Formation of lateral root meristems is a two-stage process

Marta J. Laskowski, Mary E. Williams*, H. Chad Nusbaum and Ian M. Sussex

Department of Plant Biology, Koshland Hall, Room 311, University of California, Berkeley, Berkeley, CA 94720, USA

*Present address: Biology Department, Harvey Mudd College, 301 East 12th Street, Claremont, CA 91711, USA

SUMMARY

In both radish and *Arabidopsis*, lateral root initiation involves a series of rapid divisions in pericycle cells located on the xylem radius of the root. In *Arabidopsis,* the number of pericycle cells that divide to form a primordium was estimated to be about 11. To determine the stage at which primordia are able to function as root meristems, primordia of different stages were excised and cultured without added hormones. Under these conditions, primordia that consist of 2 cell layers fail to develop while primordia that consist of at least 3-5 cell layers develop as lateral roots. We hypothesize that meristem formation is a two-step process involving an initial period during which a population of rapidly dividing, approximately isodiametric cells that constitutes the primordium is formed, and a subsequent stage during which meristem organization takes place within the primordium.

Key words: lateral root, meristem, primordium, *Arabidopsis thaliana, Raphanus sativus,* radish

INTRODUCTION

Meristems are responsible for the formation of the entire postembryonic plant body, yet our knowledge of how meristems develop is still rudimentary. Root apical meristems fall into three broad structural classes based on the number of cells or cell layers to which the files of root cells can be traced. In some plants, notably ferns, a single cell serves as the initial for the rest of the root (Gunning, 1982). In angiosperms, cell files originate in one or more cell layers. Meristems that contain cell files tracing to a single layer of cells are described as having an 'open' architecture while those having files that trace to three or four cell layers are described as having a 'closed' architecture (Clowes, 1981). The primary root meristems of both radish and *Arabidopsis* are typical closed meristems with three layers of initials (Steeves and Sussex, 1989; Dolan et al., 1993). The outermost layer produces the root cap and epidermis. A second layer produces the parenchymal and endodermal cells of the cortex, and the innermost layer produces the vascular tissue.

Observing the development of a primary root meristem is technically cumbersome because this meristem is initiated in the embryo. Meristem formation is more experimentally accessible during the development of lateral roots. Lateral roots also prove to be an excellent model system because their formation can be induced by the application of auxin, allowing the generation of large populations of developmentally synchronized meristems.

Lateral roots originate from the pericycle, a single cell layer at the periphery of the stele. The radial location of lateral roots is correlated with the internal architecture of the stele. In both radish and *Arabidopsis*, which have diarch steles, lateral roots arise from pericycle cells located in files adjacent to a xylem pole. Thus there are two columns of lateral roots 180 degrees apart (Blakely et al., 1982). We refer to the pericycle cells located adjacent the xylem poles as xylem-radius pericycle cells.

In the absence of applied auxins, lateral roots are initiated in acropetal sequence. Upon treatment with exogenous auxin, additional lateral roots are initiated along the length of the primary root (Blakely et al., 1988; Kerk, 1990). Lateral roots arising in response to auxin application are believed to have slightly different origins than those arising without added auxin. Based on estimates of the length of the cell cycle and the growth rate of the parent root it has been suggested that lateral roots arising on uninduced roots develop from pericycle cells that have been progressing through the cell cycle continuously since their inception in the apical meristem (Blakely et al., 1982). In contrast, lateral roots that develop from mature regions of the primary root are derived from pericycle cells that were previously arrested in the cell cycle (Blakely and Evans, 1979; Ferreira et al., 1994).

We identify two distinct stages in the development of a lateral root: the formation of a primordium, and the subsequent formation of a meristem that is capable of producing a lateral root. We refer to two classes of cells: founder cells and initial cells. The term founder cell has been previously used to refer to the 'cells initiating a leaf or a part of a leaf' (Poethig, 1984). In this paper, we broaden the definition of founder cells to refer to any preexisting cells that are activated and contribute to making a organ, usually by dividing to make derivative cells. Initial cells, in contrast, are those cells located in the meristem that are capable of continued division and whose derivatives form the main body of the organ. The distinction made here is important because not all founder cells or founder cell derivatives give rise to initial cells.

MATERIALS AND METHODS

Growth and auxin treatment of radish plants

Radish seeds (*Raphanus sativus* cv. Scarlet Globe, Carolina Biological Supply Company) were sterilized with a 5 minute soak in 70% ethanol followed by 3 to 5 rinses in sterile water, a 10 minute soak in 15% Clorox bleach, and another 5 rinses in water. Seeds were sown in sterilized plastic boxes containing alternate rows of vertically oriented moist germination paper and aluminum-foil baffles. These boxes were covered with foil and placed in a 25°C dark incubator. After 3 days roots that were more than 6 cm in length were selected for further experimentation.

Lateral roots were induced by transferring 10-25 sheets of germination paper complete with roots and interleaved foil baffles to a new vessel that contained MS medium (Murashige and Skoog salt base from JRH Biosciences supplemented with 3% sucrose, 55 µM inositol, 0.4 µM nicotinic acid, 0.2 µM pyridoxine·HCl, 0.15 µM thiamine·HCl, and 2.7 µM glycine) and an experimentally varied concentration of IAA. The pH of the medium was about 4.5 at which point 30 µM total IAA generates an [IAAH] (IAAH concentration) of 18 µM; addition of 90 µM total IAA lowers the pH slightly and gives an [IAAH] of about 60 µM. The roots were entirely submerged while the cotyledons were free in the air. This incubation chamber was covered with foil and left in the sterile hood on a slowly rotating (approximately 50 rpm) shaker plate. When measurements of root growth rate were to be made, small dots of carbon black dispersed in immersion oil were placed on the roots with the aid of a pulled Pasteur pipette.

Growth and auxin treatment of *Arabidopsis* plants

Seeds of *Arabidopsis thaliana* ecotype Columbia were sterilized in 15% bleach for 15 minutes, washed 5 times in water, and then vernalized at 4°C for 7 days. Seeds were resuspended in 0.1% agarose and placed individually onto square Petri plates containing MS salts (pH 5.7), 1.5% sucrose, and 0.8% agarose. Plates were sealed with parafilm and stored vertically in an incubator maintained at 22°C with constant 40 µmol m^{-2} s^{-1} light. To induce lateral roots, plates containing 5- to 7-day-old seedlings were placed horizontally and flooded with a solution of 30 µM IAA in water (pH approximately 5.7, [IAAH] about 3 µM), left for 15 minutes, rinsed with water twice, vertically oriented, and returned to the incubator.

Preparation of fixed, sectioned material

For radish, 1 cm long sections of root were fixed in FAA (5% formalin, 5% glacial acetic acid, 45% ethanol v/v) and embedded in 4:1 butyl-methyl methacrylate (see Baskin et al., 1992). Three µm thick sections were made with a Microm Microtome (Carl Zeiss, Inc.) equipped with a tungsten-carbide knife. The sections were transferred to drops of water on poly-L-lysine coated slides, dried overnight at approximately 40°C, and stained with toluidine blue O.

For *Arabidopsis*, whole plants were fixed in 4% glutaraldehyde in PBS for 4 hours, dehydrated, stained and embedded in JB-4 plastic. Plastic-embedded roots were cut into 2 µm sections and stained with toluidine blue O. Paraffin-embedded roots were also prepared and were cut into 7 µm sections and stained with safranin-O and fast green FCF.

Stained, sectioned material was observed with a Zeiss Axiophot equipped with an MTI CCD-72 video camera (Dage-MTI). Video frames from the CCD camera were digitized and displayed on a Macintosh Quadra 700 computer equipped with a Scion LG-3 frame grabber board (Scion Corp.). With the aid of the software program NIH Image 1.47 (Wayne Rasband, NIMH), the length and width of each cell was measured. For a cell to be termed a xylem-radius pericycle cell, it had to share one wall with a xylem element.

Confocal microscopy

In *Arabidopsis*, measurement of pericycle cell lengths was performed using a combination of confocal microscopy and the Image program described above. Intact *Arabidopsis* seedlings were fixed in FAA for 4 hours at 4°C, and then gradually rehydrated. Samples were treated with l N HCl for 10 minutes at 60°C, briefly rinsed in water, and then stained with 0.1% acriflavine in water. Acriflavine was saturated with SO$_2$ gas prior to using by mixing 0.1% acriflavine with 10% w/v K$_2$S$_2$O$_5$ and 20% v/v l N HCl and quickly capping the bottle. Stain was used after the color of the solution changed to a yellow hue. Stained tissue was washed with water approximately 4 times for about 1 hour each time, and then left in 15% ethanol overnight at 4°C. Tissue was dehydrated in ethanol, in steps to 100%, at 4°C. Samples were cleared in BB41/2 (1:2:2:2:2:1 benzyl benzoate: 85% lactic acid: chloral hydrate: phenol crystals: clove oil: xylene) at 4°C for a minimum of 3 hours. Samples were viewed under 530 nm irradiation on a Molecular Dynamics Sarastro 1000 confocal laser scanning microscope.

Meristem culture

For initial experiments, 3 mm sections of root were excised from intact *Arabidopsis* seedlings and cultured in MS medium plus 3% sucrose. For the subsequent experiments reported in Table 3, *Arabidopsis* seedlings were grown on sterile agar plates containing MS medium and 1% sucrose for 8 days, at which time the hypocotyls were cut with a razor blade, removing the entire shoot system from the root. The plates were resealed and returned to the incubator. Two days later, plates were opened and placed under a dissecting microscope. Segments were excised from the region just below the hypocotyl junction. These segments were transferred to sterile microscope slides on which a small quantity of MS medium plus 3% sucrose had been placed. Segments were covered with a coverslip; spacers placed on the edges of the slide held the coverslip up slightly and prevented the segments from becoming compressed. Mounted roots were viewed at 250× magnification under Nomarski optics and the developmental stage of all lateral roots was recorded. Segments that contained primordia were returned to an agar plate where they were cut into smaller pieces with clipper neuroscissors (Fine Science Tools). The primordium-containing segments were identified by reexamination under the microscope and then transferred to 1 ml of sterile MS medium plus 3% sucrose in a 24-well microtitre dish. All manipulations up to this point were carried out in a sterile hood. Dishes were sealed with micropore tape and placed on a rotating platform at ambient light and temperature for the remainder of the culture period. Three days after culture, the microtitre plates were placed under a dissecting microscope where roots were visually examined. After 7 days in culture, root segments were removed from the culture medium, placed on a slide with MS medium and a coverslip, and observed under Nomarski optics at 250× magnification.

RESULTS

Xylem-radius and phloem-radius pericycle cells are anatomically different

In radish and *Arabidopsis*, the radial location of lateral roots is invariant. Lateral roots always arise from pericycle cells located adjacent to a protoxylem pole. Cells in these files can be distinguished from cells in other files based on their length. In radish, the phloem-radius pericycle cells located behind the zone of elongation are about 3 times longer than those on the xylem radius (Table 1). To maintain the integrity of a straight root, the total length of cell files on the xylem and phloem radii must be equivalent. Thus, the shorter cells in the xylem radius files reflect a greater number of cell divisions in these files. In *Arabidopsis*, the average length of pericycle cells in the phloem radius files is approximately twice that in the xylem radius files, indicating that, on average, the xylem radius cells undergo one more round of division than do the phloem radius cells.
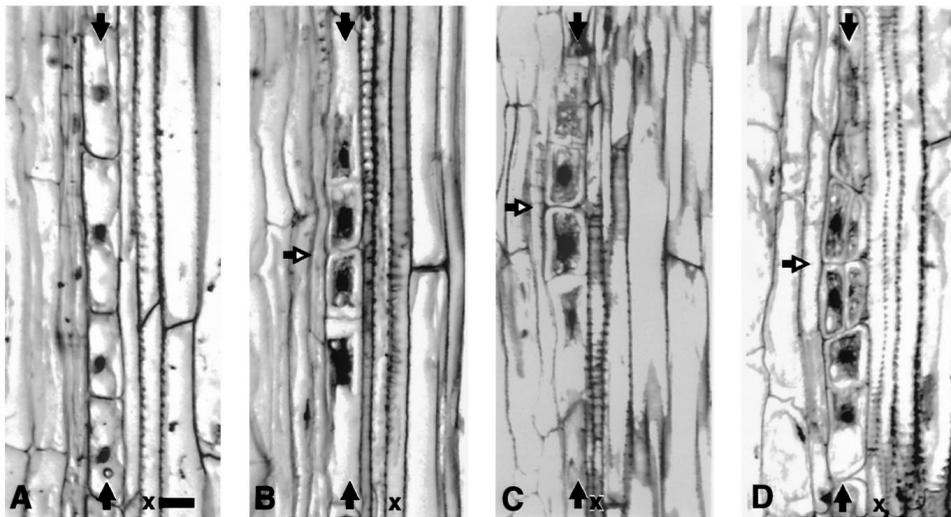
Fig. 1. Stages of lateral root initiation in radish roots to which no exogenous auxin was added. Longitudinal sections were taken from the region 0.5-1.5 cm behind the root tip. (A) Section in which lateral root primordia are not apparent. (B) Section showing transverse divisions in pericycle cells. (C) The pericycle cells in this primordium have undergone radial expansion. (D) Periclinal division has given rise to a primordium of two cell layers. The center of each developing lateral root is indicated by an open arrow. Black arrows indicate the pericycle cells, and the xylem is indicated with an x. Scale bar, 10 μm.

In *Arabidopsis*, pericycle cells in the xylem- and phloem-radius cell files also differ in cross-sectional area. The pericycle cells are arranged such that a line drawn through the plane defined by the xylem passes, at each end, through the junction of two pericycle cells. Thus, in a cross-sectional plane there are 4 pericycle cells that are in contact with the xylem. Prior to any hormonal treatment, the cross-sectional area of these cells is approximately one-third greater than the cross-sectional area of the other cells within the pericycle; the average cross-sectional area of the cells in contact with the xylem is 52.0±15.3 μm, compared with 34.8±10.9 μm for the other cells. Thus, the functional distinction between pericycle cells in the xylem radius files and phloem radius files is correlated with anatomical differences.

In addition to the restrictions on the radial location of lateral root formation, the longitudinal spacing of lateral roots also appears to be regulated. However, we have not observed any anatomical differences within the lateral-root-forming cell files that correlate with the longitudinal spacing of lateral roots.

Primordium initiation

The formation of a lateral root begins with transverse divisions in xylem-radius pericycle cells (Fig. 1A,B). These divisions are followed by radial expansion and subsequent periclinal division (Fig. 1C,D). Groups of pericycle cells that had undergone one or two periclinal divisions were observed in the region from 0.5-1.5 cm behind the tip of untreated radish roots. Based on the growth rate of the roots, the oldest cells in this region are 11-12 hours old (data not shown, see also Blakely et al., 1982).

Examining the progression of lateral root formation during its early stages is complicated by the fact that lateral roots originate infrequently along the parent root. Treatment of roots with indole-3-acetic acid (IAA), however, increases the frequency of lateral root initiation. When radish roots are incubated in 90 μM IAA, all of the xylem-radius pericycle cells undergo division, resulting in the formation of two continuous columns of pericycle-derived tissue (Fig. 2A,B). Over the course of several days, these columns give rise to a large number of closely spaced lateral roots (Fig. 2C,D). Thus, treating plants with this high concentration of IAA generates a large population of cells involved in the initial stages of lateral root formation.

Between 5 and 10 hours after treating radish roots with 90 μM IAA, xylem-radius pericycle cells undergo transverse division, decreasing the average length of the cells in that file (Table 2). In untreated roots, there is a large range of cell

Table 1. Length of pericycle cells in untreated roots

Tissue type	Average length* (μm) ± s.d.
Radish	
Xylem radius	51.9±26.1
	62.5±19.3
Xylem radius + 2d	58.4±27.0
Phloem radius	172.3±67.8
Arabidopsis	
Xylem radius	89.4±20.3
Phloem radius	167.2±42.8

*Each average is derived from 75-127 cells taken from 5-14 individual roots. The average length of the radish xylem-radius cells in the region from 0.5 to 1.5 cm behind the tip is maintained as the root matures, as indicated by the experiment marked + 2d in which tissue from the marked region was collected and fixed 2 days after the marking.

Table 2. Pericycle cell size in radish roots cultured in 90 μM IAA

Incubation period (h)	Av. length ± s.d. (μm)	Significance	Av. width ± s.d. (μm)	Significance
2	64.7±26.1	-	14.4±1.8	-
5	59.2±31.0	n.s.d.	14.3±2.4	n.s.d.
10	34.6±14.7	**	14.9±2.0	n.s.d.
13.5	20.4±8.2	**	15.9±3.2	**
15	21.2±10.0	n.s.d.	17.9±2.3	**

Each average length listed below is derived from 78-151 cells taken from 5-9 individual roots. Those cells that had divided periclinally were not included in the calculation of average width. Hence, the number of cells per average width measurement ranges from 70-115. A significance value was derived from a t-test on unpaired populations. This significance value reflects the probability that the tested mean was derived from the same population as that of the cells derived from the immediately preceding incubation time. Populations are marked below as not significantly different (n.s.d., $P>0.05$) or significantly different (**, $P<0.01$).
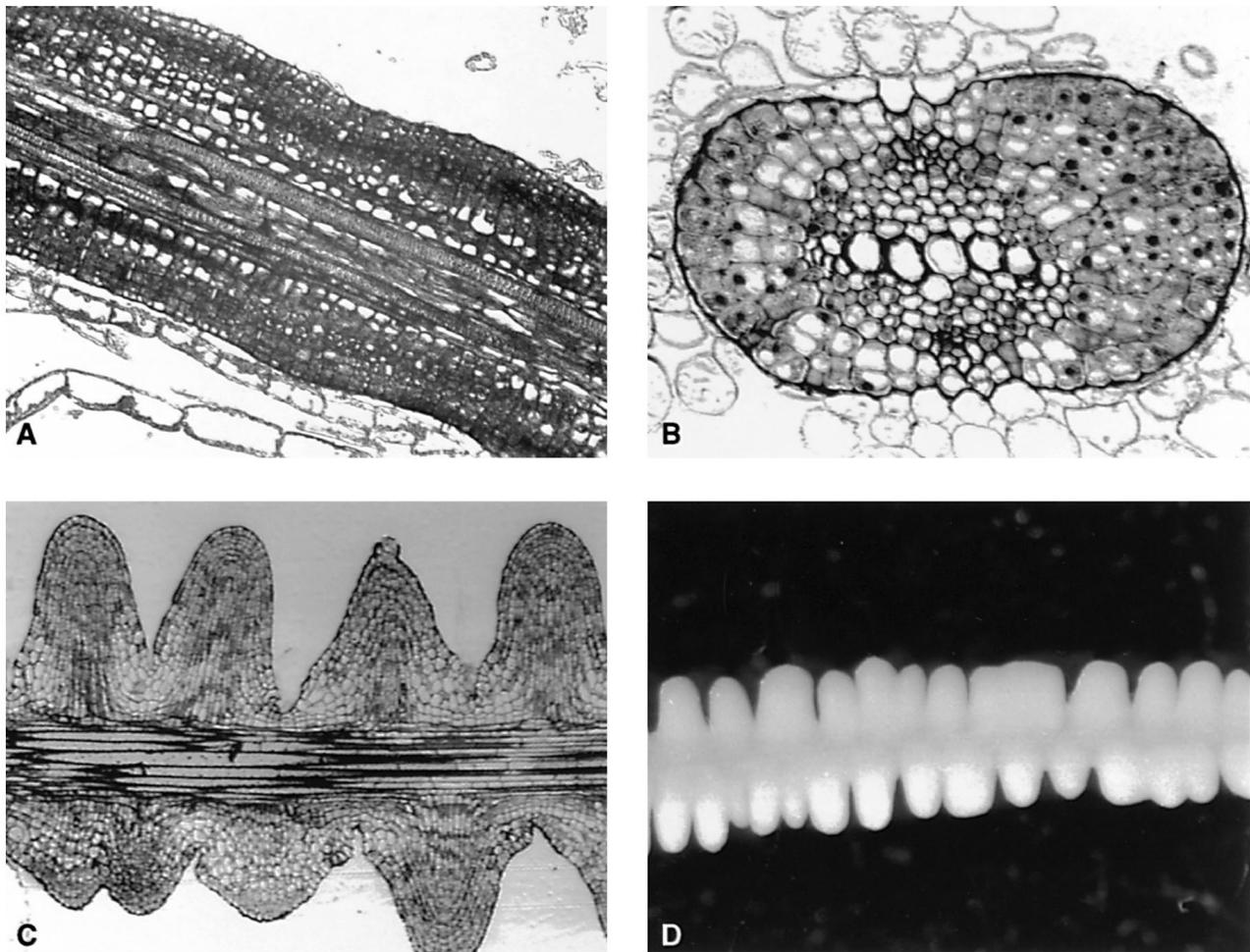
Fig. 2. Lateral root primordia in radish roots treated with 90 μM IAA. (A) Longitudinal section 48 hours after incubation. The cortical cell layers have been sloughed off leaving only the pericycle and its derivatives surrounding the vascular cylinder. (B) Cross section 48 hours after incubation. The center of each developing lateral root is in the plane of the xylem radius. (C) Longitudinal section 4 days after incubation. (D) Root, 4 days after addition of IAA. The outer cell layers have been sloughed off.

lengths observed for xylem-radius pericycle cells, however, the transverse divisions that occur during the first 15 hours after auxin treatment result in a more uniform population (Fig. 3A,B). Between 10 and 13.5 hours, the cells in these files begin radial expansion (Table 2). Radial expansion is followed by a periclinal division that is complete in 50% of the cells 15 hours after auxin application and gives rise to a group of approximately isodiametric founder cell derivatives. Occasionally, a second periclinal division was also observed 15 hours after auxin application. Thus, in a period of 15 hours these cells undergo about 3 mitoses. We estimate the length of the cell cycle to be about 5-6 hours.

The early sequence of lateral root development in *Arabidopsis* is similar to that of radish. In cross-sections of IAA-treated roots, no change is seen in tissue fixed 4 hours after auxin treatment, while radial enlargement and occasional periclinal division of the xylem radius cells is observed at 12 hours.

Meristem organization

The development of lateral root meristems in *Arabidopsis* was observed by clearing auxin-treated roots in lactic acid and
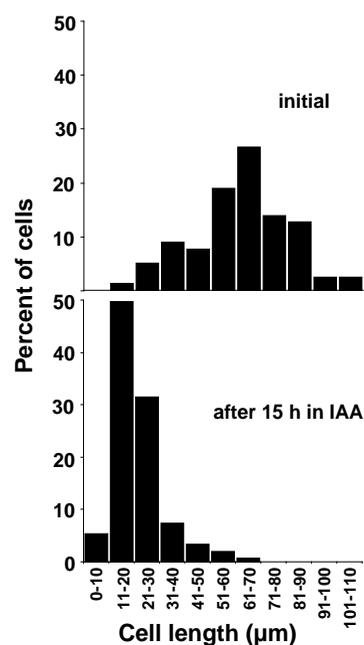

Fig. 3. Distribution of xylem-radius pericycle cell lengths before and after application of exogenous auxin. Cells were taken from the region 0.5-1.5 cm behind the tip of 3-day-old plants. (Top) initial, untreated control ($n = 179$); (bottom) after 15 hours in 90 μM IAA ($n = 151$).
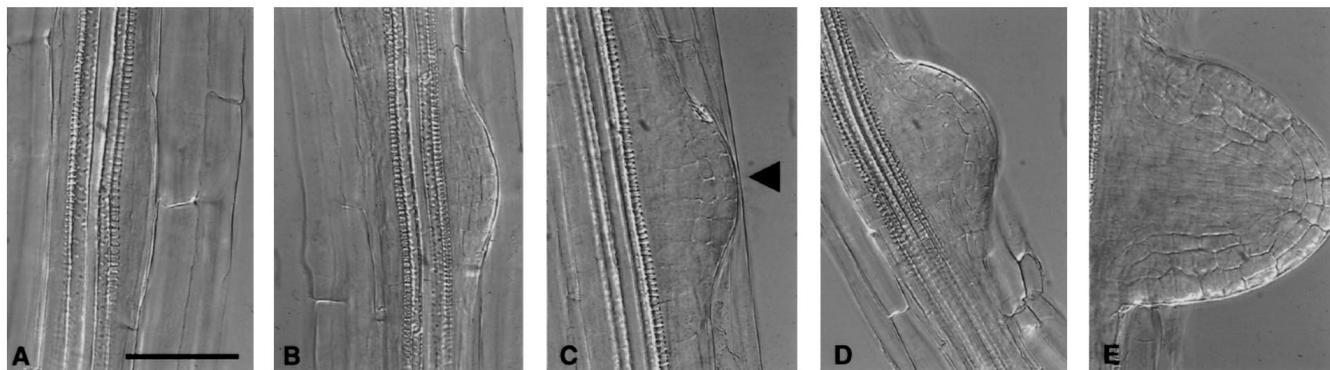
Fig. 4. Development of lateral root meristems in *Arabidopsis*. Photographs depict lateral roots at successive stages of development. (A) First periclinal division. (B) Four cell layers thick. (C) Periclinal division in cells near the apex; the recently divided cells are indicated by an arrowhead. (D) Formation of a distinct layer of cells around the apex. (E) Emerged lateral root with mature tissue pattern. Scale bar, 50 μm.

viewing them under Nomarski optics. Under these conditions, it is possible to observe most of the developing lateral roots along the length of a parent root.

Twenty four hours after IAA application, the majority of developing primordia have undergone 1-3 periclinal divisions generating primordia of 2-4 cell layers (Fig. 4A,B). These primordia generally do not extend radially beyond the endodermis. The cells of these primordia are approximately isodiametric and are arranged in parallel files extending outward from the stele (Fig. 4B). The percentage of primordia at each developmental stage is indicated in Fig. 5. The population shown there includes a large number of primordia that arose from the auxin application and a few interspersed lateral roots that were initiated prior to the auxin treatment.

As the primordia grow their radial dimension increases. Thirty two hours after auxin application, the majority of developing lateral roots have 5-8 cell layers and do not extend beyond the cortical parenchyma (Fig. 5). By 48 hours after auxin application, the majority emerge from the parent root. Lateral roots that are emerging or have recently emerged have 8-10 cell layers.

An obvious change in the pattern of cell files takes place in developing lateral roots that have about 8 cell layers. As a result of periclinal divisions in several cells located in the outer cell layers, the apex of the developing lateral root comes to be occupied by several distinct layers of cells (Fig. 4C,D). Soon thereafter, divisions behind the apex lead to the formation of long, slender cells characteristic of procambium. Continued periclinal divisions in the cells located near the apex of the developing lateral root lead to the formation of three cell layers that form the root cap (Fig. 4E). After the lateral roots emerge, starch grains can be visualized in these root cap cells. These histological changes indicate that the lateral root tissues become organized in a pattern characteristic of the root apex between 32 and 48 hours after auxin application, when the developing lateral root consists of 8-10 cell layers.

A functional test was used to determine the stage at which the meristem forms. An apical meristem can be defined as a group of cells that is capable of forming an organ directly (see also Ball, 1980). To determine the developmental stage at which lateral root primordia become functional meristems, segments of primary root that contained single primordia were excised from *Arabidopsis* seedlings that had not been treated with exogenous auxin. Segments of root that are 3 mm long are capable of initiating and developing new lateral roots in culture. As the initiation usually takes place near one of the cut ends of the segment, we hypothesize that these primordia are initiated as a result of accumulation of polarly-transported IAA. When 0.5 mm segments were cultured, however, no new primordia were initiated even after a 7-day culture period. These shorter segments were used for subsequent experimentation. 0.5 mm segments containing primordia
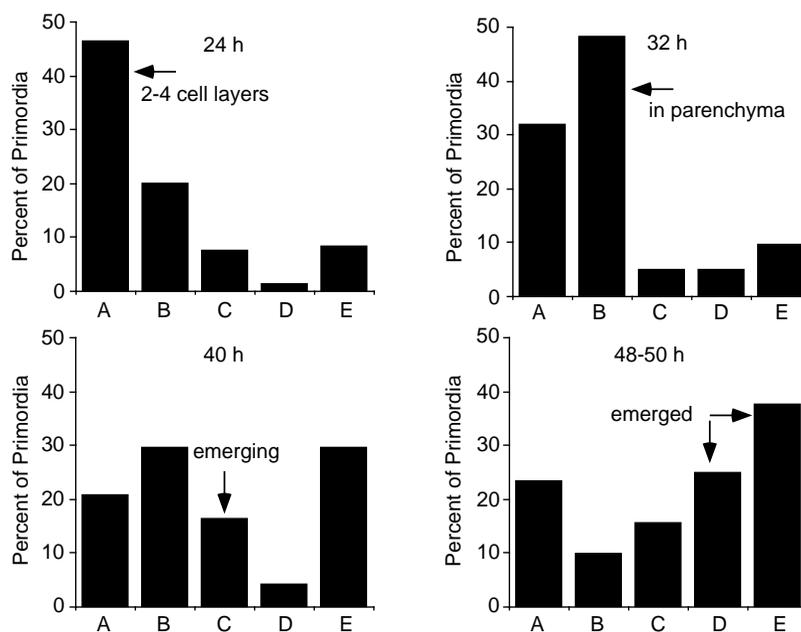

Fig. 5. Percentage of lateral roots at given developmental stages after the application of 30 μM IAA to *Arabidopsis* seedlings. Stages are as follows: A, 2-4 cell layers thick; B, in parenchyma; C, emerging; D, recently emerged lateral roots; E, emerged lateral roots that protrude from the primary root by more than one root diameter. Each graph is derived from 141-238 individual primordia.

Table 3. Results of culturing 0.5 mm sections of the primary root of *Arabidopsis* that contain lateral roots of differing developmental stages

Description of lateral root at day 0	Lateral root at day 7
no visible primordia (8)	no visible primordia (8)
transverse divisions only (2)	2-3 cell layers thick (2)
2 cell layers thick (2)	2-3 cell layers thick (2)
3-5 cell layers thick (8)	3 cell layers thick (2), 4-5 cell layers thick (1) emerged structure with multiple "hairs"(1) emerged lateral root (4)
>5 cell layers, pre-emerged (2)	emerged lateral root (2)
emerged lateral root (5)	fully emerged lateral root (5)

Numbers in parentheses refer to sample size. Results reflect the sum of three individual experiments.

of 2 cell layers also failed to form lateral roots in culture (Table 3). Developing lateral roots of 6 or more cell layers always gave rise to morphologically normal lateral roots. Segments containing developing lateral roots of 3-5 cell layers gave variable results (Table 3). The smallest primordia capable of developing lateral roots had 3 cell layers, and all of the developing lateral roots that we identified as having 4 cell layers formed emerged lateral roots. However, a few primordia identified as having 5 cell layers did not form lateral roots. In one case, a primordium of 5 cell layers remained arrested, while another gave rise to an anomalous emerged structure with a number of outgrowths that resembled swollen root hairs (Table 3). We conclude that a lateral root primordium forms an autonomous meristem when it has 3-5 cell layers. This is slightly before the cellular architecture of the developing lateral root changes.

Determination of founder cell numbers

During the early stages of lateral root development the number of preexisting pericycle cells involved in primordium formation increases. The earliest time at which lateral root primordia are histologically identifiable in *Arabidopsis* roots is approximately 12 hours after the application of IAA. At this time, the longitudinal dimension of a primordium is 101 µm, just slightly greater than the average length of a pericycle cell, and the cross-sectional dimension includes 2 to 5 radially expanded pericycle cells with an average of 3.5 (see Tables 4, 1). Thus, the initiation of a primordium starts with the activation of about 4 preexisting pericycle cells.

As lateral root development continues, neighboring pericycle cells also become activated so that the total number of preexisting pericycle cells that contribute to the formation of the mature root is larger than 4. Fully emerged lateral roots have a longitudinal dimension of about 216 µm, equivalent to the length of about 2.4 preexisting pericycle cells (Table 4). The average radial dimension is 77 µm, a space originally

Table 4. Length and radial extent of developing lateral roots in *Arabidopsis*

Treatment	Longitudinal (µm)	Radial (µm)
30 µM IAA, 12 h	101±19 ($n = 61$)	34±7 ($n = 11$)
30 µM IAA, 72 h	216±30 ($n = 80$)	77±10 ($n = 7$)

occupied by about 6 cells; two on the xylem-radius, two on the phloem-radius, and one on each side of the center which lies between the xylem and phloem radii. In an idealized rectangular lateral root primordium, the total number of founder cells could be calculated by multiplying the average number of cells in the radius, 6, by the average number of cells in length, 2.4. However, this would lead to an overestimate of the number of founder cells, because the average length of pericycle cells varies with their radial position. Taking into account the average length of the phloem-radius pericycle cells, and assuming that the cell files that lie between the xylem-radius and phloem-radius cell files are intermediate in length, we estimate that the average number of founder cells in an *Arabidopsis* lateral root is about 11.

DISCUSSION

Early stages of lateral root development

Lateral root development begins in files of pericycle cells that have distinctive morphological characteristics. The shorter average cell length of lateral-root-initiating cell files in *Arabidopsis* and radish reflects additional divisions in the xylem-radius pericycle cells as compared to the other pericycle cell files. Similar differences in average cell length of lateral-root-forming cell files were found in the roots of *Allium cepa* and *Pisum sativum* (Lloret et al., 1989) suggesting that this phenomenon occurs commonly in angiosperms. In addition, we observed that in *Arabidopsis* the average cross-sectional area of xylem-radius pericycle cells is larger than that of other pericycle cells. A comparable correlation between cell anatomy and potential for tissue formation was observed in the root epidermal cells of *Arabidopsis*. The subset of epidermal cells that form root hairs are distinguished from the neighboring files because they are more cytoplasmically dense and have a shorter average cell length (Dolan et al., 1993). It would be interesting to know if these distinctive anatomical characteristics are causally related to the developmental potentials of these cells.

A subset of the xylem-radius pericycle cells have a cuneiform, or wedge-like, shape. These cells are formed when transverse walls are laid down close to a pre-existing oblique end wall. Blakely et al. (1982) proposed that cuneiform cells have developmental significance in the early stages of lateral root development. Because we have observed a number of primordia that do not contain any such cells (see Fig. 1), we believe that the formation of cuneiform cells is not a required step in meristem formation.

The cell cycle times during the early stages of lateral root development are estimated to be 5-6 hours in radish. This is significantly shorter than the cell doubling times found in other portions of the root (Clowes, 1961). Nonetheless, the cell cycle times that we observed are typical for the early stages of lateral root development. In a previous study, estimates for cell doubling times in lateral root primordia ranged from 2.9 hours for *Pisum sativum* to 8.2 hours for *Vicia faba* (MacLeod and Thompson, 1979).

Founder cells

The distinction between founder cells, which participate in the formation of an organ but do not have an indeterminate

function, and initial cells is illustrated by the development of maize shoots. Maize shoots originate from a central group of cells that forms the initial cells of the apical meristem and at least one surrounding ring of founder cells that contributes only to part of the first leaf (Bossinger et al., 1992).

A subset of the preexisting pericycle cells serve as founder cells for each lateral root. The average number of founder cells has been estimated in several ways. Based on the frequency of polyploid cells present in lateral roots that initiated from colchicine-treated parent roots, and the assumption that all cells undergo division with equal frequency, the number of lateral root founder cells in *Vicia faba* was estimated to be 24 (Davidson, 1965). In another study, the range of founder cell numbers was calculated to be between 12 to 162, depending on the species (MacLeod and Thompson, 1979). The number given for *Vicia faba*, 162, was based on the assumption that primordium initiation takes place well behind the root apical meristem. Using direct histological observation, the number of founder cells in radish roots was estimated to be 30 (Blakely et al., 1982). Here, we used histological observation to estimate the number of founder cells in *Arabidopsis* and have concluded that there are about 11.

The origins of primary roots and lateral roots differ

Although the mature structures of embryonic and lateral root meristems are similar, during the early stages of development their structural organizations differ significantly. During embryo development, cells with the characteristic procambial shape are present at the triangular stage. This is well before periclinal division of the hypophyseal cell derivatives leads to the formation of a root cap (Scheres et al., 1994). In contrast, developing lateral roots contain cells with this characteristic shape only after periclinal division has taken place in cells at the tip of the developing root. By the time a lateral root emerges, however, its structure is similar to that of the embryonic meristem (compare Fig. 4E with Fig. 2F in Scheres et al., 1994).

Meristem formation

We have used both structural and functional approaches to identify the developmental stage at which a meristem is formed in a primordium. The results of our experiments in which excised primordia were cultured indicate that a functional, autonomous meristem forms in primordia of 3-5 cell layers. Within this range, we observed a variety of outcomes for the primordia; some became arrested while others formed lateral roots. A few formed more anomalous structures. The fact that the transition from a non-autonomous primordium to an autonomous meristem does not occur abruptly at one developmental stage may reflect the difficulty in assigning a precise number of cell layers to a primordium in an uncleared root and/or a real variability in its occurrence. The results of our histological analysis show that developing lateral roots undergo significant changes in cell architecture when they are 8-10 cell layers thick. Thus, the formation of cell patterns typical of a root meristem occurs slightly after the formation of an autonomous meristem.

We hypothesize that the formation of a meristem is a two-step process involving the establishment of a population of activated, rapidly dividing cells followed by the process of meristem organization, during which initial cells are estab-

lished. This hypothesis is based on our observation that treating roots with high concentrations of auxin causes all of the pericycle cells on the xylem radius to undergo division. Yet the result of this treatment is not a continuous sheet of lateral root. Only a subset of these dividing cells continue to differentiate into lateral root meristems indicating that there is a developmental transition between these two events (see Fig. 2).

The requirement for a population of activated cells prior to meristem formation is supported by other experiments as well. Treating leaf explants of *Convolvulus arvensis* with the proper balance of hormones induces shoot meristem formation. The length of time that the explants must be exposed to the shoot-inducing medium can be decreased, however, if the explants are first incubated on a callus-inducing medium. During this pretreatment period, cells in the explant undergo divisions leading to the formation of a population of cells from which the shoots subsequently arise. Thus, the more rapid development of shoot meristems that is observed after pretreatment may be a consequence of the establishment of an activated population of cells during the period of pretreatment (Christianson and Warnick, 1983).

While a population of activated cells may be a requirement for meristem formation, it is not a sufficient condition. Numerous experiments have indicated that plant tissue can be induced to grow as a callus that never forms discrete meristems. In addition, we found that primordia of two cell layers do not form autonomous meristems when placed in culture while more mature advanced primordia do, indicating that some developmental transition occurs between these stages. The existence of a developmental transition is also indicated by the *Arabidopsis* mutant *rml2* that undergoes cell division in the pericycle, but fails to form lateral roots (Cheng et al., 1995).

In conclusion, our studies of developing lateral roots have lead us to divide the process of lateral root formation into a number of specific stages. The first of these is the formation of populations of activated pericycle cells that we term founder cells. The derivatives of these cells form primordia within which functional, autonomous meristems form when the primordia are 3-5 cell layers thick. Shortly thereafter, the pattern of cell files in the developing lateral root changes to reflect the functioning of the new meristem.

REFERENCES

Ball, E. A. (1980). Regeneration from isolated portions of the shoot apex of *Trachymene coerulea* R. C. Grah. *Ann. Bot.* **45**, 103-112.

Baskin, T. I., Busby, C. H., Fowke, L. C., Sammut, M. and Gubler, F. (1992). Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. *Planta* **187**, 405-413.

Blakely, L. M. and Evans, T. A. (1979). Cell dynamic studies on the pericycle of radish seedling roots. *Plant Sci. Lett.* **14**, 79-83.

Blakely, L. M., Durham, M., Evans, T. A. and Blakely, R. M. (1982). Experimental studies on lateral root formation in radish seedling roots. I. General methods, developmental stages, and spontaneous formation of laterals. *Bot. Gaz.* **143**, 341-352.

Blakely, L. M., Blakely, R. M., Colowit, P. M. and Elliott, D. S. (1988). Experimental studies on lateral root formation in radish seedling roots. II. Analysis of the dose-response to exogenous auxin. *Plant Physiol.* **87**, 414-419.

Bossinger, G., Maddaloni, M., Motto, M. and Salamini, F. (1992). Formation and cell lineage patterns of the shoot apex of maize. *Plant J.* **2**, 311-320.

Cheng, J., Seeley, K. A. and Sung, Z. R. (1995). *Rml1* and *rml2, Arabidopsis* genes required for cell proliferation at the root tip. *Plant Physiol.* **107**, 365-376.

Christianson, M. L. and Warnick, D. A. (1983). Competence and determination in the process of *in vitro* shoot organization. *Dev. Biol.* **95**, 288-293.

Clowes, F. A. L. (1961) Duration of the mitotic cycle in a meristem. *J. Exp. Bot.* **12**, 283-293.

Clowes, F. A. L. (1981). The difference between open and closed meristems. *Ann. Bot.* **48**, 761-767.

Davidson, D. (1965). Cytological chimeras in roots of *Vicia faba. Bot. Gaz.* **126**, 149-154.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organization of the *Arabidopsis thaliana* root. *Development* **119**, 71-84.

Ferreiera, P. C. G., Hemerly, A. S., de Almeida Engler, J., Van Montagu, M., Engler, G. and Inze, D. (1994). Developmental expression of the *Arabidopsis* cyclin gene cyc1At. *Plant Cell* **6**, 1763-1774.

Kerk, N. M. (1990). *Gene Expression During Meristem Initiation and Organization* PhD Thesis, Yale University.

Gunning, B. E. S. (1982). The root of the water fern *Azolla*: Cellular basis of development and multiple roles for cortical microtubules. In *Developmental Order: Its Origin and Regulation*, 40th Symp. Soc. Dev. Biol. (ed. S. Subtelny and P. B. Green), pp. 379-421. AR Liss: New York.

Lloret, P. G., Casero, P. J., Pulgarin, A. and Navascues, J. (1989). The behavior of two cell populations in the pericycle of *Allium cepa, Pisum sativum*, and *Daucus carota* during early lateral root development. *Ann. Bot.* **63**, 465-475.

MacLeod, R. D. and Thompson, A. (1979). Development of lateral root primordia in *Vicia faba, Pisum sativum, Zea mays* and *Phaseolus vulgaris*: rates of primordium formation and cell doubling times. *Ann. Bot.* **44**, 435-449.

Poethig, S. (1984). Cellular parameters of leaf morphogenesis in maize and tobacco. In *Contemporary Problems in Plant Anatomy* (ed. R. A. White and W. C. Dickison), pp. 235-259. New York: Academic Press.

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the *Arabidopsis* primary root and root meristem initials. *Development* **120**, 2475-2487.

Steeves, T. A. and Sussex, I. M. (1989). *Patterns in Plant Development.* 2nd Edition. Cambridge, New York: Cambridge University Press.