A pathway for lateral root formation in Arabidopsis thaliana

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In plants, the hormone indole-3-acetic acid (IAA) can initiate the developmental program for lateral root formation. We have isolated mutants that have permitted the dissection of this program into initiation and maturation of lateral roots. The alf1-1 mutation causes hyperproliferation of lateral roots, alf4-1 prevents initiation of lateral roots, and alf3-1 is defective in the maturation of lateral roots. The alf3-1 mutant can be rescued by IAA, whereas the alf4-1 mutant is not rescued. Our data suggest a model in which IAA is required for at least two steps in lateral root development: (1) to initiate cell division in the pericycle, and (2) to promote cell division and maintain cell viability in the developing lateral root.

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Regulation of lateral root formation by IAA biosynthetic genes has increased lateral root production. First, it has been known for >40 years that exogenous application of IAA induces the formation of lateral roots (Torrey 1950; Blakely et al. 1982), and second, transgenic plants that overexpress the bacterial IAA biosynthetic enzymes have increased lateral root production (Klee et al. 1987; Kares et al. 1990). IAA plays a number of roles in plant development processes including, regulation of tropic responses, determination of cell shape, tissue differentiation, and cell division (Moore 1989; Garbers and Simmons 1994). Mutants have been isolated that are resistant to IAA and related compounds or exhibit altered tropic responses (Maher and Martindale 1980; Estelle and Somerville 1987; Okada and Shimura 1990). However, most of these mutants do not have a major impact on lateral root formation, leaving unresolved the question of whether IAA is the in planta signal for lateral root formation.

In this paper we describe the isolation of root mutants and their use in the dissection of the process of lateral root formation. Our mutants reveal that the plant growth hormone IAA is required for the initiation, morphogenesis, and continued viability of lateral roots.

Results

Isolation of mutants defective in lateral root formation

Mutagenized plants were screened on agar medium to identify strains that had defects in lateral root formation (see Materials and methods). Putative mutants with altered position, development, or number of lateral roots were isolated and designated alf (aberrant lateral root formation). Mutant strains were backcrossed to unmutagenized parental strains (see Materials and methods), and only those isolates that gave Mendelian segregation patterns in the F2 generation were considered further. By this criterion, recessive mutations in three genes were identified: alf1-1, alf3-1, and alf4-1. These three mutations map to distinct genetic loci: ALF1 on chromosome 2 near nga168, ALF3 on chromosome 5 between dihydroflavonol 4-reductase (DFR) and nga129, and ALF4 on chromosome 5 between nga249 and nga151 (see Materials and methods).

alf1-1 makes excess lateral roots and adventitious roots

The alf1-1 mutant makes an increased number of lateral roots along the primary root and makes a vast excess of adventitious roots (roots not derived from the embryonic root) from the hypocotyl (Fig. 1A), a stem-like structure between the root and the cotyledons. Though the hypocotyl has a tissue organization similar to that of the primary root (Dolan et al. 1993), it does not form adventitious roots in ALF plants unless treated with exogenous IAA. The aerial part of the alf1-1 plant is also abnormal; the cotyledons and true leaves are small and epinastic (curled) and the flowering stalks rarely develop floral organs. Because the flowers produced are not fertile, alf1-1 must be propagated as a heterozygote. In a few cases, adventitious roots have been observed to arise from the petioles and stem of alf1-1 plants (data not shown). alf1-1 appears to be a mutation in the same gene as hookless3 (hls3) [J. Ecker and A. Lehman, pers. comm.] and rootty (rty) [King 1994], two independently isolated mutations that are allelic with each other [J. Ecker and A. Lehman, pers. comm.]. Because these three mutations result in the same phenotypes, map at roughly the same location, and fail to complement (heterozygotes alf1-1/hls3 and hls3/rty display the alf1-1 adventitious root phenotype), we conclude that they are alleles of the same gene.

We observed that dark-grown alf1-1 seedlings fail to form an apical hook when germinated on unsupplemented medium (Fig. 2). In dark-grown ALF1 seedlings, a hook normally forms at the top of the hypocotyl unless the seedlings are germinated on medium containing IAA (Fig. 2) or IAA-related compounds (Ecker and Theologis 1994). Because both the failure to form an apical hook and the excess production of lateral and adventitious roots can be phenocopied in ALF1 plants by growth on IAA-containing medium, we conclude that the alf1-1 phenotype is caused by an overproduction of IAA or increased sensitivity to the hormone. This conclusion is supported by data showing that the rty mutant has higher levels of free and conjugated IAA [King 1994].

alf4-1 is defective in lateral root formation

The alf4-1 mutant fails to make lateral roots (Fig. 1B). Mutant plants fail to initiate lateral root primordia in the primary root and do not form adventitious roots from the hypocotyl. The aerial portion of the plant is small, bushy, and makes short flowering stalks. alf4-1 mutants are male sterile, but can be propagated in crosses using pollen from an ALF4 plant. The alf4-1 mutation may not be a null allele because an occasional plant can form a single lateral root. The rare lateral root, although morphologically normal, retains the alf4-1 phenotype and is unable to produce its own lateral roots.

alf4-1 fails to respond to exogenous IAA

The addition of IAA to ALF4 plants leads to the induction of many extra lateral root primordia along the entire primary root. The induction and maturation of IAA-induced primordia appear to mirror the normal sequence of events involved in the formation of lateral roots in the absence of IAA. Concentrations of IAA (1 μM) that stimulate the induction of many extra lateral root primordia in ALF4 fail to stimulate any lateral root induction in alf4-1 (Fig. 3A). Moreover, alf4-1 is not stimulated to form lateral root primordia by the putative IAA precursors, indole and tryptophan (data not shown). When alf4-1 plants are grown on high concentrations of IAA (20 μM) a few lateral roots are induced, but only near the root tip (data not shown).

In addition to the induction of lateral roots, IAA also inhibits root elongation [Evans et al. 1994]. The mutants axr1-3, axr2, and axr1-7 were isolated for their ability to make roots on media containing high levels of IAA and IAA-related compounds and their resistance to the in-
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Figure 2. Dark-grown alf1-1 mutants fail to make an apical hook. ALF1 (WS) and alf1-1 seeds were held at 4°C for 5 days and then grown in the dark for 2 days at 23°C on PNS containing 3 μM IAA or unsupplemented PNS (bar).

hibitory effects of IAA [Maher and Martindale 1980; Estelle and Somerville 1987; Wilson et al. 1990]. Unlike the alf4-1 mutant, the IAA-resistant mutants make lateral roots in response to 1 μM IAA [Fig. 3B]. Conversely, root elongation of the alf4-1 and ALF4 strains are equally inhibited by IAA [data not shown]. The different behavior of the alf4-1 mutant and the known IAA-resistant mutants [axr1-3, axr2, and aux1-7] suggests that IAA induction of lateral roots and IAA inhibition of root elongation use distinct signal transduction pathways.

alf3-1 is defective in the development of lateral root primordia into mature lateral roots

The alf3-1 mutant forms a primary root covered with arrested lateral root primordia (Fig. 1C). These primordia initiate on the alf3-1 primary root at about the same time as they do in ALF3 strains. However, whereas ALF3 primordia mature into lateral roots, most alf3-1 primordia fail to mature. Instead, alf3-1 primordia grow to the point of protruding through the epidermal cell layer and then arrest growth. Although alf3-1 primordia can grow

Figure 1. Phenotypes of the alf mutants. (A) The alf1-1 mutant makes adventitious roots from its hypocotyl. (Left) Dark-field micrographs of the transition zone between the hypocotyl and primary root. (Center) Bright-field micrographs of the hypocotyl directly above the transition zone. Plants in the left and center panels were photographed at 7 DAG. (Right) Plants at 20 DAG. ALF1 plants shown are the WS ecotype. (B) The alf4-1 mutant fails to make lateral roots. (Left) Dark-field illumination. (Center and right) Bright-field micrographs of the primary root tip and the transition zone, respectively. Plants were photographed at 10 DAG. ALF4 plants shown are the col-0 ecotype. (C) The lateral roots of alf3-1 mutants arrest growth. (Left) Dark-field illumination. The remaining sets of panels are bright-field micrographs of the primary root [center] and representative lateral roots [right]. Plants were photographed at 10 DAG. The solid arrow indicates a partially elongated alf3-1 lateral root. ALF3 plants shown are the Col-0 ecotype.
The *alf4-1* mutant is resistant to the IAA induction of lateral roots. (A) IAA fails to induce lateral roots in the *alf4-1* mutant. *ALF4* (Col-0) and *alf4-1* strains were grown on PNS medium for 4 DAG and transferred to PNS containing the indicated supplements for 3 more days. Bar, unsupplemented PNS medium. Photos are grouped in sets of three for each condition as follows: (left) dark field micrograph of root; (center and right) bright-field micrographs of the primary root tip and transition zone, respectively. (B) IAA induces lateral root formation in the IAA-resistant mutants. Indicated mutants were grown as in A and photographed under dark-field illumination.

Using time-lapse photography to follow the fate of lateral root primordia in *alf3-1* strains, we found that after the arrest of each *alf3-1* primordium, a second primordium is formed adjacent to or directly on the first (Fig. 4). One can observe a succession of as many as five lateral root primordia emerging sequentially from roughly the same site. The *alf3-1* mutant may be leaky because an occasional lateral root does emerge from the clump of primordia. However, these rare *alf3-1* lateral roots extend several millimeters and then arrest growth (Fig. 1C, arrow).

**The *alf3-1* mutant is rescued by IAA and indole**

Growth of *alf3-1* plants on IAA-supplemented medium permits the maturation of lateral root primordia into mature lateral roots (Figs. 5 and 6). The roots of *alf3-1* plants grown in IAA resemble those of *ALF3* strains grown on similar concentrations of IAA. However, because IAA also inhibits root elongation (Evans et al. 1994) it is dif-
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Figure 4. Time course of root development in the *alf3-1* mutant. An *alf3-1* plant was photographed on the days indicated. Note that primary root growth has ceased by day 8.

Figure 5. IAA rescues the *alf3-1* mutant. *ALF3* (Col-0) and *alf3-1* plants were grown on unsupplemented PNS [bar] or PNS containing 1 μM IAA. Plants were photographed at 14 DAG.

Figure 6. *alf3-1* mutants are rescued by IAA and indole but not by tryptophan. *ALF3* (Col-0) and *alf3-1* plants were germinated on unsupplemented PNS and then transferred at 2 DAG to PNS containing the indicated supplement. Bar, unsupplemented PNS medium. Plants were photographed under dark-field illumination at 14 DAG.

Difficult to determine how efficiently IAA rescues the *alf3-1* defect.

Recent work has suggested that indole is a precursor of IAA [Wright et al. 1991; Normanly et al. 1993]. Therefore, we examined the effect of indole on *alf3-1* mutants and *ALF3* plants. Indole has no effect on the morphology of *ALF3* plants: *ALF3* plants grown on indole at concentrations of 40–80 μM are indistinguishable from *ALF3* plants grown without indole. However, *alf3-1* plants grown on medium supplemented with 40 μM indole are rescued completely and make lateral roots that look like those of nonmutant plants [Fig. 6]. Unlike indole, an intermediate of the tryptophan biosynthetic pathway, tryptophan itself fails to rescue the *alf3-1* mutant [Fig. 6], indicating that *alf3-1* is not a tryptophan auxotroph. We also tested whether a precursor of indole, anthranilate, and toxic analogs of indole and anthranilate affect *alf3-1* growth. Anthranilate and 5-methylanthranilate had no effect on the mutant phenotype, whereas the toxic indole analog, 5-fluoroindole, exacerbates the *alf3-1* phenotype.

Indole is required continuously for normal development of *alf3-1* lateral roots

The ability of indole or IAA to rescue the *alf3-1* mutant suggests that the nascent lateral root primordia require either compound for both formation and continued growth. We considered two possibilities to explain the reversal of the *alf3-1* phenotype by indole (and by inference, IAA). One model is that indole is required throughout the course of lateral root development. The other model is that indole is required only to promote advancement past a certain stage in development. To distinguish between these alternatives, we performed a shift experiment [Fig. 7A]. *alf3-1* plants were germinated and grown for 5 days on medium without indole. Seedlings were
then transferred to medium containing 80 μM indole for 4 days, which allowed them to produce normal lateral roots. When the seedlings were returned to medium lacking indole, the normal lateral roots that had developed in response to indole arrested growth within several days after transfer (Fig. 7A, see arrow). The failure of the indole-initiated roots to survive suggests that alf3-1 lateral roots can undergo normal development only in the continued presence of indole.

In the course of the shift experiment described above we found that those lateral root primordia formed on alf3-1 plants during growth on medium lacking indole were not rescued by the subsequent transfer to medium containing indole. This observation was examined more closely using time-lapse photography to follow the behavior of individual primordia. After growth for 10 days on medium lacking indole, alf3-1 plants were transferred to medium containing indole and photographed daily for 5 days (Fig. 7B). Visible primordia that had formed in the absence of indole remained arrested with the alf3-1 phenotype after transfer to indole-containing medium [Fig. 7B, see arrow]. Only those primordia that formed after transfer to indole continued to develop in the presence of indole. As a control, alf3-1 plants were grown on indole-deficient medium and transferred to fresh medium also lacking indole. These plants continued to produce lateral root primordia that subsequently arrested. Interestingly, in both cases, new primordia usually appeared directly adjacent to or on top of those that had previously formed.

Cells in arrested alf3-1 lateral root primordia are dead

alf3-1 lateral root primordia that formed in the absence of indole were incapable of being rescued by the subsequent transfer to medium containing indole. This phenotype of the alf3-1 mutant, combined with its different cellular organization and morphology, led us to examine the metabolic state of the cells in the arrested primordia.

We used a double fluorescent staining procedure that distinguishes between viable and inviable cells. Fluorescein diacetate (FDA) is cleaved by esterases present in living cells and fluoresces green after it is cleaved (Widholm 1972). Propidium iodide (PI) fluoresces red in the nuclei of dead cells (Krishan 1975; Horan and Kappler 1977). PI also stains most cell walls (viable or inviable), which means that PI staining sometimes overlaps FDA staining and appears yellow. In ALF3 plants, young lateral root primordia stain with FDA (green or yellow), whereas arrested alf3-1 lateral roots fail to stain with FDA but do stain with PI (red), suggesting that the cells are not metabolically active (Fig. 8). In clusters of alf3-1 primordia the younger primordia stain with FDA (open arrows), whereas the older primordia stain only with PI (solid arrows). FDA staining begins to disappear from alf3-1 lateral root primordia at the same time that the primordia arrest growth. Only young (1-6 DAG) alf3-1 seedlings grown without indole appear morphologically normal and show a staining pattern similar to ALF3 seedlings. The staining patterns of alf3-1 roots grown on medium containing indole are indistinguishable from that of ALF3 plants, suggesting that the absence of IAA/indole is responsible for the premature cell death.

Double mutant analysis suggests that alf4-1 contains an early block in lateral root formation

Double mutant combinations between the various alf mutations were examined for their lateral root phenotypes to establish the order of action among the different ALF genes [see Materials and methods]. The alf1-1 alf4-1, and alf3-1 alf4-1 double mutants have the phenotype of the alf4-1 single mutant, a primary root devoid of lateral root primordia [Fig. 9A, B]. Therefore, alf4-1 is insensitive to the excess internal IAA produced in the alf1-1 mutant in much the same way that it is resistant to
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Figure 8. Cells in alf3-1 lateral roots die prematurely. ALF3 (Col-0) and alf3-1 seedlings were grown on unsupplemented PNS medium [bar] or PNS supplemented with 40 μM. At 12–16 DAG, plants were stained with FDA (green) and PI (red) and viewed using confocal laser microscopy as described in Materials and methods. The yellow area is where both stains overlap. The left panels show the primary root tip; the right panels show lateral roots. Solid arrows indicate older dead or dying primordia; open arrows indicate younger viable primordia. Scale bar, 100 μm.

Discussion

A model for lateral root development based on all mutants

Our data provide evidence for the role of IAA in at least two steps in the development of lateral roots that occur after formation of the pericycle in the primary root [Fig. 10]. The first step is the induction of pericycle cells by IAA to undergo mitosis and form the lateral root primordium. IAA is presumed to be transported from other parts of the plant to induce the pericycle to undergo the preliminary cell divisions that create the primordium (Charlton 1991). We assume that the ALF1 gene product modulates the level of free IAA and thus regulates the number of lateral roots that form. In the model, ALF1 acts negatively: it could be necessary for IAA catabolism or could repress the biosynthesis of IAA. The fact that neither externally added IAA nor overproduction of IAA by alf1-1 can restore root formation in the alf4-1 mutant suggests that the ALF4 gene is required either for the pericycle cells to sense IAA or to respond to it.

In the second step, either the lateral root primordium becomes unresponsive to IAA made in other parts of the plant and must produce its own IAA or the primordium requires increased transport of IAA. The lateral root primordium depends on this local rise in the IAA level for continued cell viability, cell division, and the subsequent maintenance of the lateral root apical meristem. Therefore, the ALF3 gene must function to elevate the IAA levels in the primordium. We propose two mechanisms by which this could be accomplished. The ALF3 gene product could specify an enzyme or regulator of the IAA biosynthetic pathway that is specifically turned on in cells of lateral root primordia. Alternatively, ALF3 could be required for the transport of IAA into growing...
In this case, IAA transport in the alf3-1 mutant is impaired specifically in the cells of the primordium. By either model, alf3-1 mutants require IAA/indole continuously in the root for both the proper development and continued growth of lateral roots.

Because the alf3-1 mutant can be rescued by exogenous IAA or indole and partially bypassed by the alf1-1 mutation, we conclude that alf3-1 is a root-specific IAA auxotroph. Because neither anthranilate nor tryptophan rescues the alf3-1 mutant, the alf3-1 mutant must be blocked at a branchpoint from the tryptophan pathway that can produce free indole. This block does not prevent the production of tryptophan, as the alf3-1 mutant is not a tryptophan auxotroph. The fact that indole but not tryptophan can remedy the alf3-1 mutant is consistent with recent data demonstrating that IAA is derived from an intermediate of the tryptophan pathway and not tryptophan itself (Wright et al. 1991; Normanly et al. 1993). If ALF3 is part of an IAA biosynthetic pathway, it is most likely to be a root-specific pathway. The shoots of soil-grown alf3-1 mutants appear normal for several weeks. After this time, shoot growth is stunted and the plants become anthocyanic presumably because alf3-1 plants grown in soil have an abbreviated root system as compared with that of ALF3 plants of similar age.

Because all three alf mutants are recessive we assume that the mutations cause a partial or total loss of function for their specific gene product. Several alleles of alf1 have been independently isolated. They all are recessive and have similar phenotypes suggesting that these alleles represent a loss of function [King 1994; J. Ecker and A. Lehman, pers. comm.].

Is IAA the endogenous inducer of lateral roots?

Our data are consistent with a hypothesis in which IAA or indole constitutes the endogenous signal for lateral root initiation. Mutants that are capable of lateral root initiation, alf1-1 and alf3-1, remain sensitive to the induction of lateral root formation by exogenous IAA, whereas the alf4-1 mutant, which cannot initiate lateral roots, is not responsive to exogenous IAA for lateral root induction. Although both alf1-1 and alf3-1 mutants make extra lateral root primordia without added IAA, the addition of IAA further increases the number of primordia along the primary root. We interpret these data to mean that the pericycle in the alf1-1 and alf3-1 mutants is functional and responsive to the morphogenic signals required for the initiation of lateral root formation. In contrast, the pericycle in alf4-1 has lost the ability to sense or respond to internal and external signals for lateral root initiation. Consistent with this interpretation, the alf4-1 mutant is also insensitive to another potent inducer of lateral root formation, surgical removal of the primary root tip [Torrey 1950].

The alf4-1 mutant provides evidence for at least two independent IAA signaling pathways: one that induces lateral roots and one that inhibits root elongation. alf4-1 is insensitive to the induction of lateral roots by IAA while remaining sensitive to IAA inhibition of root elongation. Most mutants that are resistant to the growth inhibitory properties of IAA do not have major defects in lateral root formation, although it has been shown recently that an axr1 axr4 double mutant is greatly reduced in production of lateral roots [Hobbie and Estelle 1995]. This result suggests that the IAA signaling pathways for induction of lateral roots and inhibition of root elongation may share components.

None of the alf mutants appears to have morphologically altered or missing pericycle cells as determined by light microscopy. Moreover, the bipolar radial orientation of lateral root formation found in ALF plants is maintained in both the alf1-1 and alf3-1 mutants [data not shown] suggesting that neither of these mutations affects the pattern of polarity. The alf3-1 mutant does affect the spacing of lateral root primordia in relation to

Primordia. In this case, IAA transport in the alf3-1 mutant is impaired specifically in the cells of the primordium. By either model, alf3-1 mutants require IAA/indole continuously in the root for both the proper development and continued growth of lateral roots.
the longitudinal axis of the primary root by making clusters of primordia separated by regions devoid of primordia. In the alf1-1 mutant or IAA-treated ALF3 roots the distribution of lateral root primordia increase in a uniform manner over most of the primary root.

Lateral root development is distinct from primary root development

Although the architecture of the lateral root is virtually identical to that of the primary root, the origins of these two structures are quite different. The primary root is formed in the embryo, whereas lateral roots are derived from groups of pericycle cells in the mature root (Steeves and Sussex 1989). The phenotype of the alf4-1 mutant suggests that the formation of lateral roots is not simply a reiteration of the program that produced the primary root—the alf4-1 mutant has a normal primary root, but fails to make lateral roots even in the presence of IAA. The phenotype of the monopteros mutant (Berleth and Jürgens 1993) supports the idea that there are different developmental antecedents for the primary and lateral roots. Plants containing this mutation fail to form a primary root but can be induced to make adventitious roots. On the basis of these considerations, we conclude either that IAA is not required for embryonic root formation or that, in the embryo, an ALF4-independent IAA transport or signaling pathway is used.

Although the early stages of primary and lateral root formation are distinct, the phenotype of the alf3-1 mutant suggests that the later stages of lateral and primary root development share components. In alf3-1 strains, both the primary root tip and lateral root primordia require IAA for viability. The growth defect of the primary root tip in alf3-1 mutants is likely the result of a defect in the actively dividing cells that form the apical root meristem. Thus, the actively dividing cells of both the root tip and lateral root primordia of alf3-1 mutants share the same defect, a requirement for IAA.

Materials and methods

Plant strains and growth conditions

A. thaliana ecotypes Columbia (Col-0), Landsberg erecta (Ler), and Wassilewskija (WS) were grown in soil as described previously [Niyogi et al. 1993]. Plants were grown aseptically on unsupplemented PNS (Plant nutrient medium with 0.5% sucrose) growth medium [Haughn and Somerville 1986] or PNS containing the supplements described in the text and figures. Plates were wrapped in gas-permeable surgical tape (3M) and grown under continuous illumination (25–45 µE m⁻² sec⁻¹) with yellow long-pass filters (Stasinopoulos and Hangarter 1990) to reduce the breakdown of indolic compounds. In transfer and time course experiments plates were incubated in a vertical orientation to facilitate straight root growth.

We compared the growth of alf3-1 and ALF3 plants on medium containing a range of toxic and subtoxic concentrations of 5-methylanthranilate and 5-fluoroindole. Mutant and nonmutant plants were equally sensitive to 5-methylanthranilate over the entire range tested, whereas the alf3-1 mutant showed greater sensitivity to 5-fluoroindole than ALF3 plants did.

Mutant screen and genetic analysis

alf1-1 and alf3-1 were isolated as follows. WS seeds (40,000) were mutagenized with ethylmethane sulfonate [EMS] [Niyogi et al. 1993]. The M1 seeds were sown in eight separate pools of 5000 seeds and allowed to self-fertilize. The resulting M2 seeds were screened on 150-mm petri dishes containing PNS supplemented with 50 nM IAA. Seeds (500) were sown per plate and they were incubated at 23°C for 2 weeks. Approximately 80,000 M2 seeds were screened in this manner, and two mutants, alf1-1 and alf3-1, were identified.

Because alf1-1 flowers are infertile, it was necessary to identify heterozygous alf1-1/ALF1 plants. Individual alf1-1/ALF1 heterozygous plants were identified after two successive rounds of subfractionation of the original M2 pool. The alf1-1 phenotype was present in 25% of these heterozygous populations indicating that the phenotype is caused by a recessive mutation. For subsequent backcrosses and outcresses, nonmutant (Alf1⁺) plants from an alf1-1/ALF1 family were crossed to the strain of interest. Two-thirds of these Alf1⁺ plants were expected to be genotypically alf1-1/ALF1, and this expectation was confirmed by examining each plant's progeny that resulted from self-fertilization. F1 seeds derived from crosses to confirmed alf1-1/ALF1 plants were allowed to self-fertilize, and the alf1-1 phenotype was then screened for in the subsequent F2 generation. For the complementation test with hls3 (which is also sterile) five nonmutant plants from heterozygous lines of both alf1-1 and hls3 were crossed to each other in pairwise combinations. Whenever both the alf1-1-derived parental line and the hls3-derived parental line were found to be heterozygous, the alf1-1 phenotype was observed in ~25% of the F2 population. When either parent was found to be homozygous for nonmutant alleles, the F1 population was 100% nonmutant.

Figure 10. A model for lateral root formation based on alf mutants. Details of the model are described in the text. The lateral root primordium is shown emerging from the right side of the primary root. Black indicates the epidermis, cortex, and endodermis, dark gray indicates the pericycle, light gray indicates the vascular tissue. The structure of the Arabidopsis root is based on data from Dolan et al. [1993].
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All (100%) of the M3 progeny resulting from the self-fertilization of the original alf3-1 isolate showed the alf3-1 phenotype. Because of variable expression patterns of the alf3-1 mutant in backcrosses to unmutagenized WS, we outcrossed alf3-1 to the Col-0 ecotype. After three backcrosses to the Col-0 ecotype, alf3-1 behaved as a recessive mutation and the resulting mutant strains were used for all subsequent crosses and growth assays. alf4-1 was found by screening through ~40,000 Col-0 M2 seeds mutagenized with γ-irradiation [Lehle Seed Company]. To identify mutants defective in lateral root production, we plated 500 seeds/150-mm plate on PNS medium containing 80 μM indole-3-acetonitrile (IAN) [an inducer of lateral root formation]. Because the original isolate of alf4-1 was male sterile, nonmutant Col-0 pollen was crossed onto the mutant and the resulting F1 plants were self-fertilized. In the F2 progeny, the alf4-1 mutant was seen in ~25% of the segregants. In subsequent outcrosses and after three backcrosses the male sterility has remained linked to the alf4-1 mutant.

Genetic mapping
The three alf mutants were mapped using a combination of SSLP (simple sequence length polymorphism) [Bell and Ecker 1994] and CAPS (C0-dominant cleaved amplified polymorphic sequences) [Konieczny and Ausubel 1993] molecular markers that detect simple sequence-length polymorphisms and restriction fragment-length polymorphisms, respectively. F2 mapping populations were constructed as follows. alf1-1 (WS ecotype) was outcrossed to Ler. DNA from alf1-1 F2 plants was isolated (see below). alf1-1 showed linkage to the SSLP marker nga168 and the visible mutation erecta on chromosome 2. This is in rough agreement with the map position of rty [King 1994]. alf4-1 (Col-0 ecotype) was outcrossed to Ler. DNA was prepared from entire alf4-1 F2 plants (see below), and the mutation was found to map between SSLP markers nga249 and nga151 on chromosome 5. alf3-1 (WS ecotype) was outcrossed to Col-0. We examined a small population of alf3-1 F1 plants generated in the first outcross to Col-0 and found that alf3-1 mapped between the SSLP marker nga129 and the CAPS marker DFR on chromosome 5. The scoring of the alf3-1 genotype was confirmed in the next generation. For more precise mapping, F2 plants from a third successive outcross to Col-0 were used. The alf3-1 plant used as the mutant parent in this cross was still homozygous for the WS alleles at the nga129 and DFR loci. In this way, these two markers could still be used in an outcross to Col-0. The advantage of this population was that alf3-1 segregated consistently as a recessive mutation and made for reliable identification of homozygous alf3-1 plants in the F2 generation.

Preparation of DNA used for genetic mapping
DNA was prepared from individual mutant F2 plants as described [Klimyk et al. 1993] except for the following modifications. Seedlings [1- to 2-week-old] were placed into the bottom of a 1.5-mL microcentrifuge tube and held on dry ice for a minimum of 5 min. The plant tissue was ground quickly with a plastic pestle after which 10 μL of 0.5 N NaOH was added to the tissue and kept at room temperature until all the samples were ready. The samples were then vacuum infiltrated for 1 min in a Speed-Vac [Savant] after which they were heated to 100°C for 30 sec. The samples were then neutralized with 100 μL of 0.2 M Tris (pH 8), 1 mM EDTA. One to two microliters of the sample was routinely used in 20- to 40-μL PCRs. Samples were stored at −20°C and successfully used after 6 months.

Double mutant construction
For the alf1-1 alf4-1 double mutant, pollen from alf1-1/ALF1 heterozygous plants was crossed onto alf4-1 flowers. F1 plants from the cross were allowed to self-fertilize and F2 populations were identified where both mutations segregated. Because neither mutation could be made homozygous by self-fertilization and the two mutations are in different ecotypes [alf1-1 in WS and alf4-1 in Col-0], we identified the double mutant by determining the genotype of individual F2 plants using closely linked molecular markers [see section on mapping]. F2 plants could all be grouped by phenotype into alf1-1, alf4-1, and nonmutant classes. The ALF4 genotype of plants in each class was determined by screening with the two SSLP markers, nga249, and nga151, which bracket the alf4-1 locus. The ALF1 genotype was identified using the SSLP marker nga168 that we found to be ~20 cm from alf1-1. Therefore we could predict that ~60% of the plants that are homozygous for the WS allele of nga168 would be homozygous for the alf1-1 mutation. All plants that were phenotypically alf1− alf4+ or nonmutant (alf1+ alf4+) were genotypically homozygous or homozygous for the WS alleles of nga249 and nga151 indicating that these plants were either alf4-1/ALF4 or ALF4/ALF4 at the ALF4 locus. On the other hand, of the 54 plants that were phenotypically and genotypically alf4-1, 13 plants were homozygous for the WS allele of nga168. Of these 13 plants, 60% are predicted to be genotypically alf1−/alf1−. This set of putative alf4-1/alf4-1 alf1-1/alf1-1 double mutants were phenotypically indistinguishable from a set of alf4-1/alf4-1 ALF1/ALF1 and alf4-1/alf4-1 alf1-1/alf1-1 ALF1 single mutants.

Because of the male sterility associated with alf4-1, we constructed the alf3-1 alf4-1 double mutant in two steps. First, alf3-1 pollen was crossed onto alf4-1 flowers and the resulting F1 plants were self-fertilized to give F2 populations that had both mutations segregating. In the second step alf3-1 F2 plants were identified and allowed to self-fertilize to create F3 families that were homozygous for alf3-1. Segregation of alf4-1, as defined clearly by the lack of lateral root formation and a short hypocotyl, was observed in several of these families.

Because of the sterility associated with the alf1-1 mutant, the alf3-1 alf1-1 double mutant was constructed by employing a scheme similar to that used to make the alf1-1 alf4-1 double mutant. alf3-1 pollen was crossed onto alf1-1/ALF1 flowers and the resulting F1 plants were self-fertilized to give F2 populations that were segregating both mutations. alf3-1 F2 plants were identified and allowed to self-fertilize to create F3 families that were homozygous for alf3-1. Segregation of alf1-1 was easily identified in several of these families.

Quantitation of lateral root primordia in the alf3-1 mutant
ALF3 and alf3-1 seeds were germinated on PNS and grown for 10 DAG. Using a dissecting microscope, the number of lateral root primordia and the length of the primary root were recorded for 70 plants from each strain. ALF3 plants made an average of 2.9 lateral roots/cm of primary root (s.e. = 0.13), whereas alf3-1 plants made an average of 7.0 lateral root primordia/cm of primary root (s.e. = 0.33).

Vital staining of roots
ALF3 and alf3-1 mutant plants were grown on unsupplemented PNS medium and PNS supplemented with 80 μM indole. At various times after germination seedlings were transferred directly to H2O containing 5 μg/ml of PI and 2 μg/ml of FDA for 15–30 min. The seedlings were then rinsed twice in H2O, once
in a mounting solution of 50% glycerol, 0.01% Triton X-100, and then mounted on a microscope slide. The samples were viewed within 15 min on a Bio-Rad MRC-600 confocal laser microscope. The same subject was recorded separately at wavelengths specific to each stain after which the two images were merged to give the final image.

Microscopes and photography

Dark-field photographs of live plants were made on a Wild M5-A microscope using Kodak Ektachrome 160T film. For bright-field microscopy, samples were fixed overnight in 70% ethanol and mounted under a coverslip in 50% glycerol and 0.01% Triton X-100. Specimens were observed on a Zeiss microscope and recorded on either Kodak Ektachrome 160T film or Kodak Technical Pan film.

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References

Aeschbacher, R.A., J.W. Schiefelbein, and P.N. Benfey. 1994. The genetic and molecular basis of root development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 25–45.
Bell, C.J. and J.R. Ecker. 1994. Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics 19: 137–144.
Berleth, T., and G. Jürgens. 1993. The role of the monopteros gene in organising the basal body region of the Arabidopsis embryo. Development 118: 575–587.
Blakely, L.M. and T.A. Evans. 1979. Cell dynamics studies on the pericycle of radish seedling roots. Plant Sci. Lett. 14: 79–83.
Blakely, L.M., M. Durham, T.A. Evans, and R.M. Blakely. 1982. Experimental studies on lateral root formation in radish seedling roots. I. General methods, developmental stages, and spontaneous formation of laterals. Bot. Gaz. 143: 341–352.
Charlton, W.A. 1991. Lateral root initiation. In Plant roots: The hidden half (ed. Y. Waisel, A. Eshel, and U. Kafkafi), pp. 103–128. Marcel Dekker, New York.
Dolan, L., K. Janmaat, V. Willemsen, P. Linstead, S. Poethig, K. Roberts, and B. Scheres. 1993. Cellular organisation of the Arabidopsis thaliana root. Development 119: 71–84.
Ecker, J.R. and A. Theologis. 1994. Ethylene: A unique plant signaling molecule. In Arabidopsis (ed. E.M. Meyerowitz and C.R. Somerville), pp. 485–521. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Estelle, M.A. and C. Somerville. 1987. Auxin-resistant mutants of Arabidopsis thaliana with an altered morphology. Mol. & Gen. Genet. 206: 200–206.
Evans, M.L., H. Ishikawa, and M.A. Estelle. 1994. Responses of Arabidopsis roots to auxin studied with high temporal resolution: Comparison of wild type and auxin-response mutants. Planta 194: 215–222.
Garbers, C. and C. Simmons. 1994. Approaches to understanding auxin action. Trends Cell Biol. 4: 245–250.
Haughn, G.W. and C. Somerville. 1986. Sulfonylurea-resistant mutants of Arabidopsis thaliana. Mol. & Gen. Genet. 204: 430–434.
Hobbie, L. and M. Estelle. 1995. The aux4 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. Plant J. 7: 211–220.
Horan, P.K. and J.W. Kappler. 1977. Automated fluorescent analysis for cytotoxicity assays. J. Immunol. Methods 18: 309–316.
Jürgens, G. 1994. Pattern formation in the embryo. In Arabidopsis (Ed. E.M. Meyerowitz and C.R. Somerville), pp. 297–312. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Kares, C., E. Prinsen, H. Van Onckelen, and L. Otten. 1990. IAA synthesis and root induction with iaa genes under heat shock promoter control. Plant Mol. Biol. 15: 225–236.
King, J.J. 1994. “Adventitious root formation and auxin homeostasis in Arabidopsis thaliana” L. Heynh.: Genetic and physiological analyses,” Ph.D. thesis. University of Wisconsin-Madison.
Klee, J.H., R.B. Horsch, M.A. Hinchee, M.B. Hein, and N.L. Hoffmann. 1987. The effects of overproduction of two Agrobacterium tumefaciens T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes & Dev. 1: 86–96.
Klimyuk, V.I., B.J. Carroll, C.M. Thomas, and J.D.G. Jones. 1993. Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J. 3: 493–494.
Konieczny, A. and F.M. Ausubel. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4: 403–410.
Krishan, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. 66: 188–193.
Maher, E.P. and S.J.B. Martindale. 1980. Mutants of Arabidopsis thaliana with altered responses to auxins and gravity. Biochem. Genet. 18: 1041–1053.
Moore, T.C. 1989. Biochemistry and physiology of plant hormones. Springer-Verlag, New York.
Niyogi, K.K., R.L. Last, G.R. Fink, and B. Keith. 1993. Suppressors of trp1 fluorescence identify a new Arabidopsis gene, TRP4, encoding the anthranilate synthase β subunit. Plant Cell 5: 1011–1027.
Normanly, J., J.D. Cohen, and G.R. Fink. 1993. Arabidopsis thaliana auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc. Natl. Acad. Sci. 90: 10355–10359.
Okada, K. and Y. Shimura. 1990. Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus. Science 250: 274–276.
Schiefelbein, J.W. and P.N. Benfey. 1991. The development of plant roots: New approaches to underground problems. Plant Cell 3: 1147–1154.
Celenza et al.

——. 1994. Root development in Arabidopsis. In Arabidopsis (ed. E.M. Meyerowitz and C.R. Somerville), pp. 335–354. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stasinopoulos, T.C. and R.P. Hangarter. 1990. Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. Plant Physiol. 93: 1365–1369.

Steeves, T.A. and I.M. Sussex. 1989. Patterns in plant development. Cambridge University Press, Cambridge, UK.

Torrey, J.G. 1950. The induction of lateral roots by indoleacetic acid and root decapitation. Am. J. Bot. 37: 257–264.

Widholm, J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47: 189–194.

Wilson, A.K., F.B. Pickett, J.C. Turner, and M. Estelle. 1990. A dominant mutation in Arabidopsis confers resistance to auxin, ethylene and abscisic acid. Mol. & Gen. Genet. 222: 377–383.

Wright, A.D., M.B. Sampson, M.G. Neuffer, L. Michalczuk, J.P. Slovin, and J.D. Cohen. 1991. Indole-3-acetic acid biosynthesis in the mutant maize orange pericarp, a tryptophan auxotroph. Science 254: 998–1000.
A pathway for lateral root formation in Arabidopsis thaliana.

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