene. However, the occurrence of multiple ground tissue layers in the double mutant does not lead to formation of an endodermis layer. Therefore absence of endodermis appears to be a direct effect of the *shr* mutation that cannot be suppressed in a *fs* background.

*Scr* single mutants contain a single ground tissue layer with a casparian strip and hence appear to lack cortex cells. Double mutants in the *scr* × *fs* cross were expected to have the overall appearance of *fs* seedlings due to the pleiotropic nature of the

*fs* mutation. We analysed seedlings with a *fs* appearance, from families segregating *scr* and *fs* seedlings, by suberin staining. A casparian strip could be visualised in a single cell layer at the same position as in *fs* single mutants in all seedlings investigated, but not in all other ground tissue layers that were present. We concluded that the presence of additional divisions in the ground tissue of a double mutant leads to the restoration of cell layers that do not contain a casparian strip and display the cell morphology of cortex cells. Hence the *fs* mutation is epistatic to the *scr* mutation.

*Wol* mutants display a narrowed vascular bundle in a specific seedling region while *fs* mutations affect the morphology of all seedling regions. Therefore we expected *wol fs* double mutants to have the *fs* seedling phenotype. In families segregating both *fs* and *wol* mutant phenotypes, we could not detect novel features suggestive of a double mutant phenotype, upon microscopic analysis. Seedlings with *fs* appearance were sectioned and they all contained a wide vascular bundle with all tissue types. We concluded that *fs* is epistatic to *wol* and that the lack of phloem in *wol* single mutants can be restored by the presence of additional vascular cells in the *wol fs* double mutant. The observation that phloem vessels are present in the widening region of the vascular bundle in the upper part of the hypocotyl is in agreement with this conclusion.

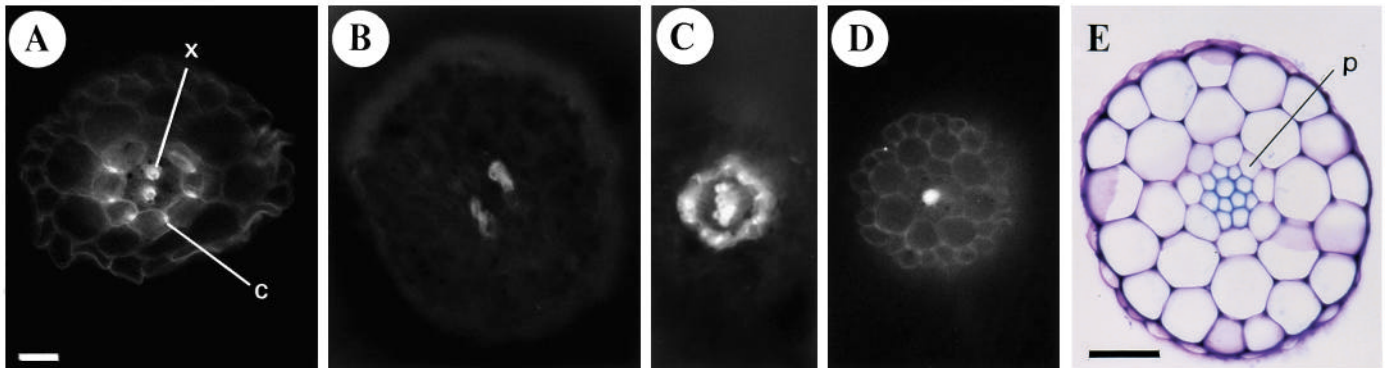
Our data indicate that restoration of cell divisions in the ground tissue of *scr* mutants as well as in the stele of *wol* mutants leads to the re-appearance of missing cell types. In both *shr* and *wol* mutants specific cell divisions during formation of the embryonic axis are absent. We conclude that the absence of layer-specific cell divisions in these mutants causes the absence of specific cell types. Since both mutations affect cell division we were interested to learn whether *shr wol* double mutants are strictly additive, which would indicate that the two genes function independently in different regions. If, on the other hand, the mutant alleles influenced a related morphogenetic pathway, enhanced phenotypes in both regions might be expected in the double mutant. Families segregating both *wol* and *shr* seedlings contained a distinctive double mutant phenotype in seedlings with *wol* appearance but with a hypocotyl of smaller diameter. The endodermis layer was lacking as judged by suberin staining (Fig. 6D). Sectioning of these seedlings revealed the absence of any vascular cell type besides xylem, as in *wol* single mutants, and the presence of only a single ground tissue layer, as in *shr* single mutants (Fig. 6E). Since the *shr* and *wol* phenotypes appear strictly additive in root and hypocotyl, independent mechanisms appear to underly cell division defects in stele and ground tissue.

The novel phenotypes found in the double mutant analyses were in all cases expressed in both the root and the hypocotyl (data not shown). This observation is consistent with the conclusion from the single mutants that cells derived from the root meristem elaborate the radial pattern laid down in the embryonic axis.

## DISCUSSION

### Embryonic pattern formation is involved in establishing cell identity of root meristem derivatives

We have shown that six different recessive mutations cause



**Fig. 6.** Analysis of double mutant seedlings. (A-D) Suberin stains on fresh transverse sections of roots. Casparian strips as well as xylem cell walls are visualised by fluorescence. (E) Toluidine-blue stained transverse section. (A) Wild type; (B) *shr*  $\times$  *fs* presumptive double mutant; (C) *fs*; (D,E) *shr*  $\times$  *wol* presumptive double mutant. c, casparian strip; p, pericycle; x, xylem. Bar, 25  $\mu$ m.

alterations of the radial pattern of root tissues, affecting the numbers and, in several cases, the identity of cells within specific layers. In all cases these radial defects originate during embryogenesis and affect all regions of the seedling derived from the embryonic axis. No mutations were found that affect the root only, so we have not obtained evidence for the existence of genes specifically involved in dictating the radial pattern of the root. Based on these observations we postulate that the radial pattern in the meristematic root is regulated by the same factors as those employed in the embryo. Several other observations lend support to the notion that radial pattern information is not exclusively generated within the meristem. First, dissected carrot somatic embryos show replacement of root tissues by cells of the hypocotyl region before a new root meristem is formed (Schiavone and Racusen, 1991). Second, the first differentiated cells of the *Arabidopsis* primary root originate from a separate embryonic tier not encompassing the meristem initials (Scheres et al., 1994).

On the basis of our data, two models for the establishment of the radial pattern of tissues in the root can be envisaged. In both models root meristem initials are positioned and specified as a result of radial pattern formation within the embryonic axis, as schematised in Fig. 7A,B. Radial pattern information could be maintained in the root meristem in several ways. One possibility is suggested by recent laser ablation experiments performed in the *Fucus* embryo which raised the possibility that stable determinants of differentiation are linked to the cell wall (Berger et al., 1994). Similarly, radial pattern information could be laid down in the cell walls of root meristem cells (Fig. 7A). A second possibility is that radial pattern information is passed down from maturing tissues to the meristem in a 'top-down' flow of information (Fig. 7B). This information could be provided by continuous expansion of the expression domain of genes required for radial pattern formation in the embryo, but also by basipetal transport of radial determinants. Our collection of mutants does not distinguish between the A and B model since in both cases a mutant pattern is perpetuated in the primary root. We are currently testing by laser ablation experiments the prediction of the B model that meristem initials have a flexible fate. Mosaic analysis, to determine whether genes defined by radial pattern mutations are continuously needed, has to await linked marker genes for detection of mutant tissue sectors. The existence of a separate genetic

system for pattern generation at the root apex, as depicted in Fig. 7C, is not in agreement with our observation that the radial pattern defects we observed have an embryonic origin.

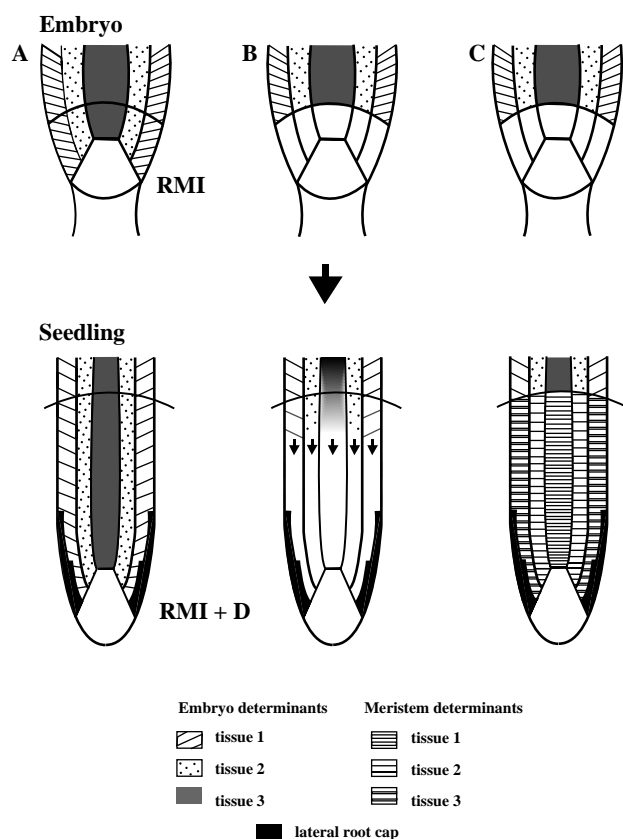
By screening for radial pattern alterations in roots with a functional root meristem we did not take into consideration mutations in a theoretical class of genes involved in a 'separate system for radial pattern formation' in the apex which, by mutation, would yield inactive meristems. We have screened extensively for mutations leading to an inactive root meristem. The cellular organisation of the remnant meristematic root is abnormal in such mutants but there are no layer-specific defects (V.W. and B.S., unpublished data). We conclude that defects in these mutants are not due to an inability of the meristem to dictate specific elements of the radial pattern.

Information to generate the radial pattern of secondary roots in our view is mediated by the same genes that are first active in the embryonic axis. Radial pattern formation and perpetuation can occur in the new root primordium by mechanisms such as those suggested in Fig. 7A,B. Descriptions of lateral root formation in *Zea mays* and *Arabidopsis* are consistent with the notion that the promeristem is not the sole source for pattern information. Such studies suggest direct formation of differentiated cell types from the root primordium without involvement of the new apical meristem (McCully, 1975; H.Nusbaum and I.Sussex, personal communication).

It must be stressed that some pattern information may have to be generated in the meristem. The formation of the root cap is specific for the distal end of the root and is likely to be regulated locally. Distortions in the organisation of the root apex, most notably in the columella root cap, have been observed in *ttg* mutants (Galway et al., 1994). An as yet unspecified role in cell signalling processes during pattern formation has been assigned to the *TTG* gene, based on several aspects of the mutant phenotype (lack of trichomes, and ectopic development of root hairs). The observed abnormalities in the root apex of *ttg* mutants may support the notion that local cell signalling is involved in organising the root cap region.

The models proposed in Fig. 7A,B are not inconsistent with the *in vitro* culture experiments in which the quiescent centre of maize was explanted and was shown to regenerate a root containing all tissues without passing through the callus phase (Feldman and Torrey, 1976). The cell population called the





**Fig. 7.** Possible mechanisms for acquisition of information regarding radial organisation within the root meristem. Only three layers, e.g. epidermis, ground tissue and stele tissue, are shown. (A) Embryonic determinants are sequestered in the complete embryonic axis at a specific stage of embryogenesis. (B) Embryonic determinants are first sequestered in the embryonic axis and the sequestering activity or the resulting information is basipetally transmitted. (C) The root meristem connects to the radial pattern information sequestered in the embryo by an inherent ability to set up this pattern again with the aid of root-specific genes. RMI, root meristem initials; D, derivatives.

quiescent centre in maize has been shown to be heterogeneous and polarised (Feldman and Torrey, 1976; Müller et al., 1993). If radial determinants are patterned during embryogenesis then this information can reside in the quiescent centre cell population without having been generated there *de novo*.

### Morphogenesis and pattern formation in the embryonic axis

The *shr*, *scr*, and *wol* mutants described in this study all lack specific cell divisions. In addition they have cell specification defects. In plants, cell division is one of the means of controlling the acquisition of structure and form, defined as morphogenesis. The specification of different cell types at appropriate positions, on the other hand, is referred to as pattern formation. We addressed the issue of whether the mutations were primarily caused by defects in morphogenesis (i.e., cell division) or pattern formation. This was done by bringing the mutations into the *fs* mutant background, which increases cell numbers in the radial dimension. The availability of only single alleles

of the region-specific mutants (the rather indistinct seedling phenotype is an unreliable criterion for efficient screening) warrants extreme caution in deducing precise roles for these genes in embryo axis organisation. Such a deduction has to await the isolation of multiple alleles and molecular cloning of the genes. Nevertheless the suppression of the cell type deletions in *scr* and *wol* but not *shr* mutants in the *fs* background allows some conclusions to be drawn. Only the *SHR* gene appears to be involved in the specification of a cell type. The *SCR* and *WOL* genes appear to influence pattern formation indirectly by regulating regional aspects of morphogenesis.

How do gene products modify patterns of cell division? The cell divisions that structure the *Arabidopsis* embryonic axis are remarkably uniform, and not species or family specific. Analysis of embryogenesis of *Stellaria media* has revealed identical division patterns. Balance between pressure from the inner cell mass and surface tension in the outer cells were postulated to be of major importance for the positioning of cell walls (Ramji, 1975). If this were the case, differences in pressure or surface tension may yield variations of the division pattern. Consequently, products of genes like *SCR* and *WOL* may program cell division via direct or indirect modification of the parameters mentioned above.

Our observations imply that distortions in morphogenesis leading to too few cells in *scr* and *wol* embryos can cause radial pattern deletions. In contrast, morphogenetic mutations leading to too many cells in the *fs* mutant have no effect on pattern formation (Torres-Ruiz and Jürgens, 1994). What mechanism underlies the influence of morphogenetic mutations leading to too few cells on pattern formation? Analysis of the *wol* mutation that affects pattern formation in the vascular cylinder, may provide some answers to this question. A relationship between size of the vascular cylinder and the number of xylem and phloem poles has been observed in pea lateral roots (Torrey, 1954) and in roots cultured from the quiescent centre of maize (Feldman and Torrey, 1976). The absence of phloem in the primary root of seedlings homozygous for the *wol* mutation may be directly due to a small cell number if it is assumed that xylem poles are specified prior to the specification of the other vascular cell types. If there are too few cells in the vascular cylinder, as in the *wol* mutant, xylem induction may 'use up' all the available vascular cells. The occurrence of phloem in *wol* seedlings where the widening vascular bundle separates to the cotyledons is consistent with this idea. The observed epistasis of *fs* in the *wol fs* double mutant is explained if it is assumed that tissue compartmentalisation in the embryo, separating for example vascular tissues and ground tissue, occurs prior to final specification of cell types. In this scenario the early additional divisions in the *fs* background lead to specification of a vascular region with more cells prior to the block of late, central cell divisions caused by the *wol* mutation. Therefore cells are available for other fates after xylem specification. The correlation between xylem pole number and position and the number and position of cotyledons in wild type, and also in *fs* mutants (Torres-Ruiz and Jürgens, 1994), tempts one to speculate that cotyledon factors are involved in this specification.

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