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Root development in *Arabidopsis*: four mutants with dramatically altered root morphogenesis

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SUMMARY

A genetic analysis of root development in *Arabidopsis thaliana* has identified mutants that have abnormal morphogenesis. Four of these root morphogenesis mutants show dramatic alterations in post-embryonic root development. The *short-root* mutation results in a change from indeterminate to determinate root growth and the loss of internal root cell layers. The *cobra* and *lion's tail* mutations cause abnormal root cell expansion which is conditional upon the rate of root growth. Expansion is greatest in the epidermal cells in *cobra* and in the stele cells in *lion's tail*. The *sabre* mutation causes abnormal cell expansion that is greatest in the root cortex cell layer and is independent of the root growth rate. The tissue-specific effects of these mutations were characterized with monoclonal antibodies and a transgenic marker line. Genetic combinations of the four mutants have provided insight into the regulation of growth and cell shape during *Arabidopsis* root development.

Key words: cell expansion, meristem, plant development, organogenesis

INTRODUCTION

A fundamental question in the field of plant development is how organ formation is regulated. Populations of meristematic cells are formed in the embryo at the shoot and root apices. Plant organs are formed from these meristems by a regulated program that specifies the timing of cell division, the orientation of the plane of cell division, and the extent of cell expansion (Steeves and Sussex, 1989).

In order to understand the process of organ development we have chosen to study the formation of roots. Roots support the plant, synthesize hormones, acquire water and minerals, and are the site of interaction with soil bacteria. Root development is a continuous process in which different cell types arise in files from the initials (Esau, 1977). The aerial part of the plant goes through a transition from vegetative to floral growth, which involves a major developmental switch in the type of organs produced and normally leads to the cessation of growth. In contrast, root development is fairly uniform with no significant developmental transition. In many species, there is also no predetermined cessation of root growth. The continuous, uniform growth of roots results in all developmental stages being present in distinguishable regions along the root (Esau, 1977).

The physiology and general developmental characteristics of roots have been described (Feldman, 1984). However very little is known about the mechanisms that control root development. In particular how the apical meristems are initiated and maintained, how cell division and expansion are regulated and how cellular differentiation is controlled are all unanswered questions. In part, this is due to the difficulty of analyzing an organ that usually grows underground. In order to understand the developmental pathways that regulate root formation, we have undertaken a genetic analysis of root development in *Arabidopsis thaliana*. We and others (Okada and Shimura, 1990; Schiefelbein and Benfey, 1991) have developed methods exploiting the small size of *Arabidopsis* that allow us to screen large numbers of roots for abnormal developmental patterns.

Among mutagenized *Arabidopsis* plants we have identified mutant lines that have abnormal root structures. These have been classified as 'root morphogenesis' or 'rom' mutants. (We will use the term 'rom' to describe the class of mutants and use descriptive names for the individual mutants.) Here we present the initial results from these screens and describe four rom mutants that show dramatic alterations in root morphogenesis. The *short-root* (*shr*) mutant exhibits a determinate growth pattern in the root and is missing internal root cell layers. The *cobra* (*cob*) and *lion's tail* (*lit*) mutants have abnormal expansion that is greatest in different cell layers and is conditional upon the rate of root growth. The *sabre (sab*) mutant has abnormal root cell-expansion that is not conditional upon the root growth rate. We used monoclonal antibodies to membrane

and cell wall components and a transgenic tissue-specific marker line to characterize the mutant phenotypes. Genetic combinations of the four rom mutants were generated. These provided insight into the regulation of growth and cell expansion during *Arabidopsis* root morphogenesis.

MATERIALS AND METHODS

Growth of plants and screening for mutants

Arabidopsis seeds were routinely sterilized by immersion in 5% sodium hypochlorite (Chlorox) for five minutes, then washed twice with distilled water. Seeds were then brought up in a solution of 0.75% low-gelling point agarose (SeaPlaque), in Murashige and Skoog (MS) salt mixture (Sigma), 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES), and the pH was adjusted to 5.7 with KOH. Seeds were aspirated into plastic 'transfer pipettes' (Fisher) so that the seeds separated in the semi-molten solution. Seeds were dropped individually from the transfer pipette onto 100 cm^2 nutrient agar plates containing 1× MS salts, 0.9% agar (BBL), 2.5 mM MES and except where noted, 4.5% sucrose. The pH of the medium was adjusted to 5.7 with KOH. The plates were placed at 4°C for 24 hours to allow for imbibition. Plates were then transferred to a room maintained at 22˚C and incubated in a near vertical position under fluorescent lamps emitting approximately 80 μeinsteins m^{-2} S^{-1} in a 16 hour light cycle.

Ethyl methane sulfonate (EMS) mutagenized M_2 seed (Columbia ecotype) were deposited on plates in three rows of approximately 10 seeds each. 'Insertion' lines (WS ecotype) that were mutagenized by co-cultivation of *Arabidopsis* seeds with *Agrobacterium tumefaciens*, which harbored a recombinant T-DNA (Feldmann, 1991), were screened as individual lines on separate agar plates. Plants were screened for phenotypic variation at 7 and 14 days using optical visors (10× magnification). Putative mutants were inspected under a Nikon stereomicroscope.

In order to analyze the growth characteristics of plants grown under different environmental conditions, the following modifications were made. For growth in the presence of varying amounts of sucrose, the sucrose concentration was varied from 0% to 6%. For growth in low light and low temperature, seeds were allowed to germinate under normal conditions and then placed in an incubator at 14˚C in the dark. For analysis of growth in soil, seeds were planted in Metromix 200 saturated with water in pots covered with plastic wrap. After 7 days the plastic wrap was removed and the plants were allowed to grow for two more weeks.

Transgenic marker lines, histological and histochemical techniques

The cauliflower mosaic virus (CaMV) 35S B2 β-glucuronidase (GUS) expression construct has been described previously (Benfey et al., 1990a). Transformation into *Arabidopsis* was by the leaf-disc method as described (Lloyd et al., 1986). Histochemical analysis of the β-glucuronidase expression was performed essentially as described (Benfey et al., 1989).

Fresh sections of roots were obtained by embedding the tissue in 3-4% molten agarose. This was performed as follows. The agarose was first allowed to boil, then cooled to approximately 50˚C and poured onto the surface of a Petri plate to form a small puddle. The plant was then drawn through the puddle so that the root was suspended in the middle of the agarose. The agarose was allowed to harden and sections were cut with a hand-held razor blade. The sections were placed in water on a microscope slide and observed with a Nikon Optiphot or a Leitz Laborlux S compound microscope.

For immunocytochemistry, roots were fixed in 2% glutaraldehyde at room temperature for 60 minutes, washed in water for 30 minutes at 0˚C, dehydrated in ethanol at low temperature, and embedded at −20˚C in LR White resin as described (Hills et al., 1987). Roots were flat-embedded, and the resin cured by UV light for 24 hours at −20˚C and 16 hours at room temperature. Sections, cut at 0.25 μm, were attached to slides and incubated for 60 minutes in the neat hybridoma supernatant of an anti-pectin monoclonal antibody, JIM7 (Knox et al., 1990). The section was washed in running water for 5 minutes then stained in FITC-conjugated goat anti-rat Ig (whole molecule, Sigma), diluted 1:60 in TBS + 3% bovine serum albumin for 60 minutes. Sections, washed for 5 minutes in running water, were mounted in Citifluor (Agar Aids, Stansted, UK) and examined in a Zeiss Universal epifluorescence microscope. Sections were also stained with another monoclonal antibody, JIM13, which recognizes a developmentally regulated oligosaccharide on arabinogalactan proteins associated with the plasma membrane (Knox et al., 1991). In *Arabidopsis* roots this antibody labels certain stele cells together with the eight endodermal cells.

Cell area and root length calculations

Slides of fresh sections and of a calibration scale were digitized using a Barneyscan slide scanner and NIH Image software. Areas were calculated by outlining cells and counting pixels using the NIH image software. Root lengths were measured with a transparent ruler held adjacent to plants growing on vertically oriented Petri dishes.

Genetic analysis

Crosses were performed essentially as described (Schiefelbein and Somerville, 1990). Homozygous plants were used for *cob*, *lit* and *shr*. Because homozygous *sabre* plants have very low fertility, heterozygous plants were used for crosses. Since the *sabre* phenotype co-segregates with the kanamycin resistance marker carried by the inserted T-DNA (P.N. Benfey and R.A. Aeschbacher, unpublished observations), the *sabre* allele could be selected in the F_1 generation by germination on nutrient agar that contained kanamycin. The semi-dominant *cob* phenotype was only observed when plants were grown on nutrient agar containing 4.5% sucrose and with a 16 hour light cycle. To simplify the genetic analysis, F_2 plants of crosses with *cob* were grown on nutrient agar containing 3% sucrose under continuous light. Under these conditions the semi-dominant phenotype was not observed.

RESULTS

Morphology of the wild-type *Arabidopsis* root

Prior to undertaking a characterization of the mutant lines it was necessary to characterize the morphology of the wild-type *Arabidopsis* root, which had not been described previously. When grown on nutrient agar medium, *Arabidopsis* roots (Columbia ecotype) exhibited fairly uniform growth. There was no apparent cessation of root growth during the period of observation. Regions or 'zones' of development were readily apparent at the root tip at low magnification (Fig. 1A). We will refer to these regions as the 'meristematic', 'elongation' and 'specialization' zones (Steeves and Sussex, 1989; Schiefelbein and Benfey, 1991). The meristematic zone is the region in which the earliest detectable progenitors or initials of the differentiated cells are located. In the elongation zone, cell division and cell expansion take place in a precisely coordinated fashion. This zone is characterized by the presence of smaller cells with dense cytoplasm (Fig. 1A). Toward the top of the elongation zone,

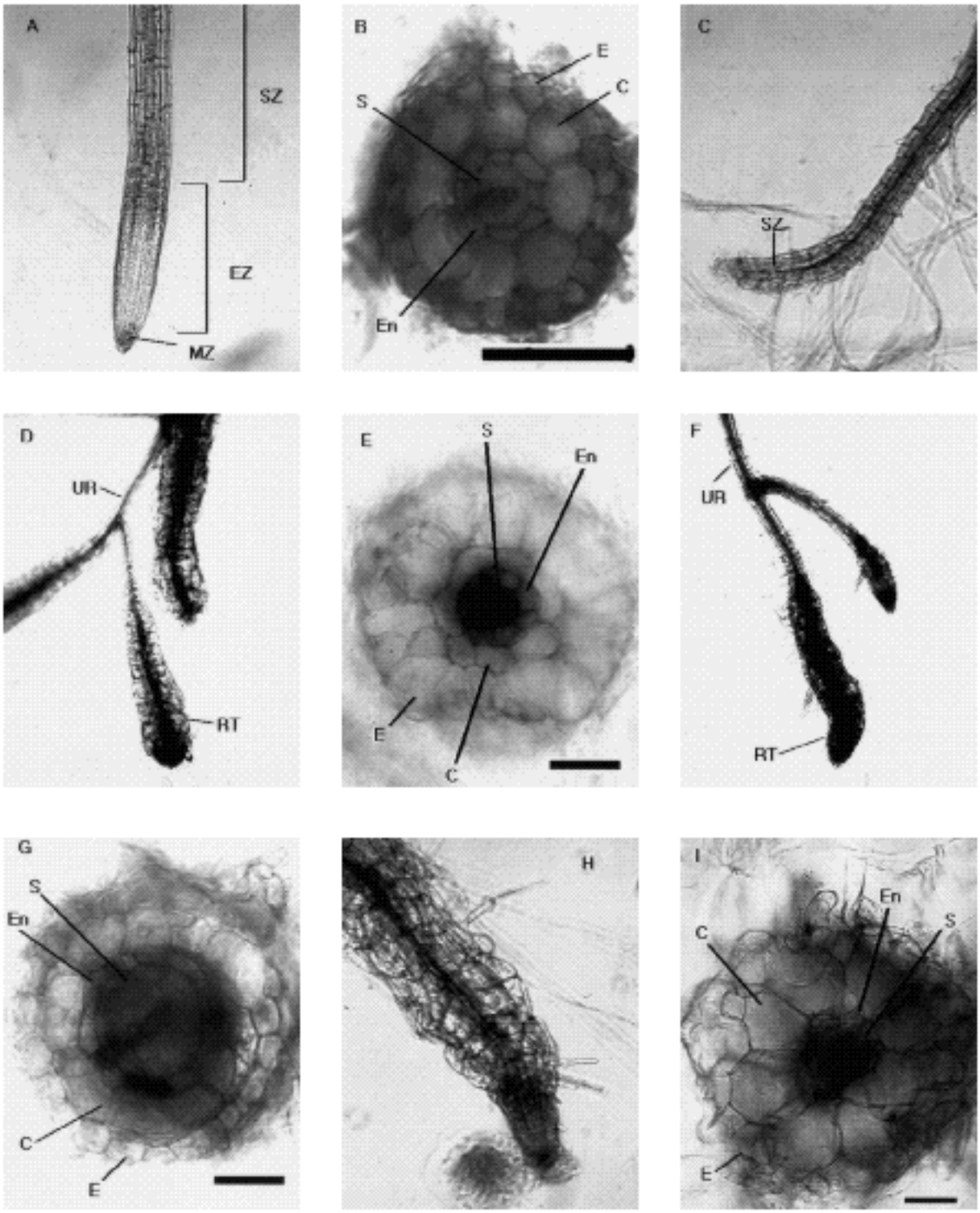

Fig. 1. Morphology of wild-type and rom mutant roots. (A) Whole mount of wild-type root tip at low magnification. The meristematic (MZ), elongation (EZ) and specialization zones (SZ) are indicated. (B) Fresh transverse section through the specialization zone of wild-type root. Note single cell layers of epidermis (E), cortex (C) and endodermis (En). (C) Whole mount of root tip of *short-root* that has ceased elongation. Note the apparent lack of the elongation and meristematic zones. (D) Whole mount of *cob* root. Note unexpanded upper root (UR) and expanded root tip (RT). (E) Fresh transverse section through the expanded specialization zone of *cob* root. Note relative expansion of epidermis (E). (F) Whole mount of *lit* root showing unexpanded upper root (UR) and expanded root tip (RT). (G) Fresh transverse section through the expanded specialization zone of *lit* root. Note relative expansion of stele (S). (H) Whole mount of *sab* root. (I) Fresh transverse section through the specialization zone of *sab* root. Note relative expansion of cortex (C). Bar, 50 μm.

cells begin to acquire their final differentiated attributes. The specialization zone is the name for the region in which cells become fully differentiated (Steeves and Sussex, 1989; Schiefelbein and Benfey, 1991; Fig. 1A).

Transverse sections of fresh tissue through the specialization zone revealed a remarkably simple pattern of cellular organization. The three outer cell layers, the epidermis, cortex and endodermis each consisted of a single layer of cells (Fig. 1B). Transverse sections of fixed tissue stained with monoclonal antibodies to the cell wall component, pectin (Knox et al., 1990), confirmed this simple pattern and revealed that the pericycle also consisted of a single cell layer (Fig. 2C). An analysis of numerous sections of primary roots led to the conclusion that the number of cells in the cortex and endodermis was nearly invariant with an eight-fold radial symmetry (Dolan et al., 1993). Longitudinal sections through the tip of the primary root stained with anti-pectin antibodies revealed the organization of the cell walls in the three zones (Fig. 2A). The same basic cellular organization was observed in the Wassilewskija (WS) ecotype.

Isolation of root mutants

We have performed a genetic screen for abnormal root development by placing mutagenized seeds in rows on nutrient agar plates that were incubated vertically to allow the roots to grow along the surface of the agar. Abnormal root growth was detected by initially observing the plants using an optical visor, then following-up with observations under a stereomicroscope. Observations were made at 7 and 14 days after germination.

We have screened approximately 40,000 EMS mutagenized M_2 seed and approximately 8,000 'insertion' lines that were generated by co-cultivation of *Arabidopsis* seeds with *Agrobacterium tumefaciens* and selection for antibiotic resistance that was conferred by transfer of a recombinant T-DNA (Feldmann, 1991). We have isolated 11 lines from the EMS mutagenized seed and three from the insertion lines for which the mutant phenotype has been shown to be stable for at least three generations. Based on the growth and histological characteristics we decided to analyze, in depth, four of these mutant lines that appeared to alter dramatically the normal morphogenetic patterns of root development.

Short-root, a mutation that causes determinate root growth and the loss of internal cell layers

The *short-root* (*shr*) mutant was identified among the insertion lines as a seedling that appeared to have relatively normal development in the aerial portions of the plant, but had a root that was noticeably shorter than in wild type (Fig. 3A). When allowed to mature (28-42 days) on nutrient agar medium the aerial portions of the plant continued to have a wild-type appearance (although the leaves were darker green) but the roots were very short compared to wild-type roots. In addition, there was a large number of secondary roots initiated primarily at the junction of the hypocotyl and the primary root (Fig. 3B). Inspection of the tips of the longest roots (both primary and secondary) revealed an absence of the small, densely cytoplasmic cells characteristic of the elongation and meristematic zones (Fig. 1C). In longitudinal sections of the root tip stained with anti-pectin antibodies, there appeared to be relatively few cells with the size and cell-wall configurations characteristic of cells in the elongation and meristematic zones (Fig. 2B, compare with Fig. 2A). The elongation and meristematic zones of newly emerging roots (either primary or secondary) resembled the equivalent wild-type regions. Primary root lengths were determined at 9 days after germination when growth had ceased. The average root length of 51 plants was 5.9±0.6 mm. At 9 days, wild-type root length exceeded 20 millimeters with no significant reduction in growth rate.

In order to characterize further this mutant we made use of a transgenic line that contained a fusion of the B2 subdomain from the cauliflower mosaic virus (CaMV) 35S enhancer, upstream of a truncated 35S promoter fused to the β-glucuronidase (GUS) coding sequence. This construct had been shown to confer expression specific to root cap cells in transgenic tobacco (Benfey et al., 1990a,b; Benfey and Chua, 1990). When introduced into *Arabidopsis*, this construct conferred expression in root cap cells as shown in the whole mount (Fig. 3C) as well as in cells in the meristematic zone as shown in a longitudinal section (Fig. 3D). Unlike tobacco, no expression was detected from this construct in aerial organs of *Arabidopsis*. This transgenic marker line was crossed with the *shr* mutant and the F_2 progeny were analyzed for expression of the marker gene in the mutant background. F_2 plants that segregated for the *shr* phenotype and showed expression of the B2 subdomain marker line had strong expression in root cap tissue even though there was no visible elongation zone (Fig. 3E compare region above the root cap with wild type in Fig. 3C). Longitudinal sections revealed a markedly different organization of the root tip in this mutant. GUS expression appeared to be restricted to the root cap and to a few cells just above the root cap (Fig. 3F).

In addition to abnormal root growth, the *shr* mutant had another striking defect. Transverse sections through the specialization zone revealed that there was no detectable layer of cells where the endodermis normally is located. This was clearly visible in sections stained with anti-pectin antibodies (Fig. 2E compare with wild type in Fig. 2C). The number of cells in the stele of the mutant also appeared to be less than that in wild type (Fig. 2E). The suberized region known as the 'Casparian strip' (Esau, 1977), present in the endodermis, also appeared to be missing from the mutant roots. We have used a monoclonal antibody to arabinogalactan proteins (Knox et al., 1991), which decorates a set of cells that includes the endodermis in wild-type roots (Fig. 2D), to characterize this defect. The antibody failed to decorate the endodermal cell layer in the *short-root* mutant (Fig. 2F). Some additional staining of stele tissue was evident in the mutant (Fig. 2F).

From this analysis there appear to be two defects in *short-root*. First, the meristem loses its ability to maintain growth, which causes the root to become determinate. Second, the root lacks internal cell layers including the endodermis and part of the stele. The mutant was crossed to wild type and the F_1 progeny were analyzed. These all had a wild-type phenotype indicating that the mutation is recessive to wild type. Segregation analysis of the F_2 progeny of these plants indicated that a single genetic locus was responsible for both the determinate growth and cell layer defects. These defects did not cause a significant change in the growth character-

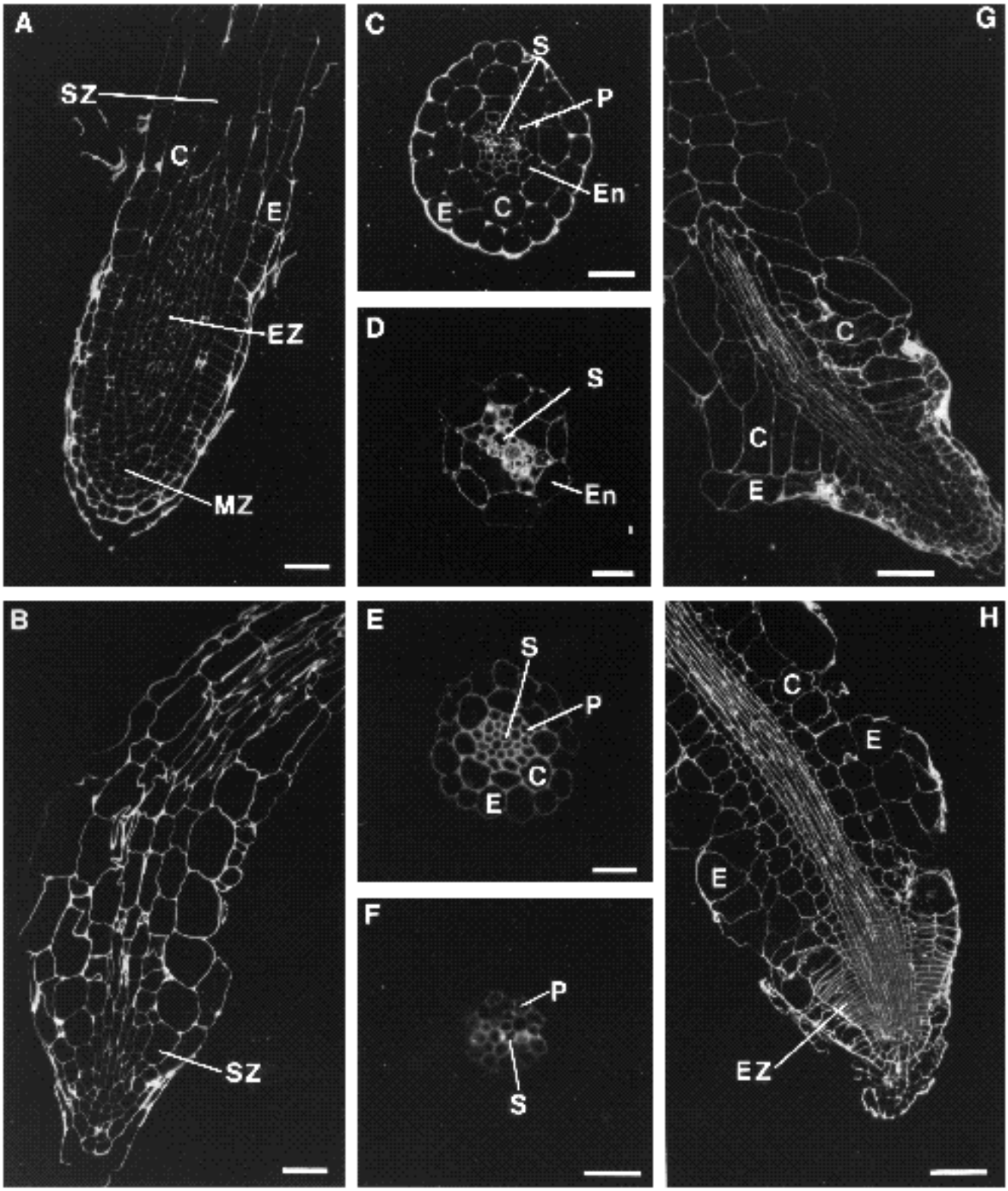

Fig. 2. Antibody-stained sections of wild-type and rom mutant roots. (A) Longitudinal section of wild-type root tip stained with JIM7, an anti-pectin antibody. Bar, 25 μm. (B) Longitudinal section of *short-root* root tip stained with JIM7. Bar, 25 μm. (C) Transverse section through the specialization zone of a wild-type root stained with JIM7. Bar, 25 μm. (D) Transverse section through the specialization zone of a wild-type root stained with JIM13, an anti-arabinogalactan antibody that stains endodermis and some stele cells. Bar, 10 μm. (E) Transverse section through the specialization zone of a *short-root* root stained with JIM7. Bar, 25 μm. (F) Transverse section through the specialization zone of *short-root* stained with JIM13. Bar, 25 μm. (G) Longitudinal section through *sabre* root tip stained with JIM7. Bar, 50 μm. (H) Longitudinal section of *cobra* root stained with JIM7. Bar, 50 μm. Abbreviations as in Fig. 1 except P, pericycle.

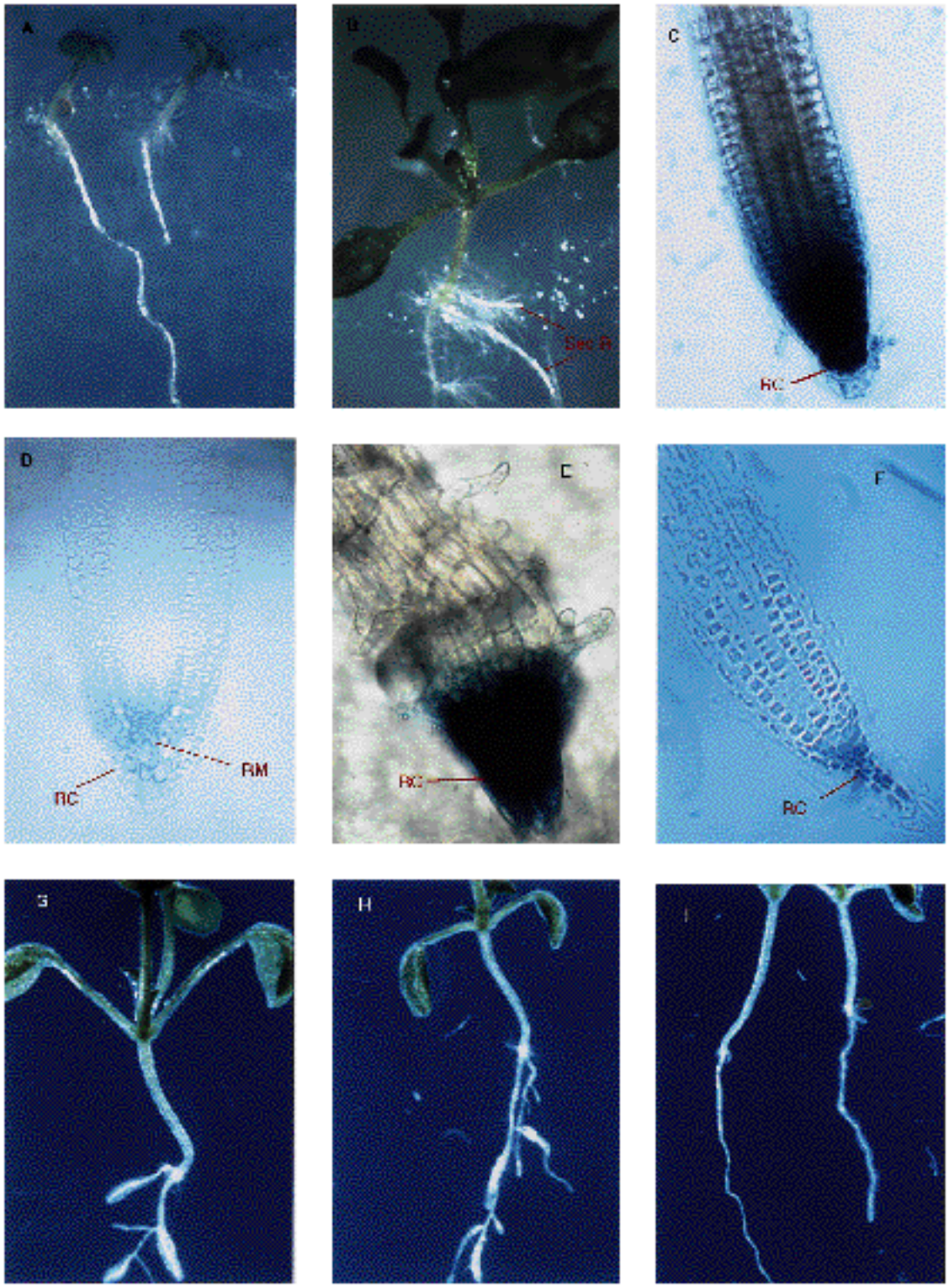

Fig. 3. (A) *Short-root* plant (right) and wild-type plant (left) at approximately 7 days after germination. (B) A *shr* plant at approximately 2 weeks grown in nutrient agar. Note large number of secondary roots (Sec R). (C) Expression conferred by the 35S B2 subdomain construct in whole mount of wild-type root. (D) Expression conferred by the 35S B2 subdomain construct in 5 μm median longitudinal section of wild-type root. (E) Expression conferred by the 35S B2 subdomain construct in whole mount of *shr* root. (F) Expression conferred by the 35S B2 subdomain construct in 5 μm median longitudinal section of *shr* root. (G) *cob* plant growing on nutrient agar medium. (H) *lit* plant growing on nutrient agar medium. (I) *sab* plant on right, wild type on left growing on nutrient agar medium. Abbreviations as above, except RC, root cap; RM, root meristem.

istics of the aerial portion of the plant (as compared to wild type) as long as the plant was maintained on nutrient agar medium. However, when *shr* mutants were transferred to soil, their growth was severely retarded resulting in a stunted phenotype. The homozygous plants were fertile but with reduced seed set. This difference in phenotype between nutrient agar-grown and soil-grown plants, is probably due to the inability of the mutant roots to assimilate sufficient nutrients in soil to maintain normal growth. This may be due to the reduced length of the mutant roots, the lack of the internal cell layers or a combination of both.

Mutations that cause abnormal cell expansion in the root

The *cobra* (*cob*) and *lion's tail* (*lit*) mutants were identified among EMS mutagenized plants as having similar phenotypes. The roots of these mutants had a noticeably larger diameter than wild type. In both mutants the degree of expansion varied along the length of the root (Fig. 1D,F). The primary root usually began to expand 3-4 days after germination. Secondary roots began to expand after emerging from the primary root. The aerial parts of *cob* were very similar to wild type (Fig. 3G). The aerial parts of *lit* also appeared similar to wild-type plants (Fig. 3H) although they were somewhat more stunted and occasionally, additional cell growth was observed on the hypocotyl of the mutant plants. This ectopic cell growth resembled callus tissue, except that the cells appeared larger than those normally found in *Arabidopsis* calli.

Initial observations suggested that sub-optimal growth conditions could reduce the degree of root expansion in these mutants. In order to characterize this response, we grew the plants under conditions that changed the rate of root growth. Wild-type and mutant seeds were planted on nutrient agar plates that contained increasing amounts of sucrose. For the wild-type plants we measured root length as a function of time. For the mutants we calculated the percentage that showed an expanded phenotype. The rate of growth of wild-type roots increased with increasing sucrose concentration to a maximum at 4.5% sucrose and then decreased slightly (data not shown). The number of mutant plants with the expanded phenotype also increased with increasing sucrose concentration (Table 1). We consistently observed expansion in the *lit* mutant at lower sucrose concentrations than expansion of the *cob* mutant (Table 1).

The appearance of the mutant phenotype with increasing sucrose concentrations could be due to the change in the osmotic potential. This would seem unlikely to be a direct effect since an expansion in cell size is the opposite effect expected in response to an increase in the external osmotic potential. To test whether the observed effect was related to the increase in growth rate that is mediated by sucrose in the media, we grew mutant plants on 4% sucrose medium but placed them at 14˚C. Under these conditions the roots remained unexpanded. In addition, plants that were grown under normal conditions and allowed to expand in the light showed a switch to the unexpanded phenotype when placed in the cold (Fig. 4B). These plants could then be returned to normal growth conditions and the expanded phenotype would reappear (Fig. 4C). This indicated that the expansion was not just a response to elevated sucrose concentrations but was a response to conditions that increased the rate of root growth.

Table 1. Response of expansion mutants to sucrose concentration (126 hours)

Percentage sucrose in plates	Percentage *lion's tail* plants with expanded roots	Percentage *cobra* plants with expanded roots	Percentage *sabre* plants with expanded roots
0	0	0	100
0.5	11	0	100
1	62	21	100
2	100	100	100
3	100	100	100
4	100	100	100
4.5	100	100	100
5	100	100	100
6	100	100	100

A third mutant, *sabre* (*sab*), was identified among T-DNA insertion lines as segregating for plants with increased root diameter and shorter roots (Fig. 1H). Unlike the *cobra* and *lion's tail* mutants, expansion was relatively uniform along the length of the root (Fig. 3I). There was also no detectable change in root expansion when the concentration of sucrose in the media was varied (Table 1). The aerial parts of the *sabre* mutant were smaller than wild type and the homozygous plants had extremely low fertility even though flowers were formed.

The three expansion mutants were crossed to wild type and the F_1 progeny analyzed. For *sab* and *lit* the F_1 progeny had a wild-type phenotype, indicating that these mutations were recessive to wild type. The progeny of the cross of *cob* and wild type initially appeared wild type, but slightly expanded root tips were observed 10-14 days after germination when grown under optimal conditions (see methods). The progeny of all the crosses were allowed to self-fertilize. For *sab* and *lit* the segregation ratios of the F_2 plants were consistent with the mutations being in a single locus and recessive to wild type. The segregation of the F_2 progeny of the cross between *cob* and wild type was: 48 wild type, 34 with a strong expanded root phenotype, and 67 with a very weak expanded root tip phenotype similar to that seen in the F_1 plants. Several plants from each class were allowed to self-pollinate. The wild-type plants gave 100% wild-type progeny, the strong expansion class gave 100% progeny with the parental phenotype. The weak expansion class of plants gave progeny that segregated in a similar manner to the F_2 progeny. We conclude that *cob* is semi-dominant.

The degree and type of cell expansion differs in the three mutants

To further characterize the three expansion mutants, transverse sections were cut through the specialization zone and longitudinal sections were made through the root tip. Cell areas were calculated from digitized images of transverse sections. For calculations of cell areas, fresh sections were used to minimize distortion of cell shape that can occur during fixation.

A visual comparison of transverse sections of the expanded regions of *cob*, *lit* and *sab* indicated that abnormal expansion occurred to different degrees in the different cell

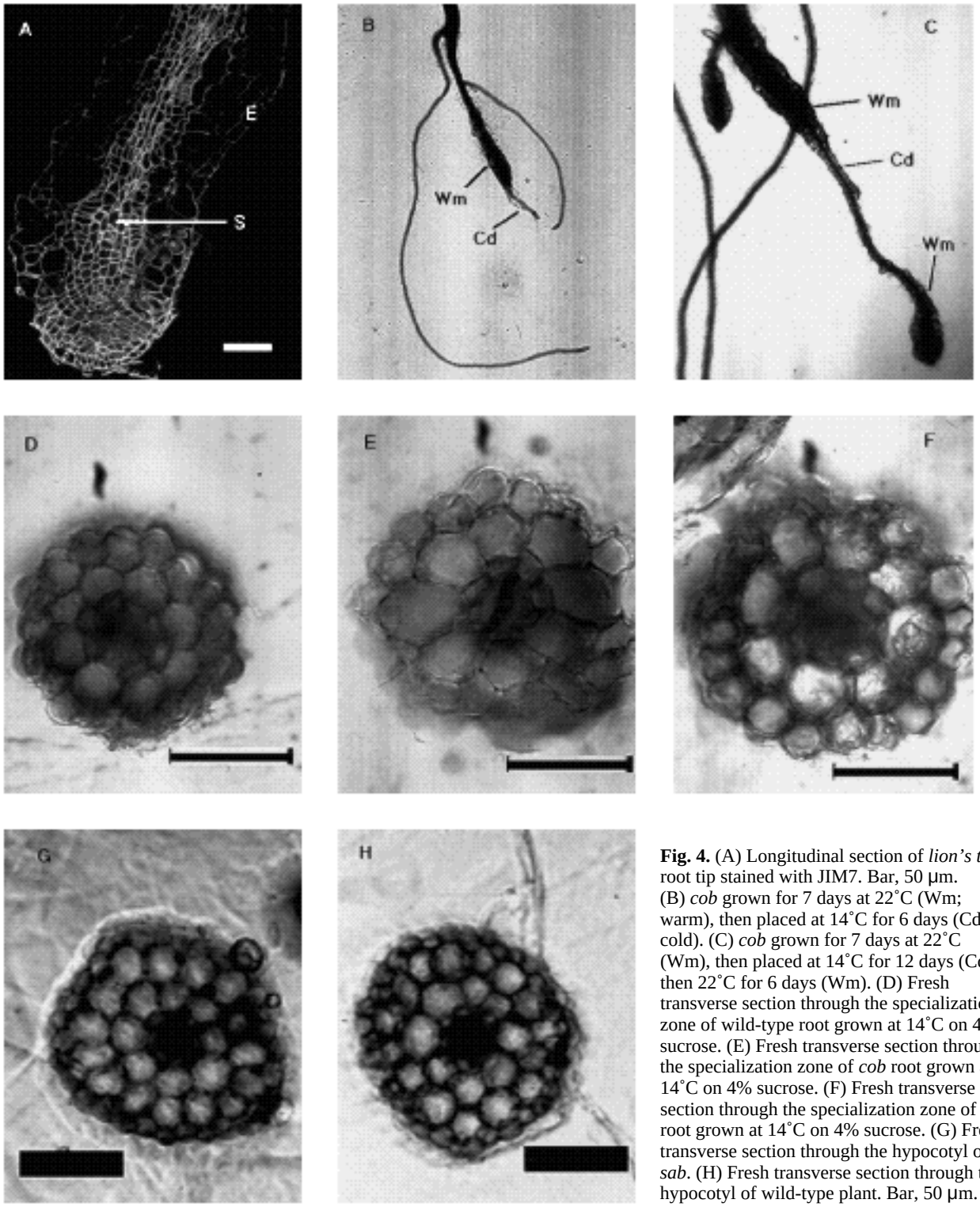

Fig. 4. (A) Longitudinal section of *lion's tail* root tip stained with JIM7. Bar, 50 μm. (B) *cob* grown for 7 days at 22˚C (Wm; warm), then placed at 14˚C for 6 days (Cd; cold). (C) *cob* grown for 7 days at 22˚C (Wm), then placed at 14˚C for 12 days (Cd), then 22˚C for 6 days (Wm). (D) Fresh transverse section through the specialization zone of wild-type root grown at 14˚C on 4% sucrose. (E) Fresh transverse section through the specialization zone of *cob* root grown at 14˚C on 4% sucrose. (F) Fresh transverse section through the specialization zone of *lit* root grown at 14˚C on 4% sucrose. (G) Fresh transverse section through the hypocotyl of *sab*. (H) Fresh transverse section through the hypocotyl of wild-type plant. Bar, 50 μm.

layers. In *cob* it appeared that the epidermal layer had the greatest expansion (Fig. 1E). In *lit* it appeared that the stele had undergone more expansion than other layers (Fig. 1G), while in *sab* it appeared that the cortex was expanded more than the other layers (Fig. 1I). This was confirmed by measurement of the cell areas of the mutants (Table 2). Fig. 5 shows a comparison of the relative cell areas in wild type and the three expansion mutants. For each cell type the cell area of the wild type was normalized to one.

The cell type with the least difference in cross-sectional area between wild type and the mutants was the endodermis. In *cob*, the epidermal cells were approximately 15 times larger in area than wild-type cells. The cortex and stele were expanded by 2.5 and 3.9 times respectively in this mutant.

Table 2. Surface area of wild type and rom mutants. The mean and standard deviation (in $\mu m^2 \times 10^{-2}$) of surface areas calculated from 5-7 fresh sections of wild type and each expansion mutant

Cell type	Epidermis	Cortex	Endodermis	Stele	Whole root
Wild type	1.8±0.38	3.5±0.63	1.4±0.38	7.2±0.47	85±3.4
cobra	27±8.2	8.9±2.9	3.8±1.1	28±7.5	620±110
lion's tail	8.3±3.7	15±5.8	3.1±1.4	65±17	400±100
sabre	10±5.5	29±11	2.6±0.93	19±6.4	470±160

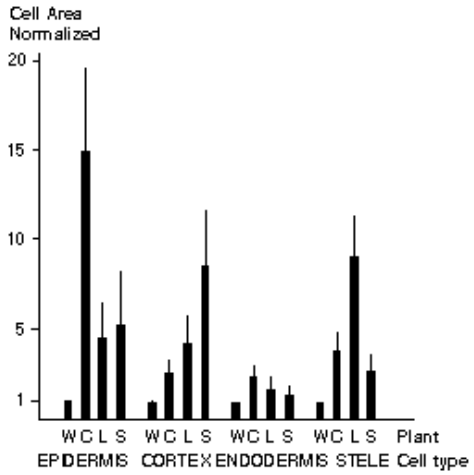

Fig. 5. Comparison of surface area of cells in wild type and the expansion mutants. The surface area of individual cells was calculated from digitized images of 5-7 fresh sections of wild type and the three expansion mutants. The average surface area of four cell types, epidermis, cortex, endodermis and stele of wild type (W) was normalized to one and compared with the average surface area for the same cell types in the three expansion mutants, *cob* (C), *lit* (L), and *sab* (S).

In *lit*, the area of the stele was approximately 9 times larger than in the wild type, while the epidermis and cortex were 4.6 and 4.3 times the wild-type size, respectively. In *sab*, the area of the cortex was approximately 8.3 times that of the wild type. The epidermis was approximately 5.6 times and the stele was approximately 2.6 times the wild-type counterpart. We conclude that abnormal expansion occurs in most cells of all three mutants. However there is a striking difference in the relative degree of expansion of the cell layers among the three mutants. When *cob* and *lit* were grown at 14˚C the transverse sections appeared very similar to wild type (Fig. 4E,F compare with Fig. 4D).

We also determined the number of cells in each of the cell layers. In all three mutants, the cortex and endodermal cell layers exhibited eight-fold symmetry in sections from the primary root. This was what was observed in wild-type roots. In *cob*, the boundaries of the epidermal cells were frequently indistinct. In one section we counted 15 cells and in another 16. In *lit* we observed 17-24 epidermal cells. In *sab* we observed 17-20 epidermal cells. In wild type the number of epidermal cells varies from 14 to 28. From these observations we conclude that these mutations do not cause significant abnormalities in the number of cells in the epidermis, cortex and endodermis.

Longitudinal sections through the root tips of the mutants were stained with anti-pectin antibodies. In sections of *sab*, it appeared that the abnormal expansion of the cortical cells was predominantly in the radial direction (Fig. 2G). In *cob*, it appeared from longitudinal sections that the elongation zone was shorter than in wild type and that the endodermal, cortical and epidermal cells were not as elongated as in the wild type (Fig. 2H). The elongation zone also appeared shorter than in the wild type in longitudinal sections of *lit* (Fig. 4A). The fact that the root cells of the three mutants are less elongated than those of wild type suggests that cell volume may not change as dramatically as the cross-sectional area.

To test whether similar shape changes occurred in the aerial organs of the expansion mutants we analyzed hypocotyls from mutant and wild-type plants. No apparent differences could be detected between cross-sections of mutant and wild-type hypocotyls. Representative sections from the hypocotyl of *sab* (Fig. 4G) and wild type (Fig. 4H) are shown. In conclusion, the three expansion mutants differ in their response to growth conditions and in the degree of expansion of their cell layers. A summary of the phenotypes of the four rom mutants is given in Table 3.

Genetic characterization of the root morphogenesis mutants

We have initiated a genetic characterization of the four root morphogenesis mutants. To place the mutants into complementation groups we performed all pair-wise crosses among the mutants. To simplify this initial analysis, the progeny of these crosses were planted under conditions in which the *cob* semi-dominant phenotype was not expressed (see methods). The F_1 progeny of these crosses all had a wild-type phenotype indicating that the mutant genes were not allelic. The F_1 progeny were allowed to self-pollinate and F_2 seed were collected. The F_2 seed were planted under similar conditions as the F_1 and the phenotype of the seedlings was observed. Table 4 shows the numbers of plants observed with the different phenotypes.

Among the F_2 progeny of the cross of *sab* with either *cob* or *lit* were plants that had the aerial phenotype of *sab* and roots that were far more expanded than homozygous *sab* roots (Fig. 6A,C). These plants were observed in the ratio expected of double homozygous mutants (Table 4). Transverse sections of the regions of the root that showed the greatest expansion indicated that there was an additive phenotype. Apparent double mutants of the cross of *sab* and *cob* had expanded cortical cells similar to those found in *sabre* and expanded epidermal cells similar to those found in *cobra* (Fig. 6B). Apparent double mutants of the cross between *sab* and *lit* had expanded cortical cells and an expanded stele similar to that found in *lit* (Fig. 6D). All of these plants had an aerial phenotype similar to *sab* and had extremely low fertility.

From the cross of *sab* and *shr*, F_2 progeny were observed that had short roots with expanded diameter, and no detectable meristematic or elongation zone at the root tip (Fig. 6E). Transverse sections revealed expanded cortical cells and no apparent endodermal cell layer (Fig. 6F). This apparent double mutant, therefore, appeared to combine the phenotypes of both the *short-root* and *sabre* mutants.

Table 3. Phenotype of rom mutants

Mutation	Root phenotype	Aerial phenotype
short-root	Determinate growth. Missing internal root cell layers.	Relatively normal when grown on nutrient agar. Stunted when grown on soil.
cobra	Expansion greatest in epidermal cells; conditional upon growth rate.	Normal.
lion's tail	Expansion greatest in stele cells; conditional upon growth rate.	Somewhat stunted. Occasional ectopic cell growth on hypocotyl.
sabre	Expansion greatest in cortex cells; not conditional upon growth rate. Expansion primarily in radial orientation.	Stunted but normal hypocotyl cell organization, very low fertility.

Expansion of the cortical cells in the root did not appear to be affected by the determinate growth pattern. The aerial portion of these plants was similar to *sab* and the plants had extremely low fertility.

Among the F_2 progeny of the crosses between *short-root* and either of the two conditional expansion mutants, *cobra* or *lion's tail*, were plants with roots that were expanded in a portion of the specialization zone and had a reduced diameter and no elongation or meristematic zone in the lower portion of the root (Fig. 6G,I). The upper parts of the root appeared similar to the expansion mutant, while the lower part appeared similar to *short-root*. Transverse sections through the expanded regions indicated an absence of the endodermal cell layer in both cases (Fig. 6H,J). In some apparent double mutants the primary root was unexpanded along its entire length resembling the root of *short-root* while the lateral roots were similar to that just described. This phenotype, which varies with the length of the root, can be explained by the phenotypes of the single mutants (see discussion).

Among the F_2 progeny of the cross between the two conditional expansion mutants, *cobra* and *lion's tail*, were plants that did not resemble either parental phenotype. These plants had aerial parts that were stunted and had abnormal levels of anthocyanin. An additional feature was the presence on some plants of ectopic cell growth on the hypocotyl or leaves. When transferred to soil these plants were sterile. We had never observed the stunted growth or ectopic cell growth in the aerial portion of *cobra* homozygotes. A far less severely stunted aerial phenotype was observed among homozygous *lit* mutants. As noted above, ectopic cell growth had been observed occasionally (approximately 1 in 200) in *lit* mutants. The roots of the double mutants were very similar in appearance to the roots of *lit* mutants (Fig. 6K), and sections through the expanded regions were indistinguishable from sections of *lit* with the greatest expansion in the stele tissue (Fig. 6L).

DISCUSSION

Roots as a model system for studying organ development in higher plants

Because of the simple, continuous, indeterminate growth pattern, roots provide an ideal model system to unravel the genetic basis for plant organ development. However, the genetic basis for root development is largely unexplored. Approximately 13 root mutants have been isolated previously from soil-grown plants but only two of these (*drt* in tomato, and *Rc* in cotton) could be classified as root morphogenesis mutants (Schiefelbein and Benfey, 1991). We and others have developed methods for screening large numbers of mutagenized *Arabidopsis* plants on Petri plates. We have also determined that the wild-type *Arabidopsis* root has a remarkably simple architecture, which has facilitated our analysis of the mutants that we have isolated.

Short root and maintenance of meristem growth potential

Root growth is maintained by division of a population of cells in the meristem known as 'initials' (Steeves and Sussex, 1989). These are the actively dividing cells that are found at the base of the files of the differentiated cell layers. In *Arabidopsis*, it has been determined that there are four sets of initials. These are the progenitors for (i) stele tissue, (ii) cortex and endodermis, (iii) epidermis and lateral root-cap cells, and (iv) columellar cells of the root cap (Dolan et al., 1993). Within the root meristem of some plants a group of cells has been demonstrated to have relatively infrequent cell divisions. These cells have been termed the 'quiescent center.' It has been proposed that the quiescent center cells may serve as a source of replacement cells for the initials (Barlow, 1976).

The *short-root* mutant has roots that cease growing after a short period of time and become differentiated at the root tip. We observed that as the root increases in length, the meristematic and elongation zones of *short-root* appeared to diminish in size. This suggests that there is a gradual loss of cells entering the differentiation pathway. One possible explanation for this inability to maintain the meristem's

Table 4. Scoring of phenotype of F_2 progeny of crosses between rom mutants

	Wild-type phenotype	Phenotype (A)	Phenotype (B)	Non-parental phenotype	χ^{2}*
sabre (A) × *cobra* (B)	347	119	93	33	4.5
sabre (A) × *lion's tail* (B)	164	51	58	15	1.0
short-root (A) × *sabre* (B)	149	49	53	10	2.6
short-root (A) × *cobra* (B)	319	89	106	38	2.8
short-root (A) × *lion's tail* (B)	174	75	52	16	6.1
cobra (A) × *lion's tail* (B)	128	53	39	18†	3.1

*χ^2 calculation is based on expected ratios of 9 wild type, 3 mutant A, 3 mutant B, 1 double mutant. $P<0.05$.
†Double mutant phenotype for *cobra* × *lion's tail* scored by aerial phenotype (see text).

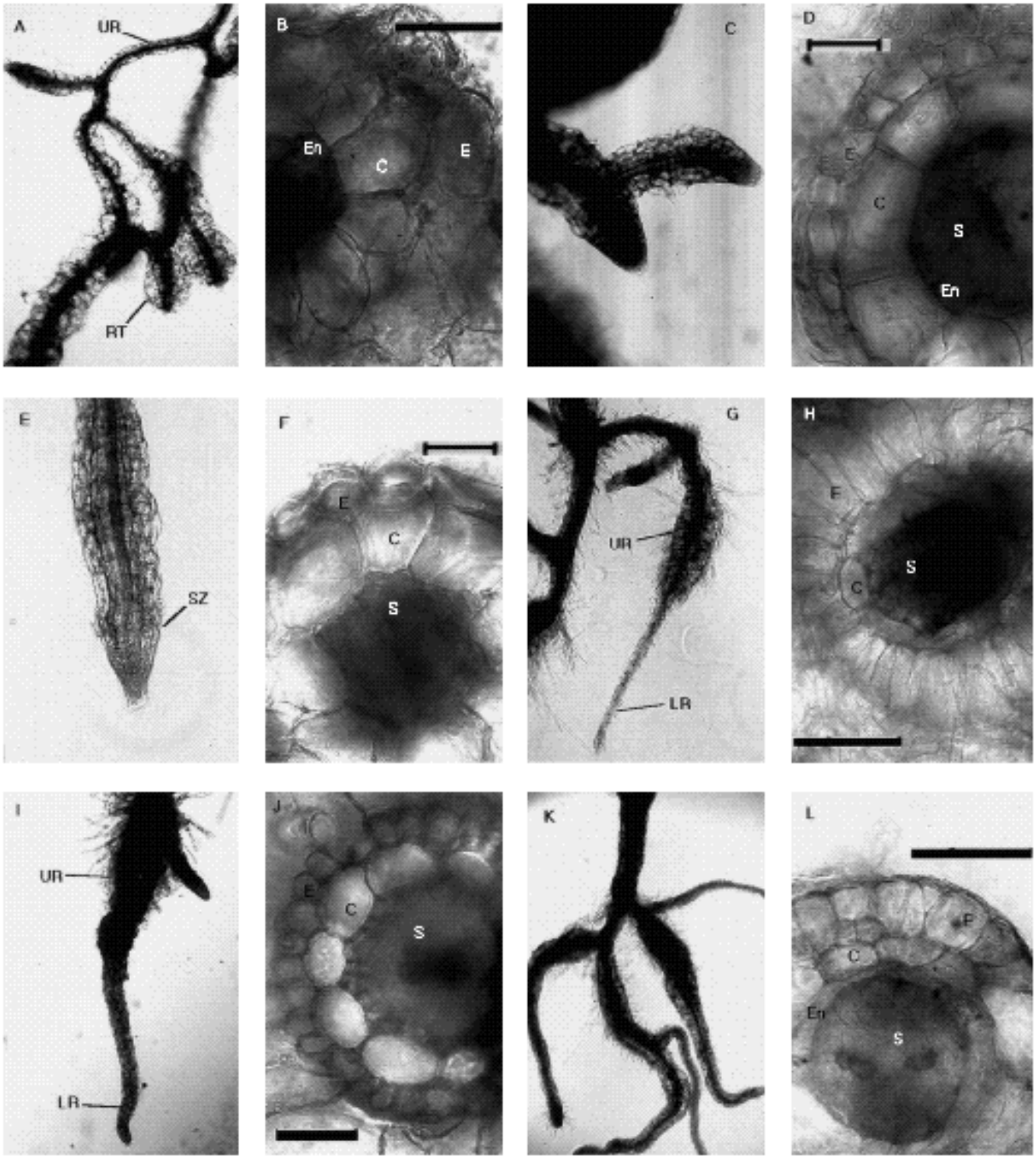

Fig. 6. Phenotype of double mutants. (A) *Sabre cobra* double mutant whole mount. Note expanded upper root (UR) and further expansion of the root tips (RT). (B) Fresh transverse section through root tip of *sab cob* double mutant. Note expanded cortical cells (C) and expanded epidermal cells (E). (C) *Sabre lion's tail* double mutant whole mount. (D) Fresh transverse section through root tip of *sab lit* double mutant. Note expanded cortical cells (C) and expanded stele (S). (E) Whole mount of root of *short-root sabre* double mutant. Note expanded specialization zone (SZ) and absence of elongation zone. (F) Fresh transverse section through root of *shr sab* double mutant. Note expanded cortical cells (C) and lack of endodermal cell layer between the cortical (C) and stele (S). (G) *Short-root cobra* double mutant. Note expanded upper root (UR) and reduced diameter of lower root (LR). (H) Fresh transverse section through upper root of *shr cob* double mutant. Note expanded epidermal cells (E) and lack of endodermal cell layer between the cortex (C) and stele (S). (I) *Short-root lion's tail* double mutant whole mount. Note expanded upper root (UR) and reduced diameter of lower root (LR). (J) Fresh transverse section through root of *shr lit* double mutant. Note expanded stele cells (S) and lack of endodermal cell layer between the cortex (C) and stele (S). (K) Whole mount of *cobra lion's tail* double mutant plant. (L) Whole mount of root of *cobra lion's tail* double mutant. Bar, 50 μm.

growth potential is that the initials are not replaced as their cell-division potential is exhausted.

The *short-root* mutant has another dramatic defect. To our knowledge, this is the first mutant of *Arabidopsis* to be shown to lack internal cell layers. In an analysis of mutations that affect body organization in the embryo of *Arabidopsis*, mutants were identified that affect the radial pattern without altering the apical-basal pattern (Mayer et al., 1991). In this class only mutations that affected the epidermal cells were isolated. Two possible reasons were proposed for the lack of mutations that affected other tissues. Either respecification occurs when internal tissues are affected or alterations of other tissues results in an inability to germinate (Mayer et al., 1991). We have shown that in *short-root*, at least one internal cell-layer is missing, indicating that respecification does not always occur. Preliminary results indicate that this defect is present in the embryo (B. Scheres, L. Di Laurenzio and P.N. Benfey, unpublished data) suggesting that the initial patterning of the root meristem is defective. It is possible that the lack of these internal cell layers leads to an imbalance in nutrient and/or hormone transport to the root tip, which results in the arrest in root growth.

Regulation of root cell expansion

In animals, cell movement plays an important role in the final determination of form. In plants, since there are no morphogenetic cell movements and cell walls are usually formed concomitant with cell division, morphogenesis is entirely dependent on how and when cells divide and expand. In addition, one of the most striking features of root development is its uniformity. There is no obvious modular growth as with the generation of stem nodes. We have characterized three mutants that have abnormal cell expansion properties. Two of these, *cobra* and *lion's tail* also show discontinuous growth with large variation in the degree of expansion along the length of the root. We have shown that the phenotype of these two mutants is conditional. Low sucrose concentrations in nutrient agar media cause the loss of the phenotype. Since sucrose has been shown to have an effect on the size of the quiescent center in maize (Feldman and Torrey, 1975) the phenotype of *lit* and *cob* may be conditional simply upon the presence of sufficient sucrose in the medium. The fact that mutant plants grown on high sucrose and in low temperature and low light do not have the expanded root phenotype suggests that the expansion phenotype may be conditional not upon sucrose concentration but upon the rate of root growth. This may also provide an explanation for the variation in degree of expansion of the mutant roots. The growth rate of the root may be slower when the root emerges from the seed and when secondary roots emerge from the primary root. The conditional phenotype raises the possibility that some component that is essential for regulated cell expansion is limiting in these mutants. Cell expansion is dependent upon changes in both the cytoskeleton and cell wall (Carpita and Gibeaut, 1993). Since the effect of these two mutations is primarily in the root, if the lesions are in a cell wall or cytoskeleton structural component then root-specific genes are likely to have been affected. The third expansion mutant, *sab* does not have a phenotype that is conditional upon the root growth rate. In addition, the aerial portion of this mutant is more severely affected than the other two mutants. Although we have shown that there is no equivalent to the root cell expansion in the hypocotyl of *sabre*, the aerial portions of the mutant are stunted as compared to wild-type plants and the mutant has extremely low fertility. This aerial phenotype of the *sabre* mutant may be caused by impaired functioning of the mutant root. Alternatively the *SABRE* gene may also play a role in the correct development of aerial organs.

Plant growth regulators are thought to play an important role in root development (Feldman, 1984). We have analyzed the phenotype of the four rom mutants when germinated on nutrient agar that contained different concentrations of auxin, cytokinin or gibberellic acid (P.N. Benfey, unpublished data). Under these conditions, *sab* and *shr* exhibited no detectable change in their root morphology except for the responses that were similar to those exhibited by wild-type plants. However, *cob* and *lit* did not show the expanded root tip phenotype at high concentrations of both auxin and cytokinin. Our interpretation of this observation is that since the documented action of these hormones is to reduce root growth (Feldman, 1984), the expanded root tip phenotype of these mutants is not expressed under these conditions. However, it is possible that these mutations have secondary effects, for example on hormone transport. Such an effect could explain the slightly stunted aerial phenotype of *lit*. These four mutations do not appear to affect the ability of the roots to sense gravity.

Cell-specific abnormalities in the mutant roots

Expansion of plant cells involves coordinate assembly of the cytoskeleton and cell wall. It is thought that orientation of microtubules plays an important role in determining the direction of expansion (Carpita and Gibeaut, 1993). Little is known about the regulation of cytoskeleton and cell wall formation during cell expansion. A striking feature of the three expansion mutants is the difference in the degree of expansion of the different root tissues as revealed by quantitation of cell areas. Expansion is proportionally greatest in the epidermis of *cobra*, in the stele of *lion's tail*, and in the cortex of *sabre*. This suggests that expansion can be differentially regulated in these tissues. The fact that cells in the different layers of the wild-type plant are different sizes and shapes indicates that there must be cell-specific regulation of cell expansion. However, in all three mutants, all cell types are expanded to some extent. Therefore, it is possible that the primary defect in these mutants is a metabolic or enzymatic process essential for regulated expansion of root cells whose effect is revealed differentially in the different cell layers. It should be noted that cell expansion in one root cell layer will almost certainly have an effect on neighboring cell layers since it is unlikely that the neighboring cells can be displaced during the expansion. Therefore the expansion of an internal cell layer is likely to cause either increased division or expansion (or both) of external cell layers.

In an independent screen of EMS mutagenized seedlings, three mutants (*rsw* 1-3) were identified that appear wild type at 18˚C, but show radial swelling at 31˚C (Baskin et al., 1992). One of these mutants showed distortion of epidermal cells but the cell expansion of internal cell layers was not characterized. It was determined that the growth rate of

wild-type plants declined dramatically at the restrictive temperature suggesting that the radial swollen phenotype was not dependent upon rapid root growth (Baskin et al., 1992).

Cell shape changes in double mutant combinations

Our genetic analysis placed the four morphogenesis mutants in different complementation groups and revealed that three of the mutations, *shr*, *lit* and *sab* were recessive to wild type. The semi-dominant phenotype of *cob* suggests that this mutation may result in haplo-insufficiency. This would be consistent with the mutation affecting a component that is limiting for regulated cell expansion.

At the present time we cannot determine whether the four rom mutations that we have analyzed represent null alleles. Preliminary results indicate that we have identified at least one additional allele of *cob* and *sab* (M.T. Hauser, R.A. Aeschbacher and P.N. Benfey, unpublished data). These both have very similar phenotypes to the alleles described here suggesting that these may be the null phenotypes. We have described the F_2 progeny of the crosses among the mutants because it provides insight into the relationship between cell shape change and developmental patterns.

The additive phenotype of combinations of *sab* with *cob* or *lit* indicated that the preferential expansion of one cell layer does not preclude expansion of another layer. This suggests that the mechanism of expansion in *sab* is in a pathway that is independent of that of the other two mutants. In addition, since the expanded root-tip phenotype of *cob* and *lit* was expressed in the apparent double mutant, this result indicated that the *sab* mutation does not cause a drastic decrease in the growth rate of the root.

The additive nature of the combination *shr* and *sab* is of interest because there is no endodermal cell layer in the apparent double mutant. As noted above, anatomical analysis has revealed that the endodermis and cortex are derived from a common precursor cell (Dolan et al., 1993). In addition, in *sab* there is little evidence of abnormal expansion of the endodermis. These two observations suggest that the *SABRE* gene product acts after the division that gives rise to the cortical and endodermal cell files.

The phenotype of the combination of *shr* and *cob* or *lit* in which the upper portion of the root was expanded and the lower part had a *shr* phenotype can be explained by the conditional nature of the two expansion mutants. The phenotype of the double mutant resembled the phenotype of a conditional expansion mutant that had been transferred into the cold, except that in the latter case the root continued to grow. The *shr* mutation causes a gradual loss of the capacity of the meristem to maintain growth. It is plausible that in the process, the rate of growth of the root slows before coming to a complete halt. We propose that the combination of the two mutations results in a root that initially grows rapidly enough to reveal the expanded phenotype of the conditional mutants but as the meristem loses its growth potential the root growth rate falls to the point at which the expansion phenotype is no longer expressed. The remarkable aspect of the double mutant is the complete change of the root from grossly expanded to normal diameter (or slightly less than normal since the internal cell layers are missing), without any change in environmental conditions. This provides additional evidence that the defect in the expansion mutants is the disruption of regulated cell expansion, which is conditional upon the rate of growth of the root.

The non-additive phenotype of the combination of *cob* and *lit* was the most difficult to interpret. The apparent epistasis in the root could be the result of a shared genetic pathway. However, given the difference in the expansion phenotype of the two mutants it seems more probable that the apparent epistasis is related to a difference in the timing of the phenotypic changes caused by the two mutations. The expansion of stele tissue in *lit* is apparent at lower sucrose concentrations than the expansion of the epidermal tissue in *cob*. This suggests that the expansion mediated by the *lit* mutation may occur prior to expansion mediated by the *cob* mutation. If expansion of the stele tissue is deleterious to growth rate (for example, causing impaired vascular function, which reduces nutrient or hormone transport), then the rate of growth may never be sufficiently high to allow the *cob* phenotype to be expressed. The aerial phenotype of the double mutant may be caused by a synergistic interaction of the two mutations in the upper part of the plant or it could be the result of a severely dysfunctional root. The latter possibility would arise if the *cobra* mutation causes functional problems in the root even when the epidermal cells are not expanded. In combination with the expanded stele tissue caused by the *lion's tail* mutation this might create a root that is unable to adequately sustain the aerial part of the plant, leading to the stunted and stressed phenotype. In addition, the presence of ectopic cell growth on some of the double mutants indicates that hormone transport or utilization may have been disrupted in these plants.

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