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.

[*Key Words*: Lateral roots; plant development; indole-3-acetic acid]

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Development in plant embryos occurs in a precise manner that is nearly identical from seed to seed for a given plant species (Jürgens 1994). In contrast, postembryonic development, although following a general pattern, is greatly influenced by environmental factors. The timing of development and choice of developmental program are affected by the climate, availability of nutrients, light, and water. Root systems increase in size by branching via lateral root formation and the extent of root branching is dependent on the growth state of the plant. For example, it has been shown that soil-grown plants produce more lateral roots in areas of high nutrient concentration (for review, see Charlton 1991). Despite the extensive studies on root development, it is unclear how root systems perceive environmental cues or what biochemical mechanisms are used to effect changes in development.

Root development in *Arabidopsis thaliana* can be observed easily on a defined agar-based medium (Schiefelbein and Benfey 1991; Aeschbacher et al. 1994). When seeds germinate, the primary root is the first part of the plant to appear. After several days the primary root reaches a length of 2–3 cm. At this time, cell division in the root is limited mostly to the growing primary tip or root apical meristem. Between 5 and 10 days after germination (DAG) foci of coordinated cell divisions occur along the primary root in the pericycle layer, a concentric layer of cells that surrounds the vascular tissue (Blakely and Evans 1979; Dolan et al. 1993; Schiefelbein and Benfey 1994). These clusters of mitotically active cells, or lateral root primordia, first appear as a ball-like structure within the primary root, increase in cell number for 1–2 days and then protrude through the epidermis (outer cell layer) of the primary root. Subsequently, the primordium differentiates into a mature lateral root that arises perpendicular to the long axis of the primary root. The maturation of the lateral root involves the formation of all the same tissues found in the primary root, including a lateral root apical meristem and a vascular system that ultimately connects into the vasculature of the primary root (Dolan et al. 1993). Because each lateral root can produce more lateral roots, a complex root system is constructed by the reiteration of a single developmental process.

Because the origins of the lateral and primary root are distinct, the initiation and establishment of the two structures could be different. The embryonic primary root arises via a stereotypic cell lineage, whereas lateral roots arise postembryonically from a differentiated tissue, the pericycle. In *Arabidopsis*, the pericycle is normally made up of 12 radially arranged cells that surround the vascular tissue (Dolan et al. 1993). The cells lie in a single layer and are organized longitudinally in files along the length of the root. By analogy to radish (a species related to *Arabidopsis*) and other plants, not all pericycle files serve as progenitors of lateral roots; lateral roots arise only from the files of pericycle cells adjacent to the two xylem poles (Blakely and Evans 1979; Steeves and Sussex 1989). It is unknown how this radially bipolar pattern is established.

What are the signals that trigger the initiation of lateral roots and thereby induce mostly quiescent cells to "dedifferentiate" and commence cell division? Two lines of evidence suggest that the plant growth regulator, indole-3-acetic acid (IAA), is a signal for lateral root ini-

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tiation in planta. 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 genes have increased lateral root production (Klee et al. 1987; Kares et al. 1990). IAA plays a number of roles in plant developmental 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 F_2 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 to 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 *rooty* (*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).

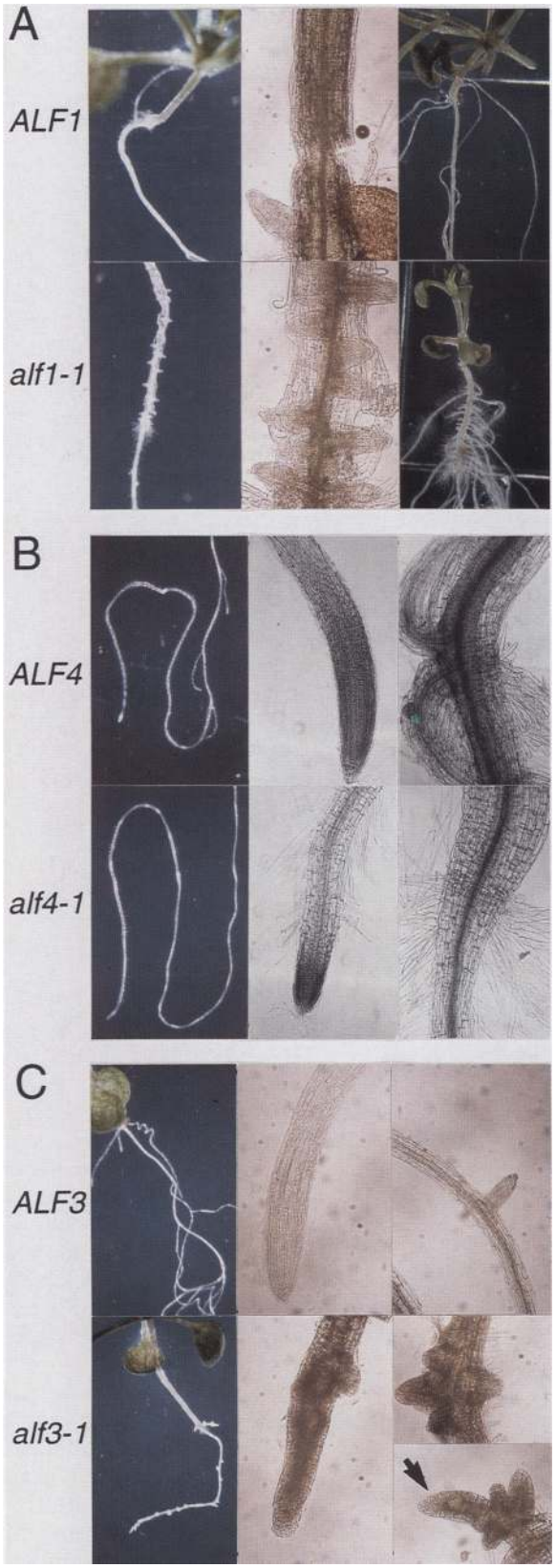
alf4-1 *is defective in lateral root formation*

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 *aux1-7* 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-



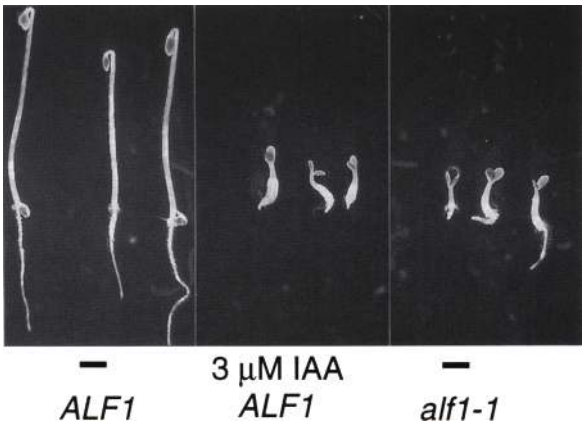


Figure 2. Dark-grown *alf1-1* mutants fail to make an apical hook. *ALF1* (WS) and *alf1-1* seeds were held at 4°C for 5 days and then grown in the dark for 2 days at 23°C on PNS containing 3 μM IAA or unsupplemented PNS (bar).

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.

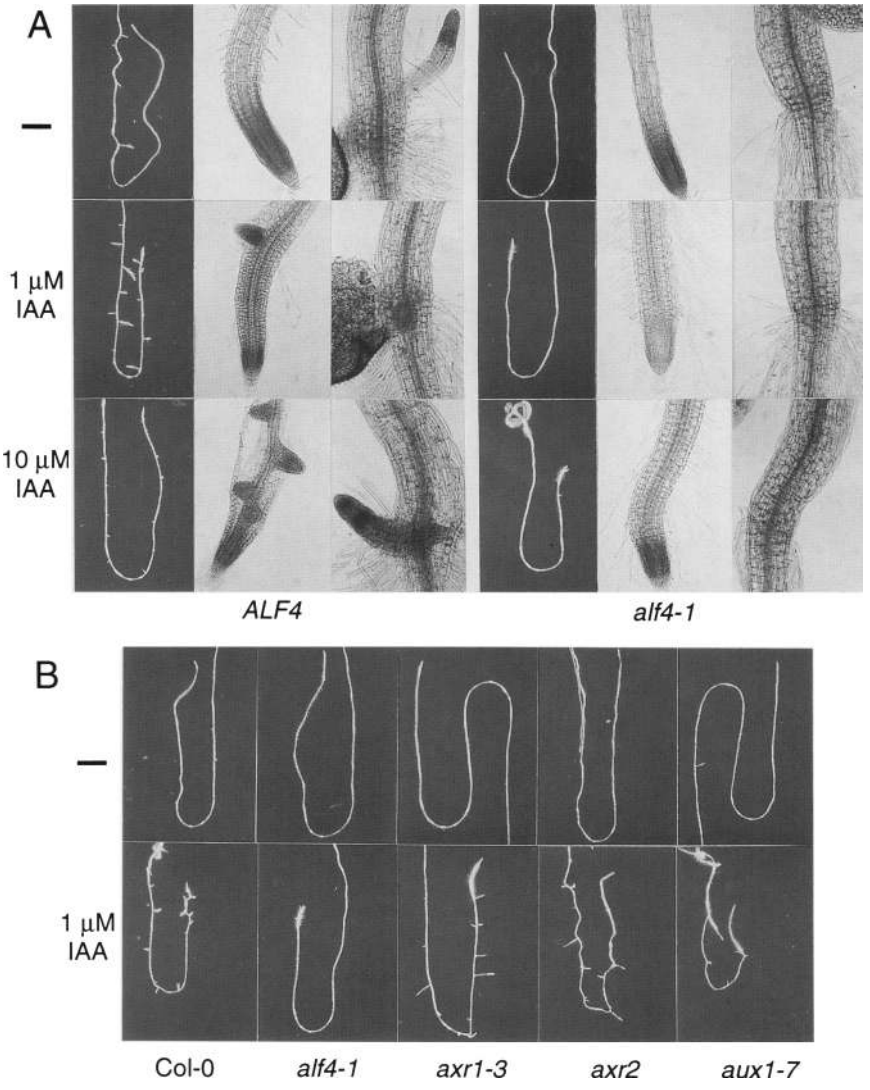


Figure 3. The *alf4-1* mutant is resistant to the IAA induction of lateral roots. (*A*) IAA fails to induce lateral roots in the *alf4-1* mutant. *ALF4* (Col-0) and *alf4-1* strains were grown on PNS medium for 4 DAG and transferred to PNS containing the indicated supplements for 3 more days. Bar, unsupplemented 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.

to 1–2 mm, they appear to remain as a bolus of undifferentiated cells and elongate or develop vascular tissue only rarely. These mutant primordia resemble the primordia of *ALF3* in their overall shape, but those from *alf3-1* generally lack the cellular organization typical of nonmutant primordia. In addition, cells in *alf3-1* lateral root primordia become much larger than cells found in *ALF3* primordia. Because mutant primordia are aberrant, it is difficult to identify the exact point in lateral root development at which *alf3-1* primordia arrest. We also found that the *alf3-1* mutant initiates more than twice the number of lateral root primordia per length of primary root when compared with *ALF3* plants of similar age (see Materials and methods).

The distribution of lateral root primordia is dramatically different between *ALF3* and *alf3-1* plants. In *ALF3* plants, primordia appear as single initiates at some distance from the next closest primordium, whereas in *alf3-1* mutants, lateral root primordia initiate adjacent to and directly upon previously arrested primordia (Fig. 1C). 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-

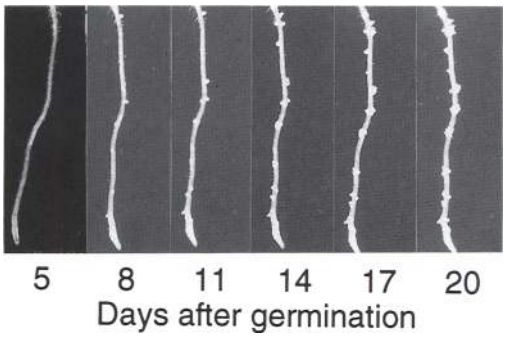


Figure 4. Time course of root development in the *alf3-1* mutant. An *alf3-1* plant was photographed on the days indicated. Note that primary root growth has ceased by day 8.

ficult 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

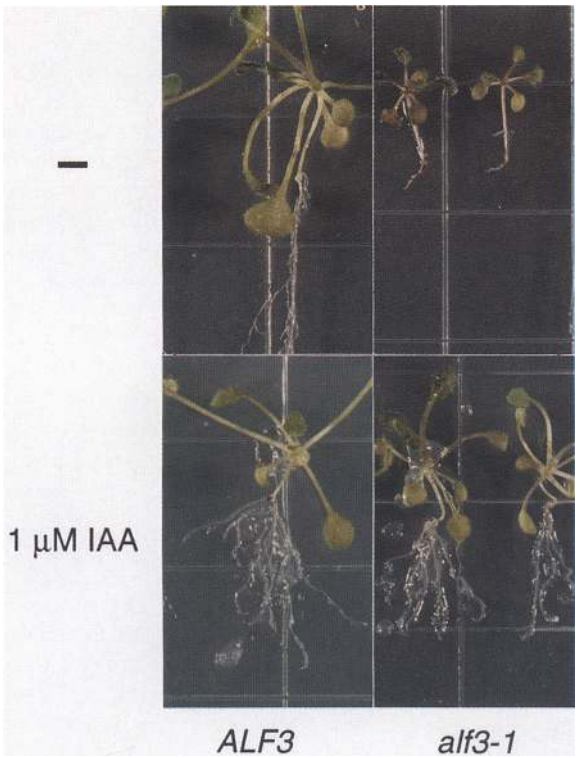


Figure 5. IAA rescues the *alf3-1* mutant. *ALF3* (Col-0) and *alf3-1* plants were grown on unsupplemented PNS (bar) or PNS containing 1 μM IAA. Plants were photographed at 14 DAG.

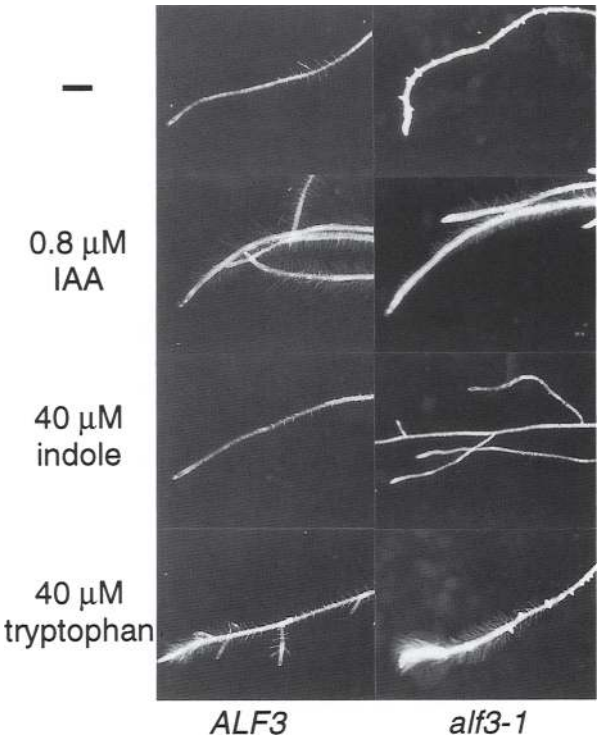


Figure 6. *alf3-1* mutants are rescued by IAA and indole but not by tryptophan. *ALF3* (Col-0) and *alf3-1* plants were germinated on unsupplemented PNS and then transferred at 2 DAG to PNS containing the indicated supplement. Bar, unsupplemented PNS medium. Plants were photographed under dark-field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

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

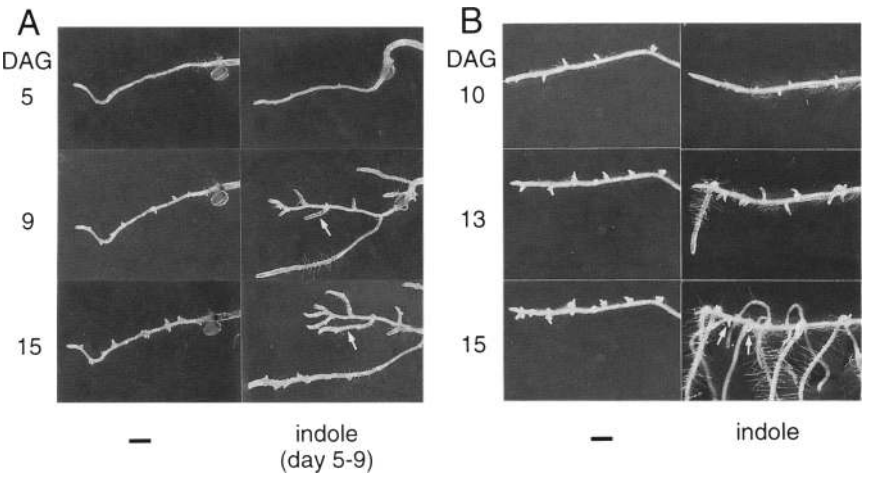


Figure 7. Time course of indole rescue of *alf3-1* roots. (*A*) *alf3-1* lateral roots rescued with indole continuously require indole for further growth. *alf3-1* plants were germinated on unsupplemented PNS medium. The plant on the *right* was transferred at 5 DAG to medium containing 80 μM indole and then returned to unsupplemented medium at 9 DAG. The arrow indicates a lateral root that formed in the presence of indole and then arrested in the absence of indole. The control plant (*left*) was transferred to unsupplemented medium at each time point. The plants were photographed on the days indicated. (*B*) Pre-existing *alf3-1* lateral root primordia are not rescued upon transfer to indole-supplemented medium. *alf3-1* plants were germinated on unsupplemented medium and transferred at 10 DAG to either medium supplemented with 80 μM indole (*right*) or unsupplemented medium (*left*) and photographed on the days shown. Arrows indicate pre-existing lateral roots that were not rescued upon transfer to indole-containing medium.

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 di-acetate (FDA) is cleaved by esterases present in live 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 roots and 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

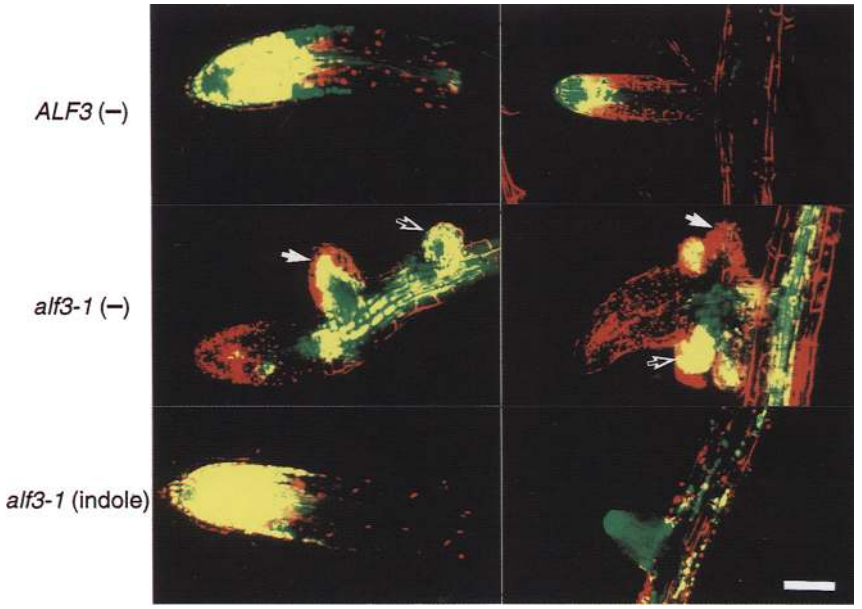


Figure 8. Cells in *alf3-1* lateral roots die prematurely. *ALF3* (Col-0) and *alf3-1* seedlings were grown on unsupplemented PNS medium (bar) or PNS supplemented with 40 μM. At 12–16 DAG, plants were stained with FDA (green) and PI (red) and viewed using confocal laser microscopy as described in Materials 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.

exogenously added IAA. Moreover, these phenotypes suggest that *ALF4* acts before *ALF3* in the formation of lateral roots.

The phenotype of the *alf1-1 alf3-1* double mutant suggests that the *alf1-1* mutation suppresses the *alf3-1* mutation. As in the *alf1-1* mutant, the *alf1-1 alf3-1* double mutant produces a vast excess of adventitious and lateral roots (Fig. 9C). Moreover, most lateral and adventitious roots in the *alf1-1 alf3-1* double mutant develop beyond the arrest point of the single *alf3-1* mutant. These data suggest that the higher levels of internal IAA caused by the *alf1-1* defect rescue the *alf3-1* defect. Suppression is not complete because both the lateral and adventitious roots arrest growth prematurely.

The alf3-1 *mutation causes growth arrest of the primary root tip*

Although the most obvious effects of our mutations are on lateral root formation, they also reveal important aspects of the relationship between the primary and lateral root. None of our mutants affects the formation of the primary root, suggesting that these genes are not involved in the formation of the embryonic root. However, *ALF3*, but not *ALF4*, is required for the continued growth of the root tip. In the *alf3-1* mutant, the primary root tip arrests growth (Figs. 1C and 4) and is inviable (Fig. 8) in the absence of IAA or indole. The presence of IAA or indole restores both cell division and viability (Figs. 5, 6A, and 8). In this sense, the primary root tip has similar properties to the lateral root primordia in *alf3-1* mutants. These observations explain the primary root tip phenotype of the *alf3-1 alf4-1* double mutant. The cells of the root tip form as they do in the *alf4-1* mutant, but lacking *ALF3* fail to elongate or divide and lose viability (Fig. 9B).

Discussion

A model for lateral root development based on alf *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

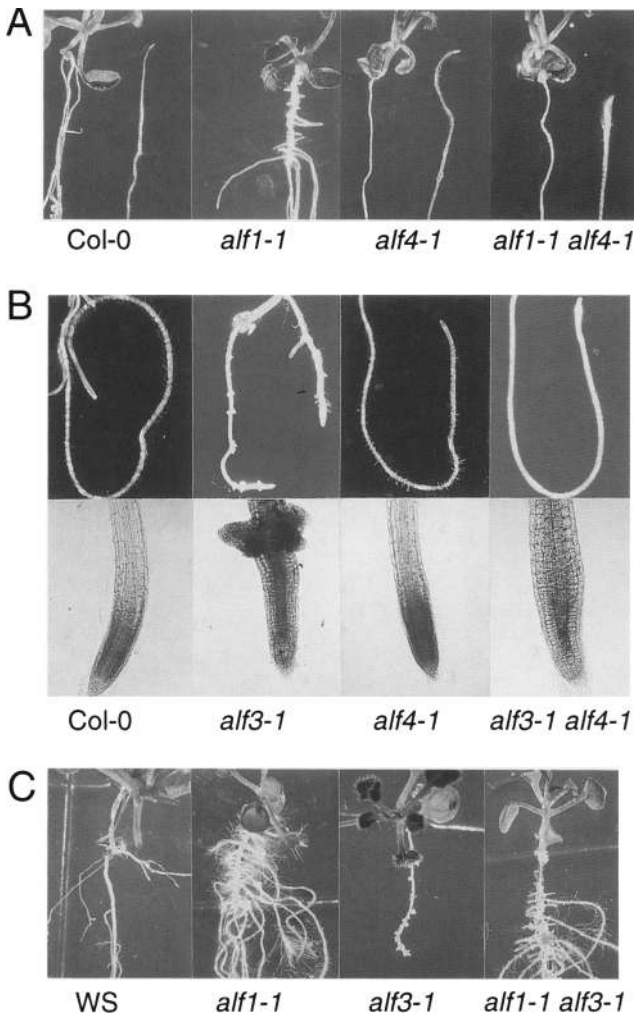


Figure 9. Phenotypes of *alf* double mutants. (*A*) *alf1-1 alf4-1* double mutant. Plants were photographed under dark-field illumination at 17 DAG. (*B*) *alf3-1 alf4-1* double mutant. Plants were photographed under dark-field and bright-field illumination at 10 DAG. (*C*) *alf1-1 alf3-1* double mutant. Plants were photographed under dark-field illumination at 16 DAG.

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.

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

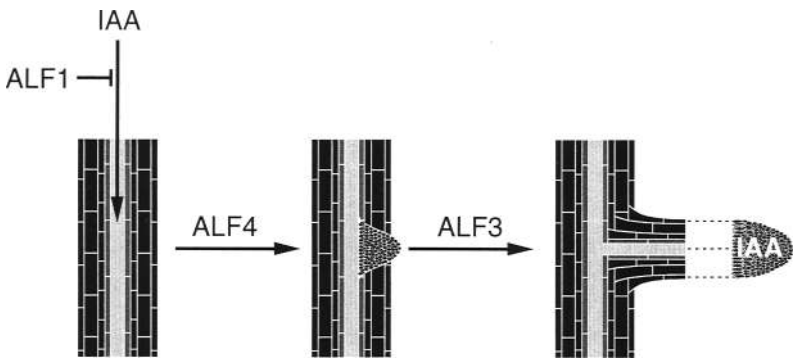


Figure 10. A model for lateral root formation based on *alf* mutants. Details of the model are described in the text. The lateral root primordium is shown emerging from the *right* side of the primary root. Black indicates the epidermis, cortex, and endodermis; dark gray indicates the pericycle; light gray indicates the vascular tissue. The structure of the *Arabidopsis* root is based on data from Dolan et al. (1993).

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 asceptically (Niyogi et al. 1993) 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) 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 M_1 seeds were sown in eight separate pools of 5000 seeds and allowed to self-fertilize. The resulting M_2 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 M_2 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 M_2 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 outcrosses, 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. F_1 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 F_2 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 F_1 population. When either parent was found to be homozygous for nonmutant alleles, the F_1 population was 100% nonmutant.

All (100%) of the M_3 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 outcrosses 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 M_2 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 F_1 plants were self-fertilized. In the F_2 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 (CO-dominant cleaved amplified polymorphic sequences) (Konieczny and Ausubel 1993) molecular markers that detect simple sequence-length polymorphisms and restriction fragment-length polymorphisms, respectively. F_2 mapping populations were constructed as follows. *alf1-1* (WS ecotype) was outcrossed to Ler. DNA from *alf1-1* F_2 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* F_2 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* F_2 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, F_2 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 F_2 generation.

Preparation of DNA used for genetic mapping

DNA was prepared from individual mutant F_2 plants as described (Klimyuk 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. F_1 plants from the cross were allowed to self-fertilize and F_2 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 F_2 plants using closely linked molecular markers (see section on mapping). F_2 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 heterozygous 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 *alf1-1/alf1-1*. 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* 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 F_1 plants were self-fertilized to give F_2 populations that had both mutations segregating. In the second step *alf3-1* F_2 plants were identified and allowed to self-fertilize to create F_3 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 *alf3-1 alf4-1* double mutant. *alf3-1* pollen was crossed onto *alf1-1/ALF1* flowers and the resulting F_1 plants were self-fertilized to give F_2 populations that were segregating both mutations. *alf3-1* F_2 plants were identified and allowed to self-fertilize to create F_3 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 H_2O containing 5 μg/ml of PI and 2 μg/ml of FDA for 15–30 min. The seedlings were then rinsed twice in H_2O, 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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