

ACTIN2 Is Essential for Bulge Site Selection and Tip Growth during Root Hair Development of Arabidopsis¹

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Root hairs develop as long extensions from root epidermal cells. After the formation of an initial bulge at the distal end of the epidermal cell, the root hair structure elongates by tip growth. Because root hairs are not surrounded by other cells, root hair formation provides an excellent system for studying the highly complex process of plant cell growth. Pharmacological experiments with actin filament-interfering drugs have provided evidence that the actin cytoskeleton is an important factor in the establishment of cell polarity and in the maintenance of the tip growth machinery at the apex of the growing root hair. However, there has been no genetic evidence to directly support this assumption. We have isolated an Arabidopsis mutant, *deformed root hairs 1* (*der1*), that is impaired in root hair development. The *DER1* locus was cloned by map-based cloning and encodes ACTIN2 (ACT2), a major actin of the vegetative tissue. The three *der1* alleles develop the mutant phenotype to different degrees and are all missense mutations, thus providing the means to study the effect of partially functional ACT2. The detailed characterization of the *der1* phenotypes revealed that ACT2 is not only involved in root hair tip growth, but is also required for correct selection of the bulge site on the epidermal cell. Thus, the *der1* mutants are useful tools to better understand the function of the actin cytoskeleton in the process of root hair formation.

Cell division, growth, and differentiation are basic processes underlying plant development. Plant cell growth is a complex process, as the cells are surrounded by a cell wall that limits enlargement of the cell. Therefore, plant cell growth requires a well-coordinated and tightly controlled expansion of the protoplast and the cell wall. Root hairs provide an excellent system for studying the process of cell growth. The root hair structure is not surrounded by other cells that would limit its expansion. As a consequence, aberrations in root hair development are readily observable. Root epidermal cells in Arabidopsis develop into root hair-forming cells (trichoblasts) or non-root hair-forming cells (atrachoblasts), depending on positional cues relative to the underlying cortical cells. Epidermal cells that are in contact with the periclinal wall between two adjacent cortical cells develop into trichoblasts, whereas those that overlie a single cortical cell will become atrichoblasts (Dolan et al., 1993). Once an epidermal cell is determined to become a trichoblast cell, the process of root hair development can be divided into three phases: First, a small bulge forms at the distal end of the epidermal cell; second, a slow-growing root hair develops by tip growth; and third, the tip-growth rate increases and the hair structure fully develops (Dolan et al., 1994). In higher plants, tip growth is found in root hairs and

pollen tubes and it constitutes an extreme form of polarized growth in which vesicles containing new cell wall material are fused to the membrane at the very extreme end (tip) of the growing cell (Cai et al., 1997; Yang, 1998; Galway, 2000). In a genetic approach to studying the mechanism of root hair development in Arabidopsis, a large number of root hair mutants has been isolated. Root hairs of these plants show different phenotypes such as reduced tip growth, development of branched root hairs, wavy root hairs, bulbous structures at the root hair basis, or burst root hairs (Schieffelbein and Somerville, 1990; Schieffelbein et al., 1993; Masucci and Schieffelbein, 1994; Grierson et al., 1997; Parker et al., 2000; for review, see Schieffelbein, 2000). Several mutant loci involved in root hair development have been cloned and encode proteins with different functions such as a GTP-binding protein (*rh3*), a putative K⁺ transporter (*trh1*), a cellulose synthase-like protein (*Kojak*), and an extracellular, chimeric Leu-rich repeat/ extensin protein (*lrx1*; Wang et al., 1997, 2001; Baumberger et al., 2001; Favery et al., 2001; Rigas et al., 2001).

Biochemical and cytological analyses of root hair and pollen tube development have revealed that a plethora of cellular components are important for tip growth, including the actin and microtubule cytoskeletons, Rac-related proteins, phosphatidylinositol kinases, phosphatidylinositol-4,5-bisphosphate, and Ca²⁺ ions (Bibikova et al., 1997; 1999; Braun et al., 1999; Kost et al., 1999; Miller et al., 1999; Emons and deRuijter, 2000; Esseling et al., 2000; Bao et al., 2001), but also extracellular cell wall-modifying proteins such as xyloglucantransferases and expansins (Baluška et al., 2000; Vissenberg et al., 2001). The

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actin cytoskeleton is a component of major importance for the cellular architecture and is involved in a variety of cellular activities such as cell polarity, division, elongation, and cytoplasmic streaming (Fowler and Quatrano, 1997; Staiger, 2000). It is a highly dynamic structure that allows a rapid response to changes in development and environmental conditions (Staiger et al., 1997). Actin monomers polymerize to form actin filaments (Holmes et al., 1990) that can aggregate to microfilament bundles. Application of actin filament-interfering compounds lead to aberrant root hair initiation and to a stop of the tip growth process during root hair elongation (Braun et al., 1999; Miller et al., 1999; Baluška et al., 2000).

Arabidopsis contains a family of eight functional actin proteins (ACT) encoded by genes that are predominantly expressed in the vegetative tissues or the reproductive organs (McDowell et al., 1996b; Meagher et al., 1999b). The first group is formed by the three actins ACT2, ACT7, and ACT8, of which ACT2 and ACT8 are most similar, differing in only one amino acid residue (McDowell et al., 1996b; Meagher et al., 1999b). The concomitant expression of several ACT in the same tissue suggests that several isoforms of highly similar but distinct ACT are important for a flexible and dynamic response to changing conditions (Meagher et al., 1999a). Although *act2*, *act4*, and *act7* T-DNA mutants (McKinney et al., 1995; Gilliland et al., 1998) have not been reported to show any obvious phenotype, the mutations have a deleterious effect that results in a lower frequency of the mutant alleles among the progeny of a heterozygous plant (Gilliland et al., 1998), suggesting that mutations in these actin genes do affect plant development. In addition, the *act7* mutant is also affected in callus formation, whereas the *act2* mutant shows wild-type development under the same conditions (Kandasamy et al., 2001), suggesting distinct functions of individual actins during plant development.

In this study, we present the isolation and characterization of *deformed root hairs 1* (*der1*) mutant Arabidopsis plants that are affected in root hair development. The mutants were isolated in a screen of an ethylmethanesulfonic acid (EMS)-mutagenized population for seedlings with a phenotype reminiscent of *lrx1* in which root hairs are frequently swollen and are also shorter than in wild type (Baumberger et al., 2001). Map-based cloning of the *DER1* locus revealed that it is mutated in the gene encoding ACT2. The three *der1* alleles all harbor missense mutations and show differences in the strength of the phenotype. A detailed characterization of the *der1* plants suggests that ACT2 is important throughout the whole process of root hair formation. The site selection of the initial bulge, the positioning of the tip-growth machinery at the bulge, and the process of tip growth per se are strongly affected. These results confirm and extend

our understanding of the role of the actin cytoskeleton in root hair development and tip growth.

RESULTS

Isolation of Root Hair Mutants

Arabidopsis seeds of the ecotype C24 were mutagenized with EMS. The seeds were propagated in 12 independent M_1 families of 1,000 plants to the M_2 generation. Seeds of the M_2 generation were grown under sterile conditions on vertical plates for 4 d and were then screened for an aberrant root hair development under the binocular. In total, 70,000 seedlings were analyzed and over 50 *der* mutants displaying a root hair phenotype were isolated. A number of *der* mutants display a phenotype with features similar to the *lrx1* mutant phenotype. An allelism test revealed that none of them was mutated in the *LRX1* locus and that only for *der1* were several allelic mutants identified from independent M_2 families, which were named *der1-1* to *der1-3*.

Characterization of *der1*

The *der1* mutant phenotype could easily be studied under the binocular, as the root hairs are very different from those of C24 wild-type plants (Fig. 1A). A clear difference in strength of the phenotype was observed among the *der1* mutants. *der1-1* displayed a rather weak phenotype with the lower one-half of the root hair proper being enlarged, whereas the upper one-half of the root hair seemed to develop normally (Fig. 1B). The root hairs were consistently shorter than in wild-type plants. *der1-2* and *der1-3* showed a

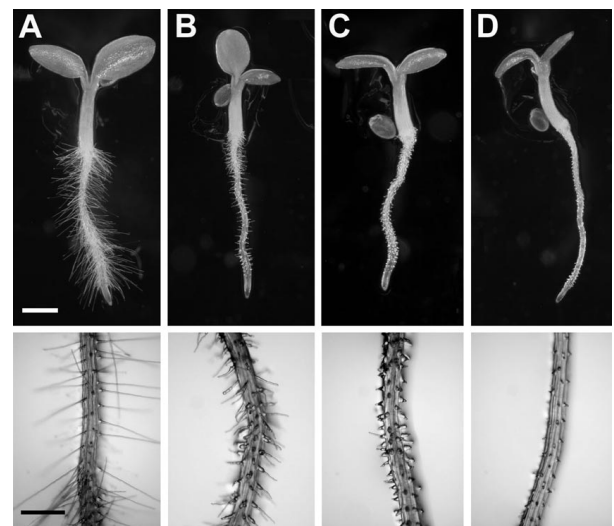


Figure 1. *der1* plants display a mutant root hair phenotype. Seedlings were grown for 4 d under continuous illumination. Top, Whole seedlings are shown. Bottom, An enlargement of the root hair zone. A, Wild type; B, *der1-1*; C, *der1-2*; and D, *der1-3*. Bars = 1 mm (top) and 0.5 mm (bottom).

comparable phenotype that was much stronger than *der1-1*. Often, the basis of the root hair was enlarged and the root hair structure was much shorter than in wild type (Fig. 1, C and D).

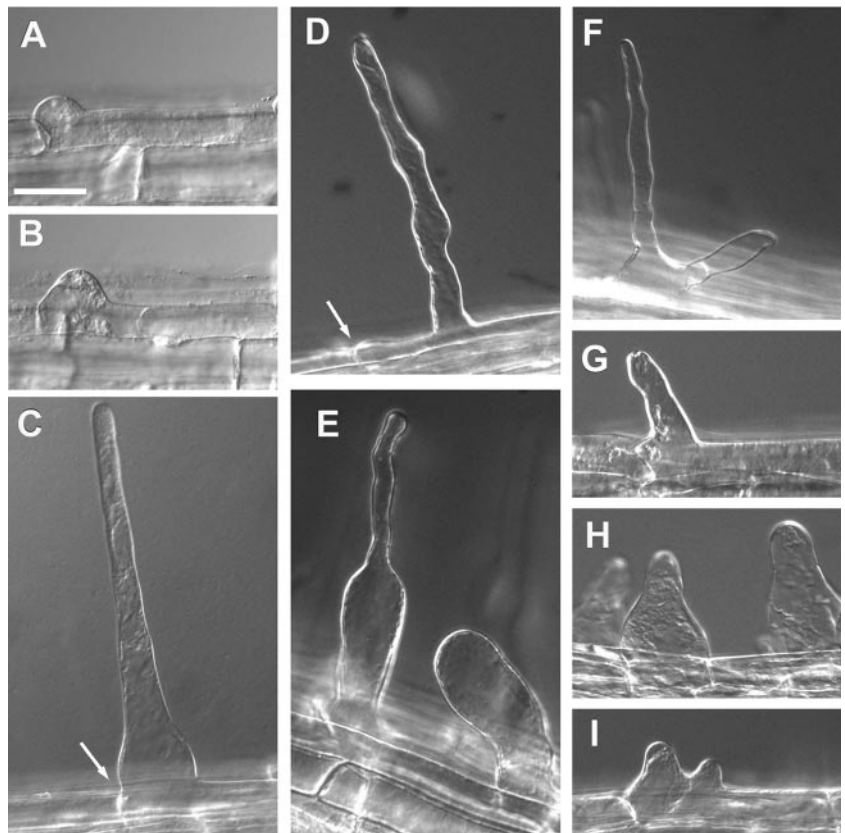
Backcrosses of the *der1* mutants into wild-type C24 revealed that *der1* is a recessive mutation, as the F_1 seedlings were not distinguishable from wild type (data not shown). F_2 seedlings of this cross segregated in a 1:3 ratio for the mutant phenotype, corroborating that *der1* is recessive. Furthermore, the observed segregation suggests that the mutant root hair phenotype is due to a mutation in one genetic locus.

Beside the root hair phenotype, *der1* plants did not display any obvious aberrations in development. Trichomes are a second cell type that is not surrounded by other cells. Thus, the effect of the *der1* mutation on trichome development was investigated. As the ecotype C24 develops very few trichomes, *der1-2* displaying a strong phenotype was crossed with plants of the ecotype Columbia. Young seedlings of the segregating F_2 population of this cross were examined, but no aberrant trichome development was observed in plants displaying the *der1* phenotype compared with plants with wild-type root hairs. Therefore, we conclude that the expression of the mutant phenotype is limited to root hairs.

Microscopic Investigation of the *der1* Phenotype

For a more detailed characterization of the *der1* mutants, differential interference contrast microscopy (DICM) was used on vertically grown, 4-d-old wild-type and *der1* seedlings (Fig. 2). In wild-type seedlings, root hair development was first observed as a bulge at the distal end of the trichoblast cell. After development of the bulge, the root hair proper was formed by tip growth that initiated at the top of the bulge (Fig. 2, A–C). In the *der1-1* mutant and also in the strong *der1-2* and *der1-3* alleles, the initiating bulge formed sometimes rather in the middle than at the distal end of the trichoblast cell (Fig. 2, C and D, arrow). This suggests misplacement of the whole process of bulge initiation. In some cases in the *der1-1* mutant, a thin root hair without an enlarged basis developed. However, the structure of these root hairs was irregular with a varying diameter, possibly as a result of asymmetric deposition of cell wall material at the root hair tip or constant slight changes of the growth direction (Fig. 2D). In an alternate manner, the lower one-half of the root hair was strongly enlarged, whereas the upper part was thinner but again very irregular (Fig. 2E). Very often, the enlarged part of the root hair formed, but did not develop further, resulting in a stump-like structure (Fig. 2E). In some cases, two root hairs developed from the initial bulge, suggesting that the initiation of tip growth

Figure 2. Root hairs observed by DICM. Roots of 4-d-old seedlings were used for the analysis. A through C, Root hair development in wild-type plants. A and B, An initial bulge is formed at the distal end of the trichoblast. C, The root hair develops by tip growth. D through F, Root hair phenotype of *der1-1* mutant. D, Root hairs often show an irregular diameter. E, The lower part of the root hair is enlarged (left) or only an enlarged, short root hair forms (right). F, Two root hairs initiate from the bulge, indicating uncontrolled initiation of tip growth on the bulge. G through I, Root hair phenotype in *der1-2* and *der1-3* plants. G, Root hairs are very short and have an irregular diameter. H, The root hair basis is frequently enlarged. I, The initial bulge extends over a large part of the epidermal cell and two root hairs appear to develop. Arrows in C and D indicate the distal end of the trichoblast cell. Bar = 40 μ m.



was disturbed and was not limited to one spot on the initial bulge (Fig. 2F). This is clearly different from branching root hairs as observed in *lrx1* mutants, for example (Baumberger et al., 2001). When a short, thin root hair structure was formed, it resembled the root hairs of *der1-1* that showed an irregular diameter along the root hair (Fig. 2G). However, the root hairs frequently had a strongly enlarged basis, implying a severely affected bulge-formation and tip-growth process in *der1-2* and *der1-3* (Fig. 2H). Many times, the initially restricted area of bulge formation grew bigger along the epidermal surface of the trichoblast, and tip growth seemed to initiate at two different positions, although it did not proceed any further (Fig. 2I). A subtle difference between *der1-2* and *der1-3* was observed in that *der1-2* developed the strongly enlarged basis at a higher frequency, whereas root hair structures of *der1-3* more often remained thin.

The structure of the wild-type and *der1* mutant root hairs was also determined by scanning electron microscopy (SEM; Fig. 3). The SEM analysis confirmed the observations made by DICM. Compared with wild type (Fig. 3A), the lower one-half of root hairs formed by *der1-1* seedlings were often enlarged and the stumps were also detectable (Fig. 3B). Again, the root hairs showed an irregular diameter as with DICM, suggesting that this phenotype is not an arti-

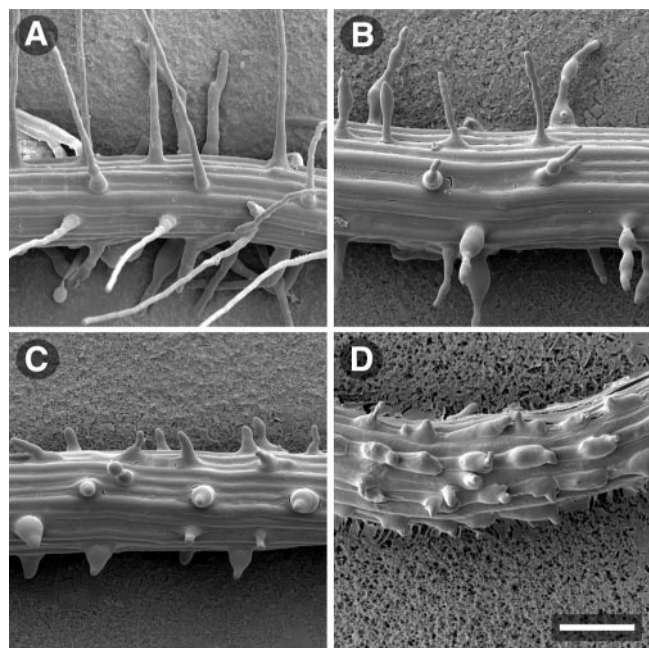


Figure 3. Root hair-phenotype analysis by SEM. Roots of 4-d-old seedlings were used for the analysis. A, Wild-type C24. B, Representative enlarged root hairs of *der1-1* and thin root hairs showing irregular diameters. C and D, *der1-2* root hairs. The root hairs are very short and often have an enlarged basis (C). The bulges often are not restricted to the distal end of the trichoblast, but extend over the whole outer cell surface and appear to initiate multiple root hairs (D). Bar = 100 μ m.

fact of sample preparation, but rather is an effect that is genuine to the *der1-1* mutation. In plants of the stronger *der1-2* and *der1-3* alleles, the very short root hair structures with the frequently enlarged basis were readily observable (Fig. 3C). In many trichoblasts, the bulge seemed no longer to be restricted to a small area at the distal end of the trichoblast cell, but rather it extended over a bigger part of the cell surface (Fig. 3D). As a possible consequence of this enlargement, root hair formation was initiated at two points. However, the actual establishment of a root hair proper formed by tip growth and starting from such a structure could never be observed. In some instances, root hair development was observed in normally hairless atrichoblast cell files, which separate the hair-forming trichoblast cell files (Fig. 3D; Dolan et al., 1993). However, this misplacement can also be observed in C24 wild-type plants and thus is not an effect of the *der1* mutation (data not shown).

Map-Based Cloning of the *der1* Mutation

The phenotype of the *der1* mutants reflects pleiotropic effects on root hair development and is of considerable interest. Therefore, we decided to clone the *DER1* gene by map-based cloning. To this end, the stronger *der1-2* allele was used for mapping. As the mutation is in the C24 background and C24 shows DNA polymorphisms with Columbia and *Landsberg erecta* at a similar frequency, two mapping populations were established. For the initial mapping, genomic DNA of 50 mutant F_2 plants of both populations was extracted and analyzed with simple sequence length polymorphism (SSLP; Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (Konieczny and Ausubel, 1993) markers. Several of the markers used for mapping were established based on the polymorphism collection available from Cereon Genetics (<http://www.Arabidopsis.org/cereon>). This preliminary analysis allowed us to map the *der1* locus on chromosome 3 to a region between the SSLP markers *nga162* and *uzu1*, at a distance of 20 and 34 cM from the top of the chromosome, respectively (Fig. 4A). The mapping population of the cross *der1-2* \times *Landsberg erecta* was subsequently increased to 1,200 mutant F_2 plants. With this population, the *DER1* locus was narrowed down to a region between two markers that were established at positions 40,000 on the BAC MVE11 and 16,500 on the adjacent BAC MCB22 (<http://www.Arabidopsis.org/chromosomes>) with three and one plants, respectively, still showing recombination (Fig. 4B). This interval spans a region of 50 kb and comprises nine annotated genes. Among those, the *ACT2* gene, encoded on MVE11 in positions 59,369 through 60,666, seemed a candidate gene as actins are known to be important for cell architecture and specifically to be involved in root hair development. Therefore, the *ACT2* gene was sequenced and missense muta-

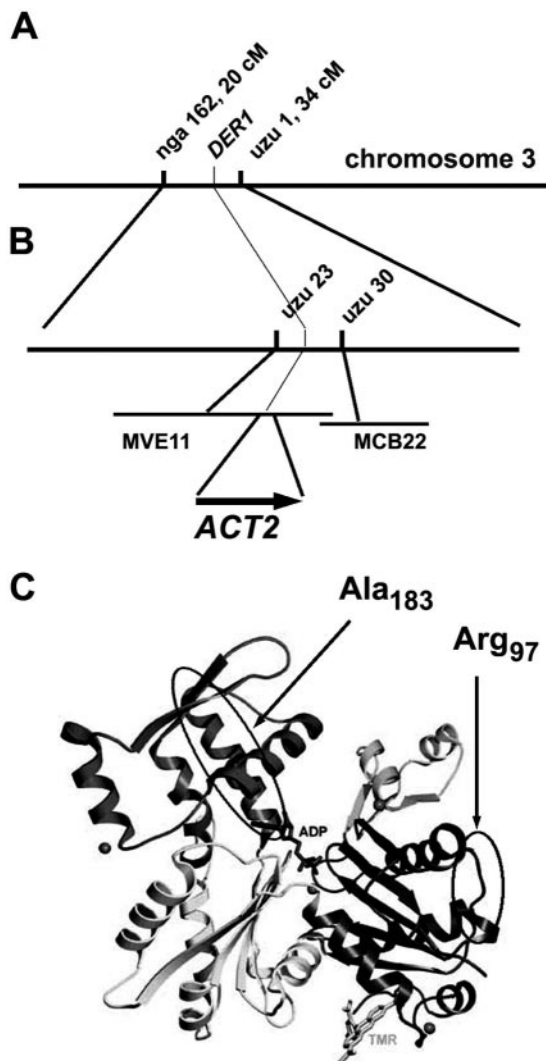


Figure 4. Map-based cloning of the *DER1* locus. A, In an initial mapping population, the *DER1* locus was located on chromosome 3, in an interval between the SSLP markers nga162 and uzu1 at 20 and 34 cM, respectively. B, Using a larger mapping population, *DER1* was mapped to a region flanked by the markers uzu23 and uzu30 and spanned by the bacterial artificial chromosome (BAC) clones MVE11 and MCB22. The *ACT2* gene is located on the BAC MVE11. C, A model of the actin protein structure in the ADP-bound form. Tetramethylrhodamine-5-maleimide shown in the right lower part was used to facilitate crystallization (Otterbein et al., 2001). The domains that harbor the two amino acids mutated in the *der1* alleles are circled.

tions were found in all three *der1* alleles compared with the wild-type C24 sequence. In the weaker *der1-1* allele, the Ala residue at position 183 (Ala-183) is changed to Val, whereas in the stronger *der1-2* and *der1-3* the Arg-97 is changed to a His and a Cys, respectively (Fig. 4C).

To examine whether the point mutations have an influence on the expression level of the ubiquitously expressed *ACT2*, gene expression was tested by northern blotting. Seedlings of wild-type C24 and the

weaker *der1-1* and stronger *der1-3* alleles were grown for 10 d on vertical plates and RNA was extracted from root tissue. Total RNA was separated on an agarose gel, blotted onto a nitrocellulose membrane, and probed with an *ACT2*-specific probe (An et al., 1996). The result clearly shows that the expression level of *act2* is not affected by the point mutations in *der1* plants and thus the *der1* mutants express the *act2* gene at wild-type levels (Fig. 5).

Complementation of the *der1* Phenotype with the *ACT2* and the Root Hair-Specific *LRX1* Promoter

To confirm that the *ACT2* gene is responsible for the *der1* phenotype, a complementation experiment was performed. To this end, the *ACT2* cDNA was isolated from reverse-transcribed and PCR-amplified C24 mRNA and was cloned downstream of a 1.6-kb *ACT2* promoter that includes the first intron of the nontranslated region of the *ACT2* gene (An et al., 1996). In a second construct, the root hair-specific *LRX1* promoter (Baumberger et al., 2001) was used to drive expression of the *ACT2* cDNA. The two constructs were transformed into *der1-2* plants and for both, segregating T_2 populations of six independent transgenic lines were analyzed. The *ACT2* cDNA expressed under both promoters complemented the phenotype (Fig. 6). This confirmed that the mutation conferring the root hair phenotype in the *der1* plants is in the *ACT2* gene. Furthermore, the complementation experiment with the *ACT2* cDNA under the control of the *LRX1* promoter shows that this root hair-specific promoter is expressed already in early stages of root hair development and confers sufficient *ACT2* expression to complement the *der1* phenotype. Thus, complementation of the *der1* phenotype provides a good system for assessing the promoter activity of a particular gene involved in trichoblast differentiation, as *ACT2* is required throughout the whole developmental process.

DISCUSSION

We have identified and characterized three alleles of *der1*, a mutant that shows severe alterations in root

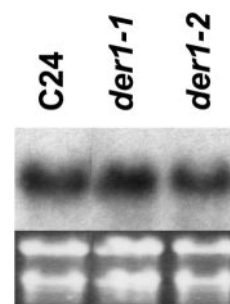


Figure 5. Northern-blot analysis of *ACT2* expression in wild-type and *der1* mutants. Seedlings were vertically grown for 10 d and root material was used for RNA extraction. Five micrograms of total RNA per lane was blotted and probed with a probe specific for *ACT2*.

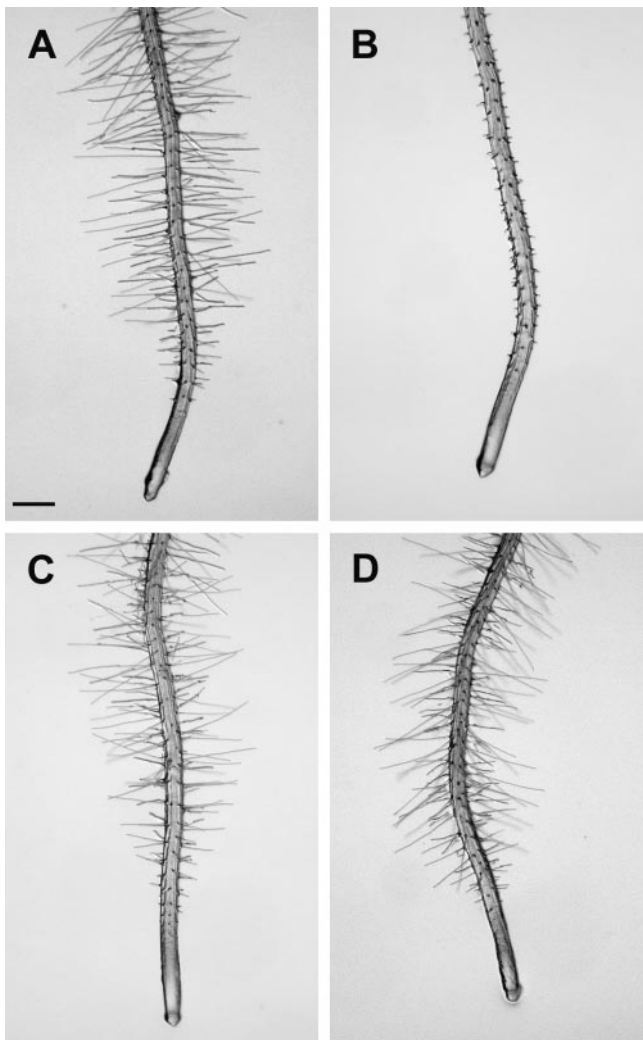


Figure 6. Complementation of the *der1* mutation. A, Wild-type C24; B, *der1-2* used for the complementation experiments. Transgenic *der1-2* seedlings expressing the *ACT2* cDNA under the control of the *ACT2* promoter (C) and the root hair-specific *LRX1* promoter (D) show wild-type root hair development. The seedlings were grown for 4 d prior to analysis. Bar = 400 μ m.

hair development. *der1-1* shows an intermediate phenotype with shorter and enlarged root hairs. The stronger *der1-2* and *der1-3* mutants have a drastically reduced root hair length and often do not develop further than to the initial bulge. The bulge-site selection is often severely affected, resulting in bulges that are sometimes misplaced and often enlarged over a large part of the cell instead of being limited to the distal end of the trichoblast. Map-based cloning of the *DER1* locus revealed that it encodes the *ACT2* gene, encoding a major actin of the vegetative tissue (An et al., 1996).

der1 plants show a phenotype that suggests that several processes are affected during root hair development. The short root hair phenotype, particularly in the strong *der1-2* and *der1-3* alleles, indicates that the root hair tip-growth process is affected. This

function for actin has been suggested by previous biochemical/cytological studies that showed that interference with the actin cytoskeleton by application of cytochalasin D or latrunculin B stops root hair growth (Bibikova et al., 1999; Braun et al., 1999; Miller et al., 1999). The phenotype of *der1-1* seedlings that develop root hairs that are severely disturbed in their structure might reflect loss of directionality of root hair growth and/or unprecise deposition of new cell wall material to the lateral walls instead of the tip region, leading to an enlarged cell. Tip growth includes transport of cell wall material-containing vesicles to the tip, followed by fusion of these vesicles with the plasma membrane. As the actin cytoskeleton is involved in the vesicle transport (Cai et al., 1997; Emons and deRuijter, 2000; Staiger, 2000), interference with this structure by a mutation in an actin protein might be expected to result in uncontrolled trafficking and deposition of vesicles during cell growth. Microtubules have also been shown to be involved in controlling directionality of root hair growth. Treatment of root hairs with microtubule-interfering drugs lead to wavy root hairs that, in contrast to *der1-1*, exhibit a more regular width (Bibikova et al., 1998). It remains to be shown whether the microtubule structure is affected in *der1-1* and thus whether there is a mutual influence between the two cytoskeletons.

In some cases, the bulges in the *der1* mutants are not properly positioned to the distal end of the trichoblast cell, and in the stronger *der1-2* and *der1-3* alleles, they frequently extend over a larger surface of the cell. Thus, *ACT2* is important in specifying the area of the cell surface involved in bulge development. This confirms the importance of the actin cytoskeleton as a component defining the cell architecture and polarization (Fowler and Quatrano, 1997). Furthermore, it is in line with previous reports that showed contact of the actin cytoskeleton with the prospective site of bulge formation (Baluška et al., 2000) and rearrangement and focusing of the microfilament bundles in the growing bulge (Braun et al., 1999; Miller et al., 1999), implying a function of the actin cytoskeleton in these processes. In future experiments, it will be interesting to study the rearrangement of the actin cytoskeleton in *der1* plants. This will possibly reveal whether *ACT2* has a particular function within the cytoskeleton that is required for proper root hair development or whether the cytoskeleton is generally affected in these plants.

It is of interest that the *der1* mutant phenotype can only be observed in root hair cells and in no other cell type. A previously reported deleterious effect on the inheritance of a mutation in *ACT2* (Gilliland et al., 1998) could not be confirmed, using the inheritance of the strong *der1-3* allele in a segregating population as a parameter (data not shown). However, this might also suggest that even this strong allele encodes a partially functional protein and that the del-

eterious effect can only be detected in an *act2*-null mutant. Root hairs might be prone to aberrations in cell development as they are not surrounded by other cells that limit their growth. Trichomes represent a cell type that is similar to root hairs in this respect and does also express *ACT2* (An et al., 1996). However, no mutant phenotype could be observed in trichomes of *der1* plants compared with wild-type C24. Thus, the root hair-specific phenotype suggests that this cell type has distinct characteristics, the composition of the actin cytoskeleton is distinct from other cell types, or the function of *ACT2* is particularly important in root hair development. *ACT2* is not required for the tip-growth process in pollen as it is not expressed in this tissue (An et al., 1996; Meagher et al., 2000; Kandasamy et al., 2002). The results presented in this work and the lack of requirement of *ACT2* for pollen development suggests that in perspective of the actin cytoskeleton, the tip-growth process in these two cell types differs significantly.

Several actin genes of *Arabidopsis* are expressed concomitantly in the same tissue. At least three actin genes, *ACT2*, *ACT7*, and *ACT8*, are expressed in root hairs (An et al., 1996; McDowell et al., 1996a). The parallel expression of several closely related proteins is thought to provide a higher degree of flexibility to the plant to respond to changes in development (Meagher et al., 1999a). Beside the possible requirement for the expression of several isoforms in the same cell, a possible redundancy in protein function among the different proteins should also prevent an aberrant phenotype upon mutation of a gene encoding one of these proteins. However, our results show that in root hair development, the *ACT* are not redundant in their function and *ACT7* and *ACT8* cannot functionally replace *ACT2*. This is particularly surprising as *ACT2* and *ACT8* differ in only one amino acid residue (McDowell et al., 1996b). Thus, even small changes in the protein sequence among actins have a tremendous effect on protein function, and the high degree of protein conservation among different actins does not reflect extreme conservation of protein function. In the case of the *act7* mutant, a strong effect of the mutation can be observed under tissue culture conditions where *act7*-derived tissue shows slower growth. However, *act2* mutant-derived tissue is not distinguishable from wild type (Kandasamy et al., 2001). Thus, although being expressed together in several tissues, individual *ACT* have at least partially nonoverlapping functions in different cell types. In an alternate manner, the expression pattern of *ACT7* and *ACT8* during root hair development might not be sufficiently overlapping with *ACT2* to allow complementation, a possibility that cannot be ruled out as the promoter β -glucuronidase fusion constructs used to study the expression pattern of the different actin genes (An et al., 1996; McDowell et al., 1996a) might not fully reflect the expressing profiles of the endogenous genes. To dif-

ferentiate between the two possibilities, a complementation test of a *der1* mutant with constructs expressing *ACT7* and *ACT8* under the control of the *ACT2* promoter will be performed. The fact that an *ACT2* cDNA under the control of the root hair-specific *LRX1* promoter allows complementation of the *der1* phenotype suggests that *ACT2* does not require an expression pattern unique to *ACT2* to allow proper function of the protein.

The availability of three different *act2* mutant alleles exhibiting a mutant phenotype to different degrees while expressing the *act2* gene at wild-type levels provides an excellent tool for studying *ACT2* function, and it allows us to relate the changes in the protein to its biological function. Both amino acid residues affected in the *der1* mutants are conserved throughout the actin family of *Arabidopsis* (except *ACT9*, which is encoded by a pseudogene; McDowell et al., 1996b) and in organisms of diverse origin such as *ACT1* of *Saccharomyces cerevisiae* and in rabbit (*Oryctolagus cuniculus*) skeletal muscle actin isoform (McDowell et al., 1996b and refs. therein). The Ala-183 that is changed to Val in the weaker *der1-1* allele is positioned in the α -helix close to the nucleotide binding cleft. Ala-183 is close to an Arg that is important in the ADP conformation of actin where it interacts with the nucleotide through hydrogen bonds (Otterbein et al., 2001). Thus, the mutation might change the structure of the protein and consequently influence the interaction with ADP. *der1-2* and *der1-3* are mutated in Arg-97, which is changed to His and Cys, respectively. Therefore, the slight difference in the phenotypes of *der1-2* and *der1-3* must be due to the different amino acids replacing the original Arg. Arg-97 is located in the subdomain1 on the protein surface (Kabsch et al., 1990) and therefore might be important for the establishment of protein-protein interactions. However, it cannot be excluded that these mutations have an influence on the overall protein structure rather than directly interfering with the binding of an interaction partner.

The intermediate phenotype displayed by *der1-1* shows that the Ala-183 to Val substitution in *ACT2* results in a partially functional protein. Comparison of the root hair phenotypes of *der1-2* and *der1-3*, respectively, with an *act2* knockout mutant that was isolated by screening a T-DNA insertion collection (McKinney et al., 1995) should reveal whether the mutations in Arg-97 result in a partially active *ACT2*.

We have isolated and characterized *der1*, a genetic locus involved in root hair development. *DER1* encodes *ACT2*, one of two major actin genes expressed in vegetative tissue. The phenotype displayed by the *der1* alleles suggests that *ACT2* is not only important for root hair tip growth-related processes, but also for the bulge site selection as a first step in root hair development. The *der1* lines are an excellent tool for studying redundancy and isoform dynamics versus complementary but distinct functions among the

different members of the actin family of Arabidopsis. The detailed analysis of the actin cytoskeleton in *der1* mutants will reveal important information on the function of ACT2 in the establishment of the actin cytoskeleton and will extend our understanding on actin function in plant cell development.

MATERIALS AND METHODS

Plant Material

Arabidopsis seeds of the ecotype C24 were EMS-mutagenized by a standard procedure (Lehle Seeds, Round Rock, TX). Mutagenized M₁ seeds were grown in families of 1,000 plants, and M₂ seeds of each family were pooled. For isolation of root hair mutants, 70,000 M₂ seeds were surface sterilized with 1% (w/v) Na-hypochlorite (Fluka, Buchs, Switzerland) and 0.03% (v/v) Triton X-100, washed three times with sterile water, and stratified for 3 d at 4°C. The seeds were subsequently plated on one-half-strength Murashige and Skoog medium and 0.4% (w/v) Phytoagar (Sigma, Buchs, Switzerland) and were grown for 4 d at 24°C under continuous light. Selected plants were grown to maturity in soil under continuous light at 24°C in growth chambers.

Microscopical Analysis

Prior to the microscopical observations, plants were grown under sterile conditions as described above. The observations were done as described by Baumberger et al. (2001). DICM was performed on an axioplan microscope (Zeiss, Jena, Germany).

Mapping of the *der1* Locus

The strong *der1-2* allele was used for mapping of the *der1* mutation. *der1-2* was crossed with Arabidopsis of the ecotypes Columbia and *Landsberg erecta*. Genomic DNA of 50 mutant F₂ plants of each population was extracted following the protocol of Fulton et al. (1995) and was used for initial mapping using SSLP and cleaved amplified polymorphic sequence markers. For the large mapping population, mutant F₂ plants were selected and grown for 1 week on soil. After extraction of genomic DNA and testing with the closely linked markers nga162 and uzu1, only F₂ plants showing recombination were grown to maturity, and F₃ seedlings were tested to confirm the phenotype. Newly generated markers were established based on the Columbia versus *Landsberg erecta* polymorphism collection of Cereon Genetics (<http://www.Arabidopsis.org/cereon>).

Cloning Procedures and Plant Transformation

For sequencing, the ACT2 gene was amplified by PCR from genomic DNA of wild-type C24 and *der1* mutant plants. To clone the ACT2 cDNA, a cDNA library from C24 (van der Graaff et al., 2000) was used as template for a PCR reaction with the primers Act2-1, AGCGCTGAGGCTGATGATATCAAC and Act2-2, TCTAGAAACATTTCTGTGAACGATC. The resulting fragment contained an Eco 47III site of which the second one-half of the recognition sequence corresponded to the second triplet of the ACT2 coding sequence and an XbaI site at the stop codon. This fragment was cloned into pCR-Blunt II-TOPO (Invitrogen, Basel) and was sequenced. The ACT2 promoter and the LRX1 promoter were amplified by PCR from genomic DNA with the primers ACT2proF1, GGTCATATGTTTCAGTTTTTAAGC; ACT2proR1, GCATGCATTTTTTATGAGCTGCAAACAC; and LRX1-1000, AAAAGTGAGGTATTTAGTCAAT; LRX1METR GCATGCATTTGCTTATGGGTCAGAAAC, respectively, cloned into pGEM-T easy (Promega, Wallisellen, Switzerland) and sequenced. For both promoters, a SphI site was introduced such that the G of the ATG start codon corresponded to the first nucleotide of the SphI site. For cloning of the promoter-ACT2 cDNA fusion construct, the promoter was digested with SphI, blunt-ended with T4 DNA-polymerase, cut with XbaI, and ligated with the vector containing the Act2 cDNA, cut with Eco 47III and XbaI. In this way, a perfect promoter-cDNA fusion was obtained without any change in the promoter or the cDNA sequence. Both promoter-ACT2 cDNA fusions were digested with BamHI/

XhoI and were cloned into pART7 (Gleave, 1992) digested by the same enzymes. The resulting promoter ACT2-cDNA ocs-termination sequence cassettes were digested with NotI and cloned into pART27 (Gleave, 1992) digested with NotI.

The resulting constructs were transformed into *Agrobacterium tumefaciens* GV3101 for plant transformation. *der1-2* plants were transformed by the floral dip method (Clough and Bent, 1998). The T₁ seedlings were germinated on one-half-strength Murashige and Skoog agar plates complemented with 50 µg mL⁻¹ kanamycin for selection of transgenic plants and 150 µg mL⁻¹ Timentin (GlaxoSmithKline, Münchenbuchsee, Switzerland) to inhibit growth of agrobacteria.

RNA Extraction and Northern Blotting

Seedlings were grown for 10 d on vertical plates, root material was collected, and RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Five micrograms of total RNA was separated by gel electrophoresis, blotted on a nylon membrane (GeneScreen Plus; PerkinElmer, Hünenberg, Switzerland), and hybridized with an ACT2-specific probe (An et al., 1996).

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