Development 124, 1789-1798 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV3632

Structural and genetic analysis of epidermal cell differentiation in *Arabidopsis* primary roots

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SUMMARY

In a screen designed to identify genes in the specification of epidermal cell fate in *Arabidopsis* primary roots we have isolated 8 new mutants that fall into 6 complementation groups corresponding to the 'root hairless' genes *RHL1*, *RHL2* and *RHL3* and the 'ectopic root hair' genes *ERH1*, *ERH2* and *ERH3*. The *erh2* mutant is allelic to *pom1*, a conditional root expansion mutant, and reveals a possible link between epidermal root hair initiation and radial cell expansion. Apart from *erh1* the mutants also show defects in shoot development, indicating a complex role for the affected genes. Mutant phenotypes in the patterning and shape of leaf trichomes in *rhl1*, *rhl2*, *rhl3* and *erh3* were particularly obvious. The root hairless mutants are only partly responsive to increased ethylene concentrations, while the ectopic root hair mutants are fully responsive to reduced concentrations of ethylene, a permissive regulator of root hair initiation. This result and the analysis of double mutants suggest a complex pathway leading to root hair initiation that requires the *RHL* and *ERH* genes for correct differentiation.

Key words: root development, root epidermis, root hair initiation, trichoblast, atrichoblast, cell fate specification, *Arabidopsis*, *ERH* and *RHL* genes

INTRODUCTION

The *Arabidopsis* primary root has a well defined and predictable structure (Dolan et al., 1993, 1994). Cells derived from rings of root meristem initials divide transversely and grow in organized files along the root axis. When they stop dividing, they continue to elongate and when they finish elongating, they differentiate. The number of cell files in each tissue layer is relatively constant. For example, there are 8 endodermal cell files surrounding the stele tissue. Outside the endodermal cells there are 8 cortical cell files surrounded in turn by about 19 epidermal cell files. The latter are divided into two types. Trichoblasts are those cells that will go on to form hairs, and these overlie the clefts between the underlying 8 cortical cell files. In between are atrichoblasts, or cells that will go on to form non-hair cells and these only contact the outer wall of one cortical cell file.

Trichoblasts can already be distinguished from atrichoblasts in the meristematic zone of wild-type roots by their denser cytoplasm. This simple and predictable pattern of cells provides a good model system to address genetically the way in which cellular pattern is established and the nature of the signalling pathways involved in cell fate specification and cell differentiation.

To understand the pathway of root epidermal cell differentiation at the genetic and molecular level a number of mutants isolated in other screens have already proved useful. Ectopic root hairs develop on the roots of the *ttg* mutant (Galway et al., 1994) that, in addition, lacks trichomes, anthocyanins and seed coat mucilage (Koorneef, 1981). However, *Arabidopsis* roots overexpressing the maize R (R-Lc) gene product, a putative *TTG* homologue (Lloyd et al., 1992), have no root hairs. Since non-hair cells are found in hair cell positions when *TTG* is constitutively expressed, this supports the idea that *TTG* is a positive regulator of non-hair cell fate.

Ectopic root hairs have also been found on the *gl2* mutant (Masucci et al., 1996). Initially this mutant was identified by its abnormally expanded trichomes and its lack of proper seed coat mucilage (Koorneef, 1981). Expression of the *GL2* gene is largely confined to the atrichoblasts in the wild-type root epidermis (Masucci et al., 1996), and the affected gene product, a homeobox containing protein (Rerie et al., 1994), is probably involved in the positive regulation of non-hair cell fate. In *ttg* mutants *GL2* expression is greatly reduced suggesting that in the wild type *TTG* positively regulates *GL2* (Di Cristina et al., 1996).

Another recessive mutant that displays ectopic root hairs is *ctr1*. The wild-type CTR1 protein was presented by Kieber et al. (1993) as a negative regulator of the ethylene response pathway that showed homology to members of the Raf family of protein kinases. After investigating *ctr1* roots Dolan et al. (1994) suggested that ethylene is a positive regulator of root hair formation and that the CTR1 protein acts as a negative regulator of the pathway. In subsequent studies Tanimoto et al. (1995) showed that indeed the exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) induced ectopic hairs, mimicking the *ctr1* phenotype, whereas root hairs disappeared upon treatment with inhibitors of either ethylene synthesis or its perception.

The first mutant specifically isolated for its defect in root hair initiation was *rhd6* (Masucci and Schiefelbein, 1994). The recessive *rhd6* mutant displays only 15-17% of the wild-type number of root hairs. However, the wild-type phenotype can be rescued by exogenous application of an ethylene precursor or auxin. In terms of reduced root hair formation *rhd6* shows phenotypic similarities to the auxin-, ethylene- and abscisic acid-resistant mutant *axr2* which, however, is dominant (Wilson et al., 1990). All the growth factor-related mutants *ctr1*, *rhd6* and *axr2* show the early structural characteristics of wild-type trichoblast development indicating that their defects are related to later stages in root hair initiation (Masucci and Schiefelbein, 1996). Moreover ectopic root hairs induced by the ethylene precursor ACC appear independently of early cell differentiation events in the meristematic zone, showing that ethylene is still effective very late in root epidermal development. A preliminary model suggests that *TTG* and *GL2* act in a position-dependent manner upstream of the ethylene-mediated processes (Masucci and Schiefelbein, 1996).

Despite recent progress many more genes remain to be identified, and since few of the known genes have been identified from a root specific screen, we conducted a visual screen of 1500 M_2 families to identify mutants that completely lack root hairs or that have root hairs in ectopic positions. We isolated 5 root hairless mutants specifying 3 genes and 3 ectopic root hair mutants specifying a further 3 genes. As most of these new mutants display a pleiotropic phenotype the affected genes are assumed to play a complex regulatory role in plant development. In particular, correlations to leaf trichome development and radial cell expansion in the root have been found. The wild-type gene products identified through the recessive root hairless mutants are assumed to be positive regulators in the pathway leading to root epidermal cell differentiation, whereas the recessive ectopic root hair mutants are assumed to represent negative regulators of root hair initiation or alternatively positive regulators of non-hair cell fate. The partial complementation of the root hairless mutants with increased levels of the ethylene precursor ACC suggests that at a later developmental stage high ACC levels can overrule the block imposed on root hair initiation by the *rhl* mutations. However, wild-type hair-cell development appears to require functional *RHL* gene products. This idea is further explored by analysing double mutant plants homozygous for both a root hairless and an ectopic root hair mutation. The results are summarized in a preliminary model for root epidermal cell differentiation.

MATERIALS AND METHODS

Plant material and growth conditions

The *rhl1* mutant (ws background) was isolated from T-DNA transformed lines (Feldmann, 1991). *rhl3-2* and *erh1* (Landsberg *erecta* background) derive from seeds mutagenized with fast neutrons (Lehle seeds) and were isolated from a screen of 500 independent families. *rhl2-1*, *rhl2-2*, *rhl3-1*, *erh2* and *erh3* were isolated by screening the progeny of 1000 'Columbia' (Col) seeds treated with 0.5% ethyl methanesulfonate (EMS). As all homozygous *rhl* mutants are lethal the mutated genes have to be maintained in segregating populations. All *erh* mutants are viable in the homozygous state. The *ctr1* allele HE14 was a gift from P. Benfey's lab, *gl2* seeds were provided by J. Schiefelbein, the *pom1-1* allele was made available by M.-T. Hauser and the *ttg* mutant and the Landsberg *erecta* (L*er*) wild type were obtained from the Ohio seed stock centre. Plates for seedling growth including ACC and 2-aminoethoxyvinyl-glycine (AVG) treatments were prepared essentially as described by Tanimoto et al. (1995).

Genetic analysis

Each mutant was outcrossed at least once to its respective wild-type ecotype. A segregation of nearly three wild types to one mutant in the F_2 generation indicated in all cases that single genes were affected and that the mutants were recessive. Pairwise crosses between mutant lines were performed to determine complementation groups. For the construction of double mutants between root hairless and ectopic root hair mutants, *erh*-like F_2 plants (homozygous for the mutation causing ectopic root hairs) were allowed to self. Two out of three were expected to be heterozygous for the *rhl* mutation as well. Therefore F_3 families were scored for segregating populations. Among these, approximately 25% of the plants were double mutants.

Mapping

The chromosomal location of the mutant genes was determined by calculating recombination frequencies of SSLP- (Bell and Ecker, 1994) and CAPS-markers (Konieczny and Ausubel, 1993). Each mutant was crossed to a different wild-type ecotype: *rhl1*, *rhl2-1*, *erh2* and *erh3* were crossed to Landsberg *erecta*; *rhl3-2* and *erh1* were crossed to 'Columbia'. Mutant F_2 plants were used for the analysis. In cases where the homozygous mutant is lethal, heterozygous siblings were used, whose progeny segregate for the mutant phenotype in the F_3 generation. Each marker was tested on approximately 30 homozygous or 60 heterozygous plants to determine linkage of the mutant genotype with the marker pattern. DNA was extracted from single seedlings or a few leaves according to a slightly modified Dellaporta protocol (Dellaporta et al., 1983). PCR conditions were essentially as described by Konieczny and Ausubel (1993) and Bell and Ecker (1994). DNA fragments were separated in 1.5% agarose gels or 5% polyacrylamide gels (Hauser et al., 1995).

Cryo-scanning electron microscopy

3-day old roots, young leaves and flower bud tissue were prepared for cryo-scanning electron microscopy essentially as described by Dolan et al. (1994).

Tissue fixation and embedding

3-day old seedlings were vacuum infiltrated with fixative consisting of 2.5% glutaraldehyde in 50 mM sodium cacodylate, pH 7.2. After 1 hour prefixing the seedlings were arranged in groups of 4 or 5 on a thin layer of 1% agarose (low gelling point; Sigma) and covered with the residual agarose at 60°C. The specimens were allowed to cool on ice and were refixed overnight. The infiltration with Historesin (Leica) was performed according to the manufacturer's instructions. To quantify ectopic root hairs thick transverse serial sections (10 μm) were cut with a Leica Autocut 2055 microtome using a 45° glass knife. Thin sections (2 μm) were examined for structural analysis of the root meristematic region. For photomicroscopy, sections were stained with toluidine blue.

RESULTS

Mutant isolation, root phenotype and genetic characterization of the new root hairless and ectopic root hair mutants

Primary roots of three differently mutagenized M_2 populations were scored 3 days after germination for a complete absence of root hairs or a 'bushy' appearance, putatively caused by ectopic root hairs. The heritability of putative mutants was examined and they were then outcrossed to their respective wild-type ecotype and crossed to each other to establish complementation groups. All putative ectopic root hair mutants

were crossed to *ttg*, *ctr1* and *gl2*, the three existing mutants known to possess root hairs in ectopic positions. Interestingly the screen identified a further allele of both *ttg* and *ctr1* which are not referred to here in any detail. Segregation data indicate that the new mutants presented here are caused by mutations in single recessive genes.

The wild-type phenotype and the mutant phenotypes are displayed in Fig. 1. On 3-day old wild-type seedlings root hairs start to emerge from above the elongation zone (Fig. 1A). They appear to be evenly distributed along the primary root with increasing length at later developmental stages. In addition there is an increased concentration of root hairs at the transition zone between root and hypocotyl, the collet region. In transverse sections through the meristematic zone the wild-type trichoblasts, situated over underlying clefts between cortical cells, are characterized by dense cytoplasm and a low degree of vacuolization compared to the atrichoblasts, with very few exceptions (Fig. 1B). In the wild type, only trichoblasts give rise to root hairs in the hair formation zone (Table 1; Fig. 3A). A few exceptions to this pattern were observed in the Landsberg *erecta* ecotype.

rhl1 (Fig. 1C,D) was isolated from a T-DNA mutagenized population (Feldmann, 1991) and we have shown that the mutation is caused by a T-DNA insertion into the gene (K. Schneider, unpublished results). This mutant has few hairs on its primary root and it is noticeable that the collet region is also bald. However, occasionally a few root hairs surround the emergence of lateral roots (data not shown). The primary roots of the *rhl1* mutant are shorter than wild-type roots and exhibit abnormal gravitropic responses. The number of cells per tissue layer is the same as the wild type. The density of the cytoplasm and the degree of vacuolization is similar in all epidermal cells of thin sections through the meristematic zone, indicating that the morphological distinction between putative trichoblasts and atrichoblasts is much less pronounced than in the wild type. Thus in *rhl1* seedlings root epidermal development is already impaired at the onset of early cell differentiation.

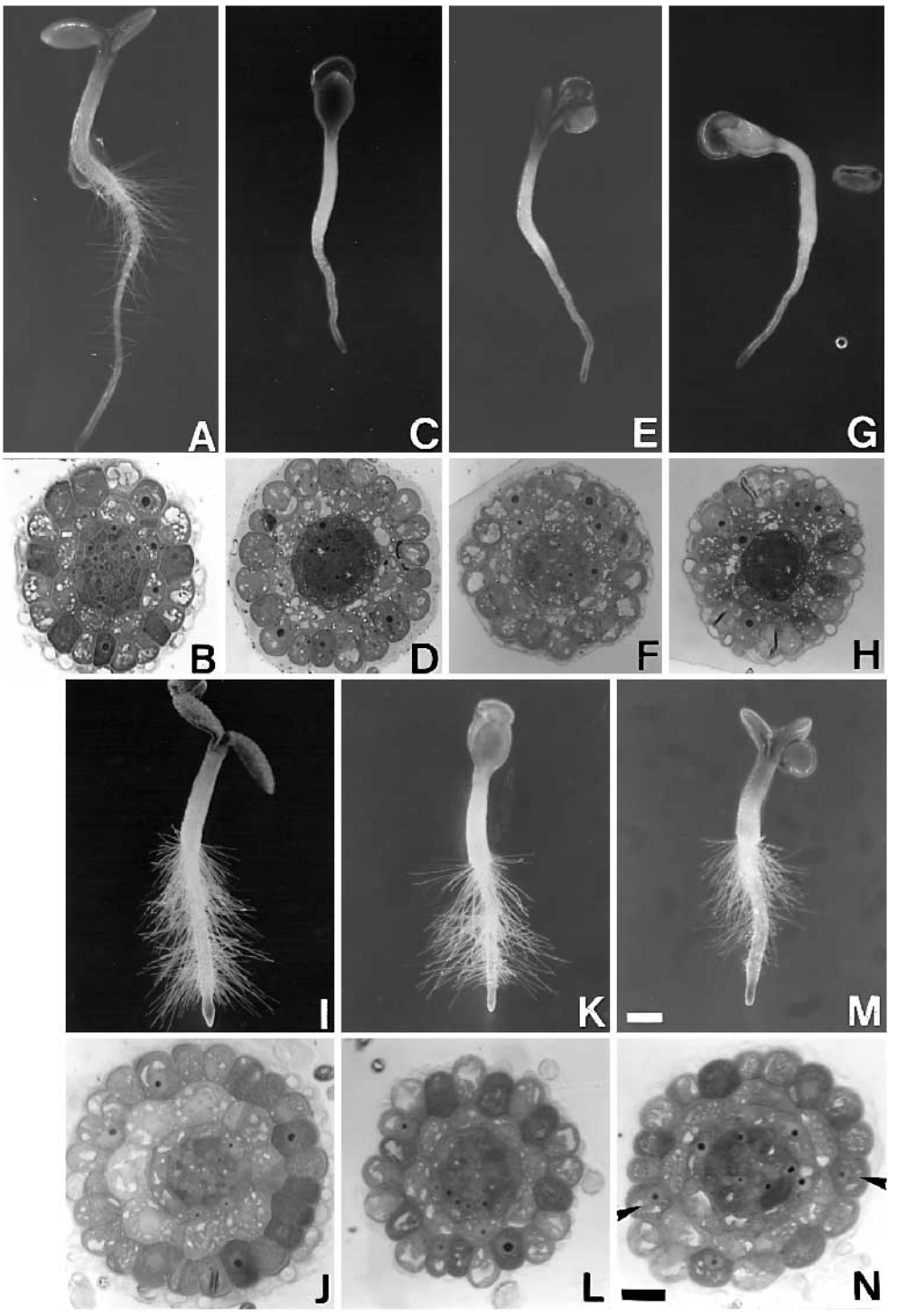

Fig. 1. Phenotypes of 3-day old seedlings and transverse sections through their primary roots in the meristematic zone. (A,B) Wild type, (C,D) *rhl1*, (E,F) *rhl2-2*, (G,H) *rhl3-1*, (I,J) *erh1*, (K,L) *erh2* and (M,N) *erh3*. The white scale bar in M equals 500 μm and refers to the magnification of all seedlings in this figure. The black scale bar in N equals 20 μm and represents the magnification of all transverse sections in this figure. Arrowheads in N indicate *erh3* cells in trichoblast positions that do not stain as wild-type trichoblasts.

Table 1. Quantification of root hairs in wildtype and mutant backgrounds

Line	Total length sectioned* (mm) [no. of roots]	Root hair density† (hair/mm)	% ectopic root hairs†‡
Col	4.2[5]	50.2±7.8	0
Ler	6.5[6]	48.2±8.9	1.2±1.8
erh1	4.1[3]	200.0±16.3	56.0±1.6
erh2/pom1	5.8[6]	95.0±15	35.3±7.9
erh3	5.8[9]	73.9±19.7	32.2±5.9

*10 μm thick serial sections were analysed.
†Values represent mean±standard deviation.
‡Ectopic root hairs are defined as root epidermal outgrowths emerging from cells facing only one cortical cell wall.

All other root hairless mutants: the EMS mutants *rhl2-1*, *rhl2-2*, *rhl3-1* and the fast neutron mutant *rhl3-2* display the same phenotype with *rhl3-2* having a maximum of around 10 root hairs per primary root (Fig.1E,G). As there was no detectable difference between the phenotypes of *rhl2-1* and *rhl2-2* either of them was used for subsequent studies. Of the two *rhl3* alleles *rhl3-2* was chosen for subsequent analysis. The outer epidermal cell walls of all *rhl*-mutants are perfectly smooth and show no sign of root hair initiation or bulge formation at the distal end of the epidermal cell (Fig. 2).

erh1 (Fig. 1I) was identified among the fast neutron treated mutants. *erh1* roots are shorter than wild-type roots and their appearance is very bushy. Serial sectioning along 3-day old seedling roots showed that 56% of hairs are in an ectopic position (Table 1). A typical transverse section through the root hair zone of *erh1* is shown in Fig. 3B. With 200 root hairs per mm *erh1* has also the highest root hair density of all the mutants (Table 1). The general root architecture in *erh1* appears to be the same as in the wild type, with 8 endodermal and 8 cortical cell files, although all cells seem to be radially expanded generating a root diameter that is 50% larger than in the wild type (Fig. 3A,B). In the meristematic zone some cells overlying the cleft between two cortical cells do not contain dense cytoplasm, on the other hand this feature is displayed by *erh1* cells in non-hair positions indicating developmental aberrations in *erh1* at very early stages (Fig. 1J). *erh2* is an EMS mutant with 35% of its root hairs in ectopic positions (Figs 1K, 3C; Table 1). In the meristematic zone, *erh2* roots show the wild-type pattern with 8 endodermal and 8 cortical cell files and 8 densely cytoplasmic trichoblast files in the correct positions (Fig. 1L). Radial cell expansion is already visible, but becomes more apparent in the hair zone (Fig. 3C). *erh2* cortical cells have a radial dimension that is 2.5-times that of the wild type. This increased radial cell expansion, and the map position on top of chromosome I (Fig. 4), eventually suggested allelism to *pom1* and reciprocal crosses proved that indeed *erh2* is an allele of *pom1*. According to accepted procedures of *Arabidopsis* nomenclature *erh2* was renamed and should be referred to as *pom1-12* in future. *erh3* (Fig. 1M), derived from the EMS mutant screen, has 32% of its root hairs in an ectopic position and the lowest ectopic and total root hair density of the *erh* mutants (Fig. 3D; Table 1). *erh3* frequently diverges from the constant wild-type primary root anatomy. In a sample of 12 individual roots, 3 specimens had only 7 cortical cell files (compare Fig. 3D), 5 roots possessed 9 cortical cell files and only 4 specimens displayed the wild-type pattern of 8 cortical cell files. Cytoplasmic density and the degree of vacuolization do not strictly correlate with the position of the epidermal cell over one or two cortical cells (Fig. 1N) indicating that the *erh3* mutation causes defects at early stages of root epidermal cell differentiation. All cell layers of *erh3* roots, and in particular the endodermis, appear to be radially expanded from the elongation zone onwards, leading to a root diameter nearly twice that of the wild type in the root hair zone (Fig. 3A,D).

All mutants have been mapped using a set of molecular markers (see Materials and Methods). Recombination fre-

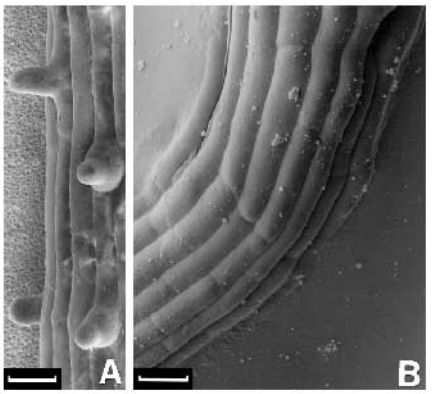

Fig. 2. Scanning electron micrographs of 3-day old primary roots of (A) wild type and (B) *rhl3-2*. Scale bar equals 30 μm.

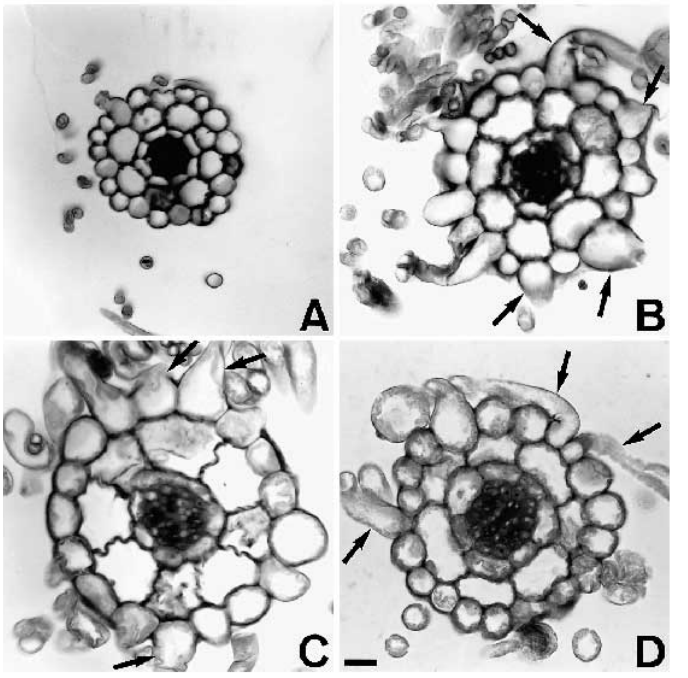

Fig. 3. Transverse sections through root hair zone of primary *Arabidopsis* roots. (A) Wild type, (B) *erh1*, (C) *erh2* and (D) *erh3*. Ectopic root hairs, i.e. outgrowths of epidermal cells facing only one cortical cell, are indicated with arrows. The ectopic localization of the root hairs was confirmed by analyzing subsequent sections. Scale bar in D equals 25 μm; all pictures were taken at the same magnification.

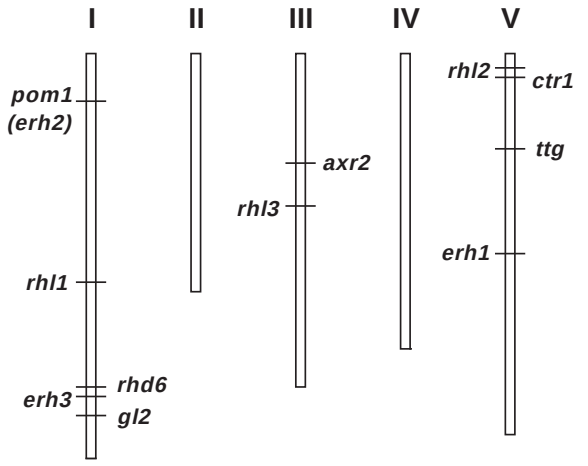

Fig. 4. Approximate chromosomal map positions of loci involved in *Arabidopsis* root hair initiation.

quencies were as follows: for *rhl1* 11.8% with GAPB (CAPS) and 25% with Athgenea (SSLP); for *rhl2* 5% with Athctr1 (SSLP) and 24% with nga225 (SSLP); for *rhl3* 5% with GL1 (CAPS) and 21% with AthCHIB (SSLP); for *erh1* 2.3% with nga76 (SSLP) and 10% with nga129 (SSLP); for *erh2* 5.9% with PVV4 (CAPS) and 7.6% with nga59 (SSLP) and for *erh3* 3% with ADH (CAPS) and 11% with nga111 (SSLP). The map positions of the affected genes, together with 5 other known root hair initiation genes are indicated in Fig. 4.

Other aspects of the mutant phenotype

The mutants were grown to maturity after the initial screen on the third day after germination. At subsequent developmental stages we observed further deviations from the wild-type phenotype (in seven out of eight mutants) that always cosegregated with the root phenotype.

The shoots of the five different *rhl* mutants were hardly distinguishable, and therefore we describe *rhl1* as the representative of the group. 3-week old *rhl1* shoots only consist of leaf rosettes as small as 1 cm in diameter which corresponds to less than half of the wild-type size (Fig. 5A). *rhl1* plants never produce flowers, show necrotic symptoms after 4 weeks and die.

In the wild-type leaf epidermis a trichome apparatus consists of 8 socket cells and the trichome, a large twice-branched single cell (Fig. 6A, Hülskamp et al., 1994). The outer trichome wall is covered with small globular deposits. In the mature wild-type leaf one trichome apparatus is separated from the next by at least 4 epidermal cells.

rhl1 trichomes are mostly unbranched cellular outgrowths near the leaf margins and do not possess any globular deposits on their surface (Fig. 6B). They are still supported by 8 socket cells, but they appear to be less organized. In sharp contrast to wild-type leaves the socket cells of different *rhl1* trichomes often occur directly adjacent to each other. *erh2/pom1-12* shoots are comparable to wild type, but their growth is reduced by about 50% as described by Hauser et al. (1995) for other *pom1* alleles, and finally *erh1* had no obvious shoot phenotype.

erh3 shows the most complex phenotype. The development of its very erect shoot is delayed in comparison with the wild type, but mature *erh3* plants are the same size as wild-type plants (Fig. 5B,C). Interestingly the *erh3* leaf epidermis also shows defects. Most obviously the trichomes are unbranched or only branch once (Fig. 6C). The initial *erh3* trichome outgrowth is wider in diameter than wild-type trichomes and it has the same surface deposits as the wild type. Occasionally the socket cells of different *erh3* trichomes directly abut each other (Fig. 6C). Occasional paired stomata were also seen in contrast to wild-type leaves where they are always separated by other epidermal cells (data not shown).

erh3 is the only mutant that displays a distinctive flower phenotype. Wildtype flower buds have an oval shape and the sepals cover the organs of the interior whorls until anther maturity (Fig. 6D). In *erh3* plants the pistil that is characterized by its short and fat shape, already protrudes through the anther, petal and sepal whorls at a very early bud stage (Fig. 6E). Occasionally we observed five sepals and five petals on *erh3* flowers instead of the four organs per whorl found in the wild type (data not shown). *erh3* siliques are only half of the wild-type length (Fig. 5C). *erh3* seeds are larger than wild-type seeds, display a darker pigmentation and their shape is round as opposed to the oval shape of wild-type seeds (data not shown). However, no reduction in fertility was observed.

Ethylene responses

Tanimoto et al. (1995) showed that ethylene stimulates ectopic root hair initiation in a concentration-dependent manner. In the

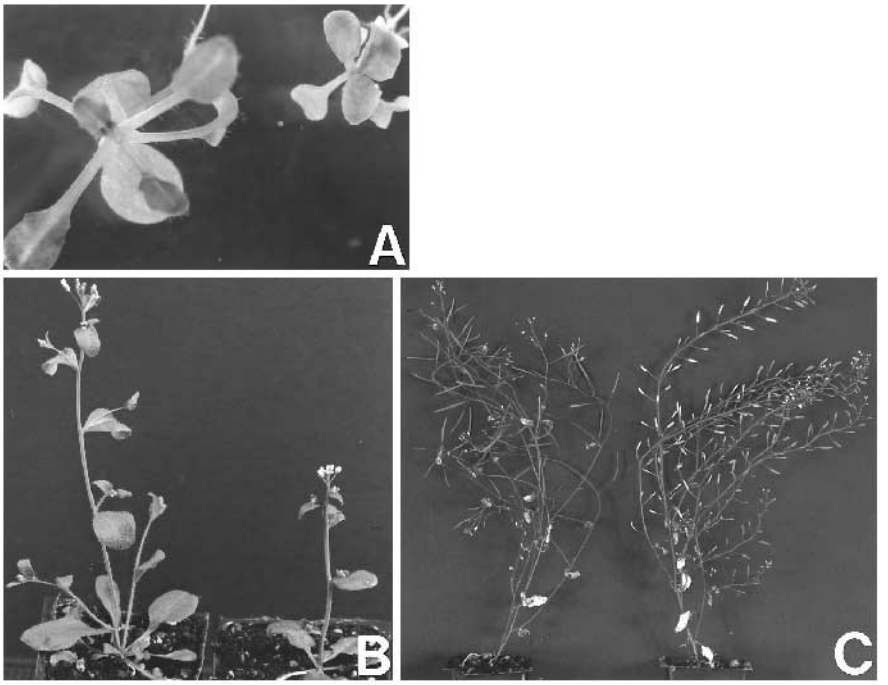

Fig. 5. Shoot phenotypes of (A) 2-week old Col wild-type and *rhl1* mutant seedlings, (B) 5-week old Col and *erh3* mutant plantlets and (C) 9-week old Col and *erh3* mutant plants.

present study we investigated the effects of the ethylene precursor ACC on the root hairless mutants and the response of the ectopic root hair mutants to the ethylene biosynthesis inhibitor AVG. The data represent the results of two independent series of experiments.

The results of the ACC treatment on the wild type and the root hairless mutants is summarized in Fig. 7. Adding 1 μM ACC to the medium has little effect on the wildtype, but upon treatment with 10 or even 100 μM ACC the hypocotyl swells and root elongation is inhibited. The density of root hairs appears to be greatly increased.

The root hairless mutants display only parts of the complex response observed in the wildtype. Again little effect is seen upon treatment with 1 μM ACC, whereas higher concentrations promote a slight swelling of the hypocotyl and inhibition of root elongation in *rhl1*, *rhl2* and *rhl3*. There are more root hairs than in the untreated mutants, but the initiation is still very limited. Even with 100 μM ACC, *rhl1* and *rhl2-2* only produce a maximum of 5-6 root hairs that emerge from below the bald collet region (Fig. 7H,L). *rhl3-2*, which is already characterized by a slightly higher root hair density in the untreated state, produces correspondingly more root hairs although the collet region still appears bald (Fig. 7M-P).

The phenotypes of AVG-treated wildtype and ectopic root hair mutant seedlings are presented in Fig. 8. In the wild-type root treated with 5 μM AVG root hair initiation below the collet region is inhibited while with 20 μM AVG very few root hairs appear below the collet region.

AVG also inhibits root hair initiation on *erh1* seedlings (Fig. 8D-F). Although *erh2/pom1-12* and *erh3* are both impaired in root hair initiation in response to AVG (Fig. 8G-L), the treatment also appears to cause extreme radial expansion in the root epidermis leading to characteristic bulging in epidermal cells of both mutants.

Double mutants

Double mutants between root hairless and ectopic root hair mutants were generated to determine the interactions between genes involved in root hair initiation.

All aspects of the phenotype of the double mutants between *rhl1* and *erh2/pom1-12* were identical to the *rhl1* single mutant, and therefore *rhl1* is formally epistatic to *erh2/pom1-12*.

Seedlings homozygous for both *rhl1* and *ttg* are completely bald on their primary roots. The shoot morphology is essentially that of *rhl1* single mutants, but doubly mutant seedlings appear to be very pale due to the lack of anthocyanins in the hypocotyl and the cotyledons. However, with respect to the phenotype of the root epidermis *rhl1* is clearly epistatic to *ttg*.

The double mutants between *rhl1* and *erh3* are characterized by hairless roots that are, however, increased in diameter in the same way as *erh3*. It is a complex phenotype, but the epidermal cell fate seems to be determined by the *rhl1* mutation, i.e. *rhl1* is epistatic to *erh3*.

Double mutants between *rhl1* and *ctr1* displayed a phenotype that was reminiscent of *rhl1* treated with 10-100 μM ACC. The root hair density was only slightly increased on primary roots whereas the collet region remained bald as in *rhl1* single mutants. The length of the root was dramatically reduced as seen in *ctr1* single mutants. The shoot phenotype was essentially the same as in *rhl1* single mutants.

Plants homozygous for the *rhl2-2* and *ctr1* mutations also showed a few more hairs on short primary roots below the collet region, but otherwise looked like *rhl2-2* single mutants.

The same result was obtained for the *rhl3-2 ctr1* double mutant that displayed the same phenotype as the *rhl3-2* single mutant treated with 10-100 μM ACC.

In all cases functional *RHL*-gene products are required for the *ctr1* root phenotype. However, for root hair formation below the collet the *rhl*- mutants are not completely epistatic to *ctr1*, indicating a more complex or indirect relationship between the affected genes. The few root hairs of *rhl ctr1*

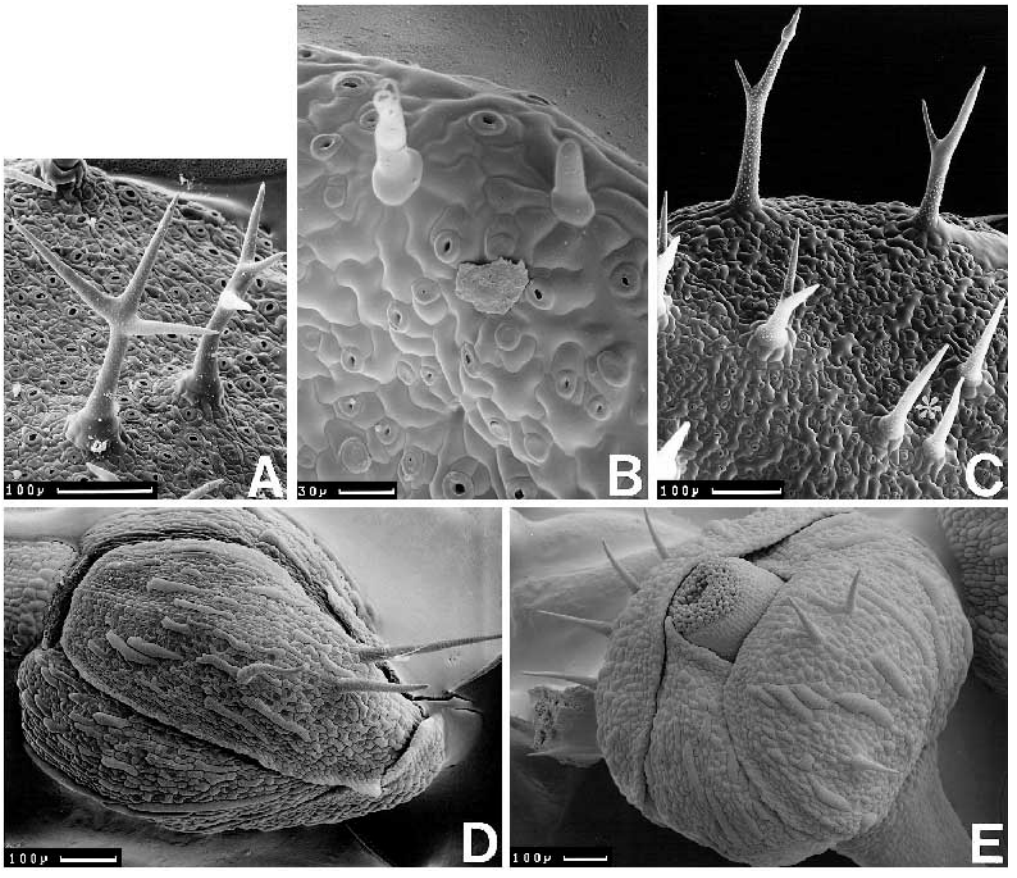

Fig. 6. Scanning electron micrographs of (A) wild-type leaf trichomes, (B) *rhl1* mutant trichomes, (C) *erh3* mutant trichomes (the asterisk indicates 2 adjacent *erh3* trichomes). (D) Col wild-type flower bud and (E) *erh3* mutant flower bud.

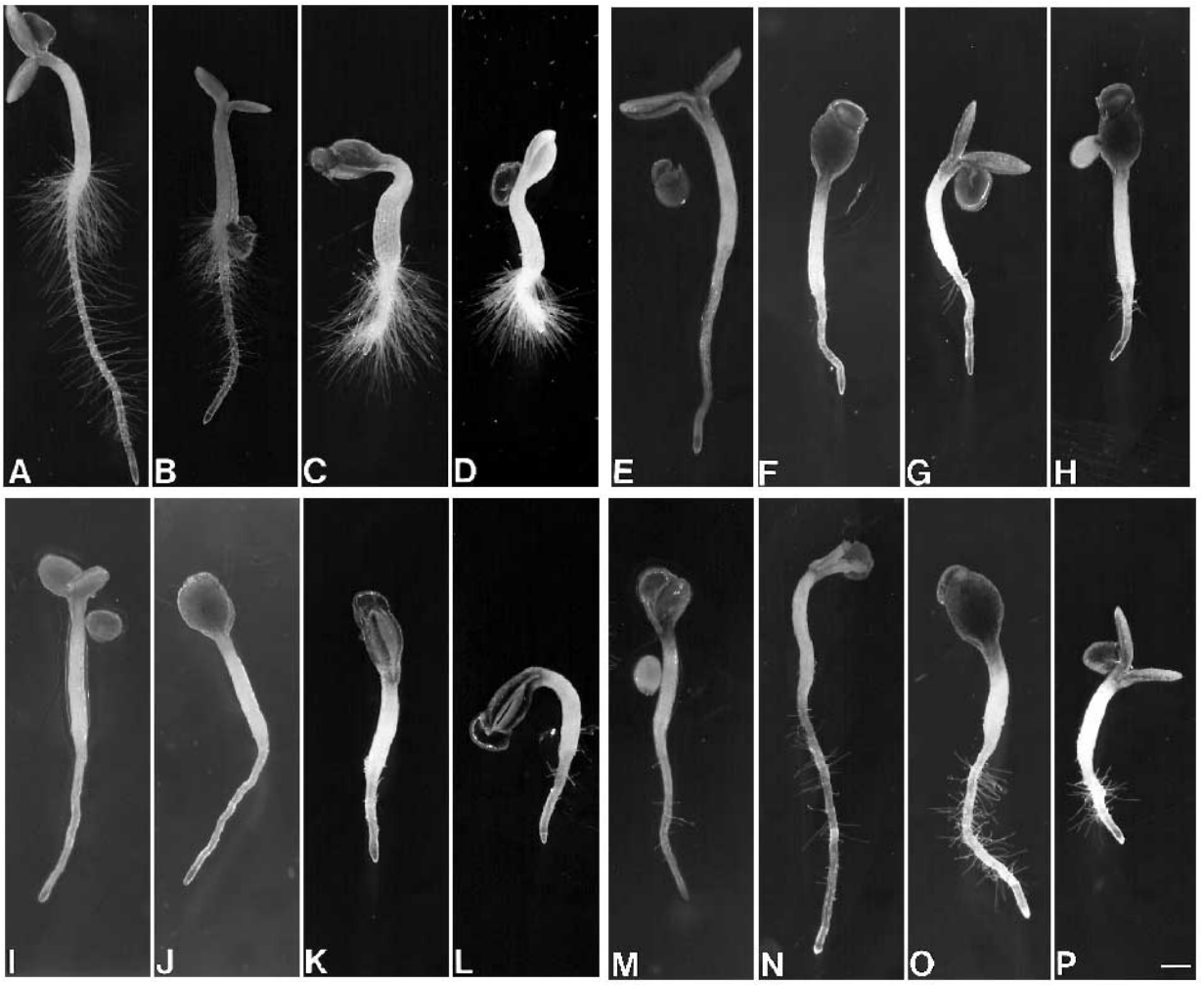

Fig. 7. ACC effects on seedlings. 3-day old seedlings of (A-D) wildtype, (E-H) *rhl1*, (I-L) *rhl2-2* and (M-P) *rhl3-2* grown on medium supplied with (A,E,I,M) no ACC, (B,F,J,N) 1 μM ACC, (C,G,K,O) 10 μM ACC and (D,H,L,P) 100 μM ACC. Scale bar in P equals 400 μm.

double mutants indicate that the *rhl* mutants appear to be equipped with a functional machinery to produce root hairs. This is consistent with the idea that the *RHL* genes act prior to cell fate specification in the meristem (Fig. 1D,F,H) whereas *CTR1* and ethylene play a role very late in the root hair initiation process irrespective of the predetermined cell fate (Masucci and Schiefelbein, 1996).

DISCUSSION

In our screen for mutants without root hairs or with root hairs in ectopic positions we have defined 6 genes that have not previously been associated with root epidermal cell differentiation. We characterize several aspects of their mutant phenotype and discuss possible functions for the affected gene products by relating both the mutants to each other and to other previously described steps in the pathway to root hair formation.

Most genes involved in root hair initiation affect shoot developmental processes as well

A new class of root hairless mutants is described here for the first time specifying the 3 genes *RHL1*, *RHL2* and *RHL3*. The mutants *rhl1*, *rhl2-1*, *rhl2-2*, *rhl3-1* and *rhl3-2* all show the same pleiotropic phenotype characterized by the complete absence of root hairs, abnormal patterning of the leaf epidermis and dwarfism. This might indicate functional redundancy of the three genes.

The formation of root hairs is regulated differently on the primary root and at the transition from the primary root to the hypocotyl region (Dolan et al., 1993), but the root hairless genes *RHL1*, *RHL2* and *RHL3* appear to be required in both pathways. The only other mutant displaying this phenotype is *axr3-1*, the strongest allele of an auxin and ethylene resistant mutant, again with a very complex phenotype (Leyser et al., 1996). However, a comparison of map positions excludes allelism between *axr3* and the root hairless mutants presented here.

Apart from the *rhl*-mutants *erh3*, an ectopic root hair mutant, shows alterations in the shoot as well as the root epidermis. Despite the opposite effects of the *erh3* and the *rhl* mutations on the root epidermal phenotype, their effects on leaf development are similar, suggesting different roles for the corresponding gene products in roots and shoots. With *gl2* and *ttg*,

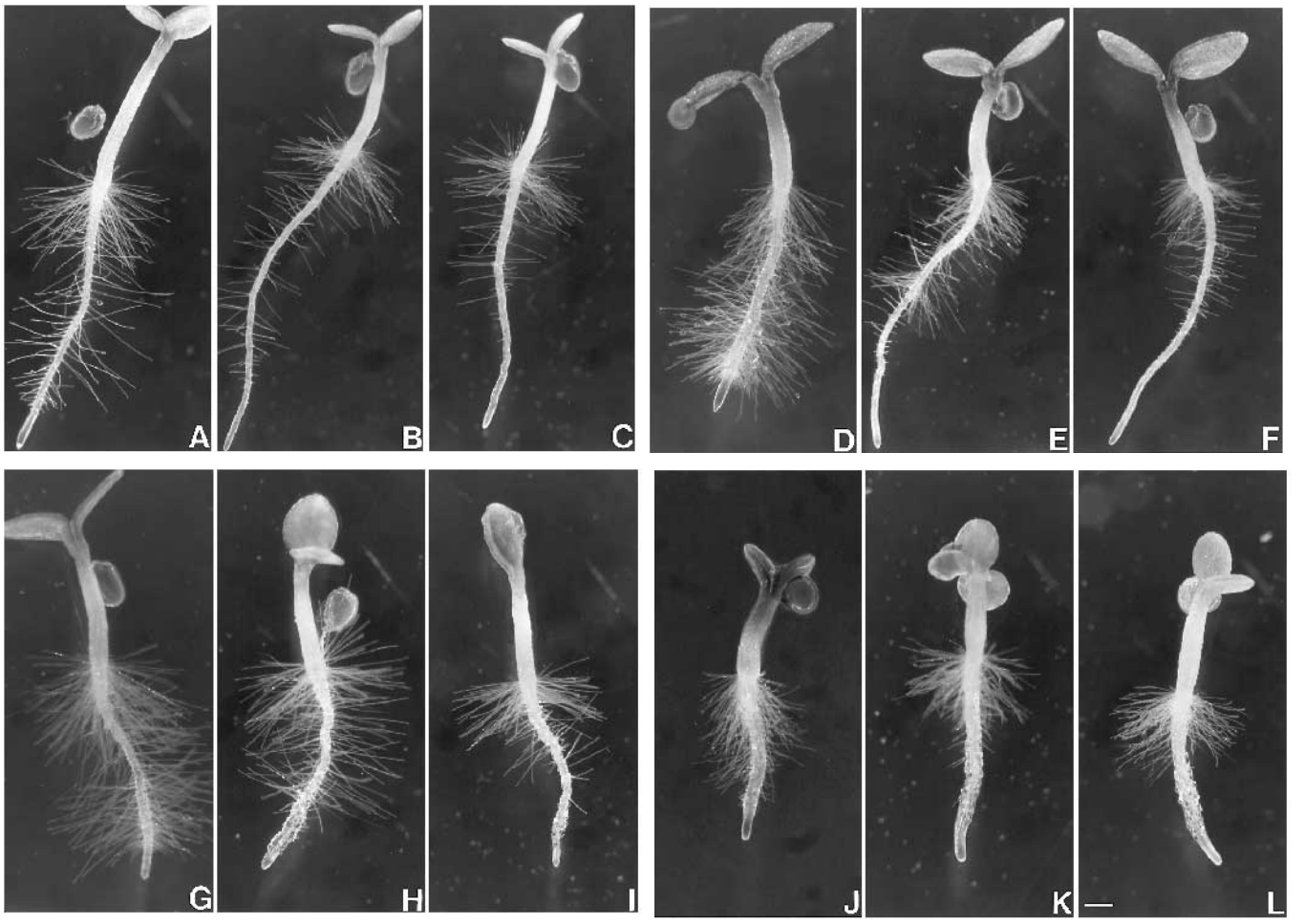

Fig. 8. AVG effects on seedlings. 3-day old seedlings of (A-C) wildtype, (D-F) *erh1*, (G-I) *erh2* and (J-L) *erh3* grown on medium supplied with (A,D,G,J) no AVG, (B,E,H,K) 5 μM AVG and (C,F,I,L) 20 μM AVG. Scale bar in L equals 400 μm.

two more loci are known to be involved in general epidermal development (Masucci et al., 1996, Galway et al., 1994) indicating a complex network of genes controlling the development of specialized epidermal cells all over the plant.

erh3 is the only known ectopic root hair mutant so far that shows a significant floral phenotype as well (Fig. 6E). Occasionally aberrant numbers of floral organs suggest a defect in primordial positioning. Despite differences between wildtype and *erh3*, siliques and seeds, the *erh3* mutant is fully fertile indicating that there is no problem with gametophyte development.

As there is only one EMS allele of the *erh3* mutation whether or not the severity of the displayed defects is due to a null mutation cannot be evaluated. However, the mutant phenotype is consistent with a role for the *ERH3* gene product in lateral inhibition mechanisms involved in specifying the position and fate of cell types or organ primordia (Bünning, 1965).

The fact that our screen based on root phenotypes mostly identified mutants with pleiotropic defects indicates that root epidermal cell differentiation is interlinked with other processes and is likely to require a number of gene products that are also involved in other processes. There appear to be only a few root specific mutants and *erh1* might be such a candidate. However, the pathway has not yet been screened to saturation.

Root hair initiation and radial cell expansion

A correlation between radial cell expansion and epidermal cell differentiation is revealed by the *erh2/pom1-12* mutant phenotype (Hauser et al., 1995). The conditional root expansion phenotype of *pom1* is dependent on a high concentration of sucrose in the growth medium (Hauser and Benfey, 1993). The growth medium used in our studies contains 3% sucrose and falls within this range. Under these conditions 3 day old *erh2* seedlings show early stages of cell differentiation comparable to the wildtype in the meristematic zone that does not yet display the fully expanded phenotype (Fig. 1A,L). This indicates that ectopic root hairs in *erh2* are likely to be initiated at late developmental stages correlated with cell expansion. The *rhl1*-like double mutant between *rhl1* and *erh2* reveals that both cell expansion and root hair formation in *erh2* are dependent on *RHL1* gene function. The radial expansion of the cortex in *pom1* is prevented at sucrose concentrations below 0.5% (Hauser and Benfey, 1993). Preliminary results indicate that *erh2*/*pom1-12* seedlings grown on medium with 0.1%

Table 2. Classification of root epidermal mutants with altered hair density

Mutant	Root epidermal phenotype	Root developmental stage of action	Relation to other processes
rhl1	No hairs	Cell fate specification at meristematic zone	Shoot development, shoot epidermis identity
rhl2	No hairs		
rhl3	No hairs		
erh3	Ectopic hairs		
ttg	Ectopic hairs		
erh1	Ectopic hairs	Cell fate specification at meristematic zone	Not identified
gl2	Ectopic hairs	After initial cell differentiation at meristematic zone	Trichome morphology, seed coat mucilage
erh2/pom1	Ectopic hairs	After initial cell differentiation at meristematic zone	Conditional cell expansion in root cortical cells
ctr1	Ectopic hairs	Differentiating root epidermal cell at a late stage of cell elongation	Growth factor mediated effects in the whole plant
rhd6	Fewer hairs		
axr2	Fewer hairs		

sucrose hardly show any ectopic root hairs (data not shown), thus suggesting that ectopic root hairs as well as the cortex cell expansion phenomenon are conditional on the growth rate of *erh2*/*pom1-12* seedlings.

A possible link between cell expansion and root hair initiation also becomes apparent by comparing transverse sections of *ctr1* roots and roots treated with the ethylene precursor ACC (Dolan et al., 1994; Tanimoto et al., 1995). The diameter of both specimens appears to be about 1.25-fold larger than the wildtype. The degree of expansion seems to be uniform in all cell layers. In both cases cell expansion is probably due to ethylene action that also promotes ectopic root hair formation, and the different ethylene pathways may or may not be linked. Masucci and Schiefelbein (1996) suggested that ethylene and auxin action do not change cell fate in the primary root epidermis, but alter cell expansion or morphogenesis at a late developmental stage to trigger hair formation. However, in *erh1*, *erh2/pom1-12* and *erh3* primary roots still appear to be expanded after blocking ethylene synthesis with AVG (Fig. 8), there even seem to be epidermal bulges on *erh2/pom1-12* and *erh3* roots whereas all root hairs below the collet have disappeared. This result indicates that radial cell expansion in *erh1*, *erh2/pom1-12* and *erh3* is likely to be caused by something other than ethylene synthesis.

Interestingly the diameters of *erh1* and *erh3* roots are also significantly wider than the diameter of wildtype roots (Fig. 3). In the case of *erh1* the radial expansion seems to affect all cell layers to a similar degree whereas in *erh3* the endodermal cells are particularly expanded. Preliminary results indicate that cell expansion of *erh1* mutants is also conditional on growth rate although *erh1* roots still produce ectopic root hairs when grown on 0.1% sucrose. Growing *erh3* mutants on 0.1% sucrose, however, appears to affect neither cell expansion or ectopic root hair initiation. Both results are consistant with the idea that *ERH1* and *ERH3* act as early as at the stage of cell fate specification and therefore the production of ectopic root hairs is not dependent on growth rate. Moreover, cell expansion of *erh3* roots is independent of growth rate altogether.

The strongly expanded bald root phenotype of the *rhl1 erh3* double mutant also argues for the independence of root hair initiation and cell expansion. It reveals that in the absence of functional *RHL1* gene product cell expansion per se does not promote root hair initiation. Still, there remains the possibility that some genes may regulate radial cell expansion and – dependent on it or not – the competence to undergo ethylene dependent hair formation at a late stage of root epidermal development.

A preliminary model for root hair formation

The variety of mutants impaired in correct root hair initiation and the different relationships between the corresponding gene products suggests a complex and not strictly hierarchical pathway regulated at different levels leading to root hair formation. To achieve an order of events in that process the mutants were grouped according to the developmental stages in which their defects were first observed (Table 2). Mutations affecting the shape of root hairs, like some members of the *rhd*-class (Schiefelbein and Somerville, 1990), do not appear to influence hair positioning and are therefore not referred to. The classification shown in Table 2 leads to the proposal of the following model for the pathway leading to root epidermal cell differentiation (Fig. 9). Very early processes in epidermal cell

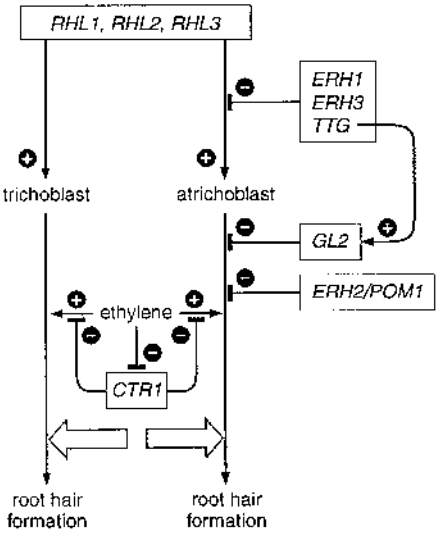

Fig. 9. Preliminary model for hair initiation in *Arabidopsis* primary roots. Open arrows show an alternative point of action of *RHL1*, *RHL2* and *RHL3* that is consistent with genetic results. Genes are all shown in boxes. Arrows indicate positive regulation (+), blunted lines indicate inhibitory action (–).

fate specification are most easily explained if the *RHL* genes promote trichoblast development in all epidermal cell files. However, the partial response of the *rhl* mutants to ACC indicate that the pathway is not strictly linear. It shows that the *rhl*-mutants can perceive the ethylene signal to a very limited degree and that there is some ethylene dependent competence to form root hairs in these mutant backgrounds. Although none of the *rhl* alleles has yet been confirmed to be a null mutation the few root hairs on ACC treated *rhl* roots are likely to be due to a root hair initiation pathway largely independent of *RHL* gene functions. The epistasis results are also formally consistent with an interpretation that places the *RHL* genes downstream of ethylene action in both cell files, although the severe pleiotropic phenotypes of the *rhl* mutants and the fact that the cell files in the *RHL* mutants are not visually distinguishable in the meristem both argue for the *RHL* genes acting earlier than this.

A number of genes, conferring ectopic root hairs when mutated, are assumed to negatively regulate hair formation or positively regulate non-hair formation in distinct cells. The earliest among these are likely to be identified by the *ttg*, *erh1* and *erh3* mutations. Their effects are already visible at the level of cell fate determination in the meristematic zone where trichoblasts and atrichoblasts are differentiating. After this initial cell fate specification the *GL2* gene product is active only in atrichoblasts to secure the hairless cell fate. The *ERH2/POM1* gene product possibly also functions at this level to control the initiated developmental program in atrichoblasts by regulating cell expansion in the adjacent cortical cells. Finally root hair formation mediated by high concentrations of growth factors, in particular ethylene, appears to affect all cells irrespective of their predetermined fate. However, trichoblasts and atrichoblasts might be distinguishable by quantitative differences in susceptibility to growth factors. By growing seedlings in the dark to lower the effective ethylene concentration it has been shown for *gl2* that root hair formation in cells in non-hair positions is dependent on a higher ethylene level than root hair formation in trichoblast positions (Di Cristina et al., 1996).

By studying 8 novel mutants that are impaired in root hair initiation and by examining the possible interactions of the corresponding genes, progress has been made towards understanding the different steps in the pathway leading to cell differentiation in the root epidermis and its links with other developmental processes.

We would like to thank Sue Bunnewell for preparing media for plates and for excellent photographic work and Nigel Orme for the graphic design of Fig. 9. We acknowledge Claire Grierson for collaboration in the initial phase of the project and Karin Metzlaff for sharing work in the first part of the screen. We are grateful to Ken Feldmann who made the *rhl1* mutant available. Our thanks also go to John Schiefelbein for providing seeds and sharing useful conversations. K. S. is grateful to members of Phil Benfey's lab and especially to Marie-Theres Hauser for sharing protocols, donating seeds and discussing important aspects throughout the work. We acknowledge both the Ohio and the Nottingham *Arabidopsis* stock centres for providing seeds. K. S. was supported by fellowships from EMBO (ALTF 568-1992), HFSP (LT-135/93) and by a grant from the Gatsby Charitable Foundation. L. D. was funded by a PMB II grant and B. W. and K. R. acknowledge funding from the BBSRC.

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(Accepted 17 February 1997)