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Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot

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SUMMARY

Mutation of the *SCARECROW* (*SCR*) gene results in a radial pattern defect, loss of a ground tissue layer, in the root. Analysis of the shoot phenotype of *scr* mutants revealed that both hypocotyl and shoot inflorescence also have a radial pattern defect, loss of a normal starch sheath layer, and consequently are unable to sense gravity in the shoot. Analogous to its expression in the endodermis of the root, *SCR* is expressed in the starch sheath of the hypocotyl and inflorescence stem. The *SCR* expression pattern in leaf bundle sheath cells and root quiescent center cells led to the identification of additional phenotypic defects in these tissues. *SCR* expression in a *pin-formed* mutant background suggested the possible origins of the starch sheath in the shoot inflorescence. Analysis of *SCR* expression and the mutant phenotype from the earliest stages of embryogenesis revealed a tight correlation between defective cell divisions and *SCR* expression in cells that contribute to ground tissue radial patterning in both embryonic root and shoot. Our data provides evidence that the same molecular mechanism regulates the radial patterning of ground tissue in both root and shoot during embryogenesis as well as postembryonically.

Key words: Meristem, Asymmetric cell division, Bundle sheath, Gravitropism, Plant embryogenesis, *Arabidopsis thaliana*, *SCARECROW* (*SCR*)

INTRODUCTION

The root and shoot systems of a plant originate from corresponding apical meristems that appear to have very different modes of function and ontogeny. There are corresponding differences in the radial pattern of tissues in shoot and root. The root has a single vascular cylinder surrounded by the ground tissue and epidermis. The radial pattern of the root is established during embryogenesis and propagated by initial cells located in the meristem. Fate mapping has shown a distinct clonal relationship between the cell layers and their initials (Scheres et al., 1994, 1995). In contrast, in the shoot there is a central pith tissue around which are found numerous vascular bundles that in turn are surrounded by ground tissue and epidermis. How these tissues are derived from the shoot meristem is not evident from the anatomy and no lineage analysis is available. Furthermore, this pattern is only observed after embryogenesis.

Despite the difference in the organization of tissues derived from the shoot and root meristems, recent findings suggest that some molecular pathways are shared. For example, development of cell fate in both root and shoot epidermis is controlled by a pathway in which the *TRANSPARENT TESTA GLABRA* and *GLABRA2* genes play analogous roles (Galway et al., 1994; Hung et al., 1998; Masucci et al., 1996; Rerie et al., 1994). We have recently demonstrated genetically that two genes, *SCARECROW* and *SHORTROOT* are essential for ground tissue organization in both root and shoot (Fukaki et al., 1998).

Mutations in the *SCARECROW* (*SCR*) gene result in roots that are missing one cell layer because of the absence of a formative cell division which normally produces cortex and endodermis (Di Laurenzio et al., 1996). The remaining mutant cell layer expresses cell-specific attributes of both cortex and endodermis. During embryogenesis there is an absence of a ground tissue layer (Scheres et al., 1995) which correlates with expression of *SCR* in this tissue prior to division (Di Laurenzio et al., 1996). These data are consistent with the idea that root radial patterning is established during embryogenesis. A screen for shoot agravitropic mutants identified alleles of *scr* (Fukaki et al., 1996, 1998). Characterization of the mutant hypocotyl and inflorescence stem revealed the origin of the inability to sense gravity. Both organs are missing a normal starch sheath (Fukaki et al., 1998; Scheres et al., 1995). This tissue contains starch-filled amyloplasts and has been hypothesized as being the site of gravity sensing in shoots (Sack, 1991).

The *scr* phenotype indicates that this gene plays a key role in radial patterning of both root and shoot. To further investigate the relationship between shoot and root radial pattern formation we analyzed *SCR* expression and mutant phenotype in mature shoot tissues and in their developmental precursors. We show that *SCR* expression in the mature inflorescence stem, leaves and embryo correlates with the pattern defects in *SCR* expressing tissues. The earliest detectable *SCR* expression is in the hypophyseal cell preceding its division to generate the quiescent center (QC) and the root cap cell lineages in the embryo. Consistent with the expression pattern we observed defects in organization of the root cap and QC in the *scr* mutant. Taken together with the observation of an L1 layer-specific *SCR* expression in the shoot meristem, this indicates that SCR's role in plant development is not limited to ground tissue formation. Similarities in the role and expression pattern of *SCR* in root and shoot suggest that the same molecular mechanism regulates radial patterning in both parts of the plant.

MATERIALS AND METHODS

Plant culture

The *scr-1* allele (in Wassilewskija background) and *scr-3/sgr1-1* allele (in Columbia background) were described previously by Di Laurenzio et al. (1996) and Fukaki et al. (1996, 1998), respectively. *Arabidopsis* seeds were surface sterilized and grown as described previously (Benfey et al., 1993). Plates for auxin transport inhibition contained 1-N-naphthylphtalamic acid (NPA) at final concentrations of 5-40 μM.

In situ hybridization

In situ hybridization was performed as described previously (Di Laurenzio et al., 1996). The antisense *STM* probe was described previously (Long et al., 1996). For microscopic analysis, slides were mounted either after a dehydration series in Permount (Fischer) or directly in Aqua-Poly/Mount (Polysciences). No signal over background was detected with the sense *SCR* probe.

Histological analysis and microscopy

To investigate the cell division patterns during embryogenesis, developing siliques were harvested and stained using Astra blue according to the method of Scheres et al. (1994) with the modification that PBS (pH 7.0) was used in fixation. Plastic sections were generated and processed as described previously (Di Lauernzio et al., 1996; Fukaki et al., 1998). Microscopic analyses were performed using Nomarski optics. Starch staining of leaves was described by Fukaki et al. (1998) except that leaves were collected from mature plants grown on soil.

GFP imaging of gene expression

The 2.5 kb region upstream of the *SCR* translational start site (Malamy and Benfey, 1997) was inserted directly upstream of the mGFP5-ER coding region in pBIN (gift from Jim Haseloff), introduced into *Agrobacterium* (LBA4404) and used to generate transgenic plants. For GFP analysis in embryos, embryo sacs were dissected out from siliques and mounted in 50% glycerol in water. Seedlings and inflorescences were placed on slides in a drop of water.

GFP fluorescence was visualized in whole mounts using a confocal laser scanning microscope (Zeiss LSM 310) with the Argon/Krypton line of 488. The later stages of embryogenesis and seedlings were imaged with a Leica confocal microscope, and the FITC channel (green: GFP) was overlaid onto the TRITC channel (red: autofluorescence and propidium iodide) to permit identification of the GFP-expressing cells.

J0571 enhancer trap line (kindly provided by Jim Haseloff), that shows specific root GFP expression in cortex/endodermis initial and all of its progeny (for description see: http:/brindabella.mrc-lmb.cam.ac.uk), was crossed with *scr-1*. Both WT and *scr-1* F_2 progeny roots were counter-stained with 10 μg/ml propidium iodide (Sigma) and imaged as above.

Plants heterozygous for the *pin-formed* mutation (*pin1-1* allele, Enkheim ecotype, kindly provided by Kiyotaka Okada) were crossed with the SCR-GFP line. Whole-mounts and fresh cross sections of *pin* inflorescences in the F_2 progeny were examined as above.

RESULTS

SCR expression in the shoot system

We have previously shown that *scr* hypocotyls are agravitropic and that this phenotype correlates with an absence of one of the ground tissue cell layers. The inflorescence stems of *scr* plants are also agravitropic and are missing a normal starch sheath layer containing sedimented amyloplasts (Fukaki et al., 1998).

To assess the role of *SCR* in shoot formation we examined its expression pattern during various stages of shoot development. This was done both by in situ hybridization and analysis of expression from a construct containing the *SCR*

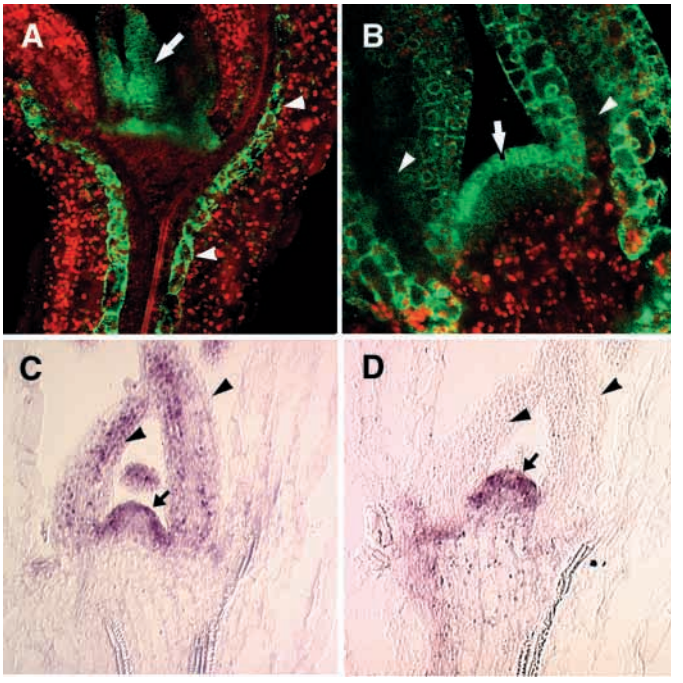


Fig. 1. *SCR* expression in WT seedling shoot. (A,B) Optical longitudinal sections through 5-day-old SCR::GFP seedling apex. GFP image (green) is overlaid on an autofluorescence image (red) showing the morphological features. (A) *SCR* expression is visible in hypocotyl endodermis (bottom arrowhead), abaxial side of cotyledon petiole (top arrowhead) and leaf primordium (arrow). (B) *SCR* expression in L1 of SAM (arrow) and in most cell layers of young leaf primordia (except presumptive vasculature, arrowheads). (C,D) In situ hybridization to consecutive longitudinal sections through 5-day-old seedling apex. (C) Antisense *SCR* probe hybridizes to SAM (arrow) and leaf primordia (arrowheads). (D) Antisense *STM* probe also hybridizes to SAM (arrow) but not leaf primordia (arrowheads; Long et al., 1996).

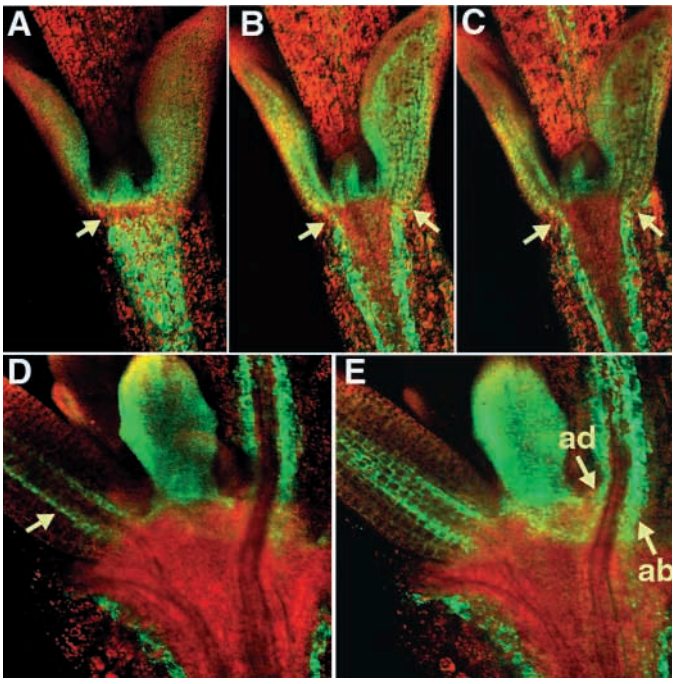


Fig. 2. Confocal images of WT SCR::GFP shoots. (A-C) Different longitudinal optical sections through 6-day-old seedling, Note, (A) a gap in green fluorescence at top of starch sheath layer (arrow); (B,C) connection between starch sheath and abaxial layer of bundle sheath (arrows). (D,E) 15-day-old seedling. (D) GFP expression in older leaf (left) is restricted to bundle sheath (arrow). (E) Connection of *SCR*-expressing cells between abaxial bundle sheath (ab, arrow) and starch sheath. Adaxial bundle sheath (ad, arrow) connects with *SCR*-expressing cells adjacent to shoot apical meristem.

promoter fused to the *Aequorea victoria* Green Fluorescent Protein (GFP) coding region (Haseloff et al., 1997). Both approaches yielded similar results. Expression in the seedling hypocotyl is confined to the endodermis or starch sheath (Fig. 1A), the cell layer affected by *scr* mutations. *SCR* expression is also detected in petioles of the cotyledons in cells that surround the vascular tissue. These *SCR*-expressing cells are contiguous to, and appear to form a single cell layer with the hypocotyl endodermis (Fig. 1A). There is no connection between hypocotyl endodermis and young leaf primordium at this stage of development (Fig. 1A).

We also studied *SCR* expression in the shoot apical meristem (SAM) and SAM-derived organs. After germination, *SCR* is expressed primarily in the L1 layer throughout the SAM including the peripheral zone (Fig. 1B,C). The expression in the L1 layer persists unchanged during seedling development (not shown).

In young leaf primordia *SCR* appears to be expressed in most tissues, except presumptive vasculature (Figs 1B, 2A-E). There appears to be a contiguity of *SCR* expression in these leaf primordia extending from the base to the tip within the epidermal and mesophyll layers (Fig. 1B). As the leaf blade expands, expression becomes more restricted to cell layers

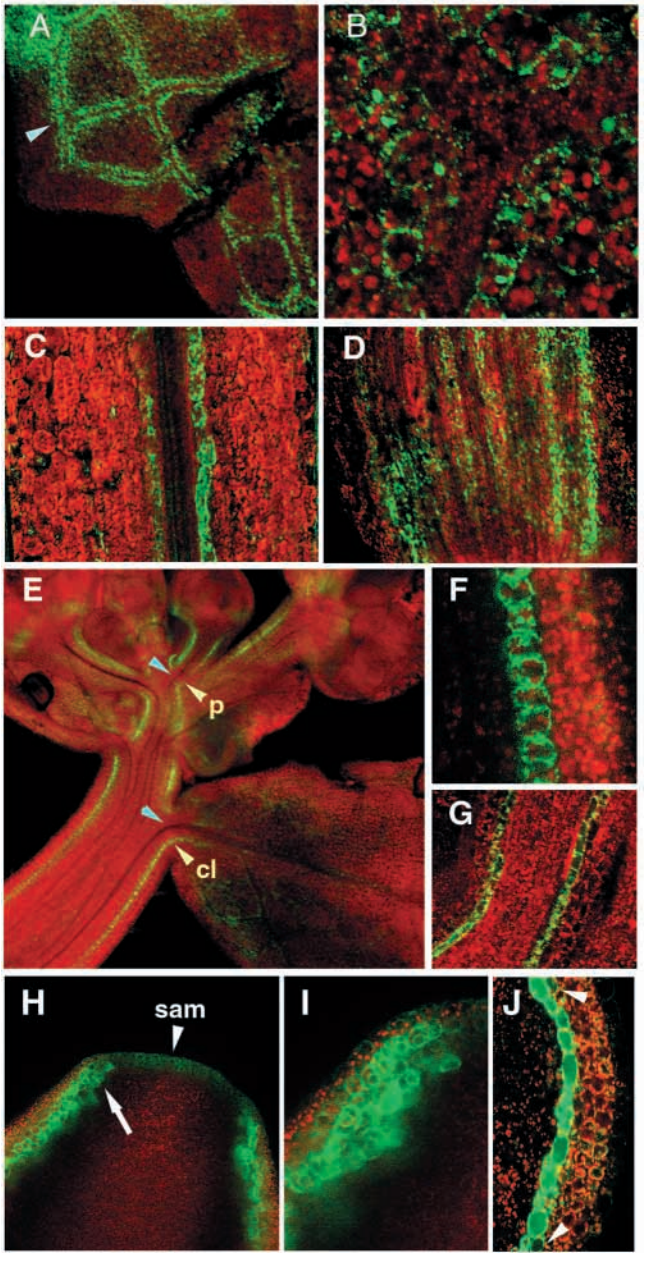


Fig. 3. *SCR* expression in wild-type (A-G) and *pin-formed* (H-J) adult shoot organs. (A) Fully expanded rosette leaf. Arrowhead indicates the region of the leaf shown in B. (B) Close-up showing highest GFP accumulation in bundle sheath (BS) cells. (C) Mature leaf petiole with GFP expression in BS cells. (D) Mature leaf petiole grown on 40 μM NPA with GFP in ectopic BS cells. (E) Inflorescence with GFP in starch sheath and contiguous internal layers in lateral organs. Blue arrowheads point to stem endodermis discontinuities at the lateral organ insertion points, yellow arrowheads indicate connections of stem endodermis with abaxial cauline leaf (cl) and abaxial pedicel (p). (F) Close-up of inflorescence stem showing expression in starch sheath layer. (G) Close-up of pedicel. (H,I) Optical longitudinal sections of *pin* inflorescence apex. (H) SAM (white arrowhead) with GFP accumulation in the L1 and L2 layers. Arrow indicates the region of the apex shown in I. (I) Close-up of *pin* inflorescence showing strong GFP accumulation in a few layers of cells. (J) Transverse section near the apex of *pin* inflorescence stem with GFP accumulation generally in a single layer of starch sheath. White arrowheads indicate outer cells with weak GFP accumulation which possibly are the result of recent cell division.

located in close proximity to vascular elements including mesophyll and bundle sheath cells. In mature leaves the strongest *SCR* expression is observed in bundle sheath cells associated with all veins (Fig. 3A,B). A similar *SCR* expression pattern was observed in fully expanded cauline leaves (Fig. 3E). In petioles expression is also found in a single cell layer surrounding the mid-vein (Figs 2D,E, 3C).

The expression of *SCR* in inflorescence stems is consistent with the agravitropic phenotype associated with an abnormal or missing starch sheath. *SCR* is expressed in a single internal cell layer corresponding to the starch sheath (Fig. 3E,F). The expression in the inflorescence stem is contiguous with the bundle sheath cell expression in the cauline leaves (Fig. 3E) and a single internal cell layer of pedicels (Fig. 3E,G).

Of particular interest is the finding that *SCR* is expressed in cells that connect the bundle sheath cells in the leaf with the shoot endodermis. The *SCR*-expressing cell layer in rosette leaves is contiguous with the hypocotyl endodermis. This connection can be first seen as a band of *SCR* expression that extends from the hypocotyl into the developing leaf primordium (Figs 1B, 2B,C). At the earliest stages of rosette leaf primordium formation there are no detectable *SCR*-expressing cells in this region (arrow in Fig. 2A). As the leaf primordium develops, cells between the endodermis and the leaf primordium begin to express *SCR* (Fig. 1B). These are contiguous with the bundle sheath cells in the developing petiole and appear to be the precursors of the cells that form the connecting tissue between the endodermis and the bundle sheath cells. However, this connection is only observed between the hypocotyl and the abaxial side of the leaf primordium and leaf petiole (Figs 1B, 2B-E). On the adaxial side of the leaf, the *SCR*-expressing band of cells appears to extend from the L1 layer of the SAM to the bases of the adjacent leaf primordia (Figs 1B, 2). The *SCR* expression pattern in the inflorescence stem is consistent with a similar process in which a direct 'connection' is made between the stem endodermis and the abaxial bundle sheath cells of cauline leaves (arrowhead in Fig. 3E). This is the first evidence suggesting that a set of cells in the developing apex is recruited to form the endodermis/bundle sheath connection on the abaxial side of leaf. These data suggest a process of specifying the abaxial bundle sheath cells which involves a progressive restriction of *SCR* expression resulting in only bundle sheath cells expressing *SCR*.

The *SCR* expression pattern raised the question of how the starch sheath is generated in tissue above each leaf. Characterization of this process is complicated in the wild-type plant because it is continually forming leaves or floral primordia from the shoot or inflorescence meristem. To circumvent this problem we analyzed *SCR* expression in the *pin-formed* (*pin*) mutant, which forms a shoot inflorescence devoid of appendages (Okada et al., 1991). In the apex of the *pin* inflorescence, weak *SCR* expression was detected in the L1 layer and even weaker expression was found in the L2 layer (Fig. 3H), in a pattern similar to that found in the SAM. Just distal to the apex, expression is detected in two or three ground tissue layers. Slightly below these tissues, expression disappears in the outermost layers remaining only in the starch sheath (Fig. 3I). In transverse sections just below the apex, expression is detected generally in a single layer of starch sheath (Fig. 3J). It is possible to find cells that appear to have recently divided in which strong expression remains in the inner cell and begins to diminish in the outer cell (arrowheads in Fig. 3J). This expression pattern of *SCR* in a *pin* background raises intriguing questions about potential similarities between the underlying processes regulating radial patterning in shoot and root (see Discussion).

Radial pattern defects in the *scr* shoot system

The *SCR* expression pattern in the shoot is consistent with the previously determined phenotypic defects (Fukaki et al., 1998). Expression of *SCR* in the SAM prior to bolting strongly suggests that it regulates a cell division process responsible for generating the inflorescence starch sheath. In addition, as with the root, there is persistent expression in the stem endodermis after all apparent cell division activity has ceased. It is possible that there is an additional role for *SCR* in the specification or maintenance of cell differentiation in this tissue.

Expression in other shoot tissues raised the possibility of additional phenotypic defects that could shed further light on SCR function. Wild-type hypocotyls contain three ground tissue cell layers: two layers of cortex and one layer of endodermis (Fig. 4A). At present there are no tissue-specific molecular markers available for the hypocotyl ground tissue. Therefore we could not definitively determine the identity of the 'mutant' cell layers in the *scr* hypocotyl. However, we have shown that the internal most ground tissue layer in *scr* hypocotyl contains amyloplasts. In contrast to wild type these amyloplasts in *scr* do not sediment (Fukaki et al., 1998) but are dispersed throughout the cell. In addition *scr* seedlings are also missing a cell layer within the cotyledon petioles (Fig. 4B). This layer surrounds the vascular tissue and is marked by *SCR* expression in wild-type plants (Fig. 1A).

Rosette leaves in *scr* plants are much smaller than wild type (Fig. 4C,D; Fukaki et al., 1996). In wild type mature rosette leaves the cell layer that surrounds the vasculature of the main vein contains amyloplasts (Fig. 4C,E). These amyloplasts are not present in *scr* leaves (Fig. 4D,F). In addition, cross sections of *scr* petioles consistently appear to contain one less cell layer (Fig. 4G). This suggests that in a manner analogous to the hypocotyl and stem, the reason for the lack of amyloplasts in the petiole (Fig. 4E,F) is that the cell layer that normally contains them is not formed. Other possible leaf defects in *scr* may be too subtle to assess at present without the availability of tissue-specific leaf markers.

Role of vasculature in *SCR* leaf expression

In both shoot and root, *SCR* expression is detected in the layer adjacent to the vascular bundles. This is particularly evident during leaf development, when *SCR* expression becomes progressively restricted to cell layers in close proximity to vascular elements (Figs 2, 3A-C). This suggests that vascularization is a prerequisite for SCR-mediated patterning of the ground tissue. To determine the relationship between vasculature and *SCR* leaf expression we grew SCR::GFP plants on NPA, an inhibitor of polar transport of auxin. Plants grown on plates containing 40 μM NPA develop small leaves with very short petioles. These petioles develop numerous ectopic veins (Mattsson et al., 1999). Cells expressing *SCR* (Fig. 3D) surround these veins. Therefore, induction of ectopic vasculature correlates with induction of *SCR* expression around these new vascular elements.

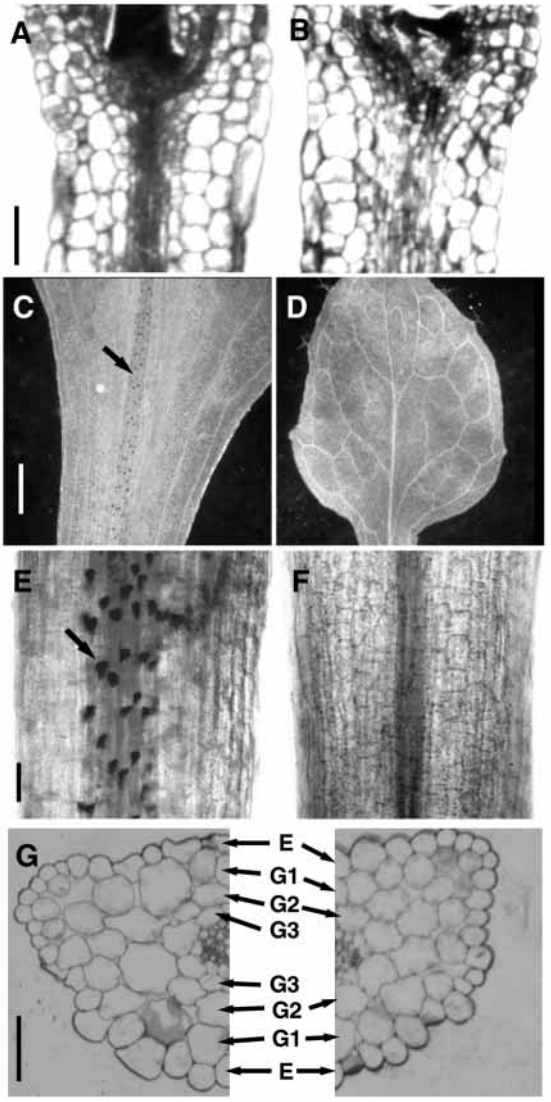


Fig. 4. Comparison of wild-type (A,C,E and left side of G) and *scr* (B,D,F and right side of G) shoot organs. (A,B) Toluidine blue stained longitudinal sections through hypocotyl/cotyledon junction of 3-day seedlings. *scr* hypocotyl and cotyledon petiole contain only two ground tissue layers as compared to three in WT. (C,D) Mature fifth rosette leaves stained for starch (shown at the same magnification). Amyloplasts are present only in WT (arrow in C). (E,F) Mature petioles stained for starch. Amyloplasts are present only in WT (arrow in E). (G) Toluidine blue stained cross sections of mature petioles. In *scr* there appears to be one ground tissue cell layer missing. G, ground tissue; E, epidermis. Genotypes were *scr-1* for B and right side of G, *scr-3* for D and F. Scale bars, 100 μm except (C,D), 600 μm.

SCR expression during embryogenesis

In our previous study we detected *SCR* expression in the prospective endodermal cell layer in nearly mature embryos and in the ground tissue at an earlier stage of embryogenesis (Di Laurenzio et al., 1996). We have also shown that mutations in the *SCR* gene result in defects in some of the periclinal cell divisions of the embryonic ground tissue that represent the prospective lower hypocotyl/root region (Scheres et al., 1995). Here we carried out a more detailed study of embryonic expression of *SCR* for three reasons. First, because hypocotyl and cotyledons are formed during embryogenesis we wished to determine the relationship between *SCR* expression and *scr* defects prior to germination. Second, *SCR* expression is detected in the SAM of seedlings – we wanted to know if expression is detectable in SAMs prior to germination. Third, to correlate root and shoot roles of SCR it is important to define at what stage of embryogenesis *SCR* expression is first established and define precisely its expression pattern throughout embryogenesis as root and shoot organs are formed.

Radial patterning of the embryonic hypocotyl occurs concomitantly with that of the embryonic root through a series of periclinal cell divisions that subdivide the embryo into an increasing number of layers (Scheres et al., 1994, 1995). The first evidence for SCR involvement in radial patterning is its expression in the ground tissue at the globular stage of embryogenesis (Fig. 5E,F and schematic). In addition to its expression in ground tissue at this early stage of embryogenesis, *SCR* expression was also detected in the hypophyseal cell (Fig. 5B,C and schematic). The hypophysis is derived from the basal cell after the first zygotic division. It subsequently divides to form an upper lens-shaped cell from which the precursors of the QC form, and a lower cell that gives rise to the columella root cap initials. After the division of the hypophyseal cell, *SCR* expression shifts to the lens shaped cell that will give rise to the central cells (Fig. 5E and schematic). After the formation of the ground tissue at the globular stage, meristems begin to form at the apical and basal poles. While there is no clear cell division event essential for SAM formation, for the root apical meristem (RAM) a key division is that of the hypophysis. No expression of *SCR* was detected in SAM precursors at this stage.

Between the triangular stage and late heart stage, the ground tissue goes through a first set of periclinal cell divisions to form a two cell-layer ground tissue in presumptive hypocotyl and embryonic root (Fig. 5G,J and arrows in schematic). The only ground tissue cells that do not undergo this division are the presumptive initials for the cortex and endodermis located at the very tip of the embryonic root. *SCR* is expressed before every one of these ground tissue divisions (Fig. 5H,I,K,L and schematic). Very consistently, after each division, *SCR* expression is restricted to the inner daughter cell (Fig. 5H,K). This appears to occur at approximately the same time in both hypocotyl and root (Fig. 5K,L and schematic). In contrast to in situ hybridization, GFP images also show a transient signal in the outer daughter cell, probably due to the relatively long half-life of GFP compared to the *SCR* mRNA (Fig. 5F,I).

At the torpedo stage, additional periclinal divisions in the hypocotyl region result in the formation of two layers of cortex and one layer of endodermis (Fig. 5M and schematic). As in the previous stages, *SCR* expression becomes restricted to the innermost, prospective endodermal cell layer following these divisions (Fig. 5N,O). At this stage, the radial organization is histologically comparable to that of the mature embryo (Fig. 5P). Moreover, because there are few if any cell divisions that take place in the hypocotyl post-embryonically, the entire hypocotyl has been formed at this stage. Strong *SCR* expression is also found in the prospective cotyledon shoulder region (Fig. 5O) that is derived from the upper lower tier (ult) region of the triangular stage embryo (Scheres et al., 1994).

This will form the bundle sheath cells of the cotyledon petiole that show strong *SCR* expression in seedlings (Fig. 1A). At later stages of embryogenesis, the *SCR* expression pattern in the ground tissue remains essentially unchanged: it is found in the prospective endodermal cell layer of the hypocotyl and root (Fig. 5Q,R and schematic).

We conclude that *SCR* expression is found in a remarkably consistent manner in each ground tissue cell prior to a periclinal division and in the inner daughter cell after the division. Remarkably, *SCR* expression was never detected in serial optical sections through SCR-GFP expressing embryos indicating that seedling expression in this region is induced upon germination.

Radial pattern defects in *scr* embryos

If SCR plays a role in regulating cell divisions in the regions where the gene is expressed, *scr* mutants would be expected to have defects in these divisions. A detailed histological analysis of *scr-1* embryos (*scr-1* is a probable null because no transcripts were detected with this allele; Di Laurenzio et al., 1996) revealed that for every division that is marked by restricted expression of the *SCR* gene in WT embryos there are corresponding defects in the mutants. Already at the triangular stage the division in the ult which will form the hypocotyl and cotyledon shoulder (Fig. 5G and schematic in Fig. 6) is occasionally defective in *scr* embryos (Fig. 6A and schematic). At the heart stage, the periclinal cell divisions in the root region are consistently defective in *scr-1* embryos, as are the divisions in the hypocotyl region at the torpedo stage (Fig. 6B,C and schematic).

A preliminary analysis of embryonic development in *scr-1* suggested that periclinal divisions of ground tissue did not occur at all (Scheres et al., 1995). However, in our detailed analysis we found that periclinal divisions in the ground tissue of *scr-1* were not totally lacking. In fact, there was considerable variability in the cell division patterns in the ground tissue of both hypocotyl and embryonic root, even within a single embryo (Fig. 6). Frequently, divisions did not result in distinct cell files as in wild type but appeared to occur in isolated cells independent of their neighbors (Fig. 6). The occurrence of some periclinal divisions in the embryonic ground tissue in *scr-1*, is consistent with the idea that the primary role of SCR is to ensure that the periclinal divisions occur in a consistent and organized manner to generate the correct radial patterning of the hypocotyl and embryonic root.

Quiescent center/root cap defects in *scr* embryos and seedlings

We have shown that *SCR* expression is also a marker for the

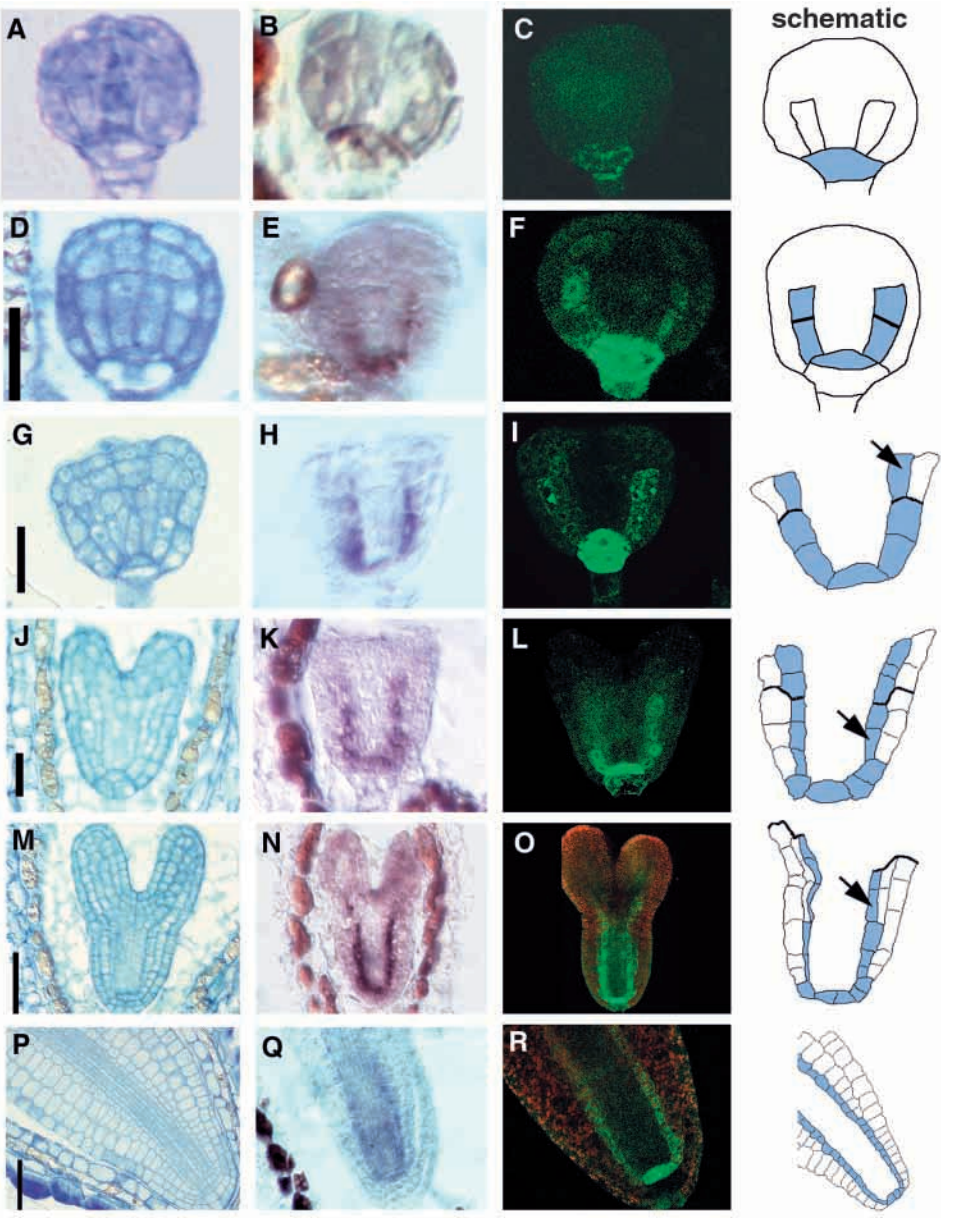


Fig. 5. *SCR* expression during embryogenesis. (A-C) Early globular stage, (D-F) late globular stage, (G-I) triangular stage, (J-L) mid-heart stage, (M-O) torpedo stage and (P-R) hypocotyl and root region of nearly mature embryos. (A,D,G,J,M,P) Astra blue stained longitudinal sections of WT embryos. (B,E,H,K,N,Q) In situ hybridization with *SCR* antisense probe. (C,F,I,L,O,R) Confocal images of GFP expression driven by *SCR* promoter. Scale bars in (A-L) 25 μm, in (M-R), 50 μm. Right: Ground tissue progenitors, derivatives and hypophysis/central cells are shown schematically traced from the leftmost panels. *SCR* expression is indicated by blue shading. Shift of *SCR* expression to the innermost cell layer after periclinal divisions indicated by arrows. Dark line indicates boundary of ult and llt.

formative division of the hypophyseal cell to generate the quiescent center/root cap cell lineages (Fig. 5A-F). To determine the role of SCR in this division we analyzed the status of these cell lineages in *scr-1* mutants.

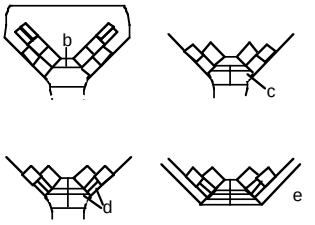
The orientation and timing of the hypophyseal division nearly always appears to proceed normally in *scr-1* during triangular stage (not shown). However, subsequent cell divisions both in the prospective QC and in the root cap are defective (Table 1; Fig. 6B,C). This phenotype can be observed as early as late heart/early torpedo stage, when periclinal cell divisions create an organized root cap and a set of root cap initials (Table 1; Fig. 5J,M; Scheres et al., 1994). In *scr-1* embryos these divisions are missing or delayed at torpedo stage, and the central cells appear to divide in a random fashion (Table 1; Fig. 6B,C).

In some nearly mature *scr-1* embryos the quiescent center/cap region remains disorganized (not shown), although in many cases a layered structure can be found (Table 1; Fig. 6D). However, even in the latter case many groups of cells lacked the stereotypical pattern of cell division planes observed in the wild type. These additional cell division defects suggest that SCR has a role in determining specific cell division planes in the QC and root cap regions.

To determine if the embryonic deficiencies associated with the QC and columella root cap persist after germination we

Table 1. Quiescent center/root cap phenotypes of *scr-1* embryos[a]

Stage	Wild type	*scr-1*
Triangular[b]	10/10	7/8
Heart[c]	9/9	3/6
Torpedo[d]	6/6	0/6
Mature[e]	8/8	7/16



[a]Values are reported as number of embryos that have undergone the indicated cell division event/total number of analyzed embryos.
[b]Embryos that have undergone the cell division leading to the formation of prospective QC and columella root cap.
[c]Embryos that have undergone divisions leading to the formation of columella root cap and its initials.
[d]Embryos that have undergone divisions leading to the formation of lateral and columella root cap.
[e]Embryos that have an organized layered structure in the QC-root cap region of RAM.

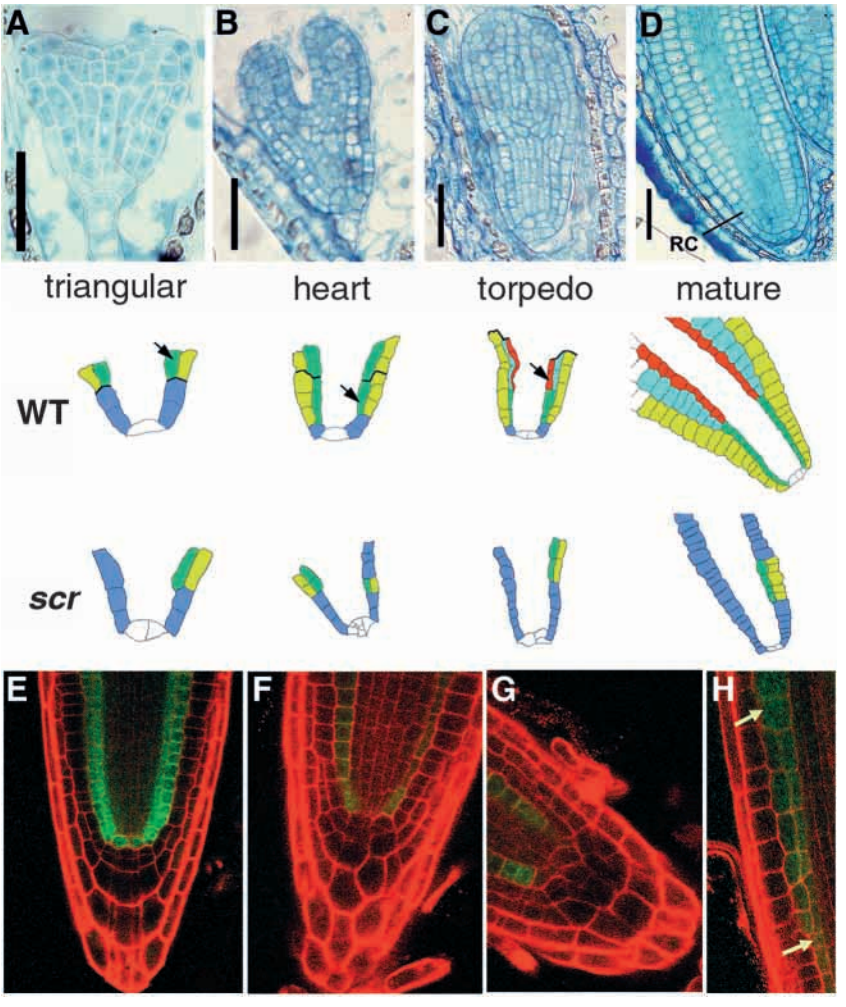


Fig. 6. Defects in *scr-1* embryos and seedling roots. Top: (A-D) Astra blue stained sections of *scr-1* embryos. (A) No periclinal cell divisions are observed on the left side, only the ult region has divided on the right side of the ground tissue. (B) Only one cell has undergone periclinal cell division on the right side of the ground tissue, and only the prospective cotyledon shoulder region has divided normally on the left side. There are no periclinal cell divisions in root cap and ectopic cell divisions in QC region. (C) Only the prospective cotyledon shoulder region has undergone periclinal cell division on the right side, no divisions on the left side and no periclinal cell divisions in the root cap. (D) Periclinal cell divisions in ground tissue on the right side result in a limited region with two cell layers. Three cell layers present in the columella root cap/QC, and lateral root cap is reduced (compare with Fig. 5P). RC, lateral root cap region. Scale bars, 50 μm. Middle: Schematic of ground tissue cell division patterns from upper panel (*scr-1*) and from Fig. 5 (wild type). Thick black line indicates boundary of prospective root/hypocotyl and cotyledon shoulder regions. The daughter cells are shown in a different color than mother cell after division. Central cells are white. Bottom: (E-H) Confocal images of primary roots (8-day-old seedlings) of J0571 line expressing GFP in ground tissue and ground tissue initials. Propidium iodide (red) used to visualize cell outlines. (E) WT primary root tip with characteristic nearly invariant cellular organization of meristem and root cap. (F-H) Representative J0571 in *scr-1*. Note disorganized meristem and columella root cap. (H) Example of occasional random divisions in ground tissue of *scr-1* root. Arrows indicate undivided cells on either side of double cell-file region.

examined primary root tips of 8-day-old seedlings. WT and *scr-1* roots were analyzed using the GFP enhancer trap line J0571 which expresses in cortex and endodermis as well as their shared initial. In *scr-1* root tips a single cell file expresses GFP, indicating the position of the mutant layer and its presumptive initial cell (Fig. 6F,G). Based on the cell division patterns in the vicinity of the initial cell, the radial pattern of the other cell layers appears normal. However, the cell shapes and their relative positions within the root tip are abnormal. The meristematic region appears disorganized. Although there is a large degree of variability in the severity of these defects, in all cases the columella root cap cells were misshapen and the number and/or the position of the central cells (QC) were abnormal (Fig. 6F,G compare to WT in Fig. 6E). In addition, as was the case in the embryonic root there are occasional, random divisions observed in the mutant ground-tissue-derived cell layer. In the majority of roots, single cell layers can be traced back to the endodermis/cortex initials (Fig. 6F,G). However, a partial double layer can be observed in some roots at different positions along its length (Fig. 6H).

DISCUSSION

Correlating mutant phenotype with gene expression data is a powerful means of deducing gene function. The finding that *scr* mutants are agravitropic in their stem and hypocotyl compelled us to perform a detailed analysis of *SCR* expression in these organs and their precursors. The *SCR* expression pattern, in turn, revealed new potential roles for SCR which we confirmed through characterization of the mutant phenotype in those tissues.

SCR regulates radial patterning of the ground tissue during embryogenesis

Because both the hypocotyl and shoot apical meristem are formed during embryonic development we were interested in determining the precise order and timing of *SCR* expression from the earliest stages of embryogenesis. We found that *SCR* is expressed in cells that are destined to undergo periclinal divisions in the ground tissue of the embryonic root, hypocotyl and prospective cotyledon shoulder regions. Following these periclinal divisions, *SCR* expression is restricted to the innermost of the two daughter cells. This supports our previous hypothesis that these divisions are asymmetric (Di Laurenzio et al., 1996). The phenotype of the *scr* mutants demonstrates that SCR plays a key role in regulating these divisions, as the same divisions that are marked by restricted *SCR* expression are defective in the *scr* mutant embryos. All periclinal divisions in the ground tissue of the prospective root, hypocotyl and cotyledon shoulder can be affected. However, the defects become progressively more prevalent over the course of embryogenesis.

It is significant that even presumably null mutations in *SCR* do not result in a complete loss of periclinal divisions in the ground tissue. Instead of the highly stereotyped division pattern in wild type, periclinal ground tissue divisions occur only occasionally. This indicates that SCR may not be essential for inducing all periclinal divisions. Instead, there may exist a SCR-independent pathway that is able to promote divisions in some of the ground tissue cells. This pathway may normally act in concert with SCR to generate correct periclinal divisions in the ground tissue, but alone results in an uncoordinated pattern of cell divisions within the layer.

SCR affects organization and development of the quiescent center and root cap

The detection of *SCR* expression in the hypophyseal cell prior to its division lead us to characterize the mutant phenotype of the cells derived from it. Consistently, we observed a disorganized QC and root cap in nearly mature *scr-1* embryos. These defects could be traced back to the late heart/early torpedo stage when cell divisions that form the root cap appear to be delayed (Fig. 6B,C). This delay often correlated with the presence of ectopic cell divisions, especially in the central cells, resulting in a disorganized QC. The observed SCR activity in the hypophyseal cell may be essential for the precision of these divisions in the root apex. In this case the role of SCR in the hypophyseal cell would contrast with that in the cortical/endodermal initial – the formative division is not affected, but the fate of the daughters is. Alternatively, SCR may be controlling these divisions in an indirect manner.

The presence of *SCR* in the hypophysis and its descendants indicates that SCR function extends beyond ground tissue patterning. Analysis of the postembryonic expression pattern in the root meristem using the SCR-GFP construct revealed persistent expression in the QC cells (data not shown). This correlates well with the phenotype of disorganization in this region of the postembryonic root.

SCR function in the shoot

We have shown that the defects in the *scr* hypocotyl (agravitropism and deletion of one of the ground tissue cell layers) correlate precisely with the pattern of *SCR* expression within this organ during embryogenesis. Our previous analysis had demonstrated a role for SCR in starch sheath development of the inflorescence stem. The *SCR* expression pattern revealed a potential role in the formation of bundle sheath cells of leaves.

The rosette leaves and inflorescence stems derive from the SAM. Although the SAM is formed during embryogenesis our analysis failed to detect any *SCR* expression in the embryonic the SAM. Expression in the seedling appeared primarily in the L1 layer of the SAM. An histological comparison of mutant and WT SAMs did not reveal any obvious differences (data not shown). This may indicate that SCR plays a redundant (or no) function in the SAM. We note that the phenotype associated with *SCR* expression in the root QC cells (which are in an analogous position to the L1 layer if one removes the root cap) is variable, suggesting some level of redundancy there as well.

As the leaf primordium expands, *SCR* expression becomes progressively restricted to the bundle sheath cells that are in direct contact with the vascular strands. Recent results indicate that bundle sheath ‘cells differentiate in a position-specific manner, rather than from a distinct cell lineage’ (Kinsman and Pyke, 1998). Our data suggest that SCR may be one of the early components involved in the formation of these cells. This role is supported by the lack of an amyloplast-containing cell layer in *scr* leaves. The idea that vascular development may be a prerequisite for bundle sheath formation is supported by the *SCR* expression pattern in petioles with ectopic vasculature.

An intriguing aspect of bundle sheath development, revealed

by the *SCR* expression pattern, is the apparent asymmetry of its contiguity with the hypocotyl endodermis. Our results indicate that *SCR*-expressing cells form a connection between leaf bundle sheath cells and existing hypocotyl endodermis only on the abaxial side of the petiole. It was shown that primary vascular strands of leaf primordia derive from the vasculature of the hypocotyl (Kinsman and Pyke, 1998). We have demonstrated that a layer of *SCR*-expressing cells follows this vasculature into the abaxial side of the primordium. It suggests that developing vascular tissue may induce *SCR* expression in the cell layer adjacent to it while the connection is being made. SCR in turn may play a role in division of these newly recruited cells to form the tightly abutting cell files seen in more mature tissue.

The induction of SCR expression in cells between the hypocotyl endodermis and leaf primordia has no apparent parallel in root development. This may be related to the differences in how appendages are formed in shoot and root. In the shoot, leaves are formed from the shoot meristem, necessitating an interruption in the continuity of vascularization and consequently ground tissue surrounding the vascular tissue. By contrast, secondary roots are formed at some distance from the root meristem and from internal pericycle tissue.

Analysis of *SCR* expression in the *pin-formed* inflorescence provides intriguing insight into the formation of the starch sheath in the absence of leaf or floral primordia formation. Previous characterization of *pin* indicated that the primary defect is in auxin transport (Okada et al., 1991). This defect in auxin transport affects vascular pattern formation in the *pin* shoot (Gälweiler et al., 1998). However, anatomical comparison indicates that, near the inflorescence apex, there is relatively little difference in the radial organization of ground tissue between *pin* and WT (Fig. 3J). Thus it is reasonable to use results from *SCR* expression in the *pin* inflorescence to elucidate normal ground tissue formation in the stem.

The *SCR* expression pattern in the apex of the *pin* inflorescence stem is remarkably reminiscent of *SCR* expression in the root meristem. Expression is found in more than one cell at the tip of the ground tissue cell files and then becomes constrained to a single cell file. Because there is no lineage analysis available for the internal layers of the stem, we can only speculate as to similarity in the underlying mechanism. One possibility is that at the apex of the stem there exist initial cells which undergo asymmetric divisions to generate the various cell files. The cells in which *SCR* is expressed at the shoot apex may be the initials for the ground tissue (or possibly just for the inner layers of ground tissue). If this is the case, then the conservation of mechanism and genes involved in ground tissue patterning argues for evolutionary homology in the radial patterning of root and shoot.

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