The **ROOT HAIR DEFECTIVE3** gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in *Arabidopsis*

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In plants, morphogenesis is largely determined by the orientation and extent of cell enlargement. To define the molecular mechanisms regulating plant cell enlargement, we have conducted a molecular genetic analysis of the **ROOT HAIR DEFECTIVE3** (*RHD3*) gene of *Arabidopsis thaliana*. Mutations affecting the *RHD3* gene were found to alter cell size, but not cell number, in tissues throughout the plant. Genetic and physiological analyses suggest that the *RHD3* gene is not required for proper cell type specification, and it is likely to act downstream of the hormones auxin and ethylene. The *RHD3* gene was cloned by a T-DNA tagging method and confirmed by the molecular complementation of the *rhd3* mutant phenotype and by the analyses of six *rhd3* mutant alleles. Consistent with the global effects of the *rhd3* mutations, the *RHD3* gene is expressed in all major plant organs. The deduced RHD3 product is a novel 89-kD polypeptide with putative GTP-binding motifs near the amino terminus. *RHD3*-like genes were identified from a protozoan (*Entamoeba histolytica*), a fungus (*Saccharomyces cerevisiae*), and another plant species (*Oryza sativa*), with the sequence identity including the putative GTP-binding motifs. These results imply that the RHD3 protein is a member of a new class of GTP-binding proteins that is widespread in eukaryotes and required for regulated cell enlargement.

[Key Words: *Arabidopsis thaliana*; cell expansion; GTP-binding protein; root hairs; cell differentiation]

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Cell enlargement in eukaryotes occurs by the regulated synthesis, secretion, and incorporation of new plasma membrane and extracellular material that serves to increase the cell surface area. In plants, cell enlargement is also coordinated with regulated modifications in the cell wall. Plant cells enlarge when the cell wall yields to the internal osmotically based turgor pressure and undergoes irreversible wall extension accompanied by water uptake and vacuole enlargement (Cosgrove 1986, 1993). Because the mature cell wall resists changes in cell shape, the morphology of plant cells and organs is largely determined by the orientation and extent of cell enlargement during cell differentiation. Although some factors important for plant cell expansion have been identified, including plant hormones, cytoskeletal components, and wall-loosening proteins (Cleland 1988; Giddings and Stachelin 1991; Abeles et al. 1992; McQueen-Mason et al. 1992), the molecular mechanisms controlling the extent and orientation of cell enlargement are largely unknown.

The *Arabidopsis thaliana* root epidermis is a useful model tissue for studying the mechanisms of regulated cell enlargement (Schiefelbein and Benfey 1991; Dolan et al. 1993; Benfey and Schiefelbein 1994). The cells of the root epidermis are accessible and undergo a large increase (>50×) in cell volume during their differentiation near the root apex. The orientation of epidermal cell enlargement is regulated; the immature cells expand principally in the longitudinal orientation to generate elongated cells (length–width ratio >10). In addition, some root epidermal cells form root hairs, which are extensions of the epidermal cells generated by the redirection of cell enlargement perpendicular to the long axis of the cell (Cormack 1962; Schiefelbein and Somerville 1990). The root hairs arise by tip growth, an extreme type of polarized cell expansion (Heath 1990), that generates long tubular-shaped cells (length–width ratio >50). Thus, the differentiating cells of the *Arabidopsis* root epidermis undergo characteristic changes in cell shape that reflect regulation of the extent and orientation of cell enlargement.
Wang et al.

In previous studies, mutations have been identified that affect *Arabidopsis* root epidermis development. These mutations define genes that influence early stages of epidermal cell type specification (e.g., TTG and GL2; Galway et al. 1994; Masucci et al. 1996), genes that affect the initiation of root hairs (e.g., *ROOT HAIR DEFECTIVE*6 [RHD6]; Masucci and Schiefelbein 1994), and genes that affect root hair enlargement (e.g., RHD1–RHD4; Schiefelbein and Somerville 1990). Mutations affecting the RHD3 gene are unique because they alter the enlargement of root hairs as well as the root proper; the root hairs exhibit a short and wavy morphology, and the roots possess a reduced length (Schiefelbein and Somerville 1990). Detailed ultrastructural analyses of growing *rhd3* root hairs have shown that vacuole formation is reduced and secretory vesicle distribution is altered (Galway et al. 1997). Because the *rhd3* mutations alter the size of roots and root hairs, the RHD3 product may be involved in a fundamental plant cell expansion mechanism.

To define the molecular mechanisms controlling cell enlargement, we have conducted a molecular genetic analysis of the RHD3 gene and characterized its role in plant development. Detailed phenotypic analysis of *rhd3* mutants revealed that the RHD3 gene is required for normal cell expansion in many tissues throughout the plant. Molecular cloning of the RHD3 gene showed that it encodes an 89-kD polypeptide with putative GTP-binding motifs. The identification of related genes from *Entamoeba histolytica*, *Saccharomyces cerevisiae* (yeast), and *Oryza sativa* (rice) suggests that these genes may encode members of a novel class of GTP-binding proteins with a common function in eukaryotic cell enlargement.

**Results**

**Genetic and phenotypic analysis of *rhd3* mutant alleles**

The identification of two recessive *rhd3* mutant alleles, *rhd3-1* and *rhd3-2*, has been reported previously (Schiefelbein and Somerville 1990). Four additional alleles have been isolated in this study, including one (*rhd3-3*) from a screen of γ-ray-treated plants and three (*rhd3-4, rhd3-5*, and *rhd3-6*) from screens of plants transformed with T-DNA insertions. Each of these was recognized initially as a putative *rhd3* mutant because of the two characteristic *rhd3* phenotypes: short and wavy root hairs and a relatively short root length (Fig. 1A, B). The analysis of *F*₁ and *F*₂ progeny derived from crossovers of these mutants with wild-type plants demonstrated that the defect in each line is inherited as a recessive mutation (data not shown).

In genetic complementation tests, each pairwise combination of these six *rhd3* mutant alleles failed to generate *F*₁ plants with normal root hairs or normal root length, demonstrating that each bears an *rhd3* allele. One exceptional complementation result was obtained; the *F*₁ individuals with the *rhd3-2/rhd3-5* combination displayed partial complementation, producing roots with an intermediate length and root hairs with an intermediate length and slightly wavy morphology (data not shown).

To determine whether the decreased root length in *rhd3* mutants was attributable to reduced cell expansion (elongation) or reduced cell number, we measured the seedling root growth rate and the root epidermis cell length. The root growth rate in the strongest allele, *rhd3-1*, is ~31% of the wild-type rate (1.44 vs. 4.66 mm/day; Table 1). Similarly, the length of the *rhd3-1* epidermal cells is ~39% of the wild-type cells (Table 1, Fig. 1C). The other *rhd3* mutant alleles also exhibited a correlation between reduced root growth rate and cell length, although the severity varied in different alleles (Table 1). The close relationship between the reduced growth rate and cell length in the *rhd3* mutants indicates that a defect in cell enlargement, rather than cell division, is primarily responsible for the reduced *rhd3* root length.

**Relationship between RHD3 and plant hormones**

The plant hormones auxin and ethylene are known to influence the extent and orientation of cell expansion in some tissues (Cleland 1988; Jackson 1991; Abeles et al. 1992). When included in plant growth media at relatively high concentrations, these hormones reduce cell expansion in wild-type *Arabidopsis* seedlings (Baskin and Williamson 1992; Masucci and Schiefelbein 1994; Table 2). Although this effect is generally similar to the effect of the *rhd3* mutations, these hormones do not phenocopy the *rhd3*-dependent abnormalities in cell shape (e.g., wavy root hairs). Nonetheless, to examine the possibility that RHD3 may promote cell elongation by counteracting the effects of ethylene (which causes greater radial expansion), *rhd3* seedlings were grown on media containing the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG). If RHD3 normally counteracts the action of ethylene in cell expansion, then AVG may be expected to suppress the *rhd3* mutant phenotype. However, when *rhd3* mutant seedlings were grown on AVG-containing media, root epidermal cell length was not affected significantly (Table 2).

To further examine the relationship between RHD3 and the plant hormones ethylene and auxin, seedlings of the strong *rhd3-1* mutant and the wild type were grown on media containing the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC), the auxins indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) or, as controls, a cytokinin (kinetin) or abscisic acid (ABA). Cell enlargement in the *rhd3* mutant roots was not affected to the same extent as the wild-type roots when seedlings were exposed to ACC, IAA, or 2,4-D (Table 2). The lack of a proportional effect in the treated *rhd3* roots indicates that the *rhd3* mutation causes enlarging epidermal cells to be less sensitive to these plant hormones, and it implies that the RHD3 gene product may participate in, or be controlled by, a hormone response pathway to influence cell expansion.
Molecular cloning of the Arabidopsis RHD3 gene

Figure 1. The RHD3 gene is required for normal cell expansion in many cell and tissue types. In each set of panels, wild type [left] and rhd3-1 mutant [right] plants are compared at the same magnification. [A] Five-day-old light-grown seedlings. Bar, 1 mm. [B] Apex of 5-day-old seedling roots. Bar, 300 μm. [C] Epidermis of 5-day-old seedling roots treated with a fluorescent cell wall stain. Bar, 50 μm. [D] Scanning electron micrograph image of the hypocotyl surface of 5-day-old seedlings. Bar, 100 μm. [E] Mature plants at 6 weeks of age. Bar, 6 cm. [F] Scanning electron micrograph image of the surface of mature leaves showing individual trichome cells. Bar, 100 μm. [G] Transverse sections of stems from mature (6 week old) plants. Bar, 100 μm. [H] Longitudinal sections of stems from mature (6 week old) plants. Bar, 120 μm. [I] Scanning electron micrograph images from the surface of petals from mature flowers. Bar, 20 μm. [J] Scanning electron micrograph images from the surface of carpels from mature flowers. Bar, 20 μm.

The RHD3 gene is not required to specify cell type

The extensive effects of the rhd3 mutations on cell enlargement during root epidermis differentiation led us to examine whether cell type specification may be affected by the RHD3 gene. As one test, we analyzed the distribution of cell types in the root epidermis, which normally produces a position-dependent pattern of root-hair-bearing cells and hairless cells (Dolan et al. 1993; Galway et al. 1994). More than 95% (56 of 58) of the

Table 1. Phenotypes of the rhd3 alleles

| Genotype | Mutagen  | Root growth rate | Epidermis cell length |
|----------|----------|------------------|-----------------------|
|          |          | mm/day           | percent wild type     | mm/cell                | percent wild type |
| Columbia | —        | 4.66 ± 0.64      | 100                   | 0.18 ± 0.03            | 100               |
| Nossen   | —        | 5.14 ± 0.49      | 100                   | 0.17 ± 0.03            | 100               |
| Wassilewskija | —    | 5.65 ± 0.51      | 100                   | 0.22 ± 0.03            | 100               |
| rhd3-1 [Col] | EMS    | 1.44 ± 0.34      | 31                    | 0.07 ± 0.01            | 39                |
| rhd3-2 [Col] | EMS    | 2.74 ± 0.22      | 59                    | 0.10 ± 0.01            | 56                |
| rhd3-3 [No] | γ      | 2.38 ± 0.25      | 46                    | 0.09 ± 0.01            | 53                |
| rhd3-4 [WS] | T-DNA  | 2.56 ± 0.25      | 45                    | 0.10 ± 0.01            | 45                |
| rhd3-5 [WS] | spontaneous | 3.88 ± 0.29 | 69                    | 0.12 ± 0.02            | 55                |
| rhd3-6 [WS] | spontaneous | 2.61 ± 0.22    | 46                    | 0.10 ± 0.01            | 45                |

*Ecotype background for each rhd3 allele is shown in parentheses; [Col] Columbia; [No] Nossen; [WS] Wassilewskija.
Wang et al.

Table 2. Effect of plant hormones on root epidermis cell length

| Media          | Columbia wild type cell length [mm] | percent unsupplemented* | rhd3-1 cell length [mm] | percent unsupplemented* |
|----------------|-------------------------------------|-------------------------|-------------------------|-------------------------|
| Unsupplemented | 0.18 ± 0.03                         | 100                     | 0.07 ± 0.01             | 100                     |
| 1 μM AVG       | 0.20 ± 0.02                         | 111                     | 0.08 ± 0.02             | 114                     |
| 25 μM AVG      | 0.12 ± 0.02                         | 67                      | 0.09 ± 0.02             | 129                     |
| 1 μM ACC       | 0.06 ± 0.01                         | 33                      | 0.05 ± 0.01             | 71                      |
| 5 μM ACC       | 0.05 ± 0.01                         | 28                      | 0.05 ± 0.01             | 71                      |
| 30 nM 2,4-D    | 0.07 ± 0.01                         | 39                      | 0.07 ± 0.01             | 100                     |
| 30 nM IAA      | 0.06 ± 0.01                         | 33                      | 0.05 ± 0.01             | 71                      |
| 300 nM IAA     | 0.03 ± 0.01                         | 17                      | 0.03 ± 0.01             | 43                      |
| 0.5 μM kinetin | 0.12 ± 0.02                         | 67                      | 0.06 ± 0.01             | 86                      |
| 5 μM ABA       | 0.12 ± 0.02                         | 67                      | 0.06 ± 0.01             | 86                      |

*Percent unsupplemented is the cell length relative to seedlings grown on unsupplemented media.

rhd3-1 mutant root hairs examined were produced on epidermal cells in the appropriate position (cells located outside an anticlinal cortical cell wall), implying that the RHD3 gene does not affect epidermal cell type specification.

To further test the possible role of RHD3 in cell type specification in the root epidermis, the relationship between the RHD3 gene and the early-acting TTG and GL2 genes was explored. The Arabidopsis TTG and GL2 loci have been proposed to act as negative regulators of the root-hair cell type, and recessive ttg or gl2 mutations cause nearly all epidermal cells to form root hairs [Fig. 2A–C; Galway et al. 1994; Masucci and Schiefelbein 1996]. The rhd3 ttg and rhd3 gl2 double mutants were constructed, and each of them produced short roots with excessive (ectopic) short and wavy root hairs [Fig. 2D–F]. This result represents an additive outcome and indicates that the effect of the ttg and gl2 mutations is independent of the effect of the rhd3 mutation. Furthermore, this result shows that the abnormal rhd3 tip growth is not restricted to cells that normally produce root hairs, but it can also occur in ectopic root hair cells.

In another test of the relationship between RHD3 and GL2, a reporter gene construct that consists of the GL2 promoter fused to the β-glucuronidase [GUS] gene [GL2-GUS; Masucci et al. 1996] was introduced into the rhd3-1 mutant background. Wild-type plants harboring the GL2-GUS construct preferentially exhibit GUS activity in the developing hairless root epidermal cells [Fig. 2G; Masucci et al. 1996]. The GL2-GUS construct was found to confer a similar pattern of GUS expression in the rhd3-1 mutant background [Fig. 2H]. This indicates that the rhd3 mutation does not affect GL2 promoter activity directly or indirectly and provides further evidence for the independence of the RHD3 and GL2 genes during epidermis development.

Role of RHD3 gene in the enlargement of other plant cells

In addition to studying the role of the RHD3 gene in roots, we wished to determine whether the RHD3 gene influences cell enlargement in other parts of the Arabidopsis plant. To examine this possibility, we analyzed plants bearing the strong rhd3 mutant allele rhd3-1 and compared them to plants of the corresponding wild-type ecotype (Columbia).

At the seedling stage of development, the rhd3-1 mutant possesses a shortened hypocotyl, which is composed of short and distorted hypocotyl epidermal cells [Fig. 1D]. We measured these cells and determined that the
length of the *rhd3*-1 hypocotyl epidermal cells is ~45% of the wild-type cells (79 \mu m/cell vs. 177 \mu m/cell). In mature plants, the *rhd3* mutation causes a dramatic reduction in plant size, characterized by reduced leaf size and stem length [Fig. 1E]. Scanning electron microscopy observations revealed that the *rhd3* mutant plants produce smaller leaf epidermis cells with abnormally shaped trichomes [Fig. 1F]. In addition, the mature stem tissues possess alterations in cell length and cell shape [Fig. 1G,H], although floral cell types are not appreciably affected [Fig. 1I,J]. Together, these data suggest that the *RHD3* gene is required for proper cell enlargement in many tissues of the *Arabidopsis* plant.

**Molecular cloning of the RHD3 gene**

A T-DNA tagging strategy was employed to isolate the *RHD3* gene. *Arabidopsis* lines harboring T-DNA insertions generated by *Agrobacterium tumefaciens* seed transformation [Feldmann 1991] were screened, and one line was identified that failed to complement *rhd3* mutants. Genetic analyses of this line [designated *rhd3*-4] demonstrated that a T-DNA insert was linked to the *RHD3* locus. Individuals heterozygous for the *rhd3*-4 mutation produced F₂ progeny in the ratio of 153 Kan⁺/rhd3 mutant—386 Kan− normal—152 Kan⁻ normal. The lack of *rhd3* mutants displaying kanamycin sensitivity suggested that a T-DNA was linked tightly to the *RHD3* gene in the *rhd3*-4 line.

A genomic DNA fragment adjacent to a T-DNA in *rhd3*-4 was isolated by plasmid rescue in *Escherichia coli*, and it was used to isolate three overlapping genomic λ clones. Using restriction fragment length polymorphism (RFLP) mapping, we determined that this set of clones is located on chromosome III of *Arabidopsis*. In independent mapping experiments with the *rhd3*-1 mutant line, we also placed the *RHD3* locus on chromosome III [see Materials and Methods]. The similar location of the genomic clones and the *rhd3*-1 mutation provided preliminary evidence that the cloned DNA may possess *RHD3* gene sequences.

The genomic DNA clones were used to isolate a set of overlapping cDNA clones from a library made from *Arabidopsis* seedling mRNA. DNA sequence analyses of the longest cDNAs (~2.8 kb) showed that the cDNAs overlapped the site of the T-DNA insertion [Fig. 3] and possessed a single long open reading frame (ORF) of 2406 bp [Fig. 4]. The AUG start codon for this ORF is preceded by an inframe stop codon. A database search of *Arabidopsis* sequences revealed that two reported expressed-sequence tagged (EST) clones (T45933, Z29807) are derived from the same gene as these cDNAs [Fig. 4].

The cDNA clone was used as a probe in genomic DNA blot hybridization (Southern) analyses. Under high-stringency conditions, one to three genomic fragments were detected, whereas under low-stringency hybridization conditions, additional genomic fragments were observed [Fig. 5]. This indicates that there may be several sequences related to the gene represented by the cDNA clone.

To establish that the cloned gene represents the *RHD3* locus, molecular complementation tests were conducted. Because none of the λ genomic clones included the entire region present in the cDNA, we isolated clones from a cosmid library made with *Arabidopsis* genomic DNA. Two cosmid clones [designated 8A1 and 24A4] were identified and found to contain large inserts that included the entire genomic region corresponding to the cDNA. These clones were introduced into *Arabidopsis* cells harboring the *rhd3*-3 allele using *Agrobacterium*-mediated plant transformation [Valvekens et al. 1988]. Tissue from these transformants produced root hairs with normal length and morphology, in contrast to the short and wavy hairs produced by *rhd3* mutant tissue [Fig. 6]. Whole plants regenerated from these transformants produced offspring that segregated normal–*rhd3* mutant phenotypes in a 3:1 ratio [not shown]. In addition to the genomic cosmid clones, a construct containing

**Figure 3.** Physical map of the *RHD3* locus. A restriction endonuclease site map of the genomic DNA from the *RHD3* gene region is shown on the top line, including the location of the T-DNA insertion in the *rhd3*-4 allele and the rescued DNA in the plasmid p10N-18. The bottom line shows the *RHD3* gene structure (exons are represented by boxes) and the molecular lesions present in each of the mutant alleles. Stars in the *rhd3*-3 and *rhd3*-6 alleles indicate translation termination induced by frameshift mutations. [N] NdeI; [R] EcoRI; [X] Xhol.
The portion of the sequence (nucleotides 1-85) obtained from Arabidopsis EST cDNA clones T45933 and Z29807. The conserved motifs (GXXXXGKS and DXXG) for GTP-binding protein domain-like sequences, the first exon, and the 5′-end of the first intron) was shown. (A) Tissue from nontransformed rhd3-3 mutant. (B) Tissue from the rhd3-3 mutant transformed with the pBIN19 vector. (C) Control tissue from nontransformed rhd3-3 mutant. (D) Control tissue from the rhd3-3 mutant transformed with the pBIN19 vector alone. Bar, 100 μm. (A,B,D) 35 μm (C).

Figure 6. Complementation of the rhd3 mutant phenotype by RHD3 genomic DNA and cDNA. The effect of RHD3 gene constructs on root hair formation in transformed plant tissue is shown. (A) Tissue from the rhd3-3 mutant transformed with the RHD3 promoter–RHD3 cDNA (construct 12A). (B) Tissue from the rhd3-3 mutant transformed with a cosmid clone containing genomic DNA encompassing the entire RHD3 gene. (C) Control tissue from nontransformed rhd3-3 mutant. (D) Control tissue from the rhd3-3 mutant transformed with the pBIN19 vector alone. Bar, 100 μm. (A,B,D) 35 μm (C).

Figure 5. Genomic DNA blot hybridization analysis of the RHD3 gene. Two DNA blots containing similar sets of restriction enzyme-digested genomic DNAs were exposed to a 32P-labeled RHD3 cDNA clone under standard stringency conditions (left) and under low stringency conditions (right) for hybridization and washing (see Materials and Methods for details). Note the presence of additional hybridizing fragments under low stringency conditions.

Figure 4. Nucleotide sequence and deduced amino acid sequence of the RHD3 cDNA. The amino acid numbers are shown at the left and the nucleotide numbers are given at the right. The portion of the sequence (nucleotides 1-85) obtained from Arabidopsis EST cDNA clones T45933 and Z29807. The conserved motifs (GXXXXGKS and DXXG) for GTP-binding protein domain-like sequences, the first exon, and the 5′-end of the first intron) was shown. (A) Tissue from nontransformed rhd3-3 mutant. (B) Tissue from the rhd3-3 mutant transformed with the pBIN19 vector. (C) Control tissue from nontransformed rhd3-3 mutant. (D) Control tissue from the rhd3-3 mutant transformed with the pBIN19 vector alone. Bar, 100 μm. (A,B,D) 35 μm (C).
In addition to sequencing the cDNA clones, the corresponding sequence of the genomic DNA was determined. The RHD3 gene spans ~6 kb of genomic DNA, and its coding region is interrupted by 22 introns (Fig. 3). The predicted RHD3 protein has 802 amino acids, with a calculated molecular mass of 89 kDa and a PI of 5.5 [Fig. 4]. It does not show significant overall similarity to any previously characterized protein with a known biochemical function. Hydropathy plots and secondary structure analyses suggest that RHD3 is largely hydrophilic with one potentially hydrophobic region near the carboxyl terminus. In the amino-terminal region, the RHD3 protein was found to contain two conserved motifs present in GTP-binding proteins (GXXGKS and DXXG), with a spacing [42 amino acids] that is typical for these motifs [Fig. 4; Dever et al. 1987].

To analyze RHD3 transcript accumulation, RNA blot hybridization (Northern) analyses were performed. Total RNAs from different tissues [whole seedlings, seedling roots, seedling aerial parts including both hypocotyls and cotyledons, mature leaves, and stems] of wild-type Arabidopsis plants [ecotype Columbia] were tested, and a single 2.8-kb RNA species was detected in all tissues examined [Fig. 7]. This result suggests that RHD3 is expressed in all major organ types and may have a general role in cell expansion throughout the Arabidopsis plant.

Molecular analysis of the rhd3 mutant alleles

To define the molecular lesions that affect RHD3 gene function, the genomic DNA sequence of the six rhd3 mutant alleles was determined. Each of the mutant alleles was found to possess a single sequence alteration in the RHD3 gene, compared with their corresponding wild-type ecotype (Fig. 3). Three of the mutant alleles [rhd3-1, rhd3-2, and rhd3-5] contain single-base-pair substitutions that lead to amino acid replacements [Ala773 to Val, Asp985 to Asn, and Ile194 to Thr, respectively] in the predicted RHD3 polypeptide. The rhd3-3 allele was generated by γ-ray irradiation, and a single-base-pair deletion was identified at Asp1283 that causes a frameshift mutation and premature translation termination. Likewise, the rhd3-6 allele exhibits a single-base-pair deletion that causes a frameshift mutation at Lys224. The rhd3-4 allele is the T-DNA insertion line, and the T-DNA was localized to the first intron [Fig. 3].

Gene expression studies were also conducted with the six mutant alleles to examine the effect of these lesions on RHD3 RNA accumulation. A dramatic reduction in the abundance of the RHD3 transcript was observed in seedlings homozygous for the rhd3-3, rhd3-4, and rhd3-6 alleles (Fig. 7). This is consistent with the molecular lesions found in these three alleles [T-DNA insertion or frameshift mutations], which are likely to disrupt gene transcription or alter transcript stability. Relatively normal accumulation of RHD3 RNA was observed in seedlings bearing the other mutant alleles, which implies that these lesions [missense mutations] alter RHD3 protein function rather than RHD3 transcript synthesis or stability.

**Figure 7.** Accumulation of RHD3-specific transcripts in diverse plant tissues and rhd3 mutants. RNA blot hybridization analysis was conducted with total RNAs, using a 32P-labeled RHD3 cDNA clone as probe. As a loading control, the blot was washed and reprobed with a 32P-labeled 18S rRNA probe. (Lanes 1–7) RNAs extracted from 1-week-old seedlings. (Lane 1) Columbia wild type; (lane 2) rhd3-1; (lane 3) rhd3-2; (lane 4) rhd3-3; (lane 5) rhd3-4; (lane 6) rhd3-5; (lane 7) rhd3-6. (Lanes 8–12) RNAs extracted from various plant tissues of wild-type plants. (Lane 8) One-week-old seedlings; (lane 9) hypocotyls and cotyledons from 1-week-old seedlings; (lane 10) roots from 1-week-old seedlings; (lane 11) rosette leaves from 3-week-old plants; (lane 12) stems from 4-week-old plants.

**RHD3-like gene products are widespread in eukaryotes**

Sequence database searches using the RHD3 gene sequence led to the identification of two nonoverlapping O. sativa [rice] EST clones [C10416, S3658] possessing similarity to the RHD3 gene product. These two clones were obtained from the Rice Genome Research Program and sequenced partially to construct a continuous ORF. The partial polypeptide predicted from this sequence [210 amino acids] is 59.5% identical to the corresponding amino acids of the RHD3 protein [Fig. 8]. The identification of a RHD3-like gene product in rice [a monocotyledonous plant] indicates that the RHD3 gene product may exist in all flowering plants.

Additional sequence database searches showed that the carboxyl terminus of the RHD3 polypeptide is similar to the product of a partially sequenced ORF [449 amino acids] from the protozoan parasite E. histolytica, designated ORF3.5 [Bruchhaus et al. 1993; GenBank accession no. X70850]. To determine whether the ORF3.5 product is also similar to the amino-terminal half of the RHD3 polypeptide, we isolated cDNA clones from an E. histolytica cDNA library using a fragment from the partial ORF3.5 genomic clone [pEh-170] as a probe. The largest cDNA clone was sequenced, and although truncated at the 5’ end, its predicted product [954 amino acids] was found to exhibit an overall amino acid sequence identity of 28.5% to the RHD3 product, with the greatest degree
Wang et al.

**Figure 8.** Amino acid sequence alignment of the RHD3 and related proteins. Compared are the Arabidopsis RHD3, O. sativa cDNA EST sequences C10416/S3658 (rice), S. cerevisiae ORF03590p (yeast), and E. histolytica ORF3.5. Residues present in at least two proteins are shaded. The conserved GTP-binding motifs (GXXXXGKS and DXXG) are double underlined, and two other conserved regions of amino acids [FVIRD and NKDLDLP] are single underlined.

In sequence database studies of the recently completed Yeast Genome Project, a predicted polypeptide (776 amino acids) from an ORF sequence (ORF03590p; GenBank accession no. U55021) was identified that possesses similarity to RHD3 throughout its entire length (28.9% identity; Fig. 8). It is noteworthy that the RHD3 product, the S. cerevisiae ORF03590p, and the Entamoeba ORF3.5 product all contain the two motifs for GTP-binding (GXXXXGKS and DXXG; Fig. 9), as well as two novel amino acid motifs, FVIRD and NKDLDLP (Fig. 8). The presence of RHD3-like polypeptides in protozoans and fungi suggests that they may be widespread in eukaryotes and required for a fundamental cellular process.

**Discussion**

The RHD3 gene is required for regulated cell enlargement

In this study we have identified a novel protein encoded by the RHD3 gene that is required for regulated cell enlargement in Arabidopsis. Plants homozygous for rhd3 mutations are small and possess organs with a reduced...
size. Microscopic observations revealed that the small stature of these plants is associated with a reduction in cell size, rather than a reduction in cell number. In addition to a major effect on the degree of cell enlargement, the rhd3 mutations alter, in a subtle fashion, the orientation of cell enlargement in many cell types. This effect is most clearly observed in cells that are not surrounded by neighboring cells, such as root hairs and trichomes, and it results in a distorted cell morphology. These findings indicate that the RHD3 gene is normally required for the proper orientation and extent of plant cell enlargement during development.

Plant cell expansion is known to be influenced by the action of plant hormones, such as auxin and ethylene (Cleland 1988; Jackson 1991). We have found that the rhd3 mutant displays a slight hormone-insensitive phenotype; auxin and ethylene treatments do not affect root epidermal cell elongation in the rhd3 mutant to the same degree as wild-type. Furthermore, we tested the possibility that the RHD3 product acts in opposition to auxin and ethylene pathways and promotes cell elongation rather than radial cell expansion. If RHD3 acted in this manner, as proposed for the SABRE gene product (Aeschbacher et al. 1995), then the reduced hormone sensitivity might be explained by the lack of the competing RHD3 pathway in the mutant cells. However, this possibility is ruled out by the finding that the ethylene inhibitor AVG does not reverse the rhd3 effects. Together, these results are consistent with the possibility that the RHD3 gene product may be required to mediate the action of the hormones in cell expansion. Because hormone treatments on wild-type plants do not phenocopy the rhd3 mutant entirely, the RHD3 product may also affect hormone-independent processes.

Despite the dramatic effect of the rhd3 mutations on cell expansion, cell type specification is not altered. The rhd3 mutants produce a normal pattern of root cell types, and they do not alter the activity of genes (e.g., TTG and GL2) that participate in epidermal cell type specification (Fig. 2). These observations are similar to those made with other morphological mutants in plants, including the Arabidopsis fass mutant, which displays abnormal cell proliferation and morphogenesis, yet exhibits relatively normal pattern formation (Torres-Ruiz and Jurgens 1994). These findings support the view that cell morphogenesis programs are controlled independently of cell type specification (patterning) programs in plants.

The RHD3 gene encodes a novel protein with GTP-binding motifs

The molecular cloning of the RHD3 gene described here has been confirmed by molecular complementation tests and by sequence analyses of six mutant rhd3 alleles. The nucleotide sequence of the RHD3 gene predicts an 89-kD protein with no significant homology to any known metabolic or regulatory proteins and lacking a signal sequence. The predicted polypeptide does contain two motifs conserved in GTP-binding proteins (GXXXGKS and DXGG) but lacks a third motif [N/TKXD] (Dever et al. 1987). Proteins with the first two motifs, such as the interferon (IFN)-induced 67-kD guanine-binding proteins (GBPs), have been shown to possess a high affinity for GTP as well as GDP and GMP but fail to bind adenosine, uracil, or cytosine or 7-methyl-GMP nucleotides (Cheng et al. 1991). Therefore, RHD3 may represent a member of a novel class of high molecular weight guanine nucleotide-binding proteins.

In eukaryotic cells, GTP-binding proteins are key players in a wide variety of cellular processes, including signal transduction, intracellular trafficking, cytoskeleton organization, and protein synthesis (Ferro-Novick and Novick 1993; Hall 1994; Stack et al. 1995). Among the best-characterized GTP-binding proteins are the small monomeric GTPases of the Ras family, which regulate the cell cycle (Lowy and Willumsen 1993), and the Rab family, which regulate vesicle trafficking (Pfeffer 1994). High molecular weight GTPases also have been identified, including a class that includes the mammalian dynamin protein and Drosophila S H I B I B E R E gene product, which are involved in endocytosis, synaptic transmission, and neurogenesis (Chen et al. 1991; van der Bieker and Meyerowitz 1991), and the yeast Vpslp, which is necessary for the proper sorting of soluble vacuolar proteins and for membrane protein retention in the late Golgi compartment (Rothman et al. 1990; Wilsbach and Payne 1993).

In a prior study, the abnormal rhd3 root hair cell morphology was found to be associated with a striking reduction in vacuole size (Galway et al. 1997). During plant cell enlargement, most of the increase in cell volume is attributable to vacuole enlargement, which is thought to occur by fusion of small vacuoles, water uptake, and incorporation of new membrane from the endoplasmic reticulum and Golgi apparatus (Cosgrove 1986; Staehelin and Moore 1995). Considering that some GTP-binding proteins participate in vesicle trafficking and that the rhd3 mutant possesses vacuolar defects, it is possible that the RHD3 product represents a member of a novel class of GTP-binding proteins required for vacuole biogenesis.

RHD3-like proteins may participate in a fundamental eukaryotic cell process

Several lines of evidence indicate that RHD3-like proteins may be involved in a fundamental cellular process. First, the rhd3 mutants alter cell enlargement in diverse cell types, including ones that display different modes of cell expansion, such as tip-growing cells (root hairs), longitudinally expanding cells (root epidermal cells), and diffusely expanding cells (leaf epidermal cells). Second, Northern blot analyses show that the RHD3 gene is expressed in all major plant tissues (root, hypocotyl and cotyledon, leaf, and stem). Finally, genes encoding RHD3-like proteins have been identified from Entamoeba, yeast, and rice, which suggests that this class of proteins is widespread in eukaryotes.

Overall, the RHD3 and RHD3-like proteins share the
greatest sequence similarity in the amino-terminal por-
tion of the protein. In particular, they all contain two 
conserved GTP-binding motifs [GXXXXGKS and DXGX] 
and two motifs of unknown function [FVIRD and NK-
DDLDP] within the amino-terminal 300 amino acids 
(Fig. 8). Similarly, the class of high molecular weight 
GTP-binding proteins composed of the Vps1p, dynamin, 
Shibire, and IFN-induced Mx proteins also possess high 
sequence similarity in their amino-terminal GTP-bind-
ing domain and diverge at their carboxy terminus 
(Horiserger et al. 1990, Rothman et al. 1990; Chen et al. 
1991; van der Bliek and Meyerowitz 1991). Mutational 
analysis of the Vps1p sequence has led to the suggestion 
that the carboxy-terminal domains of these proteins may 
be involved in protein–protein interactions and thereby 
determine their mode of action [Vater et al. 1992]. By 
analogy, the RHD3-like proteins may possess two func-
tional domains: an amino-terminal domain involved in 
guanine nucleotide binding, and a carboxy-terminal do-
main required for specific protein interactions.

The molecular analyses of the rhd3 mutants provide 
insight into some of the amino acid residues critical for 
protein function. The strong rhd3-1 allele is attributable 
to a missense mutation affecting a conserved alanine 
residue (position 575; Figs. 3 and 4) that is present in the 
carboxy-terminal region of all three RHD3-like proteins. 
One explanation for the strong rhd3-1 phenotype is that 
this alanine residue is crucial for protein interactions, 
and amino acid substitutions lead to unproductive or 
interfering interactions. The other two missense alleles 
rhd3-2 and rhd3-5 affect residues in the amino-terminal 
region that are similar in the three RHD3-like proteins 
[acidic residues and hydrophobic residues, respectively]. 
Interestingly, complementation tests with these two 
mutants give rise to F1 individuals [genotype rhd3-2/ 
rhd3-5] that possess a nearly wild-type phenotype, which 
may mean that different functional domains are affected 
by the rhd3-2 and rhd3-5 mutations.

In general, GTP-binding proteins are thought to act as 
regulatory molecules that recognize target proteins 
through conformational changes dependent on GDP– 
GTP exchange [Bourne et al. 1991]. Thus, a future goal is 
to identify proteins that interact with, or act in the same 
way as, the RHD3 protein. Potential candidates in- 
clude the products of genes defined by cell expansion 
mutant phenotypes [Schiefelbein and Somerville 1990; 
Benfey et al. 1993, Shevell et al. 1994, Aeschbacher et al. 
1995; Hauser et al. 1995; Takahashi et al. 1995] and 
genes defined by rhd3 suppressor mutations. The 
mutants in which cell enlargement can be analyzed in the 
developing Arabidopsis root epidermis should continue to 
be an advantage in further studies of the RHD3 pathway.

Materials and methods

Plant materials and growth conditions

The rhd3-1 and rhd3-2 alleles were generated by ethylmethane-
sulfonate (EMS) mutagenesis of Arabidopsis seeds [Columbia 
ecotype], as described previously [Schiefelbein and Somerville 
1990]. The rhd3-3 allele was identified in a population of γ-ir-
radiated Arabidopsis [Nossen ecotype] kindly provided by Fred 
Ausubel [Massachusetts General Hospital, Boston, MA]. The 
rhd3-4 allele was isolated from a population of Arabidopsis [WS 
ecotype] containing T-DNA insertions [Feldmann 1991] gener-
ated by the DuPont Corporation [Wilmington, DE]. The rhd3-5 
and rhd3-6 alleles were identified in a T-DNA population [WS 
ecotype] obtained from the Arabidopsis Biological Resource 
Center [ABRC] at Ohio State University [Columbus], but T-
DNA insertions were not linked to the rhd3 mutations in these 
lines. The rgg-1 and g2-1 mutants were also obtained from the 
ABRC, and the origin of the GL2-GUS transgenic line has been 
described previously [Masucci et al. 1996].

For phenotypic analyses of seedlings, Arabidopsis seeds were 
surface sterilized and grown on agarose-solidified media in 
vertically oriented petri dishes as described previously [Schiefel-
bein and Somerville 1990]. Plant hormone treatments were con-
ducted as described previously [Masucci and Schietelbein 1994, 
1996], with growth media containing either AVG [MAAG Agro-
chemicals, Vero Beach, FL], ACC [Sigma], IAA [Sigma], 2,4-D 
[Sigma], kinetin [Sigma], or ABA [Sigma]. To obtain mature 
plants, seedlings were transplanted into a potting soil–vermicu-
lite mixture and grown in environmental chambers under con-
ditions described previously [Schiefelbein and Somerville 1990].

Genetic analyses

Complementation analyses and dominance tests were con-
ducted by cross-pollinating homozygous plants and analyzing 
the root epidermis phenotype of the F1 and F2 seedlings. Double 
mutants were constructed by crossing lines homozygous for 
the single mutations, examining the F2 seedlings for putative 
double mutant phenotypes and testing these by backcrossing 
to the single mutants. The GL2-GUS construct [Masucci et al. 
1996] was introduced into the rhd3-1 mutant background by 
crossing lines homozygous for each marker and analyzing GUS 
expression in F2 seedlings exhibiting the rhd3 mutant pheno-
type. The rhd3-1 allele used for the detailed phenotypic analyses 
was backcrossed three times with the Columbia wild-type ec-
otype.

Linkage between the Kan’ marker [in the T-DNA] and the 
rhd3-4 mutation was assessed by examining at least 30 F2 fami-
lies on medium supplemented with 50 μg/ml of kanamycin. In 
the course of these genetic studies, a second T-DNA was iden-
tified in the rhd3-4 line, located within 5 cM of the T-DNA at 
the RHD3 locus.

The RHD3 locus was mapped by crossing the rhd3-1 mutant 
line with the mapping line W100 [which harbors 10 morpho-
logical mutations] and analyzing cosegregation in 240 F2 indi-
viduals. Linkage was detected with the gl1 marker [21 cM] and 
the hy2 marker [14 cM] located on chromosome III.

The RHD3 genomic DNA clones were mapped by identifying a 
RFLP between the Columbia and Niedernez ecotypes, and 
testing genomic DNAs from 58 F2 families [116 chromosomes] 
derived from a cross between these two ecotypes. Previously 
mapped molecular markers [obtained from the ABRC] were ana-
yzed with this set of genomic DNAs, and cosegregation analy-
ses showed linkage to markers HSP70-9 [2.6 cM] and PG26 [2.6 
cM] on chromosome III.

Microscopy

To calculate root growth rate, the length of the primary root was 
measured from 30 seedlings at 24-hr intervals from day 3 to day 
5 of growth. Root epidermal cell length measurements were 
made from 50 cells [10 cells from each of five roots] from 4-day-
old seedlings. Hypocotyl epidermal cell length was determined
by measuring 65 cells (13 cells from each of five hypocotyls) from 10-day-old seedlings.

Fluorescence microscopy was conducted as described by Aeschbacher et al. (1995), with minor modifications. Seedlings were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) overnight at 4°C. After incubation with 15% ethanol for 30 min, the seedlings were washed twice with distilled water, permeabilized with 1 M HCl at 60°C for 1 hr, washed with water, and stained with 0.2% acriflavine (Sigma Chemical, St. Louis, MO), 1% K3S2O8 in 0.2 N HCl at room temperature for 1 hr. After clearing and mounting with chloral hydrate, the samples were examined by fluorescence microscopy.

For most scanning electron microscopy, plant samples were fixed overnight in 4% glutaraldehyde in 20 mM NaPO4 (pH 7) buffer at 4°C, washed twice with the same buffer, and postfixed with 1% osmium tetroxide in the same buffer overnight at 4°C. After washing twice with the buffer, samples were dehydrated with an ethanol series and subjected to critical point drying. Samples were coated with gold and examined by standard scanning electron microscopy methods. For examination of some plant surfaces (e.g., hypocotyl), environmental scanning electron microscopy was employed, as described by Schiefelbein et al. (1993).

Molecular biology methods

Routine molecular biology experiments, including DNA isolation, Southern and Northern blot analysis, and DNA sequencing, were conducted essentially as described elsewhere (Sambrook et al. 1989; Kinkema et al. 1994). For standard (high) stringency Southern blots, hybridization was carried out in 7% SDS, 0.25 M sodium phosphate, 1 mM EDTA, and 1% casein at 68°C, with the final wash in 0.1X SSC and 0.1% SDS at 60°C for 30 min. For reduced stringency Southern blots, hybridization was in 6X SSPE, 0.5% SDS, 6X Denhardt’s solution, 100 μg/ml of denatured DNA, and 1 mM EDTA at 55°C, with the final wash in 0.1X SSC and 0.1% SDS at 50°C for 20 min.

For plasmid rescue, genomic DNA was isolated from the rhd3-4 line, digested to completion with either EcoRI or Ndel, ligated, and used to transform E. coli cells, as described previously (Feldmann 1992). After selection for ampicillin-resistant colonies, plasmid DNA was prepared and analyzed by restriction mapping and Southern hybridization to identify rescued plasmids containing Arabidopsis DNA.

Three genomic DNA clones were obtained from a wild-type (Columbia) genomic λ library (kindly provided by Ron Davis, Stanford University, CA) using the p10N-18 insert as a probe. Twelve cDNA clones were isolated by using a 5.2-kb EcoRI DNA fragment derived from the genomic clones as a probe (pR5.2) and screening ~300,000 plaques from a cDNA library made from 3-day-old etiolated seedlings (ecotype Columbia) and enriched for cDNAs exceeding 2 kb in length (a kind gift from J. Ecker, University of Pennsylvania, Philadelphia).

The RH33 cosmid were identified by screening 40 chapters of the Schulz binary cosmid library (obtained from the ABRC) using the insert of the cDNA clone c31 as a probe. Two cosmid clones (8A1 and 21A4) were isolated that covered the entire genomic region of the RH33 gene (determined by Southern blot analysis).

To define the molecular lesions in the rhd3 mutant alleles, genomic DNA was isolated from homozygous mutant plants and wild-type plants of the corresponding ecotypes (Columbia, Nossen, and WS), and the DNA was amplified using sets of primers that together encompassed the entire RH33 transcriptional unit. The general conditions for the PCR amplification were 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. DNA sequence information was obtained from both strands of the PCR products (including intron sequences), and sequence differences (relative to the corresponding wild-type sequence) were confirmed by performing a second independent round of amplification reactions and sequence analyses.

The rice EST clones C10416 and S3658 were obtained from Yoshiaki Nagamura (DNA Materials Management Group, Rice Genome Research Program, Ibaraki, Japan), and these were partially sequenced using primers derived from the predetermined DNA sequence. The E. histolytica ORF3.5 clone (Egh-170) and the Entamoeba χ cDNA library were provided by Egbert Tannich (Bernhard Nocht Institute for Tropical Medicine, Department of Molecular Biology, Hamburg, Germany). The cDNA clones were identified by screening the Entamoeba cDNA library with a 420-bp HindIII-BamHI fragment from the Egh-170 genomic clone as a probe.

DNA sequence analyses and searches were performed using the software package of the University of Wisconsin Genetics Computer Group [Devereux et al. 1984], and multiple sequence alignments were made with the MacDNAsis software. The complete genomic DNA sequence of the RH33 gene (Columbia ecotype) has been submitted to GenBank (accession no. U86081); the RH33 sequence from the WS and No ecotypes is available on request from us. The deduced RH33 amino acid sequence in these ecotypes is identical to Columbia except for a Val731-Ala missense in the WS and No ecotypes and a 3-bp deletion in WS leading to a Thr deletion at position 800.

Constructs and plant transformation

The RH33 promoter–RH33 cDNA construct [12A] was made by utilizing a ClaI restriction site located in the second exon of the RH33 gene (nucleotide position 178 of the RH33 cDNA). A ClaI fragment from the pR5.2 genomic clone was replaced with a ClaI fragment from the C3I cDNA clone to generate subclone PR12 [which contains 1.6 kb of DNA 5’ to the transcription start, the first exon, the first intron, and a few nucleotides of the second exon from genomic DNA linked in-frame to the remainder of the cDNA sequence]. The entire RH33 promoter–cDNA construct from PR12 was then subcloned into the binary vector pB1N19 on an XbaI–SalI fragment to generate clone 12A. This clone was then electroporated into the Agrobacterium LBA4404 strain.

The two binary cosmid clones containing the RH33 gene region (clones 8A1 and 21A4) were transferred into Agrobacterium [LBA4404] via a tripapetal mating with a helper E. coli containing the pRK2013 mobilization plasmid.

The constructs were introduced into Arabidopsis cells by Agrobacterium-mediated plant transformation essentially as described by Valvekens et al. (1988), using either hygromycin [for cosmids] or kanamycin [for clone 12A in pB1N19] to select the transformed plant cells. The effect of the constructs was assessed by analyzing the formation of root hairs on greening callus tissue, which normally occurs within 2 weeks following tissue transfer to shoot-inducing media. Following seed production, transgenic plants were confirmed by testing seeds on selective media and analyzing the integrated construct by genomic Southern blotting.

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References

Abeles, F.B., P.W. Morgan, and M.E. Saltveit, Jr. 1992. Ethylene in plant biology. 2nd ed. Academic Press, San Diego, CA.

Aeschbacher, R.A., M.-T. Hauser, K.A. Feldmann, and P.N. Benfey. 1995. The SABRE gene is required for normal cell expansion in Arabidopsis. Genes & Dev. 9: 330-340.

Baskin, T.I. and R.E. Wilhamson. 1992. Ethylene, microtubules and root morphology in wild-type and mutant Arabidopsis seedlings. Curr. Topics Plant Biochem. Physiol. 11: 118-130.

Benfey, P.N. and J.W. Schiefelbein. 1994. Getting to the root of plant development: The genetics of Arabidopsis root formation. Trends Genet. 10: 84-88.

Benfey, P.N., P.J. Linstead, K. Roberts, J.W. Schiefelbein, M.-T. Hauser, and R.A. Aeschbacher. 1993. Root development in Arabidopsis: Four mutants with dramatically altered root morphogenesis. Development 119: 57-70.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: Conserved structure and molecular mechanism. Nature 349: 117-127.

Bruchhaus, I., M. Leippe, C. Lioutas, and E. Tannich. 1993. Unusual gene organization in the protozoan parasite Entamoeba histolytica. DNA & Cell Biol. 12: 925-933.

Chen, M.S., R.A. Obar, C.C. Schroder, T.W. Austin, C.A. Poody, S.C. Wadsworth, and R.B. Vallee. 1991. Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. Nature 351: 583-586.

Cheng, Y.-S.E., C.E. Patterson, and P. Staehe. 1991. Interferon-induced guanylate-binding proteins lack an N(T)KXD consensus motif and bind GDP in addition to GDP and GTP. Mol. Cell. Biol. 11: 4717-4725.

Cleland, R.E. 1988. Auxin and cell elongation. In Plant hormones and their role in plant growth and development [ed. P.J. Davies], pp. 132-148. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Cormack, R.G.H. 1962. Development of root hairs in angio- sperms. II. Bot. Rev. 28: 446-464.

Cosgrove, D. 1986. Biophysical control of plant cell growth. Annu. Rev. Plant Physiol. Plant Mol. Biol. 37: 377-405.

——. 1993. How do plant cell walls extend? Plant Physiol. 102: 1-6.

Dever, T.E., M.J. Glynias, and W.C. Merrick. 1987. GTP-binding domain: Three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. 84: 1814-1818.

Devereux, J., P. Haerberi, and O. Smithies. 1984. A comprehen-
to interferon-induced Mx proteins performs an essential function in yeast protein sorting. *Cell* 61: 1063–1074.

Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schiefelbein, J.W. and P.N. Benfey. 1991. The development of plant roots: New approaches to underground problems. *Plant Cell* 3: 1147–1154.

Schiefelbein, J.W. and C. Somerville. 1990. Genetic control of root hair development in *Arabidopsis thaliana*. *Plant Cell* 2: 235–243.

Schiefelbein, J., M. Galway, J. Masucci, and S. Ford. 1993. Pollen tube and root-hair tip growth is disrupted in a mutant of *Arabidopsis thaliana*. *Plant Physiol.* 103: 979–985.

Shevell, D.E., W.-M. Leu, C.S. Gillmor, G. Xia, K.A. Feldmann, and N.-H. Chua. 1994. EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. *Cell* 77: 1051–1062.

Stack, J.H., B. Horazdovsky, and S.D. Emr. 1995. Receptor-mediated protein sorting to the vacuole in yeast: Roles for a protein kinase, a lipid kinase and GTP-binding proteins. *Annu. Rev. Cell Dev. Biol.* 11: 1–33.

Stachelin, L.A. and I. Moore. 1995. The plant Golgi apparatus: Structure, functional organization, and trafficking mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46: 261–288.

Takahashi, T., A. Gasch, N. Nishizawa, and N.-H. Chua. 1995. The *DIMINUTO* gene of *Arabidopsis* is involved in regulating cell elongation. *Genes & Dev.* 9: 97–107.

Torres-Ruiz, R.A. and G. Jurgens. 1994. Mutations in the FASS gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. *Development* 120: 2967–2978.

Valvecens, D., M. Van Montagu, and M.V. Lijssehettens. 1988. Agrobacterium tumefaciens-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci.* 85: 5536–5540.

van der Bie, A.M. and E.M. Meyerowitz. 1991. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature* 351: 411–414.

Vater, C.A., C.K. Raymond, K. Ekena, I. Howald-Stevenson, and T.H. Stevens. 1992. The Vps1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. *J. Cell Biol.* 119: 773–786.

Wilsbach, K. and G.S. Payne. 1993. Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. *EMBO J.* 12: 3049–3059.
The ROOT HAIR DEFECTIVE3 gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in Arabidopsis.

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