

The POLARIS Peptide of *Arabidopsis* Regulates Auxin Transport and Root Growth via Effects on Ethylene Signaling ^{OA}

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The rate and plane of cell division and anisotropic cell growth are critical for plant development and are regulated by diverse mechanisms involving several hormone signaling pathways. Little is known about peptide signaling in plant growth; however, *Arabidopsis thaliana* POLARIS (PLS), encoding a 36–amino acid peptide, is required for correct root growth and vascular development. Mutational analysis implicates a role for the peptide in hormone responses, but the basis of PLS action is obscure. Using the *Arabidopsis* root as a model to study PLS action in plant development, we discovered a link between PLS, ethylene signaling, auxin homeostasis, and microtubule cytoskeleton dynamics. Mutation of PLS results in an enhanced ethylene-response phenotype, defective auxin transport and homeostasis, and altered microtubule sensitivity to inhibitors. These defects, along with the short-root phenotype, are suppressed by genetic and pharmacological inhibition of ethylene action. PLS expression is repressed by ethylene and induced by auxin. Our results suggest a mechanism whereby PLS negatively regulates ethylene responses to modulate cell division and expansion via downstream effects on microtubule cytoskeleton dynamics and auxin signaling, thereby influencing root growth and lateral root development. This mechanism involves a regulatory loop of auxin–ethylene interactions.

INTRODUCTION

Organogenesis in plants is coordinated by complex interactions between diverse signaling systems, leading to changes in the rate and plane of cell division and in cell expansion. The activities of plant hormones, such as the auxins, cytokinins, ethylene, gibberellins, and abscisic acid, depend on cellular context and exhibit interactions that can be either synergistic or antagonistic. For example, auxin can suppress cytokinin biosynthesis (Nordström et al., 2004), auxin and cytokinin can act synergistically to induce ethylene biosynthesis (Vogel et al., 1998), and ethylene can modify auxin responses and meristem function (Morgan and Gausman, 1966; Suttle, 1988; Visser et al., 1996; Haver et al., 2002; Vandebussche et al., 2003; Souter et al., 2004; Stepanova et al., 2005). Depending on the exposure, ethylene can either inhibit or promote cell division and influence cell fate (Kazama et al., 2004), and in part it acts through interactions with DELLA proteins (Achard et al., 2003). The roles of

actin and tubulin components are also receiving much attention, both as being modified by hormones and signaling pathways (Lang et al., 1982; Cyr, 1991; Lloyd et al., 1996; Gardiner et al., 2001; Hussey, 2004) and as themselves being implicated as regulators of hormonal signaling systems (Geldner et al., 2001).

Our understanding of the molecular mechanisms that mediate developmental responses to hormones has improved enormously in recent years through the identification of mutants in *Arabidopsis thaliana*. These include mutants in hormone biosynthesis, perception, and signal transduction, and many genes have been identified that are transcriptionally upregulated or downregulated in response to hormones. However, despite these very significant advances in understanding signal transduction mechanisms in plants, we still have an incomplete picture of how the different signaling pathways interact to elicit particular developmental responses.

One question we are interested in is how hormones regulate the activity of the root meristem (Casson and Lindsey, 2003). Auxin is transported to the root tip and redistributed there by the polar auxin transport pathway, involving the activity of the AUX1 auxin influx carrier (Bennett et al., 1996; Ljung et al., 2001) and the PIN-FORMED (PIN) proteins, representing components of the efflux carrier system (Gälweiler et al., 1998; Luschnig et al., 1998; Friml et al., 2002, 2003; Bilou et al., 1998; Weijers et al., 2005). Auxin is also a positive regulator of lateral root formation after its redistribution from the root tip, thereby affecting the architecture of the root system (Marchant et al., 2002; Casimiro et al., 2003). Given that the auxin concentration is relatively high in the root tip

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(Ljung et al., 2001) and cytokinin is synthesized there (Miyawaki et al., 2004), and that both auxin and cytokinin can induce ethylene synthesis, we hypothesize that there must be a negative regulatory system in place to suppress ethylene responses that would otherwise reduce cell division and axial cell elongation and so inhibit root growth. We previously found, for example, that sterol mutants exhibiting enhanced ethylene signaling responses in the root have defective root meristem patterning and growth and that both defects can be rescued by the genetic or pharmacological inhibition of ethylene signaling (Souter et al., 2004). This finding suggests that correct root meristem function requires a tight control over ethylene responses in the root tip.

To better understand the molecular basis of plant cell division and expansion, we have used a genetic approach to identify and characterize mutants showing defective development in the root. One such mutant, identified in a screen of promoter trap transgenic plants, is the *polaris* (*p/s*) mutant (Topping et al., 1994; Topping and Lindsey 1997). Of interest is the observation that the *PLS* gene transcribes a short mRNA (~500 nucleotides in length) encoding a predicted 36-amino acid peptide, translation of which is essential for biological activity (Casson et al., 2002).

Seedlings mutant for the *PLS* gene show a semidominant phenotype, characterized by relatively short and radially expanded cells in the root with reduced division, leading to short roots; reduced leaf vascularization; and altered responses to exogenous auxins and cytokinins (Casson et al., 2002). *p/s* mutants show a partial rescue of the short-root phenotype in the presence of low (picomolar) concentrations of auxin and reduced growth inhibition by exogenous auxin compared with wild-type plants. Consistent with this reduced inhibition of root growth by auxin, seedlings show reduced expression of the auxin-regulated *INDOLE-3-ACETIC ACID1* (*IAA1*) gene in the absence of exogenous auxin.

To investigate the function of the *PLS* gene further, we used a combination of biochemical and genetic approaches to characterize the hormonal interactions in the *p/s* mutant. We show that the *PLS* gene is required for correct control of several ethylene-mediated responses, including growth in the dark, polar auxin transport, auxin homeostasis, and microtubule dynamics. These pathways require *PLS* for their integration and for two aspects of root development: growth (cell division and elongation) and architecture (lateral root formation).

RESULTS

p/s Is Defective in Ethylene Signaling

It is known that enhanced ethylene responses reduce axial growth in light-grown seedlings (Guzman and Ecker, 1990; Abeles et al., 1992; Kieber et al., 1993) and could potentially contribute to the short-root phenotype of the *p/s* mutant. Therefore, we grew *p/s* seedlings in the dark to determine whether they exhibited an abnormal etiolation response, also typical of ethylene effects on seedlings. We found that enhanced ethylene signaling is a key determinant of the short-root phenotype of the *p/s* mutant. *p/s* seedlings were found to exhibit a triple-response phenotype (a phenocopy of the ethylene response in the dark), the phenotype being similar to, but less severe than, that of the

ethylene-overproducing *eto1* (Figure 1A) or the constitutive triple response mutant *ctr1-1* (Figure 1B). To confirm the enhanced ethylene response in *p/s*, we measured the abundance of the ethylene-inducible gene transcript, *At GSTF2* (Zhou and Goldsborough, 1993; Smith et al., 2003) by RNA gel blot analysis and found it to be greater in air-grown *p/s* compared with the wild type (Figure 1C). We similarly found that expression of the primary ethylene response gene *ERF10*, which encodes a transcription factor predicted to bind the core sequence of the ethylene-responsive promoter element (Ohta et al., 2001), is increased in air-grown *p/s* seedlings (Figure 1D).

To determine whether the short-root phenotype of *p/s* can be rescued by genetic or pharmacological inhibition of ethylene signaling, *p/s* seedlings were either crossed with the gain-of-function ethylene-insensitive receptor mutant *etr1-1* (Figure 2A) or treated with 1 μ M silver ions, which inhibit ethylene signaling (Figure 2B). Silver ions may modify the conformation of ETR1 or the signal propagation to the kinase domain of the receptor (Rodriguez et al., 1999). In both cases, the length of the *p/s* primary root was restored to approximately wild-type levels. The *etr1-1* mutation was also able to suppress the triple-response phenotype of dark-grown *p/s* seedlings (Figure 2C). These data show that the *p/s* mutant exhibits enhanced ethylene responses and that this determines the short-root phenotype. This finding implicates *PLS* as a negative regulator of ethylene responses. The failure of the *p/s* mutation to suppress the *etr1-1* mutation suggests that *PLS* may act at or upstream of the ETR1 ethylene receptor.

To determine whether the enhanced ethylene responses of *p/s* are attributable to increased ethylene biosynthesis (i.e., upstream of the ethylene receptor), ethylene evolution assays were performed using gas chromatography. These experiments revealed no significant difference in ethylene production between *p/s* and wild-type seedlings (Figure 3A). Levels produced were much lower than for *eto1-4* positive controls, which are ethylene overproducers. Moreover, we found that *PLS* action does not require 1-aminocyclopropane-1-carboxylic acid (ACC) synthase5 (*ACS5*), an essential enzyme catalyzing the first committed step in cytokinin-mediated ethylene biosynthesis encoded by *ACS5/CIN5* (Vogel et al., 1998). *cin5* mutants are cytokinin-insensitive and fail to produce ethylene in response to low cytokinin concentrations, and they show no cytokinin-induced triple response in the dark (Figure 3B). However, both the *p/s* mutant and the *p/s cin5* double mutant showed a strong triple response in the presence of cytokinin, demonstrating that the *p/s* mutation promotes ethylene signaling responses in the absence of *ACS5/CIN5* function. Finally, no rescue was achieved by treatment of *p/s* seedlings with the ethylene biosynthesis inhibitor 10 μ M aminoethoxyvinylglycine (data not shown). Previously, we showed that *p/s* seedlings did not exhibit increased sensitivity to ethylene (or its precursor, ACC) (Casson et al., 2002).

Further evidence for a role for *PLS* in ethylene signaling comes from the analysis of transgenic plants strongly overexpressing the *PLS* peptide-encoding open reading frame of the *PLS* cDNA, described previously (Casson et al., 2002). Dark-grown *PLS* overexpressers failed to show the triple-response phenotype of *p/s* seedlings; instead, they developed a longer root and reduced apical hook compared with wild-type plants, similar to the

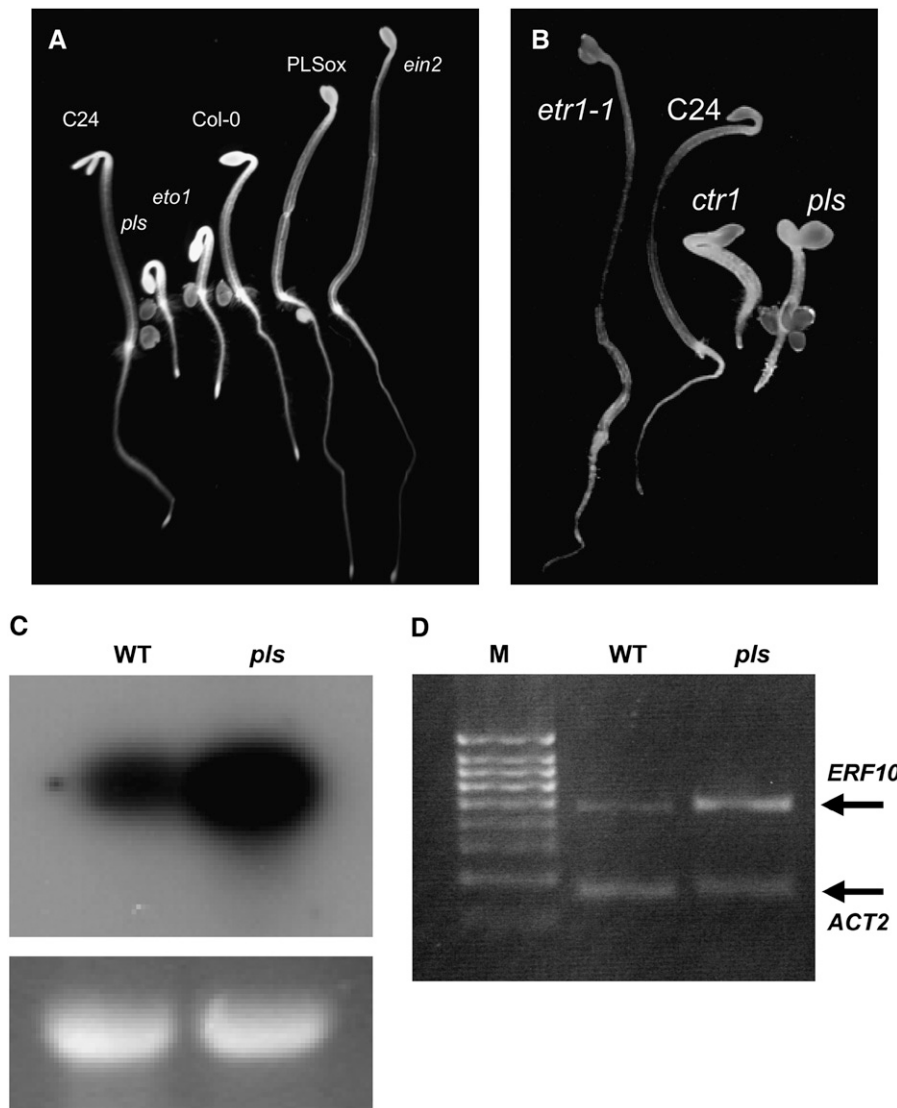


Figure 1. The *PLS* Gene Regulates Ethylene Responses.

(A) Representative seedlings of the wild type (C24 and Col-0 [for Columbia]), *pls*, *eto1-1*, *PLS* transgenic overexpressor (*PLSOx*), and *ein2* grown in the dark in air, showing the triple-response phenotype of *eto1-1* and *pls* and the etiolated phenotypes of the wild-type, *ein2*, and *PLSOx* seedlings.

(B) Representative seedlings of the wild type (C24), *etr1-1*, *ctr1*, and *pls* grown in the dark in air, showing the triple-response phenotype of *ctr1* and *pls* and the etiolated phenotypes of the wild-type and *etr1-1* seedlings.

(C) Top, RNA gel blot analysis showing increased accumulation of the ethylene-inducible *At GSTF2* mRNA in air-grown *pls* seedlings compared with wild-type seedlings. Bottom, RNA loading control (ethidium bromide-stained 28S rRNA). A total of 10 μ g of RNA was loaded per lane.

(D) Semiquantitative RT-PCR of the ethylene-inducible *ERF10* transcript in 7-d-old wild-type and *pls* seedlings. Amplification of *ACT2* as an RNA loading control is shown. M, RNA size markers.

ethylene-insensitive *ein2* (Figure 1A). In addition, although *PLS* overexpressors produce roots ~ 10 to 15% longer than wild-type plants under standard growth conditions (e.g., 19.8 ± 0.4 mm [$n = 20$] for overexpressor versus 17.9 ± 1.3 mm [$n = 20$] for the wild type), they maintain ~ 60 to 90% longer primary roots than wild-type plants when grown in the presence of the growth-inhibitory ethylene precursor ACC over a wide range of concentrations (Figure 3C). Therefore, the *PLS* gene acts to suppress the growth-inhibitory effects of ethylene signaling.

To investigate the point of *PLS* action in the ethylene signaling pathway, we crossed *PLS* overexpressors with the *ctr1-1* mutant. The rationale was that, if *PLS* functions downstream of *CTR1*, then *PLS* overexpression would be predicted to suppress the *ctr1* mutant phenotype, whereas if *PLS* acts upstream of *CTR1*, at the receptor level, the *ctr1* mutation would suppress the phenotype conferred by *PLSOx*. The results show that *PLSOx/ctr1* has an intermediate phenotype (i.e., longer roots in both light- and dark-grown seedlings than *ctr1* but much shorter

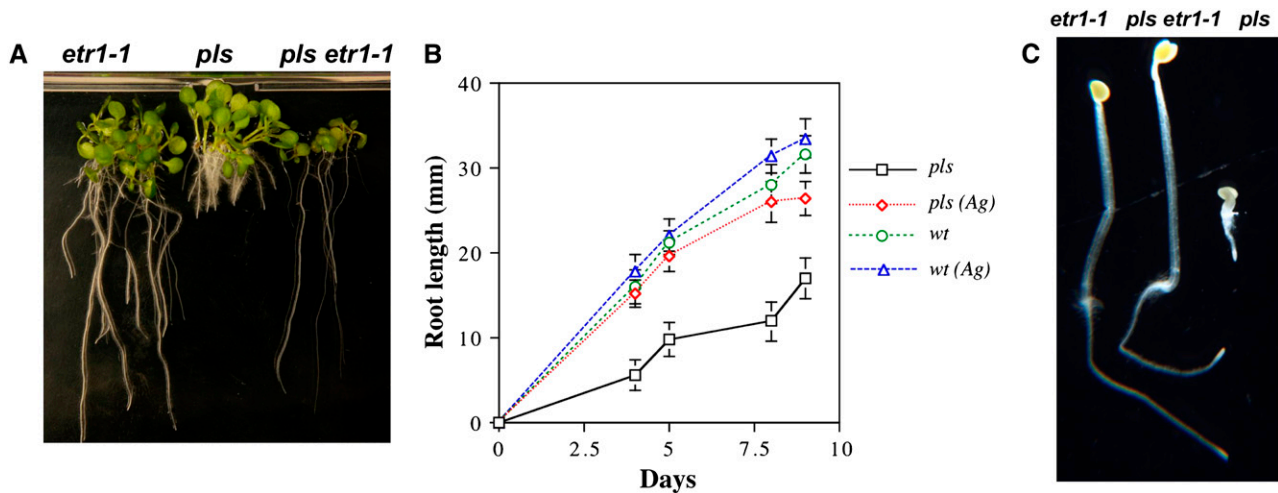


Figure 2. Restoration of *pls* Root Growth by Inhibition of Ethylene Signaling.

(A) Representative light-grown seedlings of *etr1-1*, *pls*, and the *pls etr1-1* double mutant, showing rescued root growth in the double mutant.

(B) Rescue of primary root growth in *pls* seedlings treated with 1 μ M silver ions [*pls* (Ag)], which inhibit ethylene signaling by modifying ETR1 conformation or signal propagation to the kinase domain of the receptor (Rodríguez et al., 1999). Error bars represent SE; $n = 10$.

(C) Representative dark-grown seedlings of *etr1-1*, *pls*, and the *pls etr1-1* double mutant in air, showing suppression of the triple response of *pls* in the double mutant.

than PLSOx) (Figures 3D and 3E). This finding suggests that there is not complete epistasis: PLS overexpression may be considered to partially suppress *ctr1*, or *ctr1* may partially suppress PLSOx.

If PLS acts downstream of CTR1, it would be expected that the roots and hypocotyls of PLSOx/*ctr1* seedlings grown in the dark in air would be longer than those of PLSOx seedlings grown in the presence of ethylene or saturating concentrations of ACC. The results shown in Figure 3F demonstrate that, in fact, the roots and hypocotyls of PLSOx/*ctr1* seedlings grown in the dark are $\sim 50\%$ shorter than those of PLSOx seedlings grown on 100 μ M ACC, and the hypocotyl is also shorter, suggesting that CTR1 acts downstream of PLS. By contrast, when grown in the light, air-grown PLSOx/*ctr1* seedlings have longer roots and hypocotyls than PLSOx seedlings grown on 100 μ M ACC (cf. seedlings in Figures 3D and 3G). When grown in the presence of 100 μ M ACC in the light, PLSOx/*ctr1* and PLSOx seedlings have identical phenotypes (Figure 3G). Thus, the response of *ctr1* to ACC, which leads to some hypocotyl shortening (Resnick et al., 2006), is unaffected by PLS overexpression, and the effect of PLS may be light-dependent.

Together these results suggest that PLS and CTR1 interact to modulate growth in response to ethylene, but they do not act in a single linear pathway.

Enhanced Ethylene Signaling in *pls* Represses Auxin Transport and Accumulation

It is known that ethylene can inhibit auxin transport (Suttle, 1988), so the enhanced ethylene signaling in *pls* could cause the reduced auxin responses that we observed previously (Casson et al., 2002). Root architecture is known to be affected by auxin,

and the *aux1* mutant has defective polar auxin transport, reduced auxin levels in the root tip, and reduced lateral root formation (Bennett et al., 1996; Ljung et al., 2001). Similarly, *pls* seedlings initiated $\sim 40\%$ of the number of lateral roots compared with wild-type plants (Figure 4A). To determine whether auxin levels are also affected in *pls*, the free IAA content of *pls* was measured and found to be significantly lower than in wild-type plants (Figure 4B). In particular, the peak in root auxin accumulation seen in wild-type seedlings at day 7 was not seen in *pls* seedlings. Free IAA concentrations were also $>70\%$ lower in older *pls* plants (31 ± 2 ng/100 mg dry weight [$n = 5$]; growth stage 6.0 to 6.5) (Boyes et al., 2001) than in wild-type plants (114 ± 8 ng/100 mg dry weight [$n = 5$]). In agreement with a link between PLS and auxin accumulation, transgenic PLS overexpressors showed higher free IAA concentrations than the *pls* mutant (Figure 4C).

To investigate further the role of PLS in auxin-regulated root architecture, double mutants were made by crossing the *pls* mutant with the *rooty* (*rtv*) mutant, which is characterized by high endogenous levels of auxin and the production of supernumerary lateral roots and adventitious roots on the hypocotyl (King et al., 1995) (Figures 5A and 5B). The *rtv pls* double mutants produced fewer ($<10\%$) and less elongated lateral roots than the *rtv* mutant, indicating that the *pls* mutation acts to partially suppress the phenotype conferred by *rtv* (Figures 5A and 5B). This finding is consistent with the observation that *pls* seedlings produce fewer lateral roots per unit length than wild-type plants and exhibit a low-auxin phenotype.

To determine whether there is a link between the enhanced ethylene responses in *pls* and the effects on the phenotype conferred by *rtv*, *rtv* seedlings were grown in the presence of the ethylene precursor ACC. The results presented in Figure 5C

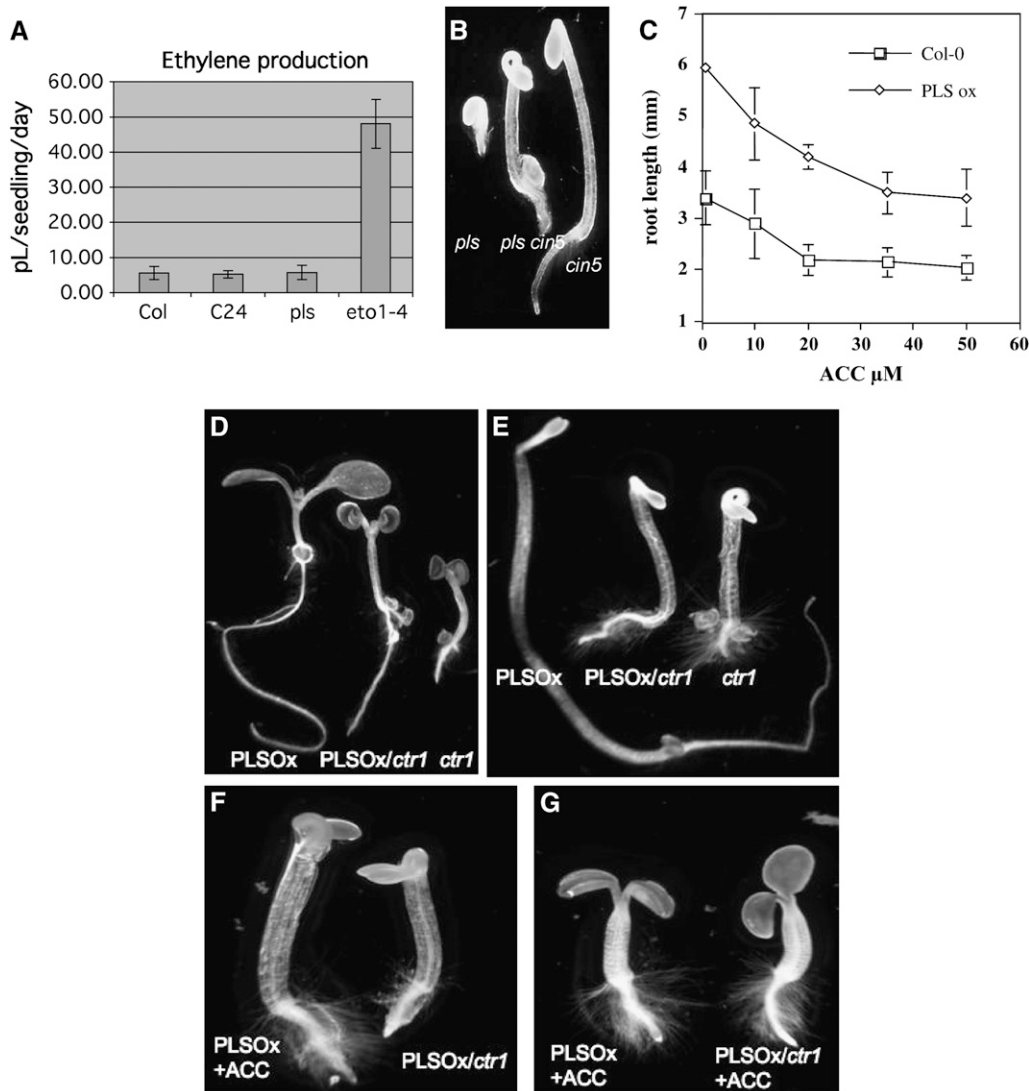


Figure 3. *pls* Is Not an Ethylene Biosynthesis Mutant.

(A) Ethylene evolution by wild-type (Col and C24), *pls*, and *eto1-4* seedlings. Error bars represent SD; $n = 6$.

(B) Phenotypes of representative seedlings of *pls*, *pls cin5*, and *cin5* mutants grown in the dark for 3 d in the presence of 0.5 μ M kinetin, showing suppression of the *cin5* phenotype by *pls* in the double mutant.

(C) Effect of growth for 7 d on ACC on primary root length of dark-grown seedlings of the wild type (Col-0) and PLS open reading frame-overexpressing line 38. The first data point is 0.5 μ M ACC. Error bars represent SE; $n = 10$.

(D) Phenotypes of representative seedlings of light-grown PLSOx (left), *ctr1-1* (right), and PLS overexpresser carrying the *ctr1-1* mutation (PLSOx/*ctr1*; middle).

(E) Phenotypes of representative seedlings of dark-grown PLSOx (left), *ctr1-1* (right), and PLSOx/*ctr1* (middle).

(F) Representative seedlings of dark-grown PLSOx (left) in the presence of 100 μ M ACC and PLSOx/*ctr1* (right) grown in air.

(G) Representative seedlings of light-grown PLSOx (left) in the presence of 100 μ M ACC and PLSOx/*ctr1* (right) grown in 100 μ M ACC.

show that treatment of *rtv* seedlings with 100 μ M ACC phenocopies the *rtv pls* double mutant, consistent with the notion that the effects of the *pls* mutation on *rtv* are mediated by ethylene signaling. Similarly, double mutants between *rtv* and the ethylene-overproducing mutant *eto1-1* (Chae et al., 2003) also show reduced lateral roots (Figure 5D). These data together show that ethylene inhibits auxin responses (i.e., suppresses the initiation

of new lateral roots by auxin) and are consistent with the view that defective ethylene responses in *pls* cause defects in auxin homeostasis.

To determine whether the low auxin levels in *pls* are attributable to defective transport from the shoot (a major site of synthesis), the transport of [3 H]IAA was measured in isolated inflorescence stem tissue. It was found that the *pls* mutant has a

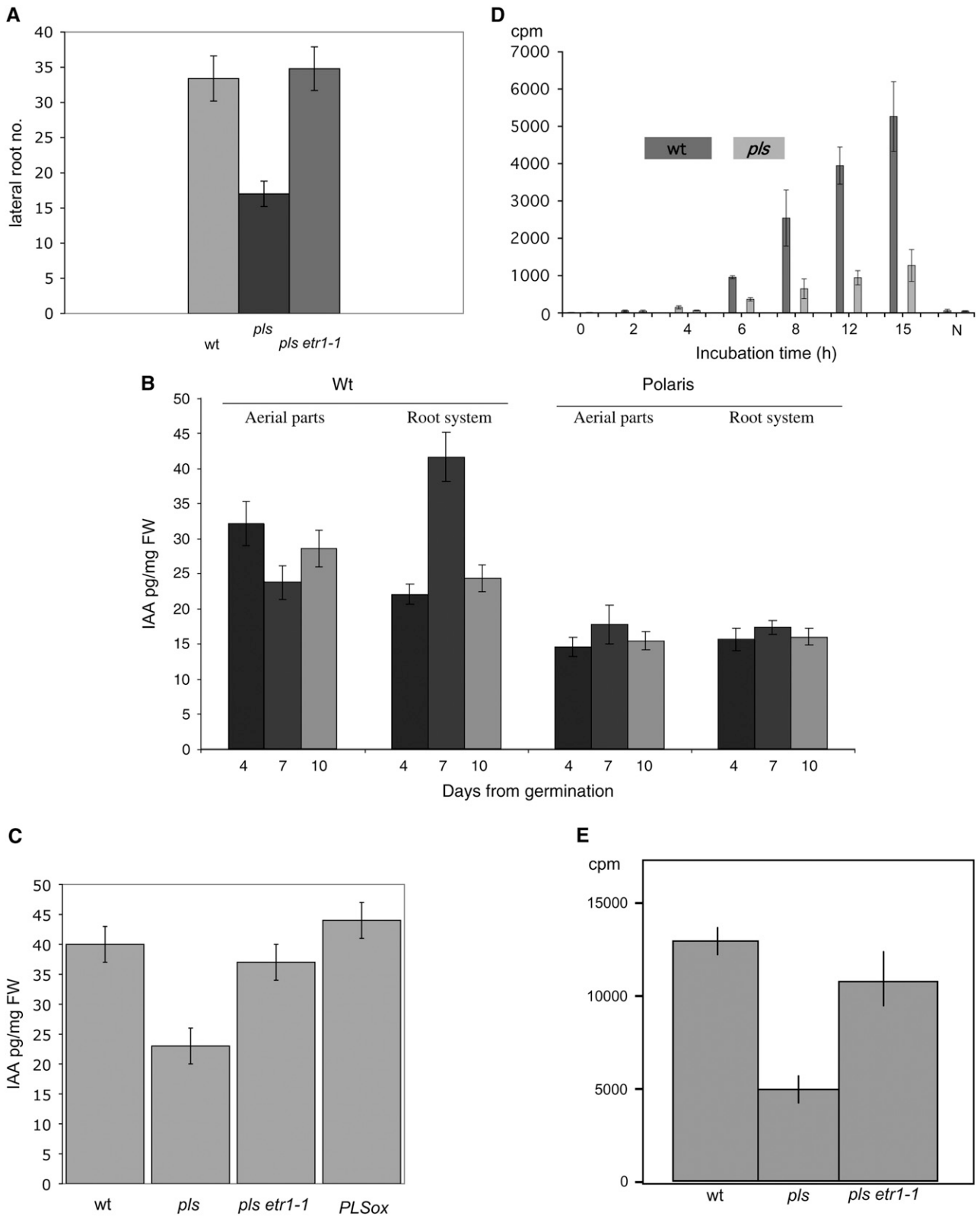


Figure 4. Auxin Transport and Accumulation Are Defective in *pls*.

(A) Lateral root numbers in *pls*, *pls etr1-1*, and wild-type seedlings at 10 d after germination. Error bars represent SE; *n* = 10.

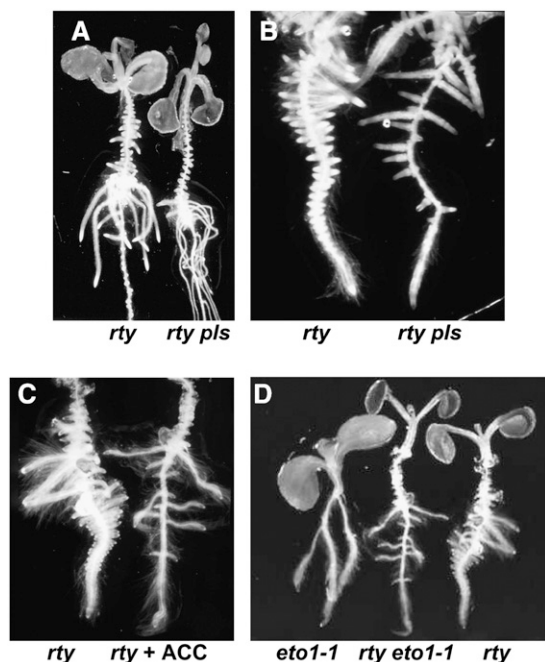


Figure 5. *pls* and ACC Suppress the *ty* Mutant Phenotype.

(A) and (B) Seedlings of the *ty* single mutant and *ty pls* double mutant, showing adventitious root formation on the hypocotyl and primary root in *ty*. In the *ty pls* double mutant (B), the reduced frequency of lateral roots compared with *ty* is apparent.

(C) *ty* seedlings treated with the ethylene precursor ACC (*ty + ACC*) show a reduced frequency of lateral roots.

(D) Seedlings of ethylene-overproducing *eto1-1*, *ty*, and the double mutant *eto1-1 ty*. The double mutant shows a reduced frequency of lateral roots.

much reduced ability for auxin transport ($\sim 24\%$ of the level of wild-type plants at 15 h) (Figure 4D). Neither *pls* nor wild-type stems transported [^3H]IAA in the wrong (i.e., basal-to-apical) direction. Therefore, the *PLS* gene is required for correct auxin transport, accumulation, and root growth.

To investigate further the role of ethylene signaling in auxin transport and accumulation, we measured auxin transport in double mutants between *pls* and the ethylene-resistant *etr1-1*. In these double mutants, free IAA levels accumulated to wild-type concentrations (Figure 4C). We also measured [^3H]IAA transport in *pls etr1-1* double mutants and found it also restored to $\sim 85\%$ of wild-type levels (Figure 4E). Moreover, lateral root numbers were restored to $\sim 80\%$ of wild-type levels in the double mutants (Figure 4A).

Figure 4. (continued).

(B) Free IAA content of *pls* and wild-type seedlings in aerial and root tissues at 4, 7, and 10 d after germination. Error bars represent SD; $n = 5$. FW, fresh weight.

(C) Free IAA content of wild-type, *pls*, *pls etr1-1*, and *PLSOx* seedlings at 10 d after germination. Error bars represent SD; $n = 3$. FW, fresh weight.

(D) Polar transport of auxin in wild-type and *pls* inflorescence stems. N indicates lack of transport in the basal-to-apical direction. Error bars represent SE; $n = 8$.

(E) Auxin transport in *pls* and *pls etr1-1* mutants. Error bars represent SE; $n = 7$ for *pls* and *pls etr1-1*; $n = 8$ for the wild type.

Together, the data presented demonstrate that the reduced auxin responses and altered root architecture in *pls* are the result of enhanced ethylene signaling. Given that ethylene is also known to have a dramatic effect on plant cell shape and division, which are processes governed by the cytoskeleton (Abeles et al., 1992; Kieber et al., 1993; Shibaoka, 1994), we investigated whether the ethylene phenotype of *pls* is responsible for the observed short-root phenotype via effects on the cytoskeleton.

PLS Is Required for Correct Responses to Tubulin Inhibitors

The effect of the *pls* mutation on the microtubule cytoskeleton was investigated by microscopy and inhibitor and genetic studies. Confocal imaging of root cell interphase microtubules revealed no gross differences in their organization between *pls* and wild-type plants. However, *pls* was found to exhibit an abnormal response to certain microtubule inhibitors. Amiprophos-methyl (APM) is an antimicrotubule herbicide that causes severe abnormalities in root architecture as a result of its disruption of the microtubule cytoskeleton (Anthony et al., 1998). *pls* seedlings grown in the presence of APM did not exhibit the typical root-swelling and growth-inhibition responses of wild-type seedlings (Figures 6A, 6B, and 6G). Furthermore, transgenic plants over-expressing the *PLS* open reading frame showed a more severe response to APM than did wild-type plants (Figure 6D). Oryzalin, a second microtubule herbicide that is chemically dissimilar to APM, although more potent (Murthy et al., 1994), but which is known to interact with the same target site on tubulin (Ellis et al., 1994), also induced a less severe response in *pls* compared with wild-type plants (Figure 6E). A third antimicrotubule herbicide, propyzamide, which is of a similar size but has a different mode of action than APM and oryzalin (Anthony et al., 1998), has identical inhibitory effects on root growth of wild-type, *pls*, and *PLS*-overexpresser plants (Figures 6F and 6H). As APM and oryzalin (but not propyzamide) inhibit the polymerization of tubulin into microtubules by a similar mechanism, these data indicate that the reduced effect of APM and oryzalin on *pls* compared with wild-type plants is attributable to altered microtubule dynamics in *pls*. This may be the result of already defective tubulin dynamics as a consequence of the enhanced ethylene signaling in *pls*.

To investigate whether the ethylene signaling defects were responsible for the resistance to APM, *pls* and *etr1-1* single and double mutants and *ein2* single mutants were grown in the presence of 5 μM APM. *ein2* (Figure 6C) and *etr1-1* (Figure 6A) single mutants showed a similar response to wild-type plants in the presence of APM. This finding shows that APM effects are not dependent upon ETR1 and EIN2, respectively, in the wild

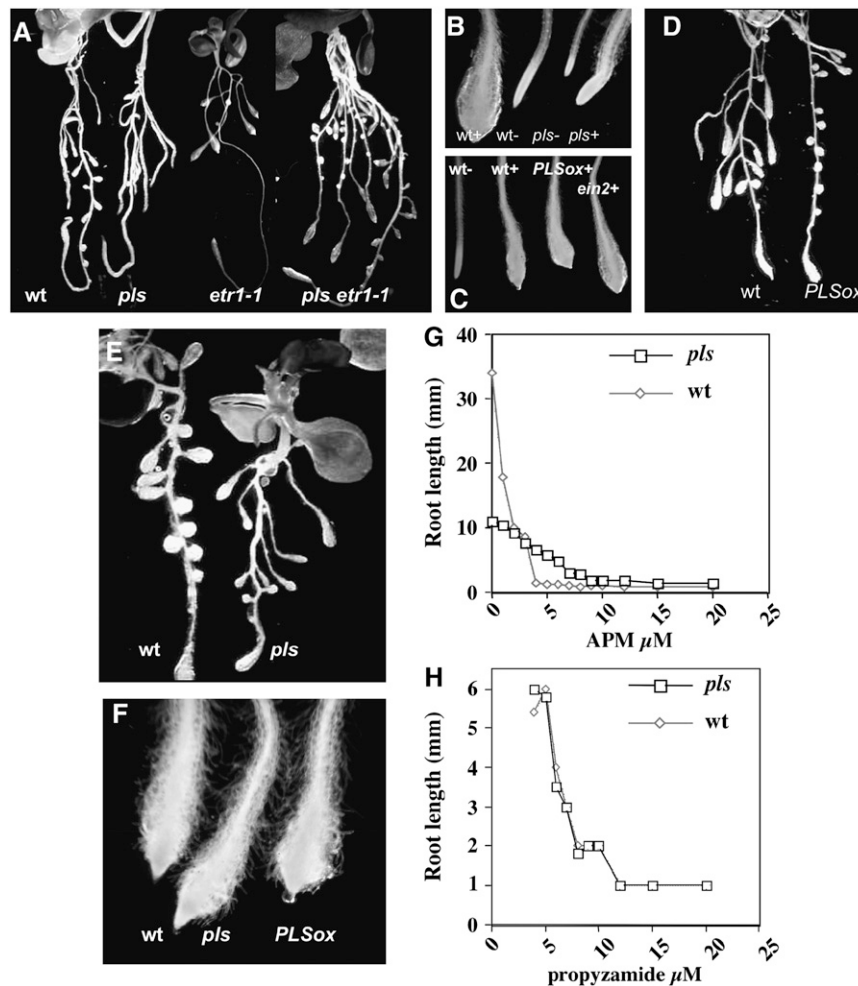


Figure 6. *pls* Has Reduced Responses to Microtubule Inhibitors.

(A) and (B) Wild-type, *pls*, *etr1-1*, and *pls etr1-1* seedlings grown for 10 d in the presence ([A] and + in [B]) or absence (– in [B]) of 5 μM APM.

(C) and (D) Wild-type, *PLSOx*, and *ein2* seedlings grown for 10 d in the presence (+) or absence (–) of 5 μM APM.

(E) Wild-type and *pls* seedlings grown for 10 d in the presence of 5 μM oryzalin.

(F) Effects of propyzamide on the primary root phenotype of wild-type, *pls*, and *PLSOx* seedlings.

(G) Kinetics of APM effects on root growth in *pls* and wild-type seedlings. The 50% inhibitory dose of APM is 1.5 μM for the wild type and 6.0 μM for *pls*. Each data point represents the mean of six measurements.

(H) Kinetics of propyzamide effects on primary root growth of wild-type, *pls*, and *PLSOx* seedlings. Each data point represents the mean of six measurements.

type. Unlike *pls* single mutants, *pls etr1-1* double mutants exhibited a strong root-swelling response that was identical to that in the wild type, confirming that the enhanced ethylene signaling in *pls* is responsible for the reduced response to APM (Figure 6A). These observations demonstrate that (1) enhanced ethylene signaling is associated with reduced responses to APM, and (2) PLS is required for correct ethylene signaling, with downstream effects on the microtubule cytoskeleton and root development. Treatment of other ethylene mutants, such as *eto1* and *ctr1*, with APM also indicated a reduced response to APM, but the effect was less pronounced because of the more severe short-root phenotypes of these mutants (data not shown).

PLS Transcription Is Negatively Regulated by Ethylene

To investigate whether the *PLS* gene is itself regulated by ethylene, both the original promoter trap line At EM101, which contains a *gusA* gene fused to the *PLS* gene promoter, and for comparison a transgenic line containing a cloned ~ 1.1 -kb fragment of the *PLS* gene promoter fused to a *gusA* gene in an otherwise wild-type background, *P_{PLS}:GUS*, were grown in the presence of the ethylene precursor ACC and the ethylene signaling inhibitor silver nitrate. Both promoter trap and *P_{PLS}:GUS* lines were found previously to respond identically to exogenous auxin (Casson et al., 2002) and were further compared here to

establish that the promoter trap activity responds in a similar way to the native promoter in β -glucuronidase (GUS) fusion studies to ethylene signaling. To confirm the GUS expression data, RNA-specific PCR was used to monitor the transcript abundance of the native *PLS* transcript in wild-type seedlings. For comparative purposes, *P_{PLS}* promoter activity was also monitored in ethylene-resistant *etr1-1* and ethylene-overproducing *eto1-1* mutant backgrounds.

Growing either At EM101 or *P_{PLS}:GUS* seedlings in the presence of 100 to 150 μ M ACC led to the loss of *PLS* gene promoter

activity, as revealed by the lack of GUS activity in seedling root tips (Figures 7A to 7F) or the reduction in the abundance of native *PLS* transcript (Figure 7G). Interestingly, 1 μ M APM had a similar effect to ACC treatment (i.e., was inhibitory) (Figure 7H). By contrast, *PLS* transcript showed an increase in abundance after treatment with 1-naphthylacetic acid (Figure 7C), as found previously (Casson et al., 2002). The slightly smaller increase than seen previously can be accounted for by the difference in the developmental stage of analysis. Similarly, treatment of seedlings with 5 to 10 μ M silver ions led to an increase in the zone of

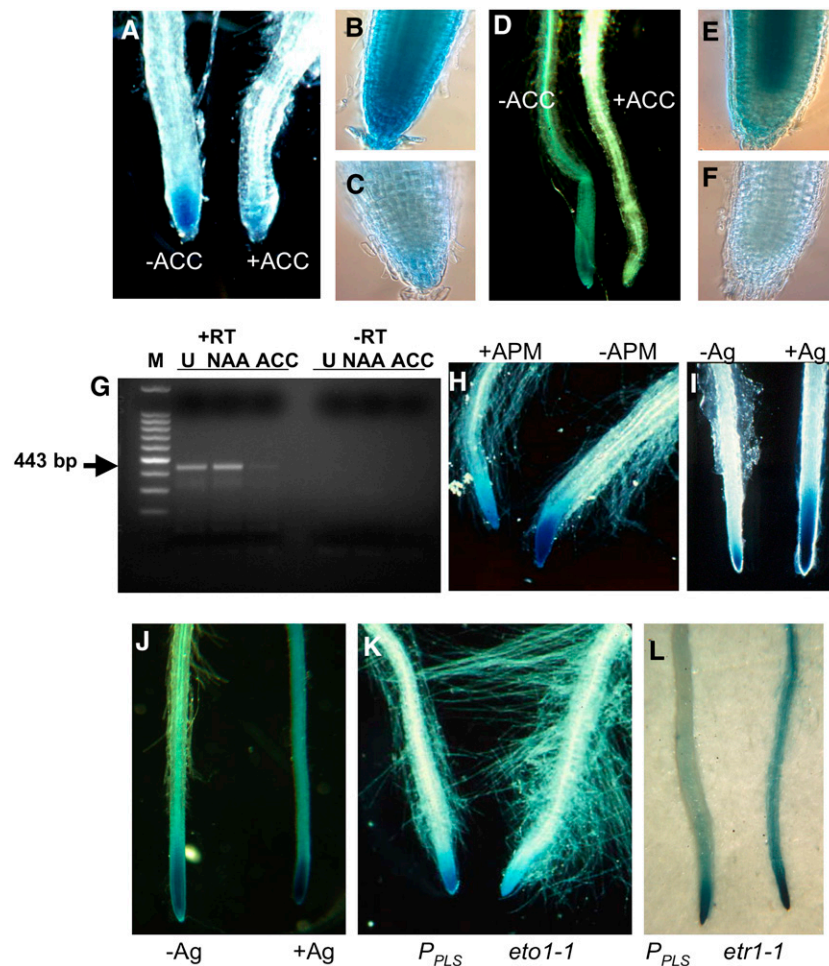


Figure 7. *PLS* Expression Is Repressed by Ethylene.

- (A) to (C) *PLS:GUS* expression in At EM101 ([A], –ACC, and [B]) is reduced on treatment with 100 μ M ACC ([A], +ACC, and [C]). (D) to (F) GUS activity in wild-type seedlings transformed with *P_{PLS}:GUS* ([D], –ACC, and [E]) is reduced on treatment with 100 μ M ACC ([D], +ACC, and [F]). (G) RNA-specific RT-PCR of the wild-type *PLS* transcript (443-bp product) in 6-d-old wild-type seedlings untreated (U), treated for 24 h with 10 μ M 1-naphthylacetic acid (NAA), or treated for 24 h with 100 μ M ACC. The –RT controls, lacking reverse transcriptase in the reaction, are shown. M, RNA size markers. (H) Effect of 1 μ M APM for 10 d (+APM) on *PLS:GUS* expression in At EM101. (I) Effect of 1 μ M silver nitrate for 5 d (+Ag) on *PLS:GUS* expression in At EM101 seedlings, leading to a spread of activity to the older part of the root. (J) Effect of 1 μ M silver nitrate (+Ag) on *P_{PLS}:GUS* transgenic plants, resulting in a spread of *PLS:GUS* activity to the older part of the root. (K) The ethylene-overproducing *eto1-1* mutation represses *P_{PLS}:GUS* expression. (L) The *etr1-1* mutation leads to a spread of *P_{PLS}:GUS* expression to the older part of the root.

GUS activity in the root, whereby it was no longer restricted to the tip but extended farther back into the older part of the root (Figures 7I and 7J). Analysis of GUS activity in the *etr1-1* and *eto1-1* mutant backgrounds gave identical results to the pharmacological studies, wherein increased ethylene signaling in the *eto1-1* background suppressed *PLS* promoter activity (Figure 7K) and the suppression of ethylene in *etr1-1* led to more extensive GUS activity in the older part of the root (Figure 7L).

DISCUSSION

The *PLS* Gene Is Required for Correct Ethylene Signaling

We present evidence that the *PLS* gene is required for the correct activity of the ethylene signaling pathway. *p/s* seedlings exhibit enhanced ethylene signaling, seen as the triple-response phenotype, and the enhanced expression of both the endogenous *At GSTF2*, an ethylene-upregulated gene (Zhou and Goldsborough, 1993; Smith et al., 2003), and the primary ethylene response gene *ERF10* (Ohta et al., 2001). A key observation is that the defective phenotype of the *p/s* mutant (e.g., the short primary root, reduced polar auxin transport, and low auxin accumulation) is rescued to wild-type status by the pharmacological or genetic inhibition of ethylene signaling. Overexpression of the *PLS* gene reduces the inhibitory effects of exogenous ACC on primary root growth, further implicating *PLS* as having a role in the perception or transduction of ethylene signaling.

The suppression by *p/s* of the *cin5* mutant's failure to undergo a triple response in the presence of cytokinins suggests that the ethylene response of *p/s* is independent of the ethylene biosynthetic enzyme ACS5, and the high-ethylene-signaling phenotype of *p/s* is unlikely to be attributable to cytokinin-induced ethylene biosynthesis (Vogel et al., 1998). Furthermore, the lack of ethylene overproduction by *p/s*, and the lack of rescue by the ethylene synthesis inhibitor aminoethoxyvinylglycine, confirm a defect in the ethylene signal perception or transduction pathway rather than in the regulation of ethylene biosynthesis. The rescue of the effects of the *p/s* mutation by *etr1-1* and the failure of *p/s* to suppress *etr1-1* suggest that *PLS* acts at or close to the ethylene receptor. Similarly, the *rte1* mutant cannot suppress the ethylene resistance phenotype of *etr1-1* (Resnick et al., 2006). RTE1 (like *PLS*) acts as a negative regulator of ethylene responses, and it appears to be required for correct ethylene receptor function.

It is likely that *PLS* acts at more than one level in the ethylene signaling pathway. Although the *p/s* mutation cannot suppress *etr1-1*, the *PLS*-overexpression phenotype is incompletely suppressed by *ctr1-1* (Figures 3D to 3G), suggesting an additional role for *PLS* downstream of *CTR1*. There also appears to be a light-mediated effect on the interaction between *p/s* and *ctr1* mutants: the light-grown *PLSOx/ctr1* has relatively longer roots than those grown in the dark (Figures 3D and 3F), perhaps suggesting a light dependence of *PLS* action.

At present, we do not know the precise mode of action of the *PLS* peptide. The semidominance of the *p/s* mutation suggests a dose-dependent effect of the peptide in suppressing ethylene responses. It is possible, for example, that the peptide interacts with an ethylene receptor upstream of *CTR1* to inhibit ethylene

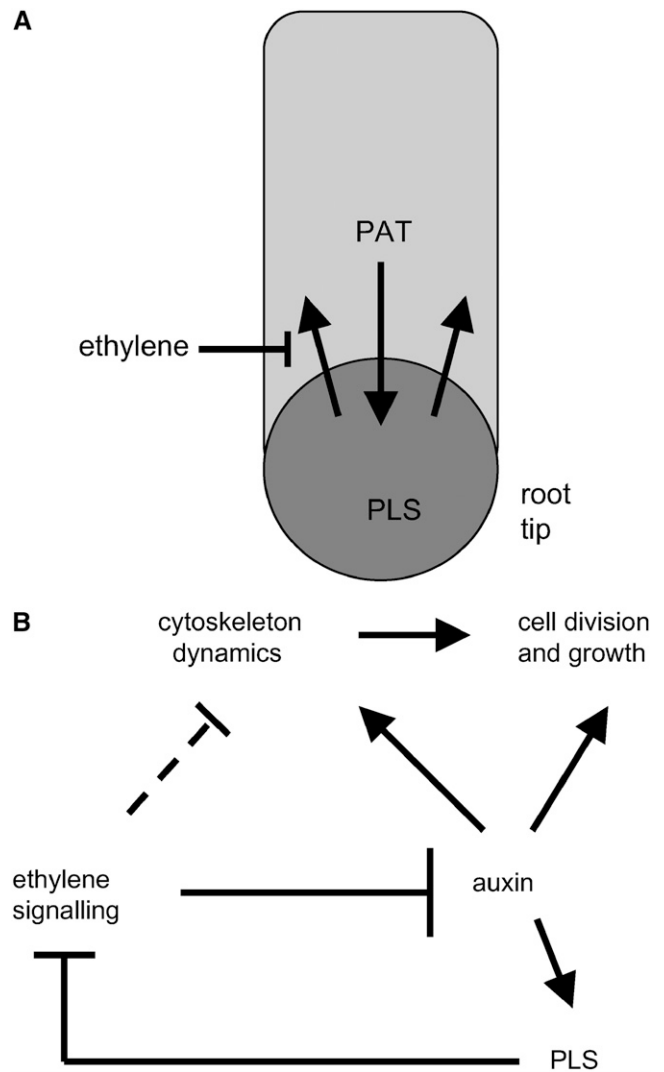


Figure 8. Model for *PLS* Function in the Root.

(A) Auxin moves into the root tip via polar auxin transport (PAT) and is also transported by this mechanism back up the root, triggering lateral root initiation. Ethylene suppresses polar auxin transport, which may prevent auxin both entering and leaving the tip.

(B) Auxin is a positive regulator of *PLS* expression and also plays a role in cytoskeleton function, cell division, and cell expansion. Ethylene suppresses these effects, and *PLS* suppresses the effects of ethylene. According to this model, ethylene treatment suppresses auxin entry and exit at the root tip, and turnover of auxin would lead to a decline in the concentration of active auxin in the root tip in ethylene-treated seedlings. This in turn would lead to a reduction in *PLS* transcription, allowing ethylene to alter root architecture (e.g., leading to the production of thicker roots and fewer lateral roots). Auxin signaling from the shoot would antagonize this effect via *PLS*, which the model suggests acts as a modulator of the auxin–ethylene interaction. It is proposed that this interaction modifies root cell division, shape, and ultimately growth through effects, at least in part, on the cytoskeleton. The inhibitory effects of ethylene on auxin transport and cytoskeleton dynamics may be independent or interdependent, but they are currently unclear mechanistically.

binding and so suppress ethylene signaling in a dose-dependent manner; or it may inhibit receptor–CTR1 interactions to suppress ethylene responses. The notion that PLS works at more than one point in the ethylene signaling pathway opens the possibility that it may represent a point of crosstalk between ethylene and other pathways of root development.

Ethylene Signaling Modifies Auxin Transport and Accumulation

It has been recognized that ethylene can reduce auxin responses and transport in other systems (Morgan and Gausman, 1966; Suttle, 1988; Haver et al., 2002), but the molecular mechanism of this effect remains obscure. The observation that the low-auxin phenotype and reduced polar auxin transport and reduced numbers of lateral roots of *p/s* can be restored to approximately wild-type levels in double mutants with the ethylene-resistant *etr1-1* demonstrates that the enhanced ethylene-signaling phenotype of *p/s* is most likely responsible for the repression of auxin synthesis and transport. This shows both that ethylene can have an inhibitory effect on auxin synthesis, transport, and biological function and that PLS is a new molecular component of this signaling interaction. We can speculate that a reduced export of auxin from the sites of synthesis might repress auxin biosynthesis in *p/s*.

The *p/s* mutant shows a reduced rate of [³H]IAA transport (Figure 4). The reduced auxin in *p/s* seedlings and older plants is consistent with the observed reduced levels of expression of the auxin-regulated *IAA1* gene, reduced leaf vasculature, and reduced growth inhibition in response to low concentrations of exogenous auxin (Casson et al., 2002). Also in agreement is the reduced frequency of lateral root initiation, a process that is regulated at least in part by auxin (Figure 4).

Although the mechanism of the relationship between ethylene signaling and auxin transport is unclear, a number of mutants have been identified in which both signaling pathways are affected. For example, the ethylene-insensitive mutant *eir1-1* is defective in the auxin efflux carrier component *PIN2* (Luschnig et al., 1998), linking auxin transport and ethylene responses. The mutant *alh1* shows a constitutive ethylene triple response and also altered responses to auxin signaling and transport, but not accumulation (Vandenbussche et al., 2003). The *hookless* mutant similarly shows defective auxin and ethylene interactions that regulate apical hook formation (Lehman et al., 1996). Recently, Stepanova et al. (2005) found that anthranilate synthases, enzymes induced by ethylene and catalyzing the synthesis of Trp and auxin, account in part at least for ethylene-mediated growth inhibition in roots. The relationship between auxin transport and ethylene is intriguing, and the *p/s* mutant offers new opportunities to investigate it.

Ethylene and APM May Affect the Same Process

Of interest is the link between signaling and the cytoskeleton revealed by the *p/s* mutant. *p/s* seedlings show reduced responses to the microtubule inhibitors APM and oryzalin. These inhibitors are members of different chemical classes (phosphor-

othioamidates and dinitroanilines, respectively), but they have the same predicted mode of action, in the binding of tubulin and the subsequent inhibition of tubulin polymerization (Murthy et al., 1994; Anthony and Hussey, 1999a). This affects microtubule dynamics (by destabilizing them), leading to an inhibition of cell division and axial cell expansion. This is phenotypically similar to the treatment of roots with ethylene, with short roots, radial cell expansion, and swollen lateral root tips in particular.

Wild-type seedlings treated with either APM or oryzalin had similar phenotypes, notably radially swollen root tips. The *etr1-1* and *ein2* mutants showed a wild-type response to APM, suggesting that ETR1 and EIN2 act upstream of the point of action of APM on microtubules. This finding is in agreement with the evidence that APM binds tubulin directly (Anthony and Hussey, 1999a). The wild-type response to APM and oryzalin is reduced dramatically in *p/s* seedlings (Figure 6), and the physiological importance of ethylene signaling in this apparent resistance is clearly demonstrated by the observation that *p/s etr1-1* double mutant seedlings showed a response to APM that is restored to wild-type levels. A central role for the *PLS* gene in this response is demonstrated by the enhanced response to APM in *PLS*-overexpressing seedlings.

A possible interpretation of these observations is that the reduced response of the *p/s* mutant to tubulin inhibitors may be the result of already defective tubulin dynamics (e.g., a stabilization of the microtubules) in the mutant, as a consequence of the observed enhanced ethylene signaling. The effects of APM and oryzalin are correspondingly reduced. PLS/ethylene signaling and APM/oryzalin both affect microtubule dynamics, but by different mechanisms, with the herbicides acting downstream of ethylene signaling.

The mechanism by which ethylene affects the cytoskeleton and growth is unclear. The role of tubulin in the control of cell division and expansion in roots of *Arabidopsis* has been shown, for example, in transgenic studies in which reduced levels of α -tubulin led to an inhibition of root elongation, among other effects (Bao et al., 2001), and in mutants defective in a range of microtubule-associated proteins, including *botero* (Bichet et al., 2001), *pleiade* (Muller et al., 2004), *mor1/gem1* (Whittington et al., 2001; Twell et al., 2002), *lefty* (Abe et al., 2004), and *spiral* (Nakajima et al., 2004; Sedbrook et al., 2004). The dwarfed *fass/tonneau* is also defective in the organization of cell division and expansion and is mutant in a protein phosphatase 2A subunit required for correct cortical microtubule regulation (Camilleri et al., 2002). Microtubule-associated proteins may play important roles in linking hormonal and other signals to the microtubule cytoskeleton (Lloyd et al., 1996; Gardiner et al., 2001). Ethylene, like mutations that affect herbicide responses (Anthony et al., 1998; Anthony and Hussey, 1999b), may affect tubulin conformation so that either herbicide binding is less efficient and/or by stabilizing tubulin heterodimer interactions in the microtubule against the destabilizing effect of the herbicides (Anthony et al., 1999). This view is consistent with the rescued response to inhibitors in the *p/s etr1-1* double mutants.

Therefore, we propose that a downstream effect of PLS is the suppression of ethylene signaling to the microtubule cytoskeleton, to control cell shape and division. Polar auxin transport requires a functional actin cytoskeleton for correct vesicle

trafficking (Geldner et al., 2001), and it is possible that interactions between actin and defective microtubule components of the cytoskeleton account for the reduced polar auxin transport in *p/s*. This possibility is supported by the evidence that auxin-regulated *PLS* expression is inhibited by both ethylene and APM, each of which acts on the microtubule cytoskeleton. Although we cannot exclude the possibility that the enhanced ethylene-signaling phenotype in *p/s* suppresses polar auxin transport by an independent mechanism from APM, it is tempting to suggest that each represses auxin transport and auxin-regulated *PLS* expression via effects on the cytoskeleton—and, by implication, that ethylene regulates auxin transport/homeostasis/responses in part, at least, by this mechanism. This could account for defects in *p/s*, such as in vascular patterning, lateral root formation, and root meristem maintenance, that depend on correct auxin transport (Mattsson et al., 1999; Casimiro et al., 2003; Bllilou et al., 2005).

Ethylene and Auxin Interact to Establish a Regulatory Loop for the Patterning of *PLS* Gene Expression in the Root

The ethylene precursor ACC has a suppressive effect on *PLS* gene expression. This has been shown by RNA-specific PCR analysis of the native gene transcript and by both promoter-GUS analysis in the original promoter trap line At EM101, in which the *PLS* gene promoter is tagged by a promoterless *gusA* gene, and in transgenic plants containing *gusA* cloned downstream of the *PLS* gene promoter sequence (Figure 7). Similarly, the ethylene-overproducing *eto1-1* mutation has an identical suppressive effect on *PLS* promoter activity. Consistent with this, the inhibition of ethylene signaling in both classes of transgenic plants, either by silver ions or by the *etr1-1* mutation, leads to an extended zone of *PLS* promoter activity back into the proximal region of the root.

We previously showed in detailed studies using quantitative competitor PCR, RNA gel blot analysis, and promoter-GUS fusion transgenic plants that the *PLS* gene is upregulated approximately threefold within 30 min after the application of auxin (Casson et al., 2002). Furthermore, we have also shown that correct patterning of *PLS-GUS* expression is dependent on GNOM activity (Topping and Lindsey, 1997), which in turn is required for correct PIN protein localization and auxin distribution via the actin cytoskeleton (Steinmann et al., 1999; Geldner et al., 2003). Therefore, we propose that the patterning and level of *PLS* expression in the root is itself regulated by an antagonistic interaction between auxin and ethylene signaling, in which auxin localization to the root tip promotes *PLS* transcriptional activation while ethylene signaling, in a more proximal position, represses expression. As ethylene signaling is suppressed, the zone of *PLS* expression is extended (Figures 7I, 7J, and 7L). This observed interaction between auxin and ethylene signaling in the root provides one possible mechanism for regulating gene expression domains, at least in a crude way.

A simple prediction from our data would be that, in a *p/s* mutant background, which exhibits enhanced ethylene signaling in the root, *PLS* transcription itself should be reduced compared with its expression in a wild-type background. The more extensive expression in the proximal region of the roots of transgenic plants

containing the *P_{PLS}:GUS* gene construct in a wild-type background, compared with the tighter *P_{PLS}:GUS* promoter trap expression in the *p/s* mutant background (Figures 7A and 7I versus Figures 7D and 7J), supports this view and suggests that ethylene suppresses *PLS* expression farther back in the root to allow ethylene signaling to take place, as is required, for example, for root hair formation at that position (Tanimoto et al., 1995).

A Model for *PLS* Function in Root Development

PLS is required for two aspects of root development: elongation (growth) and lateral root formation. We propose a model (Figure 8) in which *PLS* transcription is activated at the root tip by the relatively high auxin concentration that accumulates and is required for correct cell division at that position (Sabatini et al., 1999; Ljung et al., 2001; Friml et al., 2002; Bllilou et al., 2005). Here, *PLS* acts as a negative regulator of ethylene signaling, which is inhibitory to cell division and expansion, and therefore root growth (Souter et al., 2004). This model could account for the suppression of the inductive effects of auxin and cytokinin on ethylene biosynthesis at the root tip (cytokinin is synthesized there) (Vogel et al., 1998).

We can speculate on potential ecological implications for this mechanism. Mechanical stresses in the soil that can induce relatively high levels of ethylene synthesis/signaling could produce thicker, and potentially mechanically stronger, roots to allow better soil penetration. *PLS* expression would also suppress the ethylene-mediated inhibition of auxin transport in the root tip, again ensuring correct auxin signaling for cell division and patterning. This model suggests a number of experiments for the further elucidation of the roles of auxin and ethylene interactions at the root tip. *PLS* is also required for correct lateral root initiation, presumably via ethylene-mediated control of auxin transport to the pericycle.

In conclusion, we have identified *PLS* as an essential component in the regulation of auxin homeostasis and root growth by restricting ethylene signaling. We show that one downstream component of the cellular machinery that transduces these hormonal signals in the modulation of cell division and expansion at the root tip is the microtubule cytoskeleton. Given the paucity of identified small polypeptides that are known to be biologically functional in plants (Lindsey et al., 2002; Fiers et al., 2005) and the current interest in small RNAs in gene regulation (Kidner and Martienssen, 2005), these data add to the view that many new signaling components, unrecognized by most computational gene identification tools because of their size or genomic organization, remain to be discovered.

METHODS

Plant Materials and Growth Conditions

The transgenic line At EM101 (*Arabidopsis thaliana* ecotype C24) contains the *p/s* gene mutated by the promoter trap pΔgusBin19 (Topping et al., 1991; Casson et al., 2002). *P_{PLS}:GUS* transgenic plants have been described previously (Casson et al., 2002). *PLS*-overexpressing transgenic plants contained a partial *PLS* cDNA encoding the 36-amino acid open reading frame cloned behind the cauliflower mosaic virus 35S

promoter (Casson et al., 2002). For in vitro growth studies, seeds were vernalized, surface-sterilized, and plated on growth medium (half-strength Murashige and Skoog medium; Sigma-Aldrich), 1% sucrose, and 2.5% Phytigel (Sigma-Aldrich) at $22 \pm 2^\circ\text{C}$ at a photon flux density of $\sim 150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, as described previously (Casson et al., 2002). *etr1-1*, *eto1-1*, *ctr1*, and *cin5* mutants were obtained from the Nottingham Arabidopsis Stock Centre. For hormone/inhibitor application experiments, seeds were germinated aseptically on growth medium containing various concentrations of hormones. 2,4-D, 1-naphthylacetic acid, benzyladenine, and ACC were obtained from Sigma-Aldrich. Oryzalin, APM, and propyzamide were obtained from Fluka.

Gene Expression Analysis

Tissue localization of GUS enzyme activity was performed as described (Casson et al., 2002). For transcript analysis, RNA was extracted using the RNeasy plant RNA extraction kit (Qiagen) and the PolyAtract mRNA isolation system (Promega). RNA was blotted, hybridized, and probed as described (Casson et al., 2002). RNA gel blot analysis was performed using 50 μg of total RNA isolated from 7-d-old wild-type and *pls* seedlings. RNA markers were Promega G319. RNA-specific PCR was used to monitor *PLS* transcriptional changes, and *ACT2* was used as a control, using primers and conditions as described (Casson et al., 2002). Primers for RT-PCR of the *ERF10* transcript were 5'-GGACTTGCGTT-GAGGTCA-3' and 5'-GCCAGAGCCTACGACTC-3'.

Hormone Analysis

For each assay, ~ 50 seedlings were grown in air on half-strength Murashige and Skoog medium containing 1% sucrose in the dark for 72 h in 22-mL vials for ethylene measurement by gas chromatography as described previously (Wang et al., 2004). Each assay represents an average number from six samples/vials of the same lines and was duplicated. The ethylene production of each line was normalized accordingly, with a unit represented as picoliters per seedling per day. Auxins were assayed in in vitro-grown seedlings up to 10 d after germination, or hydroponically as described (Ljung et al., 2005), or in plants grown in controlled-environment chambers (16-h day, 23°C , 75% humidity; 8-h night, 18°C , 80% humidity; harvested at floral transition, growth stage 6.0 to 6.5) (Boyes et al., 2001). Analysis was by HPLC/gas chromatography-mass spectrometry (Micromass GCT or Quattro Ultima triple quadrupole), essentially as described (Ljung et al., 2005). The polar transport of [^3H]IAA (GE Healthcare) was measured in inflorescence stem segments essentially as described (Okada et al., 1991). The apical or (as a control) basal end of each 2.5-cm stem segment was immersed in an Eppendorf tube containing 0.08 $\mu\text{Ci}/\text{mL}$ 1.5 μM [^3H]IAA, and radioactivity accumulating in the other 0.5 cm was measured over a time course by scintillation counting (Tri-Carb 1600 TR; Packard Instruments).

Microscopy

Light micrographs were taken using a CoolSNAPcf digital camera (Photometrics; Roper Scientific) with Openlab 3.1.1 software (Improvision) on Leica MZ125 (Leica Microsystems UK), Olympus SZH10 (Olympus UK), or Zeiss Axioskop (Carl Zeiss) microscopes. Images were processed in Adobe Photoshop 5.0.

Accession Numbers

GenBank/EMBL accession numbers or Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *POLARIS*, AF285768; *At GSTF2*, At4g02520; *ERF10*, At1g03800.

ACKNOWLEDGMENTS

K.L. gratefully acknowledges funding from the Biotechnology and Biological Sciences Research Council, the Gatsby Charitable Foundation, and the County Durham Subregional Partnership, which has supported this work.

Received January 3, 2006; revised October 6, 2006; accepted November 2, 2006; published November 30, 2006.

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