Mol Gen Genomics (2003) 269: 350–360
DOI 10.1007/s00438-003-0843-1

ORIGINAL PAPER

B. Schwab · J. Mathur · R. Saedler · H. Schwarz
B. Frey · C. Scheidegger · M. Hülskamp

Regulation of cell expansion by the *DISTORTED* genes in *Arabidopsis thaliana*: actin controls the spatial organization of microtubules

Received: 5 December 2002 / Accepted: 19 March 2003 / Published online: 11 April 2003

Abstract The control of the directionality of cell expansion was investigated using a class of eight genes, the so-called *DISTORTED* (*DIS*) genes, that are required for proper expansion of leaf trichomes in *Arabidopsis thaliana*. By tracing the separation of latex beads placed on the trichome surface, we demonstrate that trichomes grow by diffuse rather than tip growth, and that in *dis* mutants deviations from the normal orientation of growth can occur in all possible directions. We could not detect any differences in intracellular organization between wild-type and *dis*-group mutants by electron microscopy. The analysis of double mutants showed that although the expression of the *dis* phenotype is generally independent of branching and endoreduplication, *dis* mutations act synthetically in combination lesions in the *ZWI* gene, which encodes a kinesin motor protein. Using a MAP4:GFP marker line, we show that the organization of cortical microtubules is affected in *dis*-group mutants. The finding that most *dis*-group mutants have actin defects suggested to us that actin is involved in organizing the orientation of microtubules. By analyzing the microtubule organization in plants treated with drugs that bind to actin, we verified that actin is involved in the positioning of cortical microtubules and thereby in plant cell expansion.

Keywords *DISTORTED* genes · Cell expansion · Cytoskeleton · Trichomes · Arabidopsis

Communicated by G. Jürgens

B. Schwab · M. Hülskamp
Zentrum für Molekularbiologie der Pflanzen (ZMBP),
Institut für Entwicklungsgenetik,
Universität Tübingen, 72070 Tübingen, Germany

J. Mathur · R. Saedler · M. Hülskamp (✉)
Institut für Botanik III, Universität zu Köln,
Gyrhofstr. 15, 50931 Köln, Germany
E-mail: martin.huelskamp@uni-koeln.de
Tel.: +49-221-4702473
Fax: +49-221-4705062

H. Schwarz
Max Planck Institut für Entwicklungsbiologie,
72076 Tübingen, Germany

B. Frey · C. Scheidegger
Schweizer Institut für Wald, Schnee und Landschaft,
8903 Birmensdorf, Switzerland

Present address: B. Schwab
Consortium für elektrochemische Industrie GmbH,
81379 München, Germany

Introduction

During differentiation, many plant cells undergo an enormous degree of expansion (Cosgrove 1997). Cell expansion requires the coordination of various processes including cell wall loosening, control of the turgor pressure necessary to provide the mechanical force for expansion, and the incorporation of new cell wall material (Carpita and Gibeaut 1993; Pritchard 1994; Cosgrove 1999). Because newly synthesized cellulose microfibrils are often coaligned with cortical microtubules (Green 1962), it has been proposed that microtubules are important for the spatial control of cellulose deposition, though various exceptions have been described which indicate that alternative mechanisms must exist (Baskin 2001). The importance of the cytoskeleton in expansion growth has been demonstrated by drug inhibitor experiments both in cells that show tip growth as well as in cells with diffuse growth zones. In tip-growing root hairs the application of actin-perturbing drugs causes the cessation of cytoplasmic streaming and root hair growth, suggesting that actin is required for the actual growth processes (Miller et al. 1999). Application of microtubule-perturbing drugs results in broadening of the very tip, wavy growth and the formation of new growth points, indicating that microtubules are important for maintaining the directionality of growth by localizing growth to the very tip (Miller et al. 1997; Bibikova et al. 1999). Similarly, diffusely growing cells respond to actin-perturbing drugs with reduced growth and to microtubule inhibitors with a change from

anisotropic growth to isotropic growth (Baskin et al. 1994; Baluska et al. 2001).

How microtubules become aligned and how the directionality of cell expansion is controlled is largely unknown. Several mutants have been described in Arabidopsis (*ton1*/ *fass*, *mor1*, *botero1*, *spk1*, *fra2* , *cobra*) that show cell elongation defects correlated with abnormalities in the organization of the microtubule cytoskeleton, and the cloning of some of these has revealed that they code for proteins with sequence similarities to microtubule-associated proteins (Traas et al. 1995; Bichet et al. 2001; Burk et al. 2001; Schindelman et al. 2001; Whittington et al. 2001; Qiu et al. 2002). The recent observation that the actin-perturbing drug latrunculin B causes a reorientation of microtubules has suggested that actin might be involved in the orientation of microtubules (Blancaflor 2000).

One goal of this work was to assess this possibility using actin-perturbing mutants that show an unexpected defect in the directionality of cell expansion in leaf hairs (trichomes) (Mathur et al. 1999; Szymanski et al. 1999). Normal trichome development is characterized by an initial expansion of the trichome cell from the epidermis, the subsequent initiation of two branch points and a phase of rapid cell expansion (Hülskamp et al. 1994; Schwab et al. 2000; Hülskamp 2000). Concomitant with this development is an increase in the DNA content, which results in a mature trichome having a DNA content of 32C. Treatment with drugs that affect microtubules leads to reduced branching (Mathur and Chua 2000). In addition, trichome cells exposed to such agents show isotropic growth, indicating a total loss of polar growth. Trichomes on plants treated with actin inhibitors show distorted shapes (Mathur et al. 1999; Szymanski et al. 1999). Branching is normal, but the proportions and directionality of growth are strongly disturbed. More than a dozen branching mutants have been described that have either more or fewer branches than normal (Marks 1997; Oppenheimer 1998; Hülskamp et al. 1999). Some of these mutants show a reduced DNA content and fewer branches or an increased DNA content and more branches in trichomes, suggesting that DNA content is correlated with branch number. In contrast to trichomes treated with microtubule inhibitors, none of the mutants showed isotropic growth. A second class of eight mutants exhibits trichomes with distorted growth and it has been shown that some of these have defects in actin organization.

In order to analyze further the role of the actin and microtubule cytoskeleton, we studied the eight *dis*-group mutants in more detail. We show that trichomes are diffusely growing cells, and that expansion growth is affected in *dis*-group mutants. Our genetic analysis revealed that the expression of the *distorted* phenotype is dependent on *ZWI*, a kinesin motor molecule, suggesting an interdependence of actin and microtubules in cell morphogenesis. This view was confirmed by the finding that the microtubule cytoskeleton itself is affected in plants exposed to actin-perturbing drugs, as well as in the *dis*-group mutants.

Materials and methods

Plant material and plant culture

The *Arabidopsis thaliana* Landsberg *erecta* ecotype was used as the wild-type reference. The following mutants have been described previously: *dis1-1*, *dis2-1*, *crk-EM1*, *ali-EM1*, *grl-EM1*, *klk-EM1*, *spi-EM1*, *wrm-EM1*, *sti-EMU*, *sti-40*, *sti-X44*, *nok-122*, *an-EM3*, *zwi-EM1*, *try-EM2* and *gl3-1* (Hülskamp et al. 1994; Folkers et al. 1997). Sixteen new *dis* mutants were isolated after mutagenesis with EMS in a screen of approximately 5000 single lines carried out as described previously (Hülskamp et al. 1994). The new mutants described in this study are: *dis2-195*, *dis2-36* , *grl-245*, *klk-73*, *spi-24*, *spi-143*, *spi-174*, *spi-89*, *spi-241*, *spi-273*, *spi-287*, *spi-291*, *spi-296, spi-302*, *wrm-54*, *wrm-305*. All plants were grown on soil under constant illumination at 25°C.

Double mutants with mutations in a *dis*-group gene and in a gene affecting branching or endoreduplication were constructed by preselecting F2 plants for the phenotype of the respective single mutant. Their self-progeny was scored for the phenotype of the *dis*-group mutant. Double mutants were confirmed by backcrosses with both parental lines.

Genetic mosaics were generated and analyzed as described previously (Hülskamp et al. 1994; Schnittger et al. 1996).

Molecular mapping of *DIS*-group genes

The map positions of *SPI*, *CRK*, *ALI* and *WRM* were determined by mapping of recombination breakpoints in the progeny of a cross between each *dis*-group mutant (Landsberg *erecta*) and wild type (Columbia-0). Details and PCR-conditions for all SSLP and CAPS markers are described in the Arabidopsis database (AtDB; http://www.arabidopsis.org). The recombination frequencies were calculated based on populations of 50 plants (*SPI*, *CRK*, *ALI*, *WRM*) and 120 plants (*KLK*), respectively: *SPI* lies 2 cM from nga59 and 8 cM from nga63; *WRM* maps 20 cM from nga162 and 30 cM from nga126; *CRK* lies 3 cM from GA1, 14 cM from nga8, and 20 cM from Det1; *ALI* is 2 cM from GA1, 12 cM from nga8, and 16 cM from Det1; *KLK* lies 9 cM from nga151, 5 cM from nga106, and 6.5 cM from NIT4.

Microbead labeling of trichomes and determination of expansion rates

To monitor the rate of distorted trichome growth, rosette leaves were dissected so that they were still attached to the hypocotyl. These were placed on a drop of MS agar in a depression slide, labeled with polylysine-coated latex beads (2 µm, Sigma 0280; Staebell and Soll 1985), immersed in water and covered with a coverslip. The rates of branch torsion were determined from micrographs taken immediately after applying the beads and after 12 h at 23°C. To determine the expansion rates along the longitudinal cell axis, the distances between between individual bead pairs were determined. Expansion rates were standardized by expressing the expansion between two beads as a percentage of the expansion growth of the whole branch. To enable a comparison between different branches, each trichome branch was subdivided into 10 equal intervals and expansion rates were determined within each of these intervals.

Results

Isolation and mapping of new *dis*-group mutations

The *DIS* group comprises eight genes: *DISTORTED1* (*DIS1*), *DISTORTED2* (*DIS2*), *CROOKED* (*CRK*),

SPIRRIG (*SPI*), *ALIEN* (*ALI*), *KLUNKER* (*KLK*), *WURM* (*WRM*) and *GNARLED* (*GRL*) (Hülskamp et al. 1994). Because most of the eight *DIS* complementation groups are represented by only one or two alleles, we performed a new screen for trichome mutants in order to search for new alleles and new complementation groups. We found 16 new *dis*-like mutants that were allelic to one or other of the known complementation groups. We identified two new *distorted2* (*dis2*) alleles, one new *gnarled* (*grl*) allele, one new *klunker* (*klk*) allele, two new *wurm* (*wrm*) alleles and 10 new *spirrig* (*spi*) alleles (see Materials and methods). In addition we have shown that the previously described *singed* mutation (Marks and Esch 1992) is an allele of the *SPI* locus.

Our molecular mapping of the *DIS*-group genes revealed distinct chromosomal map positions for each of the *DIS*-group genes. No apparent clustering on any particular chromosome was observed (Fig. 1).

Analysis of genetic interactions between *dis*-group mutants

The existence of eight *dis*-complementation groups whose mutants display similar phenotypes suggested to us that the corresponding genes act in the same molecular process. To test this genetically, we grouped the mutants into three classes according to the severity of their phenotypes, with *dis1* and *dis2* showing the strongest phenotype, *wrm*, *klk*, *cro*, *ali* and *grl* displaying a somewhat weaker phenotype, and *spi* showing the weakest phenotype. Double mutants were created between individual members of different groups or between members within a group (*dis1 klk*, *spi dis1*, *crk spi*, *crk dis2*, *crk wrm*, *spi wrm*). All double mutants displayed an additive phenotype (data not shown). These results suggest that the *dis*-group mutants analyzed indeed act in the same process and do not function in a redundant manner.

Analysis of genetic interactions between *dis*-group mutations and other mutations that cause defects in trichome morphogenesis

We created double mutants in which a *dis*-group mutation was introduced into representatives of two other classes of trichome morphogenesis mutants: branching mutants and endoreduplication/cell size mutants. Representative phenotypes are shown in Fig. 2 and branch counts for each of the mutant combinations are summarized in Table 1.

To test whether the distorted phenotype depends on the DNA content and/or the cell size, we combined *dis*-group mutations with *triptychon* (*try*) mutations (Fig. 2I and J) and with *glabra3* (*gl3*) mutations (Fig. 2K and L); *try* mutants have twice the DNA content of wild-type trichomes and have more branches, and *gl3*mutants have half the DNA content of wild-type trichomes and are less branched (Hülskamp et al. 1994). All double mutants had an additive phenotype, indicating that the expression of the distorted phenotype is independent of the DNA content/cell size.

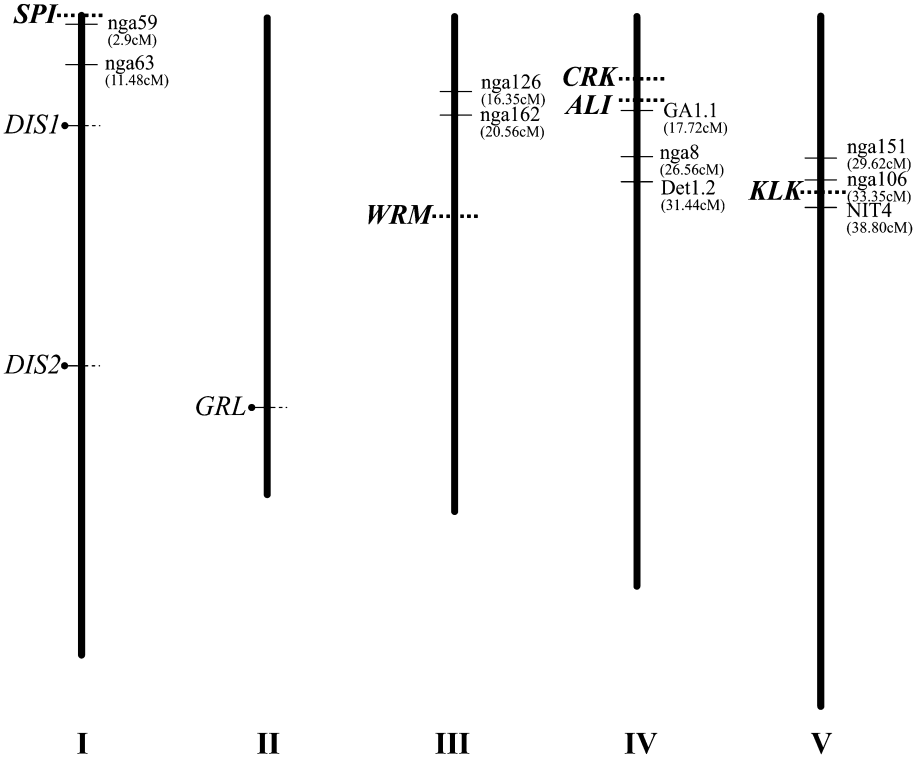
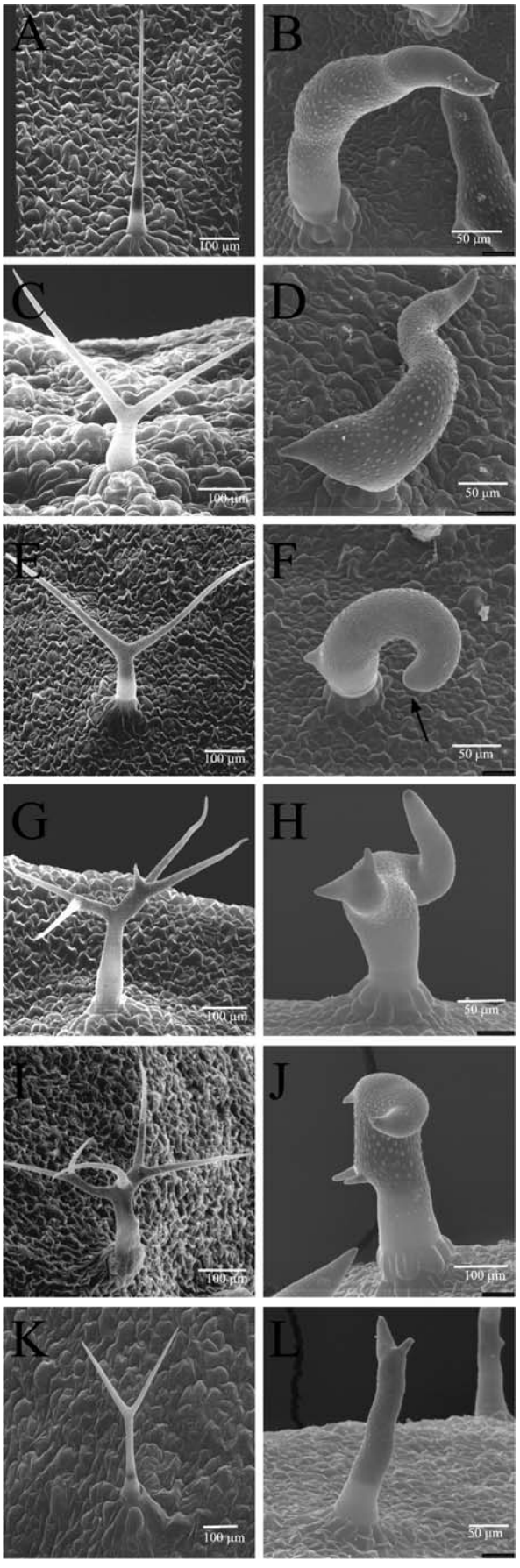


Fig. 1 Map positions of the *DIS*-group genes. The map positions of the markers according to the Arabidopsis database (AtDB; http://www.arabidopsis.org) are shown in *parentheses*. The mapped *DISa*-group genes are shown relative to the markers used for recombination mapping (for details see Materials and methods). The map positions for *DIS1*, *DIS2*, *KLK* and *GRL* have been published previously (Hülskamp et al. 1994)



◄

Fig. 2A–L Genetic interactions between *dis*-group and branching and endoreduplication/cell size mutants**.** Scanning electron micrographs of single and double mutants are shown. **A** *sti*. **B** *sti dis2*. **C** *an*. **D** *an wrm*. **E** *zwi*. **F** *zwi dis1*, note the blunted tip (*arrow*). **G** *nok*. **H** *nok crk*. **I** *try*. **J** *try klk*. **K** *gl3*. **L** *gl3 wrm*

Table 1 Number of branch points in single and double mutants

Mutant	Percentage of trichomes with the indicated number of branch points[a]							
	0	1	2	3	4	5	6	7
sti dis1	**97**	**3**	-	-	-	-	-	-
*sti-EMU*b	86	14	-	-	-	-	-	-
try dis1	-	**1**	**6**	**46**	**35**	**11**	**1**	
try-EMU1	-	*4*	*25*	*44*	*24*	*4*	-	-
nok dis1	-	-	**7**	**52**	**36**	**4**	**1**	-
*nok-122*b	-	-	*< 1*	*18*	*51*	*20*	*10*	*1*
an dis1	**3**	**96**	**1**	-	-	-	-	-
an-EM1	*2*	*97*	*1*	-	-	-	-	-
zwi dis1	**7**	**93**	-	-	-	-	-	-
zwi-EM1	*7*	*93*	*< 1*	-	-	-	-	-
gl3 dis1	**16**	**80**	**4**	-	-	-	-	-
gl3	*13*	*85*	*2*	-	-	-	-	-

[a]The values for the double mutants (shown in bold) were obtained in the present study: the other data are from Folkers et al. (1997)

Four representative trichome branching mutants were chosen that display either more branches such as *noeck* (*nok*), fewer branches such as *angustifolia* (*an*), no branches such as *stichel* (*sti*) or reduced branching combined with reduced branch growth such as *zwichel* (*zwi*). Double mutant combinations of *dis*-group mutants with *an*, *sti* and *nok* exhibited an additive phenotype (Fig. 2A, D, G and H). The number of initiated branches was unchanged as compared to the respective branching mutant and growth distortion was indistinguishable from that seen in *dis*-group mutants, suggesting that the *DIS*-group genes do not interact genetically with the branching genes. This was also observed for all *dis*-group *zwi* double mutants; however, in this case we also found a new phenotype (Fig. 2E and F). While in the single mutants the tips of branches were always pointed, the tip of the main branch in double mutants had a rounded, almost blunt form. This synthetic phenotype thus suggests a connection between the *DIS* and *ZWI* pathways.

Clonal analysis of *DIS*-group genes

In a previous study (Hülskamp et al. 1994), a clonal analysis of two *DIS* genes was reported. Starting from a heterozygous *dis* mutant plant, the wild-type allele was mutated by EMS mutagenesis in somatic sectors. Under these conditions, two aspects of the function of the respective genes could be analyzed: first, whether the genes acted cell autonomously, and secondly, whether more severe phenotypes were observed in small sectors. The latter would, for example, be expected if strong

mutations were lethal and only weak mutations were compatible with survival to give adult plants. Here we subjected alleles of the genes *CRK*, *ALI*, *WRM*, *GRL*, *KLK* and *SPI* to clonal analysis. Homozygous mutant sectors were generated by EMS mutagenesis of the respective heterozygous *dis*-group mutant seeds. Cells in which the single wild-type allele has undergone a mutation become homozygous mutant, and if the gene concerned acts locally, their descendants can be recognized as a distorted mutant sector (Fig. 3). Because sectors can only be recognized on the basis of mutant trichome cells, the resolution of this type of analysis is limited, though it was sufficient to classify *DIS1*and *DIS2*with respect to their local requirements (Hülskamp et al. 1994). In order to control the efficiency of the mutagenesis, we used double heterozygous combinations of the *dis*-group mutants with *stichel* (*sti*), which has been shown previously to act locally (Schnittger et al. 1996). Distorted mutant sectors were found for all six *dis*-group mutants (Table 2) suggesting that mutations in a group of cells could not be rescued by wild-type cells nearby. A more detailed analysis of the sectors revealed that the trichome phenotypes in mutant sectors were no stronger than those associated the corresponding *dis* germline allele. Thus, none of the newly induced mutations enhanced the phenotype. The assumption that strong alleles are incompatible with survival and the known alleles represent hypomorphic alleles is therefore unlikely to be true.

dis- group mutants affect the directionality of cell expansion

To determine whether trichomes are tip-growing cells or diffusely growing cells we applied 2 μm polylysine-coated latex beads to the surface of young trichome cells and determined growth rates using the beads as surface markers (Fig. 4A). The relative growth rates were calculated separately for 10 regions along the growth axis. As shown in Fig. 4B, growth was found along the whole cell axis. Although growth rates were higher in the tip region than at the base, the growth mode is clearly different to that of tip-growing cells, where growth is found exclusively at the very tip. Trichome cells can therefore be considered to be diffusely growing cells.

Growth of *dis*-group mutant trichomes is indistinguishable from wild type until branch initiation is complete (Fig. 5). Later stages are characterized by highly variable growth distortions. Trichome cells may bulge out, branches may twist around their axis and often only one branch elongates at the expense of the others. Except for the *spi* mutant, which has a slightly weaker phenotype, the phenotypic range was similar in all *dis*-group mutants.

The random growth of *dis*-group mutant trichomes suggested to us that they are affected in the directionality of cell expansion. To assess this, we used the latex-bead technique to monitor growth directionality in *dis*-group

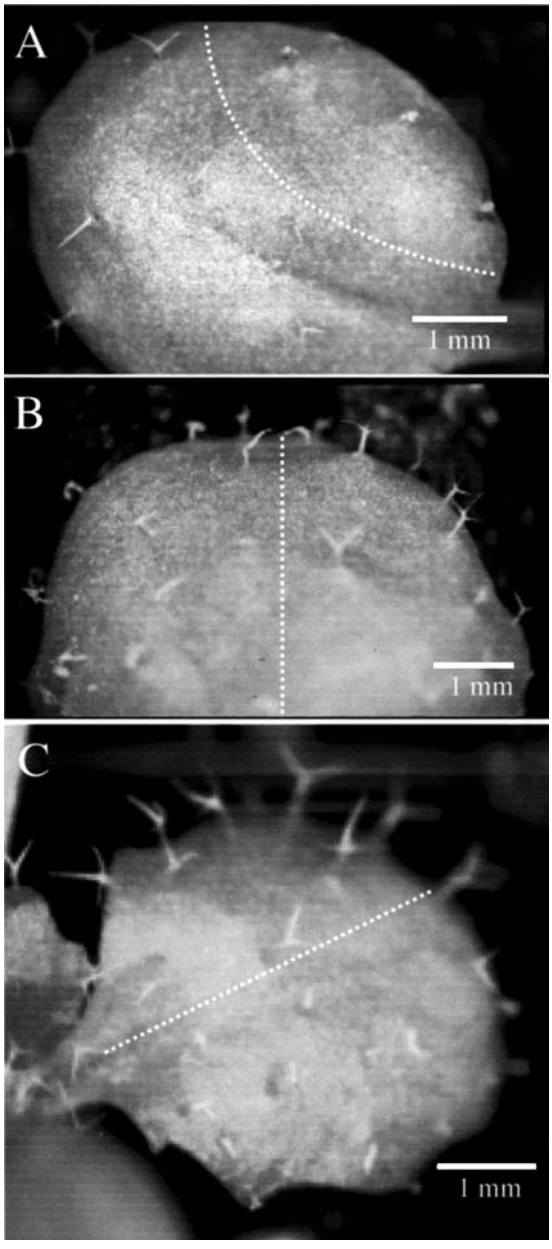


Fig. 3A–C Clonal analysis of *dis*-group mutants. Examples of *dis*-group mutant trichome sectors. The *dotted lines* mark clone boundaries. **A** An *ali* mutant trichome sector. **B** A *crk* mutant trichome sector. **C** A *spi* mutant trichome sector

Table 2 Frequency of distorted and stichel sectors

Genotype	Number of *dis* sectors	Number of *sti* sectors	Ratio of *dis* sectors to *sti* sectors
ali	21	5	4.2
klk	21	14	1.5
grl	10	6	1.6
spi	6	2	3.0
crk	6	17	0.4
wrm	6	11	0.5

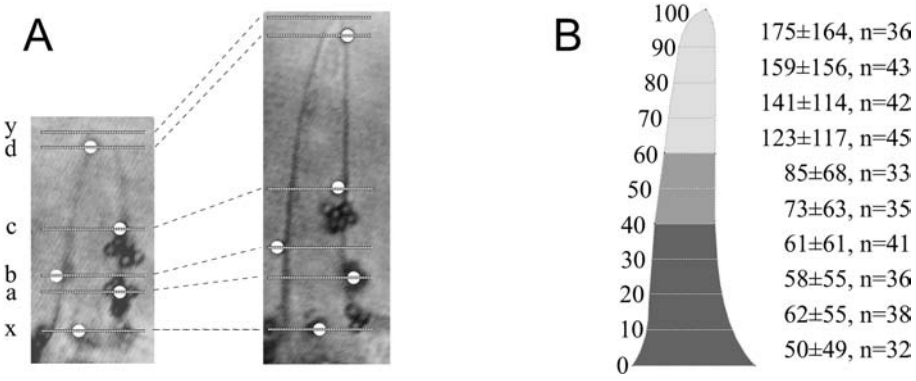
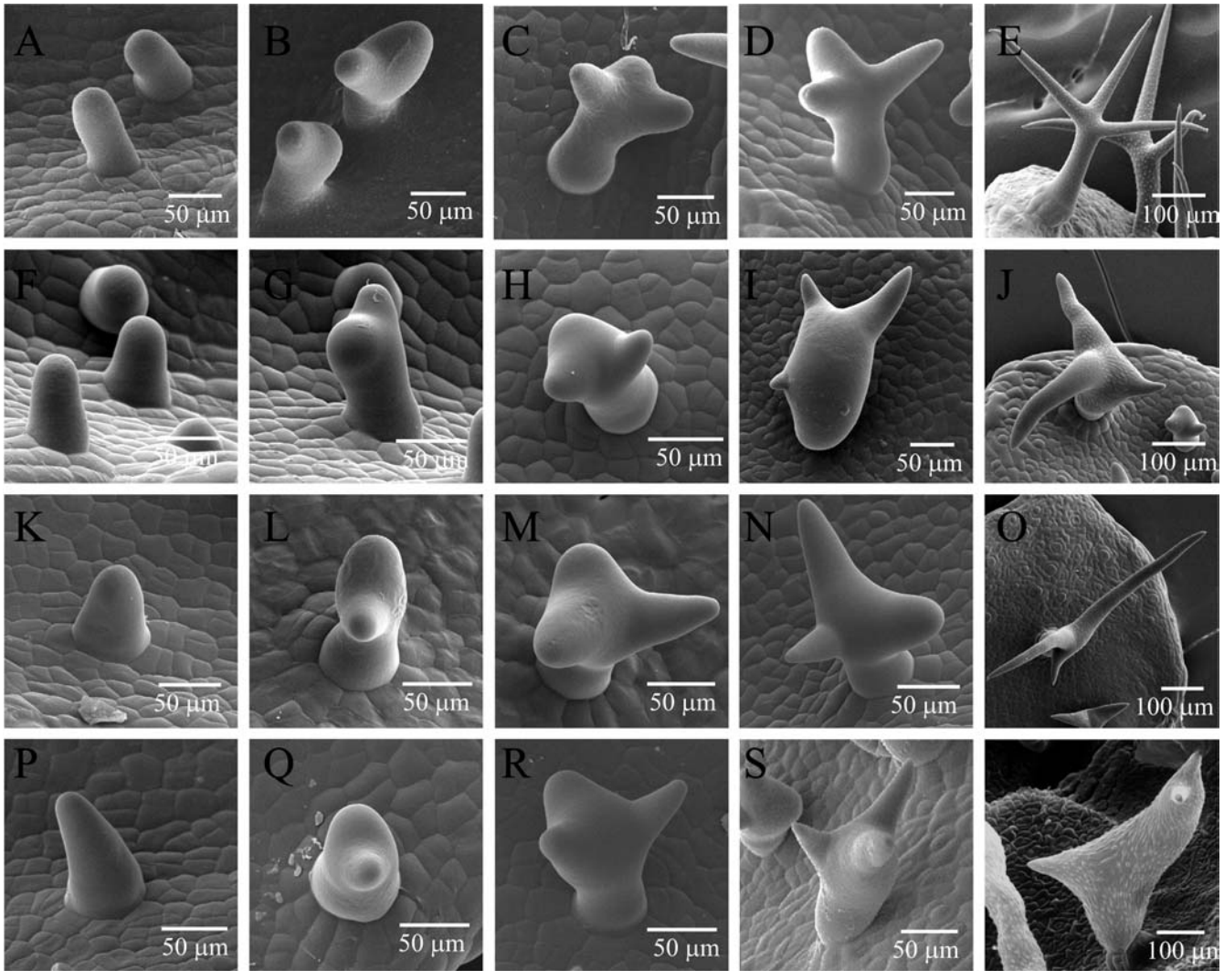


Fig. 4A, B Growth mode of trichomes. **A** The positions of individual latex beads (examples a,b and c marked in *white*) were mapped within a defined interval (x,y). Their positions are shown immediately after application (*left*) and 12 h later (right). **B** The percentage contribution (relative growth rates) to total growth of 10 separate regions along the leaf axis is plotted in a schematic representation

Fig. 5A–T Development of *dis*-group mutants. Scanning electron micrographs of various developmental stages are shown. **A–E** Wild type. **F–J** *crk* mutant. **K–O** *spi* mutant. **P–T** *wrm* mutant. The *first column* shows incipient trichomes before branching. The *second column* shows trichomes with one branch. In the *third column* trichomes are shown that already have three branches. Note that the first subtle deviations from wild type may be seen in these stages. The *fourth column* shows trichomes with elongated branches. Here, growth distortions are already quite pronounced. The *fifth column* shows mature trichomes

mutants. While in wild-type trichomes growth was strictly aligned with the growth axis, *dis*-group mutants showed various alterations (Fig. 6). First, the tip of the expanding branch remains unchanged and the region below begins to expand like a balloon in all directions perpendicular to the growth axis. In this situation beads move apart along the radial axis (Fig. 6A). Second, a rotation of the branch around its growth axis can occur,



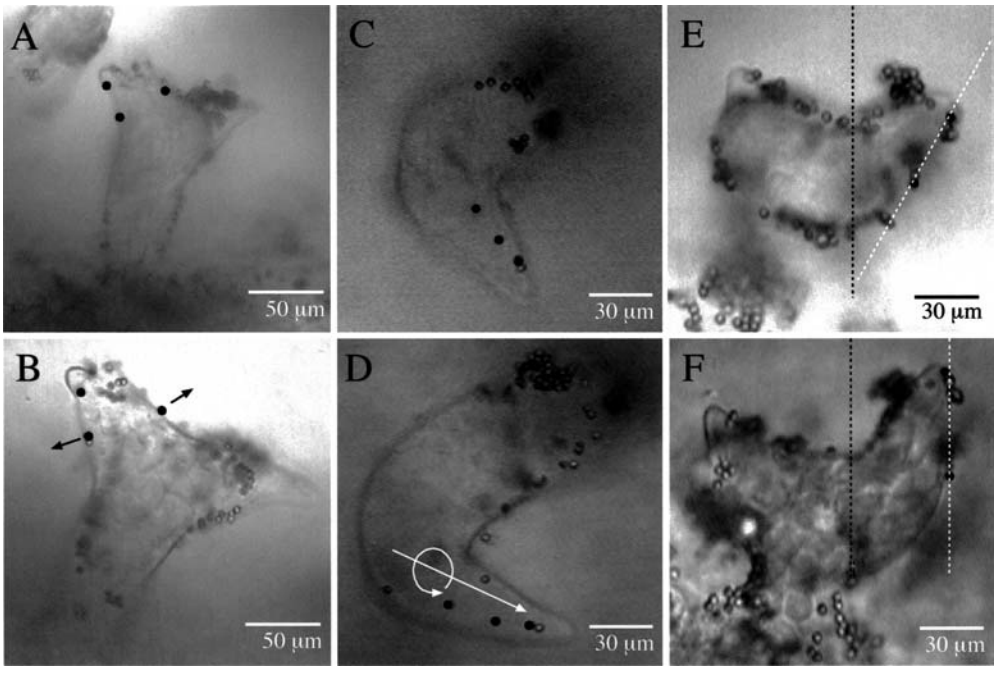


Fig. 6A–F Directionality of expansion growth in *dis*-group mutants. Trichomes labeled with latex beads are shown immediately after bead application (**A**, **C** and **E**) and 12 h later (**B**, **D** and **F**). **A, B** The trichome branch widens as indicated by the two marked latex beads moving apart. **C, D** The trichome branch rotates about its axis as revealed by the marked latex beads moving to the left. **E, F** The trichome branch bends toward the center as seen by the shift in the axis between two marked beads

and the three labeled beads move from left to right relative to the branch axis (Fig. 6B). Third, the whole branch bends away from the normal direction of growth (Fig. 6C). Generally, all these variations are observed in all mutants and on the same leaf, suggesting that they occur at random.

Interestingly, the pattern of papillae on the trichome cell surface is similar to that seen in our latex-bead experiments, suggesting that the positions of the papillae in the cell wall are laid down early in trichome morphogenesis and hence can be used as a marker for the morphogenetic ontogeny of the individual trichome cell (Fig. 5J and T).

dis2 but not *spi* mutants are affected in the organization of the actin cytoskeleton

In previous studies it has been shown that drug treatments that destabilize the actin cytoskeleton result in a phenocopy of *dis*-group mutants, and conversely that the *dis*-group mutants have defects in the organization of the actin cytoskeleton (Mathur et al. 1999; Szymanski et al. 1999). This has been shown for all *dis*-group mutants except *dis2*and *spi*. We used a transgenic GFP-talin line to analyze the actin organization in *dis2*and *spi* mutants. As shown in Fig. 7D, the actin organization in *dis2*mutants was aberrant, actin filaments were shorter than normal and not oriented longitudinally. In contrast, the actin cytoskeleton in *spi* mutants was indistinguishable from that of the wild type (Fig. 7B and C).

As the distortion of the actin cytoskeleton appears to be the primary defect in most of the distorted mutants, we wished to determine the earliest time point at which the actin defects could be recognized. For practical reasons, we used transgenic *crk* mutants containing the GFP-talin construct for this analysis, because this line exhibited the best labeling in our hands. The earliest time point during development at which we could recognize convincing deviations from wild type was after all branches had been initiated and when trichome branches were already expanding (Fig. 7E). This developmental stage roughly corresponds to the growth phase during which the distorted growth was observed in the bead experiments. As a certain degree of randomness is also observed in wild type, we lack clear-cut criteria that would allow us to rule out the possibility that subtle actin defects may occur even earlier.

Distortions of the actin cytoskeleton affect the organization of the microtubules

It is well established that changes in the directionality of cell expansion involve rearrangements of the cortical microtubules, and that these in turn direct rearrangements of cellulose microfibrils and thereby alter cell shape. We wanted to know whether the observed cell shape changes in *dis* mutants also involved changes in microtubule organization. In order to study the organization of the microtubule cytoskeleton directly we used a 35S:MAP:GFP line. As shown in Fig. 8A and B, microtubules in *dis*-group mutants did not show the typical longitudinal arrangement seen in wild type. Instead, cortical microtubules closely followed all twists and curves of the cell.

In order to demonstrate that this difference in microtubule organization is related to the actin phenotype of most of the distorted mutants and not related to other as yet undefined functions of the *DIS*-group genes, we

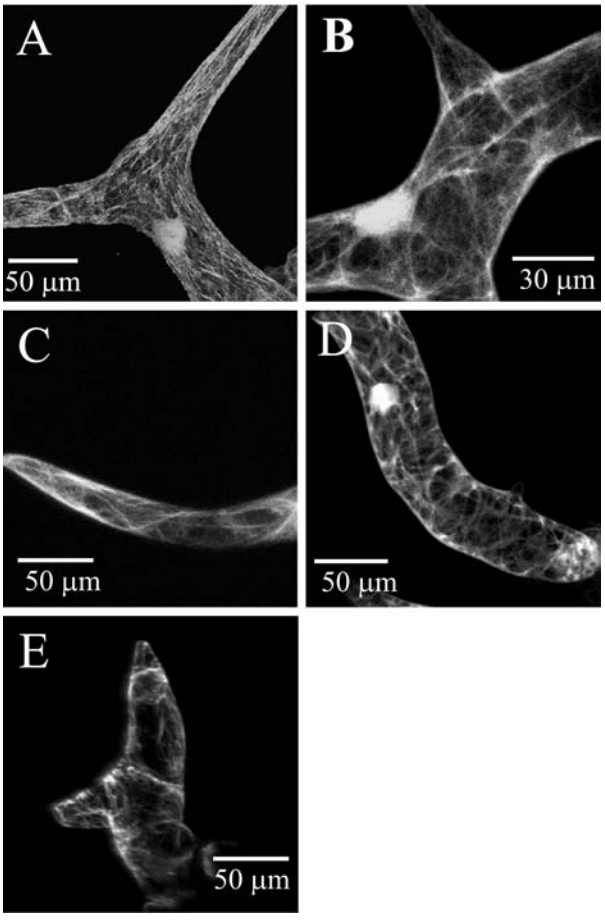


Fig. 7A–E Actin organization in *dis*-group mutants. Fluorescence micrographs of trichome cells in which the actin cytoskeleton was labeled by the incorporation of talin:GFP. **A** Wild-type branch of a trichome. **B** A *spi* mutant; central region of a trichome showing the nucleus. **C** A *spi* mutant trichome with an intact actin cytoskeleton. **D** A *dis2*mutant trichome. Note that the actin organization is aberrant. **E** A *crk* mutant; the youngest stage found to exhibit actin distortions

tested whether drug induced perturbation of actin also results in alterations in the organization of the microtubule cytoskeleton. Using the actin perturbing drug cytochalasin B we found that trichomes showed a distorted phenotype, and as was found in *dis*-group mutants, the microtubules closely followed the outlines of the cells (Fig. 8C and D).

Cell biological analysis of *dis*-group trichomes

Transmission electron microscopy was used to compare the distribution of cellular components in wild-type and *dis*-group trichomes (Fig. 9). The density of cortical microtubules was similar in wild type and *dis*-group mutants (Fig. 9D and H). Closely associated with the microtubules we found vesicles in both wild type and *dis*-group mutants (Fig. 9C and G). Also the thickness and structure of the cell wall was the same in wild-type and *dis*-group mutant cells.

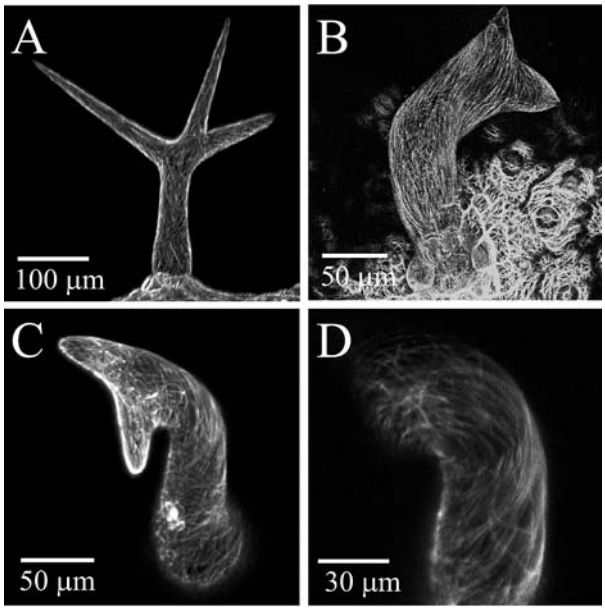


Fig. 8A–D Microtubule organization in *dis*-group mutant trichomes and cytochalasin B-treated trichomes. Fluorescence micrographs of trichome cells in which the microtubule cytoskeleton was labeled by the incorporation of GFP:MAP4. **A** Wild-type trichome. **B** A *dis2* mutant. Note that the microtubules closely follow the outlines of the trichome cells. **C** Cytochalasin B-treated wild-type trichome. **D** Higher magnification of **C**

Discussion

The organization of both fibrous actin and microtubules plays an important role in the growth of tip-growing as well as diffusely growing cells, though their relative contribution seems to be different in each case. While microtubules are involved in the spatial control of growth, actin seems to play a more general role in the actual process of growth (Miller et al. 1997; Bibikova et al. 1999; Baluska et al. 2001; Dong et al. 2001). It was therefore surprising to find that actin perturbations in trichomes resulted in a defect in growth directionality.

dis- group mutants are affected in the directionality of expansion growth

During trichome cell growth two growth phases can be distinguished (Hülskamp 2000). An initial growth phase during which cell form is established, including the formation of three branches, is followed by rapid cell expansion. *dis*-group mutants specifically affect the second growth phase. This is suggested by two observations. First, the genetic analysis indicates that the expression of the *dis*-group phenotype is independent of the branching and endoreduplication genes, which act earlier. Second, in *dis*-group mutants the first deviations from wild type are seen only after branch initiation is complete.

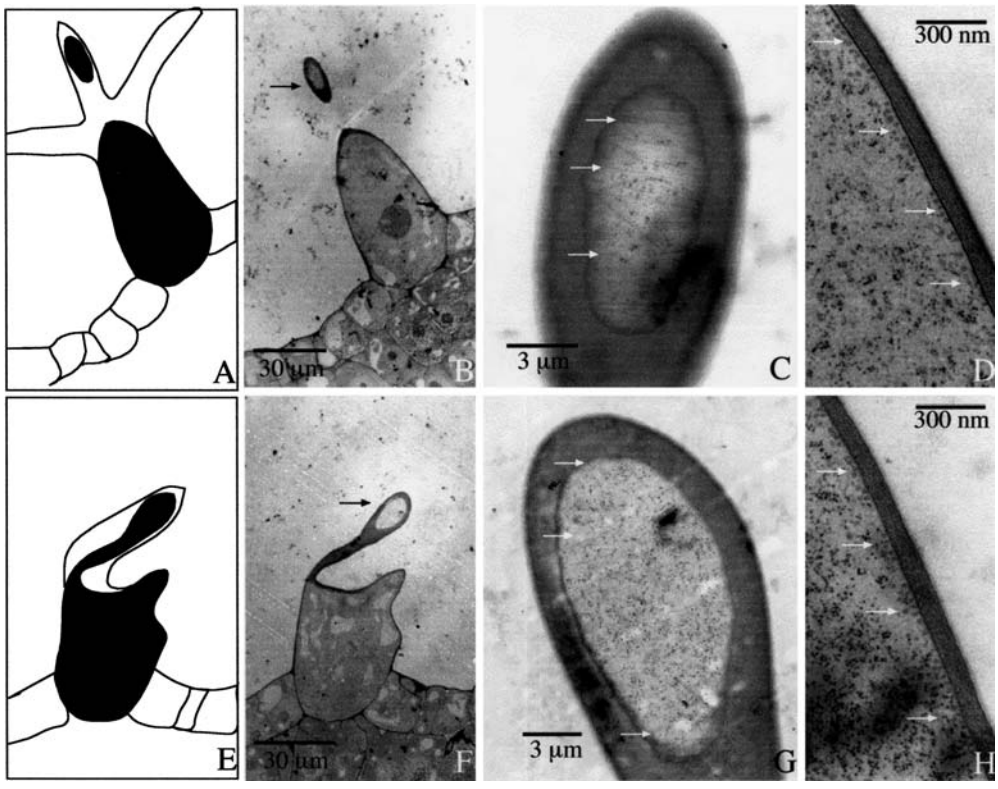


Fig. 9A–H Ultrastructural analysis of wild-type and *dis*-group mutant trichomes. Transmission electron micrographs of wild-type (**B–D**) and *dis2*mutant trichomes (**F–H**) are shown. **A** Schematic drawing depicting the section shown in **B** and **C** (shown in *black*) relative to the whole trichome. **B** Wild-type trichome showing the central body of the trichome cell. The *arrow* indicates the area shown at higher magnification in **C**. **C** Higher magnification of B. Note the cortical microtubules and vesicles (*arrows*). **D** Higher magnification of the cortical area. Note the cortical microtubules. **E** Schematic drawing showing the sections displayed in **F** and **G** (shown in *black*). **F** Central region of a *dis2*mutant trichome. The region shown at higher magnification in **G** is indicated by an *arrow*. **H** Higher magnification of a cortical region of a *dis2*mutant trichome. The *arrows* indicate microtubules

It has previously been proposed that trichomes grow by diffuse growth because late stages of trichome growth involve increases in both length and diameter (Szymanski et al. 1999). Although the finding that radial growth does not take place only at the tip showed, by definition, that the trichome cell grows by a diffuse growth mechanism, it remained unclear whether the enormous growth along the main growth axis takes place only at the tip or over the entire cell surface. We assessed the mode of cell expansion directly by labeling the cell surface with small latex beads and following their movement during cell growth. Our experiments clearly demonstrate that trichome expansion involves the whole cell body, and that the relative contribution of growth is approximately four-fold higher in the tip region than at the base of a trichome branch. Using the bead-labeling approach we could also resolve the growth distortions in the *dis*-group mutants in more detail. By focusing on a particular surface area we found that in the mutants expansion growth could be altered in all possible dimensions, indicating that expansion growth is randomly altered at the subcellular level.

The *dis*-group growth phenotype in root hairs and trichomes: a comparison

At least four mutants, *rhd3*, *rhd4*, *tip1*and *cow1*, have been identified that show a root-hair phenotype reminiscent of the trichome phenotype observed in *dis*-group mutants (Schiefelbein and Somerville 1990; Schiefelbein et al. 1993; Grierson et al. 1997; Ryan et al. 1998). Root hairs are wavy and vary in diameter. These mutants show no defects in leaf trichome morphogenesis, indicating that the control of the directionality of cell expansion differs between the tip-growing root hairs and the diffusely growing trichome cells. Only the *rhd3*mutant has been studied in more detail. The *RHD3*gene encodes a protein containing putative GTP-binding motifs, and it was shown that *rhd3*mutant root hairs have a smaller vacuole and an abnormal vesicle distribution in the tip region (Galway et al. 1997; Wang et al. 1997). As none of the eight distorted mutants shows a root hair phenotype under normal growth conditions, it is conceivable that the *DISTORTED* genes act in a different pathway from that mediated by the root-hair morphogenesis genes.

The roles of the actin and microtubule systems appear to differ in root hairs and trichomes, judging from the results of inhibitor experiments. In trichomes, both microtubules and actin are important for growth

directionality. In root hairs, microtubules are involved in restricting growth to the tip, whereas actin is absolutely required for growth as such. However, it has been observed that the actin perturbing drug latrunculin B has a more profound effect on the growth of pollen tubes than on cytoplasmic streaming, indicating that localized actin polymerization plays a role in growth and therefore actin distribution contributes to growth directionality (Vidali and Hepler 2001). Whether, as has been suggested for other actin-based growth systems (Mitchison and Cramer 1996), actin is involved in the positioning of microtubules in plants is unknown.

Does actin control the organization of microtubules in trichomes?

In maize roots, the application of the actin-perturbing drug latrunculin B results in the swelling of cells in the elongation zone (Blancaflor 2000). Concomitantly with this change from anisotropic to isotropic growth, the microtubules also reorient, forming oblique instead of transverse arrays, suggesting that actin is involved in the positioning of microtubules. In our study we made two observations that support the idea that actin is also involved in the spatial control of microtubules during trichome cell expansion or at least some aspects of it. The first observation is that all *dis*-group mutants show blunted tips when combined with mutations in the microtubule-based kinesin motor molecule ZWI (Reddy et al. 1996, 1997; Oppenheimer et al. 1997; Song et al. 1997). As all single mutants exhibit pointed tips, this phenotype is new. Such a genetic interaction is generally interpreted as indicating that the two genes act together in a given process. Given the molecular nature of ZWI, it is therefore attractive to speculate that actin and microtubules act together during cell expansion leading to tip formation. This interpretation is supported by the fact that the ZWI protein contains a domain with sequence similarity to the actin-binding protein talin, which suggests that ZWI is directly involved in cross-talk between microtubules and the actin cytoskeleton (Reddy 2001).

The second observation that supports a role for actin in microtubule organization during trichome cell expansion is that microtubule orientation is altered in *dis*-group mutants and also in trichome cells treated with actin-perturbing drugs. Because actin microfilaments are known to serve as intracellular tracks for secretory vesicle transport to the plasma membrane at growth sites (Fowler and Quatrano 1997; Schmidt and Hall 1998), it has been speculated that the distorted phenotype is caused by inappropriate intracellular trafficking (Mathur et al. 1999; Szymanski et al. 1999). Cell wall areas that receive more material would expand at the expense of other regions. In this scenario one would expect that cell form changes should not be linked to the organization of microtubules, such that cortical microtubules should be arranged longitudinally irrespective of the actual cell form. This is not the case, as is particularly obvious in situations where the branch twists like a corkscrew. Here one would expect that the twisting of the cell caused by inappropriate actin based intracellular transport would not be accompanied by a twisted microtubule network. In such situations, however, microtubules closely follow the outline of the cell, suggesting that actin is involved in the organization of microtubules. It would therefore be very exciting to perform a high resolution analysis of the relative position of actin microfilaments and microtubules during their reorientation in the course of trichome development.

Acknowledgements We would like to thank members of the lab for critically reading the manuscript. B.S. was supported by the Friedrich-Naumann-Stiftung. This work was supported by a grant to M.H. from the Volkswagen Stiftung. This work has been carried out in compliance with the current laws governing genetic experimentation in Germany.

References

Baluska F, Jasik J, Edelmann HG, Salajova T, Volkmann D (2001) Latrunculin B-induced plant dwarfism: plant cell elongation is F-actin dependent. Dev Biol 231:113–124

Baskin TI (2001) On the alignment of cellulose microfibrils by cortical microtubules: a review and a model. Protoplasma 215:150–171

Baskin TI, Wilson JE, Cork A, Williamson RE (1994) Morphology and microtubule organzation in Arabidopsis roots exposed to oryzalin or taxol. Plant Cell Physiol 35:935–942

Bibikova TN, Blancaflor EB, Gilroy S (1999) Microtubules regulate tip growth and orientation in root hairs in *Arabidopsis thaliana*. Plant J 17:657–665

Bichet A, Desnos T, Turner S, Grandjean O, Höfte H (2001) BOTERO1 is required for normal orientation of cortical microtubules and anisotropic cell expansion in Arabidopsis. Plant J 25:137–148

Blancaflor EB (2000) Cortical actin filaments potentially interact with cortical microtubules in regulating polarity of cell expansion in primary roots of maize (*Zea mays* L.). J Plant Growth Reg 19:406–414

Burk DH, Liu B, Zhong R (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell 13:807–828

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1–30

Cosgrove DJ (1997) Assembly and enlargement of the primary cell wall in plants. Annu Rev Cell Dev Biol 13:171–201

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev Plant Physiol 50:391–417

Dong CH, Xia GX, Hong Y, Ramachandran S, Kost B, Chua N-H (2001) ADF proteins are involved in the control of flowering and regulate F-actin organization, cell expansion, and organ growth in Arabidopsis. Plant Cell 13:1333–1346

Folkers U, Berger J, Hülskamp M (1997) Cell morphogenesis of trichomes in *Arabidopsis*: differential regulation of primary and secondary branching by branch initiation regulators and cell size. Development 124:3779–3786

Fowler JE, Quatrano RS (1997) Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. Annu Rev Cell Dev Biol 13:697–743

Galway ME, Heckman J, Schiefelbein JW (1997) Growth and ultrastructure of *Arabidopsis* root hairs: the *rhd3*mutation alters vacuole enlargement and tip growth. Planta 201:209–218

Green PB (1962) Mechanism for plant cellular morphogenesis. Science 138:1404–1405

Grierson CS, Roberts K, Feldmann K, Dolan L (1997) The *COW1*locus of Arabidopsis acts after *RHD2*, and in parallel with *RHD3*and *TIP1*, to determine the shape, rate of elongation, and number of root hairs produced from each site of hair formation. Plant Physiol 115:981–990

Hülskamp M (2000) How plants split hairs. Curr Biol 10:R308–R310

Hülskamp M, Misera S, Jürgens G (1994) Genetic dissection of trichome cell development in *Arabidopsis*. Cell 76:555–566

Hülskamp M, Folkers U, Schnittger A (1999) Trichome development in *Arabidopsis thaliana*. Int Rev Cytol 186:147–178

Marks MD (1997) Molecular genetic analysis of trichome development in *Arabidopsis*. Annu Rev Plant Physiol Plant Mol Biol 48:137–163

Marks MD, Esch JJ (1992) Trichome formation in *Arabidopsis* as a genetic model for studying cell expansion. Curr Top Plant Biochem Physiol 11:131–142

Mathur J, Chua N-H (2000) Microtubule stabilization leads to growth reorientation in *Arabidopsis thaliana* trichomes. Plant Cell 12:465–477

Mathur J, P. Spielhofer P, Kost B, Chua N-H (1999) The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in *Arabidopsis thaliana*. Development 126:5559–5568

Miller DD, Norbert CA, de Ruijter NCA, Emons AMC (1997) From signal to form: aspects of the cytoskeleton-plasma membrane-cell wall continuum in root hair tips. J Exp Bot 48:1881–1896

Miller DD, Bisseling T, Emons AMC (1999) The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin pertubing drug. Plant J 17:141–154

Mitchison TJ, Cramer LP (1996) Actin-based cell motility and cell locomotion. Cell 84:371–379

Oppenheimer D (1998) Genetics of plant cell shape. Curr Opin Plant Biol 1:520–524

Oppenheimer DG, Pollock MA, Vacik J, Szymanski DB, Ericson B, Feldman K, Marks MD (1997) Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis. Proc Natl Acad Sci USA 94:6261–6266

Pritchard J (1994) The control of cell expansion in roots. New Phytologist 127:3–26

Qiu JL, Jilk R, Marks MD, Szymanski DB (2002) The Arabidopsis *SPIKE1*gene is required for normal cell shape control and tissue development. Plant Cell 14:101–118

Reddy AS (2001) Molecular motors and their functions in plants. Int Rev Cytol 204:97–178

Reddy AS, Narasimhulu SB, Safadi F, Golovkin M (1996) A plant kinesin heavy chain-like protein is a calmodulin-binding protein. Plant J 10:9–21

Reddy ASN, Narasimhulu SB, Day IS (1997) Structural organization of a gene encoding a novel calmodulin-binding kinesin-like protein from *Arabidopsis*. Gene 204:195–200

Ryan E, Grierson CS, Cavell A, Steer M, Dolan L (1998) *TIP1*is required for both tip growth and non-tip growth in *Arabidopsis*. New Phytologist 138:49–58

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

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

Schindelman G, Morikami A, Jung J, Baskin TI, Carpita NC, Derbyshire P, McCann MC, Benfey PN (2001) *COBRA* encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. Genes Dev 15:1115–1127

Schmidt A, Hall MN (1998) Signaling to the actin cytoskeleton. Annu Rev Cell Dev Biol 14:305–338

Schnittger A, Grini PE, Folkers U, Hülskamp M (1996) Epidermal fate map of the *Arabidopsis* shoot meristem. Dev Biol 175:248–255

Schwab B, Folkers U, Ilgenfritz H, Hülskamp M (2000) Trichome morphogenesis in Arabidopsis. Phil Trans R Soc Lond B 355:879–883

Song H, Golovkin M, Reddy ASN, Endow SA (1997) In vitro motility of AtKCBP, a calmodulin-binding kinesin protein of *Arabidopsis*. Proc Natl Acad Sci USA 94:322–327

Staebell M, Soll DR (1985) Temporal and spatial differences in cell wall expansion during bud and mycelium formation in *Candida albicans*. J Gen Microbiol 131:1467–1480

Szymanski DB, Marks MD, Wick SM (1999) Organized F-actin is essential for normal trichome morphogenesis in Arabidopsis. Plant Cell 11:2331–2348

Traas J, Bellini C, Nacry P, Kronenberger J, Bouchez D, Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375:676–677

Vidali L, Hepler PK (2001) Actin and pollen tube growth. Protoplasma 215:64–76

Wang H, Lockwood SK, Hoeltzel MF, Schiefelbein JW (1997) The *ROOT HAIR DEFECTIVE3*gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in *Arabidopsis*. Genes Dev 11:799–811

Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613