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# The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in *Arabidopsis thaliana*

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## SUMMARY

***Arabidopsis thaliana*** **trichomes provide an attractive model system to dissect molecular processes involved in the generation of shape and form in single cell morphogenesis in plants. We have used transgenic** ***Arabidopsis*** **plants carrying a** ***GFP-talin*** **chimeric gene to analyze the role of the actin cytoskeleton in trichome cell morphogenesis. We found that during trichome cell development the actin microfilaments assumed an increasing degree of complexity from fine filaments to thick, longitudinally stretched cables. Disruption of the F-actin cytoskeleton by actin antagonists produced distorted but branched trichomes which phenocopied trichomes of mutants belonging to the 'distorted' class. Subsequent analysis of the actin cytoskeleton in trichomes of the distorted mutants,** ***alien*****,** ***crooked*****,** ***distorted1*****,** ***gnarled*****,** ***klunker*** **and** ***wurm*** **uncovered actin organization defects in each case. Treatments of wild-type seedlings with microtubule-interacting drugs elicited a radically different trichome phenotype characterized by isotropic growth and a severe inhibition of branch formation; these trichomes did not show defects in actin cytoskeleton organization. A normal actin cytoskeleton was also observed in trichomes of the** ***zwichel*** **mutant which have reduced branching.** ***ZWICHEL,*** **which was previously shown to encode a kinesin-like protein is thought to be involved in microtubule-linked processes. Based on our results we propose that microtubules establish the spatial patterning of trichome branches whilst actin microfilaments elaborate and maintain the overall trichome pattern during development.**

Key words: Trichome, Actin, Cytoskeleton, Morphogenesis, *Arabidopsis thaliana*

## INTRODUCTION

Single cell systems frequently offer a convenient way to dissect the complex phenomenon of cell morphogenesis. In plants, three attractive models have become established, namely, root hairs, pollen tubes and trichomes. Root hairs and pollen tubes which grow predominantly at the tip develop into simple, tubular and relatively uncomplicated structures. Trichome cells, on the other hand, progress through a complex series of morphogenetic events before achieving their final stellate form (Marks, 1997; Huelskamp et al., 1998). We are interested in the role of the cytoskeleton in the generation and maintenance of plant cell shape and form. Whereas the involvement of cytoskeletal elements in polarized tip growth of pollen tubes and root hairs has been actively investigated (Cai et al., 1997; Miller et al., 1997; Franklin-Tong, 1999; Kost et al., 1999; Bibikova et al., 1999), the role of the cytoskeleton in trichome morphogenesis has not been explored. There is some evidence, based on genetic and molecular studies of the *zwichel* mutant, that microtubule function is required for normal trichome development (Oppenheimer et al., 1997; Oppenheimer, 1998).

*Arabidopsis thaliana* produces both unbranched and branched trichomes during its vegetative and reproductive growth phases. Unbranched trichomes are found primarily on leaf petioles, inflorescence-stems and floral sepals, whereas the majority of trichomes on leaves possess 3-4 branches. Based on morphological criteria the development of *Arabidopsis* trichomes has been divided into a number of stages (Szymanski et al., 1998). These stages (Fig. 1A), include the initiation of a trichome precursor cell (stage 1), its growth into a tubular form (stage 2), followed by the splitting of the tube into a branched structure (stage 3), extension of the branches and their proper orientation with regard to the proximodistal axis of the leaf blade (stages 4, 5), and maturation of the trichome (stage 6). As the trichome cell progresses through these 6 developmental stages it undergoes radical changes in shape, size and spatial-patterning suggesting the requirement of multiple gene activities. It is therefore not surprising that at least 36 genes have been implicated in trichome morphogenesis (Huelskamp et al., 1999), and a large collection of *Arabidopsis* mutants affecting different aspects of trichome morphogenesis has been generated (Huelskamp et al., 1994; Marks, 1997). The various mutants can be broadly

categorized as those affecting trichome initiation, trichome growth/development and trichome maturation.

*Arabidopsis* mutants altered in trichome growth and development can be further sub-divided into several classes which show specific defects in branch initiation, branch number, trichome size and/or shape. An entire class of 8 morphological mutants characterized by a common 'distorted' trichome phenotype has been described (Feenstra, 1978; Huelskamp et al., 1994). Although the mechanism responsible for the mutant phenotype is unknown, cytoskeletal malfunctioning leading to defective wall deposition (Huelskamp et al., 1994), and/or mis-regulation of wall loosening events necessary for cell expansion (Oppenheimer et al., 1993), have been proposed as possible causes.

The paucity of information on the role of the cytoskeleton in trichome morphogenesis prompted us to characterize, in detail, changes in the actin cytoskeleton during trichome morphogenesis of *Arabidopsis thaliana* using a GFP-talin fusion protein as an in vivo marker for F-actin (Kost et al., 1998). We found that the 'distorted' trichome phenotype (Huelskamp et al., 1994) can be recapitulated by treating wild-type (WT) seedlings with a number of actin inhibitors. Consistent with this finding the actin cytoskeleton in 6 mutants belonging to the 'distorted' class displayed varying degrees of aberrations in their organization. Microtubule inhibitors, on the other hand, produced unbranched trichomes which contained a normal actin cytoskeleton. Taken together, our observations provide insights into the role of the actin cytoskeleton in extension growth of trichome branches and define the relative roles of microfilaments and microtubules during trichome morphogenesis.

## MATERIALS AND METHODS

### Plant materials and growth conditions

*Arabidopsis thaliana* (ecotype Landsberg) was used for this study. Transgenic lines expressing *35S-GFP-talin* were generated previously (Kost et al., 1998). Seeds of mutants belonging to the 'distorted' trichome class and the *zwichel* mutant were provided by Dr Martin Huelskamp, Tuebingen, Germany and Dr D. Oppenheimer, Tuscaloosa, USA, respectively. Crosses were made between a *35S-GFP-talin* line (pBA005#50) and all trichome mutants, which served as female recipients. Trichome mutants carrying the *35S-GFP-talin* transgene were selected from T3 progeny and used for F-actin analysis.

Seeds were surface sterilized with 15% bleach and washed three times with sterile deionized water. After the final wash, the seeds were plated on MS medium (Murashige and Skoog, 1962; Sigma Co. M5524) supplemented with 3% sucrose and 0.2% Phytagel (Sigma, P-8169). Plates were incubated at 4°C for 2 days and then placed in a tissue culture room at 22-25°C, under 16-hour light/8-hour dark conditions.

### Treatment with inhibitors

Twenty seedlings (4- to 6-day old) with first pair of open primary leaves were placed on a 1 cm wide sterilized Whatman filter paper strip in a 5 cm plastic Petri dish containing 2 ml of liquid MS medium. Actin antagonists, Cytochalasin D (0.1, 1, 5 and 10 μM; Sigma), Latrunculin B (0.1, 1, 2 and 5 μM; Calbiochem), Phalloidin (5 and 10 μM; Sigma), Jasplakinolide (1, 5 and 10 μM; Molecular Probes) and microtubule inhibitors, Oryzalin (1 and 10 μM; Reidel Co. Germany), Colchicine (10, 100 and 1000 μM; Sigma), Propyzamide (5 and 10 μM, Sumitomo Chemical Co, Japan) and Paclitaxel (1 and 10 μM; Calbiochem) were used. The different concentrations of actin-microfilament- or microtubule-interacting drugs were added directly to the liquid MS medium. Because trichomes develop continuously over a period of days in an asynchronous manner, we used drugs at minimum concentrations that would allow an observable effect within a maximum period of 6 days from the day of the treatment. Plates were kept upright in a tissue culture room. Plants were observed at 1, 2, 4 and 6 days after placing in the different solutions. As controls, seedlings were placed in liquid MS medium containing 0.1, 1.0, 10.0 μl of DMSO or absolute ethanol, the solvents used for most inhibitors served as controls.

To observe the recovery of plants following Lat-B treatments, drug-treated seedlings were washed with liquid MS medium 3 times and then placed on plates containing fresh MS basal medium. Another set of seedlings was maintained on the drug-containing medium as controls. Observations on recovery were made from 24 hours onwards, after the transfer. All experiments were repeated at least four times.

### Microscopy and image processing

In any given experiment, one half of the samples were randomly chosen, placed on double-sided sticky tape, frozen in liquid nitrogen and scanned at 30 Pascal partial pressure at 15 KV with a scanning electron microscope (JEOL-JSM-5310-LV; Ahlstrand, 1996). The remaining plants were observed using a Kr/Ar laser confocal microscope (Biorad MRC-1024). Images were stored as PICT files and processed using the Adobe Photoshop 3.0 programme (Adobe Systems Inc. Mountain View, CA).

## RESULTS

### Changes of the actin cytoskeleton during *Arabidopsis* trichome development

The availability of *Arabidopsis thaliana* transgenic lines carrying a chimeric *GFP-talin* gene (Kost et al., 1998) facilitated the visualization and analysis of F-actin in living trichome cells during their morphogenesis. In the present study the homozygous *GFP-talin* transgenic line pBA005#50 was used as the wild type (WT) with respect to trichome development. Trichomes develop asynchronously over a period of 3 to 4 days on *Arabidopsis* leaves (Marks and Esch, 1994). Fig. 1A shows the stages of development. At the initiation stage, a committed cell expands radially relative to its neighboring epidermal cells and attains a swollen appearance (stage 1; Fig. 1A, 1). This stage-1 cell on the leaf epidermis shows a relatively diffuse actin cytoskeleton, increased nuclear size and a perinuclear actin cage (Fig. 1B). After the first radial expansion the trichome precursor cell initiates growth in a direction nearly perpendicular to the epidermal plane (Fig. 1A, stages 2 and 3). The F-actin organization appears quite diffuse in stage 2 trichomes (Fig. 1B), but tip directed actin filaments become organized during the next stage. These appear to radiate upwards from the nucleus, which at this stage, is still located in the basal half of the cell (Fig. 1C). At the next stage, two foci of F-actin congregation are formed which mark the primary branch initiation points and lead the cell into stage 4 of development (Fig. 1A, stage 4). A marked increase in the size of the subapical vacuolar compartment as well as an expanding vacuole in the stalk region are seen at stages 3 and 4. The foci denoting branch point initials display organized actin bundles which connected the initials to the perinuclear cage (Fig. 1C). During the subsequent rapid extension phase, trichomes display a sharp tip with high fluorescence and characteristic cortical and sub cortical F-actin organization (Fig. 1D,E,F). A fine cortical actin mesh girdles the trichome stalk and branches (Fig. 1D,E). The long F-actin cables exhibit a typically unidirectional orientation with respect to the

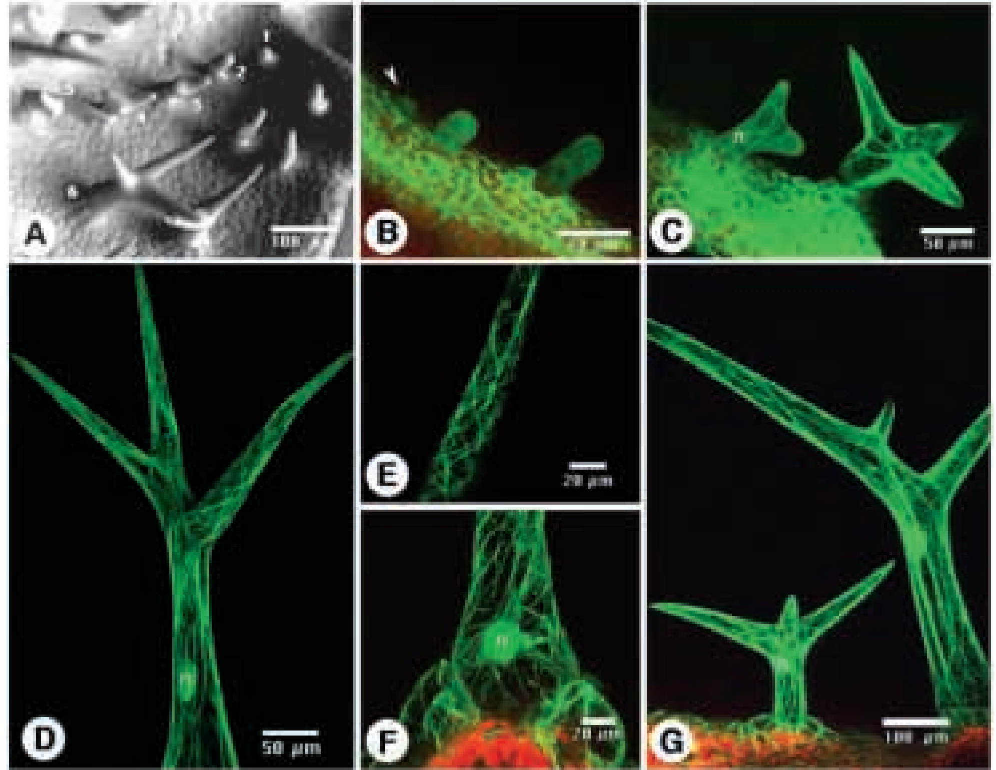


**Fig. 1.** Trichome development on an *Arabidopsis* leaf and the actin cytoskeleton at different stages of development. (A) Stages (1 to 6) in trichome development on WT leaf. Stage 1 denotes the initiation of a trichome precursor cell whereas stage 6 indicates a mature trichome cell with characterstic papillate surface decorations. (B) WT trichomes exhibit a diffuse actin cytoskeleton during stages 1 and 2 of development. Arrowhead points to an emerging trichome initial on the leaf epidermis. (C) Stages 3 and 4 of trichome development in WT is characterised by initiation of branches and their extension growth. F-actin bundles stretch longitudinally, appearing to cross at the perinuclear cage (n; stage 3) and the extending branch tip. Actin bundles become conspicuous and further branch extension and secondary branching occurs during stage 4. (D) Prominent sub-cortical F-actin cables stretched parallel to the long axis of the trichome during stage 5. The nucleus (n) may occupy a position in the stalk or higher up (G). (E) Cortical F-actin filaments girdle the branch tip. (F) Sub-cortical actin cables in an unbranched trichome cell with a basally placed nucleus (n). (G) General F-actin organization in a mature (stage 6) WT trichome exhibits strong, regular, F-actin cables stretching longitudinally, and a fine cortical meshwork.

long axis of the developing trichome (Fig. 1D). A similar actin organization was seen in unbranched trichomes as well (Fig. 1F).

Overall, during the different morphogenetic stages, the cortical F-actin strands maintained their meshwork appearance (Fig. 1D,E) whereas the subcortical F-actin cytoskeleton displayed an increasing level of complexity with successive stages of development. Thus, trichomes at early developmental stages (1 and 2) showed relatively short actin microfilaments, which became organized into thicker bundles as the trichome matured into stage 3. At stages 4 and 5 the actin cytoskeleton consisted predominantly of longitudinally stretching F-actin cables (Fig. 1G). These observed changes in F-actin arrays during the different stages of trichome development prompted us to speculate that perturbing the actin organization might have an effect on trichome morphogenesis. We tested this hypothesis by treating seedlings with various actin interacting drugs.

### Treatment with actin interacting drugs distorts trichomes

Four well characterized actin interacting drugs; cytochalsin-D (CD), latrunculin-B (Lat-B), phalloidin (Pha) and jasplakinolide (Jas) were used to treat seedlings of *GFP-talin* transgenic plants. Considering the non-synchronous and continuous emergence of trichomes on a leaf, a range of inhibitor concentrations was used to determine the lowest concentration that could elicit an effect within a 6-day period. CD concentrations lower than 1 μM failed to evoke visible abnormalities in trichome cell morphology or the actin cytoskeleton during this period. Treatment with 1-5 μM CD or 1-2 μM Lat-B for 48 hours or longer resulted in distorted trichomes, with no effect on branch initiation (Fig. 2A). Similar trichome distortions were observed upon treatment with 5-10 μM Pha or Jas (Fig. 2C,D). Control treatments with ethanol/DMSO did not elicit any trichome phenotypes.

The actin disorganization evoked by the two inhibitors CD and Lat-B was different between 6 and 24 hours. Early in the treatment, Lat-B-treated trichome cells showed a diffuse actin pattern (Fig. 2G), whereas short, thick rods or bundles were apparent even at an early stage of treatment in CD-treated trichomes (Fig. 2B). After 48 hours of treatment, however, short actin bundles were common features in both drug-treated trichomes. Furthermore, peripheral actin bundles were conspicuously absent from these trichomes which contained short actin bundles randomly scattered in the cytoplasm without any apparent connections to the plasma membrane, as were observed in untreated WT controls (Fig. 1C,D,E,F,G). Considering the asynchronous nature of trichome formation and development on a leaf, the final morphology of a trichome cell reflected the developmental stage of a particular cell at the time of drug application. Trichomes that had already passed stage 5 and embarked on the maturation phase of their development merely displayed a slight bending or waviness within 36 hours of treatment, even though a disrupted actin cytoskeleton was clearly visible in these cells (Fig. 2E,F). Morphologically, the early stage of trichome initiation and stalk development did not appear to be affected by the drug treatments even though the actin filaments were short and bundled (arrow, Fig. 2B).

### Actin inhibitor effects on extension growth are specific and reversible

To further confirm the specific inhibitory effects of actin drugs

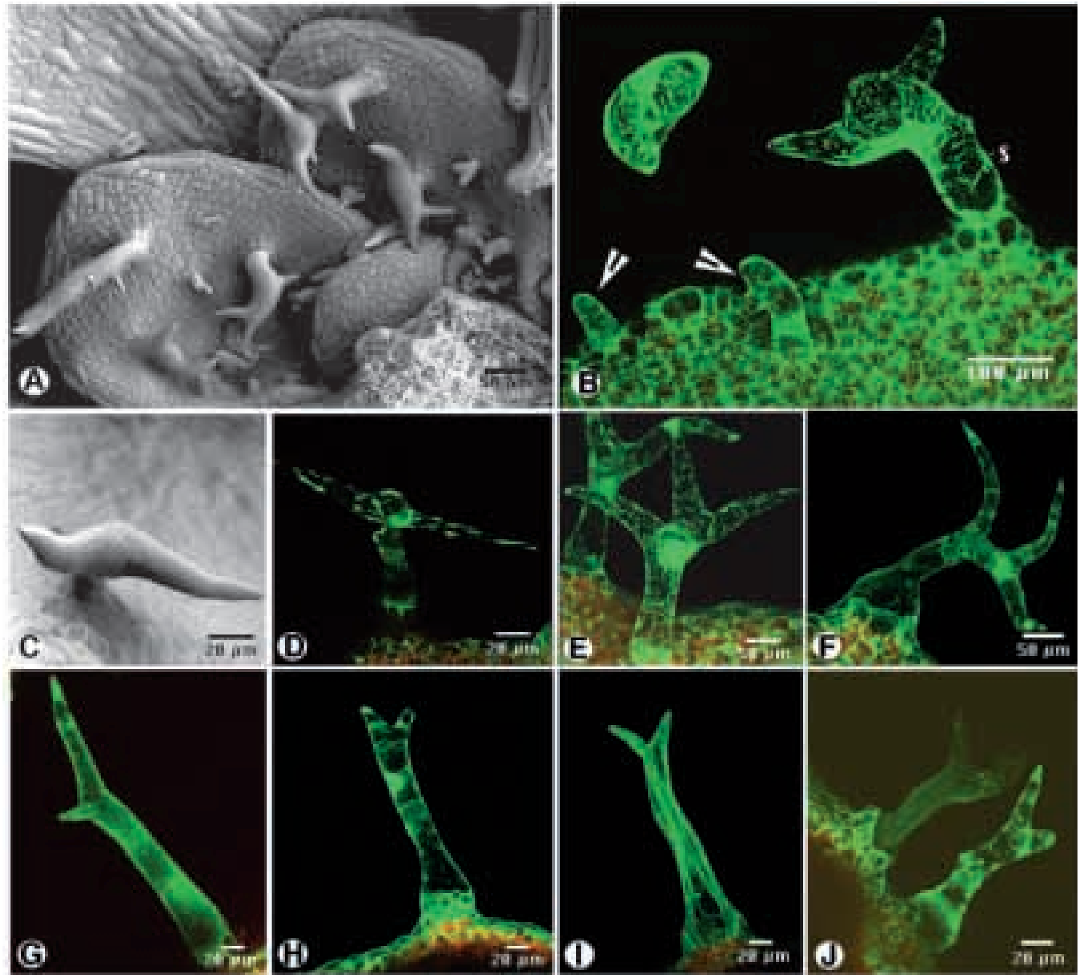


**Fig. 2.** Effect of actin-interacting drugs on trichome morphology and actin cytoskeleton. (A) Treatment with 1 µM cytochalasin-D (CD) or latrunculin-B (Lat-B) for 48 hours or longer. Note the distorted trichome phenotype. (B) Treatment with CD for 48 hours. The first two stages of trichome development do not seem to be affected (arrowheads) and a proper trichome stalk 's' is present, but trichome branches are distorted. Note the short, thick actin bundles in trichome cells. (C) Distorted trichome obtained after treatment with phalloidin or jasplakinolide for 72 hours.(D) Treatment with 5 µM phalloidin for 72 hours results in short, thick actin rods. (E) Trichomes with already extended branches (stage 5) show thick actin bundles similar to those seen on CD-containing medium, but only a slight bending after 48-96 hours of Lat-B-treatment. (F) A late stage 4 trichome treated with Lat-B for 24 hours. Note the diffuse actin cytoskeleton and a distortion of the branches. (G) A trichome treated with Lat-B for 24 hours, before being placed on MS basal medium for recovery exhibits a totally destroyed F-actin cytoskeleton. (H) A trichome treated with Lat-B for 48 hours shows a completely broken down F-actin organization and branches that fail to extend properly. (I) Trichomes recovered their F-actin organization after 24 hours on the recovery medium and reinitiated branch extension. (J) Longer drug treatments resulted in trichomes with thick actin bundles. These trichomes did not recover extension growth even after 96 hours on the drug-free recovery medium.

on extension growth of trichomes, we treated the seedlings with either CD or Lat-B for different time periods before transferring them to an inhibitor-free medium. Taking cognizance of the fact that trichomes developed asynchronously and continuously even as the treatment continued, the resumption of normal trichome morphogenesis after removal of the drug was taken to indicate a specific role for the target actin microfilaments during the process. Treatment with 1 µM Lat-B for 6-12 hours was sufficient to disorganize actin filaments in trichome cells (Fig. 2G). At about 24 hours the actin microfilaments had become totally disorganized and extension of branches was inhibited (Fig. 2H). The first signs of recovery after transfer to inhibitor-free medium were observed with the reappearance and alignment of F-actin cables along the long axis of growth (Fig. 2I). This was followed by the emergence of trichomes with morphology including extended branches. Nearly 98% of the seedlings removed from the inhibitor after a 24-hour treatment recovered and resumed normal trichome morphogenesis after transfer to drug free medium (Fig. 2I), which rules out a general toxicity effect of the inhibitor. In contrast, longer treatments (24-48 hours) with Lat-B resulted in 78% of the trichomes emerging distorted and the effects were carried over to the next pair of developing leaves. Treatments lasting longer than 48 hours and extending up to 96 hours resulted in totally distorted trichomes with carry-over effects lasting for the next 2 to 3 pairs of leaves (Fig. 2J). Moreover, following this long treatment with the inhibitor, the plants became dwarfed in stature, developed multiple lateral roots and required 10-16 days after drug removal to resume normal growth and development. As expected, a subset of trichomes that matured during the treatment never regained their normal shape, even though their actin microfilaments resumed the normal configuration. Treatment with CD produced similar results (data not shown).

### 'Distorted' mutants exhibit defective actin organization in trichomes

The distorted trichomes obtained upon treatment of WT seedlings with actin drugs phenocopied trichomes of mutants belonging to the 'distorted' group. This prompted us to investigate the actin cytoskeleton in 6 distorted mutants: *alien*, *crooked*, *distorted1*, *gnarled*, *klunker* and *wurm*. Trichomes in these mutants show a general morphological distortion with

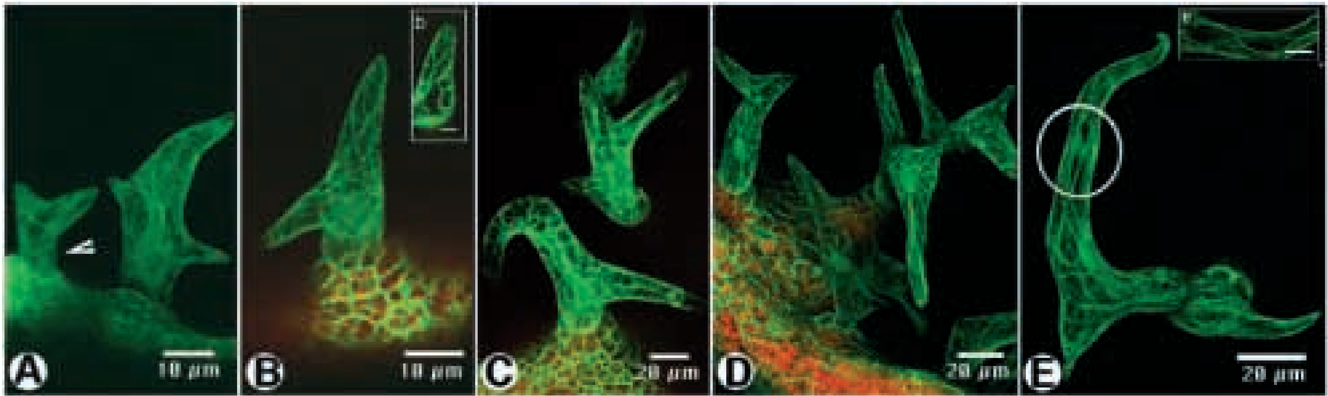


**Fig. 3.** The F-actin cytoskeleton during different stages of trichome development in the *crooked* mutant. (A) Stage 3 (arrowhead) trichomes of *crooked* exhibit a tendency towards random transverse linking of actin filaments compared to the growth of more longitudinal growth of actin filaments observed in the WT (Fig. 1C). (B) Stage 4 trichome showing a small amount of primary branch extension but already possessing an aberrantly organized actin cytoskeleton (b) seen in a single confocal section through the middle of the trichome cell. Bar in b, 15 µm. (C) Late stages 4 and 5 trichomes display thick, transversely linked actin bundles and a distorted morphology. (D) Mature trichomes exhibit numerous intracellular compartments created by random cross-linked actin bundles. Longitudinally running F-actin cables are conspicuously absent. (E) A single trichome showing a disproportionately growing primary branch and random cross-links between the actin filaments; (e) F-actin strands appear to be longitudinally stretched over a short distance corresponding to a relatively normally extending portion of the branch (encircled). Bar in e, 5 µm.

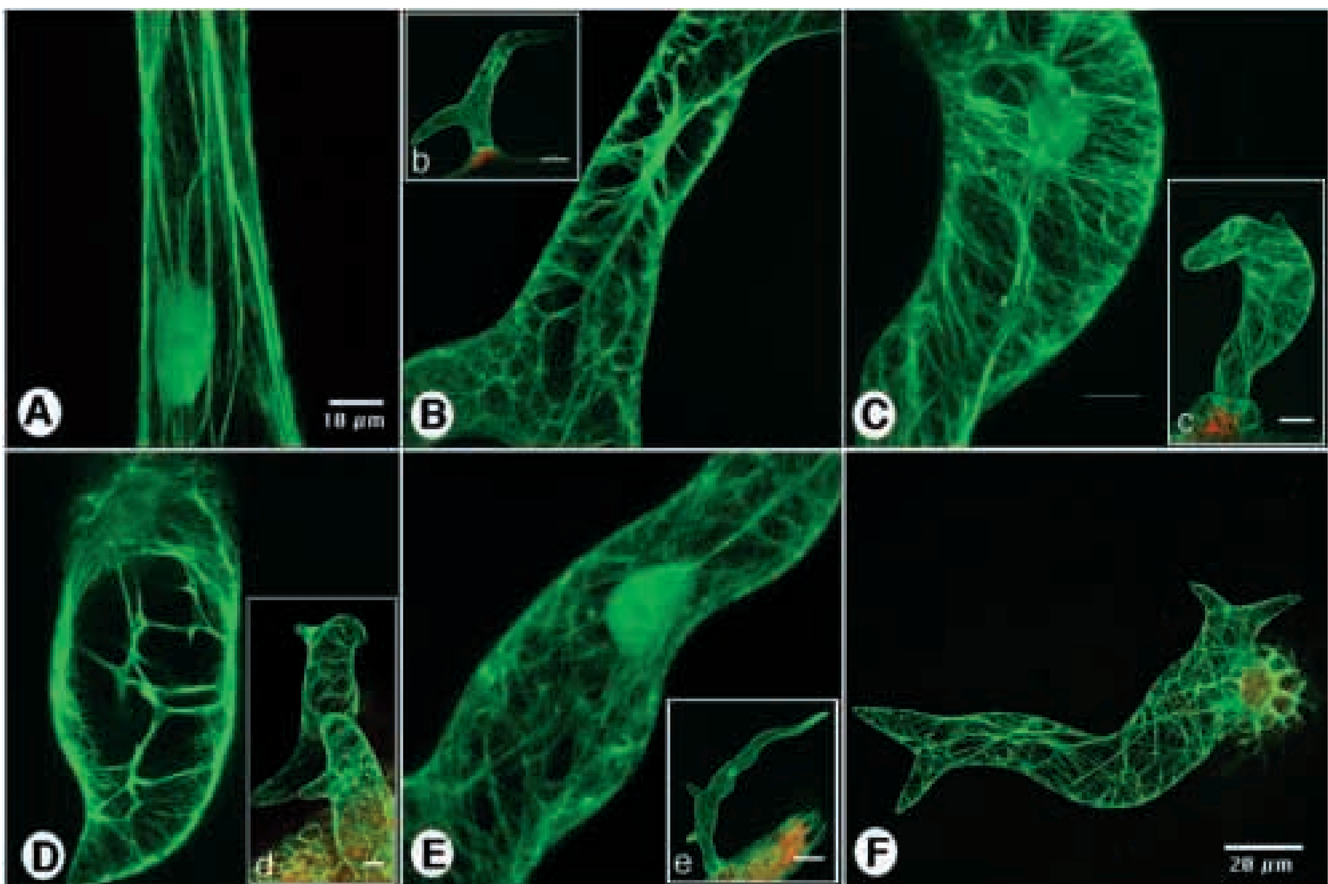


**Fig. 4.** The actin cytoskeleton in mature trichomes of *Arabidopsis* WT and some 'distorted' mutants. (A) Subcortical F-actin cables in WT are regular in diameter and run parallel to the long axis of the trichome. Note that very few transverse links can be observed. (B) A mature trichome of *alien* (b) exhibits an apparent increase in the number of actin microfilaments and numerous transverse links. Long F-actin cables are conspicuously absent. (C) A mature trichome of *gnarled* (c) showing increased actin microfilaments with transverse links and spirals. Long F-actin cables are absent. (D) A trichome of *wurm* displaying an internal zig-zag F-actin organization and relatively few actin filaments (d) compared to *alien* (b) and *gnarled* (c) trichomes. Long actin cables are absent. (E) *Klunker* trichomes exhibit a diffuse actin cytoskeleton (e) with microfilaments of variable diameter. (F) A *distorted1* trichome showing long F-actin cables stretching randomly throughout the cell. A-E are at same magnification: Bars in b to e, 50 µm.

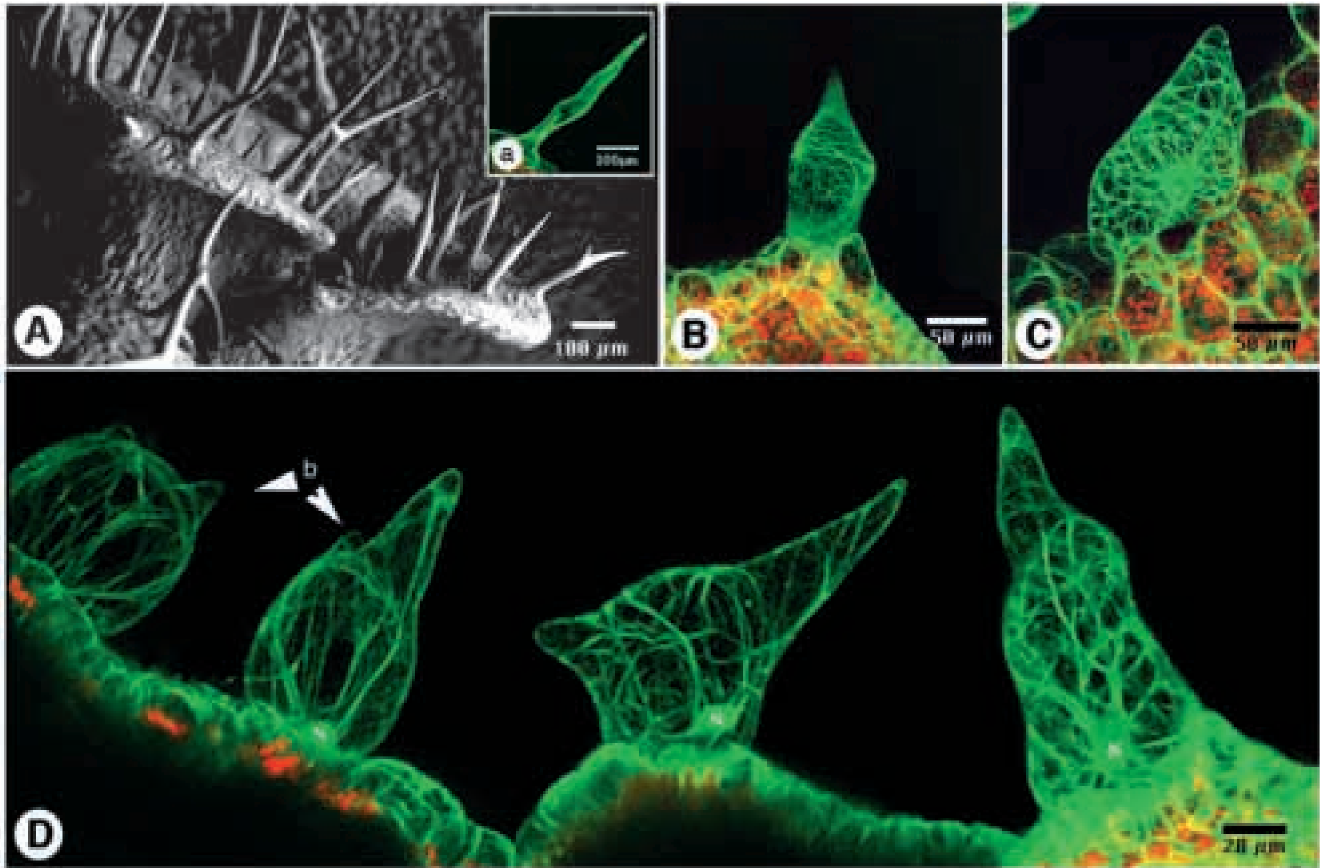


**Fig. 5.** Effects of microtubule-inhibiting drugs on trichome morphology and actin cytoskeleton. (A) A SEM picture of a leaf from WT seedling that was treated with 1 µM paclitaxel for 6 days. The asynchronous development of trichomes allowed some branched trichomes to appear whereas the majority of trichomes appear unbranched and spike-like. (a) The actin cytoskeleton is intact and cables extended longitudinally. (B) The cortical F-actin organization in a trichome treated with 5 µM oryzalin for 96 hours. Trichomes assume a spike-like appearance with a pronounced, characteristic subapical bulge. (C) Top view of an isotropically expanding trichome obtained after treatment with 10 µM colchicine. Note the thick actin bundles. (D) Treatment with propyzamide resulted in radially swollen trichomes with intact but relatively thick actin cables. Indicated branch initials (b) were probably formed prior to the onset of isotropic growth. The nucleus 'N' in all trichomes treated with MT inhibitors remained in the lower half of the cell.

variable phenotypes, even on the same leaf, ranging from a completely 'ballooned' cell with no distinct branching point, to trichomes in which one primary branch extends unidirectionally and disproportionately at the expense of the other. Alternatively, both primary branches may extend partially in a disoriented manner. Although secondary branches are usually initiated, they fail to extend in most distorted trichomes.

Figs 3, 4 show the F-actin cytoskeleton in trichomes of 'distorted' mutants. It is striking that all distorted trichomes investigated showed a defect in actin organization when compared to the actin cytoskeleton in WT trichomes. The most apparent aberration consisted of thick, randomly cross-linked F-actin cables in the distorted trichomes as opposed to the normal tip oriented, stretched and compact actin cables of WT trichomes. This anomaly is most visible in trichomes of *crooked* which was analyzed in some detail (Fig. 3). We found that actin organization defects in *crk* became apparent even during early stages (2 and 3) of trichome development, prior to the onset of any visible morphological deformations. Rather than being stretched along the long axis of the trichome the F-actin filaments in *crk* trichomes started forming multiple transverse cross-links (Fig. 3A,B). Consequently, at later developmental stages, numerous thick actin bundles dissected the intracellular trichome space into irregular compartments (Fig. 3C,D). Whereas mature *crk* trichomes predominantly showed this F-actin organization, a few trichomes with disproportionate branch extension (Fig. 3E) displayed transverse links between subcortical bundles without any thick polygonal compartmentation found in other trichomes (Fig. 3C,D).

*Klunker* trichomes, on the contrary, did not exhibit thick F-actin bundles but displayed an increased number of randomly linked, sub-cortical actin filaments of varying thickness (Fig. 4e,E). In *gnarled* (Fig. 4c,C), and *alien* (Fig. 4b,B), relatively more actin filaments were observed and loops and spirals of F-actin predominated over tip-oriented actin cables. *Wurm* trichomes exhibited numerous actin bundles arranged in a random zig-zag orientation (Fig. 4d,D) and similar defects were observed in trichomes of *distorted1* (Fig. 4F). However, apart from these gross anomalies in F-actin organization in the different mutants, the overlapping actin phenotypes did not allow specific actin organization defects to be linked to a particular trichome mutant. All distorted trichome mutants show a few trichomes in which primary branches extend for some length without major distortions. Further investigation of these exceptional trichomes

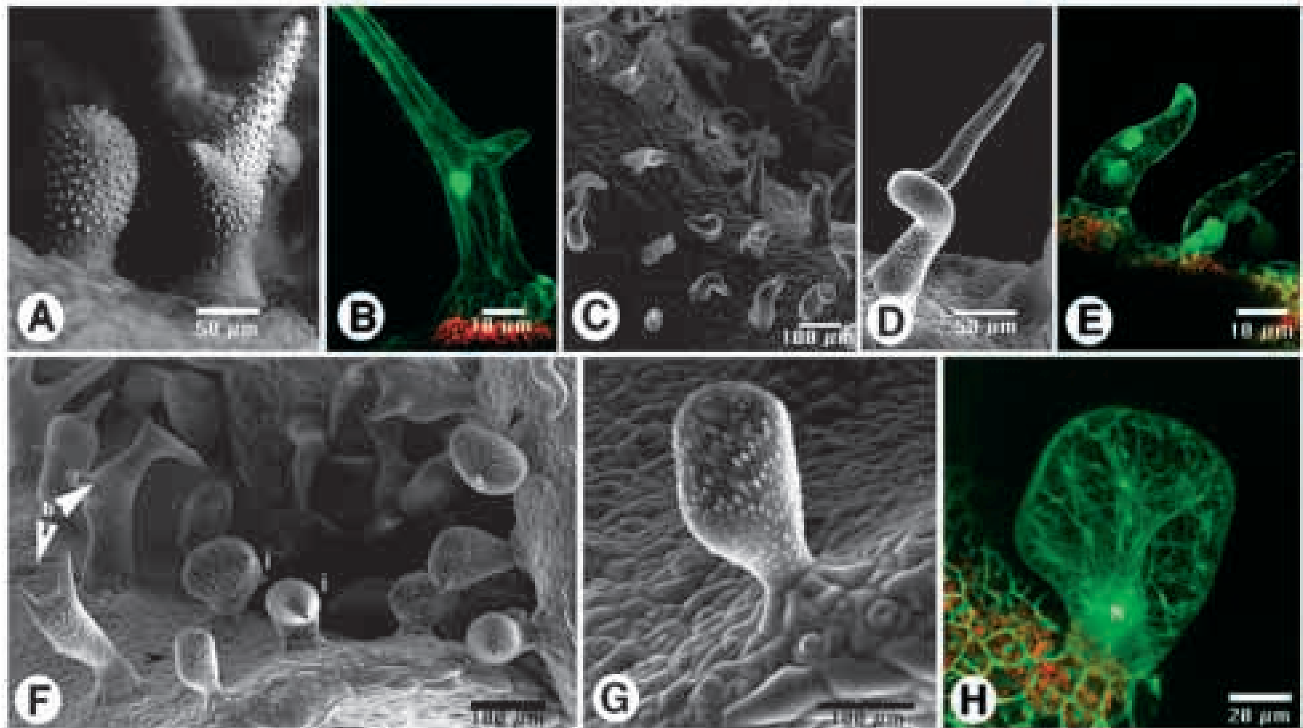

**Fig. 6.** Effects of microfilament- and microtubule-interacting drugs on *zwichel*, a branching mutant and *wurm*, a member of the distorted group. A. SEM of *zwichel* trichomes showing short stalk and reduced branching. (B) The F-actin cytoskeleton in a *zwichel* trichome is unbroken and similar to the WT. (C) *Zwichel* seedlings treated with 1 μM Lat-B exhibit distorted trichomes. Note that branch extension is almost completely inhibited in the majority of trichomes. (D) A single distorted *zwi* trichome obtained after Lat-B treatment for 48 hours. (E) The actin cytoskeleton is totally disturbed in the distorted *zwi* trichome. (F) SEM of trichomes obtained after treatment of *wurm* seedlings for 48 hours with 1 μM paclitaxel. Depending upon the stage at which they were exposed to the drug, trichomes with branch initials (i), partly extended primary branches (b), or no branches at all (black arrowheads), are observed. (G) Magnified view of a single *wurm* trichome from F, showing radial swelling and no branch initials. Similar trichomes were observed on new leaves if the seedlings were maintained on 1 μM paclitaxel for longer than 6 days. (H) The actin cytoskeleton in paclitaxel-treated *wurm* trichomes remained disorganized like that in untreated trichomes indicating that the swollen morphology of the trichome was not due to a further disturbance of actin microfilaments.

confirmed that the actin organization in these small isolated patches within trichomes was indeed normal. However, disorganized actin was always correlated with regions/individual trichomes displaying maximum distortion. Therefore, on a single leaf it is possible to observe trichomes exhibiting severe as well as less severe actin organization defects.

### Microtubule-interacting drugs produce a different trichome phenotype

As controls, WT *Arabidopsis* and *GFP-talin* transgenic plants were treated with colchicine, oryzalin, propyzamide and paclitaxel (taxol) which are well known for their effects on the microtubular cytoskeleton (Fukuda, 1989; Morejohn, 1991; Baskin et al., 1994). Despite differences in their mode of action, all four inhibitors produced similar trichome phenotypes which varied only in their severity and the rapidity with which they could be elicited (Fig. 5). The phenotype clearly differed from that of the 'dis' mutants or trichomes obtained after actin drug treatment. In general, there was a shift towards isotropic growth and neither disproportionate, one sided growth of cells, nor their bending and distortion was observed. Trichome cells at an early stage of development (stages 1 to 3) merely formed distended bulges, whilst trichome cells at later stages of development characteristically produced a subapical or mid-line bulge. In all cases branching of the trichomes was severely impaired. At 1 μM paclitaxel trichomes resembling straight spike-like structures with a subtle sub-apical bulge at the incipient branching point were seen (Fig. 5A). The F-actin cytoskeleton remained intact and both cortical and long, tip directed sub-cortical F-actin bundles were seen in the unbranched trichomes (Fig. 5A).

Treatment with 1-10 μM oryzalin produced an essentially similar phenotype with no disruptions in the F-actin cytoskeleton (Fig. 5B). Propyzamide treatments at 5 and 10 μM produced mildly swollen trichome phenotypes (Fig. 5D), whereas colchicine at 10-100 μM generated large bloated trichome cells which had lost their normal shape completely and could be distinguished from other epidermal cells only by their enlarged size and a roughly triangular appearance (Fig. 5C). In these deformed cells as well, the F-actin strands remained intact but sometimes appeared as thicker bundles (Fig. 5) rather than the fine filaments observed in mature untreated trichomes (Fig. 1G). However, no random, transverse cross-links between thick actin bundles or an actin organization reminiscent of that observed in mutants of the 'dis' group was ever observed in these trichomes. In each of these treatments the nucleus remained in the lower half of the trichome-cell stalk (Fig. 5D) and failed to move to its characteristic position near the branching point.

### *Zwi* trichomes display normal actin organization and become distorted upon treatment with actin inhibitors

The *zwichel* mutant exhibits both reduced branching and stalk length and its WT gene product has been implicated in microtubule interactions (Fig. 6A; Huelskamp et al., 1994; Oppenheimer et al., 1997; Oppenheimer, 1998). We found that the actin cytoskeleton in *zwi* trichomes was normal with longitudinally stretching actin cables (Fig. 6B), similar to those in WT trichomes (Fig. 1G). This result indicates that the observed *zwichel* phenotype does not involve any actin cytoskeleton defects. Treatment of *zwichel* mutant plants carrying the *GFP-talin* transgene with Lat-B, however, produced distorted trichomes which lost their branch extension capability, concomitant with the expected alterations in their actin cytoskeleton (Fig. 6C-E). The actin drug-induced phenotype in *zwichel* confirms a general role for actin in executing a proper extension growth of trichomes.

### Treatment of *wurm* with microtubule inhibitor blocks trichomes at the pre-extension stage

As a member of the ‘distorted’ group of mutants *wurm* shows an aberrant actin cytoskeleton in trichomes as compared to the WT (Fig. 4D). In an attempt to establish the relative temporal order for microtubule and actin-microfilament action during trichome morphogenesis, seedlings of *wurm* were treated with 1 μM paclitaxel and oryzalin for 48 hours. Depending upon the stage of trichome cell development at the time of treatment, an overall radially swollen morphology with no branches or short unextended branches was expected if microtubules functioned before the extension growth phase. A range of phenotypes was elicited after the 48-hour treatment (Fig. 6F) including unbranched, radially swollen trichomes as well as trichomes where branches initiated but failed to extend (Fig. 6F,G). No changes were observed in the actin microfilaments in comparison to the untreated controls (Fig. 4D versus Fig. 6H). *Wurm* seedlings maintained on 1 μM paclitaxel for longer durations, up to 6 days, produced only ballooned new trichome cells similar to those shown in Fig. 6G. Branch initiation was totally inhibited in these cells. As a further confirmation we found that simultaneous treatment of *urm* and WT seedlings with 1 μM each of paclitaxel and Lat-B resulted in severely ballooned, unbranched trichome cells. Seedlings incubated with these two drugs also showed general malformations and did not survive prolonged treatment (data not shown). These results reinforce our view that microtubule involvement precedes the active involvement of actin microfilaments.

## DISCUSSION

Trichome cell morphogenesis in *Arabidopsis* progresses through an orderly series of developmental events (Fig. 1; Szymanski et al., 1998), marked by certain key ‘turning points’. These include the emergence of a specified trichome cell from the epidermal plane as a vertically growing tube, the splitting of the tube at the tip to produce primary branches and the rapid extension of the straight branches in nearly diagonally opposite directions (Fig. 7, upper panel). Secondary branching and trichome maturation may occur as independent events as suggested by the isolation of *Arabidopsis* mutants impaired specifically in these processes (Huelskamp et al., 1994). Our results reported here implicate both microtubules and microfilaments in trichome morphogenesis and highlight their role reversal during the process.

### Early stages in trichome morphogenesis are microtubule dependent

Observations on seedlings treated with microtubule or actin-microfilament interacting drugs indicated that microtubules play an important role during early trichome development. Treatment with microtubule drugs has clear effects on trichome cell initials, their tubular growth and branch initiation (Figs 5, 6). Thus, trichome cells may be converted to mere swellings on the epidermis, with little polarized growth, following colchicine/oryzalin treatment (Fig. 5B,C) or into spikes with no branch formation after paclitaxel treatment (Fig. 5A). None of the microtubule-drug treatments produce gross changes in F-actin architecture, except that the normally longitudinally stretched actin cables (Fig. 1G) followed the periphery of the radially expanded trichome cells (Fig. 5B,C,D). It appears that during early stages of trichome morphogenesis in *Arabidopsis* the initial grows in a polar fashion by diffuse growth in a manner similar to that described for cotton trichomes (Tiwari and Wilkins, 1995). The direction of diffuse growth is likely determined by the orientation of cellulose microfibrils in the cell wall, which in turn are guided and aligned by cortical microtubules (Kropf et al., 1998). In plant cells that expand anisotropically the cortical microtubule array is organized transverse to the axis of most rapid extension growth (Baskin et al., 1994). Treatment with chemicals such as colchicine/oryzalin causes depolymerization of this cortical microtubule array resulting in the isotropic expansion of trichome initials and the appearance of swollen forms.

The formation of branch points on the tubular trichome intial (Fig. 1, stages 2 and 3) likely entails a reorientation of growth directionality. This may be brought about by a reorientation of existing microtubules or more likely by a reorganization of microtubules de novo. In either case, the depolymerization/stabilization of microtubules by the appropriate drugs would interfere with such reorganization, resulting in unbranched or sparsely branched trichomes (Figs 5, 6).

A role for microtubules during trichome branching is further supported by observations on three *Arabidopsis* mutants. The *ZWICHEL* gene encodes a kinesin-like calmodulin-dependent protein which has been implicated in microtubular interactions (Oppenheimer, 1998) and *zwichel* mutant trichomes have a short stalk and reduced branching. Similarly, *fass* and *ton* mutants have a disturbed cortical microtubular cytoskeleton and possess unbranched trichomes (Torres-Ruiz and Jurgens, 1994; Huelskamp et al., 1999).

We note that the F-actin in early stages of trichome cell development is relatively diffuse and not organized into the characteristic actin bundles seen in later stages. In addition, WT seedlings treated with actin-interacting drugs as well as mutants of the ‘distorted’ class are able to initiate a trichome stalk as well as secondary branches (Figs 2, 3). Taken together, our observations indicate a dominant role for the microtubular cytoskeleton as compared to that of actin microfilaments during early stages of trichome cell morphogenesis.

### Trichome extension growth requires actin cytoskeleton function

Following the delineation of branch points the trichome cell

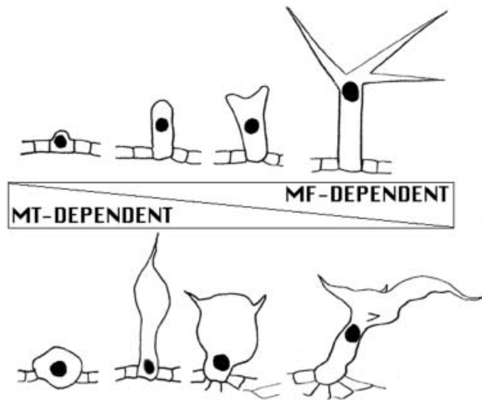


**Fig. 7.** A model indicating major 'turning points' and the relative roles of microtubules (MT) and actin-microfilaments (MF) during different stages of trichome cell morphogenesis in *Arabidopsis thaliana*. All stages of trichome development are affected by treatments with inhibitors indicating that both MTs and MFs act interdependently. However, as seen by the terminal trichome morphology obtained upon treatment with MT (left part, lower panel) or MF-inhibiting drugs (right side, lower panel) the early stages involving the establishment of the spatial pattern for trichome cell morphogenesis, apparently rely more upon MT-dependent processes. Disruption of MTs during these early stages results in a shift towards isotropic growth. Later stages when the established pattern is elaborated and maintained by extension growth of the trichome stalk and branches, predominantly involve MFs. Trichome maturation appears to be unaffected by MT or MF cytoskeletal functions *per se* and occurs as an independent event.

embarks on a phase of rapid extension growth. Trichomes in 'distorted' mutants initiate branching but the stalk and the branches are distorted. Moreover, the branches frequently develop in a lopsided manner with the growth of one branch taking precedence over the other. Although secondary branches are initiated they do not extend properly, in most cases being limited to short spiky outgrowths (Figs 2, 3). At maturity, distorted trichomes acquire a randomly undulating and recumbent form rather than the stellate, erect appearance of the WT trichomes.

Different causes have been suggested for the distorted trichome phenotype, such as general defects in the biosynthesis of new membrane/cell wall components, changes in intracellular osmotic and vacuolar properties, or improper trafficking of newly synthesized materials to their final destination (Oppenheimer et al., 1993; Huelskamp et al., 1994). Whereas biosynthetic/osmotic defects could potentially contribute to the distorted phenotype they may be expected to yield more pleiotropic phenotypes. However, in most mutants of the 'distorted' class the morphological abberations appear to be limited to the epidermis (Oppenheimer et al., 1993; Huelskamp et al., 1994, 1999; Marks, 1997). Whilst the most dramatic and sustained changes in morphology are observed in trichomes, the epidermal surface of rapidly elongating/expanding structures such as cotyledons and the first pair of leaves and their petioles also display scruffiness (Mathur and Chua, unpublished observations). Even between the distorted trichomes there is a high degree of phenotypic randomness which suggests inappropriate intracellular trafficking to be the most likely causal factor. We consider it likely that formation of the regular, stellate WT trichome results from a polarized, symmetrical and targeted delivery of secretary vesicles to the extending trichome cell wall. A disturbance in this process would be expected to lead to asymmetrical deposition of cell wall materials and consequently a distorted phenotype. Actin microfilaments are known to serve as intracellular tracks for rapid delivery of secretary vesicles to specific integration sites on the plasma membrane (Fowler and Quatrano,1997; Schmidt and Hall, 1998), and in plants, studies on root hairs and pollen tubes have also implicated the actin cytoskeleton during elongation by tip growth (Cai et al., 1997; Hable et al., 1998). Our results here confirm and extend a conserved role for actin microfilaments in polarized extension growth in eukaryotes (Drubin and Nelson,1996). As a consequence of actin cytoskeletal malfunctioning the cell ends of the fission yeast fail to extend properly (Mitchison and Nurse, 1985) and aberrant cell wall deposition occurs in *Saccharomyces cervisiae* cells (Gabriel and Kopecka, 1995). Accumulated evidence from these studies supports a general role for actin microfilaments in the stabilization of tip localized ion channels as well as transport and exocytosis of secretory vesicles (Fowler and Quatrano, 1997; Kropf et al., 1998).

In support of the above hypothesis involving targeted delivery of secretary vesicles as a likely cause for the distorted phenotype, we showed that the distorted trichomes can be phenocopied by treatment of the WT with different well characterized actin inhibitors. These included CD and Lat-B which affect actin polymerization specifically and phalloidin and jasplakinolide, which are F-actin stabilizers (Cooper, 1987; Spector et al., 1989; Ayscough et al., 1997). Our results directly implicate the actin cytoskeleton in the rapid extension growth phase of trichome morphogenesis and further highlight the importance of appropriate actin dynamics in this process. We consider it likely that mutations in different actin-interacting proteins would produce similar distorted trichome phenotypes.

The isolation of at least 8 mutants belonging to the 'distorted' group strongly suggests the involvement of multiple genes and processes during the extension growth phase of trichome morphogenesis. That 6 of the 'distorted' mutants display actin cytoskeleton aberrations indicates that many of the possible functional defects leading to trichome growth distortions may occur as a result of malfunctioning of the actin cytoskeleton. The actin drug experiments which target the actin cytoskeleton in a gross way reinforce this notion.

Although direct proof for the involvement of actin-interacting proteins in trichome morphogenesis is not available at this time, results from *Drosophila* have clearly demonstrated such a link in bristle development (FlyBase, 1994; Tilney et al., 1996). Thus, the absence of bundling proteins required for bundling of single F-actin strands into longer cables results in the '*singed*' and '*forked*' bristle mutants of *Drosophila* (Cant et al., 1994; Petersen et al., 1994). Mutations in profilin, actin-depolymerization factor (ADF) or β-subunit of capping protein also produce defective bristles (Verheyen and Cooley, 1994; Gunsalus et al., 1995; Hopmann et al., 1996). Given these precedents and the results described here, it would not be surprising if the 6 distorted mutant genes encoded proteins directly or indirectly involved in actin cytoskeleton functions.

## Relative roles of microfilaments and microtubules during trichome morphogenesis

Our general observations on the relative roles of microfilaments and microtubules during trichome cell morphogenesis in

*Arabidopsis* are summarized in a model outlined in Fig. 7 (bottom panel). The various morphogenetic 'turning points' (Fig. 7, upper panel) suggest multiple mechanistically and temporally distinct check-points which likely involve many different cytoskeletal proteins and their interactions. The foundation of a spatial pattern appears to be laid down early in trichome morphogenesis and our results clearly show that this can be largely attributed to the microtubular cytoskeleton. Further elaboration and maintenance of this pattern in succeeding developmental stages, however, involve predominantly, actin cytoskeleton based processes. The model does not propose exclusive functions for either microtubules or microfilaments during trichome development but emphasizes a reversal in their relative roles with maturation. Our results add a cell biological perspective to the existing genetic models on branch formation and shape determination in *Arabidopsis* trichomes (Huelskamp et al., 1994; Folkers et al., 1997; Oppenheimer, 1998).

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## REFERENCES

**Ahlstrand, G.** (1996). Low temperature low-voltage scanning microscopy (LTLVSEM) of uncoated frozen biological materials: a simple alternative. In *Proceedings of Microscopy Microanalysis*. (ed. G. Bailey, J. Corbett, R. Dimlich, J. Michael and N. Zaluzec), pp. 918. San Francisco: San Francisco Press.

**Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P. and Drubin, D. G.** (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin -A. *J. Cell Biol*. **137**, 399-416.

**Baskin, T. I., Wilson, J. E., Cork, A. and Williamson, R. E.** (1994). Morphology and microtubule organization in *Arabidopsis* roots exposed to oryzalin or taxol. *Plant Cell Physiol*. **35**, 935-944.

**Bibikova, T. N., Blancaflor, E. B. and Gilroy, S.** (1999). Microtubules regulate tip growth and orientation in root hairs of Arabidopsis thaliana. *Plant J.* **17**, 657- 665.

**Cai, G., Moscatelli, A. and Cristi, M.** (1997). Cytoskeletal organization and pollen tube growth. *Trends Plant Sci*. **2**, 86-91.

**Cant, K., Knowles, A., Mooseker, M. S. and Cooley, L.** (1994). *Drosophila singed*, a fascin homologue, is required for actin bundle formation during oogenesis and bristle extension. *J. Cell Biol.* **125**, 369-380.

**Cooper, J.A.** (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473-1478.

**Drubin, D. G. and Nelson, W. J.** (1996). Origins of cell polarity. *Cell* **84**, 335-344.

**Feenstra, W. J.** (1978). Contiguity of linkage groups 1 and 4, as revealed by linkage relationships of two newly isolated markers dis-1 and dis-2. *Arab. Inform. Serv.* **15**, 35-38.

**FlyBase** (1994). The Drosophila Genetic Database. *Nucleic Acids Res.* **22**, 3456-3458.

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

**Fowler, J. E., and Quatrano, R. S.** (1997). Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. *Annu. Rev. Cell Dev. Biol.* **13**, 697-743.

**Franklin-Tong, V. E.** (1999). Signaling and the modulation of pollen tube growth. *Plant Cell* **11**, 727-738.

**Fukuda, H.** (1989). Regulation of tubulin degradation in isolated zinnea mesophyll cells in culture. *Plant Cell Physiol.* **30**, 243-252.

**Gabriel, M. and Kopecka, M.** (1995). Disruption of the actin cytoskeleton in budding yeast results in formation of an aberrant cell wall. *Microbiology* **141**, 891-899.

**Gunsalus, K. C., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M. and Goldberg, M. L.** (1995). Mutations in *twinstar*, a *Drosophila* gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J. Cell Biol*. **131**, 1243-1259.

**Hable, W. E., Bisgrove, S. R. and Kropf, D. L.** (1998). To shape a plant – the cytoskeleton in plant morphogenesis. *Plant Cell* **10**, 1772-1774.

**Hopmann, R., Cooper, J. A., and Miller, K. G.** (1996). Actin organization, bristle morphology, and viability are affected by actin-capping protein mutations in *Drosophila*. *J. Cell Biol.* **133**, 1307- 1320.

**Huelskamp, M., Misera, S. and Jurgens, G.** (1994). Genetic dissection of trichome cell development in *Arabidopsis*. *Cell* **76**, 555-566.

**Huelskamp, M., Folkers, U. and Grini, P. E.** (1998). Cell morphogenesis in *Arabidopsis*. *BioEssays* **20**, 20-29.

**Huelskamp, M., Schnittger, A. and Folkers, U.** (1999). Pattern formation and cell differentiation: Trichomes in *Arabidopsis* as a genetic model system. *Int. Rev. Cytol.* **186**, 147-178.

**Kost, B., Spielhofer, P., and Chua, N.-H.** (1998). A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes actin cytoskeleton in growing pollen tubes. *Plant J.* **16**, 393-402.

**Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C., and Chua, N.-H.** (1999). Rac homologues and compartmentalized phosphatidylinositol 4, 5-bisphosphate act in a common pathway to regulate polar pollen tube growth. *J. Cell Biol.* **145**, 317-330.

**Kropf, D. L., Bisgrove, S. R. and Hable, W. E.** (1998). Cytoskeletal control of polar growth in plant cells. *Curr. Opin. Cell Biol.* **10**, 117-122.

**Marks, M. D. and Esch, J. J.** (1994). Morphology and development of mutant and wild type trichomes on the leaves of *Arabidopsis thaliana*. In *Arabidopsis*: *an Atlas of Morphology and Development*. (ed. J. Bowman), pp. 56-73. New York: Springer-Verlag.

**Marks, M. D.** (1997). Molecular genetic analysis of trichome development in *Arabidopsis*. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 137-163.

**Miller, D. D., deRuiijter, N. C. A. and Emons, A. M. C.** (1997). From signal to form: aspects of the cytoskeleton-plasma membrane-cell wall continuum in root hair tips. *J. Exp. Bot*. **48**, 1881-1896.

**Mitchison, J. and Nurse, P.** (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe. J. Cell Sci.* **75**, 357-376.

**Morejohn, L. C.** (1991). The molecular pharmacology of plant tubulin and microtubules. In *The Cytoskeletal Basis of Plant Growth and Form* (ed. C. Lloyd), pp. 29-44. London: Academic Press Ltd.

**Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.

**Oppenheimer, D. G., Esch, J. and Marks M. D.** (1993). Molecular genetics of Arabidopsis trichome development. In *Control of Plant Gene Expression*. (ed. D. P. S. Verma), pp. 275-286. Boca Raton: CRC Press.

**Oppenheimer, D. G., Pollack, M. A., Vacik, J., Szymanski, D. B., Ericson, B., Feldmann, K. and Marks, M. D.** (1997). Essential role for a kinesin-like protein in *Arabidopsis* trichome morphogenesis. *Proc. Nat. Acad. Sci. USA* **94**, 6261-6266.

**Oppenheimer, D. G.** (1998). Genetics of plant cell shape. *Curr. Opin. Plant Biol.* **1**, 520-524.

**Petersen, N. S., Lankenau, D. H., Mitchell, H. K., Young, P. and Corces, V. G.** (1994). Forked proteins are components of fiber bundles present in developing bristles of *Drosophila melanogaster*. *Genetics*. **136**, 173-182.

**Schmidt, A. and Hall, M. N.** (1998). Signaling to the actin cytoskeleton. *Ann. Rev. Cell Dev. Biol.* **14**, 305-338.

**Spector, I., Shochet, N. R., Blasberger, D. and Kashman, Y.** (1989). Latrunculins-novel marine macrolides that disrupt microfilament organisation and affect cell growth: I. Comparison with cytochalasin D. *Cell Mot. Cytoskeleton*. **13**, 127-144.

**Szymanski, D. B., Jilk, R. A., Pollock, S. M. and Marks, M. D.** (1998). Control of GL2 expression in *Arabidopsis* leaves and trichomes. *Development* **125**, 1161-1171.

**Tilney, L. G., Connelly, P., Smith, S. and Guild, G. M.** (1996). F-actin bundles in *Drosophila* bristles are assembled from modules composed of short filaments. *J. Cell Biol*. **135**, 1291-1308.

**Tiwari, S. C. and Wilkins, T. A.** (1995). Cotton (*Gossypium hirsutum*) seed trichomes expand via diffuse growing mechanism. *Can. J. Bot*. **73**, 746-757.

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

**Verheyen, E. M. and Cooley, L.** (1994). Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. *Development* **120**, 717-728.