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Genetic Control of Root Hair Development in *Arabidopsis thaliana*

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Visual examination of roots from 12,000 mutagenized *Arabidopsis* seedlings has led to the identification of more than 40 mutants impaired in root hair morphogenesis. Mutants from four phenotypic classes have been characterized in detail, and genetic tests show that these result from single nuclear recessive mutations in four different genes designated *RHD1*, *RHD2*, *RHD3*, and *RHD4*. The phenotypic analysis of the mutants and homozygous double mutants has led to a proposed model for root hair development and the stages at which the genes are normally required. The *RHD1* gene product appears to be necessary for proper initiation of root hairs, whereas the *RHD2*, *RHD3*, and *RHD4* gene products are required for normal hair elongation. These results demonstrate that root hair development in *Arabidopsis* is amenable to genetic dissection and should prove to be a useful model system to study the molecular mechanisms governing cell differentiation in plants.

INTRODUCTION

In the root systems of higher plants, some of the epidermal cells form long, tubular outgrowths called root hairs. By greatly increasing the total surface area of the root system, root hairs are believed to play an important role in the absorption of water and nutrients from the soil (Clarkson, 1985). These structures also serve as attachment sites for soil-borne microbes, including nitrogen-fixing bacteria like *Rhizobium* (Bauer, 1981). Because of their rapid growth and accessibility, root hairs have been widely used in studies of plant cell expansion. The constituents of the root hair cell wall have been analyzed (Belford and Preston, 1961; Cormack, 1962; Mort and Grover, 1988), and ultrastructural studies of cellulose microfibril and microtubule orientation in growing hairs have been reported (Newcomb and Bonnett, 1965; Sievers and Schnepf, 1981; Lloyd, 1983; Emons and Wolters-Arts, 1983).

Despite the interest in root hair functions and cell expansion, there is relatively little known about the developmental mechanisms involved in the formation of root hairs. At its simplest, the overall process of root hair development can be divided into two stages: initiation and elongation. Root hair initiation encompasses all the processes required for a root epidermal cell to produce a localized "bulge" in its cell wall. These processes presumably include a developmental decision that determines whether or not a particular cell will form a hair, and also includes those factors required for the cell wall loosening that must occur for the wall to yield to internal pressure. The elongation stage of root hair development is known to depend on growth at the root hair tip (Sievers and Schnepf, 1981). For tip growth to occur, new cell wall components need to be directed to, and deposited at, the tip of the growing tube. The mechanism responsible for establishing and maintaining this polarity in cell growth is not understood, although studies on pollen tubes (which also develop by tip growth) suggest that calcium-associated ion currents play an important role (Weisenseel, Nuccitelli, and Jaffe, 1975; Herth, 1978; Picton and Steer, 1983).

As with many other aspects of plant development, the isolation of mutations affecting various steps in root hair development may be a useful method of identifying the genes that regulate this process. Previous studies have indicated that some aspects of root hair development are under genetic control. For example, selection for root hair length within a single cultivar of white clover resulted in populations with differences in mean root hair length (Caradus, 1979). In addition, mutations that affect root hairs have been identified through screens for auxin-resistant *Arabidopsis* plants [*Dwf* (Mirza et al., 1984), *axr2* and *axr3* (M. Estelle, personal communication)] and a screen for phosphate uptake efficiency in tomato [cottony root (Hochmuth, Gabelman, and Gerloff, 1985)]. However, perhaps because of the difficulties involved in observing large numbers of plant root systems, there does not appear to have been an attempt to screen directly for root hair mutants. *Arabidopsis* is well suited to the analysis of root pheno-

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types because relatively large numbers of plants can be grown on the surface of agar-solidified medium in vertically oriented Petri dishes (Caspar and Pickard, 1989). We have exploited this characteristic to screen visually a population of mutagenized seedlings for mutations that affect root hair morphology. In this report, we describe this screening procedure and the isolation and characterization of four classes of root hair mutants.

RESULTS

Mutant Isolation

A simple visual screening protocol was employed to identify *Arabidopsis* mutants with altered root hair development. When *Arabidopsis* seedlings are grown on Petri plates in a vertical orientation, the roots grow along the surface of the agar medium, permitting observation of the developing roots. Figure 1A illustrates the growth of *Arabidopsis* roots under these conditions; root hairs are easily visible on the primary root after only 3 days to 4 days of incubation (Figure 1B). A total of approximately 12,000 M_2 seedlings descended from mutagenized seed were screened by placing seeds on 100 cm^2 nutrient agar plates (100 seeds per plate), incubating the plates at 22°C for 4 days in a vertical orientation, and examining the roots with a dissecting microscope. More than 70 plants that produced root hairs differing from the wild type in either length or morphology were identified. Following transfer of the seedlings to soil, most of the plants survived to maturity and set seed. After retesting the root hair phenotype of these putative mutants in the following (M_3) generation, more than 40 true-breeding mutant lines were retained. In addition to the root hair mutants, approximately 30 mutants that exhibit other abnormalities in root development were recovered by this method (J. Schiefelbein and C. Somerville, unpublished results).

Examination of the root hair mutants allowed them to be subdivided into several different phenotypic classes. Representative mutants from the four main classes were chosen for more detailed study. These four mutant lines (JS3, JS9, JS29, and JS44) produce root hairs that are distinctly different from the wild type and one another. In each line, the mutation responsible for the root hair defect has been stably inherited through four successive selfing generations. Despite the root hair abnormalities, each mutant is vigorous and fertile. Furthermore, although some of the root hair phenotypes appear to be similar to the phenotypes of trichome (leaf hair) mutants of *Arabidopsis* (Haughn and Somerville, 1988; Marks and Feldmann, 1989), none of the root hair mutations affect trichome morphology. A description of the root hairs produced by each of these mutants is presented in a later section.

Genetic Analyses

The genetic basis for each of the root hair mutant phenotypes was determined by manually cross-pollinating each mutant to the wild type and analyzing the F_1 and F_2 progeny. The progeny were scored by examining their root hair phenotypes after growth for 4 days on vertically oriented nutrient agar plates. In each case, the F_1 plants produced hairs of wild-type phenotype, and the F_2 plants segregated for the appropriate mutant phenotype in a manner consistent with the 3 wild type:1 mutant hypothesis. These data are presented in Table 1, and they indicate that each of the mutant phenotypes is caused by a single nuclear recessive mutation.

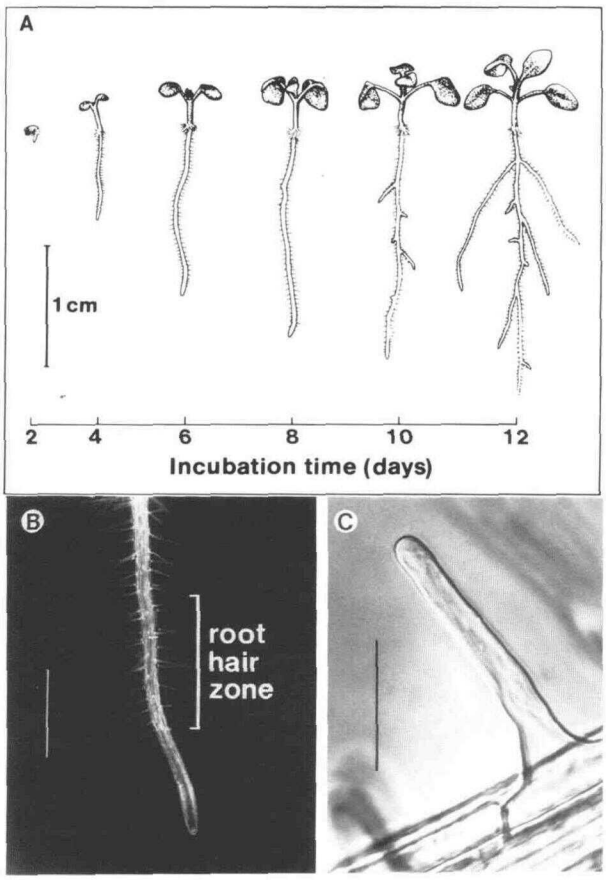


Figure 1. Growth of Wild-Type *Arabidopsis* Roots and Root Hairs on Nutrient Agar.

(A) Drawings of typical seedlings after 2 days to 12 days of incubation. Bar equals 1 cm.

(B) Apex of 4-day-old root with the zone of root hair development indicated. Bar equals 1 mm.

(C) Immature, elongating root hair examined with Nomarski optics. Bar equals 50 μm.

Table 1. Genetic Segregation of Root Hair Mutations

Cross	Number of Plants: Wild-Type Hairs	Number of Plants: Mutant Hairs	χ^{2a}
Wild type × JS44 (*rhd1*), F_1	32	0	
Wild type × JS44 (*rhd1*), F_2	202	63	0.212[b]
Wild type × JS9 (*rhd2*), F_1	30	0	
Wild type × JS9 (*rhd2*), F_2	204	62	0.406[b]
Wild type × JS3 (*rhd3*), F_1	27	0	
Wild type × JS3 (*rhd3*), F_2	270	87	0.076[b]
Wild type × JS29 (*rhd4*), F_1	25	0	
Wild type × JS29 (*rhd4*), F_2	183	60	0.012[b]

[a] χ^2 calculation based on an expected ratio of 3 wild type:1 mutant.
[b] $P > 0.5$.

To determine whether the mutations identified in these lines affected the same or different genes, complementation tests were performed. Each mutant was cross-pollinated to the others, and the F_1 plants were examined for their root hair phenotype. In each instance, the F_1 plants produced hairs of wild-type phenotype, indicating that the mutations reside in four different complementation groups, each of which has a distinctive mutant phenotype. Because the mutations affect various aspects of root hair development, we have designated the genes defined by these complementation groups *RHD1*, *RHD2*, *RHD3*, and *RHD4*.

By performing complementation analyses with other root hair mutants in the collection, additional lines with mutations in these *RHD* genes have been isolated. In all, one *rhd1* mutant, three *rhd2* mutants, two *rhd3* mutants, and two *rhd4* mutants have been recovered. Mutants with defects in the same gene produce root hairs with the same phenotype, with one exception. The root hairs produced by the two *rhd3* mutants have slightly different phenotypes; the mutant with the most severe phenotype has been used in the studies reported here. Because a single M_2 seed source was used in the mutant screen (with an M_1 population size of 12,000 plants), the additional *rhd* mutants do not necessarily represent independent isolates containing different alleles of these genes.

Root Hair Phenotypes

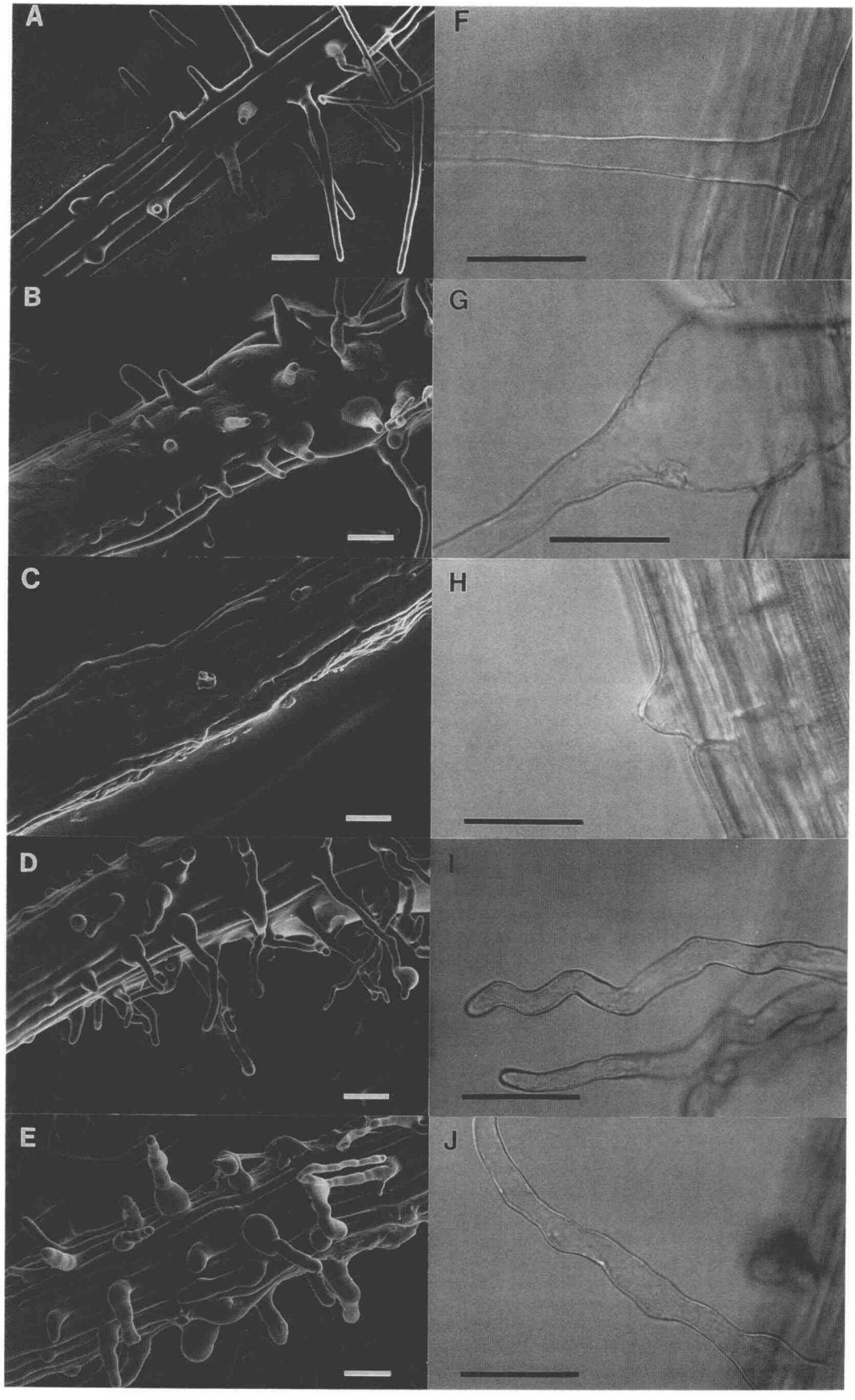
Root hairs normally develop near the root apex, just behind the zone of maximum root elongation, in a region often referred to as the root hair zone. In wild-type *Arabidopsis* seedlings grown on nutrient agar, the hairs begin to emerge approximately 1 mm behind the root tip and they become progressively longer throughout the root hair zone until they reach their mature length (approximately 1 mm) at the basal end of the root hair zone, as shown in Figure 1B. Each epidermal cell does not produce a hair, but in those that do, the hair emerges at the apical end of the cell (the end nearest the root tip). Although generally described as cylindrical in shape, root hairs are not perfectly cylindrical; rather, the diameter of the hair decreases slightly from the base to the tip. Examples of wild-type hairs are illustrated in Figures 1C and 2F (Nomarski optics) and in Figure 2A (scanning electron microscopy).

The phenotypes of the root hair mutants indicate that, in each case, the mutations affect the morphology of the individual root hair cells and do not noticeably alter the spacing or distribution of root hairs. In addition, mutations in three of the four genes (the exception is *RHD3*) are root hair-specific; these mutant plants cannot be distinguished visually from the wild type in any other way. The morphology of the hairs along the root is uniform in each of the mutants, except for *rhd1* hairs, which are somewhat variable in phenotype. The descriptions of root hair mutants and double mutants that follow are based on the most common phenotypes observed.

The *rhd1* mutant produces hairs that are similar in length to the wild type. However, the distinctive feature of these hairs is the presence of a wide ("bulbous") region at the base of the hair (Figures 2B and 2G). This condition appears to be confined to the hair-forming cells; epidermal cells that do not produce root hairs do not seem to be affected. As mentioned previously, the phenotype of *rhd1* hairs is variable; some of this variation appears to be related to the age of the plant at the time of hair emergence. Generally, hairs that are formed early are less affected (possess a smaller bulge at the hair base) than hairs formed after 3 days to 4 days of root growth (the phenotype depicted in Figures 2B and 2G). In some *rhd1* hairs, the entire epidermal cell wall appears to be forced outward to form the basal portion of the hair.

Plants homozygous for mutations in the *RHD2* gene produce very short root hairs. As illustrated in Figures 2C and 2H, it appears that hairs begin to emerge from the epidermal cells, but they do not elongate, which leads to a "stubby" hair phenotype. Despite the severe root hair phenotype, mutant plants are normal in appearance and exhibit no other morphological abnormalities.

Homozygous mutations in the *RHD3* gene cause plants to produce root hairs that are shorter than the wild type (approximately 0.5 mm in the most severe mutant and 0.6 mm to 0.7 mm in the less severe mutant), exhibit a wavy appearance, and are occasionally branched (Figures 2D and 2I). Rather than elongating in a single direction perpendicular to the root axis, *rhd3* hairs appear to elongate in slightly different directions over time to produce the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wavy hairs. This may be caused by asymmetric deposition of cell wall material at the root hair tip. As previously mentioned, *RHD3* is unique among the four *RHD* genes because mutations in this gene do not appear to be root hair-specific. In addition to the root hair deformations, each of the *rhd3* mutants produces a root that is slightly shorter than the wild type (approximately 70% of wild type after 4 days of incubation in the most severe mutant), and there is a corresponding reduction in the overall size of mature *rhd3* plants relative to wild type.

Plants homozygous for mutations in the *RHD4* gene produce hairs that are shorter than wild-type hairs (approximately 0.5 mm to 0.6 mm) (Figure 2E). The hairs also vary in diameter along their length, forming bulges and constrictions during the elongation process (Figures 2E and 2J). Occasionally, these mutant plants also produce hairs that are branched. Mutations in *rhd4*, like mutations in *rhd3*, seem to alter the process of cell wall deposition at the root hair tip.

Double Mutant Analyses

To define possible epistatic interactions between the *RHD* genes, all six double mutant combinations of the four genes were constructed. The F_1 plants used for complementation analysis (heterozygous for each pair of mutations) were allowed to self-pollinate to produce F_2 seed. Because the phenotype of the double mutants was not known beforehand, the genotypes of putative double mutant plants from the F_2 population were tested by cross-pollinating them to each of the homozygous-mutant parent plants. The double mutant phenotypes were revealed when the progeny from these testcrosses were examined; those F_2 plants whose testcross progeny were homozygous for each of the parental mutations were retained as confirmed double mutants.

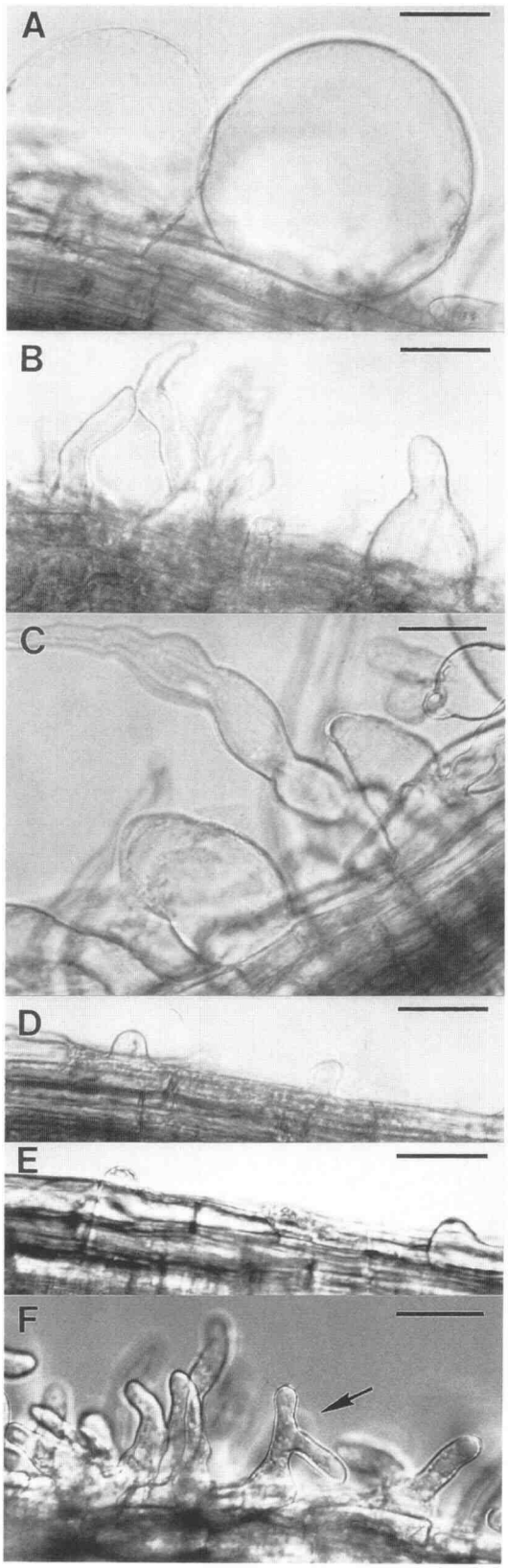
The root hair phenotypes of the double mutants indicate that the combination of two homozygous *rhd* mutations in a single plant results in either an additive or a completely epistatic interaction. The phenotypes of typical root hairs from these double mutants are shown in Figure 3 and the results outlined in Table 2. The double mutant combinations *rhd1 rhd2*, *rhd1 rhd3*, *rhd1 rhd4*, and *rhd3 rhd4* each produce root hairs that display the phenotype expected from the simple addition of the effects of the two single mutations (an additive interaction). For example, the *rhd1 rhd2* double mutant produces hairs with a bulbous base (due to the effect of the *rhd1* mutation), but normal elongation is prevented by the *rhd2* mutation, resulting in large, spherical-shaped hairs (Figure 3A). On the other hand, plants homozygous for mutations in the *RHD2* gene and either the *RHD3* or *RHD4* genes produce root hairs identical in phenotype to those produced by *rhd2* plants (an epistatic interaction).

DISCUSSION

By using a simple visual screening procedure to isolate plants with altered root hairs, we have identified four different genes of *Arabidopsis thaliana* that are involved in root hair development. Because mutations in each of the four genes (*RHD1*, *RHD2*, *RHD3*, and *RHD4*) result in the formation of root hairs with distinct phenotypes, it was possible to use a developmental genetic approach (Botstein and Maurer, 1982) to begin to dissect the process of root hair development. In Figure 4, an outline of *Arabidopsis* root hair development is presented that indicates the stage at which each *RHD* gene appears to be required. Of these four genes, *RHD1* appears to be required at the earliest step. The bulbous base phenotype of the *rhd1* mutant is interpreted to mean that the *RHD1* product is normally involved in regulating the degree of epidermal cell wall loosening during root hair initiation. Thus, when the normal function of the *RHD1* gene is altered, a larger portion of the cell wall yields to the internal pressure, resulting in hairs with a bulbous base. Each double mutant combination involving *rhd1* also produces hairs with a

Figure 2. Phenotype of Wild-Type and Mutant *Arabidopsis* Root Hairs.

(A) to **(E)** Scanning electron micrographs of elongating hairs in the root hair zone. Each micrograph is oriented such that the direction of root growth (and, thus, the location of the root tip) is toward the lower left in each panel. Bars equal 50 μm.
(A) Wild type.
(B) *rhd1*.
(C) *rhd2*.
(D) *rhd3*.
(E) *rhd4*.
(F) to **(J)** Light micrographs of typical, mature root hairs using Nomarski optics. The direction of root growth is toward the bottom of each panel. Bars equal 50 μm.
(F) Wild type.
(G) *rhd1*.
(H) *rhd2*.
(I) *rhd3*.
(J) *rhd4*.



bulbous base, a further indication that the *RHD1* gene product is required early in hair development. Each of the three other genes appears to be involved in some aspect of root hair elongation. Of these, the *RHD2* gene product appears to be required before either *RHD3* or *RHD4* because the *rhd3* and *rhd4* mutants produce hairs that are able to elongate (albeit abnormally), whereas the *rhd2* hairs remain very short. Furthermore, the *rhd2 rhd3* and *rhd2 rhd4* double mutants produce hairs identical in phenotype to the *rhd2* mutant alone. The present data do not provide evidence to distinguish between the developmental timing of the *RHD3* and *RHD4* genes. Each appears to be necessary for proper cell expansion at the tip, and the *rhd3 rhd4* double mutant produces hairs that exhibit the combined effect of each single mutation. In summary, we interpret these results to mean that the *RHD1* gene encodes a product that acts during root hair initiation, whereas the *RHD2*, *RHD3*, and *RHD4* gene products are required for normal root hair elongation (Figure 4).

These results also provide some clues about the nature of the products encoded by the *RHD* genes. It is likely that three of the four gene products (the exception is *RHD3*) are root hair-specific, and that each of the mutant phenotypes is caused by loss-of-function mutations. Furthermore, the double mutant analysis indicates that at least three independent factors required for root hair development have been identified by mutation. This conclusion is derived from the additive effect observed in each of the double mutant combinations between *rhd1*, *rhd3*, and *rhd4*.

Because of the complicated nature of cell differentiation, there are many possible explanations for the roles of the *RHD* gene products. One possibility is that one or more of these genes may be required for the synthesis of structural elements of the root hair cytoskeleton or cell wall (e.g., a root hair-specific tubulin, actin, or extensin). Alternatively, some of these genes may be involved in establishing or maintaining the characteristic polar cell growth of root hairs (Schnepf, 1986). Little is known about the biochemical basis of this process, although general factors have been identified that alter root hair growth, including calcium ions and the plant hormones auxin and ethylene (Cormack, 1962; Mirza et al., 1984; M. Estelle, personal communication; J. Schiefelbein and C. Somerville, unpublished re-

Figure 3. Root Hair Phenotypes of Double Mutants.

The direction of root growth is toward the left of each panel.
(A) to **(F)** Light microscopy using Nomarski optics.
(A) *rhd1 rhd2*.
(B) *rhd1 rhd3*.
(C) *rhd1 rhd4*.
(D) *rhd2 rhd3*.
(E) *rhd2 rhd4*.
(F) *rhd3 rhd4*. A branched hair is indicated with an arrow.
Bars = 50 μm.

Table 2. Summary of Double Mutant Phenotypes

Genotype	Phenotype	Genetic Interaction
rhd1 rhd2	Bulbous, stubby hairs (*rhd1* + *rhd2*)	Additive
rhd1 rhd3	Bulbous, wavy hairs (*rhd1* + *rhd3*)	Additive
rhd1 rhd4	Bulbous, short hairs (*rhd1* + *rhd4*)	Additive
rhd2 rhd3	Stubby hairs (*rhd2*-like)	Epistatic
rhd2 rhd4	Stubby hairs (*rhd2*-like)	Epistatic
rhd3 rhd4	Very short, wavy hairs (*rhd3* + *rhd4*)	Additive

sults). By the further characterization of these mutants, including analyses at the molecular level, it may be possible to gain a better understanding of the nature of the *RHD* gene products and their roles in root hair development. These studies may also lead to new insights regarding developmental processes in other cells that exhibit polar growth, such as pollen tubes (Sievers and Schnepf, 1981), fungal hyphae (Wessels, 1986), *Funaria* caulonema tip cells (Schmiedel and Schnepf, 1980), and *Fucus* zygotes (Quatrano, 1978).

An interesting observation from these studies is that, although these mutant plants have severe root hair abnormalities, each of them is healthy and fertile. It appears, therefore, that fully developed root hairs are not required for the growth of *Arabidopsis* under the experimental conditions used here. It should be informative to subject these mutants to water and nutrient deprivation to assess the importance of root hair length and morphology in absorption.

Some of the root hair phenotypes exhibited by these mutants are similar to the root hair deformations induced by nitrogen-fixing bacteria on compatible hosts. In many cases, the *Rhizobium*-legume symbiosis is initiated by the formation of infection threads in the root hairs and curling of the hairs (Bauer, 1981; Callaham and Torrey, 1981). Root hair deformations are also induced by *Frankia* during infection of the roots of certain woody angiosperms (Newcomb and Wood, 1987). These reported root hair deformations include wavy hairs (apparently similar to the *rhd3* hairs) and branched hairs (occasionally seen in the *rhd3* and *rhd4* mutants; see Figure 3F). Thus, an interesting possibility is that genes homologous to the ones identified in this study are involved in, or affected by, the host plant's early interactions with these nitrogen-fixing bacteria.

The process of root hair development in *Arabidopsis* should provide a useful model system for studying cell differentiation at the molecular genetic level. First, because the phenotype can be observed at the seedling level, large numbers of individual plants can be examined in a small amount of space. In addition, it appears that all root hair mutants, including ones that lack root hairs, are viable. Root hairs develop in a simple and predictable manner, without the complications of cell division. Furthermore, root hair development occurs continuously in a specific zone that allows observation of each stage of development (from emergence to full-length hair) in one section of root. Finally, the development of tools in *Arabidopsis* to clone genes identified only by mutant phenotype, such as chromosome walking using restriction fragment length polymorphism markers (Chang et al., 1988; Nam et al., 1989), should permit analyses of root hair genes at the molecular level.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* (L.) Heynh. lines used in these experiments were all derived from the Columbia wild type. For growth of plants in Petri dishes, seeds were surface-sterilized as described (Estelle and Somerville, 1987) and placed on the surface of agarose-solidified nutrient medium in 100 cm^2 Petri dishes. The medium contained MS salts (GIBCO) supplemented with 20 g/L sucrose, 0.5 g/L Mes (pH 5.8), and 0.6% agarose. The plates

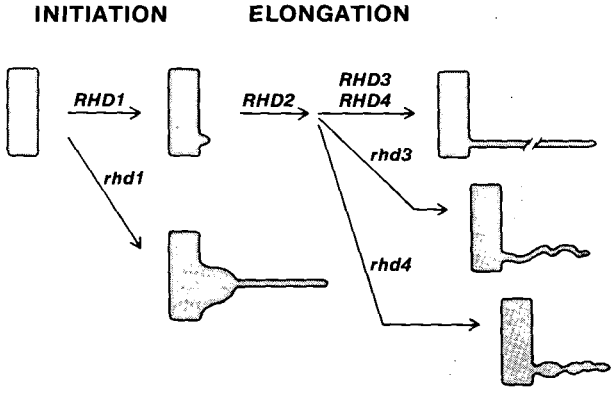


Figure 4. Proposed Genetic Pathway of *Arabidopsis* Root Hair Development.

The top row of root epidermal cell drawings indicate the normal stages of root hair development. Each *RHD* gene is positioned at the point within this developmental pathway where its normal product is initially required, based on the experiments described in the text. Mutations in each gene result in the production of hairs with the phenotype immediately preceding the gene symbol (e.g., *rhd2*) or hairs of a phenotype not normally encountered during root hair development (e.g., *rhd1*, *rhd3*, *rhd4*), in which case the phenotype is schematically drawn below the normal pathway. The drawing of the mature wild-type root hair is not to scale; its actual length, relative to the other drawings, is approximately 2 times greater than indicated.

were incubated in a horizontal orientation for 48 hr at 4°C, and then sealed with Parafilm and incubated in a vertical orientation at 22°C under continuous fluorescent illumination (100 μmol to 150 μmol/m²/sec in the 400 nm to 700 nm range). Plants selected from Petri dishes were grown to maturity in a support medium consisting of a 1:1:1 mixture of spaghnum:perlite:vermiculite irrigated with mineral nutrients (Estelle and Somerville, 1987).

Mutant Screening

Mutagenesis of *Arabidopsis* with ethyl methane sulfonate was carried out by previously described procedures (Estelle and Somerville, 1987). After sterilization, the M_2 seeds were distributed in two rows on the agar surface at a density of 5 seeds/cm (100 seeds/plate) using a sterile transfer pipette. Root hairs were inspected with a Wild M5 dissecting microscope after 4 days of incubation and were reexamined 3 days later, at which time putative mutants were transferred to the potting medium and grown to maturity. Twenty selfed (M_3) seeds from each of the putative mutants were retested on nutrient agar plates and the true-breeding lines retained.

Microscopy

All microscopic observations of roots were made with seedlings grown for 4 days in Petri dishes under the conditions described above. Low-magnification observations of the roots on agar were made with a Wild M5A dissection microscope equipped with an MPS12 camera system. For light microscopy, the root was placed in a drop of liquid nutrient media (as above) on a slide and examined with a Leitz Laborlux 12 microscope with Nomarski Differential Interference Contrast optics. For scanning electron microscopy, the terminal 1 cm of root was excised, placed on embedding medium (Tissue Tek, Miles Laboratories), frozen in a liquid nitrogen slush, coated with gold, and examined in the frozen state (approximately −150°C) on a JSM-35C scanning electron microscope equipped with an EMscope SP-2000 cryo system.

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