The *SCARECROW* Gene Regulates an Asymmetric Cell Division That Is Essential for Generating the Radial Organization of the Arabidopsis Root

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Summary

In the Arabidopsis root meristem, initial cells undergo asymmetric divisions to generate the cell lineages of the root. The *scarecrow* mutation results in roots that are missing one cell layer owing to the disruption of an asymmetric division that normally generates cortex and endodermis. Tissue-specific markers indicate that a heterogeneous cell type is formed in the mutant. The deduced amino acid sequence of SCARECROW (SCR) suggests that it is a member of a novel family of putative transcription factors. *SCR* is expressed in the cortex/endodermal initial cells and in the endodermal cell lineage. Tissue-specific expression is regulated at the transcriptional level. These results indicate a key role for *SCR* in regulating the radial organization of the root.

Introduction

Asymmetric cell divisions, in which a cell divides to give two daughters with different fates, play an important role in the development of all multicellular organisms (Horvitz and Herskowitz, 1992). In plants, the regulation of asymmetric cell divisions is of heightened importance in organ development because there is no cell migration. In contrast to animal organogenesis, plant organs are not formed primarily during embryogenesis. Rather, cells that form the apical meristems are set aside at the shoot and root poles of the early embryo. These reservoirs of stem cells are considered to be the source of all postembryonic organ development. A fundamental question in developmental biology is how cell division is regulated in meristems to direct organization and growth of plant organs. More specifically, it is of paramount importance to understand the regulation of the asymmetric divisions of meristematic cells that give rise to distinct tissues within the organ.

We are using the Arabidopsis root as a model to study organ development in plants. The radial organization of the mature tissues in the Arabidopsis root consists of the epidermis, cortex, endodermis, and pericycle, which surround the vascular cylinder (Dolan et al., 1993) (Figure 1A). These mature tissues are derived from four sets of stem cells or initials: the columella root cap initials; the pericycle/vascular initials; the epidermal/lateral root cap initials; and the cortex/endodermal initials (Dolan et al., 1993) (Figure 1A). It has been shown that at least some of these initials undergo asymmetric divisions (Dolan et al., 1993). The cortex/endodermal initial, for example, first divides anticlinally (in a transverse orientation) (Figure 1B). This asymmetric division produces another initial and a daughter cell. The daughter cell then divides periclinally (in a longitudinal orientation) (Figure 1B). This second asymmetric division produces the progenitors of the endodermis and the cortex cell lineages (Figure 1B).

We have identified and characterized mutations that disrupt the asymmetric divisions of the cortex/endodermal initial (Benfey et al., 1993; Scheres et al., 1995). The *short-root* (*shr*) and *scarecrow* (*scr*) mutations result in the loss of a cell layer between the epidermis and pericycle. In both mutants, the cortex/endodermal initial divides anticlinally, but the subsequent periclinal division that increases the number of cell layers does not take place (Benfey et al., 1993; Scheres et al., 1995). The defect is first apparent in the embryo and extends the length of the embryonic axis, which includes the embryonic root and hypocotyl (Scheres et al., 1995). This is also true for the other radial organization mutants characterized to date, suggesting that radial organization that occurs during embryonic development may influence the postembryonic organization generated by the meristematic initials (Scheres et al., 1995).

Although both *shr* and *scr* have only a single cell layer between the epidermis and pericycle, they differ in the identity of the resulting mutant cell layer. In *shr*, two endodermis-specific markers are absent from the mutant layer (Benfey et al., 1993), suggesting that the *SHR* gene may be involved in specification of endodermis identity. In contrast, we have now determined that the mutant cell layer in *scr* has differentiated characteristics of both cortex and endodermis. This is consistent with a role for *SCARECROW* (*SCR*) in the regulation of the asymmetric cell division of the cortex/endodermal initial rather than in specification of the identity of either cortex or endodermis.

We have cloned the *SCR* gene and determined its structure and expression pattern. The deduced amino acid sequence of the *SCR* gene product contains a number of potential functional domains similar to those found in transcription factors. Closely related sequences have been found in both dicots and monocots, indicating that SCR is a member of a novel protein family. The expression pattern of the *SCR* gene has been characterized by means of in situ hybridization and by an enhancer trap insertion upstream of the *SCR* gene. The expression pattern is consistent with a key role for SCR in regulating the asymmetric division of the cortex/endodermis initial, which is essential for generating the radial organization of the root.

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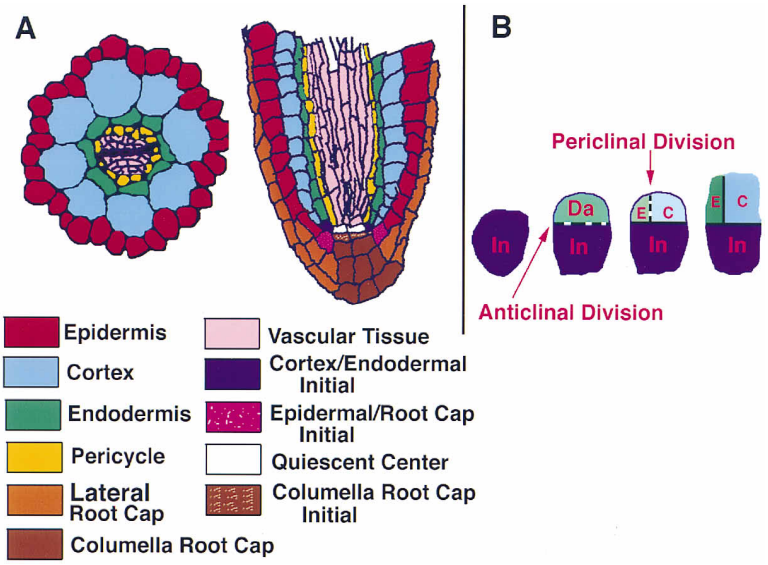

Figure 1. Schematic of Arabidopsis Root Anatomy and Division Pattern of the Cortex/Endodermal Initial

(A) Schematic of Arabidopsis root anatomy. Transverse section showing epidermis, cortex, endodermis, and pericycle surrounding the vascular tissue. In the longitudinal section, the epidermal/lateral root cap initials and the cortex/endodermal initials are shown at the base of their respective cell files.

(B) Schematic of division pattern of the cortex/endodermal initial. The initial expands and then divides anticlinally to reproduce itself and a daughter cell. The daughter then divides periclinally to produce the progenitors of the endodermis and cortex cell lineages. C, cortex; Da, daughter cell; E, endodermis; In, initial.

Results

Characterization of the *scr* Phenotype

The *scarecrow* mutation (*scr-1*) was identified initially in a screen of T-DNA-transformed Arabidopsis lines (Feldmann, 1991) as a seedling with greatly reduced root length compared with wild type (Scheres et al., 1995). A second mutant (*scr-2*) with a similar phenotype was subsequently identified among T-DNA-transformed lines. Analysis of cosegregation between the mutant phenotype and antibiotic resistance carried by the T-DNA indicated tight linkage for *scr-1* and no linkage for *scr-2* (see Experimental Procedures). An antibiotic-sensitive line of *scr-2* was isolated and crossed with *scr-1*. The F1 and F2 progeny of this cross were all mutant, with the F2 segregating 3:1 for antibiotic resistance, confirming allelism (see Experimental Procedures). The principal phenotypic difference between the two alleles was that *scr-1* root growth was more retarded than that of *scr-2*, suggesting that it is the stronger allele (Figure 2A). For both alleles the aerial organs appeared similar to wild type and the flowers were fertile (Figures 2A and 2B). The progeny of backcrosses of *scr-1* or *scr-2* to wild-type plants segregated 3:1 for the root phenotype, indicating that the mutation is monogenic and recessive.

Analysis of transverse sections through the primary root of seedlings revealed only a single cell layer between the epidermis and the pericycle (Figure 2C) instead of the normal radial organization consisting of cortex and endodermis (Figure 2D). This radial organization defect was not limited to the primary root, but was also present in secondary roots (Figure 2E) and in roots regenerated from calli (Figure 2F). Occasionally, more than the normally invariant eight cells were observed in the mutant cell layer in the primary root. We have also observed abnormal placement or numbers of epidermal cells (see Figure 2E). These abnormalities were more frequently observed in *scr-1* than in *scr-2*. Nevertheless, organization of the mutant root closely resembled that of wild type except for the consistent reduction in the number of cell layers. In longitudinal optical sections of *scr* roots, the mutant cell layer could be identified by its position between epidermis and pericycle and could be traced back to a single initial cell within the meristem (Figure 2G). The putative initial cell was in a similar position within the meristem as the wild-type cortex/endodermal initial (Figure 2H). Because the endodermis and cortex are normally generated by an asymmetric division of the cortex/endodermal initial, this indicates that the primary defect in *scr* is disruption of this asymmetric division.

Cortex and endodermis are also formed from the asymmetric division of embryonic ground tissue at the early torpedo stage. This division occurs along the length of the embryonic axis, which encompasses the embryonic root and hypocotyl. We have shown that the radial organization defect in *scr-1* first appears in the developing embryo and manifests itself as a failure of the embryonic ground tissue to undergo the asymmetric division into cortex and endodermis (Scheres et al., 1995). Other embryonic tissues appear similar to wild type (Scheres et al., 1995). Similar results were obtained for *scr-2* (data not shown). Hence, this mutation identifies a gene required for the asymmetric division that produces cortex and endodermis from ground tissue in the embryo and from the cortex/endodermal initials in primary and secondary roots.

Characterization of Cell Identity in *scr* Roots

To understand the role of the *SCR* gene in regulating this asymmetric division, it was necessary to determine the identity of the mutant cell layer. Tissue-specific markers were used to distinguish among several possibilities. The cell layer could have differentiated attributes of either cortex or endodermis. Alternatively, it could have an undifferentiated initial cell identity or a heterogeneous identity with differentiated attributes of both endodermis and cortex in the same cell.

Transverse sections of *scr-1* and *scr-2* roots were assayed for the presence of tissue-specific markers. The casparian strip, a deposition of suberin between

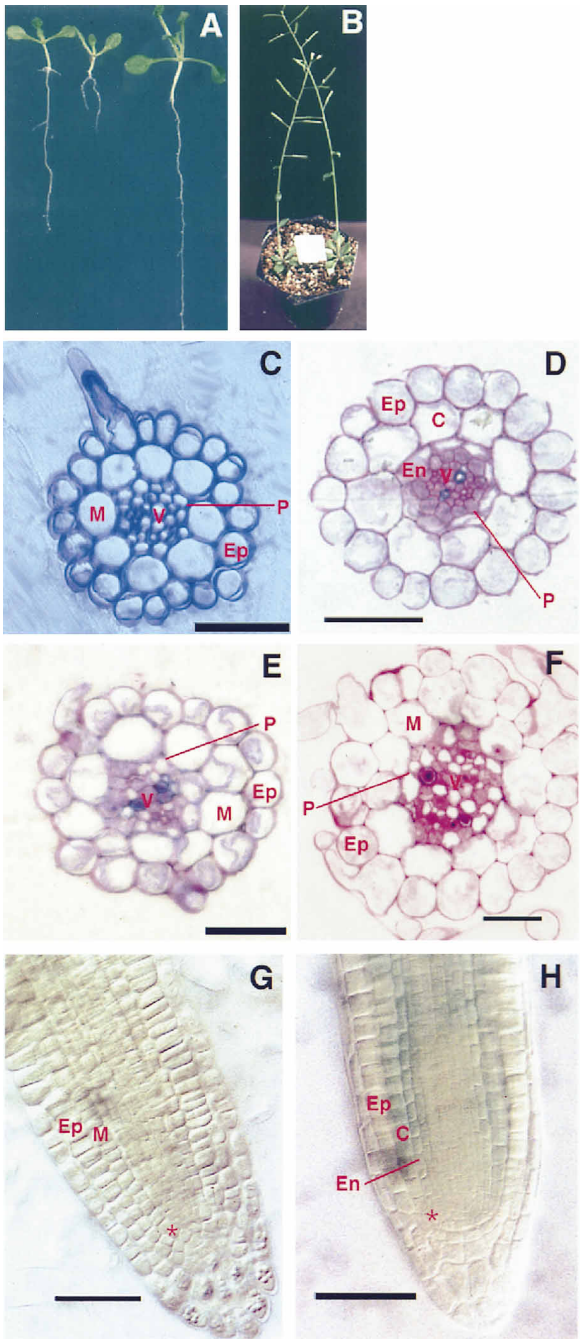

Figure 2. Phenotype of *scr* Mutant Plants

(A) 12-day *scr-2*, *scr-1*, and wild-type seedlings grown vertically on nutrient agar medium.

(B) *scr-2* plants after seed set.

(C–F) Transverse plastic sections of primary root of *scr-2* (C), primary root of wild type (D), lateral root of *scr-1* (E), and root regenerated from *scr-1* callus (F).

(G and H) Nomarski optical sections through the root meristems of *scr-2* (G) and wild type (H).

Bar is 50 μm. C, cortex; En, endodermis; Ep, epidermis; M, mutant cell layer; P, pericycle; V, vascular tissue; asterisk, cortex/endodermal initial.

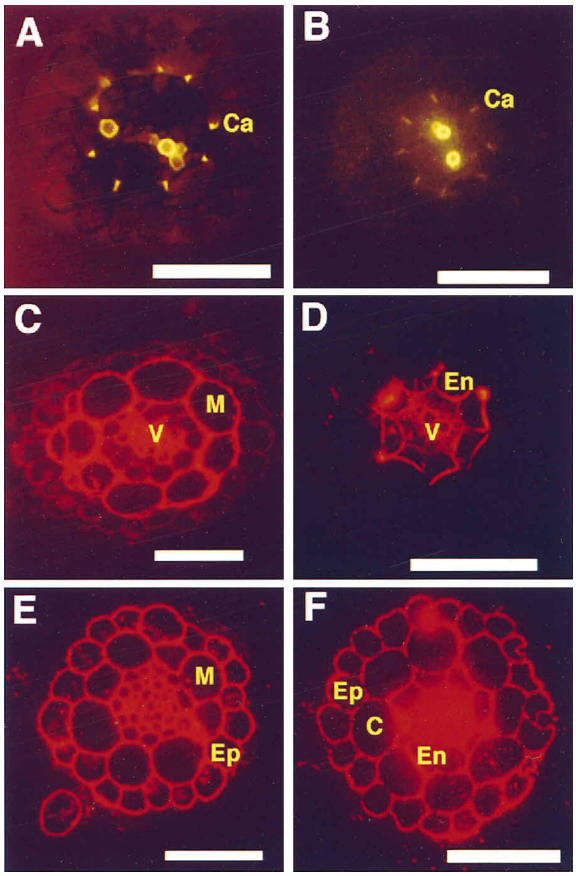

Figure 3. Characterization of the Cellular Identity of the Mutant Cell Layer

(A) Endodermis-specific casparian strip staining of transverse section through the primary root of *scr-1*. (Note that the histochemical stain also reveals xylem cells in the vascular cylinder).

(B) Casparian strip staining of transverse section through the primary root of wild type.

(C) Immunostaining with the endodermis-specific (and a subset of vascular tissue–specific) JIM13 monoclonal antibody on transverse root section of *scr-2*.

(D) JIM13 antibody on transverse root section of wild type.

(E) Immunostaining with the JIM7 monoclonal antibody, which stains all cell walls on transverse root section of *scr-2*.

(F) JIM7 antibody on transverse root section of wild type.

Bar, 50 μm. Abbreviations are as in Figure 2. Ca, casparian strip.

radial cell walls, is specific to the endodermal cells and is believed to act as a barrier to the entry of solutes into the vasculature (Esau, 1977). Histochemical staining revealed the presence of a casparian strip in the mutant cell layer (Figure 3A, compare with the normal endodermal staining pattern in wild type in Figure 3B). The presence of lignin in the vascular cylinder was also revealed by this histochemical stain, indicating the presence of differentiated xylem cells in both mutant (Figure 3A) and wild type (Figure 3B). Another marker of the differentiated endodermis is the arabinogalactan epitope recognized by the monoclonal antibody JIM13 (Knox et al., 1990). The mutant cell layer showed staining with this antibody (Figure 3C, compare with wild type in Figure 3D). As a positive control, we used the JIM7 antibody,

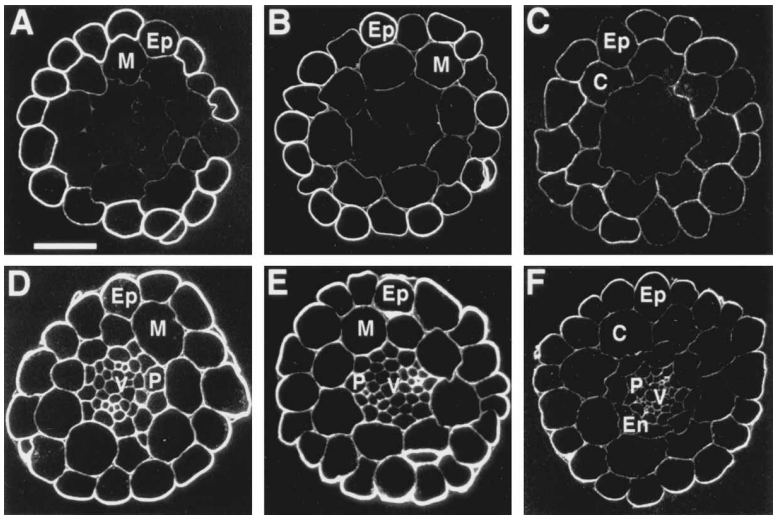

Figure 4. Characterization of Cortex Attributes in the Mutant Cell Layer

(A–C) Immunostaining with the cortex-specific (and epidermis-specific) CCRC-M2 monoclonal antibody on transverse root sections of *scr-1* (A), *scr-2* (B), and wild type (C). (Apparent differences in intensity of staining between *scr* and wild-type epidermal cells were variable.)

(D–F) Immunostaining with the CCRC-M1 monoclonal antibody (specific to a cell wall epitope found on all cells) on transverse sections (from the same roots as in [A]–[C]) of *scr-1* (D), *scr-2* (E), and wild type (F).

Bar is 30 μm. Abbreviations are as above.

which recognizes pectin epitopes in all cell walls (Figures 3E and 3F). These results indicate that the cell layer between the epidermis and the pericycle has differentiated attributes of the endodermis.

As a marker for the cortex, we used the CCRC-M2 monoclonal antibody, which recognizes a cell wall carbohydrate epitope found only on differentiated cortex and epidermis cells (Freshour et al., 1996). In sections from the differentiation zone of *scr-1* and *scr-2*, both the mutant layer and the epidermis showed staining (Figures 4A and 4B) that was similar to that of wild type (Figure 4C). In *scr-1*, staining of both cell types was apparent, but staining of the mutant layer was somewhat weaker than wild-type cortex. As a positive control, we used the CCRC-M1 monoclonal antibody, which recognizes a different carbohydrate epitope (Puhlmann et al., 1994) found on all cells (Figures 4D–4F).

With the CCRC-M2 antibody we observed one interesting difference between the staining pattern of the mutants and wild type. The appearance of this carbohydrate epitope correlates with differentiation in epidermis and cortex. In wild-type plants, there is no staining in sections close to the root tip, including the meristematic region. In sections higher up in the root, atrichoblasts (epidermal cells that do not make root hairs) stain. In sections from more mature root tissue, all epidermal cells as well as cortex cells stain for this epitope (Freshour et al., 1996). In both *scr-1* and *scr-2*, sections could be found in which all epidermal cells stained, while there was little detectable staining of cortex cells (data not shown). The significance of this difference in staining is not readily discernible. Although not precisely identical to the wild-type staining pattern, the fact that the mutant cell layer clearly stains for this cortex marker indicates that there are differentiated cortex attributes in these cells.

Taken together, these results indicate that the mutant cell layer has differentiated attributes of both the endodermis and cortex. We can thus rule out the possibility that there has been a simple deletion of a cell type or that the resulting cell type remains in an undifferentiated initial-like stage. This result is consistent with a role for the *SCR* gene in regulating the asymmetric cell division rather than a role in directing cell specification.

Molecular Cloning of the *SCR* Gene

To elucidate further the function of the *SCR* gene, we made use of the inserted T-DNA sequences to clone the gene. Plant DNA flanking the insertion site was obtained from *scr-1* by plasmid rescue and used to isolate the corresponding wild-type genomic DNA (see Experimental Procedures). Several cDNA clones were isolated from a library made from silique tissue. Comparison of the sequence of the longest cDNA and the corresponding genomic region revealed an open reading frame interrupted by a single small intron (Figure 5A). A potential TATA box and polyadenylation signal that matched the consensus sequences for plant genes were also identified (Heidecker and Messing, 1986; Joshi, 1987; Mogen et al., 1990).

Comparison of the nucleotide sequence of the genomic clone and the rescued plasmid placed the site of the T-DNA insertion in *scr-1* at codon 470 (Figures 5A and 5B). For *scr-2*, although no linkage was found between the mutant phenotype and antibiotic resistance, DNA blot and polymerase chain reaction (PCR) analysis of antibiotic-sensitive lines revealed the presence of T-DNA sequences that cosegregated with the mutant phenotype. The insertion position in *scr-2* was determined by sequencing the PCR products amplified from its genomic DNA using a combination of T-DNA- and *SCR*-specific primers (Figure 5B). In *scr-2*, the T-DNA insertion point is at codon 605 (Figures 5A and 5B).

To verify linkage between the cloned gene and the mutant phenotype, we identified the chromosomal location of both the *scr* locus and the *SCR* gene. To map the *scr* locus, we used molecular markers on F2 progeny of crosses between *scr-2* (ecotype Wassilewskija [Ws]) and Columbia (Col) wild type. These placed the *scr* locus at the bottom of chromosome III, approximately 0.5 cM away from each of the two closest markers available, cdc2b (J. Celenza) and BGL1 (Konieczny and Ausubel, 1993). To map the *SCR* gene, we identified a polymorphism between Col and Landsberg using the *SCR* probe b (Figure 5B). Southern analysis of 25 recombinant inbred lines (Jarvis et al., 1994) mapped the cloned gene to the same location as the *scr* locus on chromosome III.

The determination of the molecular defects in two

A

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      10        20        30        40        50        60        70        80        90        100       110       120
     -41                                      +1
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                                             M  A  E  S  G  D  F  N  G  G  Q  P  P  P  H  S  P  L  R  T  T  S  S  G   24

AGTAGCAGCAGCAACAACCGTGGTCCTCCTCCTCCTCCTCCTCCTTTAGTGATGGTGAGAAAAAGATTAGCTTCCGAGATGTCTTCTAACCCTGACTACAACAACTCCTCTCGTCCT
S  S  S  S  N  N  R  G  P  P  P  P  P  P  P  P  L  V  M  V  R  K  R  L  A  S  E  M  S  S  N  P  D  Y  N  N  S  S  R  P   64

CCTCGCCGTGTCTCTCACCTTCTTGACTCCAACTACAATACTGTCACACCACAACAACCACCGTCTCTTACGGCGGCGGCTACTGTATCTTCTCAACCAAACCCACCACTCTCTGTTTGT
P  R  R  V  S  H  L  L  D  S  N  Y  N  T  V  T  P  Q  Q  P  P  S  L  T  A  A  A  T  V  S  S  Q  P  N  P  P  L  S  V  C  104

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G  F  S  G  L  P  V  F  P  S  D  R  G  G  R  N  V  M  M  S  V  Q  P  M  D  Q  D  S  S  S  S  S  A  S  P  T  V  W  V  D  144

GCCATTATCAGAGACCTTATCCATTCCTCAACTTCAGTCTCTATTCCTCAACTTATCCAAAACGTTAGAGACATTATCTTCCCTTGTAACCCAAATCTCGGTGCTCTTCTTGAATACAGG
A  I  I  R  D  L  I  H  S  S  T  S  V  S  I  P  Q  L  I  Q  N  V  R  D  I  I  F  P  C  N  P  N  L  G  A  L  L  E  Y  R  184

CTCCGATCTCTCATGCTCCTTGATCCTTCCTCTTCCTCTGACCCTTCTCCTCAAACTTTCGAACCTCTCTATCAGATCTCCAACAATCCTTCTCCTCCACAACAGCAACAGCAGCACCAA
L  R  S  L  M  L  L  D  P  S  S  S  S  D  P  S  P  Q  T  F  E  P  L  Y  Q  I  S  N  N  P  S  P  P  Q  Q  Q  Q  Q  H  Q  224

CAACAACAACAACAGCATAAGCCTCCTCCTCCTCCGATTCAGCAGCAAGAAAGAGAAAATTCTTCTACCGATGCACCACCGCAACCAGAGACAGTGACGGCCACTGTTCCCGCCGTCCAA
Q  Q  Q  Q  Q  H  K  P  P  P  P  P  I  Q  Q  Q  E  R  E  N  S  S  T  D  A  P  P  Q  P  E  T  V  T  A  T  V  P  A  V  Q  264

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T  L  W  L  L  Q  R                                                                                                    511

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2163
AAT
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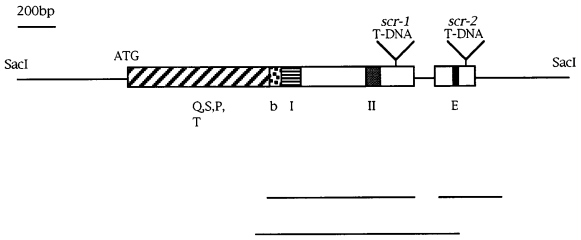
B



C

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SCR bZIP-like domain      PAVQTNTAEALRERKEEIKRQKQ  D
                                  ||  | | |   : | ||
GCN4        (yeast)       LKRARNTEAARRSRARKLQRMKQ  L
TGA1        (Arabidopsis) RRLAQNREAARKSRLRKKAYVQQ  L
C-Fos       (mouse)       IRRERNKMAAAKCRNRRRELTDT  L
c-JUN       (human)       RKRMRNRIAASKCRKRKLERIAR  L
CREB        (human)       VRLMKNREAARECRKKKEYVKC  L
Opaque-2    (maize)       KRKESNRESARRSRYRKAAHLKE  L
OBF2        (maize)       MRQIRNRDSAMKSRERKKSYIKD  L
RAF-1(rice)               RRMVSNRESARRSRKKKQAHLAD  L
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D

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SCR VHIID domain

SCR       AFEKEDSVHIIDLDIMQGLQWPGLFHILASRPGGPPHVRLTGL

F13896    AVKNESFVHIIDFQISQGGQWVSLIRALGARPGGPPNVRITGI

Z37192    AMEGEKMVHVIDLDASEPAQWLALLQAFNSRPEGPPHLRITGV

Z25645    AIKGEEEVHIIDFDINQGNQYMTLIRSIA

D41474              IHVIDFXLGVGGQWASFLQELAHRRG

T18310              VHIIXFXLMQGLQWPALMDVFSARKGGPPKLRITGI
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Figure 5. Structure of the *SCR* Gene

(A) Sequence of the *SCR* genomic region. Regulatory sequences including TATA box, ATG start codon, and polyadenylation signal are underlined. Within the amino terminus of the deduced amino acid sequence, the residues found in homopolymeric repeats are underlined and in bold.

(B) Schematic diagram of genomic clone indicating possible functional motifs, T-DNA insertion sites, and subclones used as probes. Abbreviations are as follows: Q, S, P, and T, region with homopolymeric repeats of these amino acids; b, region with similarity to the basic region of bZIP factors; I and II, regions with leucine heptad repeats; E, acidic region.

(C) Comparison of the charged region found in *SCR* with the basic DNA-binding region found in bZIP transcription factors.

(D) Translations of EST clones with similarities to SCR. F13896, Z37192, and Z25645 are from Arabidopsis, D41474 is from rice, and T18310 is from maize.

independent alleles and the colocalization of the cloned gene and the mutant locus confirm that we have identified the *SCR* gene.

The *SCR* Gene Product Has Motifs That Suggest It May Act as a Transcription Factor

The *SCR* gene product is a 653 amino acid polypeptide that contains several motifs suggestive of function (Figure 5B). The amino terminus has homopolymeric stretches of glutamine, serine, threonine, and proline residues, which account for 44% of the first 267 residues. Domains rich in these residues have been shown to activate transcription and may serve such a role in SCR (Johnson et al., 1993). A charged region between residues 265 and 283 has similarity to the basic domain of the basic–leucine zipper (bZIP) family of transcriptional regulatory proteins (Figure 5C) (Hurst, 1994). The basic domains from several bZIP proteins have been shown to act as nuclear localization signals (Varagona et al., 1992), and this region in SCR may act similarly. This charged region is followed by a leucine heptad repeat (residues 291–322). A second leucine heptad repeat is found toward the carboxyl terminus (residues 436–473). Since leucine heptad repeats have been demonstrated to mediate protein–protein interactions in other proteins (Hurst, 1994), the existence of these motifs suggests that SCR may function as a dimer or a multimer. The second leucine heptad repeat is followed by a small region rich in acidic residues, also present in a number of defined transcriptional activation domains (Johnson et al., 1993). While each of these domains has been found within proteins that do not act as transcriptional regulators, the fact that all of them are found within the deduced SCR protein sequence strongly suggests that SCR is a transcriptional regulatory protein.

SCR Is a Member of a Novel Protein Family

The deduced SCR protein sequence was compared with the sequences in the available databases. Five expressed sequence tags (ESTs), three from Arabidopsis, one from rice, and one from maize, showed significant similarity to residues 394–435 of the SCR sequence, a region immediately amino terminal to the second leucine heptad repeat. We have designated this region the VHIID domain (Figure 5D). Subsequent analysis of these EST sequences has revealed that the sequence similarity extends beyond this region; in fact, the similarity extends throughout the entire gene products of the available clones (L. P., unpublished data). The combination and order of the motifs found in these sequences do not show significant similarity to the general structures of other established regulatory protein families (e.g., bZIP, zinc finger, MADS domain, and homeodomain), indicating that these proteins comprise a novel family.

SCR Is Expressed in the Cortex/Endodermal Initial and in the Endodermis

RNA blot analysis revealed expression of *SCR* in siliques, leaves, and roots of wild-type plants (Figure 6A). No hybridization was detected to RNA from *scr-1* plants (Figure 6B, lane 2). This indicates that *scr-1* has a reduced level of *SCR* RNA expression and may represent

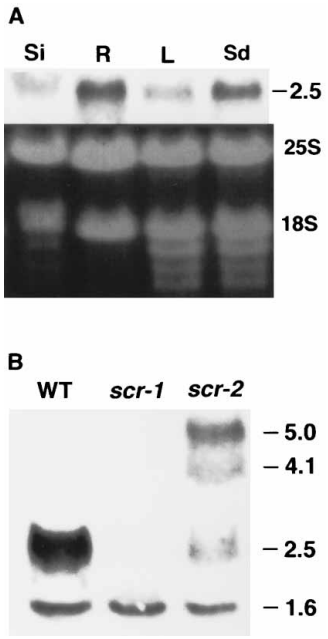

Figure 6. Expression of the *SCR* Gene

(A) Northern blot of total RNA from wild-type siliques (Si), roots (R), leaves (L), and whole seedlings (Sd) hybridized with *SCR* probe a (Figure 5B). Ribosomal RNA is shown as a loading control.

(B) Northern blot of wild-type, *scr-1*, and *scr-2* total RNA from seedlings, probed with *SCR* probe a (Figure 5B) and with a probe from the Arabidopsis *Glutamate Dehydrogenase 1* (*GDH1*) gene (Melo-Oliveira et al., 1996) as an internal loading control. The 1.6 kb band corresponds to *GDH1*. In *scr-2*, additional bands of 4.1 kb and 5.0 kb were detected with the *SCR* probe.

the null phenotype. In *scr-2*, we detected hybridization to RNA species larger than the normal size. This indicates that abnormal *SCR* transcripts are made in this allele, suggesting that proteins with some residual function may be produced.

To determine whether expression was localized to any particular cell type, we performed RNA in situ hybridization on sections of root tissue. In mature roots, expression was localized primarily to the endodermis (Figure 7A). Expression appeared to start very close to or within the cortex/endodermal initials and continue up the endodermal cell file as far as the section extended.

During embryonic development, expression was detected in the ground tissue of late heart-stage embryos prior to the division into cortex and endodermis (Figure 7B). After the division of the ground tissue, expression was only detected in the endodermis (Figure 7C). Sense strand controls showed only background hybridization (Figure 7D).

To determine whether the localization of *SCR* RNA was regulated at the transcriptional or posttranscriptional level, we examined enhancer trap (ET) lines in which the β-glucuronidase (uid-A or GUS) coding sequence with a minimal promoter (Malamy and Benfey, submitted) was expressed in the root endodermis. Restriction fragment length polymorphisms were observed when DNA from one of these lines, ET199, and from wild type were probed with *SCR*. PCR and sequence analysis confirmed that the enhancer trap construct had inserted

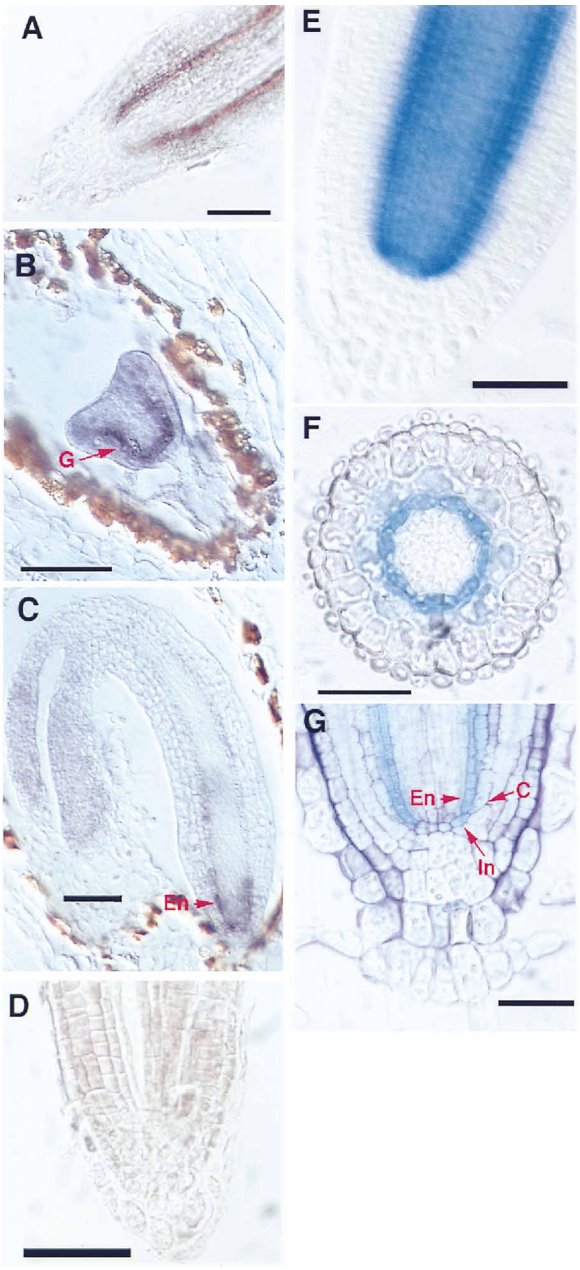

Figure 7. Tissue-Specific Expression of *SCR*

(A) *SCR* RNA expression detected by in situ hybridization of *SCR* antisense probe to a longitudinal section through the root meristem.

(B) In situ hybridization of *SCR* antisense probe to a heart-stage embryo.

(C) In situ hybridization of *SCR* antisense probe to bent-cotyledon-stage embryo.

(D) Control in situ hybridization using a *SCR* sense probe to a longitudinal section through the root meristem.

(E) GUS expression in a whole mount in the enhancer trap line ET199 in primary root tip.

(F) GUS expression in the ET199 line in transverse root section in the meristematic region. (Note that the light staining in the cortex is probably due to diffusion of the product of the histochemical reaction.)

(G) GUS expression in ET199 detected in a section through the root meristem. GUS expression is observed in the cortex/endodermal initial and in the first cell in the endodermal cell lineage, but not in the first cell of the cortex lineage. Expression in two endodermal layers is observed higher up in the root because the section was not median at that point.

Bar is 50 μm. Abbreviations are as above. G, embryonic ground tissue.

approximately 1 kb upstream of the *SCR* start site and in the same orientation as that of *SCR* transcription. When homozygous for the insertion, no mutant phenotype was observed in this line.

In mature roots, expression in ET199 whole mounts showed a similar pattern to that revealed by the in situ hybridizations, with the strongest staining present in endodermal cells (Figure 7E). Transverse sections indicated that expression was primarily in endodermal cells in the elongation zone (Figure 7F). Longitudinal sections through the meristematic zone revealed that expression could be detected in the cortex/endodermal initials (Figure 7G). Of particular interest was the restriction of expression to the endodermal daughter cell after the periclinal division (Figure 7G). This indicated that the expression pattern observed in the in situ analysis was not due to posttranscriptional partitioning of *SCR* RNA. Rather, it suggests that after the periclinal division of the cortex/endodermis initial only one of the two cells is able to transcribe *SCR* RNA.

Discussion

The *SCR* Gene Regulates an Asymmetric Division Required for Root Radial Organization

The formation of the cortex and endodermal layers in the Arabidopsis root requires two asymmetric divisions. In the first, an anticlinal division of the cortex/endodermal initial generates two cells with different developmental potentials. One will continue to function as an initial, while the other undergoes a periclinal division to generate the first cells in the endodermal and cortex cell files. This second asymmetric division is eliminated in the *scr* mutant, resulting in a single cell layer instead of two. The *scr* mutation appears to have little effect on any other cell divisions in the primary root, indicating that it is involved in regulating a single asymmetric division in this organ. Several other mutations have been characterized that appear to affect specific cell division pathways in Arabidopsis. These include *knolle*, in which formation of the epidermis is impaired (Lukowitz et al., 1996), *wooden leg*, in which vascular cell division is defective (Scheres et al., 1995), and *fass*, in which there are supernumerary cortex and vascular cells (Scheres et al., 1995; Torres Ruiz and Jurgens, 1994). In *gnom*, the asymmetric division of the zygote becomes symmetric (Mayer et al., 1993). However, *scr* and *shr* are the only characterized mutations that appear to eliminate a specific asymmetric division.

Mutational analyses in several organisms have revealed that the genes that regulate asymmetric divisions can be specific to a single type of division or can affect divisions that are not clonally related (Horvitz and Herskowitz, 1992). In most cases, these mutations result in the formation of two identical daughter cells with similar developmental potentials (Horvitz and Herskowitz,

1992). One example is the *swi*$^-$ mutation in Saccharomyces cerevisiae (Nasmyth et al., 1987). However, there are also examples of mutations that result in the formation of heterogeneous cell types, such as the *ham-1* mutation in Caenorhabditis elegans (Desai et al., 1988).

Is *SCR* Involved in Cell Specification or Cell Division?

Genes that regulate asymmetric cell divisions can be divided into those that specify the differentiated fates of the daughter cells and those that function to effect the division of the mother cell (Horvitz and Herskowitz, 1992). The tissue-specific markers clearly indicate that the aberrant cell layer formed in the *scr* mutant has differentiated features of endodermis. Because we had only one marker for cortex that also stains epidermis, we can not state unequivocally that the aberrant layer has cortex attributes. However, because this marker never stains endodermis in wild type, we can conclude that the mutant cell layer is heterogeneous, with differentiated attributes of both endodermis and either cortex or epidermis. Thus, *scr* is in the rare class of asymmetric division mutants in which a heterogeneous cell type is created. The ability to express differentiated characteristics of endodermis and cortex (or possibly epidermis) implies that the differentiation pathways for these cell types are intact and do not require the functional *SCR* gene. This suggests that *SCR* is involved primarily in regulating division of the mother cell and that the correct occurrence of this division can be uncoupled from cell specification. This is in contrast with the *shr* mutant, in which the periclinal division of the cortex/endodermal initial also fails to occur and the resulting cell has characteristics of cortex but not endodermis (Benfey et al., 1993; L. Di L., J. W.-D., and P. N. B., unpublished data).

A Role for *SCR* in Embryonic Development

At least one additional cell division appears to be affected in the *scr* mutant. During embryonic development, the ground tissue does not divide to form the endodermal and cortex layers of the embryonic root and hypocotyl. We have detected expression of *SCR* in the ground tissue prior to division and in the endodermal tissue after this division occurs. Thus, *scr* may play a direct role in regulating both this division and the division of the cortex/endodermal initial in the root apical meristem. Alternatively, the embryonic role of *SCR* may represent its primary function, in that the radial organization established in the embryo may somehow act as a template that directs the division of the cortex/endodermal initials, thus perpetuating the pattern. This is consistent with the finding in the *scr* mutant that the aberrant pattern established in the embryo is perpetuated in the primary root. An intriguing observation was the detection of *SCR* expression in the embryonic derivatives of the hypophysis. The mutant phenotype is not informative as to a possible role for *SCR* in these cells.

The possibility that *SCR* expression in the embryo may represent its primary function is also consistent with a recent study in which the daughter cells of the cortex/endodermal initial were laser ablated. When a single daughter cell was ablated, it was replaced by a cell that followed the normal asymmetric division pattern. When three adjacent daughter cells were ablated, the central initial divided anticlinally, but failed to perform the periclinal division (van den Berg et al., 1995). This provided evidence that information from mature cells is required for the correct division pattern of cortex/endodermal initials, suggesting a "top-down" transfer of information. The absence of a cell layer in lateral roots and callus-derived roots of the *scr* mutant suggests that embryo events are not unique in their ability to establish radial organization. Whatever the origin of radial pattern information, these observations indicate that *SCR* plays an important role in regulating both embryonic and post-embryonic root radial organization.

Tissue-Specific Expression of *SCR* Is Regulated at the Transcriptional Level

The mechanism of SCR action is still unclear. However, the cloning of the gene and its expression pattern provide some clues as to its role in the regulation of a specific asymmetric division. The *SCR* gene is expressed in the cortex/endodermal initial, but immediately after division is restricted to the endodermal lineage. Because this expression pattern was observed in an enhancer trap line in which *SCR*-regulatory elements are in proximity to a marker gene, we can conclude that restriction of *SCR* to the endodermal cell file is due to differential regulation of transcription. Another marker line in which expression of GUS is detected only in the cortex daughter cell provides a control for differential degradation of GUS RNA or protein (Malamy and Benfey, submitted). We can thus rule out partitioning of *SCR* RNA as a means of achieving this segregation of expression.

What remains to be determined is whether the observed difference in transcriptional activity of the two daughter cells is due to internal polarity of the mother cell prior to division, such that cytoplasmic determinants are unequally distributed, or to external polarity that influences cell fate after division. Since *SCR* is expressed in the initial prior to cell division, an attractive hypothesis is that it is involved in establishing polarity in the cortex/endodermal initial. The sequence of the SCR protein strongly suggests that it acts as a transcription factor. Hence, it may act to regulate the expression of genes within the mother cell essential for the establishment of unequal division. Alternatively, it is conceivable that its primary role is in the more mature cells, creating an external polarity that provides a signal to the mother cell to divide asymmetrically. This would be consistent with the detection of expression in more mature endodermal cells.

The insertion sites of T-DNA in the two *scr* alleles raised the possibility that the mutant phenotype is due to the production of truncated proteins. Northern blot analysis indicated that *SCR* RNA is undetectable in *scr-1*. This suggests that the phenotype is either the null or due to highly reduced RNA expression. In *scr-2*, we detected an alteration in RNA size, which would be consistent with the presence of a protein with residual function. The sequence at the junction of the T-DNA insertion in *scr-2* indicates that, if a protein were made, it would

be terminated at codon 607, which is two residues beyond the point of T-DNA insertion. The production of a small amount of truncated but still functional protein could provide an explanation for the observation that *scr-2* appears to be the weaker allele. At present, we have no evidence for a role for *SCR* in shoot formation, even though we detected *SCR* in shoot RNA.

A Novel Family of Transcriptional Regulators

Analysis of five EST clones reveals that their encoded proteins share a high degree of homology with SCR. Further analysis of these ESTs indicated that sequence similarity extends from at least the highly conserved VHIID domain to the carboxyl terminus of the gene products. Comparison of the amino termini of these deduced polypeptides was precluded by the fact that the ESTs are incomplete. The high degree of similarity among these encoded proteins, in combination with the motifs observed in the *SCR*-encoded protein (homopolymeric motifs, two leucine heptad repeats, and a bZIP-like basic domain that may also function as a nuclear localization sequence), indicates that they form a novel class of regulatory proteins. At present, the function of the EST gene products is unknown.

In summary, we have identified a gene involved in the regulation of a specific asymmetric division in plants. Sequence analysis shows that the *SCR* gene has many hallmarks of transcription factors. In situ and marker line expression studies show that it is expressed in the cortex/endodermal initial cell before asymmetric division occurs. Together, these findings suggest that the *SCR* gene product is a key regulator of the molecular events that establish the asymmetric division that generates separate cortex and endodermal cell lineages. The establishment of these lineages is not required for cell differentiation to occur, because in the absence of division the resulting cell acquires mature characteristics of both cortex (or possibly epidermis) and endodermal cells. It is possible that *SCR* functions to establish the polarity of the initial before cell division or that it is involved in generating an external polarity that has an effect on asymmetric cell division.

Experimental Procedures

Plant Culture

Arabidopsis ecotypes Wassilewskija (Ws), Columbia (Col), and Landsberg erecta (Ler) were obtained from Lehle. Arabidopsis seeds were surface sterilized and grown as described previously (Benfey et al., 1993). Generation of the enhancer trap lines is described elsewhere (Malamy and Benfey, submitted).

Genetic Analysis

For the *scr-1* allele, cosegregation of the mutant phenotype and kanamycin resistance conferred by the inserted T-DNA was determined as described previously (Aeschbacher et al., 1995). Because kanamycin affects root growth, 1557 seeds from heterozygous lines were germinated on nonselective media, scored for the appearance of the mutant phenotype, and subsequently transferred to selective media. All (284) phenotypically mutant seedlings showed resistance to the antibiotic, whereas 834 of 1273 phenotypically wild-type seedlings showed resistance to kanamycin. Phenotypically wild-type plants (83) were also transferred to soil and allowed to set seed. The progeny of these plants were plated on selective and nonselective media and scored for the cosegregation of the mutant phenotype and antibiotic resistance. A majority (48) of the plants segregated for the mutant phenotype and for kanamycin resistance, whereas 35 were wild type and sensitive to kanamycin. The original *scr-2* line (initially named *pinocchio*) contained at least two T-DNA inserts. Cosegregation analysis revealed a lack of linkage between the antibiotic resistance marker carried by the T-DNA and the mutant phenotype. Antibiotic-sensitive lines were identified that segregated for mutants. Lines that were homozygous for *scr-2* and antibiotic sensitive were crossed to *scr-1*. All F1 antibiotic-resistant progeny exhibited a mutant phenotype. All F2 progeny (from independent lines) were mutant, and there was a 3:1 segregation for antibiotic resistance, indicating that the two mutations were allelic.

Mapping

Mutant plants of *scr-2* (Ws background) were crossed to Col wild type. DNA from individual mutant F2 plants was analyzed for cosegregation with microsatellite (Bell and Ecker, 1994) and CAPS markers (Konieczny and Ausubel, 1993). The closest linkage was found to two CAPS markers located at the bottom of chromosome III. Out of 238 mutant chromosomes, 1 was recombinant for the BGL1 marker (Konieczny and Ausubel, 1993), and 1 out of 210 chromosomes was recombinant for the cdc2b marker (J. Celenza).

A restriction fragment length polymorphism for the *SCR* gene was identified between Col and Ler ecotypes with XhoI endonuclease. Genomic DNAs from independent RI lines (Jarvis et al., 1994) were digested with XhoI, and blots were hybridized to *SCR*. Using the segregation data obtained for 25 RI lines, the *SCR* gene was mapped relative to molecular markers by C. Lister. The *SCR* gene was assigned to the bottom of chromosome III, closest to BGL1.

Phenotypic Analysis

Morphological characterization of the mutant roots was performed as follows: 7–14 days postgermination, phenotypically mutant seedlings were fixed in 4.0% formaldehyde in PIPES buffer (pH 7.2). After fixation, the samples were dehydrated in ethanol, followed by infiltration with Historesin (Jung-Leica). Plastic sections (3–7 μm) were mounted on Superfrost slides (Fisher). The sections were either stained with 0.05% toluidine blue and photographed using Kodak 160T film or used for casparian strip detection or antibody staining.

For clearing of whole mounts for optical sections, tissues were incubated for 15 min at 55°C to 57°C in acidified methanol: 10 ml of methanol, 2 ml of concentrated HCl, and 38 ml of water. Tissues were then transferred to a 1:1 mixture of 14% hydroxylamine in 60% ethanol and 14% NaOH in 60% ethanol and incubated for 15 min at room temperature. Tissues were then rehydrated through 40%, 20%, and 10% ethanol, 10 min each, followed by 30 min in 25% glycerol in 5% ethanol. Cleared tissues were mounted in 50% glycerol and viewed with Nomarski optics on a Leitz Laborlux S microscope.

Casparian strip detection was performed as described previously (Scheres et al., 1995), with the following modifications. Plastic sections were used, and the counterstaining was done in 0.1% aniline blue for 5–15 min. The sections were visualized with a Leitz fluorescent microscope with FITC filter. Pictures were taken using a Leitz camera attached to the microscope and Kodak HC400 film. Images were digitized with a Nikon slide scanner and manipulated in Adobe Photoshop.

For staining with the JIM monoclonal antibodies, plastic sections were blocked for 2 hr at room temperature in 1% BSA in PBS containing 0.1% Tween 20 (PBT). Samples were incubated with primary antibodies at 4°C in 1% BSA in PBT overnight and then washed three times for 5 min each with PBT. Samples were incubated for 2 hr with biotinylated secondary antibodies (Vector Laboratories) in PBT and washed as above. Samples were incubated with Texas red–conjugated avidin D for 2 hr at room temperature, washed as before, and mounted in Citifluor. Immunofluorescence was observed with a Leitz fluorescent microscope with a rhodamine filter. Tissue fixation, embedding, and staining with the CCRC antibodies was performed on transverse sections (250 nm) as described previously (Freshour et al., 1996).

Molecular Techniques

Genomic DNA preparation was performed using the Elu-Quik kit (Schleicher & Schuell) protocol. Nonradioactive DNA probes were

labeled with either random-primed labeling or PCR-mediated synthesis according to the Genius kit manual (Boehringer Mannheim). Escherichia coli cells were transformed using a Bio-Rad gene pulser. Plasmid DNA was purified using the alkaline lysis method (Maniatis et al., 1982).

For plasmid rescue, approximately 2 μg of genomic DNA from phenotypically mutant seedlings was digested with 10 U of EcoRI (for the T-DNA right border) or SalI (left border) overnight at 37°C in the presence of 5 μg of RNase A in a final volume of 50 μl. After phenol–chloroform extraction and ethanol precipitation, DNA pellets were resuspended in 99 μl of 1× ligation buffer and 1 U of T4 DNA ligase (Boehringer Mannheim). After overnight ligation at 25°C, 1 more unit of ligase was added, and incubation proceeded for 8 hr. The ligation mixtures were phenol–chloroform extracted and ethanol precipitated, and pellets were resuspended in 20 μl of distilled water. We used 1 μl for electroporation of 30 μl of E. coli cells (WM1100, Bio-Rad). Among the resulting colonies, one EcoRI clone gave the expected restriction pattern, and its sequence was determined. None of the SalI clones showed the expected restriction pattern.

A probe made from a rescued fragment of 1.2 kb was used to screen a wild-type (Col) genomic library (Clontech). One genomic clone containing an insert of approximately 23 kb was isolated. A 3.0 kb SacI fragment, which hybridized to the 1.2 kb probe, was subcloned and sequenced. Comparison of the nucleotide sequence between the genomic clone and the rescued plasmid revealed the site of the T-DNA insertion. Approximately 600,000 plaques from a cDNA library, obtained from inflorescences and siliques (Ws) and therefore enriched in embryos, were screened with the 1.2 kb probe. Four cDNA clones were isolated. The dideoxy sequencing method was performed using the Sequenase kit (United States Biochemical). Sequence-specific internal primers were synthesized and used to obtain the entire sequence of the SacI genomic as well as the cDNA clones. Total RNA from plant tissues was obtained using phenol–chloroform extractions as described in Berry et al. (1985) with minor modifications. Northern blot hybridization and detection were performed according to the Genius kit manual (Boehringer Mannheim).

Antibiotic-sensitive lines of *scr-2* were found to contain a rearranged T-DNA insert as determined by Southern blots and PCR using T-DNA-specific probes and primers, respectively. The insertion of this T-DNA in the *SCR* gene was confirmed by Southern blots using *SCR* probes. A combination of T-DNA- and *SCR*-specific primers was used to amplify both 5′ and 3′ T-DNA/*SCR* junctions. The PCR fragments were cloned using the TA cloning kit (Invitrogen) and sequenced.

To identify the site of insertion of the enhancer trap T-DNA, genomic DNA from ET199 homozygous plants was amplified using primers specific for the T-DNA left border and the *SCR* gene. An approximately 2.0 kb fragment was amplified. This fragment was sequenced, and the site of insertion was found to be approximately 1 kb from the ATG start codon.

In Situ Hybridization

Antisense and sense *SCR* riboprobes were labeled with digoxigenin-11-UTP (Boehringer Mannheim) using T7 polymerase following the protocol of the manufacturer. Probes contained a 1.1 kb 3′ portion of the cDNA. Probe purification, hydrolysis, and quantitation were performed as described in the Boehringer Mannheim Genius System user's guide.

Tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C and rinsed two times in PBS (Jackson et al., 1991). They were subsequently preembedded in 1% agarose in PBS. The fixed tissue was dehydrated in ethanol, cleared in Hemo-De (Fisher), and embedded in ParaplastPlus (Fisher). Tissue sections (10 μm thick) were mounted on SuperfrostPlus slides (Fisher). Section pretreatment and hybridization were performed according to Lincoln et al. (1994), except that proteinase K was used at 30 mg/ml and a 2 hr prehybridization step was included. A probe concentration of 50 ng/ml/kb was used in the hybridization.

Slides were washed, and the immunological detection was performed according to Coen et al. (1990), with the following modifications. Slides were first washed for 5 hr in 5× SSC, 50% formamide. After RNase treatment, slides were rinsed three times (20 min each) in buffer (0.5 M NaCl, 10 mM Tris–HCl [pH 8.0], 5.0 mM EDTA). For immunological detection, antibody was diluted 1:1000. Levamisole (240 ng/ml) was included in the detection buffer, and after the reaction was stopped in 10 mM Tris, 1 mM EDTA, sections were mounted directly in Aqua-Poly/Mount (Polysciences).

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References

Aeschbacher, R.A., Hauser, M.-T., Feldmann, K.A., and Benfey, P.N. (1995). The *SABRE* gene is required for normal cell expansion in *Arabidopsis*. Genes Dev. *9*, 330–340.

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics *18*, 137–144.

Benfey, P.N., Linstead, P.J., Roberts, K., Schiefelbein, J.W., Hauser, M.-T., and Aeschbacher, R.A. (1993). Root development in *Arabidopsis*: four mutants with dramatically altered root morphogenesis. Development *119*, 57–70.

Berry, J.O., Nikolau, B.J., Carr, J.P., and Klessing, D.F. (1985). Transcriptional and post-transcriptional regulation of ribulose 1,5-bisphosphate carboxylase gene expression in light and dark grown amaranthus cotyledons. Mol. Cell. Biol. *5*, 2238–2246.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *FLORICAULA*: a homeotic gene required for flower development in Antirrhinum majus. Cell *63*, 1311–1322.

Desai, C., Garriga, G., McIntire, S., and Horvitz, H.R. (1988). A genetic pathway for the development of the *Caenorhabditis elegans* HSN motor neurons. Nature *336*, 638–646.

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.

Esau, K. (1977). Anatomy of Seed Plants, Second Edition (New York: John Wiley & Sons).

Feldmann, K.A. (1991). T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. Plant J. *1*, 71–82.

Freshour, G., Clay, R.P., Fuller, M.S, Albersheim, P., Darvill, A.G., and Hahn, M.G (1996). Developmental and tissue-specific structural alterations of the cell wall polysaccharides of *Arabidopsis thaliana* roots. Plant Physiol. *110*, 1413–1429.

Heidecker, G., and Messing, J. (1986). Structural analysis of plant genes. Annu. Rev. Plant Physiol. *37*, 439–466.

Horvitz, H.R., and Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell *68*, 237–255.

Hurst, H.C. (1994). Transcription factors. I. bZIP proteins. Protein Prof. *1*, 123–168.

Jackson, D., Culianez-Macia, F., Prescott, A.G., Roberts, K., and Martin, C. (1991). Expression patterns of *MYB* genes from *Antirrhinum* flowers. Plant Cell *3*, 115–125.

Jarvis, P., Lister, C., Szabo, V., and Dean, C. (1994). Integration of CAPS markers into the RFLP map generated using recombinant inbred lines of *Arabidopsis thaliana*. Plant Mol. Biol. *24*, 685–687.

Johnson, P.F., Sterneck, E., and Williams, S.C. (1993). Activation domains of transcriptional regulatory proteins. J. Nutr. Biochem. *4*, 386–398.

Joshi, C.P. (1987). An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucl. Acids Res. *15*, 6643–6653.

Knox, J.P., Linstead, P.J., King, J., Cooper, C., and Roberts, K. (1990). Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta *181*, 512–521.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. *4*, 403–410.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K., and Hake, S. (1994). A *knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell *6*, 1859–1876.

Lukowitz, W., Mayer, U., and Jurgens, G. (1996). Cytokinesis in the Arabidopsis embryo involves the syntaxin-related KNOLLE gene product. Cell *84*, 61–71.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development *117*, 149–162.

Melo-Oliveira, R., Oliveira, I.C., and Coruzzi G. (1996). *Arabidopsis* mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc. Natl. Acad. Sci. USA, *93*, 4718–4723.

Mogen, B.D., MacDonald, M.H., Graybosch, R., and Hunt, A.G. (1990). Upstream sequences other than AAUAAA are required for efficient messenger RNA 3′-end formation in plants. Plant Cell *2*, 1261–1272.

Nasmyth, K., Stillman, D., and Kipling, D. (1987). Both positive and negative regulators of HO transcription are required for mother-cell-specific mating-type switching in yeast. Cell *48*, 579–587.

Puhlmann, J., Bucheli, E., Swain, M.J., Dunning, N., Albersheim, P., Darvill, A.G., and Hahn, M.G. (1994). Generation of monoclonal antibodies against plant cell-wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal α-linked fucosyl-containing epitope. Plant Physiol. *104*, 699–710.

Scheres, B., Di Laurenzio, L., Willemsen, V., Hauser, M.-T., Janmaat, K., Weisbeek, P., and Benfey, P.N. (1995). Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the radial axis. Development *121*, 53–62.

Torres Ruiz, R.A., and Jurgens, G. (1994). Mutations in the *FASS* gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. Development *120*, 2967–2978.

van den Berg, C., Willemsen, V., Hage, W., Weisbeek, P., and Scheres, B. (1995). Cell fate in the *Arabidopsis* root meristem determined by directional signaling. Nature *378*, 62–65.

Varagona, M.J., Schmidt, R.J., and Raikhel, N.V. (1992). Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. Plant Cell *4*, 1213–1227.

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