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LRP1, a Gene Expressed in Lateral and Adventitious Root Primordia of Arabidopsis

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We describe a gene that is expressed in lateral and adventitious root primordia of Arabidopsis. The gene was identified by expression of a transposon-borne promoterless β-glucuronidase gene in lateral root primordia. The gene, designated *LRP1* for lateral root primordium 1, and its corresponding cDNA were cloned and sequenced. The expression pattern of the gene in lateral root primordia was confirmed by in situ hybridization with LRP1 cDNA probes. The *LRP1* gene encodes a novel protein. *LRP1* expression is activated during the early stages of root primordium development and is turned off prior to the emergence of lateral roots from the parent root. Insertion of the transposon in the *LRP1* gene disrupted its expression. To evaluate the homozygous insertion line for a mutant phenotype, several aspects of wild-type lateral root development were analyzed. A mutant phenotype has not yet been identified in the insertion line; however, there is evidence that the gene belongs to a small gene family. *LRP1* provides a molecular marker to study the early stages of lateral and adventitious root primordium development.

INTRODUCTION

Primary and lateral roots of most plants carry out the same functions and often resemble each other in cellular and tissue organization; however, they differ markedly in their ontogeny and meristem establishment (Peterson and Peterson, 1986; Charlton, 1991). Lateral root initiation typically occurs acropetally behind the meristem at regular intervals along the length of a parent root (McCully, 1975; Klepper, 1987; Barlow and Adam, 1988). In most angiosperms, lateral roots arise primarily from the pericycle, a uniserate cylindrical layer of cells surrounding the central vascular cylinder of a mature root (Blakely et al., 1982; Peterson and Peterson, 1986; Torrey, 1986). Multiple rounds of cell division of pericyclic derivatives produce a dome-shaped primordium. A lateral root meristem is established de novo from cells within the primordium prior to or at the emergence of the lateral root through the epidermis (Peterson and Peterson, 1986; Doerner, 1993). The new lateral root elongates and matures following establishment of the lateral root meristem and connection of the vascular tissues.

Although the tissue arrangement in mature lateral roots of Arabidopsis is the same as it is in primary roots, the number of cell files that comprises each tissue differs and varies from one lateral root to the next (Dolan et al., 1993). The inconstancy in lateral root cell file number suggests that the regulation of cellular patterning in the meristem also differs between primary and lateral roots (Dolan et al., 1994; Scheres et al., 1994). Although lateral root initiation and development have been studied in a number of plants, little is known about lateral root physiology and development in Arabidopsis (Peterson and Peterson, 1986; Dolan et al., 1993, 1994; Benfey and Schiefelbein, 1994; Scheres et al., 1994), and there is little molecular information about lateral root development in any plant (Taylor and Scheuring, 1994; Vera et al., 1994).

Several molecular and genetic approaches have been taken to study lateral root development. Taylor and Scheuring (1994) have identified and cloned an auxin-inducible gene, designated *RSI-1* for root system inducible-1, which is expressed in lateral root primordia of tomato. This gene's function is not known, and *RSI-1* is believed to be expressed at a low level in shoot and other root tissues. Vera et al. (1994) have recently described the cell cycle regulation of a hydroxyproline-rich glycoprotein gene, designated *HRGPnt3*, expressed in both adventitious and lateral root primordia of tobacco. Although the function of the *HRGPnt3* gene is not known, it is expressed immediately after the first cell division of lateral root primordium formation (Keller and Lamb, 1989; Vera et al., 1994). Moreover, a number of genes (e.g., the Arabidopsis cyclin gene *cyc1At* and cell cycle–dependent genes *cdc2* and *cdc2a*) expressed in both primary root tips and lateral root primordia as well as in other tissues have been cloned by homology to other known genes; however, further identification of genes expressed only during lateral root primordia development has been elusive (Hemerly et al., 1992, 1993; Martinez et al., 1992; Ferreira et al., 1994; Taylor and Scheuring, 1994).

In Arabidopsis, mutant screens have produced three nonallelic mutations that cause aberrant lateral root formation (*alf*; J.L. Celenza and G.R. Fink, personal communication). The phenotypes of these mutants are (1) one that has too many lateral roots (*alf1-1*), (2) one that has lateral roots that initiate

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but fail to mature (*alf3-1*), and (3) one that fails to make lateral roots (*alf 4-1*). All three mutants have pleiotropic phenotypes in that either the primary root is also affected (*alf3-1*) or the shoot is also affected (*alf1-1* and *alf4-1*). Possible reasons for the scarcity of Arabidopsis mutations that exclusively affect lateral root development are as follows: (1) genes needed for lateral root development have similar roles in primary root development; (2) pleiotropy results from the involvement of genes expressed during lateral root development in other developmental processes; and (3) genes expressed in lateral roots belong to gene families with overlapping functions.

Insertional mutagenesis with gene-trapping constructs can identify genes that belong to families or have multiple functions during development. Whether derived from transposable elements or Agrobacterium T-DNA, gene trap constructs carry a promoterless (promoter trap) or core promoter-containing (enhancer or promoter trap) reporter gene and a marker gene flanked by sequences that specify the DNA borders to be inserted into genomic DNA (Wilson et al., 1989; Kertbundit et al., 1991; Fedoroff and Smith, 1993; Springer et al., 1995). Insertion of a gene trap in the proper orientation to and near a promoter or enhancer region results in regulated expression of a reporter gene, facilitating the identification of regulatory sequences of interest and cloning of genomic DNA flanking the insertion site. Due to the versatility of maize transposons used in insertional mutagenesis systems, we and others have developed systems utilizing internally deleted gene trap constructs derived from the maize *Activator* (*Ac*) transposon for use in Arabidopsis (Fedoroff and Smith, 1993; Springer et al., 1995).

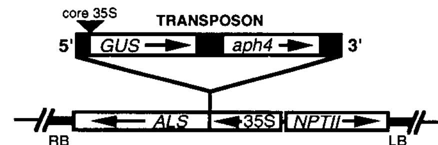
Here, we describe a lateral and adventitious root primordium-specific gene identified by insertional mutagenesis with a gene trap transposon. The gene was initially identified by the expression of a transposon-borne β-glucuronidase (*GUS*) reporter gene in lateral root primordia. We cloned the gene and its corresponding cDNA and confirmed that the gene is expressed in lateral root primordia by in situ hybridization with cDNA probes. The locus has been designated lateral root primordium 1 (*LRP1*). Although insertion of the transposon at *LRP1* disrupts gene expression, no mutant phenotype has been observed in plants homozygous for the inserted transposon under the conditions tested to date. *LRP1* expression is activated early during primordium development, providing a molecular marker for the early stages of lateral root development.

RESULTS

GUS Activity Is Restricted to Lateral Root Primordia in Enhancer Trap Line 125-3

The transposon-tagging system used to generate the line discussed later in the text has been described previously (Fedoroff and Smith, 1993). Of importance here is the structure of the tagging transposon. Briefly, the ends of the transposon are derived from an internally deleted *Ac* transposable element and consist of 325 bases and 500 bases from the 5′ and 3′ ends of the element, respectively (Figure 1A). Within the transposon is a coding sequence for *GUS* and the bacterial aminocyclitol phosphotransferase (*aph4*) gene expressed from the cauliflower mosaic virus (CaMV) 19S promoter. The *GUS* gene is preceded by a short core sequence from the CaMV 35S promoter, making it an enhancer or promoter trap if the transposon inserts in the proper orientation in or near a plant enhancer or promoter. A dominant marker for hygromycin resistance, such as the *aph4* gene, permits the identification of transposon-containing plants on selective medium. In the original T-DNA configuration used to transform plants, the transposon is inserted between a mutant Arabidopsis acetolactate synthase (*ALS*) gene and the CaMV 35S promoter (Figure 1A). Excision of the transposon unites the promoter with the *ALS* gene, resulting in resistance to the herbicide chlorsulfuron (Figure 1B). Therefore, chlorsulfuron resistance permits the identification of germinal excision events and provides a selectable marker at the empty donor site. During a large-scale screen of lines containing transposed elements, we identified an enhancer trap-containing line (hereafter referred to as line 125-3) in which GUS activity was restricted to lateral root primordia.

A *Ds-GUS* T-DNA BEFORE TRANSPOSITION



B AFTER TRANSPOSITION

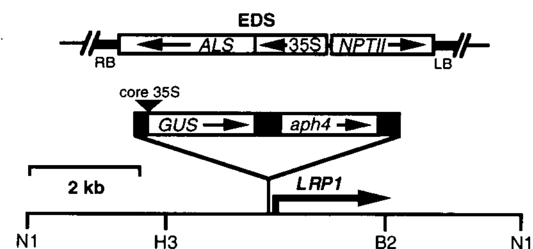


Figure 1. Transposon Insertion at *LRP1*.

(A) Structure of the *Dissociation* (*Ds*)–*GUS* T-DNA. The transposon consists of a promoterless *GUS* gene preceded by a core CaMV 35S promoter and the hygromycin resistance gene *aph4*. The transposon is inserted between a mutant *ALS* gene and the CaMV 35S promoter. The neomycin phosphotransferase II (*NPTII*) gene confers kanamycin resistance and is used as the transformation marker. RB and LB, right and left T-DNA borders, respectively.

(B) The structure of the empty donor site (EDS) and the transposon inserted just upstream of the *LRP1* transcribed region with *GUS* in the same transcriptional orientation. B2, BglII; H3, HindIII; N1, NcoI.

Expression of the Affected Gene Is Root Specific and Eliminated by Transposon Insertion

To establish that insertion of the transposon was responsible for the root-specific GUS activity pattern, we determined the relationship between the transposon-borne hygromycin resistance marker and the GUS activity pattern. All 100 of the hygromycin-resistant (Hyg^r) progeny analyzed exhibited GUS activity in all lateral root primordia. Linkage of the donor and insertion sites was determined by measuring the crossover frequency between the hygromycin resistance marker on the transposon and a chlorsulfuron resistance marker at the empty donor site. Among 300 progeny from a selfed parent heterozygous for both the donor site and the transposed element, six were chlorsulfuron resistant but hygromycin sensitive, showing that the transposon had moved to a nearby site on the same chromosome ~4 centimorgans from the donor site (Figure 1B).

Genomic sequences flanking the transposon insertion site were cloned and used to detect homologous mRNAs by RNA gel blot analysis (see Methods for details). No hybridizing RNAs were detected when the 3-kb segment adjacent to the 5′ end of the transposon was used as a probe (data not shown). However, the 5-kb fragment adjacent to the 3′ end of the transposon detected a single RNA of ~2 kb in roots but not in aerial tissues (Figure 2A). The 3′ flanking probe was then used to isolate cDNA and genomic clones from Arabidopsis cDNA (ecotype Columbia) and genomic (ecotype Nossen) libraries.

cDNA clones of four different lengths were isolated, including one that appeared to contain a full- or nearly full-length 1.9-kb insert. When used as a probe, the cDNA insert detected a 2-kb mRNA in wild-type roots but not in roots from plants homozygous for the 125-3 transposon insertion (Figure 2B). Insertion of the transposon therefore disrupted expression of *LRP1*. To distinguish the wild-type locus from the transposon-disrupted locus in line 125-3, we refer to the latter as the transposon-containing *LRP1* gene.

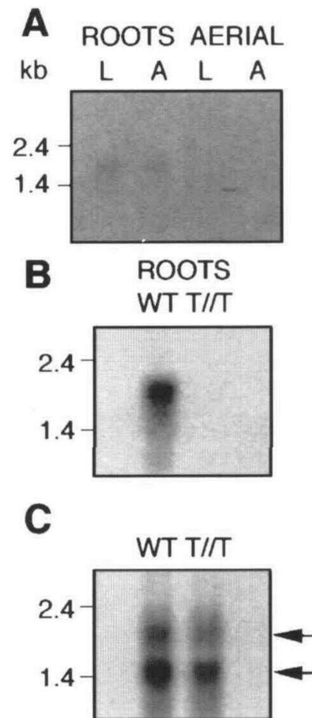


Figure 2. Autoradiographs of RNA Gel Blots.

(A) Detection of *LRP1* transcripts in poly(A) RNA from roots and aerial tissues of Arabidopsis plants grown in liquid (L) or on agar (A) for 4 weeks.

(B) Detection of *LRP1* transcripts in poly(A) RNA from roots of wild-type (WT) plants and line 125-3 homozygous for the transposon insertion at *LRP1* (T//T).

(C) Identical blot as shown in **(B)** which was stripped and reprobed using reduced stringency during hybridization and for the final wash. Arrows at right indicate two additional bands detected under reduced-stringency wash conditions. Length markers are given at left in kilobases.

GUS Expression in Lateral Root Primordia Results from Insertion of the Transposon at the *LRP1* Locus

The LRP1 cDNA was used as a probe to determine the gene copy number by DNA gel blot hybridization (Figure 3A). Under stringent conditions, the LRP1 cDNA detected a single gene in all three ecotypes, and no polymorphisms were found with the enzymes used. Bands of the same size as those observed with the LRP1 cDNA were detected with cloned genomic DNA flanking the 3′ end of the transposon insertion site.

To show that *GUS* expression in lateral root primordia was due to insertion of the transposon at the *LRP1* locus, we analyzed transposon-containing derivatives that no longer expressed the *GUS* gene that arose in plants containing a transposase gene. We compared the transposon insertion site in plants that were Hyg^r and had the primordium-specific GUS activity pattern to the insertion site in siblings that were Hyg^r but lacked detectable GUS activity. Hyg^r plants with the specific GUS activity pattern were crossed to lines homozygous for a source of *Ac* transposase, and 10 Hyg^r F_1 progeny from the *Ac* cross were grown and allowed to self. The F_2 progeny were then selected for resistance to hygromycin and screened for GUS activity. Three siblings from one F_1 parent that were Hyg^r but had no GUS activity were identified, and genomic DNA from these siblings was used for DNA gel blot analysis (Figure 3B). The analysis showed that although the transposon, linked to the primordium-specific GUS activity pattern, increased the distance between the NcoI sites flanking the *LRP1* locus, it reduced the NcoI fragment length detected on the blot by introducing a new NcoI site present in *aph4*. In progeny plants containing the transposon but lacking GUS activity in primordia, the NcoI fragment was restored to its wild-type length due to the departure of the transposon from the *LRP1*

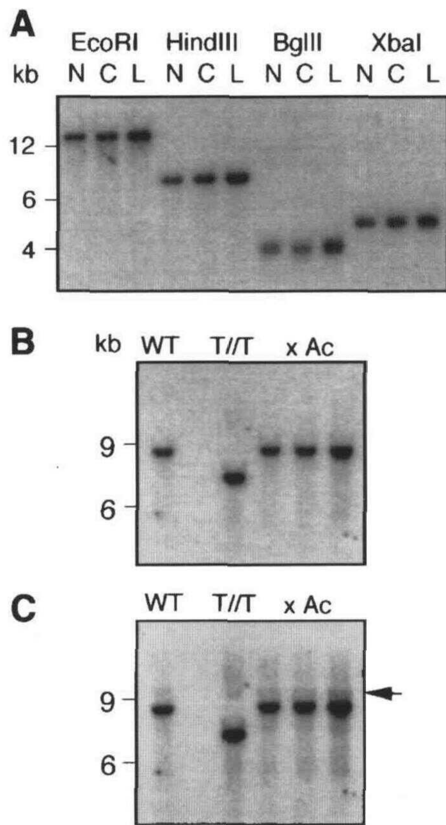


Figure 3. Autoradiographs of DNA Gel Blots.

(A) Detection of restriction fragments at the *LRP1* locus in genomic DNA from Arabidopsis ecotype Nossen (N), Columbia (C), or Landsberg *erecta* (L). DNA was digested with the enzymes indicated at top.
(B) Detection of NcoI restriction fragments at the *LRP1* locus in genomic DNA from wild-type (WT) plants, line 125-3 homozygous for the transposon insertion (T//T), and three Hyg^r, GUS^- progeny from line 125-3 after remobilization of the transposon (× Ac).
(C) Identical blot shown in **(B)** that was stripped and reprobed using reduced stringency during hybridization and for the final wash. The arrow at right indicates an additional band detected when lower stringency conditions were used. Length markers are given at left in kilobases.

locus. Hence, insertion of the transposon in the *LRP1* gene is responsible for the lateral root primordium–specific expression of the *GUS* gene.

Genomic DNA flanking the 3′ end of the transposon was used as a probe to map the *LRP1* locus. The probe hybridized to two overlapping yeast artificial chromosomes, which were previously mapped to the top arm of chromosome 5 corresponding to λ clone M224 (P. Dunn and J. Ecker, personal communication).

Sequence Analysis of the LRP1 cDNA and Genomic DNA

The longest cDNA (λ clone from Arabidopsis ecotype Columbia), the genomic DNA (λ clone from Arabidopsis ecotype Nossen), and DNA flanking both sides of the transposon insertion site (λ clone from Arabidopsis ecotype Nossen) were sequenced. The sequences of the genomic DNA and cDNA at the *LRP1* locus are shown in Figure 4. Two possible consensus transcription start sites were found; both lie within the usual distance (32 ± 7 bp) downstream of the putative TATA box indicated in Figure 4 (Joshi, 1987a). These transcriptional start sites do not correspond to the cDNA start site, suggesting that the cDNA is not full length. Sequence comparison of the genomic and cDNA clones revealed the presence of one 435-bp intron flanked by consensus intron donor and acceptor sites (Shapiro and Senapathy, 1988).

The translational start site and longest open reading frame were identical in the cDNA and genomic DNA except for a single base difference. This base difference results in a nonconservative amino acid difference such that the cDNA and genomic DNA encode tryptophan and proline, respectively (amino acid 52 in Figure 4). The 5′ untranslated region (UTR) within the cDNA is 676 bp long, and 16 base differences were found in this region when compared to the genomic DNA. The 5′ UTR contains five stop codons. The 3′ UTR also differs by three bases between the cDNA and genomic DNA. The 3′ end of the gene was identified by the presence of a canonical poly(A) addition sequence located 51 bases upstream of the polyadenylation site, which was inferred from the sequence preceding the poly(A) tail in the cDNA (Joshi, 1987b).

The nucleotide sequence discrepancies between the 5′ and 3′ untranslated regions of the *LRP1* genomic clone and its corresponding cDNA were probably due to the different ecotypes from which they were isolated. The nucleotide difference between the genomic and cDNA sequences in the coding region of *LRP1* that results in an amino acid difference at position 52 was perhaps also due to ecotype differences or cloning artifacts. The amino acid difference at position 52 was not in a region found to have homology with other proteins.

LRP1 Codes for a Novel Protein

The *LRP1* open reading frame codes for a 321–amino acid protein with a predicted molecular mass of 33.6 kD. The protein is rich in glycine (12.5%) and serine (11.2%). The N-terminal third of the protein is rich in hydrophobic amino acids. The center third of the protein contains two serine/threonine–rich regions separated by a short region containing seven cysteines. The C-terminal third of the protein consists of a short stretch of acidic amino acids followed by a region rich in hydrophobic amino acids and glycine. A search of data bases revealed no significant DNA homology to any known genes but did reveal some amino acid similarities (Altschul et al., 1990). The cysteine

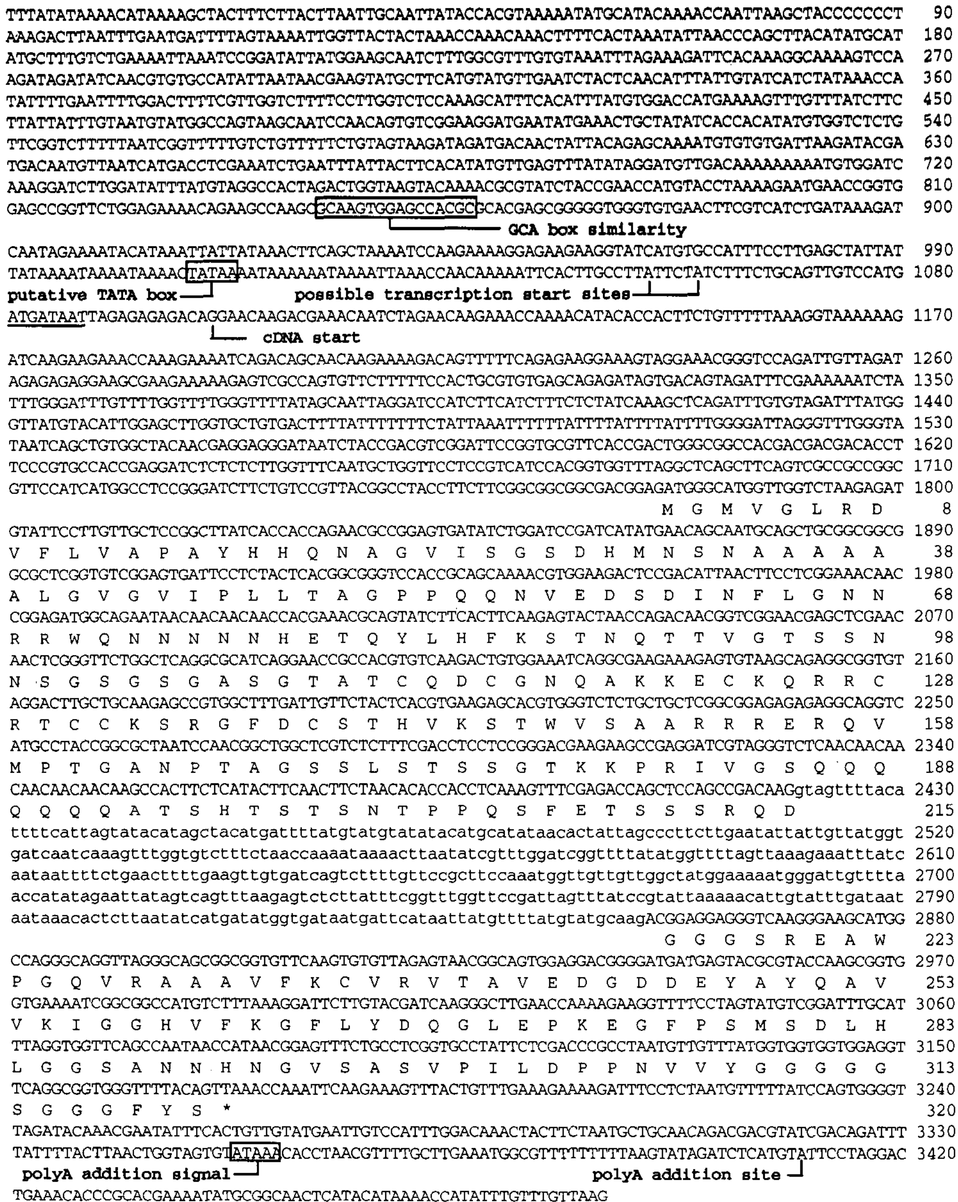


Figure 4. Sequence of Genomic DNA at the *LRP1* Locus from Arabidopsis Ecotype Nossen.

The 8-bp duplication caused by the transposon insertion in the enhancer trap line 125-3 is underlined. A putative TATA box, likely transcriptional start sites, and cDNA start site are indicated. The longest open reading frame is indicated by the single-letter amino acid code. The single intron is presented in lowercase letters. A poly(A) consensus addition sequence and the cDNA poly(A) addition site are indicated. The GenBank accession number for this sequence is U24702.

arrangement in LRP1 (amino acids 111 to 139) can be shown as $C\text{-}X_2\text{-}C\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C\text{-}X_7\text{-}C$. This is similar to $H\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C\text{-}X_{10\text{-}14}\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_7\text{-}C$ (where X is a variable amino acid), which is the conserved arrangement of cysteines and histidines in the zinc binding sites of the activation domains of the protein kinase C family of proteins (Hubbard et al., 1991). No motifs, such as a signal or plant organelle localization sequence, were found that would suggest the secretion or cellular location of the protein (Taylor and Scheuring, 1994).

It was determined by sequence comparison that the transposon inserted 12 bases upstream of the 5′ end of the longest cDNA and resulted in the characteristic *Ac*-associated 8-bp

duplication at the insertion site (Figure 4). The *GUS* gene within the transposon at *LRP1* is in the same transcriptional orientation as the *LRP1* gene (Figure 1B). The transposon insertion site and orientation are consistent with the observed loss of endogenous *LRP1* mRNA expression and the lateral root–specific expression pattern of the *GUS* gene.

The *LRP1* Gene Is Expressed Early in Lateral Root Primordium Development

To determine whether the GUS activity pattern accurately reflects the expression pattern of *LRP1*, in situ hybridization was done with roots from the Nossen ecotype, using single-stranded RNA probes. Several regions of the cDNA were tested as probes, but only probes from the center one-third of the cDNA gave a strong positive signal (data not shown). Therefore, a 300-bp probe corresponding to the center of the translated region (insert in pDS110) was used for further analysis (Figure 5).

The *LRP1* gene is expressed in cells derived from the pericycle during early primordium development but not in the neighboring, nonproliferative pericycle (Figure 5A). Earlier stages of primordium development were not observed during these experiments. The *LRP1* gene continues to be expressed during primordial cell proliferation. Just prior to the emergence of a lateral root, expression appears concentrated in cells at the base of the primordium (Figure 5B). In contrast to the GUS activity pattern in line 125-3 (Figure 6G), *LRP1* is no longer detectable at the time of lateral root emergence (Figure 5C). A probe corresponding to the sense strand of *LRP1* gave no hybridization signal (Figure 5D); nor did the antisense probe detect transcripts in lateral root primordia from line 125-3 plants homozygous for the transposon inserted at *LRP1* under high-stringency wash conditions (Figure 5E).

Homology of *LRP1* to Other Genes

Several observations suggest that *LRP1* is a member of a small gene family with overlapping or redundant functions. First, elimination of *LRP1* gene expression by the transposon insertion did not produce a discernible mutant phenotype. Second, during RNA gel blot analysis, cross-hybridization of the cDNA

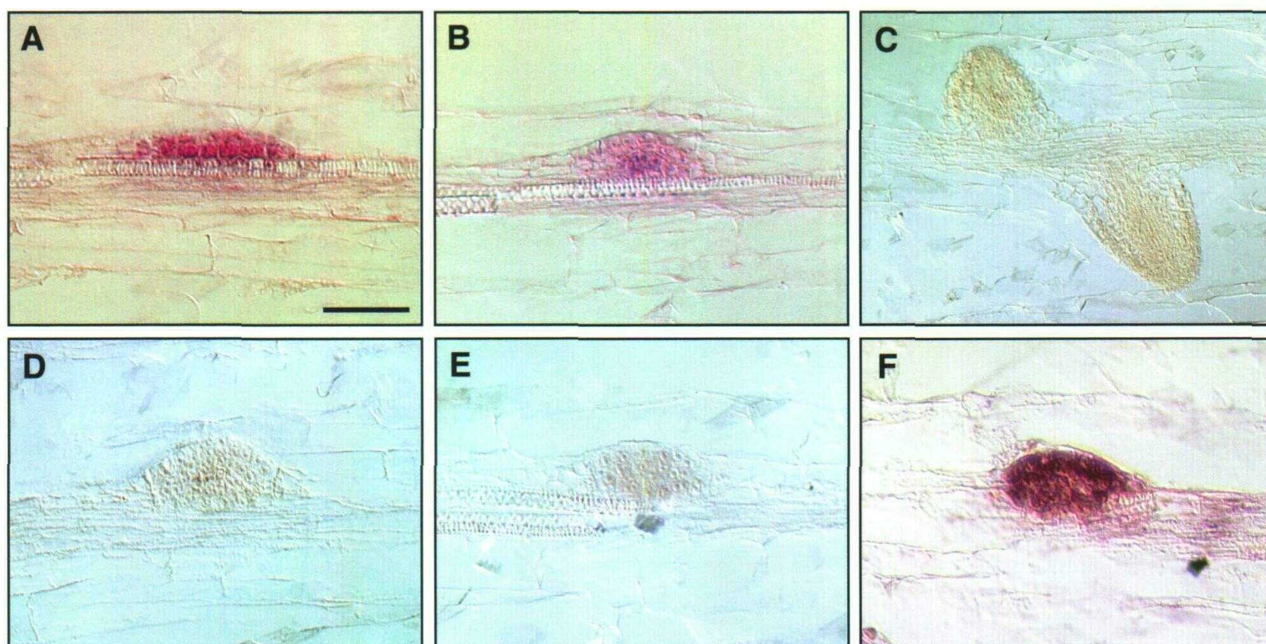


Figure 5. Localization of *LRP1* Transcripts by in Situ Hybridization.

The hybridized RNA probe is shown by purple staining. Longitudinal sections of roots from Arabidopsis plants are shown.
(A) Young wild-type lateral root primordium; antisense probe; high-stringency wash. Bar = 50 μm.
(B) Older wild-type lateral root primordium; antisense probe; high-stringency wash.
(C) Two young wild-type lateral roots; antisense probe; high-stringency wash.
(D) Older wild-type lateral root primordium; sense probe; high-stringency wash.
(E) Older lateral root primordium of a plant homozygous for the transposon insertion at *LRP1*; antisense probe; high-stringency wash.
(F) Older lateral root primordium of a plant homozygous for the transposon insertion at *LRP1*; antisense probe; low-stringency wash.

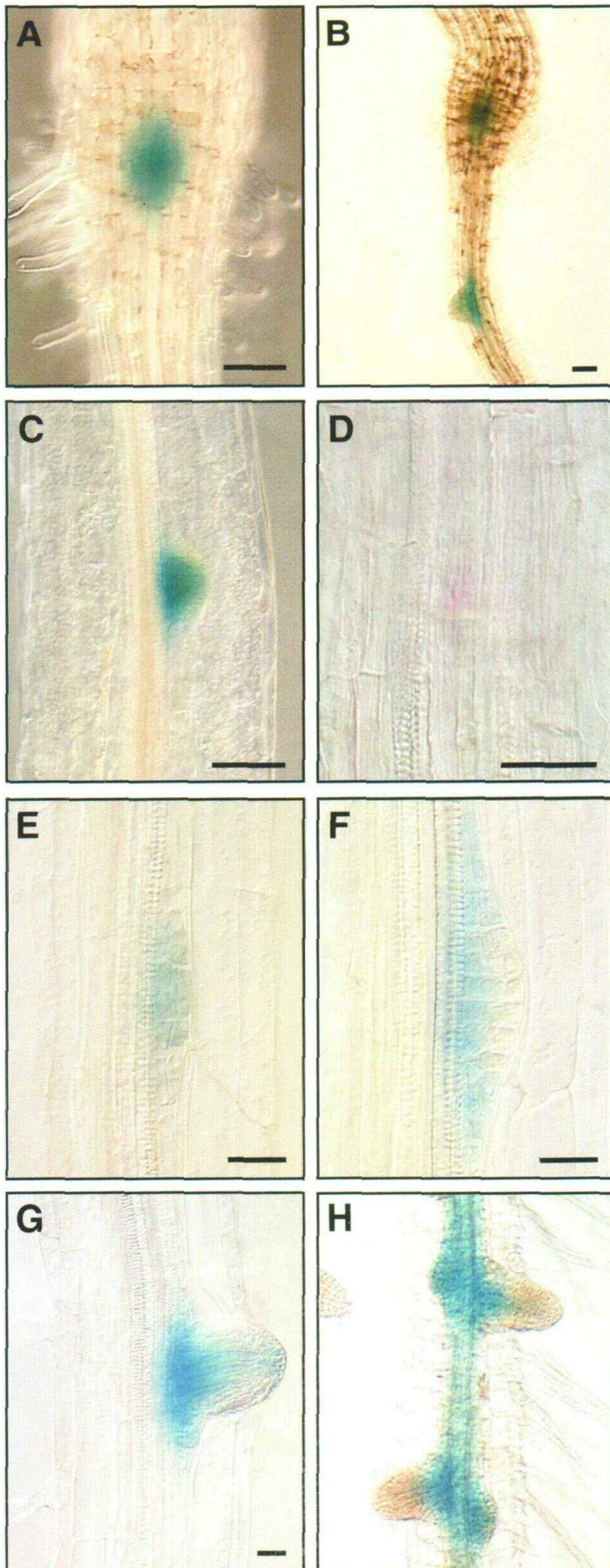


Figure 6. Histochemical Detection of GUS Activity Patterns and Lateral Root Primordia Development for Enhancer Trap Line 125-3.

Blue and red staining indicate GUS activity.

(A) Hypocotyl–root junction of a 4-day-old Arabidopsis seedling with the two, first-formed lateral root primordia stained blue.

(B) Hypocotyl–root region of a seedling 6 days after germination with three stained primordia.

(C) Hypocotyl region of a 4-week-old seedling 4 days after the removal of the primary root tip. The adventitious root primordium is stained blue.

(D) Optical section of root with the plane of focus at the pericycle. No primordium was observed at the red-stained site.

(E) Lateral root primordium where GUS activity is consistently detectable.

(F) Mature lateral root primordium prior to emergence from the parent root tissues.

(G) Lateral root just after emergence.

(H) Root segment from a 4-week-old seedling 6 days after constant exposure to α-naphthaleneacetamide.

Scale bars in **(A)** to **(C)** = 100 μm; bars in **(D)** to **(H)** = 20 μm.

probe to two additional bands was observed when lower hybridization and washing stringencies were used (Figure 2C). Third, at least one additional genomic fragment was clearly detected when DNA gel blotting was performed under reduced stringency conditions (Figure 3C). Fourth, under moderately stringent washing conditions during in situ hybridization, strong hybridization was observed in lateral root primordia of both wild-type plants (data not shown) and plants homozygous for the 125-3 transposon (Figure 5F). When the washing stringency was increased, the hybridization signal was eliminated in root primordia of plants homozygous for the 125-3 transposon but not from wild-type roots (compare Figure 5A and 5B with Figure 5E). Fifth, a search of the expressed sequence tag (EST) data base revealed significant homology between the *LRP1* coding region and an Arabidopsis EST (GenBank accession number T88542). The *LRP1* sequence (nucleotides 2082 to 2214 in Figure 4) is 68% identical at the amino acid level and 75% identical at the nucleotide level to the EST sequence (nucleotides 9 to 143). Thus, *LRP1* appears to belong to a small family of homologous genes with similar expression patterns. The absence of a mutant phenotype in the *LRP1* insertion homozygote suggests that at least some members of the gene family have redundant or overlapping functions.

Phenotype of Plants Homozygous for the *LRP1* Insertion

The observations detailed in the following discussion for GUS activity were the same whether the transposon insertion at *LRP1* was heterozygous or homozygous. Furthermore, the morphological observations made for line 125-3 and described later in the text are consistent with observations made for wild-type Arabidopsis plants of the Nossen ecotype. GUS activity was first detected in line 125-3 4 days after germination. The activity was confined to the first two lateral root primordia that

developed simultaneously and opposite to one another at the hypocotyl–root junction (Figure 6A). Even though the first two primordia are clearly present, they often remain dormant and do not produce a lateral root until several days or even weeks after germination. Formation of all subsequent lateral root primordia was followed without delay by the emergence of a lateral root (Figure 6B). GUS activity was detected in each lateral root primordium of plants from line 125-3 throughout its life cycle.

We tested *LRP1* insertion homozygotes for the ability of exogenous auxin and primary root tip removal to induce additional lateral roots and adventitious roots and found no defect in comparison to wild-type plants (data not shown). GUS activity was also detected in all adventitious root primordia, regardless of the method used for induction (Figure 6C). Adventitious root primordia clearly consisted of pericyclic derivatives in the hypocotyl, although it was unclear whether other tissues also participated in the formation of adventitious roots (Figure 6C; data not shown). We have also observed that the *LRP1* insertion line produces normal lateral roots when the plants are grown in different agar concentrations and soil. We have not directly tested ethylene's effect on lateral root development but have grown insertion homozygote plants under such adverse conditions as water flooding the plates and seedlings growing on filter paper with limited amounts of medium. Neither condition revealed a mutant phenotype. However, we observed that the GUS activity pattern in line 125-3 changes under adverse growth conditions and after treatment to induce lateral root initiation. Under these conditions, GUS activity was detected in very young leaf primordia of some but not all plants (data not shown).

GUS Activity Suggests *LRP1* Is Expressed Early in Primordium Development

On two occasions, GUS activity was detected prior to any morphologically distinguishable changes in the pericycle (Figure 6D). However, GUS activity was not consistently detectable until somewhat later in the development of the primordium. Once a primordium consists of a very small dome, GUS activity is invariably found in all primordial cells (Figure 6E). GUS activity continued to be found during the later stages of primordium development (Figure 6F) and after the emergence of a lateral root (Figure 6G). During later stages of primordium development, GUS activity was strongest at the base of the primordium and not in the lateral root tip (Figures 6F and 6G). The persistence of GUS activity at the bases of primordia was observed to vary widely and ranged from disappearance immediately after lateral root emergence to persistence for 1 week after lateral root emergence (~1 cm or greater in length). If plants were grown on medium containing auxin (α-naphthaleneacetic acid) or an auxin analog (α-naphthaleneacetamide), GUS activity could be detected throughout the pericycle at the most mature end of the primary root toward the hypocotyl and persisted strongly at the base of each lateral root (Figure 6H).

DISCUSSION

To date, few mutations affecting only lateral root development have been identified, and *LRP1* is one of few genes cloned whose expression in roots is restricted to lateral root primordia. Here, we describe the use of a transposon-borne enhancer trap to identify *LRP1* in Arabidopsis. Identifying a gene in plants based solely on the activity pattern of a transposon-based reporter gene used as an enhancer trap is a novel use of transposons. *LRP1*'s expression pattern in root primordia clearly does not include the new meristem and is therefore unlike previously cloned lateral root–specific genes (Taylor and Scheuring, 1994; Vera et al., 1994).

Based on our knowledge of other developmental systems, lateral root initiation probably relies on one or more signaling pathways and cellular competence to respond to a signal. Auxin has long been suspected of playing a direct role in the induction of lateral root initiation, although nothing is known about the underlying molecular mechanisms (Hinchee and Rost, 1986; Charlton, 1991; Taylor and Scheuring, 1994). Although speculative, the presence of a potential GCA box (*cis*-acting auxin-responsive element) in the promoter region suggests that *LRP1* expression may be regulated by auxin during early lateral root primordium development (An et al., 1990). Additional experimentation is required to determine whether *LRP1* is auxin inducible and whether it is directly or indirectly inducible.

Two hypotheses have been proposed to explain the competence of pericycle cells to produce a lateral root. One holds that all of the pericycle cells are competent, but additional environmental cues are necessary for initiation of lateral root formation. The other hypothesis is that some pericycle cells are predetermined in the meristem and that only these can form lateral roots. A combination of the two hypotheses appears to be necessary to explain the observation that a predictable density of lateral roots is produced under most growing conditions, but under certain conditions, many more laterals can be induced (Torrey, 1986; Charlton, 1991; Taylor and Scheuring, 1994). GUS activity was detected in line 125-3 in all lateral root primordia under all growth conditions tested. Therefore, *LRP1* function is not likely to be part of a unique pathway necessary for increased lateral root induction but rather is part of a common pathway leading to the production of all lateral and adventitious root primordia.

The earliest GUS activity we detected in line 125-3 was prior to any obvious primordium formation. However, consistent and strong GUS activity was not observed until primordia consisted of a small mass of cells. This is also the earliest stage of primordium development that we ever observed in sections and that stained during in situ hybridization experiments. Primordia at earlier stages were never seen in the sectioned material, and, therefore, we are not sure whether *LRP1* is expressed in pericycle cells prior to cell division. Regardless, *LRP1* is expressed very early during primordium development, suggesting that it might be involved in initiating or continuing primordium development.

GUS activity was detectable at the base of lateral roots in line 125-3 long after emergence (Figure 6G). However, the results of in situ hybridization experiments clearly showed that *LRP1* was no longer expressed by the time lateral root primordia reached the epidermis (Figure 5C). We conducted in situ hybridization experiments with roots from line 125-3 using the *GUS* gene as a probe. The results of these experiments showed that *GUS* mRNA expression in line 125-3 was identical to *LRP1* expression in wild-type roots (data not shown). In this example, GUS activity persisted for up to several days after RNA synthesis stopped, and the pattern of GUS activity clearly differed from the *GUS* RNA expression pattern.

We did not detect a mutant phenotype in plants that were homozygous for the transposon insertion at *LRP1*. Many physiological and environmental conditions are known to affect lateral root development, and it is possible that the conditions under which the *LRP1* insertion exhibits a mutant phenotype have not yet been identified (Torrey, 1986; Klepper, 1987; Barlow and Adam, 1988). We have tested several different growth conditions, although we have by no means exhausted all possibilities. Because the transposon was inserted near the transcription start site at *LRP1* and not in the coding region, it is possible that the gene is expressed at a low but adequate level for the *LRP1*-encoded protein to function at wild-type levels. However, we have also presented evidence that homologous genes are expressed in the same tissues as *LRP1*, which may explain the absence of a mutant phenotype.

One of the most striking aspects of lateral root development is the plasticity of lateral root initiation and maturation. As described previously, for example, the first two lateral root primordia develop simultaneously and directly opposite one another at the hypocotyl–root junction. This occurs several days after germination and is invariably associated with *GUS* expression in the *LRP1* insertion line. However, elongation and maturation of lateral roots from these first two primordia are not necessarily immediate. Lateral roots can emerge from these primordia immediately after they are formed, or one or both primordia can remain dormant for many days or weeks prior to lateral root elongation. Thus, primordium formation and associated changes in gene expression can substantially precede lateral root outgrowth. The availability of the *LRP1 GUS* expression line will now permit us to investigate the relationship between lateral root gene expression and the morphologically observable cell division events that give rise to the primordium.

METHODS

Plant Growth Conditions

Arabidopsis thaliana seed were surface sterilized for 10 min in an aqueous solution of 0.5% sodium hypochlorite and 0.01% Tween 20 and rinsed five times with sterile distilled water before sowing. The culture medium consisted of Murashige and Skoog salts (Murashige and Skoog, 1962) supplemented with 0.4 mg/L thiamine-HCl, 100 mg/L myo-inositol, 1% sucrose, and 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.7. The medium was made semisolid with 0.8% agar (Difco). All plants were grown under a cycle of 16 hr of light and 8 hr of darkness. To induce adventitious root formation and increase lateral root density, plants were grown in Petri dishes held in a vertical position for 2 weeks. The root tips were then cut, and the plants were moved to fresh basal medium for 6 more days. To increase lateral root density without cutting off the primary root tip, plants were moved after 2 weeks on basal medium to medium containing either 0.2 μM α-naphthaleneacetic acid or 10 μM α-naphthaleneacetamide. For some RNA extractions, 30 plants were grown for 4 weeks in 30 mL of liquid medium per each 250 mL Erlenmeyer flask, shaking on a rotary platform at 100 rpm.

Detection of Marker and Reporter Gene

Hygromycin (20 mg/L final concentration) and/or chlorsulfuron (5 ppm final concentration) was filter sterilized and added to the germination medium. β-Glucuronidase (GUS) activity was detected by incubation of tissues in an assay buffer (Jefferson, 1987) containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (X-GlcA CHX) or 6-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (salmon-GlcA CHX) (Biosynth International, Skokie, IL) for 24 hr at 37°C and incubation at 4°C overnight. Hypocotyl tissues were cleared by several washes in 70% ethanol.

Genomic and cDNA Libraries

Libraries were constructed using genomic DNA from wild-type Arabidopsis (Nossen ecotype) plants (λ No-1) and transposon-containing enhancer trap line 399-8-4-125-3 (λ 125-3). Genomic DNA (1 μg) was subjected to partial Sau3AI endonuclease digestion and fractionated on an agarose gel. Fragments between 9 and 23 kb were gel purified, partially filled in with dATP and dGTP, and ligated to XhoI cut Lambda Fix II arms that were partially filled in with dCTP and dTTP according to the supplier's instructions (Stratagene). Both libraries were maintained using the bacterial strains provided and following the supplier's instructions (Stratagene).

Clones from the λ 125-3 library containing the transposon of interest were identified by hybridization with a probe homologous to the bacterial aminocyclitol phosphotransferase (*aph4*) gene contained within the transposon (Fedoroff and Smith, 1993). A clone (λ 125-3-1) was isolated that had a 15-kb insert composed of the 5-kb-long transposon and the 5 kb of genomic DNA flanking both the 5′ and 3′ ends. A 6.5-kb NcoI fragment containing 1.5 kb of the 3′ end of the transposon and 4.5 kb of genomic DNA was subcloned into pBluescript II KS+ (Stratagene) to make pDS100. Using a probe made from the pDS100 insert, a clone (λ No-1-2) from the λ No-1 library containing a 13-kb insert was isolated. Clone λ No-1-2 contained an 8.5-kb NcoI fragment corresponding to 4.25 kb of genomic DNA on either side of the transposon insertion site. The NcoI fragment was subcloned into pBluescript II KS+ to make pDS101. A 3.5-kb HindIII-BglII fragment (from 1 kb upstream of the gene to the poly[A] addition sequence of lateral root primordium 1 [*LRP1*]) from pDS101 was subcloned into pBluescript II KS+ to make pDS102.

Four different cDNA clones were isolated by screening 10^6 plaques from a λ library (PRL-2; from T. Newman, Michigan State University, East Lansing, MI) made from poly(A) RNA of Arabidopsis ecotype Columbia with the insert of pDS100.

Sequence Analysis

Both a 1.9-kb insert from cDNA clone pZLDS-5 and a 3.5-kb genomic subclone from pDS102 were subjected to ExoIII and S1 nuclease digestion to produce a deletion series using an Erase-A-Base kit (Promega). Both strands of the cDNA and genomic clone were sequenced using a polymerase chain reaction–based dideoxynucleotide terminator protocol and an ABI automated sequencer following the manufacturer's instructions (Applied Biosystems, Foster City, CA). Nucleotide and deduced amino acid sequence comparisons were done using BLAST searches (Altschul et al., 1990).

RNA and DNA Gel Blot Analyses

DNA gel blot analysis was done essentially as described in Fedoroff and Smith (1993). For lower stringency DNA gel blot analysis conditions, the hybridization temperature was reduced from 65°C to 55°C, and the final wash stringency was reduced from 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 65°C to 2 × SSC at 55°C. RNA gel blot analysis was carried out using poly(A) RNA extracted from liquid-grown root cultures and aerial tissues of agar- or soil-grown plants ~4 to 6 weeks after germination using a Quick Prep Micro mRNA purification kit according to the supplier's instructions (Pharmacia Biotech, Piscataway, NJ). For RNA gel blot analysis, 2 to 5 μg of poly(A) RNA was separated in a formaldehyde/3-(*N*-morpholino)propanesulfonic acid/agarose gel, transferred to a Hybond-N+ membrane (Amersham, Arlington Heights, IL), hybridized in a hybridization incubator (Robbins Scientific, Sunnyvale, CA) using a buffer described by Church and Gilbert (1984), washed to the highest stringency recommended, and autoradiographed essentially as described in Ausubel et al. (1987). An RNA ladder standard (Life Technologies, Gaithersburg, MD) was used to estimate the length of the RNAs. Probes were prepared using the cDNA insert in pZLDS5 or the genomic DNA insert in pDS100 and a random primer kit with ^{32}P-dCTP as the label (U.S. Biochemical Corp.). For RNA gel blot analysis at reduced stringency, the hybridization temperature was reduced from 65°C to 55°C, and the final wash stringency was reduced from 0.1 × SSC at 65°C to 0.5 × SSC at 60°C. For loading controls, RNA gel blots were stripped and reprobed with the Arabidopsis α-tubulin gene *TUA3* (Ludwig et al., 1987). To permit the cross-hybridization of *TUA3* to all six Arabidopsis α-tubulin family members, hybridization was performed at 55°C using the aforementioned buffer; the final wash conditions were 2 × SSC at 48°C (Kopczak et al., 1992).

Histology and in Situ Hybridization

Whole plants were fixed for 1 hr in an aqueous solution of 10% formaldehyde, 5% acetic acid, and 50% ethanol by pulling and releasing a gentle vacuum three times for 20 min. Plants were rinsed in 50% ethanol, the root systems were severed, and 15 root systems were arranged together in a single line. Root bundles were cut into 1-cm-long pieces, and the shorter bundles were collected together and placed into a sheet of lens paper (Eastman Kodak, Rochester, NY). The lens paper was folded over the roots to hold them in place during processing but was removed prior to sectioning. Tissues were dehydrated through an ethanol series and Hemo-De (Fisher Scientific, Pittsburgh, PA), infiltrated with Polyfin wax (Polysciences, Warrington, PA), embedded, and cut into 10-μm sections using a rotary microtome. Sections were fixed to Probe-on-Plus (Fisher Scientific) slides. Preparation for and washes after hybridization were essentially performed as described by Jackson (1991), except that protease K (Sigma) at 20 mg/mL was used instead of pronase, and the final wash after hybridization was performed in 50% formamide and 0.3 × SSC at 42°C. Hybridization was done overnight at 55°C in a humid chamber using a buffer consisting of 50% formamide, 4 × SSC, 1 mM EDTA, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.1% SDS, 10% dextran sulfate, 1 mg/mL yeast tRNA, and probe at 0.5 ng/μL/kb of probe complexity. Digoxigenin-UTP was incorporated into RNA probes and detected following the supplier's instructions (Boehringer Mannheim; hybridization application manual [1992]). The final antisense and sense probes used were derived from a 382-bp SacI-MscI insert in pDS110 corresponding to nucleotides 963 to 1345 of the cDNA insert in clone pZLDS5.

Microscopy

Sectioned material subjected to in situ hybridization was quickly dehydrated through an ethanol series and Hemo-De, and then mounted using Permount (Fisher Scientific). Whole-root mounts were incubated in 25% glycerol in 1 × PBS at 4°C for at least 8 hr and then in 50% glycerol in 1 × PBS for 8 hr more before mounting on slides. Coverslips were cut and placed at the edge of each slide as spacers for the final coverslip to prevent tissue damage.

Specimens were viewed using Nomarski (differential interference contrast) optics on a Zeiss Axioplan microscope (Zeiss, Thornwood, NY). Images were captured to Photoshop software (Adobe Systems, Mountain View, CA) on a Macintosh computer using a Sony DXC-760MD video camera and a NuVista+ video production card (Truevision, Indianapolis, IN). Image composites were arranged using Pagemaker (Aldus, Seattle, WA) prior to printing.

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