Maturation of the Ground Tissue of the Root Is Regulated by Gibberellin and *SCARECROW* and Requires *SHORT-ROOT*[1][w]

Alice J. Paquette and Philip N. Benfey*

Department of Biology, Duke University, Durham, North Carolina 27708

As the root of Arabidopsis (*Arabidopsis thaliana*) ages, a third layer of ground tissue forms that we find rapidly takes on cortex character. We show that GA and *SCARECROW* (*SCR*) regulate the timing of this developmental transition and that *SHORT-ROOT* (*SHR*) is required for it.

The Arabidopsis root is usually described as having four concentric single-celled layers surrounding the central vascular tissue (Benfey and Scheres, 2000). From the outside of the root, these are the epidermis, cortex, endodermis, and pericycle. Together the cortex and endodermis form the ground tissue (Fig. 1A, brackets). This radial pattern is established in the apical meristem, and *SHR* and *SCR*, which are members of the GRAS family of putative transcription factors (Pysh et al., 1999), have been shown to play a central role in the patterning process (Nakajima and Benfey, 2002). In fact, as the Arabidopsis root ages, a third layer of ground tissue forms, which rapidly takes on cortex character. Baum et al. (2002) first reported progressive changes over time in the Arabidopsis root meristem cytoarchitecture. These changes occur later than the early post-embryonic stage that is commonly analyzed in developmental-genetic studies. Here, we replicate their findings with respect to the ground tissue, in Columbia (Col) seedlings grown on agar plates, and extend them using molecular markers. We find that GA and *SCR* additively regulate the timing of formation of the new ground tissue layer, termed the middle cortex, and that *SHR* is required independently of *SCR* for this developmental transition to occur.

FORMATION OF A DISTINCT STEM CELL FOR EACH OF THE TWO GROUND TISSUE LAYERS

After sterilizing the seeds and imbibing them in the dark at 4°C for 2 to 3 d, we sowed them on 1% agar plates containing 1× Murashige and Skoog salts, 0.5 g/L MES, and 1% Suc at pH 5.7 (for details, see supplemental materials). We sealed the plates with Parafilm and incubated them in a nearly vertical position in a growth chamber at 22°C with a 16-h-light/8-h-dark photoperiod. Time of transfer to the growth chamber was designated d0. We analyzed primary root apical meristems at d3, d7, and d14 by staining roots with propidium iodide and imaging them using a confocal microscope. When observed in midlongitudinal section, only the ground tissue cell files on either side of the root are visible, and cell divisions were scored only in these two sets of cell files. As divisions in files out of the plane of section were not recorded, quantitative data is an underestimate of the amount of division throughout the meristem. Transverse sections were imaged only to illustrate qualitative data.

It has been reported that in Col the cortex and endodermis of the primary root are derived from a common progenitor, the cortex/endodermal (C/E) initial, or C/E stem cell (Benfey et al., 1993; Dolan et al., 1993). While this is the case at very early stages of postembryonic development, these stem cells soon begin to divide periclinally to generate separate stem cells for each of the two layers of ground tissue. Under our conditions, by d3 many roots had at least one undivided C/E stem cell of the two visualized (Fig. 1A and inset, arrowheads); by d7, however, significantly fewer roots displayed the immature state (d7: 12%, $n = 50$ versus d3: 33%, $n = 60$; $P < 0.025$). Both stem cells were divided in almost all of the d7 roots (Fig. 1B, arrows).

INITIATION OF MIDDLE-CORTEX FORMATION AND MOLECULAR IDENTITY OF THIS NEW CELL LAYER

Under our conditions, by d14 a third layer of ground tissue, termed the middle cortex (Baum et al., 2002), begins to form (34% of primary root meristems at d14 had at least one middle-cortex cell, $n = 50$). At later time points generation of this layer is apparently maintained by a specific set of divisions of the inner ground tissue stem cell (previously the endodermal stem cell) and its derivatives (Baum et al., 2002). The first cells of the middle cortex, however, form distant from the quiescent center (QC; Fig. 1C, arrow)

[1] This work was supported by the National Institutes of Health (postdoctoral fellowship no. GM20566 to A.J.P. and grant no. GM43778 to P.N.B.).

* Corresponding author; e-mail philip.benfey@duke.edu; fax 919–613–8177.

[w] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.104.058362.

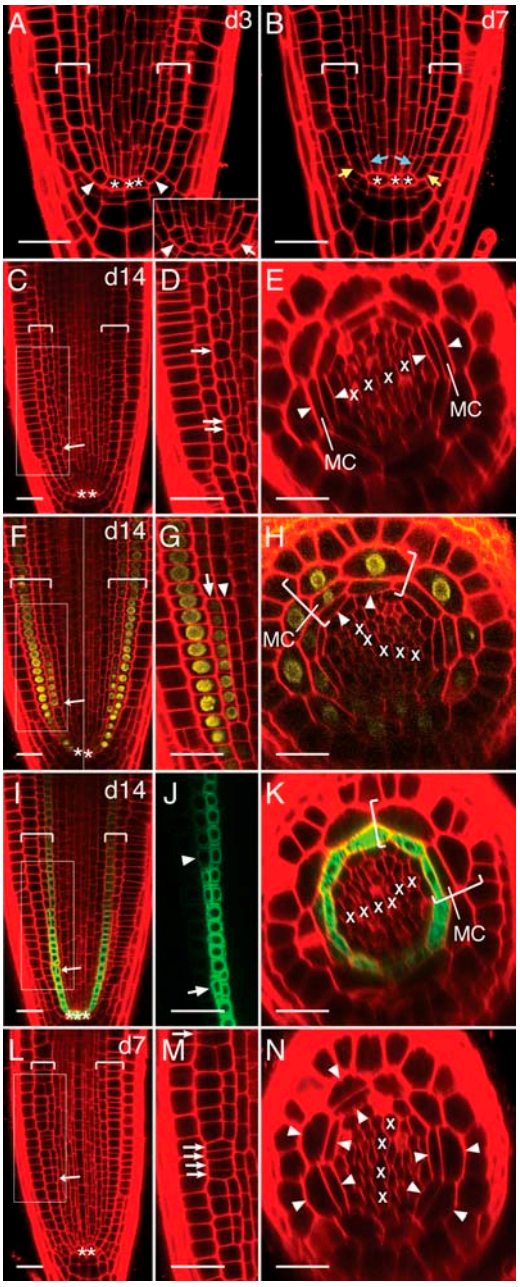

Figure 1. Maturation of the wild-type Arabidopsis primary root and regulation of the process by *SCR*. A and B, Over time, separate stem cells form for each of the two layers of ground tissue by periclinal division of the C/E stem cell. A, At d3 many C/E stem cells are not divided (arrowheads), but the process is beginning (inset, arrow). B, By d7 almost all roots show separate cortex stem cells (yellow arrows) and endodermis stem cells (blue arrows). C to K, By d14 middle cortex is beginning to form. Areas boxed in C, F, and I are shown magnified in D, G, and J, respectively. C to E, The first cells of the middle cortex arise from periclinal division of endodermal-layer cells that are not near the QC (C, arrow) and that are adjacent to the xylem poles (E, arrowheads). At this stage, within a cell file, divided cells are frequently interspersed with undivided cells (D, arrows). F to H, Cells of the middle cortex rapidly take on cortical identity. F, Composite image of two focal planes of the same root. The cortex-specific marker *pCo2*::YFP_{H2B} is expressed de novo in all outer derivatives of divided endodermal-layer cells (G, arrow). *pCo2*::YFP_{H2B} is induced in middle-cortex cells but not in adjacent endodermal cells (G and H, arrowheads). I to K, Cells of the middle cortex lose endodermal identity. The endodermis-specific marker *SCR*::*green fluorescent protein* is eventually downregulated in middle-cortex cells that are distant from the QC (J, compare arrowhead to arrow; K, MC). K, Shows a different root than in I and J. L–N, *SCR* is not required for the divisions that normally form middle cortex. *SCR* may, in fact, normally act to repress these divisions in both time and space. At d7 divisions of ground tissue cells in the *scr-5* null mutant resemble middle-cortex-forming divisions in wild type with respect to their distance from the QC (compare L to C, arrows) and their discontinuous pattern within a cell file (compare M to D, arrows). In a *scr* null mutant the first divisions occur at an earlier time point and in a position different from that in wild type (compare N to E, arrowheads relative to Xs). Longitudinal (A–D, F, G, I, J, L, M) and transverse (E, H, K, N) optical sections of the apical meristematic zone. Brackets, Ground tissue; asterisk, QC cell; X, xylem progenitor. Scale bars, 25 microns.

and arise from asynchronous periclinal division of individual endodermal-layer cells, indicated by divided and undivided cells being interspersed both within a cell file (Fig. 1D, arrows) and within a ring of endodermis in cross section (Fig. 1E). Interestingly, it appears that the first endodermal cells to divide tend to be adjacent to the xylem poles (Fig. 1E, arrowheads). This matches the pattern and approximate timing of initiation of middle-cortex formation reported in roots of sand-grown plants of the Wassileweskija ecotype (Baum et al., 2002).

The new layer of ground tissue was termed the middle cortex because of its anatomical position. Here we demonstrate that cells of this new layer have cortex character by analyzing the expression of an early marker of cortex and another of endodermis in the ground tissue of d14 roots (Fig. 1, F–K). Within the meristem the pCo2::YFP_{H2B} line specifically marks the nuclei of cortex cells (Heidstra et al., 2004). We also detected expression in the middle cortex, even in those cells that were closest to the QC (Fig. 1, F and G, arrows; H, middle cortex [MC]). As these cells are derived from nonexpressing cells of the endodermal layer, this indicates that new cells of the middle cortex rapidly take on cortex identity. Further indicating that the middle cortex is, indeed, a new layer of cortex, the endodermal marker SCR::green fluorescent protein (Sabatini et al., 1999; Wysocka-Diller et al., 2000) is gradually down-regulated in new middle-cortex cells as they are displaced farther from the QC (Fig. 1J, compare arrowhead to arrow; K, MC).

SCR IS NOT REQUIRED FOR MIDDLE-CORTEX-FORMING DIVISIONS AND INHIBITS THEIR PREMATURE INITIATION

With a nonsense mutation in codon number 239 in *SCR*, *scr-5* is likely a null allele. We chose this allele for our study because it is in Col. It has been reported that the *scr* mutant has only a single layer of ground tissue (Scheres et al., 1995), indicating that *SCR* is required specifically for the proximal daughter of the C/E stem

cell to divide periclinally to generate the original two layers of ground tissue. It has been noted, however, that sporadic periclinally divided ground tissue cells not near the QC occasionally appear in the *scr* mutant (Wysocka-Diller et al., 2000). Under our conditions in the *scr-5* allele, these divisions first appeared at d3 (10 out of 30 roots; Fig. 2D, arrowhead) and were extensive by d7 (37 out of 40; Fig. 1, L–N). With respect to the position relative to the QC and the interspersion of divided and undivided cells in the same cell file, these divisions look very similar to the middle-cortex-forming divisions found at d14 in wild-type roots (compare Fig. 1, C and D to L and M). In addition to being detected in *scr* at a much earlier time point than in wild type, the position of the first divisions in *scr* relative to the xylem axis appears to be approximately 90° away from that in wild type, i.e. in *scr* these divisions tend to take place in ground tissue cells near the phloem poles (Fig. 1N, arrowheads). These two differences between wild type and an *scr* null mutant suggest, intriguingly, that *SCR* may actually have a negative role in regulating middle-cortex formation: *SCR* may normally act to prevent its early initiation and to repress divisions in ground tissue cells at the phloem poles, resulting in the first divisions in wild type forming at d14 and at the ends of the xylem axis.

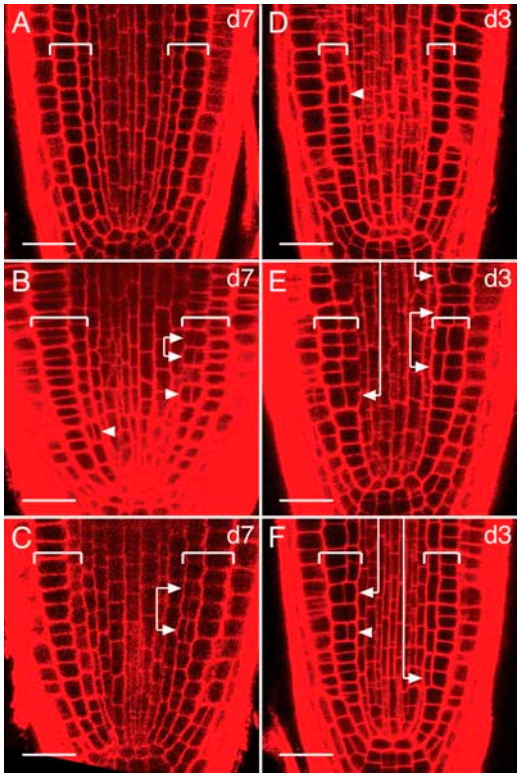

Figure 2. Effect of inhibition of GA biosynthesis on timing and extent of middle-cortex formation in wild type and its stronger effect in *scr*. Longitudinal optical sections of the primary root apical meristem. A to C, Wild type at d7. D to F, *scr-5* at d3. A, Mock-treated wild-type seedlings show no formation of middle cortex at d7. Inhibition of GA biosynthesis by growth on 2 μM pac (B) and by removal of copalyl diphosphate synthase function (i.e. introduction of the *ga1-3* mutation into Col) (C) each lead to premature initiation of middle-cortex formation (arrowheads and joined arrows). D, Middle-cortex-forming divisions are just beginning at d3 in mock-treated *scr-5* seedlings (arrowhead). Growth on 0.5 μM pac (E) and addition of the *ga1-3* mutation (F) each lead to more extensive middle-cortex formation in a greater proportion of roots (arrowhead and joined arrows). Brackets, Ground tissue. Scale bars, 25 microns.

REGULATION OF THE TIMING AND EXTENT OF MIDDLE-CORTEX-FORMING DIVISIONS BY GA

The growth regulator GA controls a wide range of developmental processes including root elongation (Fu and Harberd, 2003). In the shoot, GA biosynthesis and meristem activity are intimately related (for review, see Barley and Waites, 2002; Doerner, 2003; Hay et al., 2004), and, in the root, genes encoding enzymes that represent control points in both early and late steps in GA biosynthesis are expressed in the meristem during early postembryonic growth (Silverstone et al., 1997; T.-p. Sun, personal communication). In addition, two genes that regulate GA signaling, *GA INSENSITIVE* (Peng et al., 1997) and *REPRESSOR OF gai-1* (Silverstone et al., 1998), act in the root (Fu and Harberd, 2003) and have the same protein structure as *SCR* and *SHR*, all four being putative transcription factors of the GRAS family (Pysh et al., 1999; Doerner, 2003; Thomas and Sun, 2004). We decided, therefore, to test whether changes in GA levels affect the timing of the developmental transition in the ground tissue during root maturation.

To assess the potential interaction of regulation of this process by GA and *SCR*, we performed all analyses in both wild type and *scr-5* (for details, see supplemental materials). First, we treated both wild type and *scr* with GA_3 or paclobutrazol (pac, an inhibitor of GA biosynthesis). We found that high doses of GA (50 μM) delayed the onset of middle-cortex formation in wild-type seedlings: At d14, while 28% of mock-treated roots showed middle-cortex cells ($n = 50$), only 4% of GA-treated roots did ($n = 50$, $P < 0.005$). This effect was stronger in the *scr* background, in that 5-fold less GA (10 μM) gave a similar reduction, from 83% ($n = 40$) to 8% ($n = 40$, $P < 0.001$) at d7. Surprisingly, pac treatment also had a stronger effect on *scr* than on wild type. Two micromolar pac induced precocious formation of middle cortex in wild type: 10% of 70 pac-treated roots showed middle-cortex cells already at d7 (Fig. 2B), while none of 70 mock-treated roots did (Fig. 2A, $P < 0.025$). In contrast, 4-fold less pac (0.5 μM) was sufficient to increase both the extent of middle-cortex formation in *scr* at d3 (compare Fig. 2, D and E) and the rate, from 44% ($n = 45$) to 100% ($n = 45$, $P < 0.001$).

Second, we used a genetic means to reduce the amount of GA biosynthesis in both wild type and *scr-5*. *ga1-3*, originally isolated in Landsberg *erecta*, is mutant in the gene encoding copalyl diphosphate synthase,

which catalyzes the first step dedicated to GA biosynthesis (Sun et al., 1992). We analyzed segregating seed that was the product of six outcrosses of this mutant into Col (with the *ga1-3* mutants of this population showing very strong GA-related phenotypes) and also analyzed *scr* mutant F3 progeny of a cross of *scr* into these Col-outcrossed *ga1-3* plants. The effect on middle-cortex formation of the *ga1-3* mutation on Col was somewhat weak but significant ($P < 0.05$): At d7 11% of the *ga1-3* mutants showed middle-cortex cells already ($n = 57$; Fig. 2C), while only 1 of 79 heterozygous and homozygous wild-type siblings did. The effect of *ga1-3* on *scr* was, again, stronger than on wild type in both rate and extent of middle-cortex formation: At d3 93% of *scr-5 ga1-3* double mutants showed middle-cortex formation ($n = 45$; Fig. 2F), while only 38% of *scr-5* single mutants did ($n = 40$, $P < 0.001$).

INITIATION OF MIDDLE-CORTEX FORMATION REQUIRES *SHR* ACTIVITY

SHR is upstream of *SCR* in that the root phenotypes of *shr* encompass those of *scr*, and the *shr* mutant has greatly reduced *SCR* expression (Helariutta et al., 2000). Since *SCR* delays middle-cortex formation, one might expect precocious divisions in the *shr* mutant as well. Instead, however, these divisions fail to take place in *shr* mutants, suggesting that *SHR* is required for formation of the middle cortex and that its role in this process is independent of its ability to up-regulate *SCR*. We asked whether we could bypass the requirement for *SHR* function by reducing GA biosynthesis. Treatment of *shr-2* with 2 μM pac left the ground tissue pattern unchanged at d7 ($n = 35$; Fig. 3, A and B). Divisions occurred neither adjacent to the phloem poles (Fig. 3B, arrows) nor at the ends of the xylem axis (Fig. 3B, arrowheads). *shr-2 ga1-3* double mutants also showed no middle-cortex-forming divisions at d7 ($n = 35$; Fig. 3C). Thus, initiation of middle-cortex formation appears to require *SHR* function, even in the context of inhibited GA biosynthesis. This suggests that, normally, changes in GA levels may alter the timing and extent of middle-cortex formation through their effect on *SHR* or a *SHR*-dependent (and *SCR*-independent) process.

DISCUSSION

We have replicated and extended the observations of Baum et al. (2002) regarding the ground tissue changes that occur during the maturation of the Arabidopsis root. First, the C/E stem cells divide periclinally to generate separate stem cells for each of the first two layers of ground tissue. Then, individual endodermal cells divide periclinally to begin formation of the middle cortex. New middle-cortex cells lose endodermal character and rapidly take on cortex character.

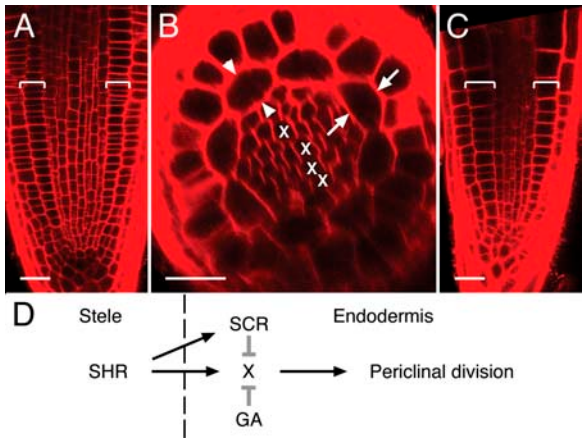

Figure 3. Lack of effect of inhibition of GA biosynthesis on the *shr* mutant and a model. Longitudinal (A and C) and transverse (B) optical sections of the primary root apical meristem of *shr-2* at d7. *shr-2* shows no middle-cortex formation, even when grown on 2 μM pac (A and B) or when combined with the *ga1-3* mutation (C). Divisions occur neither adjacent to the phloem poles (B, arrows) nor at the ends of the xylem axis (B, arrowheads). D, One possible model for the role of SHR, SCR, and GA in the developmental transition of initiation of middle-cortex formation by periclinal divisions of endodermal cells. The divisions require X function in the endodermis, which, in turn, requires SHR (which originates in the innermost tissue of the root, the stele) but does not require SCR. SCR and GA both independently inhibit X function (grey T lines) to varying degrees relative to each other at different stages of root maturation. Brackets, Ground tissue. X, Xylem progenitor. Scale bars, 25 microns.

Thus, the primary root comes to have more than a single layer of cortex. Regulating the timing of this process may allow the plant to adapt to changes in its environment. In other plant species, such as maize (*Zea mays*) and rice (*Oryza sativa*), even young roots have multiple cortex layers. The mechanisms regulating middle-cortex formation in Arabidopsis may play a role in these species to generate their initial radial pattern or to add more cortex layers at other stages of development.

Initiation of middle-cortex formation does not require *SCR* function. In fact, *SCR* appears to normally act both to prevent early formation of middle cortex and to keep the first divisions from occurring adjacent to the phloem poles. GA also negatively regulates the timing of initiation of middle-cortex formation: GA treatment of wild-type seedlings delays this developmental change, and reduction of GA biosynthesis results in the transition occurring precociously. Do the repressive signals of *SCR* and GA interact to influence this process, or do they each contribute to the regulation independently? It appears to be the latter (Fig. 3D). Reducing GA levels in the *scr* null mutant results in even more extensive middle-cortex formation than in untreated *scr* roots, and crossing *scr-5* into the *ga1-3* mutant background results in middle-cortex divisions beginning even earlier than in the *ga1-3* mutant alone.

The relative contribution of these two negative regulators in achieving proper timing and positioning of this developmental transition appears to change over time. At early stages, the influence of *SCR* appears to be more important, because in *scr* mutants precocious divisions begin at d3 (when GA levels are presumably still high), while in *ga1-3* divisions only begin at d7, suggesting that the activity of *SCR* is sufficient to repress the transition earlier. GA perturbations can still affect the *scr* mutant ground tissue at d3, however, suggesting that GA must play some role at early stages. The strong early repression of middle-cortex-forming divisions by *SCR* may normally act as a buffer against minor fluctuations in GA levels/signaling, as suggested by the fact that the *scr* mutant is more sensitive to both increases and decreases in GA levels than wild type. The relative role of GA to *SCR* in regulating initiation of middle-cortex formation may increase with age, as the effects of GA manipulation can be seen at d7 and d14 even when *SCR* is not mutated. The first divisions still do not occur adjacent to the phloem poles in *ga1-3*, however (data not shown), suggesting that *SCR* must play some role at later stages.

In contrast to *scr*, the ground tissue in *shr* appears to be insensitive to inhibition of GA biosynthesis, suggesting that GA levels/signaling may normally suppress premature ground tissue maturation by repressing *SHR* function or inhibiting a process that requires *SHR* but not *SCR*. One such process is already known: the inner ground tissue layer acquiring endodermal character (Benfey et al., 1993; Scheres et al., 1995). Thus, a possible model (Fig. 3D) is that a gene *X* is induced in the endodermis by *SHR*, and it is required for periclinal division and repressible by GA.

ACKNOWLEDGMENTS

The authors thank Masao Tasaka for the generous gift of the *scr-5* allele, David Welch for invaluable discussions, Tai-ping Sun for helpful advice and segregating *ga1-3* seed, Renze Heidstra for the pCo2::YFP_{H2B} line, Anita Fernandez, Kimberly Gallagher, Ji-Young Lee, and Tai-ping Sun for critical reading of the manuscript, Ignatius Tan for exceptional management of the confocal facility at New York University, and Juliette Colinas for calling our attention to Baum et al. (2002).

Received December 15, 2004; revised March 16, 2005; accepted March 17, 2005; published June 13, 2005.

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