

The *POLARIS* Gene of *Arabidopsis* Encodes a Predicted Peptide Required for Correct Root Growth and Leaf Vascular Patterning

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The *POLARIS* (*PLS*) gene of *Arabidopsis* was identified as a promoter trap transgenic line, showing β -glucuronidase fusion gene expression predominantly in the embryonic and seedling root, with low expression in aerial parts. Cloning of the *PLS* locus revealed that the promoter trap T-DNA had inserted into a short open reading frame (ORF). Rapid amplification of cDNA ends PCR, RNA gel blot analysis, and RNase protection assays showed that the *PLS* ORF is located within a short (~500 nucleotides) auxin-inducible transcript and encodes a predicted polypeptide of 36 amino acid residues. *pls* mutants exhibit a short-root phenotype and reduced vascularization of leaves. *pls* roots are hyperresponsive to exogenous cytokinins and show increased expression of the cytokinin-inducible gene *ARR5/IBC6* compared with the wild type. *pls* seedlings also are less responsive to the growth-inhibitory effects of exogenous auxin and show reduced expression of the auxin-inducible gene *IAA1* compared with the wild type. The *PLS* peptide-encoding region of the cDNA partially complements the *pls* mutation and requires the *PLS* ORF ATG for activity, demonstrating the functionality of the peptide-encoding ORF. Ectopic expression of the *PLS* ORF reduces root growth inhibition by exogenous cytokinins and increases leaf vascularization. We propose that *PLS* is required for correct auxin-cytokinin homeostasis to modulate root growth and leaf vascular patterning.

INTRODUCTION

Hormone signaling systems coordinate plant growth and development through a range of complex interactions. It is not clear how interactions are coordinated between different classes of the classic hormones (auxin, cytokinin, ethylene, gibberellin, and abscisic acid) and between other signal molecules such as brassinosteroids (Altmann, 1999) or peptides (Lindsey et al., 2002). However, significant progress has been achieved in understanding the molecular basis of plant signaling systems through extensive genetic screens using *Arabidopsis*. A number of studies have identified diverse components in the signaling pathways of auxin (Leyser and Berleth, 1999), ethylene (McGrath and Ecker, 1998; Woeste and Kieber, 1998), gibberellins (Fridborg et al., 1999), abscisic acid (Finkelstein and Lynch, 2000), and cytokinins (Kakimoto, 1998; Vogel et al., 1998; Riou-Khamlichi et al., 1999; Hwang and Sheen, 2001; Inoue et al., 2001).

One experimental system, which has attracted much attention recently as a model in which to study hormonal in-

teractions in development, is the *Arabidopsis* root (Dolan et al., 1993), which is readily amenable to mutational screens. For example, genes such as *MONOPTEROS* (Hardtke and Berleth, 1998) and *BODENLOS* (Hamann et al., 1999) are required for normal root development and encode components of the auxin signaling pathway. The *MONOPTEROS* gene, which encodes an auxin response factor transcription factor, also is required for correct patterning of vascular tissues in the cotyledon and leaf (Hardtke and Berleth, 1998). The *aux1* mutant was identified on the basis of a defective root gravitropic response and encodes a putative component of the auxin influx carrier system (Bennett et al., 1996).

The *EIR1/AGR1/AtPIN2* gene also is required for a normal gravitropic response and for ethylene sensitivity and encodes a likely component of the auxin efflux complex (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998). The *axr1* mutant is resistant to both auxin and ethylene and exhibits altered root branching (Lincoln et al., 1990). This and other mutants, such as *axr3* and *monopteros*, also exhibit abnormal auxin distribution (Sabatini et al., 1999).

However, we still have an incomplete notion of the molecular mechanisms of root formation and growth control and of how hormones interact to elicit the diverse developmental pathways found in plants. For example, cytokinins can act

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as important regulators of cell division and appear to be synthesized in root tips, although exogenous cytokinins can inhibit root growth (Sossountov et al., 1988; Riou-Khamlichi et al., 1999; Chiappetta et al., 2001). Cytokinins can act antagonistically with auxin in some developmental contexts, such as in lateral root development, but they also can act synergistically, as in ethylene biosynthesis (Vogel et al., 1998).

Key components of the cytokinin signaling pathway have been identified on the basis of resistance to the growth-inhibitory effects of exogenous cytokinins (Brandstatter and Kieber, 1998), including the identification of a predicted cytokinin receptor (Inoue et al., 2001). Nevertheless, the mechanisms by which hormonal interactions occur remain unclear.

To gain new insight into the control of root growth and development, we screened a population of *Arabidopsis* promoter trap transgenic lines for genes expressed in, and required for, root development. Promoter trapping can facilitate expression analysis of genes by the characterization of *in vivo* fusion gene expression, the investigation of tagged gene function by mutant analysis, and the cloning of tagged genes (Lindsey et al., 1998).

Previously, we identified a promoter trap transgenic line that has β -glucuronidase (GUS) fusion activity detectable from the heart stage of embryogenesis, in the basal region of the embryo, and in the seedling root tip (Topping et al., 1994; Topping and Lindsey, 1997). Here, we describe the cloning and characterization of the tagged gene, designated *POLARIS* (*PLS*). It encodes a short transcript and a predicted small polypeptide. It is required for correct responses to cytokinins and auxins, for correct cell expansion in the root, and for vascular patterning in the leaf.

RESULTS

pls Is Defective in Root Growth and Leaf Vascularization

A screen of GUS-expressing promoter trap lines of *Arabidopsis* identified the line AtEM101. It contains a single-copy T-DNA and exhibits GUS fusion activity predominantly in the embryonic root from the heart stage and in the seedling primary and lateral root tips (Topping et al., 1994) (Figures 1A and 1B).

A time course of histochemical staining of the root of AtEM101 revealed that, after a short period (5 min) of incubation in 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, GUS activity was detectable only in the columella initials and lateral root cap (Figure 1B), similar to the pattern observed for the auxin-regulated *DR5::GUS* gene fusion (Sabatini et al., 1999). Longer incubation periods in 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (>1 h) revealed GUS activity more widely in the root tip, notably in the meristematic region (Figure 1C). Overnight staining revealed GUS activity predominantly in both the primary and lateral root tips, but

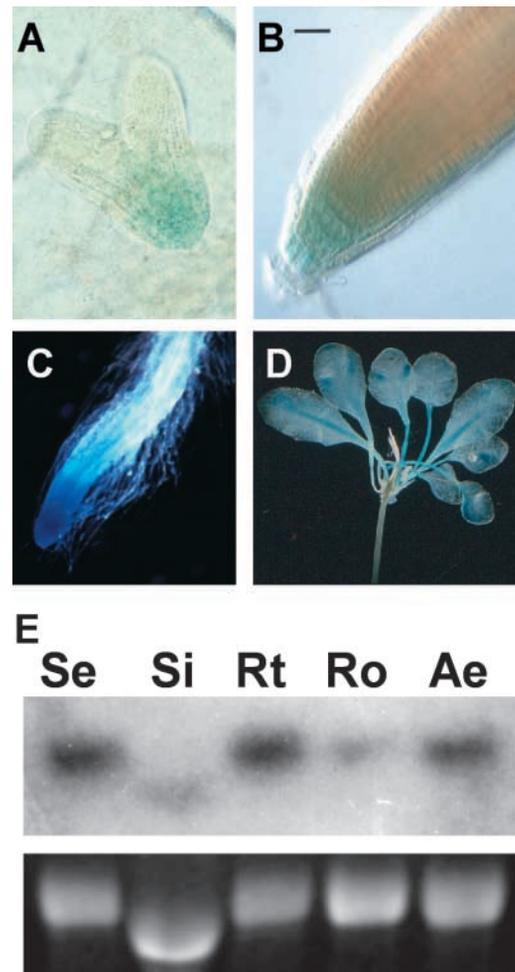


Figure 1. GUS Expression in the *PLS-GUS* Promoter Trap Line AtEM101.

(A) Heart-stage embryo, with 5 h of GUS staining, showing GUS activity in the basal region. Magnification $\times 100$.

(B) Seven-day-old seedling root tip, with 5 min of GUS staining, showing activity in columella and, weakly, in young vascular cells. Bar = 50 μ M.

(C) Seven-day-old seedling root tip, with 1 h of GUS staining, showing activity throughout the root tip. Magnification $\times 7$.

(D) Aerial parts of a 12-day-old seedling, with 8 h of GUS staining, showing activity in leaf vascular tissues. Magnification $\times 5$.

(E) RNA gel blot analysis showing *PLS-GUS* fusion transcript levels in the *pls* mutant using 10 μ g of RNA from 7-day-old total seedlings (Se), siliques (Si), root tips (Rt), roots with tips removed (Ro), and aerial parts (Ae). The top gels show a GUS fusion transcript, and the bottom gels show ethidium bromide-stained 25S rRNA. Note that siliques RNA runs more rapidly through gels than that from other tissues.

lower levels of activity also were detectable in aerial parts of the seedling. In leaves, GUS activity was detected principally in vascular tissues of the lamina and petiole (Figure 1D).

To investigate further the transcriptional regulation of the *PLS-GUS* fusion gene, RNA gel blot analysis was performed using total RNA from 7-day-old seedlings and from siliques of 21-day-old plants. The results are presented in Figure 1E. A *gusA* probe identified an ~3-kb transcript, which is ~300 nucleotides larger than the predicted *gusA* mRNA plus T-DNA left border region, indicating a transcriptional fusion. The relative abundance of the fusion transcript in different regions of the root, and in different organs, was consistent with the histochemical staining pattern of GUS activity. In particular, root tips showed greater transcript abundance compared with the older, proximal region of the root.

To determine whether there is a defective seedling phenotype associated with the T-DNA insertion, AtEM101 seedlings homozygous for the T-DNA were backcrossed to the wild type to reveal segregating mutants. Seedlings of selfed heterozygotes were grown on vertical agar plates for 14 days in the light, and three classes of seedlings were identified. Approximately 25% (43 of 186) were GUS negative and exhibited a relatively long root, comparable to that of wild-type seedlings (mean length, 75 ± 4 mm [$n = 10$]); 25% (41 of 186) were GUS positive and exhibited a significantly shorter root than the wild type (mean length, 38 ± 2 mm [$n = 10$]; i.e., ~51% at 14 days after germination) (Figure 2A). These seedlings were homozygous for the T-DNA insertion, as demonstrated by 100% of the progeny showing this GUS-positive short-root phenotype on selfing.

The third class, which comprised ~50% of the seedlings (102 of 186), was GUS positive, although with reduced GUS activity, as determined by histochemical staining compared with the previous class. It exhibited an intermediate rate of root growth (mean length, 53 ± 3 mm; i.e., ~71% the length of wild-type roots at 14 days after germination). These seedlings were heterozygous for the T-DNA insertional mutation, as shown by segregation of the GUS-associated root phenotype on selfing. The mutant was designated *pls*.

To investigate further the root phenotype of the *pls* mutant and heterozygote, seedlings were germinated and grown in the light on vertical agar plates and primary root length was measured over a time course. The results presented in Figure 2B show that the rate of increase in root length of seedlings homozygous for the T-DNA insertion was reduced compared with that in the wild type, such that by day 14 after germination, the length of the mutant primary root was ~50% of that of the wild type. The rate of elongation of the primary roots of seedlings heterozygous for the T-DNA insertion was intermediate between those of wild-type and homozygous seedlings, indicating that the *pls* mutation is semidominant. Additionally, *pls* mutants typically produced more anchor roots at the root-hypocotyl junction (three or four) than did wild-type seedlings (usually one; Figure 2C).

Microscopic analysis of cleared *pls* roots (day 7 after germination) revealed that the cells of the root meristem and

the cortex of the primary root were shorter and more radially expanded than in the wild type. Cortical cells in the maturation zone were a mean length of 123.2 ± 1.2 μm ($n = 68$) for the wild type and 98.8 ± 0.9 μm ($n = 68$) for *pls*. The *pls* root was wider than the wild-type root, both across the region of the quiescent center (wild type, 69.0 ± 1.5 μm [$n = 10$]; *pls*, 74.8 ± 1.7 μm [$n = 10$]) and 100 μm farther back from the root tip (wild type, 86.8 ± 1.0 μm [$n = 10$]; *pls*, 96.5 ± 1.9 μm [$n = 10$]). The reduction in axial cell elongation in the root would only partially account for the *pls* short-root phenotype, indicating that the mutant meristem cells divide less frequently than wild-type cells.

Aerial parts of seedlings and mature plants were not obviously abnormal in their morphology. However, microscopic analysis of the rosette leaves of 12-day-old plants showed that the extent of vascularization of the rosette leaves was reduced compared with that in the wild type, with fewer higher order veins arising from the major strands (Figure 2D). Plants hemizygous for the *pls* mutation showed levels of venation intermediate between those in homozygous mutants and the wild type (data not shown).

Together, these data suggest that the T-DNA insertion had caused a semidominant mutation affecting root growth and architecture, and vascular patterning in leaves, under the conditions studied.

***PLS* Encodes a Short Transcript**

To investigate the molecular basis of the mutation, part of the *PLS* locus (166 bp of T-DNA flanking sequence) was cloned by inverse PCR (Topping et al., 1994) and used as a molecular probe to identify a corresponding Arabidopsis genomic clone. A 3.6-kb EcoRI genomic subclone, hybridizing to the inverse PCR fragment, and another 1.7 kb of contiguous genomic region, were sequenced. The T-DNA had inserted in a small open reading frame (ORF) of 108 bp in a 755-bp region between two larger genes (Figure 3). There was no rearrangement of the adjacent genomic DNA. One gene, 450 bp upstream of the T-DNA, encodes a predicted protein of 92 amino acid residues of unknown identity (designated *GENE X*). The second gene is *BRI1*, which locates *PLS* to the bottom of chromosome 4 (Li and Chory, 1997).

It was suspected at first that the ORF into which the promoter trap had inserted (designated the *PLS* ORF) was an exon of one of the adjacent two genes. However, it was found to be part of a separately transcribed gene. 3' rapid amplification of cDNA ends (RACE) of *GENE X* demonstrated that its transcription terminated with a polyadenylation sequence 356 bp upstream of the T-DNA insertion site (data not shown). RNA gel blot analysis of 7-day-old AtEM101 seedlings, probed with the ^{32}P -labeled 3' RACE product and a *gusA* gene probe, revealed two distinct transcripts: the *GENE X* transcript (~0.5 kb) and the GUS fusion transcript (~3 kb; data not shown). Furthermore, *BRI1* is located on the nontranscribed strand (i.e., in the wrong

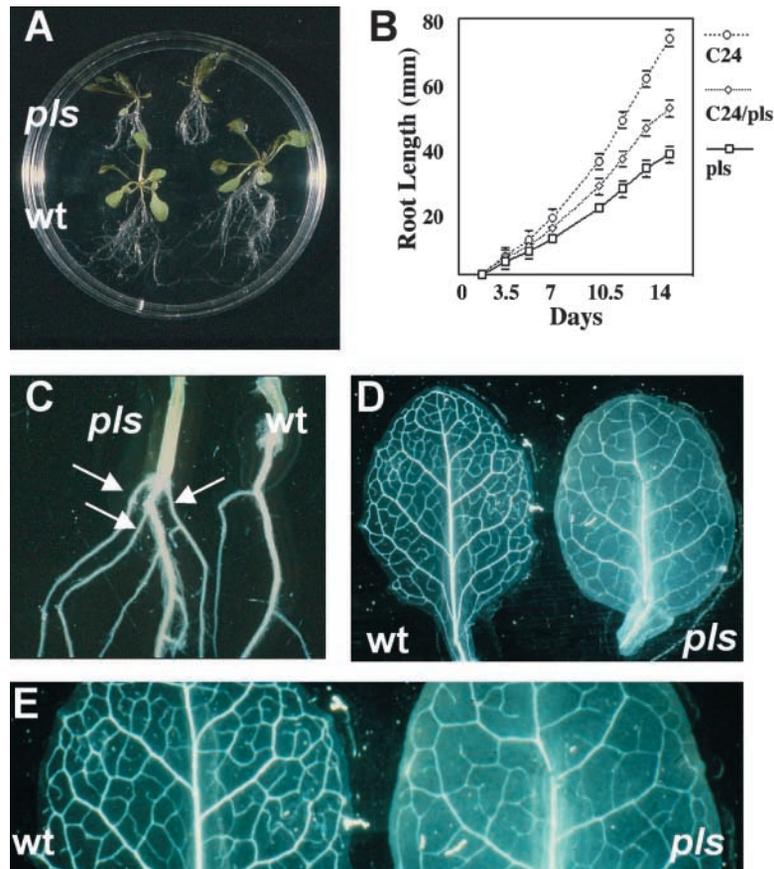


Figure 2. *pls* Mutant Phenotype.

(A) *pls* (top) and wild-type (*wt*; bottom) seedlings, 14 days after germination, showing the short-root phenotype of the *pls* mutant.

(B) Primary root growth of wild-type C24, *pls* mutant, and C24/*pls* heterozygote seedlings.

(C) The hypocotyl-root junction of *pls* (left) and wild-type (right) seedlings, 14 days after germination, showing the increased frequency of anchor roots (arrows) in the *pls* mutant.

(D) Rosette leaves of wild-type (left) and *pls* mutant (right) seedlings, 12 days after germination, showing the decreased frequency of higher order vascular strands in the *pls* mutant.

(E) Enlargement of leaves shown in **(D)** showing detail of the reduced vascularization of the *pls* mutant.

orientation to activate the T-DNA promoter trap) and is ~400 bp downstream of the T-DNA insertion site.

5' RACE PCR from the GUS fusion transcript was used to clone part of the *PLS* cDNA, and this sequence was used to clone the remainder by 3' and 5' RACE PCR from the wild-type plant. RNA gel blot analysis, using *PLS* cDNA as a probe, revealed the presence of a single low-abundance transcript of ~500 nucleotides, representing the predicted *PLS* transcript (Figure 4A). The transcript was detectable only when gel blots of at least 1 μ g of poly(A)⁺ RNA were probed and autoradiographed for at least 4 weeks. Cloning and sequencing of an RNA-specific PCR product corresponding to this transcript confirmed its identity. DNA gel blot analysis revealed a single hybridizing band, indicating a single copy per haploid genome (data not shown).

***PLS* Gene Transcription Initiates within an Upstream Gene**

The site of transcript initiation was determined by 5' RACE PCR and RNase protection assays. It was found that the transcription start site is the same for both wild-type *PLS* and *PLS-gusA* fusion transcripts (Figure 4B). Interestingly, two transcription start sites were found ~95 bp apart. Start site 1, ~117 bp upstream of the *GENE X* poly(A) site, has the sequence ATCCGTAT (the G represents the initiation site). Start site 2, which is ~23 bp upstream of the poly(A) site, has the sequence CCACTTAATA; RACE results were unable to resolve which of the three underlined bases is likely to represent the initiation site.

The RNase protection assay results show that start site 2,

which generates the shorter transcript, is used more frequently than start site 1. Start site 2 has the predicted TATA sequence TATATAA (positions -32 to -26). Start site 1 has a poorer TATA-like sequence, AATAATA (positions -35 to -29). Sequencing of 3' RACE products revealed that the *PLS* transcript had a variable 3' end, with the transcript being between 427 and 606 nucleotides long, according to the transcription start and polyadenylation sites used.

To confirm that *PLS* transcript initiation is driven by sequences within *GENE X*, transgenic lines were produced containing an upstream genomic fragment fused to the *gusA* reporter gene. This fragment, which comprised 370 bp upstream of the predicted transcription initiation start site 2 and lacked the ATG codon of the putative 92-amino acid *GENE X* ORF, was designated pPLS::GUS. T1 and T2 transformants were identified on the basis of T-DNA-mediated resistance to kanamycin. Six families of independent pPLS::GUS transgenic lines were analyzed histochemically for GUS activity in 7-day-old seedlings. All showed GUS activity in the root tip (Figure 4C). A GUS fusion with a longer promoter region, 1190 bp upstream of the predicted transcription initiation start site 2, showed stronger GUS activity than the shorter promoter (Figure 4D), with activity also detectable in the aerial parts of the seedling, as in line AtEM101 (data not shown).

The *PLS* Transcript Encodes a Predicted 36-Amino Acid Polypeptide

Sequencing of the *PLS* locus in C24 (Figure 3) showed that the T-DNA inserted into the 25th codon (Leu) of an ORF encoding a predicted 36-amino acid residue *PLS* polypeptide, with a predicted molecular mass of 4.6 kD and no significant homology with known proteins. The Columbia allele also was cloned and found to be identical in sequence. The T-DNA is expected to disrupt *PLS* function. The *PLS* ORF is preceded by an in-frame 27-bp ORF encoding a predicted nine-amino acid polypeptide; its TGA stop codon immediately precedes the ATG of *PLS*. A predicted eight-amino acid ORF overlaps with *PLS* 4 bp upstream of the *PLS* ATG.

No other ATG codons appear in the 5' untranslated region (UTR) initiating from transcriptional start site 2. The N-terminal 24 amino acids of the *PLS* putative polypeptide are predicted to form two β -sheets, whereas the remaining 12 amino acids are likely to form an α -helix. Between the two β -sheets are three basic Arg residues (positions 10 to 12) that may form a turn region and a possible cleavage site. The second β -sheet contains a repeated SIS separated by four residues. The proximity of these Ser residues to the basic Arg residues of the β -turn may signify a cAMP- and cGMP-dependent protein kinase phosphorylation site.

The C-terminal α -helix also contains the repeat KLFKLFK. The Lys residues and the terminal His residue represent the only charged residues in this helical region. The three-amino acid spacing between each of the Lys residues indicates



Figure 3. *PLS* Encodes a Small Transcript.

(A) *PLS* locus. Closed rectangles indicate transcripts, and arrows indicate ORFs (for *GENE X* and *PLS*). The T-DNA insertion site is indicated. **(B)** The *PLS* gene sequence shows transcription start site 2 (inverted arrowhead), the *GENE X* polyadenylation site (boldface), the 9-amino acid uORF (underlined), the 36-amino acid *PLS* ORF (amino acids shown), and the *PLS* polyadenylation sites (arrows). The asterisk marks the position of the T-DNA insertion site (in the 25th codon; L).

that they would all lie on the same face of the α -helix, creating an amphipathic helix with both hydrophobic and charged faces. The fact that the predicted helical region is Leu rich indicates the potential for a Leu zipper motif, suggesting the possibility for protein-protein interactions. Two of the Leu residues form a heptad (Leu residues 25 and 32), which may be significant given the small size of the polypeptide.

PLS Gene Expression Is Upregulated Rapidly by Exogenous Auxin

PLS-GUS activity is upregulated in response to a polarizing signal in the embryo and root, a candidate for which is auxin (Topping and Lindsey, 1997). To investigate this further, RNA

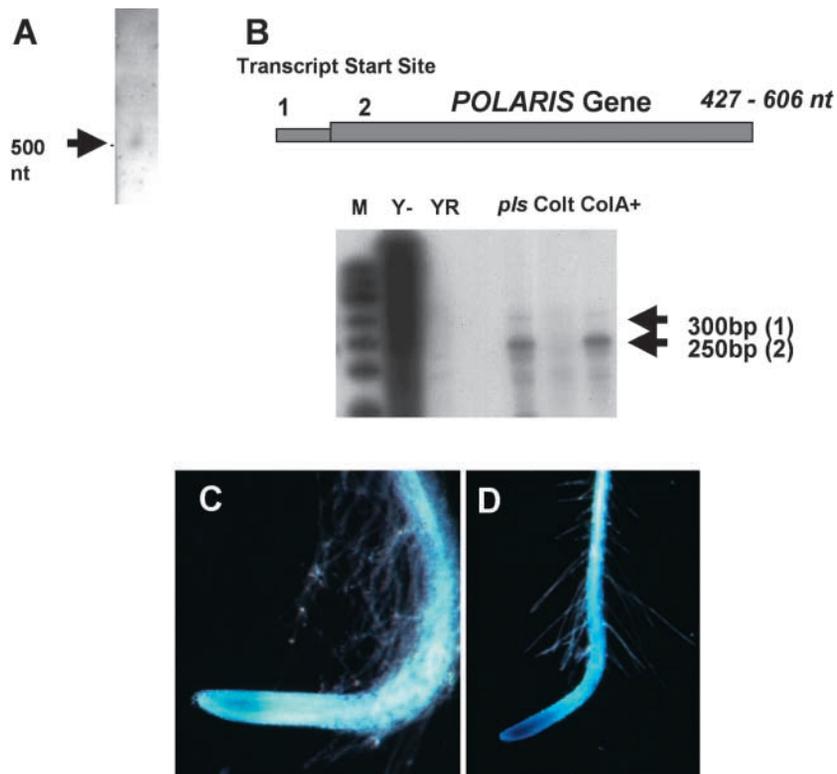


Figure 4. *PLS* Expression.

(A) RNA gel blot [5 μ g of poly(A)⁺ RNA] showing the \sim 500-nucleotide (nt) *PLS* transcript.

(B) RNase protection assays to map the *PLS* transcript initiation site in wild-type and AtEM101 seedlings using a radiolabeled antisense *PLS* transcript. Transcript start sites (1 and 2) are shown in the bottom panel (arrows) and in diagrammatic form above. M, size markers (radiolabeled phiX174 Hinf1 DNA); Y⁻, yeast RNA control; YR, RNase-treated yeast RNA control; *pls*, 50 μ g of total RNA from AtEM101 seedlings; Colt, 50 μ g of total RNA from wild-type seedlings (Columbia); ColA⁺, 5 μ g of poly(A)⁺ RNA from wild-type seedlings (Columbia).

(C) Activity of the 370-bp promoter region (p*PLS*::GUS expression) in the root tip of a 7-day-old transgenic seedling with 24 h of GUS staining. Magnification $\times 7$.

(D) Activity of the 1190-bp promoter region in the root tip of a 7-day-old transgenic seedling with 24 h of GUS staining. Magnification $\times 5$.

gel blot analysis of the *PLS*-GUS transcript was used to examine the effects of exogenous auxin, cytokinin, and inactive auxin analogs on the activity of the *PLS* gene promoter. AtEM101 seedlings were grown for 7 days after germination on hormone-free medium and then transferred to medium containing 10 μ M 1-naphthalene acetic acid (1-NAA) or 10 μ M kinetin for 30 min to 24 h before RNA extraction. Total RNA was fractionated, blotted, and probed with a *gusA* gene fragment.

The transcript abundance increased approximately threefold within 30 min and approximately sevenfold by 24 h of treatment with auxin (Figure 5A). This effect also was seen with 2,4-D but not with the nonfunctional auxin analogs 2-NAA and 2,3-D (Figure 5B). Histochemical analysis showed that when AtEM101 seedlings were treated with high auxin concentrations (e.g., 10 μ M 2,4-D), GUS activity was detectable in all root tissues and in leaves (data not shown). By contrast, treatment with kinetin led to reduced

gusA transcript abundance, to a level of \sim 40% of that in the untreated seedlings, within 2 h (Figure 5A).

To determine whether transcription of the native *PLS* gene also is regulated by auxin, competitor reverse transcription (RT)-PCR was used to determine *PLS* mRNA relative abundance in seedlings either treated or untreated with auxin. The results, presented in Figure 5C, showed increased *PLS* transcript abundance in seedlings treated with 10 μ M 1-NAA for 2 or 24 h compared with untreated seedlings. The relative increase in the native *PLS* amplicon was clear compared with the abundance of amplicon derived from a synthetic cDNA fragment, which was identical to the *PLS* sequence except for an introduced *Clal* site and was introduced into the PCR as an internal standard. Actin controls showed that no general increase in mRNA abundance occurred in response to auxin treatment.

The cloned *PLS* promoter region in pPLS:GUS also showed auxin inducibility after treatment of seedlings with 10 μ M 1-NAA, as shown by GUS histochemistry and confirmed by RNA gel blot analysis (Figure 5D, pS). There was an \sim 2.5-fold increase in transcript levels at 24 h after auxin treatment compared with untreated controls. Therefore, the 370-bp region within pPLS:GUS is sufficient for auxin-inducible transcription. The 1190-bp promoter::GUS also showed auxin inducibility after treatment of transgenic seedlings with 10 μ M 1-NAA (Figure 5D, pL).

The pPLS promoter contains a number of TGTCTC-like putative auxin-responsive elements (AuxREs) (Ulmasov et al., 1997). Two sequences, TGTCTC and TGTCGG, occur on the opposite strand (AuxREs are functional on both DNA strands). Two overlapping elements were identified starting at position -146 (TGTCTTGTCTA) relative to transcriptional start site 1. These were just upstream of the sequence AATAAT (position -130), which is similar to the sequence AATAAG found close to the TGTCTC element in two AuxREs in the soybean *GH3* promoter (Liu et al., 1994). Furthermore, at positions -85 and -61 are putative TGTCTC-like elements, with the sequence TGTTTC separated by a T-rich tract. Because conservation at positions 1 to 4 is more important than at positions 5 and 6, these elements may be nonfunctional (Ulmasov et al., 1997).

p/s Mutants Show Hyperresponsiveness to Cytokinin and Reduced Responsiveness to Auxin

Reduced longitudinal cell expansion and increased radial expansion, seen in the *p/s* mutant root, can be caused by increased accumulation or sensitivity to any of several hormones, including ethylene (Abeles et al., 1992), auxin (Ljung et al., 2001), or cytokinin (Beemster and Baskin, 2000). To investigate the sensitivity of *p/s* root growth to exogenous hormones, homozygous mutant and wild-type seedlings were grown in the presence of different concentrations of exogenous auxin (2,4-D, 100 pM to 100 nM; 1-NAA, 100 pM to 250 nM), cytokinin (benzyladenine [BA], 100 pM to 1 μ M), and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; 10 pM to 10 nM).

For auxin treatment, 3-day-old seedlings were transferred from hormone-free medium to plates containing auxin for another 3 days in the light. For cytokinin and ACC treatments, seedlings were grown continuously in the presence of either BA or ACC for 7 days after germination in the dark (conditions used previously to identify mutants defective in cytokinin and ethylene signaling) (Vogel et al., 1998). Changes in primary root lengths of mutant and wild-type seedlings were determined (Table 1).

Both cytokinin and ACC treatments resulted in reduced primary root growth in both mutant and wild-type seedlings. Interestingly, the *p/s* roots were proportionally shorter than wild-type roots in the presence of exogenous BA over a range of concentrations. On the lowest BA concentration tested (100 pM), there was an \sim 20% reduction in *p/s* root

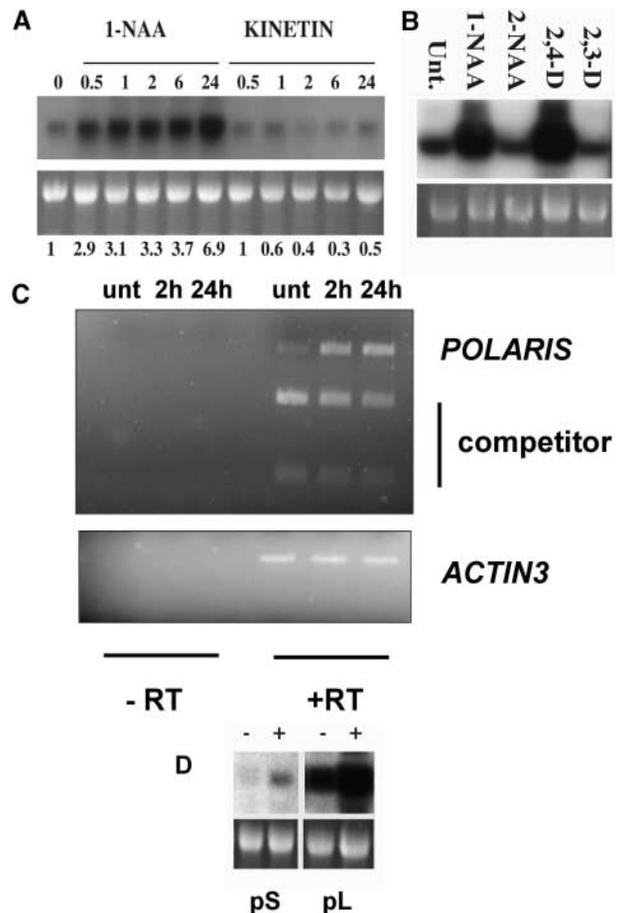


Figure 5. The *PLS* Gene Promoter is Auxin Inducible.

(A) RNA gel blot showing *PLS-GUS* fusion transcript levels after treatment with auxin (10 μ M 1-NAA) or cytokinin (10 μ M kinetin). Seedlings were grown for 7 days on hormone-free medium and then transferred to the hormones for the times indicated. The top gel shows the *PLS-GUS* transcript, and the bottom gel shows ethidium bromide-stained 25S rRNA. Relative transcript levels were normalized compared with untreated controls, and values are shown below. Each lane contained 10 μ g of total RNA.

(B) *PLS-GUS* fusion transcript levels after treatment of 7-day-old AtEM101 seedlings with 10 μ M active auxins (1-NAA and 2,4-D) and inactive analogs (2-NAA and 2,3-D) for 24 h. Each lane contained 20 μ g of total RNA. Unt., untreated.

(C) Competitor PCR analysis showing the increase in abundance of the native *PLS* transcript (*POLARIS*) in untreated 7-day-old wild-type seedlings (unt) and after treatment of 7-day-old wild-type seedlings with 10 μ M 1-NAA for 2 and 24 h. The competitor lanes show the abundance of amplicon derived from a cleaved (with *Cla*I) synthetic *PLS* cDNA fragment that was identical to wild-type *PLS* sequence except for an introduced *Cla*I site and that was introduced into the PCR as an internal standard. The *ACTIN3* transcript was amplified as a control, and control amplifications lacking reverse transcriptase are shown (-RT).

(D) *PLS-GUS* fusion transcript levels in transgenic lines containing either the 370-bp (pS) or 1190-bp (pL) promoter fragments linked to *gusA* after growth on 10 μ M 1-NAA for 24 h (+) or unsupplemented medium (-). Each lane contained 20 μ g of total RNA.

Table 1. Changes in Primary Root Lengths of *p/s* and Wild-Type C24 Seedlings after 7 Days of Continuous Growth on Cytokinin (BA) and ACC and after Transfer at 3 Days after Germination from Hormone-Free to Auxin (2,4-D, 1-NAA)-Containing Medium for another 3 Days

	C24 (<i>n</i> = 10)		<i>p/s</i> (<i>n</i> = 10)	
	Mean Root Length (mm ± SE)	Percent Change from Control	Mean Root Length (mm ± SE)	Percent Change from Control
1/2MS10	18.0 ± 0.7	100	11.6 ± 0.4	100
BA (100 pM)	17.9 ± 0.7	99.4	9.2 ± 0.4	79.3
BA (1 nM)	17.3 ± 0.8	96.1	9.4 ± 0.4	81.0
BA (10 nM)	16.9 ± 0.8	93.9	8.3 ± 0.4	71.6
BA (100 nM)	9.6 ± 0.2	53.3	4.9 ± 0.3	42.2
BA (1 μM)	9.0 ± 0.3	50.0	3.3 ± 0.3	28.4
ACC (10 pM)	14.6 ± 0.8	81.1	8.9 ± 0.3	76.7
ACC (100 pM)	11.3 ± 0.5	62.8	7.4 ± 0.4	63.8
ACC (1 nM)	7.2 ± 0.2	40.0	4.8 ± 0.3	41.4
ACC (10 nM)	3.5 ± 0.2	19.4	1.9 ± 0.2	16.4
1/2MS10	36.0 ± 1.0	100	11.9 ± 0.7	100
2,4-D (100 pM)	32.8 ± 0.6	91.1	15.1 ± 0.7	126.5
2,4-D (1 nM)	30.5 ± 0.8	84.6	12.9 ± 0.6	108.4
2,4-D (10 nM)	7.6 ± 0.4	21	5.5 ± 0.3	46
2,4-D (100 nM)	2.3 ± 0.1	6.4	1.8 ± 0.1	15.1
NAA (100 pM)	33.6 ± 0.8	93.3	15.1 ± 0.5	126.9
NAA (1 nM)	34.5 ± 0.6	95.8	14.4 ± 0.6	120.6
NAA (10 nM)	30.3 ± 0.6	84	12.7 ± 0.3	106.3
NAA (100 nM)	11.4 ± 0.3	31.7	5.1 ± 0.2	42.9
NAA (250 nM)	5.6 ± 0.1	15.6	3.4 ± 0.1	28.2

The relative change in primary root length is shown as percent change compared with control seedlings grown on hormone-free medium (1/2MS10). The seedlings treated with BA and ACC were grown on horizontal agar plates, whereas the seedlings treated with auxins were grown on vertical agar plates, accounting for root length differences between controls of the respective treatments.

elongation, although the wild type was unaffected. There was no difference between the relative growth inhibition of *p/s* and wild-type roots in the presence of ACC over the concentration range tested, although significant inhibitory effects were seen in both sets of seedlings.

However, there was a significantly reduced growth-inhibitory effect of auxin on the *p/s* root compared with the wild-type root. Indeed, at 100 pM and 1 nM 2,4-D and 1-NAA, respectively, the roots of *p/s* mutant seedlings were significantly longer than those of seedlings grown in the absence of exogenous auxin. These results suggest that the growth of the *p/s* mutant primary root is hyperresponsive to the inhibitory effects of exogenous cytokinin, whereas the *p/s* mutation causes a suppression of the growth-inhibitory effects of auxin on root length and, at the lowest auxin concentrations tested, leads to root growth enhancement.

One prediction from the observation that the *p/s* mutant exhibits increased cytokinin responsiveness would be that the expression of cytokinin-regulated genes might be increased in *p/s* seedlings. To test this possibility, the expression levels of the *IBC6/ARR5* and *IBC7/ARR6* genes (Brandstatter and Kieber, 1998; D'Agostino et al., 2000), which are upregulated transcriptionally by cytokinin, were investigated in *p/s* and wild-type seedlings in the absence of exogenous cytokinin.

Densitometric scanning of RNA gel blots showed that *IBC6/ARR5* was upregulated threefold in the *p/s* mutant, whereas *IBC7* transcript was unchanged (Figures 6A and 6B). Consistent with this finding, the expression of a green fluorescent protein (GFP) gene driven by the *IBC6/ARR5* gene promoter and introduced into the *p/s* mutant background by crossing (to avoid position effects on the level of expression) was increased in the root tips of seedlings in a *p/s* mutant background compared with expression in a wild-type background (Figures 6C and 6D). This result indicated that at least one gene, specifically regulated by cytokinins, is upregulated in the *p/s* mutant.

A prediction from the observation that the *p/s* mutant showed reduced responses to exogenous auxin would be that the expression of auxin-inducible genes might be downregulated in *p/s* seedlings compared with the wild type. To test this prediction, the expression levels of the *IAA1* and *IAA2* genes (Abel and Theologis, 1996), which are upregulated transcriptionally by auxin, were investigated in *p/s* and wild-type seedlings in the absence of exogenous auxin. Densitometric scanning of RNA gel blots showed that *IAA1* transcript abundance in the *p/s* mutant was downregulated to ~45% of the level found in wild-type seedlings, whereas the expression of *IAA2* was unchanged (Figures 6E and 6F).

PLS cDNA Complements the *pls* Mutant Phenotype

To confirm that mutation of the *PLS* gene is responsible for the observed short-root phenotype, the wild-type cDNA was introduced into the homozygous *pls* mutant under the transcriptional control of the 370-bp p*PLS* promoter. This promoter contained only a small region of the 3' end of the *GENE X* ORF, which would not be expected to produce a functional protein. T1 transformants were selfed, and T2 and T3 seedlings representing individual families were analyzed for root phenotype after growth on hormone-free medium for 9 days. To ensure that no wild-type contaminants had been included accidentally, T2 seedlings from each potential complementing line were stained for *PLS*-GUS activity. All lines examined showed GUS staining in the root tips. RNA-specific PCR analysis of potentially complementing transgenic lines demonstrated that each showed expression of the wild-type *PLS* transcript, which was absent in the *pls* mutant (data not shown).

The complementation experiment showed that the short-root mutant phenotype was partially rescued by expression of the wild-type *PLS* allele (Figures 7A and 7B). For example, at 9 days after germination, *pls* mutant roots had a mean length of 14.7 ± 0.8 mm compared with 25.1 ± 2.1 mm for the wild type ($n = 10$). The mean root length of eight independent T3 complementing transgenic lines (Figure 7B) ranged from 19.3 ± 0.3 mm for line 77 to 21.65 ± 0.8 mm for line 87. These lines represent a mixture of homozygotes and hemizygotes for the complementing T-DNA. All eight transgenic lines had longer roots than those of the *pls* mutant. These data demonstrate the functionality of the *PLS* cDNA.

To determine whether the *PLS* ORF is required for complementation activity, a mutant *PLS* cDNA was constructed in which the *PLS* ORF ATG codon was mutated to ATC, cloned behind the 35S promoter of *Cauliflower mosaic virus*, sequenced, and introduced into *pls* mutant plants. Mutation of the *PLS* ATG would prevent its recognition as a translation initiation codon. The sequence of the nine-amino acid ORF immediately upstream of the *PLS* ORF (Figure 3) was unchanged. Primary root length measurements were determined at 7 days after germination for seedlings that were (1) *pls* mutants, (2) transgenic for the wild-type *PLS* ORF transcript, and (3) transgenic for the mutant *PLS* ORF transcript.

The results, presented in Table 2, showed that although the wild-type *PLS* ORF transgenic seedlings had significantly longer roots than the *pls* mutant seedlings, all five independent lines transgenic for the mutant *PLS* ORF (designated m*PLS* ORF1 to ORF5) showed no significant difference in root length compared with the *pls* mutant seedling root. RT-PCR analysis confirmed that each of these lines expressed the mutant transcript, whereas the control (untransformed) *pls* seedlings showed no amplification product (Figure 7C). This finding strongly suggests that the functionality of the *PLS* gene requires a functional translation initiation codon in the *PLS* ORF.

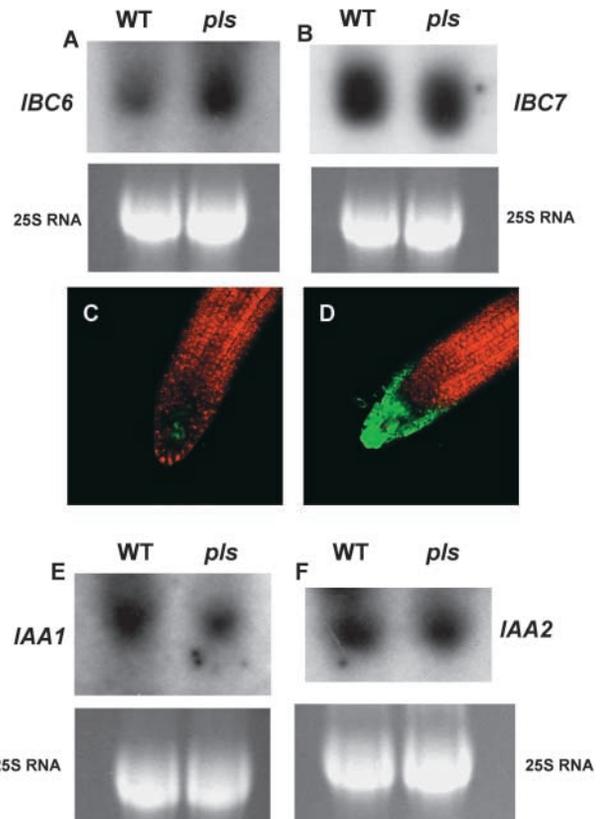


Figure 6. Gene Expression in a *pls* Mutant Background.

(A) and (B) RNA gel blot analysis of transcript abundances of *ARR5/IBC6* (A) and *ARR6/IBC7* (B) genes in wild-type (WT) and *pls* mutant seedlings 7 days after germination. 25S RNA is shown as a loading control. Each lane contained 10 μ g of total RNA.

(C) and (D) *ARR5/IBC6::GFP* gene fusion activities in the root tips of transgenic wild-type (C) and *pls* mutant (D) seedlings 7 days after germination, viewed with the confocal microscope. Magnification $\times 100$.

(E) and (F) RNA gel blot analysis of transcript abundances of *IAA1* (E) and *IAA2* (F) genes in wild-type and *pls* mutant seedlings 7 days after germination. 25S RNA is shown as a loading control. Each lane contained 10 μ g of total RNA.

Overexpression of a Partial *PLS* cDNA Confers Reduced Root Growth Inhibition in the Presence of Exogenous Cytokinin and Increased Leaf Venation

The enhanced response of *pls* mutant roots to exogenous BA suggested that the *PLS* gene can negatively regulate cytokinin responses. A prediction would be that seedlings overexpressing the wild-type *PLS* gene would exhibit reduced responses to cytokinin.

To test this possibility, several independent transgenic lines (Columbia ecotype background) were produced that contained a partial (3' region of the *PLS*) cDNA of 270 bp, which included the 9- and 36-amino acid ORFs, under the

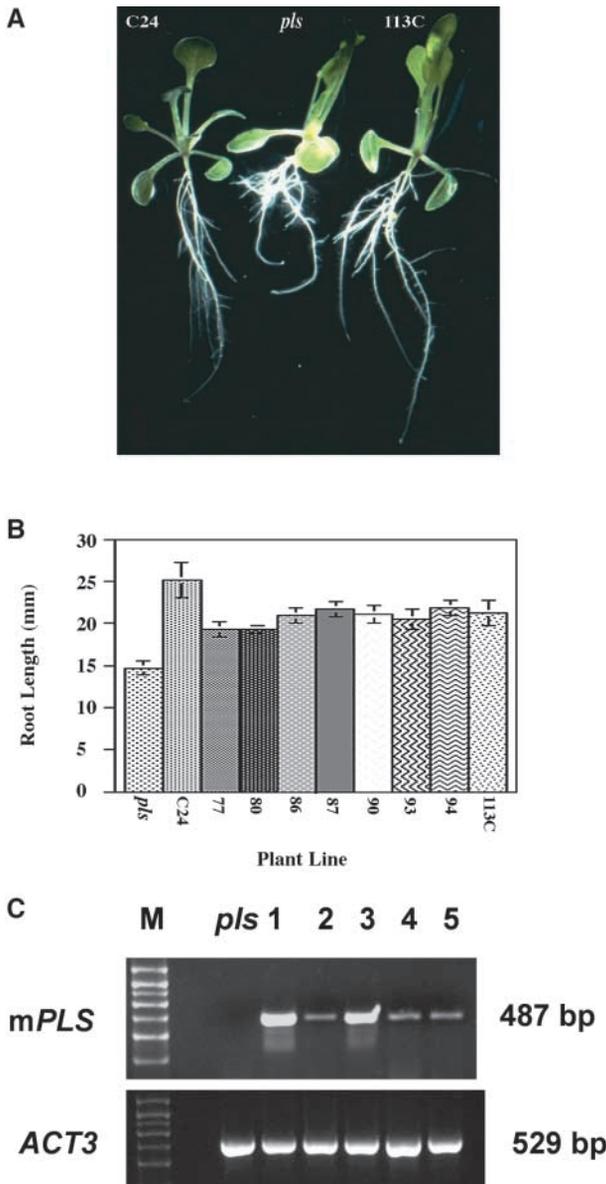


Figure 7. Complementation of *pls* with the *PLS* ORF-Containing Partial cDNA.

(A) Fourteen-day-old seedlings of the wild type (C24), *pls* mutant, and complementation line 113C showing restoration of wild-type root architecture to the complemented mutant.

(B) Primary root length of *pls*, the wild type (C24), and eight independent complementation lines (77 to 113C) containing the partial *PLS* cDNA at 9 days after germination. All lines show significantly longer roots than the *pls* mutant.

(C) RT-PCR analysis of seedlings of the *pls* mutant (*pls*) and transgenic lines mPLS ORF 1 to 5 (lanes 1 to 5, respectively). The top gel shows amplification of the 487-bp mPLS ORF cDNA from RNA extracted from each of the five mPLS ORF transgenic lines but not from the *pls* mutant. The bottom gel shows amplification of the control *ACT3* cDNA in *pls* and each mPLS ORF transgenic line. Control experiments lacking reverse transcriptase showed no amplification products (data not shown). M, molecular mass markers (kD).

transcriptional control of the 35S promoter of *Cauliflower mosaic virus*. The short cDNA was used to determine whether the ORF-containing region of the transcript, rather than the entire cDNA, was sufficient for biological activity. Root length in the presence of exogenous BA was measured as an indicator of sensitivity to cytokinin.

T4 seedlings of each of five independent transgenic lines overexpressed *PLS* transcript to relatively high levels (up to ~100-fold) compared with the wild type (Figure 8A). Wild-type seedlings and T4 *PLS* overexpressers were grown for 7 days in the presence and absence of exogenous cytokinin. When grown in the absence of exogenous BA, there was no significant difference in primary root length between the control wild-type and transgenic seedlings (mean of 17.9 ± 2.4 mm [$n = 20$] for wild-type compared with 20.6 ± 1.9 mm [$n = 100$] for the overexpressers).

However, each of the *PLS* overexpressers exhibited significantly longer roots when grown in the presence of BA (Figure 8B). For example, on 1 μ M BA, line 59 showed a mean root length of 7.6 ± 0.2 mm compared with 5.3 ± 0.3 mm for the wild type ($n = 20$; $P = 5.4 \times 10^{-6}$); that is, the root was 43% longer under these conditions. In particular, the slope of the graph (Figure 8B) suggests that the *PLS* overexpressers had the most reduced response to BA at between 0.5 and 1.0 μ M. This finding demonstrates that the *PLS* gene acts to partially suppress the growth-inhibitory effects of cytokinins.

When grown for 7 days on 10 pM to 5.0 μ M 1-NAA in the light, there was no significant difference in primary root length of the *PLS* overexpressers compared with that of wild-type seedlings (data not shown). However, a comparison of the vascular patterning of rosette leaves of wild-type lines (Columbia ecotype) and the *PLS*-overexpressing transgenic lines (also Columbia background) showed clearly that the overexpressers had more complex venation, with more higher order strands than the wild type (Figure 8C). It should be noted that Columbia ecotype rosette leaves had less complex venation than did leaves of C24 wild-type plants (cf. Figure 2D), so direct comparisons between different ecotypes cannot be made.

These results are consistent with the observation that *pls* mutant leaves had less complex venation than wild-type leaves (Figure 2D). In addition, the *PLS*-overexpressing lines also showed a dramatic loss of leaf trichomes compared with the Columbia wild type (Figure 8C). Although the wild-type Columbia leaves typically had ~30 to 40 trichomes on the adaxial rosette leaf surface under the growth conditions used, the *PLS* overexpressers had none.

DISCUSSION

In this article, we describe the cloning of the Arabidopsis *PLS* gene that, when mutated, results in a short-root phenotype, altered vascular patterning in the leaf, and altered responses to exogenous cytokinins and auxins. Analysis of *pls* mutants, genetic complementation studies, and charac-

Table 2. Root Lengths of *pls* Seedlings, *pls* Transgenic Lines Containing a Wild-Type *PLS* ORF (*PLS* ORF), and Five Independent *pls* Transgenic Lines Containing a Mutated *PLS* ORF ATG Codon (mPLS ORFs) at 7 Days after Germination

	<i>n</i>	Mean Primary Root Length mm \pm SE
<i>pls</i>	20	16.6 \pm 0.6
<i>PLS</i> ORF	20	20.3 \pm 0.8
mPLS ORF1	20	16.9 \pm 0.5
mPLS ORF2	20	16.4 \pm 0.7
mPLS ORF3	13	16.9 \pm 1.1
mPLS ORF4	21	17.7 \pm 0.6
mPLS ORF5	20	16.5 \pm 0.4

n = number of seedlings measured per line. There was no significant difference between the *pls* mutant and any of the five *pls* transgenic lines containing the mutated *PLS* ORF ATG codon.

terization of transgenic overexpressers strongly suggest that the gene encodes a small polypeptide that is required for correct development and cytokinin and auxin responses.

***PLS* Shows Unusual Organization**

Eukaryotic genes typically are separated by noncoding regions containing regulatory elements. However, the *PLS* and *GENE X* transcripts overlap at their respective 5' and 3' UTRs. Partial complementation of the *pls* mutant phenotype was achieved using a genomic clone of *PLS* that lacked the predicted translational start of the *GENE X* ORF. Furthermore, analysis of the pPLS::GUS transgenic lines showed that the *GENE X* coding region contains sequences having promoter activity that correlates with the expression of *PLS* with respect to spatial pattern and auxin inducibility (Figures 3 to 5); *GENE X* and *PLS* encode transcripts of different sizes. It should be noted that this short promoter region is relatively weak (Figure 4C), accounting at least in part for the incomplete complementation, although transgene position effects also might affect the extent of complementation.

A similar gene organization has been reported for the *EhMCM3* and *EhPAK* genes of *Entamoeba histolytica* (Gangopadhyay et al., 1997), which overlap and also may have two transcriptional start sites. The *OTC* and *AUL1* genes of Arabidopsis also have a 22-bp overlap of their 3' UTRs (Quesada et al., 1999). The *CCT8* and *TRP1* genes of *Candida albicans* overlap across the ORFs of both genes (Gerads and Ernst, 1998). Examples of gene overlap also exist in mouse and human (Speek et al., 1996; Koskimies et al., 1997).

Active RNA Versus Polypeptide?

An interesting question is whether the *PLS* gene encodes one or more functional polypeptides or a biologically active RNA molecule. Very few genes have been identified in plants

that encode peptides that function in signaling, but *PLS* adds to the growing list of putative and actual plant peptide factors (reviewed in Lindsey et al., 2002). These include CLAVATA3 (Fletcher et al., 1999), the phytoalexins (Matsubayashi et al., 1999), ENOD40 (Sousa et al., 2001), systemin (Pearce et al., 1991, 2001), the SCR proteins (Schopfer et al., 1999), and analogs of mammalian atrial natriuretic peptides (Billington et al., 1997).

We have yet to detect a *PLS* polypeptide by protein gel blot analysis using polyclonal antibodies to the N-terminal 18 amino acids. However, the genetic evidence demonstrates that a partial *PLS* cDNA that contains only the sequence encoding the three short ORFs (9, 8, and 36 amino acids) is functional and that the functionality of the cDNA requires that the *PLS* ORF has an ATG codon. Therefore, these data suggest that the *PLS* gene encodes a functional polypeptide rather than a biologically active RNA molecule.

The region of 5' UTR between start sites 1 and 2 contains three very small ORFs of six, three, and three amino acids, respectively. Because start site 1 is used relatively infrequently and the ORFs are so small, they may have little functional significance. However, the 5' UTR extending downstream from start site 2 contains two small ORFs of predicted nine and eight amino acids. Upstream ORFs (uORFs) are rare in eukaryote messages. The small sizes of the nine- and eight-residue polypeptides at the *PLS* locus suggest that they are unlikely to represent functional gene products. The position of the uORFs closer to the 5' end of the *PLS* transcript means that they are likely to be identified by the ribosome before the larger *PLS* ORF.

Studies by Sousa et al. (2001) indicate that ORFs smaller than ~30 amino acids may not be translated and do not inhibit the translation of a larger downstream ORF, and certainly the *gusA* ATG in the *PLS* promoter trap obviously is recognized and is functional. A number of studies in both plants (Wang and Wessler, 1998) and other eukaryotes (Kozak, 1989; Oliveira and McCarthy, 1995; Linz et al., 1997) indicate a role for uORFs in the regulation of both transcript stability and the expression of the main ORF. Therefore, the uORFs may play a role in controlling both *PLS* translation and also *PLS* transcript stability.

Polypeptide signaling molecules are common in animals and typically are cleaved from larger proproteins via such enzymes as proprotein convertases, endoproteases, and carboxypeptidases (Canaff et al., 1999). Similar processing enzymes now are known to be required for correct development in plants (Berger and Altmann, 2000; Helliwell et al., 2001; Li et al., 2001). The amino acid sequence and predicted structure of *PLS* suggest processing of the N terminus, with the C terminus mediating interactions with other proteins. Studies using the yeast two-hybrid system suggest that the C-terminal domain of *PLS* interacts with other proteins (P.M. Chilley and K. Lindsey, unpublished data). The potential for cleavage, and the possible interaction with other proteins that might produce an immunologically distinct complex, may explain why the predicted polypeptide was undetectable.

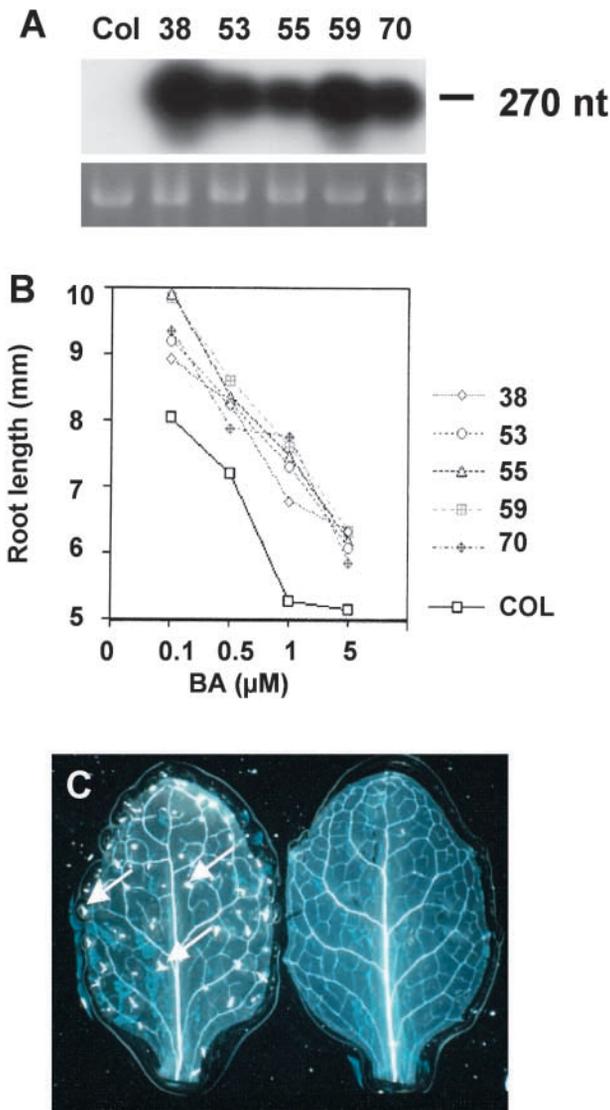


Figure 8. Effects of Overexpressing a PLS ORF-Containing Partial cDNA in Wild-Type Seedlings.

(A) RNA gel blot analysis of wild-type (Columbia [Col]) and five independent transgenic overexpressors of the partial *PLS* cDNA (designated 38 to 70) probed with radiolabeled *PLS* cDNA. 25S RNA is shown as a loading control. Under the exposure conditions used, the autoradiograph does not reveal the low-abundance *PLS* transcript in the wild-type sample. Each lane contained 10 μg of total RNA.

(B) Primary root length of wild-type (Columbia) and five independent *PLS*-overexpressing lines grown for 7 days in the presence of 0.1 to 5 μM BA. Note that wild-type and overexpressing seedlings, grown in the absence of exogenous BA but under otherwise identical conditions, exhibited no significant difference in primary root length.

(C) Rosette leaves of wild-type (left) and *PLS*-overexpressing (right) seedlings 12 days after germination showing the increased frequency of higher order vascular strands and lack of trichomes in the overexpressors. Arrows indicate trichomes.

PLS Gene Expression Is Auxin Inducible

The *PLS* gene promoter, which contains potential TGTCTC-like AuxREs, is activated by functional auxins but not by nonfunctional analogs. This auxin inducibility is consistent with the pattern of *PLS* gene transcription. *PLS* promoter activity is strongest in the root tip, and the root tip is a site of relatively high auxin concentration or sensitivity (Sabatini et al., 1999). GUS histochemistry indicates that the greatest activity of the *PLS* promoter is in the columella initials, similar to that of the auxin-inducible DR5 promoter (Sabatini et al., 1999). *PLS* also is expressed strongly at the site of lateral root initiation (Topping and Lindsey, 1997), a process that is induced by auxin (Celenza et al., 1995; Tian and Reed, 1999). *PLS* also is expressed in aerial organs, albeit at lower levels than in the root. We conclude that auxin is a key component of the signaling system that regulates the spatial patterning of *PLS* expression.

PLS Function

Phenotypic analysis of the *pls* mutant and *PLS* overexpressors indicates that *PLS* is required for correct responses to exogenous cytokinins and auxins, correct root growth, and correct vascular patterning and trichome development of the rosette leaf. The *PLS*-overexpressing transgenic lines (in the Columbia background) were found to lack trichomes, indicating that *PLS* acts as a negative regulator of trichome development in this ecotype, but at present, we do not understand the mechanistic basis for this. Ecotype C24, the background in which the *pls* mutant was identified, has no trichomes, and no effect of the mutation was discernible. Reduced levels of *PLS* expression were found to limit root growth, as seen by the semidominant effect of the mutation.

However, there was not a linear relationship between *PLS* expression and root growth: the *PLS* overexpressors did not have longer roots in the presence of exogenous BA in linear proportion to the level of *PLS* expression. Furthermore, the *PLS* overexpressors did not show enhanced responsiveness to the root-inhibitory effects of exogenous 1-NAA. It is possible that the *PLS* gene product interacts with one or more other components to modulate root growth, which may become rate limiting at high *PLS* concentrations (such as in the *PLS* overexpressors).

Because cytokinins are synthesized in root tips and are at relatively high concentrations in roots (Sossountov et al., 1988; Chiappetta et al., 2001), we can speculate that a mechanism exists to reduce the sensitivity of cells at the root tip to cytokinins to suppress their growth-inhibitory effects. This system may involve the gene *STUNTED PLANT1* (Baskin et al., 1995), and *PLS* also may encode a component of this mechanism.

Supporting this hypothesis are the observations that (1) the *PLS* gene is expressed strongly in root tips, (2) the *pls*

mutant exhibits enhanced root growth inhibition in the presence of the cytokinin BA, (3) overexpression of the partial *PLS* cDNA confers reduced growth inhibition by exogenous BA, and (4) the *p/s* mutant exhibits increased expression of the cytokinin-inducible *ARR5/IBC6::GFP* gene, which is expressed in the root tip (Brandstatter and Kieber, 1998). Therefore, the mutant is altered in its responses to cytokinins, so *PLS* might play a role in suppressing cell sensitivity to cytokinins or in cytokinin biosynthesis.

Plant development involves complex interactions between the "classic five" hormones: cytokinin, ethylene, auxin, gibberellin, and abscisic acid. In the case described here, *PLS* is activated transcriptionally by auxin, and the gene product appears to be required for correct responses to cytokinins and auxin. Only subsets of auxin- and cytokinin-regulated genes are altered in the *p/s* mutant (i.e., *ARR5/IBC6* but not *ARR6/IBC7*, and *IAA1* but not *IAA2*), suggesting specificity of action. Furthermore, both cytokinins and auxin can induce ethylene biosynthesis (Vogel et al., 1998), and both cytokinins and auxin can inhibit root growth at high concentrations (Cary et al., 1995).

It also is possible that the observed increase in cytokinin-mediated responses in the *p/s* mutant may be mediated by downstream ethylene effects or reduced auxin responses, because auxins and cytokinins interact and often have apparently antagonistic effects, such as in shoot branching, root branching, and vascularization (Ljung et al., 2001).

This antagonism with auxin is suggested by the enhanced responsiveness of *p/s* to exogenous cytokinin, the reduced growth inhibition in the presence of exogenous auxins, and the upregulation of the *ARR5/IBC6* gene and the downregulation of the *IAA1* gene in the *p/s* mutant. Because it is known that auxin plays an essential role in the patterning of vascular tissues (Przemeck et al., 1996; Hardtke and Berleth, 1998), possible alterations to auxin-cytokinin interactions in the *p/s* mutant, which are attributable to increased cytokinin levels or sensitivity or reduced auxin levels or sensitivity, could account for the observed reduced vascularization in the *p/s* leaf.

In support of this model of modified cytokinin-auxin responses in the *p/s* mutant, we have found (P.M. Chilly, S.A. Casson, and K. Lindsey, unpublished data) that the *p/s* mutation acts as a phenotypic suppressor of the *rooty/superroot* mutant, which is defective in auxin homeostasis (Boerjan et al., 1995; King et al., 1995). Furthermore, the cytokinin-insensitive *cin5* mutant (Vogel et al., 1998) has a phenotype showing characteristics that contrast with those of the cytokinin-sensitive *p/s* mutant (e.g., no triple response in the presence of exogenous cytokinins in the dark, compared with the enhanced triple response of *p/s* in these conditions; P.M. Chilly, S.A. Casson, and K. Lindsey, unpublished data). The results presented here support the model that the *PLS* gene is required for correct auxin-cytokinin homeostasis to modulate root growth and leaf vascular patterning. Current experiments are aimed at further distinguishing between the effects of *PLS* expression on auxin, cytokinin, and ethylene interactions.

METHODS

Materials and Growth Conditions

The transgenic line AtEM101 (*Arabidopsis thaliana* ecotype C24) contains the promoter trap p Δ gusBin19 (Topping et al., 1991, 1994). *Arabidopsis* seeds transgenic for the *IBC6/ARR5::green fluorescent protein* gene fusion were kindly provided by Joe Kieber (University of North Carolina, Chapel Hill). For in vitro growth studies, seeds were vernalized and surface-sterilized (Clarke et al., 1992) and plated on growth medium (half-strength Murashige and Skoog [1962] medium [Sigma], 1% Suc, and 0.8% agar [Difco, Detroit, MI]) at $22 \pm 2^\circ\text{C}$ at a PPF of $\sim 150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For hormone application experiments, seeds were germinated aseptically on growth medium containing various concentrations of hormones and assayed according to the particular experiment.

Gene Expression Analysis

Tissue localization of β -glucuronidase (GUS) enzyme activity was performed as described (Topping and Lindsey, 1997). For transcript analysis, RNA was extracted using the RNeasy Plant RNA Extraction kit (Qiagen Ltd., Surrey, UK) and the PolyAtract mRNA isolation system (Promega, Southampton, UK). RNA was blotted, hybridized, and probed as described (Wei et al., 1997). RNA markers were Promega G319. *PLS* gene sequences were used to design PCR primers for rapid amplification of cDNA ends (RACE) PCR and to generate a 299-bp sequence for RNase protection assays (RPAs).

RPAs were performed using the RPA III kit (Ambion, Austin, TX) using either 50 μg of total RNA or 5 μg of poly(A)⁺ RNA mixed with 4×10^5 cpm of labeled RNA probe. Reaction samples were separated on a mini denaturing polyacrylamide gel, transferred to Whatman paper, and exposed to x-ray film for 1 to 7 days without drying. RNA probes for RPAs were made using the MAXscript T7/T3 In Vitro Transcription kit (Ambion). Templates for the transcription reaction were prepared by cloning DNA fragments into the pCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands).

For RNA-specific (RS)-PCR, the following oligonucleotide primers were used: 5'-CTTATACGGATATCCTGGCAATTCGGACTTGAT-AGGGTGATCAATGGA-3' (the underlined region is complementary to the 3' end of the *PLS* transcript), 5'-CTTATACGGATATCCTGGCAATTCGGACTT-3', and 5'-GGAGACTAAAGCGAACATATAAAACC-3'. Genes for RNA gel blot analysis were isolated by reverse transcription (RT)-PCR from RNA isolated from 7-day-old C24 seedlings. Reverse transcription was performed with an oligo(dT) primer using 15 μg of total RNA essentially as described previously (Althorpe et al., 1999).

Primers used for amplification were as follows: for *ARR5/IBC6*, 5'-CACGAGTCACGATCCTACTC-3' and 5'-CAGGACATGCATGTGTGTG-3'; for *ARR6/IBC7*, 5'-CATCGAGAGATTGCTTCG-3' and 5'-CGACGACGACGTC AACAC-3'; for *IAA1*, 5'-CTTAAGGACACAGAGCTTCG-3' and 5'-GATCCTTTCATGATTCTGAG-3'; and for *IAA2*, 5'-GAGGCAATAGAGATGGAC-3' and 5'-GTCTAGAGCAGGAGCGTCG-3'. For mutant (m)*PLS* open reading frame (ORF) RT-PCR, the primers 5'-TATCTAGACCTTTATCGAGACATAAGATTG-3' and 5'-ATGGATCCACTTAATATATTAGTATTGG-3' were used; and for *ACT3*, ACT For (5'-GATCCTAACCGAGCGTGGTTAC-3') and ACT Rev (5'-GACCTGACTCGTCATACTCTGC-3') were used.

m*PLS* ORF and *ACT3* RT-PCR reaction conditions were 94°C for 2 min followed by 30 cycles of 94°C denaturation for 30 s, 60°C primer

annealing for 30 s, 72°C extension for 60 s, and a final extension at 72°C for 7 min. RNA gel blot analysis was performed using 50 µg of total RNA isolated from 7-day-old wild-type and *pls* seedlings.

Gene Cloning and DNA Analysis

The Arabidopsis Columbia λGEM 11 genomic library (Ronald Davis, Stanford, University, Stanford, CA) was provided by Jeff Dangl (formerly of the European Commission Arabidopsis T-Project DNA Centre, Köln, Germany) and screened as described previously (Wei et al., 1997). Arabidopsis DNA was extracted using cetyl-trimethyl-ammonium bromide (Ausubel et al., 1996) purified with the Qiagen genomic-tip 100/G, as described previously (Wei et al., 1997). For 3' and 5' RACE-PCR cDNA synthesis, the oligo(dT) primer was 5'-CCAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTT-3'. 5' RACE-PCR of the *PLS* transcript was performed on poly(A)⁺ and total RNA from 7-day-old seedlings using primers 5'-CCAGGTGTTCCGGCGTGGTGTAGAGC-3' (for the GUS fusion transcript) and 5'-GGTTTCATT-CATGTTTCAGTGAG-3'. Products were cloned as described previously (Wei et al., 1997).

3' RACE of the *PLS* transcript was performed on total RNA from 7-day-old seedlings using primers 5'-CCAAGCTTCTGCAGGAGCTC-3' (3' RACE anchor primer) and 5'-GGAACACGAAATCCGAAGAGC-GAG-3' as follows: 94°C for 2 min followed by 35 cycles of 94°C denaturation for 30 s, 60°C primer annealing for 30 s, 72°C extension for 45 s, and a final extension at 72°C for 7 min. DNA sequencing was performed using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) and dye terminator labeling reactions as described previously (Wei et al., 1997). DNA was prepared for DNA gel blot analysis and PCR amplification from ~1 g wet weight of plant tissue using the Phytopure kit (Nucleon Biosciences, Lanarkshire, UK). DNA gel blot analyses were performed as described (Wei et al., 1997).

Nucleotide and deduced protein sequences were used to search for homologies in the National Center for Biotechnology Information peptide sequence databases using the BLAST network service (Altschul et al., 1990). Protein motif homologies were determined using the Scan-Prosit program (<http://www.expasy.ch/tools/scnpsit1.html>). The putative *PLS* polypeptide secondary structure was modeled using the Protean program (Lasergene Navigator; DNASTar, Madison, WI).

Competitor RS-PCR

Construction of the *PLS* Competitor

A *Clal* restriction site was introduced into *PLS* by PCR. This also mutated the *PLS* ORF ATG codon for functional analysis of the ORF in transgenic studies. Using a plasmid genomic clone of the *PLS* locus, two overlapping fragments of the *PLS* gene were produced by PCR. The first fragment of ~300 bp was amplified with the primers BamHI Transc. For (5'-ATGGATCCACTTAATATATTAGTATTGG-3') and *Clal* Mut Rev (5'-CTGGGTTATCGATTTCATGTTTCAGTGAGAC-3'); the engineered *Clal* site is underlined, and the second fragment of ~150 bp was amplified with the primers *Clal* Mut For (5'-CATGAATCG-ATAACCCAGACTTTGTTTAAATTCAG-3') and XbaI Transc. Rev (5'-TATCTAGAGTAGATATTGAAAATGATAGG-3').

The fragments were digested with *Clal*, ligated, and used to amplify a mutant form of *PLS* carrying a *Clal* site using the primers BamHI Transc. For and XbaI Transc. Rev. Sequencing confirmed the inclusion of a *Clal* site. The *PLS* competitor was cloned into the

pCR2.1-TOPO vector (Invitrogen) in the sense orientation with respect to the T7 RNA polymerase promoter. In vitro transcription of the *PLS* competitor was performed using the Maxiscript kit (Ambion) according to the manufacturer's instructions. Dilutions of the *PLS* competitor RNA were titrated against a fixed quantity of total RNA (10 µg), according to the method described below, to determine the dilution at which the quantity of both *PLS* and competitor were approximately the same after PCR amplification.

DNase Treatment of RNA

DNase treatment of RNA for PCR amplification was based on the method of Sanyal et al. (1997). To 10 µg of total RNA was added 3 µL of the *PLS* competitor RNA (between 1×10^5 and 1×10^6 dilution), followed by 2 µL of 5 × RT buffer (supplied with reverse transcriptase from *Moloney murine leukemia virus*; Promega) and 2 units of RQ1-DNase (RNase free; Promega). The volume was made up to 10 µL, and the mixture was incubated at room temperature for 15 min. DNase was inactivated by the addition of 1 µL of 25 mM EGTA, pH 8.0, and heating at 65°C for 10 min.

Reverse Transcription

For reverse transcription, 2 µL of the primer POL-RS-PCR (5'-CTTATACGGATATCCTGGCAATTCGGACTTGATAGGGTGATCAAT-GGA-3') (10 pmol/µL) was added to the DNase RNA, heated for 10 min at 70°C, and placed on ice. To a RT master mixture (3 µL of 5 × *Avian myeloblastosis virus* buffer, 2 µL of deoxynucleotide triphosphate [12.5 mM], 1 µL [40 units] of RNasin, 2 µL [20 units] of reverse transcriptase from *Moloney murine leukemia virus*, and 6.5 µL of water) was added 6.5 µL of the RNA:primer mixture, and the reaction was incubated at 42°C for 1 h. A negative RT reaction was performed on the remaining RNA:primer mixture. cDNA was purified using the High Pure Product Purification kit (Roche Biochemicals, Mannheim, Germany) and eluted in a volume of 50 µL.

PCR

Five microliters of the eluted cDNA was used as a template for PCR amplification using the primers (10 pmol/µL) 5'TEST (5'-GGAGAC-TAAAGCGAACATATAAAACC-3') and RS-PCR AD (5'-CTTATACGG-ATATCCTGGCAATTCGGACTT-3'). Hot-start PCR was used with 40 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 60 s, and a final extension of 72°C for 10 min.

Purification and Digestion of PCR Products

PCR products were purified using the High Pure Product Purification kit (Roche Biochemicals) and eluted in a volume of 100 µL. The products then were precipitated in ethanol (one-tenth volume of sodium acetate 4M, pH 6, and 2.5 volumes of ethanol) and resuspended in 10 µL of water. Digestion was performed in a total volume of 25 µL of 1 × reaction buffer and 10 units of *Clal* at 37°C for 2 h. Products then were analyzed by electrophoresis on a 2% TAE gel stained with ethidium bromide.

PCR Amplification of *ACTIN3* as a Loading Control

To determine that RNA loading was correct for each reaction, PCR amplification of the *ACTIN3* cDNA was performed. DNase treatment

of the RNA was performed as described. Reverse transcription was performed with an oligo(dT)₁₅ primer, and the cDNA was purified as described. Five microliters of the eluted cDNA was used as a template for PCR amplification using the primers ACT For and ACT Rev (10 pmol/μL). Hot-start conditions were used with 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 10 min.

Gene Constructs and Plant Transformation

Promoter fragments were amplified using the Expand High Fidelity PCR system and ligated into T-tailed pBluescript SK⁻ or pCR2.1-TOPO vector (Invitrogen), then transferred to pΔGUS (Topping et al., 1991). The promoter-GUS-nopaline synthase terminator cassette was transferred as a HindIII-EcoRI fragment into the binary vector pCIRCE (a gift from M. Bevan, John Innes Centre, Norwich, UK). For complementation analysis, a 1.15-kb DNA fragment, cognate to the *PLS* transcript and native promoter region, was amplified from a 3.6-kb genomic clone, introduced into the pCR2.1-TOPO vector (Invitrogen), and then transferred to the binary vector pMOG1006 (a gift from Mogen, Leiden, The Netherlands). For overexpression studies, a 270-bp partial *PLS* cDNA containing the 36-amino acid ORF was cloned behind the 35S promoter of *Cauliflower mosaic virus* in pCIRCE. All constructs were validated by sequencing. Plant transformation was performed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* C58C3 (Dale et al., 1989).

Microscopy

Tissues were cleared and mounted for light microscopy in chloral hydrate (Topping and Lindsey, 1997) or 20% glycerol. To reveal leaf vascular tissues, leaves were cleared by incubating at 70°C for 30 min in 90% ethanol, followed by incubation at 70°C for 30 min in lactic acid:phenol:glycerol:water (1:1:1:1) and viewed under dark-field illumination (Telfer and Poethig, 1994). Photographs were taken on Ektachrome 160 tungsten-balanced film using Nikon Optiphot (Tokyo, Japan), Zeiss Axioskop (Jena, Germany), and Olympus SZH10 (Tokyo, Japan) microscopes. Confocal images were taken with a Bio-Rad Radiance 2000 microscope after counterstaining of tissues with 10 μg/mL propidium iodide. Images were processed in Adobe Photoshop 5.0 (Mountain View, CA).

Accession Number

The GenBank accession number for the predicted *PLS* transcript is AF285768.

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The *POLARIS* Gene of *Arabidopsis* Encodes a Predicted Peptide Required for Correct Root Growth and Leaf Vascular Patterning

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