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Cell specification in the *Arabidopsis* root epidermis requires the activity of *ECTOPIC ROOT HAIR 3* – a katanin-p60 protein

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SUMMARY

The *Arabidopsis* root is composed of radial cell layers, each with distinct identities. The epidermal layer is composed of rows of hair cells flanked on either side by rows of non-hair epidermal cells. The development of hair and non-hair cells is dependent on domains of positional information with strict boundaries. The pattern of cell differentiation and the expression of molecular markers of cell fate is altered in the *ectopic root hair 3* (*erh3*) mutant epidermis indicating that ERH3 is required for the specification of cell fates from early in development (in the meristem) through differentiation. Furthermore the expression of molecular markers indicates that the specification of cell identities is defective within other radial cell layers. *ERH3* encodes a p60 katanin protein that is expressed throughout the plant. Katanin proteins are known to sever microtubules, and have a role in the organisation of the plant cell wall since mutants with decreased katanin activity have been shown to have defective walls. We suggest that microtubules are involved in the specification of cell identities in cells of the *Arabidopsis* root. Microtubules may be required for the localization of positional cues in the wall that have previously been shown to operate in the development of the root epidermis. Alternatively microtubules may be involved in another as yet undefined process required for the specification of cell identity in plants.

Key words: Epidermal pattern, Radial pattern, Cell specification, Katanin, *ectopic root hair3*, *Arabidopsis thaliana*

INTRODUCTION

Multicellular organisms are composed of different specialised cells with distinct functions in stereotypical arrangements. The spatial organisation of the constituent cell types results from a plethora of activities including cell-cell communication, cell division and expansion. The *Arabidopsis* root is composed of concentric rings of different cell types including from the outside to the inside: root cap, epidermis, cortex and endodermis around the pericycle, which encloses the stele (Dolan et al., 1993). Spatial development of the cortex and endodermis from a common stem cell requires the activities of the *SCARECROW* and *SHORT ROOT* genes (Helariutta et al., 2000; Wysocka-Diller et al., 2000). The development of the epidermis and root cap from the protodermal initial requires the activities of the *TORNADO1* and *TORNADO2* genes (Cnops et al., 2000). Once the radial arrangement of layers is specified, the epidermis is subspecified into cells of two distinct types. There are longitudinal stripes of hair cells and non-hair cells. Hair cells (H) are derived from meristematic trichoblasts (T) and located over the junction between two underlying cortical cells, while non-hair (N) epidermal cells are derived from meristematic atrichoblasts (A) that are located over a single cortical cell (Dolan et al., 1994). Laser ablation and clonal analysis has shown that the positional information specifying cell fate in N and H cells is organised in domains with strict boundaries (Berger et al., 1998a; Berger et al., 1998b). Furthermore laser ablation experiments indicate that these spatially restricted positional cues are located outside the cell, in the wall (Berger et al., 1998a).

Mutants in which the spatial arrangement of epidermal cell identities is defective have been instructive in defining a molecular mechanism for epidermal cell development. *GLABRA2* (*GL2*) encodes a homedomain protein that is expressed in A/N cells and promotes A/N fate (Masucci et al., 1996). *WEREWOLF* (*WER*) is a Myb-related transcription factor that is expressed in N cells and promotes N fate by positively regulating *GL2* expression (Lee and Schiefelbein, 1999). *TRANSPARENT TESTA GLABRA* (*TTG*) is a WD40-repeat-containing protein that is also required for *GL2* transcription (Walker et al., 1999). *CAPRICE* (*CPC*) theoretically acts as a positive regulator of H cell identity as *cpc* mutants lack root hairs (Wada et al., 1997). Since *CPC* is a Myb-related protein that lacks a transcriptional activation domain (Wada et al., 1997), it is assumed that CPC acts by negatively regulating the expression of the N cell promoting gene *GL2*. It has been proposed that WER and CPC compete for the same binding sequences in promoters of target genes. Because of the similarity between CPC and WER and given the opposite role played by each in the development of the epidermis, a model has been proposed in which the ratio of WER to CPC determines the fate of any individual epidermal

cell. A cell with a high level of WER relative to CPC will develop as an N cell while one with higher levels of CPC will develop as an H cell (Lee and Schiefelbein, 1999).

It has been reported that *erh3-1* mutants develop H cells in the N position indicating that ERH3 is required for a process during position-dependent cell differentiation in the root epidermis (Schneider et al., 1997). Here we show that ERH3 is required for the differentiation of cells in both the H and N positions. Furthermore we show that ERH3 encodes a katanin p60 catalytic subunit, a protein that severs microtubules (McNally and Vale, 1993). In other systems katanin activity is correlated with increased microtubule dynamics (Quarmby, 2000). Katanin has recently been shown to be required for the formation of the cell wall in plants – *fra2* mutants have defects in the composition of their cell walls (Burk et al., 2001). *FRA2* encodes the p60 katanin and is therefore identical to *ERH3* (Burk et al., 2001). We suggest that the katanin-mediated microtubule reorganisation is central to the stability of cell identities in the *Arabidopsis* root.

MATERIALS AND METHODS

Plant materials

Generation of mutants

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col) were mutagenised using EMS (ethane methyl sulfonate). The self-fertilised progeny of these seeds were collected as individual M_2 families, and screened for ectopic hair phenotypes after 5 days growth in vitro. Putative ectopic hair mutants were backcrossed to Col plants through three successive cycles before phenotypic analysis. Independent lines with similar mutant phenotypes were crossed to these putative mutants and examined in the F_1 generation to assess complementation.

Growth conditions

The seeds were surface sterilised in 5% bleach for 10 minutes, rinsed three times in sterile distilled water then pipetted onto the surface of growth medium in Petri dishes. The growth medium was 0.5% (w/v) phytagel, 1% (w/v) sucrose and MS salts at pH 5.8. The seeds were stratified at 4°C for 2 days in the dark and then incubated at 25°C (±2°C) under continuous illumination. The plates were inclined at an angle of 80 degrees to allow the roots to grow along the surface of the medium. The roots were observed after 4 to 5 days incubation.

Seed stocks

The enhancer trap lines J0481, J0571, J0671 and J2301 were obtained from J. Haseloff (Department of Plant Science, Cambridge, UK). John Schiefelbein, University of Michigan, USA kindly provided *wer* and *GL2::GUS* lines. Other mutant seed were provided by the Nottingham Arabidopsis Stock Centre (NASC).

Histochemical localisation of β-glucuronidase (GUS)

The expression pattern of the *GL2* promoter::*GUS* fusion gene was visualised using the following GUS protocol. Four- to five-day old seedlings were incubated in GUS staining solution pH 7.0 (0.1 M $NaPO_4$ buffer, pH 7.0; 1 mM $K_3Fe(CN)_6$, pH 7.0; 1 mM $K_4Fe(CN)_6.3H_2O$, pH 7.0; 1 mM X-glucuronide; 0.08 mM EDTA, pH 8.0) at 21°C for 3 hours. The seedlings were transferred to 70% ethanol to stop the reaction.

Imaging

Confocal microscopy: Seedlings (4- to 5-day old) were stained with either 1 mg/ml or 200 μg/ml propidium iodide (PI) solution for 5-60 minutes. Roots were imaged with a Leica TC5 SP confocal microscope using the 488 nm excitation and 590-765 nm emission lines for PI. The 488 nm excitation and 510-550 nm emission lines were used to image GFP expression in the enhancer trap lines. Images were processed using NIH image (http://rsb.info.nih.gov/nih-image/) and assembled using Adobe Photoshop 5.

Tissue fixation and embedding

Roots (4- to 5-day old) were fixed for 1 hour in 2% (w/v) glutaraldehyde in 50 mM sodium cacodylate buffer, pH 6.8. The roots were placed on a thin slab of 1% (w/v) agarose and covered with agarose for easier handling. These root-agarose sandwiches were refixed in 2% (w/v) glutaraldehyde overnight. Samples were washed twice in water for 5 minutes, dehydrated in an ethanol series (25%, 50%, 75% and 95%) for 10 minutes each and infiltrated with 50% (w/v) LR white (medium grade plus 0.5% benzoin methyl ether): 50% ethanol and twice in 100% resin for at least 2 hours each. The samples were transferred to resin-filled capsules and polymerised at 60°C for 24 hours. The sections were cut on a Reichert Jung Ultracut Microtome. For light microscopy, 0.5 μm thick sections were collected on glass slides and viewed on a Nikon E800 microscope.

Genetic mapping

The *ERH3* gene was cloned using a map based cloning strategy. A segregating F_2 family was made from a cross between an *erh3-3* homozygote in the Columbia background and wild-type Landsberg *erecta*. The rough map position of *erh3-3* was obtained using SSLP and CAPS markers by analysing DNA from 107 F_2 *erh3-3* mutants of the mapping population. For fine mapping, DNA polymorphism Cereon Genomics database (http://www.arabidopsis.org/Cereon/index.html) was used to generate SSLP markers. The following primers were used for the fine mapping: CER449943, 5′-CCGAGCTTAGCAACCGCCC-3′ and 5′-CATCGTCACATTTATGAAGC-3′; CER461145, 5′-CAAGAACCTCATACCTTGC-3′ and 5′-GACTCTCCCACCACCACACG-3′; CER464737, 5′-CCTTGAACAGAATCCACGGC-3′ and 5′-GTTGTGTTGCCCTTACAGAG-3′; AAA1, 5′-CAATTTCTTCAAGCCCTAAGC-3′ and 5′-CGTCAAGGGTGTTTAAATGC-3′. DNAs from 267 F_2 *erh3-3* mutants from the mapping population were analysed using these primers.

Sequencing of mutant alleles

To identify mutations in the *erh3* alleles, the *ERH3* coding region was amplified from wild-type and mutant plant DNAs by PCR using a mix of Taq polymerase (Gibco BRL) and Pfu polymerase (Promega). Primers have been designed based on the wild-type sequence. The amplification conditions were as follows: 3 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, then a final extension at 72°C for 10 minutes. After purification, PCR products were used directly for sequencing. Sequencing was carried out using Big Dye Terminator Sequencing Kit (Perkin-Elmer) in conjunction with the Applied Biosystems 3700 DNA Sequencer.

Sequence analysis

The BLAST search programme (Altschul et al., 1997) was used for sequence analysis and comparisons in the GenBank, EMBL, and swissProt databases. Multiple sequence alignments and relationship tree based on Neighbour-Joining method was done with CLUSTAL X (Thompson et al., 1997) and TreeView programmes. All the sequences of the AAA domain-containing proteins can be retrieved from the web site server (http://yeamob.pci.chemie.uni-tuebingen.de/Default.html).

RT-PCR analysis

First-strand DNA synthesis was carried out from 4 μg of *A. thaliana* total RNA, using a Superscript First strand DNA Preamplification Kit (Gibco-BRL) with an oligo(dT) primer. Specific primers for RT-PCR amplification of *ERH3* transcripts, flanking an intron sequence, were designed: 5′-GATGCCCGAATACTTTCAGG-3′ and 5′-CAGATTGATATTGATAAGAGCC-3′. These primers give a 501 bp fragment

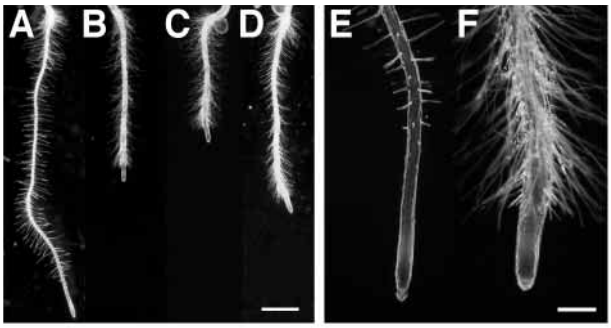

Fig. 1. *erh3* mutants are short and hairy. Root phenotypes of 4-day old seedlings. Wild type (A,E), *erh3-1* (B), *erh3-2* (C,F), *erh3-3* (D). Scale bar, 1000 μm (A-D), 250 μm (E,F).

with cDNA as template and a 830 bp fragment using genomic DNA as template. PCR reactions were done using 1 μl of RT reaction products as a template in a 50 μl PCR reaction according to the following conditions: 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 1 minute at 72°C. RT-PCR products were loaded on a 2% (w/v) agarose gel and visualised by ethidium bromide fluorescence. The specificity of the amplification was checked by sequencing of the PCR products. The reproducibility of the results was tested by repeating the experiment with both the same reverse transcripts and with a different batch of reverse transcripts prepared from the same total RNAs.

RESULTS

Isolation of two new *erh3* mutant alleles

To further characterise the function of *erh3* in the development of the root epidermis we identified two new alleles, designated *erh3-2* and *erh3-3*, from an EMS-mutagenised population in Columbia. *erh3-2* is the strongest allele; plants homozygous for *erh3-2* have the shortest roots and most severe defects in root epidermal morphology (Fig. 1). Mutant roots are radially swollen compared to wild type. This radial swelling is not confined to the mature regions of the root but is also evident in the meristem (Fig. 2B,D). Just above the central cells the *erh3-2* mutant root is approximately 15% wider than wild type and this difference is maintained in the upper regions of the meristem, 100 μm from the central cells. Root diameter of the *erh3-3* mutant is intermediate between wild type and the severe *erh3-2* mutant. The phenotype of mutant plants becomes progressively more severe with age. By 7 days, the *erh3-2* mutant roots have severe elongation defects. *erh3-2* mutants were used for all phenotypic characterisations described here.

Cell walls are misaligned in *erh3* mutants

The positioning of cell plates and walls in the *erh3-2* meristem is abnormal. When viewed in longitudinal section the majority of walls in the wild-type meristem are aligned either transversely across the root or longitudinally along the root (Fig. 2A). This regular arrangement of walls is altered in *erh3* mutants, in which there are patches where the walls are obliquely oriented (Fig. 2B). The arrangement of cell walls becomes more defective and by 7 days the organisation of cells in the meristem is severely disrupted (data not shown).

Cell files in the wild-type epidermis are arranged in a brickwork pattern where T/H cells are morphologically

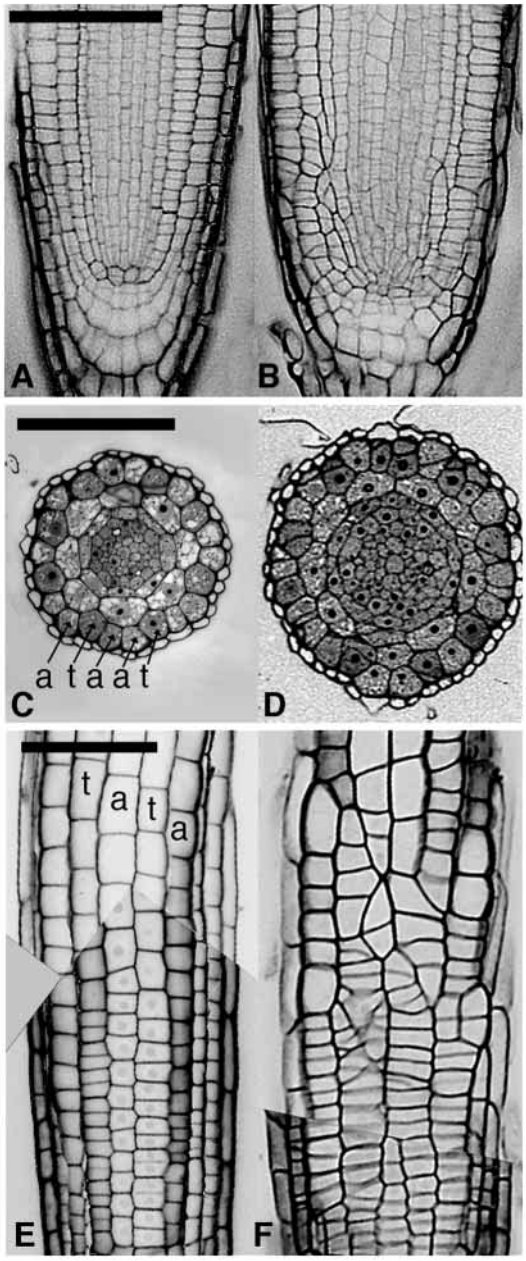

Fig. 2. Alignment of cell walls is defective in the meristems of *erh3* mutant roots. Wild type (A,C,E), *erh3-2* (B,D,F). Medial longitudinal sections (A,B). Transverse sections (C,D). Organisation of epidermal cells (E,F). a, atrichoblast (meristematic cell that will form a non-hair cell); t, trichoblast (meristematic cell that will form a hair cell). Scale bar, 100 μm.

distinguishable from A/N cells (Fig. 2E). The majority of divisions in the epidermis are transverse in orientation, adding more cells to each row (Berger et al., 1998b). The orientation of walls is abnormal in the epidermis of *erh3-2* mutant roots – many of the new walls are oblique and not transverse (Fig. 2F). This indicates that ERH3 is required to orient cell plates during cytokinesis.

The pattern of epidermal cell differentiation is defective in *erh3* roots

We showed previously that hairs form on a subset of cells in

Table 1. Epidermal specification in *erh3* alleles

	N position				H position				Np/Hp position		
	N	eH	*n*	% ectopic hairs	eN	H	*n*	% ectopic non-hairs	N	H	*n*
Wild type	38	1	39	2.56	0	24	24	0	1	0	1
erh3-1	38	5	43	11.63	5	31	36	13.89	13	8	21
erh3-2	325	99	424	23.35	53	272	325	16.31	23	18	41
*erh3-2**	126	39	165	23.64	9	71	80	11.25	–	–	–
erh3-3	42	4	46	8.70	3	20	23	13.04	0	2	2

N position, cells overlying periclinal wall of single cortical cells; H position, cells overlying anticlinal wall between two cortical cells; Np/Hp position, cells that are in both N and H position due to oblique cell walls.

N, non root hair; eH, ectopic hair; eN, ectopic non-hair; H, root hair; *n*, number of cells scored in each position.

*erh3-2**, analysis that excludes all epidermal cells and/or underlying cells with misaligned cell walls.

the N position on 4-day-old *erh3-1* seedlings (Schneider et al., 1997). We have called these misplaced hair bearing cells "ectopic hair cells". We determined the location of hair and non-hair cells in 4-day-old *erh3-2* roots (in which the mutant phenotype is not yet severe). Hairs formed on 23% of cells located in the N position. In addition 16% of cells in the H position remained hairless, i.e. they were ectopic non-hair cells (Table 1). Lower numbers of ectopic hairs and non-hairs were seen in the *erh3-1* and *erh3-3* mutants, consistent with their weaker allelic strengths. The presence of both ectopic hairs and non-hairs suggests that ERH3 is required for the specification of cell identity in both N and H locations.

To determine if the presence of ectopic hair and non-hair epidermal cells in *erh3* roots is a consequence of the misaligned cell walls, we identified patches of epidermal cells where cells walls were not obliquely orientated and therefore the cellular organisation was identical to wild type. Ectopic hair and non-hair cells were identified in these patches (Table 1), indicating that epidermal fate changes are not a consequence of altered planes of cell division.

To determine when *ERH3* is required for the specification of epidermal cells, we examined the expression of marker genes that are preferentially expressed in atrichoblasts. The *GL2* promoter::GUS fusion gene (Masucci et al., 1996) is strongly expressed in A/N cells in the root meristem of wild type plants (Fig. 3A). The pattern of *GL2* expression is altered in patches along the roots of 4-day-old *erh3-2* mutants, resulting in breakdown of 'atrichoblast stripes'. Cells in the T/H position express *GL2* and occasionally cells in the A/N position fail to express the marker indicating a requirement of *ERH3* function for the A/N-specific patterning of *GL2* expression (Fig. 3B). J2301 is an enhancer trap line that expresses GFP in the A/N cells of the wild-type epidermis (Fig. 3C) (Berger et al., 1998c). The pattern of J2301 expression is also defective in patches along the 4-day-old *erh3-2* epidermis. GFP is visible in cells in the T/H position and expression is often absent from cells in the A/N position (Fig. 3D). This indicates that cell specification is defective in the *erh3-2* mutant epidermis from a very early stage in development and supports the view that ERH3 is required for cell specification in the root epidermis.

ERH3 may act in the same pathway as *CPC* and *RHD6*

To characterise the interactions between *erh3* and other genes involved in epidermal cell specification we characterised the phenotypes of double mutants (Fig. 4). *erh3-2, ttg* double mutants produce fewer ectopic hairs than the *ttg* single mutant, and more ectopic hairs than the *erh3-2* single mutant (Table 2). This intermediate (additive) genetic interaction between *ttg* and *erh3* suggests that these genes act in independent pathways in cells in the A/N position. A similar interaction was seen in the T/H position. The double mutant had an intermediate number of ectopic A/N cells. We observed the same phenotypes in *erh3-2 wer* double mutants, data not shown. The root epidermis of *erh3-2 cpc* seedlings is largely hairless. In the A/N position, the percentage of ectopic T/H cells was very low (Table 2), which corresponds to the Cpc$^-$ phenotype. This masking of one phenotype by the other phenotype is typical of an epistatic interaction. This suggests that these genes act in the same pathway. In the T/H cells of the *erh3-2 cpc* double mutant, a different genetic interaction exists. The double mutant has more ectopic A/N cells than seen in both the *erh3-2* and *cpc* single mutants (Table 2). This synergistic relationship suggests

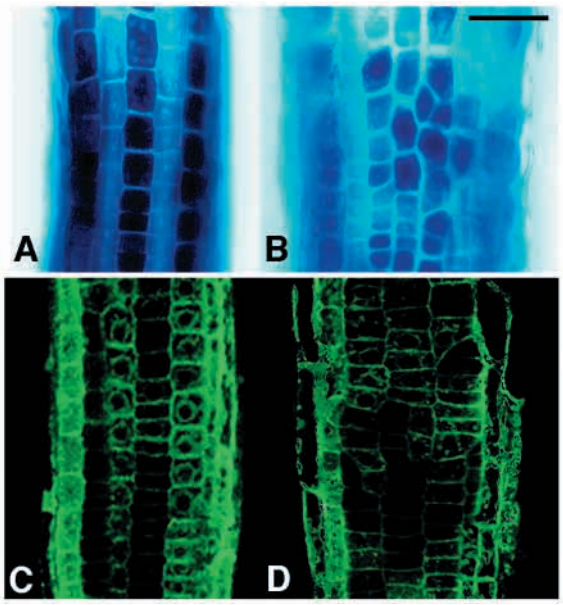

Fig. 3. Marker lines that are preferentially expressed in atrichoblasts, exhibit patchy expression in *erh3-2* mutants, causing breakdown in 'atrichoblast stripes'. *GL2::GUS* expression in wild-type (A) and *erh3-2* mutant epidermis (B). Expression of the *J2301::GFP* enhancer trap in wild-type (C) and *erh3-2* mutant (D) epidermis. Scale bar, 50 μm.

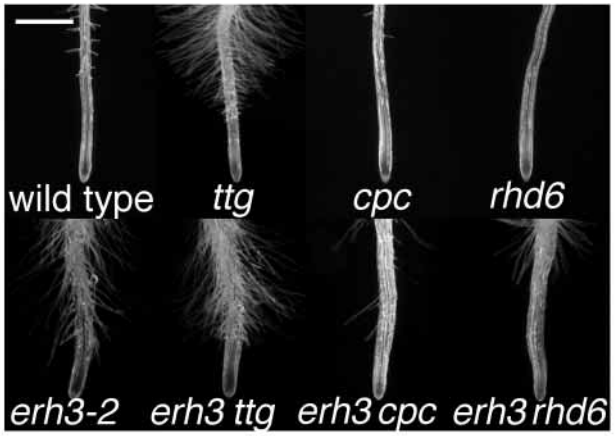

Fig. 4. Double mutant phenotypes indicate that *erh3* is epistatic to *cpc* and *rhd6* for epidermal patterning and root-hair initiation. Scale bar, 500 μm.

that *ERH3* and *CPC* are acting in the same pathway. *ROOT HAIR DEFECTIVE6* (*RHD6*) is a gene involved in root hair initiation (Masucci and Schiefelbein, 1994). The root epidermis of *erh3-2 rhd6* double mutants has a reduced number of T/H cells (Table 2), which is characteristic of the Rhd6$^-$ phenotype. Taken together these data suggest that *ERH3* acts in the same pathway as *CPC* and *RHD6*, but independently from *TTG* and *WER*. This conclusion remains tentative since *erh3-2* may not be a complete loss of function allele.

Cell specification is defective throughout the root in *erh3* mutants

To determine if *ERH3* is required for the specification of cell identities throughout the root, we examined the expression of enhancer traps that are expressed in specific cell types in mutant roots. The enhancer trap J0481 is expressed in the lateral root cap of wild-type roots (Fig. 5A). A sub-population of cells in the lateral root cap fail to express the enhancer trap marker in *erh3* mutants indicating that cell identity may be altered (Fig. 5B). Transverse sections of the meristem show that no increased propidium iodide uptake occurs in non-GFP expressing cells in the lateral root cap of *erh3-2* mutants (Fig. 5D), indicating that these cells are viable, and that lack of GFP expression is not a consequence of premature cell death. In wild-type roots the enhancer trap J0571 is expressed in the endodermis and cortex (Fig. 6A,C) whilst J0671 is expressed in the mature cortex (Fig. 7A,C). In *erh3-2* mutants these markers are ectopically expressed in the epidermis (Fig. 6B,D and Fig. 7B,D). Further support for a requirement for *ERH3* in the specification of cell identities in the lateral root cap, epidermis and cortex is provided by J2104, a vascular and endodermal marker, and J3411, a lateral root cap marker. Whilst these enhancer trap lines are not strictly tissue specific, they are also misexpressed in the *erh3-2* mutant background (data not shown).

Molecular cloning of the *ERH3* gene and sequencing of the *erh3* mutant alleles

The *ERH3* gene was isolated using a map based cloning strategy. A segregating F_2 population was made from a cross between an *erh3-2* homozygote, in the Columbia background and wild-type Landsberg *erecta*. *erh3-2* was initially mapped to the south of chromosome I, downstream from the SSLP marker nga111 (Bell and Ecker, 1994) and the ADH CAPS marker (Konieczny and Ausubel, 1993) using a population of 60 chromosomes (Schneider et al., 1997). By analysing 534 *erh3* chromosomes with additional SSLP markers, *erh3* was located to within a single BAC, F5I6 (Fig. 8A). Examination of BAC sequence (accession number AC018848) identified a gene encoding a p60 katanin-like protein, *AAA1* (accession number AF048706) as a possible candidate. Using an SSLP marker (AAA1, see Materials and Methods) in the *AAA1* gene, no recombinants were found between *AAA1* and *erh3* in the population of 534 chromosomes.

To show that *AAA1* encodes the gene responsible for the Erh3$^-$ phenotype, the genomic DNA sequence of the three *erh3* mutant alleles was determined. Each possesses a single base substitution causing amino acid modification in the gene product (Fig. 8A,B): *erh3-1* contains a C to T transition which results in a histidine (H^{353}) change to a tyrosine (Y); *erh3-2* contains a G to A transition resulting in a glycine (G^{274}) change to an arginine (R); *erh3-3* contains a C to T transition resulting in an alanine (A^{406}) change to a valine (V).

ERH3 encodes a p60 katanin-like protein

The *AAA1-ERH3* gene is organised in 7 exons and encodes a predicted polypeptide of 523 amino acids. The analysis of the deduced amino acid sequence showed that this protein is

Table 2. Double mutant analysis of epidermal specification

	N position				H position				Np/Hp		
	N	eH	*n*	% ectopic hairs	eN	H	*n*	% ectopic non-hairs	N	H	*n*
Wild type	38	1	39	2.56	0	24	24	0	1	0	1
erh3-2	325	99	424	23.35	53	272	325	16.31	23	18	41
ttg	3	113	116	97.41	0	50	50	0	0	0	0
ttg erh3-2	96	213	309	68.93	9	137	146	6.16	0	9	9
cpc	265	1	266	0.38	124	87	211	58.77	0	0	0
cpc erh3-2	523	2	525	0.38	378	25	403	93.80	10	2	12
rhd6	150	0	150	0	112	2	114	97.21	6	0	6
rhd6 erh3-2	244	0	244	0	200	0	200	100.00	41	0	41

N position, cells overlying periclinal wall of single cortical cells; H position, cells overlying anticlinal wall between two cortical cells; Np/Hp position, cells that are in both N and H position due to oblique cell walls.

N, non root hair; eH, ectopic hair; eN, ectopic non-hair; H, root hair; *n*, number of cells scored in each position.

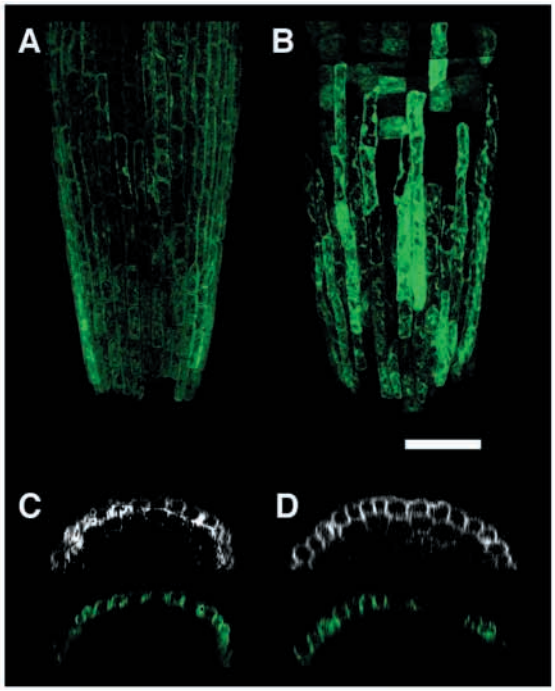

Fig. 5. Cells in the lateral root cap fail to express a lateral root cap marker in *erh3* mutants. Expression of the *J0481::GFP* enhancer trap in wild-type (A,C) and *erh3-2* mutant (B,D) meristems. (A,B) Projected images of GFP expression in the meristems. (C,D) Transverse sections approximately midway through the lateral root cap: top, PI-stained cell walls; bottom, GFP expression. Scale bar, 50 μm.

a member of the large AAA (*A*TPases *A*ssociated with various cellular *A*ctivities) protein superfamily, and has an AAA domain between positions 231 to 466 (Fig. 8B). The most similar proteins to AAA1-ERH3 are the p60 subunits of katanin proteins from *Drosophila melanogaster*, *Homo sapiens* (McNally and Vale, 1993), *Mus musculus* (Syu and Saltiel, 1999), *Xenopus laevis*, *Strongylocentrotus purpuratus* (Hartman et al., 1998), *Caenorhabditis elegans* (Clark-Maguire and Mains, 1994) and *Chlamydomonas reinhardtii* (Lohret et al., 1999) (Fig. 8C). These proteins all share a similar organisation (size, AAA domain position) and alignment analysis shows that several regions are conserved between ERH3/AAA1 and other p60 katanin proteins (data not shown). The percentage identities between ERH3/AAA1 and the p60 katanin range from 48 to 51% throughout the whole protein and from 64 to 66% within the AAA domain.

ERH3/AAA1 expression was examined by RT-PCR analysis. This showed that *ERH3/AAA1* is expressed throughout the wild-type plant. Signal was detected in leaves, roots, stems, flowers and siliques (Fig. 8D). Normal levels of *AAA1-ERH3* mRNA was observed in seedlings homozygous for each mutant allele, implying that these mutations alter the protein function but not the transcript synthesis or stability.

DISCUSSION

We have identified that ERH3 is required for the specification

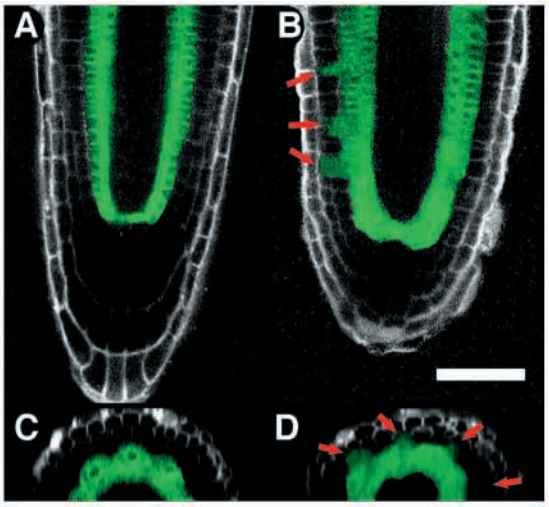

Fig. 6. Cortical- and endodermal-specific marker is ectopically expressed in the epidermis of *erh3* mutants. Expression of the *J0571::GFP* enhancer trap in wild-type (A,C) and *erh3-2* mutant (B,D) meristems. (A,B) Medial longitudinal sections. (C,D) Transverse sections. Red arrowheads indicate ectopic expression in epidermal cells. Images of GFP expression were superimposed over images of PI stained cell walls. Scale bar, 50 μm.

of cell identities in the *Arabidopsis* root. ERH3 activity is required for the specification of both non-hair and hair cells in the epidermis – mutants develop hair cells in non-hair positions and non-hair cells form in hair locations. Expression of lateral root cap-specific markers indicates that ERH3 is required for the specification of cell identities in the lateral root cap. The epidermal expression of cortical and endodermal markers in *erh3* mutants suggests that ERH3 may be required to repress cortical and endodermal cell identity in the epidermis. *ERH3* encodes an *Arabidopsis* katanin p60 homologue, a protein that is known to sever microtubules in animal cells (McNally and Vale, 1993). It is possible that EHR3 is required for a microtubule-dependent cell wall biosynthetic process that is involved in the spatial organisation of positional information

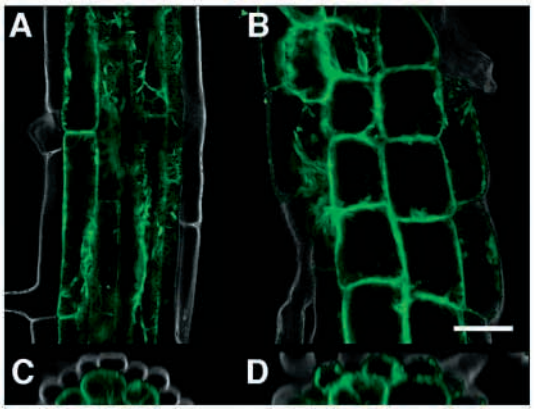

Fig. 7. A mature cortical cell-specific marker is ectopically expressed in the epidermis of *erh3* mutants. Expression of the *J0671::GFP* enhancer trap in wild type (A,C) and *erh3-2* mutants (B,D). (A,B) Longitudinal sections. (C,D) Transverse sections. Images of GFP expression were superimposed over images of PI stained cell walls. Scale bar 50 μm.

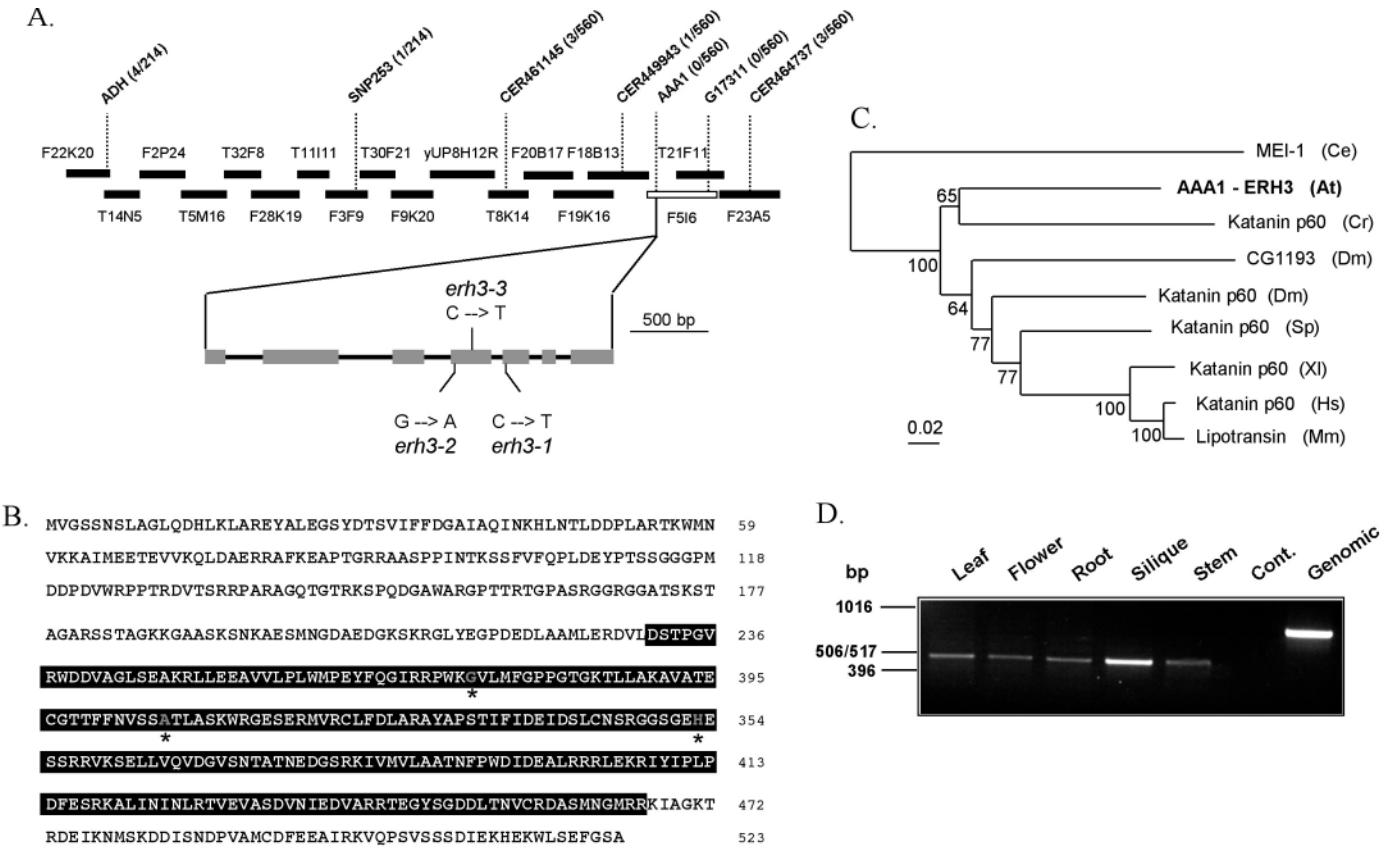

Fig. 8. Mapping and organisation of the *ERH3* gene encoding a p60 katanin-like protein. (A) *ERH3* is located on the south of chromosome 1. Adjacent SSLP and CAPS markers, with the number of recombinant chromosomes identified, are indicated. The position of BAC clones spanning this region is indicated. The *ERH3* gene is localised on BAC F5I6 (white box). The intron and exon organisation of the *ERH3* gene, and the *erh3-1*, *erh3-2* and *erh3-3* mutations are shown. (B) Deduced amino acid sequence of the *ERH3* gene product. The black box indicates the AAA domain. The mutated amino acids are indicated by asterisks. (C) Relationship analysis based on deduced amino acid sequence alignment of p60 katanin AAA domains using the neighbour-joining method (Thompson et al., 1997). The species origin of the sequence is indicated in brackets: Ce, *C. elegans*; At, *A. thaliana*; Cr, *C. reinhardtii*; Dm, *D. melanogaster*; Sp, *S. purpuratus*; Xl, *X. laevis*; Hs, *H. sapiens*; Mm, *M. musculus*. The GenBank accession number of the sequences are: At, AF048706; Ce,L25423; Cr, AF205377; Dm, AF223064, AE003601; Sp, AF052191; Xl, AF177942; Hs, AF056022; Mm, AF153197. Bootstrap values (100 replicates) are shown at the nodes. (D) Analysis of *ERH3* expression by RT-PCR. Total RNA was isolated from leaves, flowers, roots and siliques of wild-type plants. A PCR reaction on a sample lacking the RT matrice was used as a negative control (Cont). Wild-type genomic DNA was used as a positive control.

in the root. An alternative view is that microtubules are required for the orientation of spatial determinants of cell fate in epidermal cells and that ERH3 is required for this organisation.

Katanin proteins have been extensively characterised in sea urchins and *Caenorhabditis elegans* (McNally, 1998; Srayko et al., 2000). Katanins are heterodimeric proteins comprising a p60 subunit that severs microtubules and a p80 subunit that is required for the targeting of the katanin protein complex to its site of action within the cell (Hartman et al., 1998; McNally, 1998; McNally and Vale, 1993). Since katanin-mediated severing of microtubules increases the number of MT ends at which polymerisation or depolymerisation can take place, the dynamic state of mitotic microtubules may be at least in part dependent on katanin activity.

Defects in katanin activity result in the formation of abnormal spindles in *Caenorhabditis elegans* (Clark-Maguire and Mains, 1994; Srayko et al., 2000). The catalytic subunit p60 is encoded by *mei1*, and *mei2* encodes the p80 subunit. Dominant mutations in *mei1* result in the appearance of p60 katanin protein in mitotic cells, where the protein is normally absent. Spindles in these cells are shorter than wild type suggesting that the extra katanin activity in these cells results in the severing of the microtubules that constitute the spindle (Clark-Maguire and Mains, 1994). In addition, misexpression of *mei1* (katanin p60) and its partner *mei2* (p80) results in a reduction in the length of microtubules in HeLa cells (Srayko et al., 2000). Together these data suggest that katanins are active in severing of microtubules in a diverse range of animal cell types.

The defect in the pattern of epidermal cell differentiation in *Arabidopsis erh3* mutants suggests that the katanin complex is required during the specification of cell fate in the root epidermis. The altered *GL2::GUS* expression pattern in *erh3* mutants and the epistatic nature of the double mutant phenotypes suggests that ERH3 is active early and is required for the correct positioning of cues for cell identity. Mutations in katanin p60 have recently been isolated in *Arabidopsis* (Bichet et al., 2001; Burk et al., 2001). These alleles of *erh3* (*botero1* (*bot1*) and *fragile fibre2* (*fra2*)) have not been

characterised for defective specification of cell identities. As might be expected, the organisation of microtubules is altered in these mutants compared to wild type. Plants homozygous for the *fra2* mutation are slow to reorganise microtubules during cell elongation (Burk et al., 2001). As wild-type root cells elongate, perinuclear microtubules disappear and a largely transverse cortical array develops. This reorganisation is delayed in the *fra2* mutants and the resulting cortical arrays show defective cortical microtubule arrangements. The cell walls of *fra2* mutants are drastically different from those of wild type and contain little cellulose and hemicellulose (Burk et al., 2001). These microtubule and wall defects suggest that the katanin p60 is required for the reorganisation of the microtubule cytoskeleton, which is in turn necessary for the formation of the cell wall during cellular morphogenesis.

The defects in cell identity observed in *erh3* mutants indicate that katanin-dependent processes are involved in cell specification in the root. We can rule out the possibility that this mis-specification is a secondary consequence resulting from misalignment of cell walls in mutant roots: patches of cells with normal patterns of cell organisation occasionally form on mutant roots and exhibit the cell specification defects (see Table 2). It has previously been postulated, on the basis of clonal analysis and laser microsurgical experiments, that positional information directing cell fate in the root epidermis is organised in the cell wall with strict boundaries (Berger et al., 1998a). Since *erh3/fra2/bot1* mutants have been shown to have defective cell walls, it is formally possible that a katanin-dependent cell wall biosynthetic process is required for the stable fixation of positional cues that specify cell identity. According to this model, molecules conferring positional information would be incorporated into the wall in a spatially regulated manner that depends on the integrity of cell wall structure. Disruption in this structure results in mislocalisation of positional cues and consequent defects in the spatial pattern of cellular differentiation. Changes in the expression of marker genes in *erh3* mutants indicates that the p60 katanin requiring process is active in the specification of identities in other cell types including the lateral root cap, cortex and endodermis. It is therefore likely that positional cues specifying cell identity in these cell types are also located in the wall and that the defects in cellular specification result from a defective spatial organisation of the signals in the *erh3* mutant roots.

An alternative view is that microtubules are directly active in the specification of cell identity in the root and that disruption of the microtubules in *erh3* mutants results in the development of defective identities. The specification of cell identity and polarity has been shown to involve microtubules in a range of animal systems [for reviews see Gotta and Ahringer (Gotta and Ahringer, 2001) and Doe and Bowerman (Doe and Bowerman, 2001)]. For example, sperm asters play a critical role in the establishment of anterior-posterior polarity in the nematode zygote (O'Connell et al., 2000; Wallenfang and Seydoux, 2000). Disruption of normal aster formation results in the symmetrical distribution of proteins such as PAR-2 which are normally located in the posterior region of the embryo. This results in mis-specification of the anterior-posterior embryo axis. Similar mechanisms operate in the polarization of mammalian epithelial cells and the development of neuroblasts in insects [for review see Doe and Bowerman (Doe and Bowerman 2001)]. The mispecification of cell identities in *erh3* mutants suggests that an analogous mechanism involving microtubules may operate in plant cells.

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