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Genetic Interactions during Root Hair Morphogenesis in Arabidopsis

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Root hairs are a major site for the uptake of water and nutrients into plants and form an increasingly important model system for studies of development of higher plants and cell biology. We have identified loss-of-function mutations in eight new genes required for hair growth in Arabidopsis: *SHAVEN1* (*SHV1*), *SHV2*, and *SHV3*; *CENTIPEDE1* (*CEN1*), *CEN2*, and *CEN3*; *BRISTLED1* (*BST1*); and *SUPERCENTIPEDE1* (*SCN1*). We combined mutations in 79 pairs of genes to determine the stages at which these and six previously known genes contribute to root hair formation. Double mutant phenotypes revealed roles for several genes that could not have been predicted from the single mutant phenotypes. For example, we show that *TIP1* and *RHD3* are required much earlier in hair formation than previous studies have suggested. We present a genetic model for root hair morphogenesis that defines the roles of each gene, and we suggest hypotheses about functional relationships between genes.

INTRODUCTION

Root hairs contribute as much as 77% of the root surface area of cultivated crops, forming the major point of contact between the plant and the rhizosphere. Each hair is a long, narrow tube produced from a single cell by tip growth (the deposition of new membrane and cell wall material at a growing tip). Large collections of viable and fertile Arabidopsis root hair mutants are available (Schiefelbein and Somerville, 1990; Masucci and Schiefelbein, 1994; Grierson et al., 1997; Pitts et al., 1998) with all of the advantages of the Arabidopsis genome project. In Arabidopsis, root hairs form in a predictable pattern, so developing hair cells can be observed from the beginning of their life in the root meristem throughout the developmental process. Mechanisms that control this patterning have been identified (Dolan et al., 1994; Galway et al., 1994; Tanimoto et al., 1995; DiCristina et al., 1996; Masucci et al., 1996; Masucci and Schiefelbein, 1996; Schneider et al., 1997, 1998; Wada et al., 1997; Dolan and Scheres, 1998; Lee and Schiefelbein, 1999).

The selection of an initiation site within the hair cell depends on the gene *RHD6* and is influenced by the plant growth regulators auxin and ethylene (Masucci and Schiefelbein, 1994, 1996). Once an initiation site has been selected, a small swelling forms (sometimes called a bulge). This requires local acidification of the cell wall (Bibikova et al., 1998) and is thought to be due at least in part to local wall loosening. Mutations in the *RHD1* and *TIP1* genes result in hairs with larger swellings at their bases than those of wild-type hairs. This is thought to result from increased loosening of the wall around the base of the hairs and suggests that the degree of cell wall loosening in wild-type plants is restricted by the RHD1 (Schiefelbein and Somerville, 1990) and TIP1 (Ryan et al., 1998) gene products.

Once a swelling has formed, the hair begins to elongate by tip growth. This transition to tip growth takes place when the hair is between 20 and 40 μm long (Dolan et al., 1994). Elongation is accompanied by the generation of a tip-high calcium gradient that can be observed throughout the remainder of root hair growth (Schiefelbein et al., 1992; Wymer et al., 1997). Mutations at the *RHD2* locus result in the formation of swellings that do not elongate successfully (Schiefelbein and Somerville, 1990). Calcium gradients are not observed in swellings on most hair cells on *rhd2* mutant plants (Wymer et al., 1997), supporting the idea that the calcium gradient is associated with the ability to elongate.

Plants homozygous for mutations in *COW1* and *TIP1* produce short, wide root hairs, and sometimes multiple hairs form at a single site of hair formation (Schiefelbein et al., 1993; Grierson et al., 1997; Ryan et al., 1998). Plants homozygous for mutations in *RHD3* and *RHD4* have short, wavy root hairs (Schiefelbein and Somerville, 1990; Galway et al., 1997, 1999; Wang et al., 1997). The *RHD3* gene has been characterized at the molecular level. *RHD3* encodes a

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protein with GTP binding motifs that may be required during vacuole enlargement (Wang et al., 1997). The phenotypes of double mutants have previously suggested that *COW1* acts after *RHD2* and that *TIP1* and *RHD3* act in parallel with *COW1* (Grierson et al., 1997).

To identify more genes involved in root hair formation and to isolate new alleles of previously known genes, we performed a new screen for mutants that affect root hair shape. This identified eight new loci and additional alleles of two previously characterized genes. We combined pairs of mutations at 14 genes to determine the stages at which each is required during hair cell morphogenesis and to identify the genetic interactions that take place as root hairs develop. The double mutant phenotypes revealed new roles for genes that could not have been predicted from their single mutant phenotypes and suggested hypotheses about functional relationships between genes. We combined our results with previously published data, in a model that defines roles for 15 genes during root hair morphogenesis.

RESULTS

Eight New Loci Involved in Root Hair Morphogenesis

We isolated 29 new mutants affecting root hair morphogenesis, 20 of which defined eight new loci: *SHAVEN1* (*SHV1*), *SHV2*, and *SHV3*; *CENTIPEDE1* (*CEN1*), *CEN2*, and *CEN3*; *BRISTLED1* (*BST1*); and *SUPERCENTIPEDE1* (*SCN1*). All of our new mutants formed root hairs on a similar proportion and arrangement of epidermal cell files and in a similar location on each hair-forming cell as in wild-type plants (data not shown), suggesting that processes governing the patterning of hair cells and the location of hairs on cells were not affected by the new alleles. We chose one allele at each locus to study in more detail or chose multiple alleles if they had obviously different phenotypes.

Figure 1A shows mature root hairs on homozygous mutant plants, and Table 1 lists phenotypes of mutants at each locus included in this study. The new mutants fell into two phenotypic groups.

The first group included mutants in the *RHD2*, *SHV1*, *SHV2*, and *SHV3* genes. Root hairs on all mutants at these loci except *rhd2-2* seldom were $\geqslant$40 μm long, indicating that they failed to develop beyond the transition to tip growth, which takes place when hairs are 20 to 40 μm long (Dolan et al., 1994). This suggests that the *RHD2, SHV1, SHV2, and SHV3* genes are required for tip growth to be successfully established. When hairs $\geqslant$40 μm long formed on mutants at the *RHD2*, *SHV1*, and *SHV2* loci, they were much shorter than wild-type hairs (Figure 1A and Table 1), supporting the conclusion that this group of genes is required for tip growth.

The second group included mutants in the *BST1*, *CEN1*, *CEN2*, *CEN3*, *RHD3*, and *SCN1* genes. Root hairs on these plants were $\geqslant$40 μm, suggesting that they could develop beyond the transition to tip growth. These hairs were short and often had different shapes from those of the wild type (Figure 1A and Table 1), suggesting that these genes are required to control the shape of the root hair during tip growth. Several mutants in this group sometimes produced multiple hairs (Table 1), implicating the *SCN1*, *BST1*, *CEN2*, and *CEN3* genes in mechanisms that restrict the number of root hairs produced by tip growth from each hair-forming site.

Where we have quantitative data for more than one allele (Table 1), we can draw conclusions about allele strength. Root hair length is more strongly affected by the *cen1-2* mutation than by *cen1-1*, suggesting that *cen1-2* is the stronger allele. The proportion of root hairs $\geqslant$40 μm indicated that the *rhd2-2* and *rhd2-3* alleles are weaker than *rhd2-1*. Root hairs were longer on *rhd3-7* plants than on *rhd3-1*, suggesting that *rhd3-7* is the weaker allele. A higher proportion of multiple hairs formed on plants homozygous for *tip1-2* than *tip1-1*, suggesting that the former was the stronger allele.

In a statistical test for 3:1 segregation in F_2 populations, $\chi^2 \leqslant 3.84$ (and hence $P \geqslant 0.05$) for *shv1-1*, *shv1-4*, *shv2-1*, *cen1-1*, *cen1-2*, *cen1-3*, *cen2-1*, *cen3-1*, *cen3-2*, *scn1-1*, *bst1-1*, *rhd2-3*, and *rhd3-7*; similar results were produced whether the mutant parent was male or female. This suggests that all of these alleles are recessive, single locus, nuclear mutations. *shv3* was difficult to score because the phenotypic range was close to that of wild-type plants; the segregation of F_2 populations (e.g., 175 wild type:94 Shv3$^-$) probably included some wild-type plants misscored as Shv3$^-$, but we cannot rule out the possibility that a Shv3$^-$ phenotype might be exhibited by a minority of heterozygous plants.

Mutations in the *RHD3* and *TIP1* loci affect the growth of many cell types (Wang et al., 1997; Ryan et al., 1998), whereas the *COW1* locus appears to be specific for root hair cells (Grierson et al., 1997). *TIP1* affects pollen tube growth, and both *TIP1* and *RHD3* affect the stature and architecture of the plant (Galway et al., 1997; Wang et al., 1997; Ryan et al., 1998). Our segregation data show that none of the mutations in the eight new genes exhibited reduced transmission, which suggests that they do not affect pollen tube function, although we cannot exclude the possibility that these mutations impair pollen tube growth in a way that does not affect pollen competition during fertilization. The results of an extensive survey of plant architecture showed that mutations at the new loci did not have strong pleiotropic phenotypes (data not shown). Under our growth conditions, which provided high levels of nutrients and water, the stature and vigor of plants homozygous for mutations in each of the eight new genes were similar to those of the wild type.

Figure 1B shows the map locations of the new loci along with those of other loci included in this study. Representatives of each new complementation group map to a different

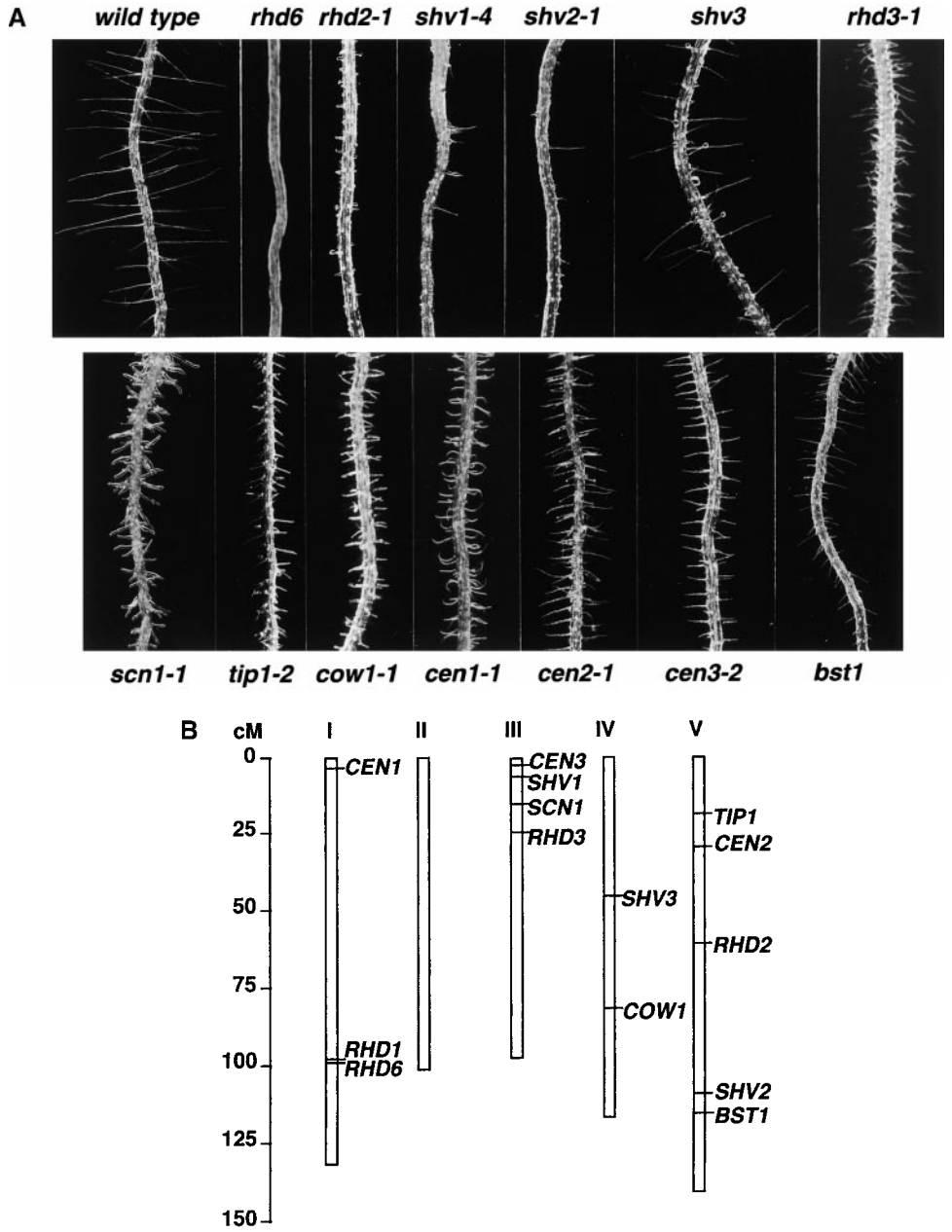

Figure 1. Mutant Phenotypes and Map Positions of Genes Involved in Root Hair Morphogenesis.

(A) Root hairs of wild-type and single mutant seedlings. Mature root hairs of mutants at each new locus are shown alongside those of plants mutated at previously identified loci (*rhd6*, *rhd2-1*, *rhd3-1*, *tip1-2*, and *cow1-1*).

(B) Locations on the Arabidopsis genetic map of genes involved in root hair formation. Shown for comparison are previously published map positions of other loci included in this study: *RHD1*, *RHD2*, and *RHD3* (Schiefelbein and Somerville, 1990); *RHD6* (Masucci and Schiefelbein, 1994); *TIP1* (Ryan et al., 1998); and *COW1* (Grierson et al., 1997).

Table 1. Root Hair Phenotypes of Arabidopsis Lines

	Phenotype[b]			
Genotype[a]	% Hairs ⩾40 μm Long[c]	Length of Hairs ⩾40 μm Long (% Wild-Type Length)[d]	% Multiple Hairs[c,e]	Other Features[f]
Wild type	96 SD ± 4 (5)	100 (5)	0 (5)	Straight; about half of epidermal cells form hairs
bst1	100 (6)	19.2 SD ± 3.8 (6)	4 SD ± 2 (6)	Straight
cen1-1	100 (5)	25.9 SD ± 0.1 (5)	0 (5)	Sometimes curled
cen1-2	95 SD ± 7 (5)	12.6 SD ± 1.0 (5)	0 (5)	Sometimes curled
cen2-1	100 (8)	29.1 SD ± 6.1 (6)	7 SD ± 4 (8)	Sometimes curled
cen3-1	100 (5)	21.5 SD ± 6.1 (5)	14 SD ± 10 (5)	Sometimes bulbous bases
cen3-2	100 (8)	23.4 SD ± 4.2 (8)	8 SD ± 5 (8)	Some bulbous bases; sometimes curled
cow1-1	100 (5)	25.7 SD ± 2.3 (5)	7 SD ± 3 (5)	Sometimes slightly crooked; wide
cow1-2	100 (6)	20.4 SD ± 3.2 (6)	20 SD ± 2 (6)	Sometimes slightly crooked; wide
rhd1	ND	ND	ND	Large swelling at base
rhd2-1	0 (8)	None	0 (5)	
rhd2-2	100 (5)	8.2 SD ± 1.2 (5)	0 (5)	Straight; wide
rhd2-3	22 SD ± 3 (5)	13.0 SD ± 1.3 (5)	0 (5)	Straight
rhd3-1	100 (6)	15.6 SD ± 1.3 (6)	0 (6)	Wavy; crooked
rhd3-7	100 (8)	21.7 SD ± 0.8 (8)	0 (8)	Wavy; crooked
rhd6	100 (5)	89 SD ± 18 (5)	ND	Very few epidermal cells form hairs
scn1-1	100 (5)	26.8 SD ± 2.8 (5)	61 SD ± 7 (5)	Sometimes curled; wide
shv1-1	15 SD ± 5 (5)	10.3 SD ± 2.3 (5)	0 (5)	Straight
shv1-4	4 SD ± 2 (8)	29.8 SD ± 8.3 (5)	0 (8)	Straight
shv2-1	18 SD ± 9 (6)	15.8 SD ± 1.8 (6)	0 (6)	Straight
shv3	30 SD ± 12 (6)	68.2 SD ± 11.6 (5)	0 (6)	Straight
tip1-1	97 SD ± 6 (5)	19.1 SD ± 5.4 (5)	26 SD ± 4 (5)	Straight; wide
tip1-2	91 SD ± 13 (5)	14.6 SD ± 3.7 (5)	53 SD ± 6 (5)	Straight; wide

[a] Mutants are listed in alphabetical order to facilitate comparison with other tables in this article.
[b] The numbers within parentheses are number of roots. ND, not determined.
[c] Fifty mature hairs were assessed on each root, except *rhd6*, where three hairs were assessed on each root.
[d] Ten mature hairs were measured on each root, except on *rhd6*, where two hairs were measured on each root. Wild-type length was ~900 μm.
[e] Multiple hairs that appeared at a single site of hair formation.
[f] At least 1000 roots of each genotype were examined.

position on the Arabidopsis genome, confirming that each represents a different genetic locus.

Double Mutant Analysis

We combined pairs of mutations at the eight new loci with each other and with those at six previously characterized loci affecting root hair morphogenesis (*RHD1*, *RHD2*, *RHD3*, *RHD6*, *TIP1*, and *COW1*) to make 79 double mutant combinations. We grouped the double mutants according to type of genetic interaction.

Epistatic Interactions at the Beginning of Root Hair Formation and during the Transition to Tip Growth

Epistatic interactions, where the phenotype of a double mutant was essentially the same as that of a single mutant parent, are listed in Table 2. Photomicrographs of a subset of the results are shown in Figure 2. Because the mutations used in our study are recessive, they are probably loss-of-function alleles. Loss-of-function mutations in a gene that is required early in the process might be expected to be epistatic to those in genes that act later.

The *rhd6* mutation was epistatic to mutations in all 10 genes we tested (Table 2), suggesting that *RHD6* acts before any of these genes to permit hair formation.

Mutations in *RHD2*, *SHV1*, *SHV2*, and *SHV3* were epistatic to mutations in several other genes, resulting in double mutant roots with very few hairs ⩾40 μm long (Table 2 and Figure 2; Schiefelbein and Somerville, 1990; Grierson et al., 1997). These results support our conclusion from the single mutant phenotypes that these genes are required for the transition from swelling formation to tip growth. That *rhd2-1* is epistatic to *shv3* suggests *RHD2* may be required earlier in this transition than *SHV3*. Similarly, epistasis of *shv1-1* to both *shv2-1* and *shv3-1* suggests that *SHV1* may be required earlier than *SHV2* or *SHV3*.

Partially Suppressed Phenotypes Implicate *TIP1*, *RHD3*, *CEN1*, and *SCN1* in the Transition from Swelling Formation to Tip Growth

Results in Figure 3 and Table 3 show that a second set of genes, *TIP1*, *RHD3*, *CEN1*, and *SCN1*, are also involved in the transition to tip growth. When mutations of some members of this group are present, a higher proportion of hairs on Rhd2$^-$, Shv1$^-$, and Shv2$^-$ plants grow ⩾40 μm long, partially suppressing the phenotypes of Rhd2$^-$, Shv1$^-$, and Shv2$^-$ single mutants.

In three cases (*rhd3-1 shv2-1*, *shv1-1 scn1-1*, and *shv2-1 scn1-1*), the proportion of hairs ⩾40 μm long was similar to that of the wild type. This was not considered to be epistasis because some effects of the *shv1-1* and *shv2-1* mutations were evident in the shapes of hairs (Figure 3, and Tables 1 and 3).

The *tip1-2* and *tip1-2 shv2-1* phenotypes were indistinguishable except that the proportion of hairs ⩾40 μm long was slightly less in *tip1-2 shv2-1* (Tables 1 and 3, and Figure 3). This almost complete epistasis suggests that the growth of root hairs on *tip1-2* plants does not require *SHV2*.

Three Classes of Additive Phenotypes Revealed Genetic Relationships Affecting the Transition to Tip Growth, the Number of Hairs Produced at Each Hair-Forming Site, Hair Length, and Hair Shape

Genes that function independently of each other can be identified because the effects of mutant alleles combine in double mutant individuals to give an additive phenotype. By comparing data about double mutants with the single mutant phenotypes in Table 1, we identified three categories of additive double mutants. In class 1 additive double mutants, all of the phenotypes of both single mutant parents were present and were combined to some extent. Class 2 phenotypes had some additive and some synergistic features. Double mutants in class 3 lacked at least one phenotypic feature of a parental line, although other aspects of both parental phenotypes were present. Double mutants in each class are listed in Table 4, and example phenotypes are shown in Figure 4.

Class 2 additives had synergistic features affecting three phenotypic traits. The first was a decrease in the proportion of hairs ⩾40 μm long, so that fewer proceeded to successful tip growth. For example, only half of the root hairs on *cen3-1 cen1-1* and *cen2-1 cen3-2* double mutants exceeded 40 μm (Table 4), whereas in the corresponding single mutants, the proportion of hairs ⩾40 μm long was similar to that of the wild type (Table 1). Double mutants with this phenotype (Table 4 and Figure 4) included mutations in the *CEN1*, *TIP1*, and *SCN1* genes, supporting our conclusions from the partly suppressed phenotypes (Table 3) that these genes are involved in the transition from swelling formation to tip growth. The *cen1-1 bst1*, *tip1-1 bst1*, *scn1-1 bst1*, *cen2-1 scn1-1*, and *shv2-1 bst1* double mutants also had this phenotype, which suggests that *BST1*, *CEN2,* and *CEN3* can also affect this step.

The second trait affected by synergy was the production of multiple hairs from hair-forming sites. For example, 61% of hair cells on *scn1-1* single mutant roots had multiple hairs, and *cen1-1* single mutants had no multiple hairs (Table 1), but when these mutations were combined, every hair cell on *scn1-1 cen1-1* double mutant roots produced multiple hairs. Other double mutants with synergistic phenotypes affecting this trait had mutations in the *CEN2*, *CEN3*, *COW1*, and *SCN1* genes, confirming our conclusions from single mutant phenotypes that these genes are required to restrict the number of hairs formed at each hair-forming site.

The third feature affected by synergy was hair shape. In the most dramatic cases, plants carrying combinations of the *scn1-1* mutation with the *bst1*, *cen1-1*, or *cen2-1* mutations produced hairs that were almost shapeless balloons (Table 4). Double mutants with synergistic phenotypes affecting hair shape were homozygous for mutations in the *CEN1*, *CEN2*, *CEN3*, *SCN1*, *COW1*, *TIP1*, *BST1*, *SHV2*, and *SHV3* genes, confirming conclusions from the single mutant phenotypes that *CEN1*, *CEN2*, *CEN3*, *SCN1*, *TIP1*, and *COW1* are involved in this process (Table 1; Schiefelbein et

Table 2. Combinations of Mutations Producing Epistatic Phenotypes

Gene	Phenotype[a]	Double Mutants with the Phenotype
RHD6	Very few epidermal cells form hairs	*rhd6 rhd2-2*, *rhd6 shv1-4*, *rhd6 shv3*, *rhd6 tip1-2*, *rhd6 cen2-1*, *rhd6 rhd3-7*, *rhd6 rhd3-9*, *rhd6 cen3-2*, *rhd6 bst1*, *rhd6 cen1-2*, *rhd6 scn1-1*
RHD2	Very few hairs exceed 40 μm long; those that do are short	*shv3 rhd2-1*, *rhd2-3 cow1-1*, *rhd2-1 cen3-2*, *rhd2-1 cen2-1*, *rhd2-3 cen1-1*, *rhd2-1 cen1-1*, *rhd2-1 bst1*
SHV1	Very few hairs exceed 40 μm long; those that do are short	*shv1-1 shv3*, *shv2-1 shv1-1*, *cen3-1 shv1-4*, *shv1-1 cow1-2*, *rhd3 shv1-1*, *bst1 shv1-4*, *cen2-1 shv1-4*
SHV2	Very few hairs exceed 40 μm long; those that do are short	*shv2-1 cen1-2*, *shv2-1 cen3-2*
SHV3	Few hairs exceed 40 μm long	*rhd3 shv3*, *bst1 shv3*, *cen1-2 shv3*, *cen3-1 shv3*

[a] See Table 1.

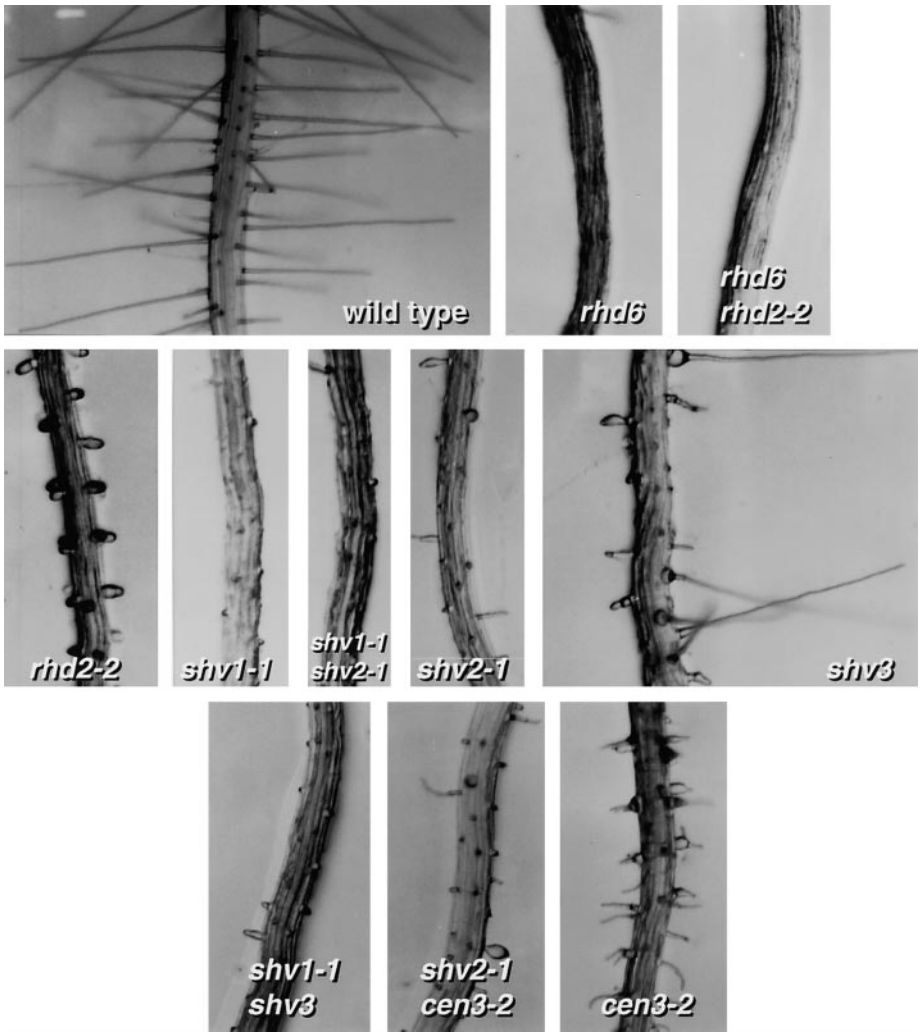

Figure 2. Mature Root Hairs of Double Mutant Plants with Epistatic Phenotypes.

Root hairs of wild-type and single mutant plants are shown for comparison. The *rhd6 rhd2-2* double mutant is mostly bald, as is the *rhd6* single mutant. This is an example of epistasis, where the *rhd6* mutation is epistatic to *rhd2-2*. Other results shown here demonstrate that *shv1-1* is epistatic to both *shv2-1* and *shv3* and that *shv2-1* is epistatic to *cen3-2*.

al., 1993; Grierson et al., 1997; Ryan et al., 1998) and also implicating *BST1*, *SHV2*, and *SHV3*.

In class 3 additive double mutants, both parental lines had short hairs, but these effects did not combine in the double mutants. Instead, double mutant hairs were similar in length to the hairs of one parental line (cf. results in Tables 1 and 4). For example, root hairs on *cen2-1 cen1-2* plants were of similar length (Table 4) to those on *cen1-2* single mutants (Table 1), suggesting that *CEN2* may not be able to contribute to root hair elongation in the absence of the *CEN1* gene. The results in Table 4 suggest a similar dependency of *CEN2* on *BST1* and *RHD3*, of *CEN3* on *RHD3*, and of *RHD3* on *TIP1*. In three cases, hair shape was also closer to one parent than the other, such that the curled (*cen2-1 rhd3-7*) or wavy (*rhd3-1 cen3-2* and *rhd3-1 tip1-2*) phenotype of a parent was not seen in the double mutant.

Role of *TIP1* during Swelling Formation

Ryan et al. (1998) previously suggested that mutations in the *TIP1* gene could increase the size of the swelling formed during root hair initiation. Figure 5 shows swellings formed on wild-type and on *tip1-2*, *shv1-4*, and *tip1-2 shv1-4* mu-

tant plants. Statistical analysis (Figure 5) confirmed that the swellings formed on *tip1-2* and *tip1-2 shv1-4* mutant plants were markedly larger than those on wild-type or *shv1-4* plants. The root hairs that we measured had not undergone elongation growth, demonstrating that increased swelling caused by the *tip1-2* mutation had been produced early in root hair development and probably in the absence of tip growth.

To investigate the relationship between *TIP1* and *RHD1* during swelling formation, we measured the length (along the root axis) and height (perpendicular to the root) of five swellings on each of five roots of *rhd1* (length 92.5 μm, SD ± 16.3; height 82.0 μm, SD ± 13.2) and *tip1-2 rhd1* (length 83.7 μm, SD ± 13.7; height 62.1 μm, SD ± 9.7) plants. The results suggest that the increase in swelling size conferred by the *tip1-2* mutation (Figure 5) is not seen in the presence of the *rhd1* mutation, indicating that *TIP1* may restrict swelling size by a mechanism that requires *RHD1*.

Synergistic Phenotypes Affect the Beginning of Root Hair Formation

The most dramatic results of our study are the three synergistic double mutant phenotypes shown in Figure 6. In all three cases, the double mutant roots are almost hairless, even though the corresponding single mutants have normal numbers and arrangements of hair cells. These results indicate that the *TIP1*, *CEN2*, *RHD3*, *SCN1*, and *SHV3* genes have critical roles affecting the very beginning of root hair formation.

DISCUSSION

Eight New Genes Affecting Root Hair Morphogenesis

In a large, visual screen, we identified 29 new mutants and eight new loci involved in root hair morphogenesis. The new mutants identify recessive, nuclear, single-gene mutations with strong root hair phenotypes. Single mutant phenotypes suggest roles for *RHD2*, *SHV1*, *SHV2*, and *SHV3* in the transition from swelling formation to tip growth; for *BST1*, *CEN1*, *CEN2*, *CEN3*, *RHD3*, and *SCN1* in controlling hair shape and length; and for *BST1*, *CEN2*, *CEN3*, and *SCN1* in preventing the formation of multiple hairs at each hair-forming site (Table 1). Visual screens have not reached saturation (no more than nine alleles from all screens so far for

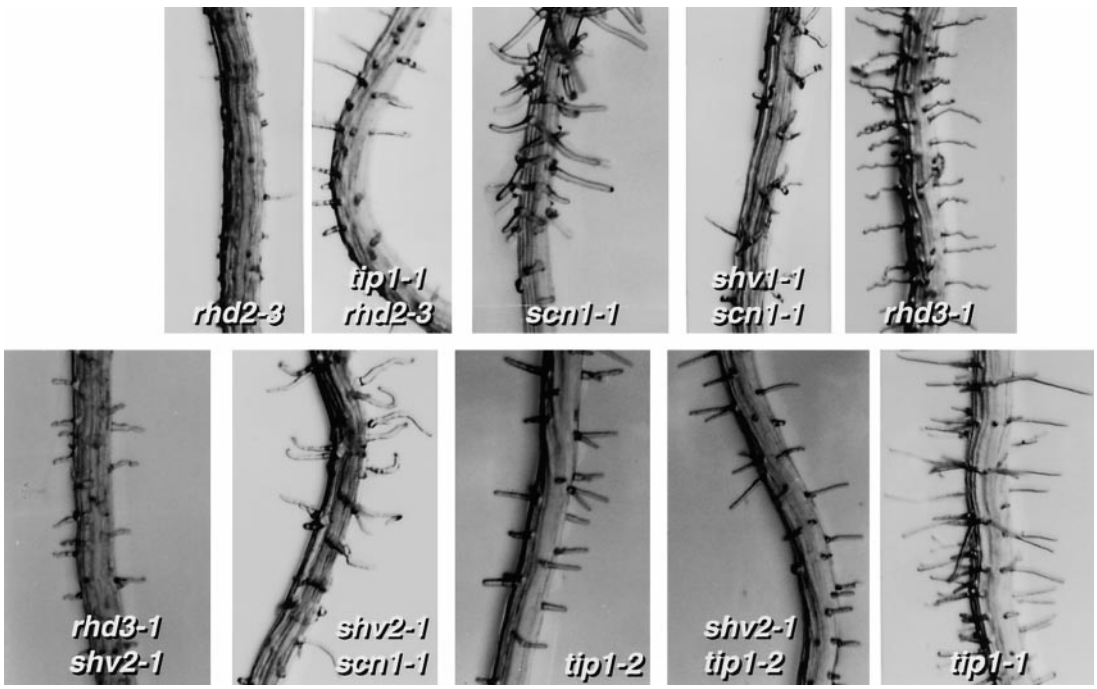

Figure 3. Mature Root Hairs of Double Mutant Plants with Partly Suppressed Phenotypes.

Root hairs on *tip1-1 rhd2-3* double mutant roots are frequently longer than those on *rhd2-3* single mutant roots. This phenotype suggests that the wild-type function of *TIP1* contributes to the short root hair phenotype of the *rhd2-3* mutant. Results shown here demonstrate similar roles for *RHD3* in the *shv2-1* phenotype, and for *SCN1* in the *shv1-1* and *shv2-1* phenotypes. The phenotypes of *tip1-2* and *shv2-1 tip1-2* are almost indistinguishable, suggesting that *tip1-2* is almost completely epistatic to *shv2-1*. The *shv1-1* and *shv2-1* single mutant phenotypes are shown in Figure 2.

Table 3. Partly Suppressed Root Hair Phenotypes of Double Mutants

Genotype	Phenotype[a]			
	% Hairs ⩾40 μm Long[b]	Length of Hairs ⩾40 μm Long (% Wild-Type Length)[c]	% Multiple Hairs[b,d]	Other Features
tip1-2 rhd2-1	**29 SD ± 8** (5)	11.3 SD ± 2.0 (5)	36 SD ± 6 (5)	Straight; wide
tip1-1 rhd2-3	**36 SD ± 4** (5)	12.6 SD ± 4.7 (5)	0 (5)	Straight; wide
rhd3-1 shv2-1	**94 SD ± 9** (5)	9.4 SD ± 0.1 (5)	2 SD ± 3 (5)	Wavy; crooked
cen1-1 shv1-4	**25 SD ± 2** (5)	9.6 SD ± 3.2 (5)	0 (5)	Sometimes curled
tip1-2 shv2-1	**75 SD ± 5** (5)	13.6 SD ± 3.8 (5)	54 SD ± 6 (5)	Straight; wide
tip1-1 shv2-1	**58 SD ± 1** (5)	10.2 SD ± 2.8 (5)	ND (5)	Straight; wide; bulbous tips
shv1-1 scn1-1	**96 SD ± 6** (5)	13.7 SD ± 2.0 (5)	43 SD ± 25 (5)	Sometimes curled; wide
shv2-1 scn1-1	**96 SD ± 6** (5)	28.9 SD ± 5.4 (5)	**25 SD ± 1** (5)	Sometimes curled; wide; crinkled

[a] Features in boldface are closer to the wild type than they were in one of the parental lines. Numbers within parentheses are number of roots.
[b] Fifty mature hairs were assessed on each root.
[c] Ten mature hairs were measured on each root. Wild-type length was ~900 μm.
[d] Multiple hairs that appeared at a single site of hair formation. ND, not determined.

RHD3; Wang et al., 1997; this work), which suggests that more genes remain to be found.

Double Mutant Analysis Reveals a Complex Genetic Network Controlling Root Hair Morphogenesis

The phenotypes of double mutant plants confirmed roles for genes suggested by the single mutant phenotypes, revealed additional roles that were not apparent from single mutant phenotypes, and suggested hypotheses about the functional relationships between genes. In some cases, the mutations cause incomplete loss of function, and our interpretations are models that can be tested as new molecular and genetic tools become available.

The Beginning of Root Hair Formation

Previous studies have shown that *RHD6* acts downstream of *TTG* and *GL2*, and it is believed to be one of the first genes to act in root hair cells to promote hair formation (Masucci and Schiefelbein, 1996). The *rhd6* mutation was epistatic to mutations in all 10 genes we tested (Table 2), which suggests that our new genes act downstream of *RHD6*, and hence probably downstream of *TTG*, *GL2*, and *CPC* (Masucci and Schiefelbein, 1996).

We observed synergistic effects between mutations in the *TIP1* and *CEN2*, *CEN2* and *SHV3*, and *SCN1* and *RHD3* pairs of genes that prevented root hair formation (Figure 6). The results demonstrate that those genes can influence root hair development much earlier in the process than has previously been suspected. For example, *RHD3* had previously been placed after *RHD2* in a genetic pathway of root hair formation (Schiefelbein and Somerville, 1990). However, the roots of *scn1-1 rhd3-1* double mutant plants are almost completely bald (Figure 6), indicating that in the absence of *SCN1* function, *RHD3* is required at the very beginning of hair formation. *tip1-1 cen2-1* and *cen2-1 shv3* double mutant plants have phenotypes similar to *scn1-1 rhd3-1* (Figure 6), showing that *TIP1*, *CEN2*, and *SHV2* can also affect root hair formation much earlier than suggested by their single mutant phenotypes. Further anatomical and genetic (triple mutant) studies are required to clarify the relationships between these genes and other genes affecting the beginning of hair formation.

We have two alternate hypotheses concerning this group of double mutants. In the first, two genes could have overlapping functions and partly compensate for each other's absence; but in double mutants, where the functions of both genes are impaired, they might fail to compensate for each other, and a severe root hair phenotype would result. In the second, the mutant alleles used to make each double mutant are not complete loss of function, but because they affect the same linear pathway, the function of the pathway is critically impaired in each double mutant, resulting in a severe root hair phenotype. Further genetic and molecular information is needed to enable us to distinguish between these hypotheses.

Swelling Formation

Root hairs first appear as swellings on the surface of hair-forming cells. Previous research has suggested that these swellings are formed by cell wall loosening and that the *TIP1* and *RHD1* genes restrict the amount of loosening in wild-type plants (Schiefelbein and Somerville, 1990; Ryan et al., 1998). We showed that the *tip1-2 shv1-4* double mutant has an additive phenotype (Figure 5), confirming that *TIP1* acts

during swelling formation to limit the size of the swelling. We also made a series of double mutants that included the *rhd1* mutation. These had additive phenotypes (Table 4), suggesting that *RHD1* acts independently of the other genes studied here. The increase in swelling size conferred by the *tip1-2* mutation (Figure 5) is not seen in the presence of the *rhd1* mutation, indicating that *TIP1* may restrict swelling formation by a mechanism that involves *RHD1*. A more careful analysis of swelling volume and shape is required to verify these observations.

Transition from Swelling Formation to Tip Growth

Previous work has suggested that swelling formation does not involve the same mechanisms as root hair elongation and might require different genes (Schiefelbein and Somerville, 1990; Grierson et al., 1997). This is supported by physiological evidence that a calcium gradient is not established until after the swelling forms and that the gradient does not form in Rhd2$^-$ cells (Wymer et al., 1997). Our results uphold and expand on this view. Eleven genes (*RHD2*, *SHV1*, *SHV2*,

Table 4. Additive Root Hair Phenotypes of Double Mutants

	Phenotype[b]			
Genotype[a]	% Hairs ⩾40 μm Long[c]	Length of Hairs ⩾40 μm Long (% Wild-Type Length)[d]	% Multiple Hairs[c,e]	Other Features[f]
Class 1[g]				
cow1-2 bst1	96 SD ± 6 (5)	*10 SD ± 0.6* (5)	23 SD ± 2 (5)	Sometimes crooked or curled
cow1-2 cen2-1	90 SD ± 9 (6)	*11.2 SD ± 2.1* (5)	34 SD ± 5 (6)	Often crooked or curled
cen3-2 cow1-1	100 (5)	*15.8 SD ± 2.9* (5)	*8 SD ± 3* (5)	Some bulbous bases; sometimes crooked or curled; wide
rhd3-7 cow1-1	100 (5)	*13.6 SD ± 2.2* (5)	8 SD ± 6 (5)	Wavy; crooked; wide
tip1-1 cen3-2	100 (5)	*13.4 SD ± 1.8* (5)	*27 SD ± 9* (5)	Some bulbous bases; sometimes curled
tip1-2 shv1-4	9 SD ± 3 (5)	*14.4 SD ± 8.7* (5)	ND	Straight; wide; length variable
Class 2				
cen1-1 bst1	100 (5)	*9.2 SD ± 1.5* (5)	10 SD ± 3 (5)	Sometimes curled; **some bulbous bases**
cen3-1 cen1-1	**51 SD ± 11** (5)	*9 SD ± 0.8* (5)	18 SD ± 9 (5)	Sometimes curled
cen2-1 cen3-2	**50 SD ± 2** (5)	8.0 SD ± 2.9 (5)	**36 SD ± 4** (5)	Sometimes curled; occasional bulbous bases
tip1-1 bst1	**71 SD ± 11** (5)	*8.3 SD ± 2.6* (5)	**44 SD ± 2** (5)	**Bulbous bases; hair diameter varies**
scn1-1 bst1	**19 SD ± 5** (5)	*6.7 SD ± 0.9* (5)	*0* (5)	**Most hairs bloated, formless balloons**
scn1-1 cen1-1	**13 SD ± 4** (5)	7.0 SD ± 0.5 (5)	**100** (5)	**Multiple hairs almost merged into one large balloon**
cen2-1 scn1-1	**12 SD ± 4** (5)	5.2 SD ± 0.2 (5)	**100** (5)	**Multiple hairs almost merged into one large balloon**
scn1-1 cen3-2	100 (5)	7.3 SD ± 1.8 (5)	**100**	**Some hairs have side branches or branch at tip;** wide; sometimes curled
scn1-1 cow1-1	100 (5)	*10.3 SD ± 0.6* (5)	**100** (5)	Wide; crooked; curled
scn1-1 shv3	**1 SD ± 1** (5)	ND	ND	**Single, large swelling**
shv2-1 bst1	**5 SD ± 1** (5)	*8.2 SD ± 2.5* (5)	ND	**Often crooked**
shv2-1 cow1-2	19 SD ± 4 (5)	*9.5 SD ± 4.2* (5)	29 SD ± 6 (5)	Crooked or curled; **sometimes wide toward the tip**
tip1-1 scn1-1	**37 SD ± 5** (5)	*13.4 SD ± 1.9* (5)	98 SD ± 3 (5)	**Some hairs bloated, others spiky**
Class 3				
bst1 cen2-1	99 SD ± 1 (5)	20.6 SD ± 1.6 (5)	5 SD ± 3 (6)	Wide; often curled; sometimes crooked
cen2-1 cen1-2	100 (5)	15.0 SD ± 2.9 (5)	0 (5)	Often curled
cen2-1 rhd3-7	100 (6)	22.2 SD ± 1 (6)	5 SD ± 4 (6)	Wavy; crooked; not curled
rhd3-1 bst1	100 (6)	13.8 SD ± 1.8 (5)	3 SD ± 5 (6)	Wavy; crooked; hair length variable
rhd3-1 cen1-2	100 (5)	10.4 SD ± 1.8 (5)	0 (5)	Wavy; sometimes curled
rhd3-1 cen3-2	100 (5)	16.1 SD ± 0.3 (5)	3 SD ± 1 (5)	Some bulbous bases, sometimes curled; not wavy
rhd3-1 tip1-2	100 (5)	14.9 SD ± 2.1 (5)	44 SD ± 10 (5)	Wide; occasionally crooked; not wavy

[a] Single mutant phenotypes for comparison are listed in Table 1.

[b] Features in boldface are synergistic. Features in italic are less severe than one would predict from combining the parental phenotypes (although some influence of both parental mutations can still be seen). Features underscored are much closer to those of one parent than one would predict from combining the parental phenotypes. Numbers within parentheses are number of roots. ND, not determined.

[c] Fifty mature hairs were assessed on each root.

[d] Ten mature hairs were measured on each root. Wild-type length was ~900 μm.

[e] Multiple hairs at a single site of hair formation.

[f] Between 50 to 500 roots of each genotype were examined.

[g] *rhd1 scn1-1*, *rhd1 cen1-2*, *rhd1 rhd2-3*, *rhd1 cen2-1*, *rhd1 shv3*, *rhd1 cen3-2*, *rhd1 tip1-2*, *rhd1 shv2-1*, *rhd1 bst1*, *rhd1 rhd3-7*, and *rhd1 shv1-4* all had additive phenotypes with large swellings characteristic of *rhd1* at the base of hairs that were similar to the hairs on the other parental line.

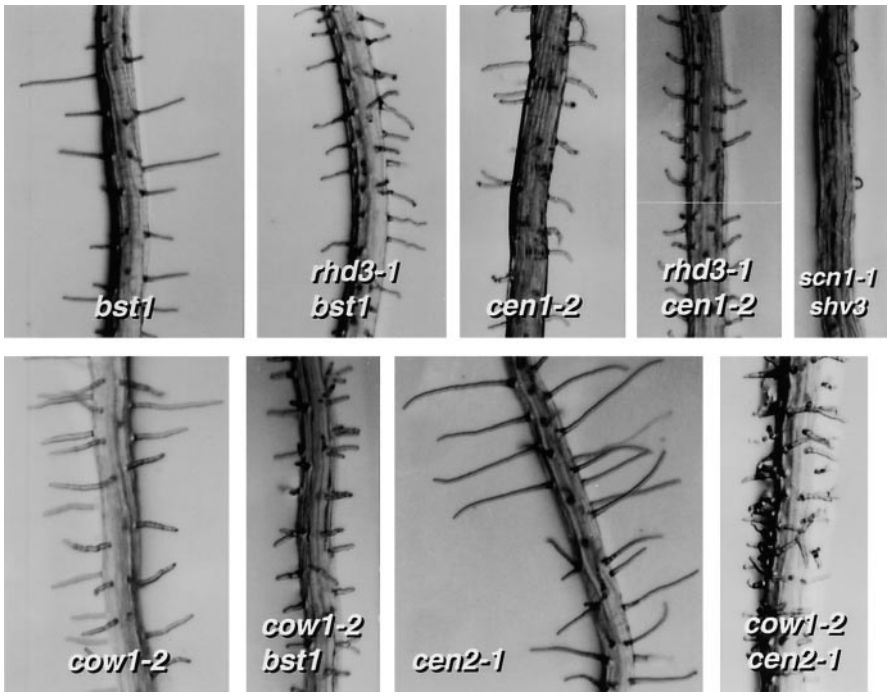

Figure 4. Mature Root Hairs of Double Mutant Plants with Additive Phenotypes.

Examples of three categories of additive double mutants are shown. *cow1-2 bst1* and *cow1-2 cen2-1* have class 1 additive phenotypes, in which the defects of the parental lines affecting hair length, hair shape, and the production of multiple hairs from a single site of hair formation are combined in the double mutants. *scn1-1 shv3* has a class 2 phenotype; synergistic effects greatly reduce the proportion of hairs >40 μm long, and a very high proportion of cells produce only a large swelling. *rhd3-1 bst1* and *rhd3-1 cen1-2* have class 3 additive phenotypes; both mutations affect hair length, but root hair lengths on double mutants are similar to that of one of the single mutant parents. Comparable pictures of the wild type and *shv3* are shown in Figure 2, and of *scn1-1* and *rhd3-1* in Figure 3.

SHV3, *TIP1*, *BST1*, *RHD3*, *CEN1*, *CEN2*, *CEN3*, and *SCN1*) are involved at the transition to tip growth, and six of these (*RHD2*, *SHV1*, *SHV2*, *BST1*, *CEN1*, and *CEN3*) are involved for the first time at this stage.

The *RHD2* gene was previously shown to be required for the transition from swelling formation to tip growth (Schiefelbein and Somerville, 1990; Wymer et al., 1997). We have identified a similar requirement for *SHV1*, *SHV2*, and *SHV3*. Epistasis (Figure 2 and Table 2) suggests that *SHV2* contributes later than *SHV1* and that *SHV3* contributes later than *RHD2*. Because the phenotypes were so similar, it was not possible to differentiate double mutants carrying mutations in the *RHD2* gene and in the *SHV1* or *SHV2* genes, so the relationship between *RHD2* and *SHV1* and *SHV2* could not be elucidated.

The requirement for *RHD2*, *SHV1*, and *SHV2* is partly overcome by mutations in a second set of genes—*TIP1*, *RHD3*, *CEN1*, and *SCN1* (Table 3 and Figure 3). In the case of *TIP1* and *SHV2*, the *tip1-2* mutation is almost completely epistatic to *shv2-1* (Table 2 and Figure 3), suggesting that a function of *SHV2* may be to negatively regulate *TIP1*. If this is the case, the phenotype of Shv2$^-$ mutants may be attributed to unregulated TIP1 activity. This model predicts that a double mutant carrying *shv2-1* and a weak loss-of-function allele of *TIP1* would have a phenotype closer to the *shv2-1* single mutant phenotype because of the greater levels of residual TIP1 activity. The *tip1-1 shv2-1* double mutant carries the weaker of the two Tip1$^-$ alleles; its partly suppressed phenotype is closer to that of *shv2-1* than is the *tip1-2 shv2-1* phenotype (Tables 1 and 3). This is consistent with our hypothesis. In other cases of partial suppression, we did not observe epistasis, suggesting that the corresponding genes are not involved in the same biochemical pathway. In those cases, we hypothesize that each partly suppressed phenotype identifies two genes with opposing effects on a process required for tip growth.

In some double mutants with class 2 additive phenotypes, we observed synergistic effects that reduced the proportion of hairs proceeding to successful tip growth to ≥ 40 μm long (Table 4). This provided further evidence that *CEN1*, *TIP1*, and *SCN1* are involved during the transition to tip growth and showed that the *BST1*, *CEN2*, and *CEN3* genes can also affect this step.

Number of Hairs Produced from Each Site of Hair Formation

Tip1⁻ and Cow1⁻ mutants have previously been shown to produce multiple hairs from a proportion of hair-forming sites (Schiefelbein et al., 1993; Grierson et al., 1997; Ryan et al., 1998). This suggests that *TIP1* and *COW1* prevent multiple hairs from forming on wild-type plants. Single and double mutant phenotypes show that *BST1*, *CEN2*, *CEN3*, and *SCN1* are also required to repress the production of multiple hairs (Tables 1 and 4). Synergistic phenotypes (Table 4, class 2 additives) also implicate *CEN1*. The proportions of multiple hairs on single and double mutants carrying the *scn1-1* mutation were especially high (Tables 1 and 4), suggesting that *SCN1* plays a particularly important role in preventing the production of multiple hairs.

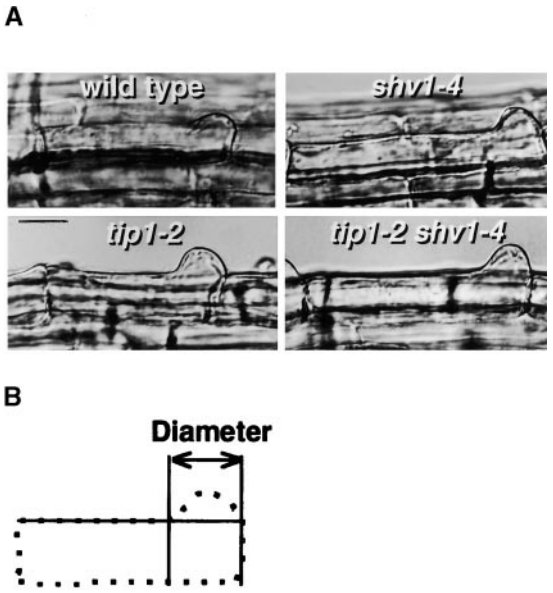

Figure 5. *tip1-2* Affects the Diameter of the Swelling Formed during Root Hair Initiation.

(A) Young root hairs of wild-type, *shv1-4*, *tip1-2*, and *tip1-2 shv1-4* plants. Most root hairs on *shv1-4* (4%, SD ± 2%) and *tip1-2 shv1-4* (9%, SD ± 3) plants did not progress beyond the stage shown (see Tables 1 and 4). For this character, *shv1-4 tip1-2* plants had the *shv1-4* phenotype (Table 1). Bar = 20 μm.

(B) Measurement of the diameters of young root hairs similar to those shown in **(A)**. The shape of a root hair cell is shown with a dotted line. A horizontal line indicates the shape of the cell that had no hair formed, identifying the limits of the swelling that had formed at the beginning of hair formation. Student's *t* tests showed that the diameters of wild-type (21.55 μm, $n = 26$) and *shv1-4* (21.82 μm, $n = 55$) hairs were not significantly different from each other but were significantly smaller than those of *tip1-2* (27.29 μm, $n = 99$) and *shv1-4 tip1-2* (24.73 μm, $n = 63$). These results show that before growth ceases (a characteristic of *shv1-4*), root hairs on *shv1-4 tip1-2* plants have an increased diameter, which is a characteristic of *tip1-2*.

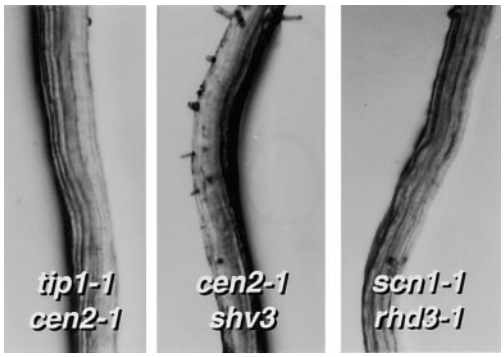

Figure 6. Synergistic Phenotypes Affecting the Beginning of Hair Formation.

The double mutants shown have a severe hairless phenotype that is not seen in the parental single mutant lines. Comparable pictures of the wild type and *shv3* are shown in Figure 2, *tip1-1* and *scn1-1* in Figure 3, and *cen2-1* in Figure 4.

The *cen2-1 cen1-2* double mutant produced no multiple hairs (Table 4), in contrast to the *cen2-1* single mutant phenotype in which ~7% of the hair-forming sites produced multiple hairs (Table 1). These results raise the intriguing possibility that either *CEN2* or *CEN1* must be present for multiple hairs to form.

Root Hair Elongation by Tip Growth

Previous studies identified roles for *RHD3*, *RHD4*, *TIP1*, and *COW1* during root hair elongation. The *cow1-1* mutation was shown to be epistatic to *rhd4*, suggesting that *RHD4* cannot contribute to tip growth in the absence of the *COW1* gene (Grierson et al., 1997). We did not find any other examples of epistasis affecting tip growth. This indicates that most of the genes in our study that are involved in tip growth contribute at least partly independently during root hair elongation. That said, class 3 additive phenotypes identified several pairs of genes that do not contribute independently to hair length (Table 4). Many double mutants with mutations in the *RHD3* gene had phenotypes in this class (Table 4 and Figure 4), suggesting that *RHD3* contributes to tip growth by a process that involves several other genes, including *BST1*, *CEN1*, *CEN2*, *CEN3*, and *TIP1*. Similar results suggest that the contribution of *CEN2* to hair length is dependent on *CEN1* and *BST1* (Table 4).

When tip growth took place in double mutants homozygous for mutations in the *COW1* gene, the phenotypes were often class 1 additive. These results suggest that *COW1* acts independently of most other genes. We found no evidence that *COW1* contributes to hair formation at any stage other than to control the shape and number of hairs formed during tip growth.

Broader Features of the Network That Underpins Root Hair Morphogenesis

Mutations in the *TIP1*, *CEN1*, *CEN2*, *CEN3*, and *BST1* genes formed double mutants in many categories, with phenotypes affecting a range of stages of hair formation (Figures 3 to 6 and Tables 1, 3, and 4). This suggests that these genes directly or indirectly affect multiple processes throughout root hair development.

The synergistic phenotypes of class 2 additive double mutants identify either functional redundancy between the corresponding genes or incomplete loss-of-function mutations in components of a linear pathway. We are not able to distinguish between these interpretations without more genetic or molecular information about the mutations involved. However, combinations of mutant alleles of *CEN3* with *CEN1*, *CEN2* with *CEN3*, and *TIP1* with *BST1* all have strong phenotypes (Table 4) that suggest functional relationships and that merit further analysis.

Mutations in the *SCN1* gene had particularly strong effects. *rhd6* was epistatic to *scn1-1*, preventing root hair formation (Table 2), but all of the other double mutants that included *scn1-1* had partly suppressed, class 2 additive, or synergistic phenotypes (Tables 3 and 4, and Figure 6). Apparently *SCN1* either takes part in direct interactions or has overlapping functions with many other genes, including *RHD3*, *BST1*, *CEN1*, *CEN2*, *CEN3*, *COW1*, *SHV3*, and *TIP1*.

Conclusions

Figure 7 summarizes contributions of 15 genes to root hair growth. Hair formation requires *RHD6*. *TIP1*, *CEN2*, *RHD3*, *SCN1*, and *SHV3* are active at the beginning of hair formation. In the absence of tip growth, *TIP1* acts to restrict the size of the swelling that forms at the beginning of hair growth, possibly by a mechanism that requires *RHD1*. The transition to tip growth requires *RHD2*, *SHV1*, *SHV2*, and *SHV3*. The effects of *RHD2*, *SHV1*, and *SHV2* oppose those of genes from the group *TIP1*, *RHD3*, *CEN1*, and *SCN1* on the same processes of tip growth. We hypothesize that *SHV2* negatively regulates *TIP1* during tip growth. *BST1*, *CEN2*, and *CEN3* are also required for tip growth. Mechanisms that restrict the number of root hairs produced from each site of hair formation require *TIP1*, *COW1*, *BST1*, *CEN2*, *CEN3*, and *SCN1*. *CEN1*, *CEN2*, *CEN3*, *RHD3*, *SCN1*, *TIP1*, *COW1*, *BST1*, *SHV2*, and *SHV3* are all required to control hair shape. *RHD3* contributes to tip growth by a process that involves other genes, including *BST1*, *CEN1*, *CEN2*, *CEN3*, and *TIP1*. The contribution of *CEN2* to hair length is dependent on *CEN1* and *BST1*. *COW1* acts largely independently during elongation and does not contribute to any other stage of root hair development. In contrast, *SCN1* and *TIP1* interact with many other genes and affect many stages of morphogenesis.

We will test our specific hypotheses about the functions of genes as soon as molecular tools become available. The genetic framework that we have provided sets the scene and provides genetic resources for future studies of root hairs, a cell type that is very well suited to research into cell biology and cell physiology and that plays critical roles affecting the human food chain.

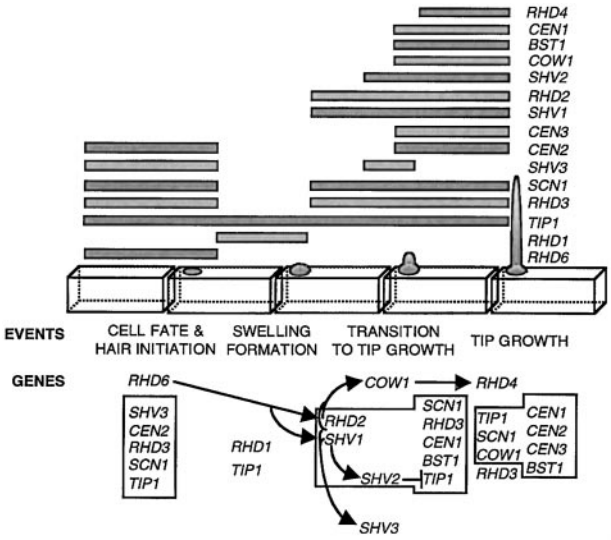

Figure 7. Genetic Contributions to Root Hair Development.

This diagram combines the information obtained from this study with that from previous publications (Schiefelbein and Somerville, 1990; Schiefelbein et al., 1993; Masucci and Schiefelbein, 1994; Grierson et al., 1997; Ryan et al., 1998). Wild-type development is summarized by a line drawing. Horizontal bars represent stages at which each gene is involved. The sequence of gene action is indicated below. A mutation in a gene immediately before an arrow is epistatic to a mutation in the gene immediately after an arrow (complete epistasis results are listed in Table 2). A blunted line indicates hypothetical negative regulation of *TIP1* by *SHV2* during the transition to tip growth. Boxed areas indicate double mutant results that identify functional relationships between genes during a particular stage of morphogenesis.

METHODS

Arabidopsis Lines

The isolation of *tip1-2* (Ryan et al., 1998) and *cow1-1* and *cow1-2* (Grierson et al., 1997) has been described previously. *bst1* was isolated from a fast-neutron-mutagenized population obtained from Lehle Seeds (Round Rock, TX). To isolate other mutants, we used the chemical mutagen ethyl methanesulfonate to induce heritable changes in the genome of 6000 *Arabidopsis thaliana* ecotype Columbia seeds (treated in a 0.5% solution for 12 hr). Approximately 75,000 M_2 seedlings were screened for root hair abnormalities. Heritable mutants were crossed to the wild type and self-fertilized to demonstrate that we had isolated 29 new recessive, single-locus, nuclear muta-

tions. Lines with heritable root hair phenotypes were grouped according to phenotype and intercrossed with other members of the same group to test for complementation. Representatives of each complementation group were then intercrossed with each other to confirm the final number of complementation groups. Additional alleles of two monoallelic loci (*scn1-2* and *shv2-2*) were identified by crossing to unpublished lines kindly provided by Katharina Schneider and John Schiefelbein. The 29 lines isolated in this study were *shv3*; *cen3-1*, *cen3-2*, *cen3-3*; *shv1-1*, *shv1-2*, *shv1-3*, *shv1-4*, *shv1-5*; *shv2-1*, *shv2-2*; *bst1*; *cen2-1*, *cen2-2*; *scn1-1*, *scn1-2*; *cen1-1*, *cen1-2*, *cen1-3*, *cen1-4*; *cow1-3*, *cow1-4*, *cow1-5*; *rhd2-2*, *rhd2-3*, *rhd2-4*; and *rhd3-7*, *rhd3-8*, *rhd3-9*. *cen1* alleles have a trichome phenotype and map near to *WAVY/SINGED*, but no test for allelism has been conducted. In a side-by-side comparison, the phenotypes of our mutants were noticeably different from those of *axr1-12*, *axr3*, *axr4-2*, *etr1*, and *aux1-7*.

Mutant lines generated after two rounds of backcrossing to the wild type were used for phenotypic characterization. Root hair phenotypes of >1000 5-day-old seedlings of each line were assessed and photographed by using bright-field and dark-field microscopy with a Leica MZ6 stereozoom microscope. Root hairs on photographs were counted and measured to generate quantitative data. The proportion of hairs ⩾40 μm long was counted to indicate whether hairs could develop beyond the transition to tip growth, which takes place when hairs are 20 to 40 μm long (Dolan et al., 1994). Hairs ⩾40 μm long were presumed to be tip growing, and their length at maturity was measured to assess their ability to elongate. The lengths of five mature hairs on each of five wild-type roots grown under the same conditions as the mutants were also measured, and the results were used to calculate the mutants' percentage of the wild-type length. Several of the new mutants produced multiple hairs at a proportion of hair-forming sites. We counted the proportion of hair-forming sites producing multiple hairs to allow us to compare the strengths of these phenotypes. The Landsberg *erecta* ecotype was obtained from the Nottingham Arabidopsis Stock Centre. Seeds of *rhd1-1*, *rhd2-1*, *rhd3-1*, *tip1-1*, and *rhd6* were the kind gift of John Schiefelbein. Mutants represented 10 complementation groups, two of which (*RHD2* and *RHD3*) have been described before (Schiefelbein and Somerville, 1990). A mutant that is the only known representative of its complementation group is referred to without an allele extension (e.g., *rhd6*, not *rhd6-1*).

We recorded the number of days to bolting, the number of rosette leaves at bolting, trichome shape and density, the shape and area of the largest leaf, the number and length of main and cauline shoots and branches, the number and length of fruiting and nonfruiting stems, the distance between siliques, and silique length. Replicate observations were made of six homozygous mutant plants from each line after two rounds of backcrossing to the wild type. Because mutations at one locus, *CEN1*, strongly affected the shape of trichomes, the possibility that this locus is allelic to the *WAVY* locus (Marks and Esch, 1992) should be investigated. Mutations at *SHV1* and *CEN2* reduced the apparent hairiness of leaves by approximately half, although the shape of the trichomes that were present appeared to be normal.

Growth Conditions

Seedlings shown in Figure 1A were grown on Murashige and Skoog medium (Flow Laboratories, Irvine, Scotland, UK) containing 3% sucrose and 1% agarose (unless otherwise stated); those shown in Figures 2 to 4 and 6 were grown on another medium (Wymer et al., 1997) containing 1% sucrose and 1% agarose. Seeds were germinated with the plates in a vertical orientation at 22°C with constant illumination. For self-fertilization and crossing experiments, plants were transferred at the eight-leaf stage to compost supplemented with slow-release fertilizer (Osmocote; Sierra UK, Nottingham, UK) and kept well watered; the seedpods were bagged before seeds were shed.

Genetic Mapping

Mutants of the Columbia cultivar of Arabidopsis were crossed with wild-type plants of the Landsberg cultivar. DNA of mutant seedlings from the second (F_2) generation was analyzed using cleaved amplified polymorphic sequences (CAPS) and microsatellite markers to determine the location of each mutated gene. CAPS primers (Research Genetics, Huntsville, AL) were used as described (Konieczny and Ausubel, 1993). The results were analyzed using Mapmaker 3.0 (Lander et al., 1987; Lincoln et al., 1992) and the Kosambi function. Loci were linked to markers as follows: *shv1-1*, 1.9 centimorgans (cM) from GAPC; *shv2-1*, between LFY3 (13.3 cM) and nga139 (35.2 cM); *shv3*, between AG (15.6 cM) and DHS1 (21.0 cM); *scn1-1*, 6.8 cM from GAPC; *cen1-1*, no recombinants with NCC1 in 26 plants; *cen2-1*, between nga151 (12.8 cM) and nga139 (10.9 cM); *cen3-2*, between GAPC (5.5 cM) and GL1 (31.2 cM); and *bst1*, 24 cM south of DFR.

Isolation and Characterization of Double Mutants

Mutant plants were intercrossed, and F_2 progeny that displayed one of the mutant phenotypes were self-fertilized to produce multiple F_3 families that segregated for the double mutant phenotype. Confirmation of the identities of *cen3-2 cow1-1*, *scn1-1 cen1-1*, *cen2 scn1-1*, *rhd3 scn1-1*, *scn1-1 bst1*, *bst1 cen2*, *cen1-1 bst1*, *rhd3 cen3-2*, *scn1-1 shv1-1*, *shv2-1 tip1-2*, and *tip1-1 cen3-2* was obtained by backcrossing to single mutants and by crossing to the wild type and examining the F_2 progeny for both parental single mutant phenotypes. The latter test was used to confirm the identities of *cen3-2 scn1-1* and *tip1-1 scn1-1*. Null alleles are ideal for determining epistasis. We used the strongest loss-of-function alleles available to us for most of the double mutants described here. While this work was ongoing, Wang et al. (1997) published the sequence of *rhd3-1*, revealing that although it is the strongest loss-of-function allele of *RHD3*, it is not null.

Root hair phenotypes of 50 to 500 5-day-old seedlings of each double mutant were assessed and photographed by using bright-field and dark-field microscopy with a Leica MZ6 stereozoom microscope. Root hairs on photographs were counted and measured to generate quantitative data.

The diameters of initiating root hairs of wild-type, *tip1-2*, *shv1-4*, and *tip1-2 shv1-4* double mutant plants were measured using differential interference photomicroscopy of roots grown on glass slides, as previously described (Grierson et al., 1997).

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