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Arabidopsis Trichome Morphogenesis: A Genetic Approach to Studying Cytoskeletal Function

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Abstract

Arabidopsis trichome (leaf hair) development is being used to genetically dissect cytoskeletal organization and function in plant cells. The highly constrained and distinct requirements for microtubule and microfilament function during trichome morphogenesis make it possible to screen for mutations that affect cytoskeletal function. Pharmacological drugs that disrupt normal microtubule dynamics block the establishment of polarized trichome growth. In contrast, drugs that alter F-actin organization or dynamics affect the maintenance and coordination of the normal pattern of cell growth at later stages of morphogenesis. There are distinct classes of trichome morphology mutants that appear to affect either the microtubule or the microfilament-dependent phases of trichome morphogenesis. For example, the *zwichel* (*zwi*) mutant displays altered trichome stalk expansion and branch initiation that is similar to the effects of microtubule-disrupting agents. The *ZWI* gene encodes a kinesin-like microtubule motor protein. The cell shape defects of the "distorted group" of trichome morphology mutants are phenocopied by exposing developing trichomes to drugs that disrupt the actin cytoskeleton, but not those that affect microtubule-disrupting agents. Distorted mutants fail to maintain polarized growth during discrete stages of trichome development. The combination of molecular genetic and cell biology tools will help to uncover the mechanisms of cytoskeletal organization and function in plant cells.

Key words: Trichome; Cytoskeleton; *Arabidopsis*; Distorted mutants; *ZWI*; Microtubules; Microfilaments

Introduction

The plant cytoskeleton is comprised of an intricate filamentous network of both microtubules and microfilaments. In interphase cells, the organization of the microtubule cytoskeleton and the cellulose microfibrils are thought to control cell shape changes during growth (Cyr 1994). It has long been hypothesized that the physical properties of the circumferential cell wall microfibrils limit lateral expansion and promote cell elongation. A circumferential coalignment of both microtubules and cellulose microfibrils that are perpendicular to the long axis of the cell has been observed in many elongating cell types. However, the mechanisms by which cortical microtubules and cellulose microfibrils are organized and the function of each polymer during polarized cell growth are poorly understood. In interphase cells the actin cytoskeleton consists of an intricate three-dimensional scaffolding of both transvacuolar and cortical arrays. A primary func-

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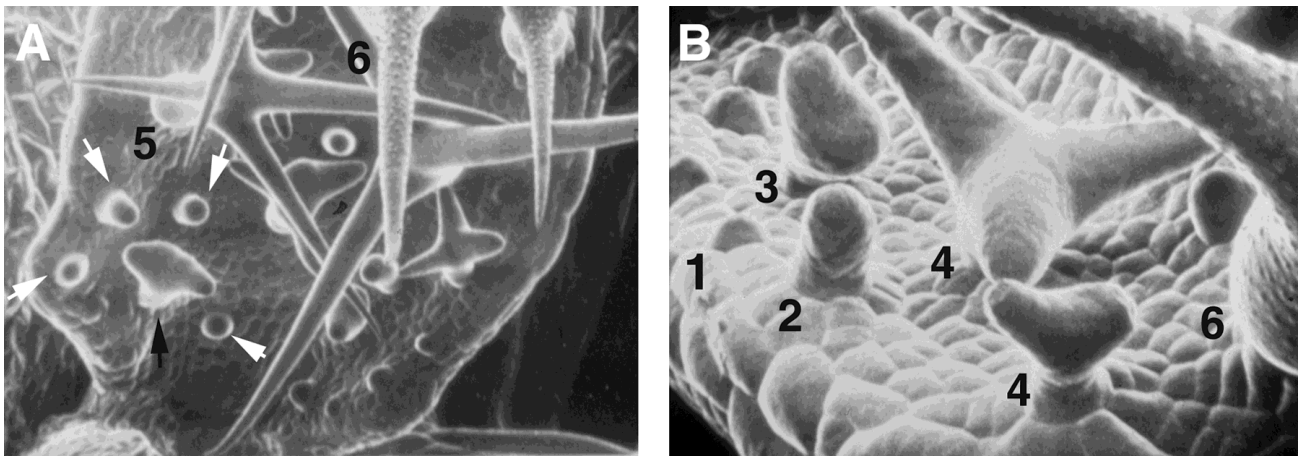

Figure 1. Scanning electron micrographs of the adaxial surface of *Arabidopsis* leaves that illustrate aspects of trichome spacing and morphogenesis. Numbers to the left of each labeled trichome indicate its developmental stage. (**A, B**) An illustration of additional stages of trichome development: stage 1, isodiametric expansion in the plane of the epidermis; stage 2, stalk emergence and polar expansion; stage 3, branch initiation; stage 4, expansion of the stalk and branches with a blunt tip morphology; stage 5, trichome expansion with pointed branch tips; stage 6, mature trichome with a papillate cell wall (Reproduced with permission from Szymanski and others 2000.)

tion of the actin cytoskeleton in plant cells is to provide the roadways upon which organelles and vesicles traffic (Boevink and others 1998). This is not to say that the actin cytoskeleton is a static structure. The organization of the actin cytoskeleton is modulated in response to a diverse set of intrinsic and extrinsic cues, and understanding its regulation is an important question in plant biology (Staiger 2000). A genetic analysis of microtubule and microfilament function during *Arabidopsis* trichome morphogenesis has the potential to improve our understanding of how the cytoskeleton is organized and its function.

The power of a genetic approach to study the cytoskeleton has been established in organisms from yeast to flies and worms (Ayscough 1998). The feasibility of using genetic techniques to study cytoskeletal function during interphase growth has also been demonstrated in plants. Identification of mutations in the *Arabidopsis* actin gene family members by reverse genetics will provide important tools to understand the function of actin isoforms in plant cells (McKinney and others 1995). Actin organization is important for normal coleoptile elongation (Thimann and others 1992; Waller and Nick 1997). The rice *ying yang* mutant displays an altered actin organization in coleoptile epidermal cells during cell elongation in response to auxin (Wang and Nick 1998). Mutations in the *FASS* gene affect the organization of interphase microtubules (McClinton and Sung 1997), whereas mutation of the *Arabidopsis ZWICHEL* (*ZWI*) gene, which encodes a kinesin-like motor protein, leads to defects in trichome morphogenesis (Oppenheimer and others 1997). An analysis of the "distorted group" of trichome mutants provides a unique experimental window into actin-dependent cell morphogenesis (Szymanski and others 1999). This review summarizes current progress in using *Arabidopsis* trichome morphogenesis to unravel the complexities of cytoskeletal organization.

Arabidopsis Trichome Development

Trichome Morphogenesis

Arabidopsis leaf hairs are unicellular stellate trichomes that usually contain three branches. Historically, *Arabidopsis* trichome development has been used to address the question of how cell fate and pattern formation is regulated in the plane of the epidermis (Larkin and others 1997; Marks 1997). The cell fate decision making is complex and involves the concerted activity of at least seven genes that regulate trichome initiation. Many of the early acting genes encode putative transcriptional regulators (Szymanski and others 2000). Once an epidermal precursor enters the trichome pathway, it undergoes a complex morphogenetic program (reviewed in Hülskamp and others 1998). Trichome growth has been divided into discrete stages based on morphological landmarks (Szymanski and others 1998; Figure 1A,B). The first sign of trichome formation is isodiametric expansion within the plane of the epidermis (stage 1). In cross section, the tri-

chome precursor appears as an enlarged spherical cell with a greatly enlarged nucleus. During stage 1 of trichome development, neighboring socket cells form a tight association with the precursor cell. Stage 2 trichomes display obvious polarized elongation perpendicular to the leaf surface until the cell reaches a length of approximately 30 μm. The cellular organization and cell shape changes that occur during stage 2 are similar to tip growing cells, but the actual growth mechanism is not known. During stage 3, branch initiation occurs sequentially on the developing stalk, often yielding a cell with three branches. The elongating branch buds initially have a blunt tip morphology (stage 4), but after the branches elongate to about 15 μm, the tip morphology becomes more pointed (stage 5). The vast majority of the cell volume is generated during stage 5 (Szymanski and others 1999). Once cell expansion has ceased, the cell wall acquires a papillate surface (stage 6). Although there is some variation between trichomes in the shape changes that occur during morphogenesis, each cell executes a similar developmental program. Based on the shape changes and cellular organization of developing trichomes, both tip growth and diffuse growth appear to be involved (Szymanski and others 1999).

Pharmacology

Exposure of developing trichomes to drugs that specifically alter either microtubule or microfilament-dependent function provides strong evidence for stage-specific requirements for each cytoskeletal array. This was clearly shown using a dexamethasone-regulated form of the maize R gene to induce trichome formation in the presence or absence of cytoskeleton-disrupting agents (Lloyd and others 1994). Using this experimental system trichome initiation is dexamethasone-dependent (Figure 2A,B). The induced trichomes are highly polarized and contain either two or three branches (Figure 2B). Exposure of the developing leaves to high concentrations of oryzalin prior to initiation causes isotropic cell expansion without branch formation (Figure 2C). Mathur and others (1999) also showed that at high drug concentrations, agents that either stabilize or depolymerize microtubules can inhibit polarity establishment in developing leaves. However at lower drug concentrations some polar expansion is observed. Precise actin organization is not required for polarity establishment: stalk elongation and branch initiation during stages 2 and 3 are not affected noticeably by F-actin-disrupting agents (Szymanski and others 1999). Branch formation occurs even when induced trichomes develop in the presence of high concentrations of F-actin-disrupting drugs (Figure 2D). The apparent unimportance of F-actin during growth pattern establishment is not due to an absence of F-actin during these stages of development. Using conventional fixation techniques coupled with the freeze shattering permiabilization approach described by Wasteneys and others (1997), anti-actin antibodies detected intricate networks of F-actin in both stage 1 and stage 2 cells (Szymanski and others 1999). Perhaps during stage 2 F-actin plays a minor role in facilitating the apical transport of vesicles, but in its absence, diffusion or microtubule-dependent function is sufficient. In this regard trichome cell elongation during stage 2 is unlike the actin-dependent tip growth that is observed in pollen tubes, root hairs, and fungal hyphae (Jackson and Heath 1993; Miller and others 1999; Picton and Steer 1981). At this time the mode of trichome expansion during stage 2 is not clear. Shaw and others(2000) have developed high resolution methods to directly measure cell surface growth rates in root hairs over time. Similar cell wall marking experiments will have to be conducted on developing trichomes to determine the modes of cell expansion at different developmental stages.

Inhibitor studies demonstrate that both microtubule and F-actin-dependent functions are required to coordinate cell expansion following the initiation of polarized stalk and branch structures. In untreated cells, cell expansion during stages 4 and 5 is highly regulated and includes diffuse growth of the stalk and branches as well as potential tip-directed growth in the elongating branches. Stage 4 and 5 trichomes that are treated with microtubule-disrupting agents display reduced branch elongation and are often swollen and twisted (Figure 3A,B). Agents that affect F-actin organization severely disrupt trichome morphogenesis following the transition from branch initiation (stage 3) to branch and stalk expansion (stage 4). Cell shape in the presence of F-actin-disrupting drugs is disorganized: stalks are often swollen (Figure 3 C,D), and while branch elongation and cell expansion occur, branch morphology varies from an aborted stub to a highly elongated structure that is swollen and twisted. Unlike microtubule-disrupting agents, cytochalasin D and latrunculin B do not block cell expansion or branch initiation. Even at high drug concentrations trichomes resembling those of the "distorted group" of mutants develop over a period of days when either drug is applied to the leaf surface. The expansion observed in cells with disrupted F-actin appears

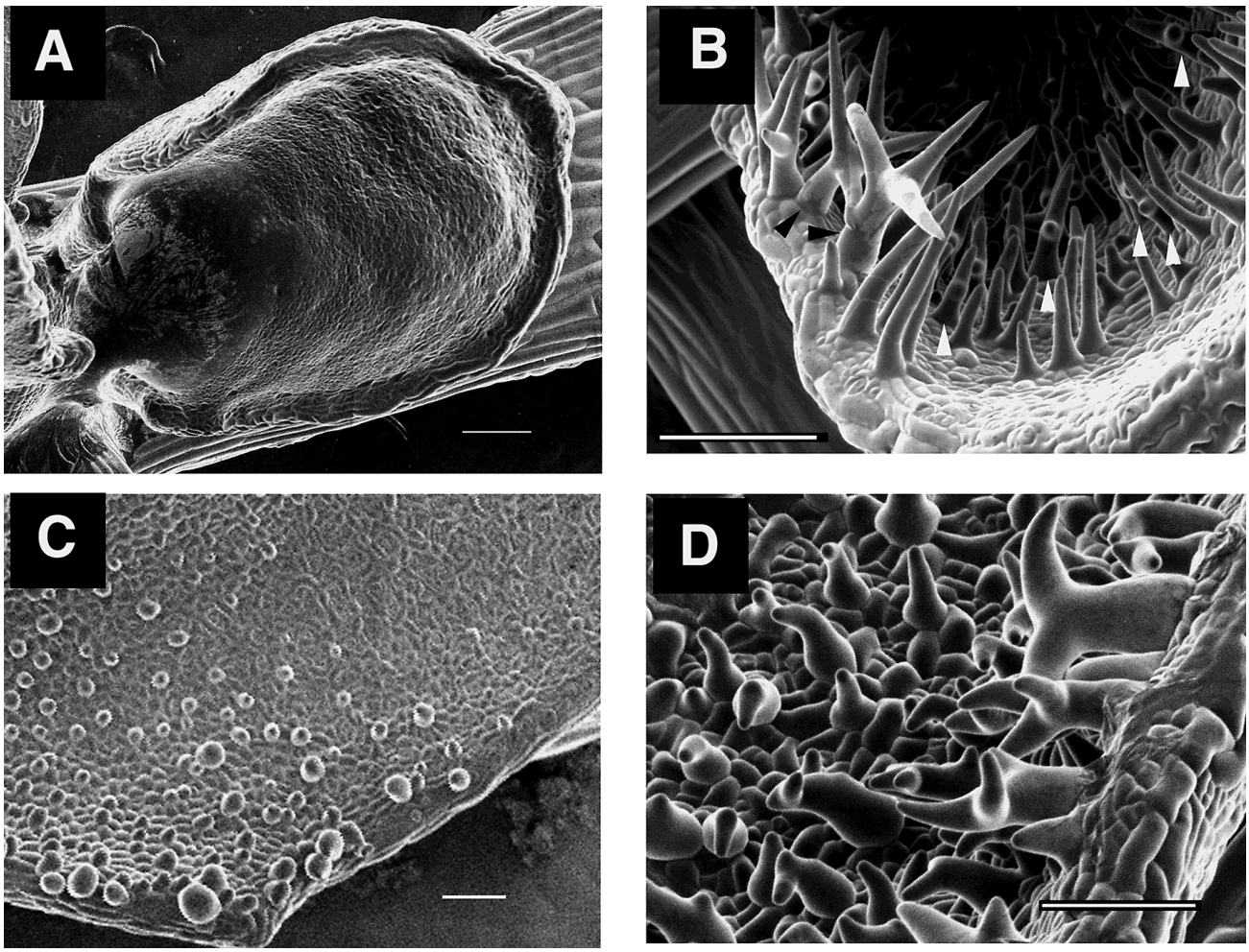

Figure 2. Effects of cytoskeleton inhibitors on polarized outgrowth, branch initiation, and morphogenesis in developing trichomes. Effects of cytochalasin D and oryzalin on early morphogenetic events were examined in a transgenic line in which trichome initiation is dexamethasone inducible. All plants in this experiment are *transparent testa glabrous (ttg)* and contain the 35S::*R-GR*.transgene, and thus lack trichomes in the absence of dexamethasone. (**A**) Developing leaf of an uninduced plant 48 h after treatment with buffer containing 0.1% DMSO. (**B**) Developing leaf 48 h after dexamethasone induction. (**C**) Developing leaf 48 h after dexamethasone induction in the presence of oryzalin. (**D**) Developing leaf 48 h after dexamethasone induction in the presence of cytochalasin D. White-arrowheads, two-branched trichomes, black arrowheads, three-branched trichomes. Bar A-D, 100 μm. (Reproduced with permission from Szymanski and others 1999.)

to be microtubule-dependent: the presence of both F-actin and microtubule inhibitors severely inhibits cell growth (Szymanski and others 1999). A graphical summary of the stage-specific microtubule and actin-dependent requirements during trichome development is shown in Figure 4.

Interestingly, Mathur and others (1999) showed that drugs that either stabilize or destabilize the cytoskeleton have similar effects on trichome morphogenesis. For example, the effects of the F-actin stabilizing agents phalloidin and jasplakinolide on trichome morphogenesis were indistinguishable from those of cytochalasin D and Latrunculin B, which destabilize microfilaments. The authors suggested that each actin-binding drug either disorganized, fragmented, or depolymerized F-actin in developing trichomes (Mathur and others 1999). It is possible that the actin-dependent reinforcement of an organized growth pattern requires actin polymerization and the activity of F-actin binding proteins, both of which can be affected by different classes of actin-binding agents. Microtubule stabilizing and depolymerizing drugs also had similar effects on trichome morphogenesis. Interestingly, pulse chase

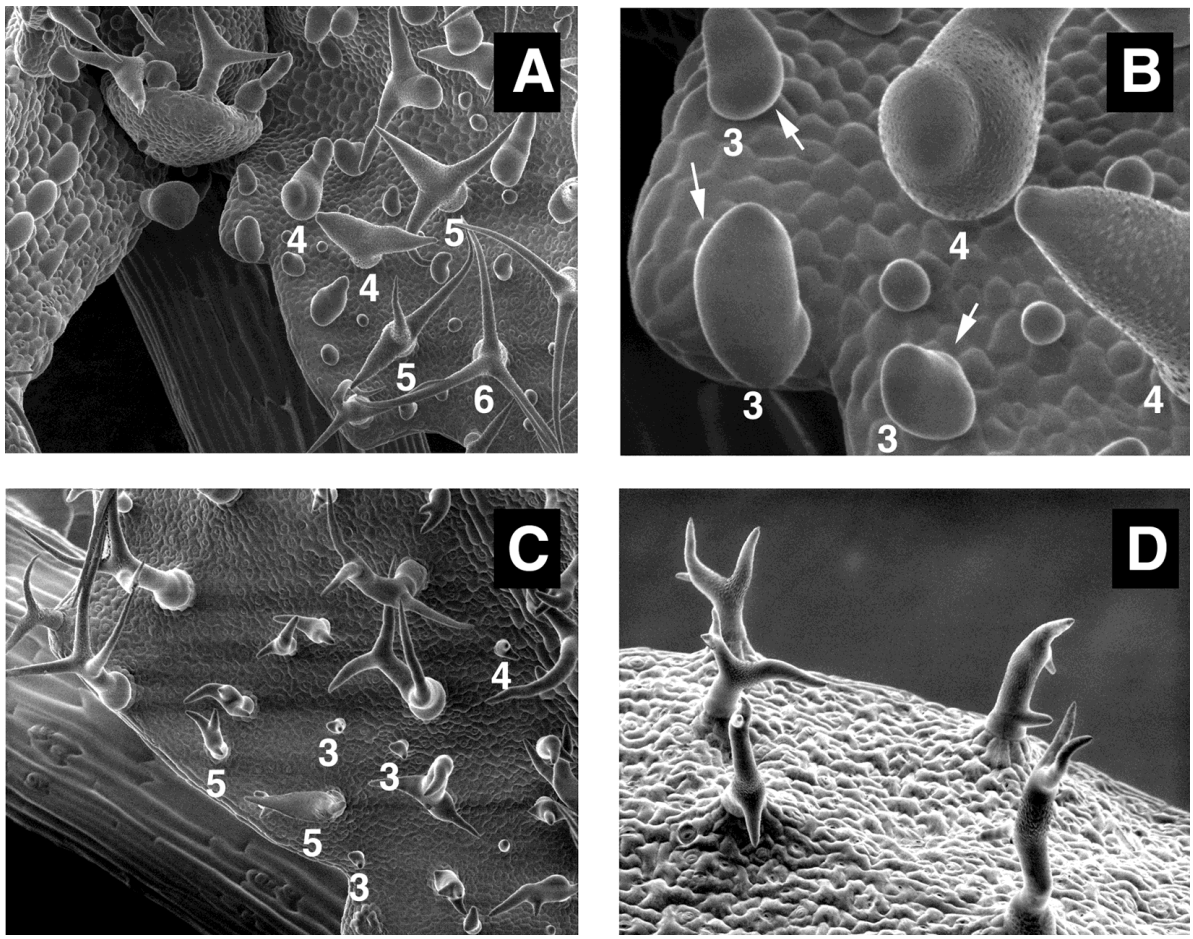

Figure 3. Effects of cytoskeletal inhibitors on trichome morphogenesis. (**A**) Shape defects of cells treated with 100 μM oryzalin for 48 h. (**B**) High magnification of (A) demonstrating swollen branch morphology and inhibition of branching in stage 3 and 4 oryzalin-treated cells. (**C**) Trichome morphology after 48 h treatment with 50 μM Latrunculin B. (**D**) Trichome morphology after treatment with 50 μM cytochalasin D for 6 days. Arrows indicate bulges that may represent defective trichome branches. Numbers printed below each trichome indicate the developmental stage (Reproduced with permission from Szymanski and others 1999, 2000.)

experiments with microtubule-stabilizing drugs increased trichome branching in *zwi* and *stichel* (*sti*) mutant trichomes, both of which display reduced branch initiation (Mathur and Chua 2000). Perhaps stabilization of microtubules promotes branching, but is not sufficient to cause increased branch formation in wild-type cells. *zwi* and *sti* trichomes may contain a subset of components that are required for branch initiation, and transient stabilization of microtubules may partially substitute for *ZWI* or *STI* gene function. A genetic analysis of the control of trichome branching has revealed a highly redundant regulatory pathway that involves the activity of several genes (Luo and Oppenheimer 1999). The mechanistic details of branch initiation will require further molecular genetic analysis.

Cytoskeletal Organization in Developing Trichomes

An analysis of the cytoskeletal organization in developing trichomes has provided some insight into the possible functions of each array, but more importantly has allowed comparison of cytoskeletal organization in wild-type and mutant cells. To maximize the probability of detecting direct effects of the mutation of interest on cytoskeletal organization, the cytoskeleton localization experiments should be conducted at the stage of development at which the mutant phenotype is first apparent. This is easier said than done. Using fixed samples and immunolocalization, thoroughly reconstructing a developmental time series is a time-consuming and ulti-

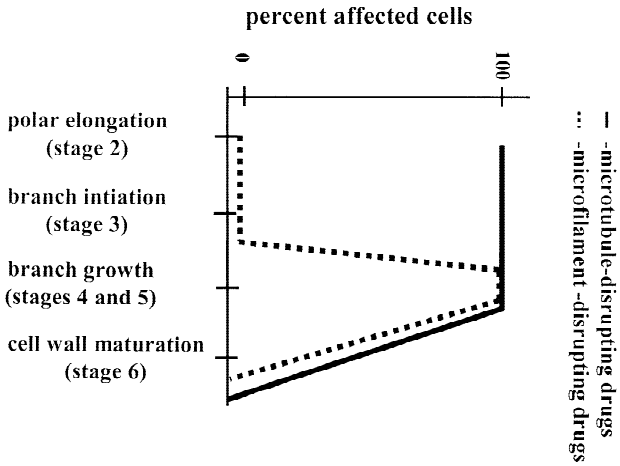

Figure 4. Summary of the stage-specific effects of microtubule and microfilament-disrupting agents on trichome morphogenesis. Scanning electron microscopy was used to detect the cell shape defects of drug-treated cells 24–48 h after treatment. The percent of affected cells is plotted on the y-axis and the stages of trichome development are plotted on the x-axis. Solid line, percent of trichomes at a given stage that are affected by microtubule-disrupting drugs. Dashed line, percent of trichomes at a given stage that are affected by microfilament-disrupting drugs.

mately incomplete exercise. In some experimental systems, direct GFP fusions to tubulin or actin or to proteins that bind either cytoskeletal array have been used to obtain a time series of cytoskeletal organization in living cells. For example, Marc and others (1998) constructed a fusion between green fluorescent protein (GFP) and the microtubule-binding domain of microtubule-associated protein 4 (GFP-MAP4) that can report on microtubule dynamics in living plant cells. Granger and Cyr (2000) went on to show that the reporter does not significantly alter cell cycle parameters in dividing BY-2 cells and can be used to noninvasively monitor microtubule organization throughout the cell cycle. GFP-MAP4 was subsequently used to examine microtubule organization in *Arabidopsis* trichomes (Mathur and Chua 2000).

The GFP-MAP4 signal in living cells reveals a complex organization of microtubules throughout trichome development. Although it is difficult to resolve the microtubule cytoskeleton during the early stages of trichome development, GFP-MAP4 has detected extremely fine arrays of short microtubules in the stalks of trichomes (Figure 5 A,B). The orientation of microtubules in trichome stalks varies from transverse to longitudinal, with some cells displaying helical arrays of opposite handedness (Figure 5A). The relationship between microtubule orientation and growth is complex and may not adhere to the existing paradigm of transverse microtubules and anisotropic growth. It would be very helpful to have a complete time series of microtubule organization throughout trichome development to more closely relate cytoskeletal reorganization to specific morphogenetic events.

The microtubule organization during branch formation has not been resolved. However, in mature trichomes Mathur and others (2000) detected microtubules in the branch-stalk junction that intersect at different angles. The authors suggest that these alterations in microtubule organization are remnants of the arrays that were previously associated with branch formation. Indeed, specialized microtubule-containing structures may be associated with branch formation. GFP-MAP4 detects aster-like microtubule configurations in trichomes that are recovering from paclitaxel-treatment (Mathur and Chua 2000). Perhaps these specialized structures promote branch formation.

A survey of the actin organization in trichomes at each developmental stage has been conducted using both immunolocalization of actin in fixed samples (Szymanski and others 1999) and GFP fused to the actin-binding domain of mouse TALIN (GFP-TALIN) to detect actin organization in living cells (Mathur and others 1999). In some cases the description of actin at specific developmental stages differed. For example, antibodies clearly detected F-actin structures in fixed stage 1 and 2 trichomes (Szymanski and others 1999), but no similar structures were detected with GFP-TALIN (Mathur and others 1999). Clearly, both chemical fixation of material and GFP-based technologies have limitations. For example, the controversial dense actin patch at the tip of growing pollen tubes that was detected using chemical fixation methods appears to be an artifact (Miller and others 1996). Steady state actin organization in pollen tubes reported with GFP-TALIN (Kost and others 1998) resembles the structures revealed using cryofixation and improved chemical fixation methods coupled with rhodamine-phalloidin staining (Gibbon and others 1999; Miller and others 1996). In many systems GFP fusions to cytoskeletal proteins also have limitations. For example, GFP fusions to actin fail to complement actin mutants, and can cause defects in cytokinesis (Doyle and Botstein 1996; Westphal and others 1997). GFP fusions to actin-binding proteins such as TALIN may display similar toxicity or may alter actin organization in living cells. Therefore, a combination of localization strategies is the safest approach to describe the *in vivo* organization of the cytoskeleton most accurately.

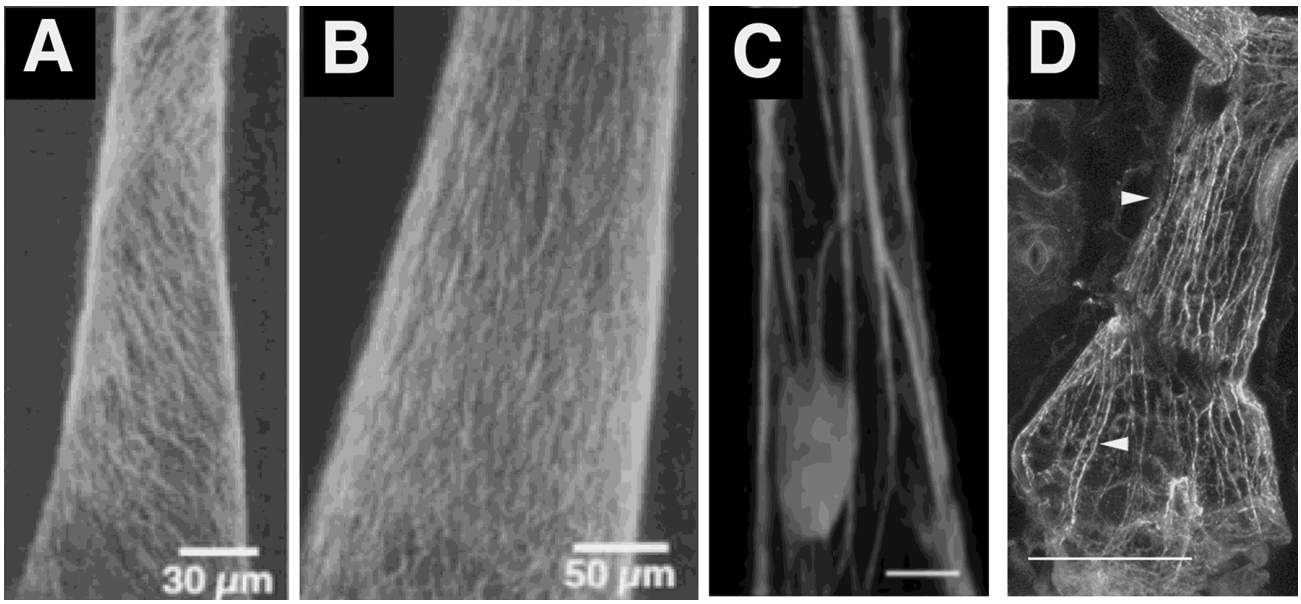

Figure 5. Localization of the microtubule and microfilament cytoskeletons in the stalks of late-stage trichomes. (**A, B**) MAP4-GFP fluorescence reporting microtubule organization in the stalks of stage 5 or stage 6 trichomes. (**C**) TALIN-GFP fluorescence reporting the microfilament organization in the stalk of a stage 5 or stage 6 trichome. (**D**) Actin immunofluorescence in the stalk of a stage 6 trichome. All images are maximum projections of optical sections obtained using a confocal fluorescence microscope. Arrowheads, longitudinal bundles of microfilaments. Bars: A, 30 μm; B, D, 50 μm; C, 10 μm. (A-C, reproduced with permission from Mathur and others 1999 , Mathur and Chua 2000 , D, reproduced with permission from Szymanski and others 1999).

At later stages of trichome development, GFP-TALIN and actin antibodies revealed a similar general relationship between actin organization and the morphological transitions that occur. In regions of the cell in which growth patterns are being established, such as branch buds and the apical regions of stage 3 branches, the actin signal is strong and diffuse (Mathur and others 1999; Szymanski and others 1999). Subsequently, in other cellular domains in which the growth pattern is established, such as the central branch domains at stages 4 or 5 or in the stalks of stage 6 cells, F-actin dominates the anti-actin signal, a subset of which is aligned with the growth axis. Figures 5C and 5D illustrate the organization of F-actin in the stalk of mature trichomes using GFP-TALIN and actin antibodies, respectively. The polarized alignment of F-actin in trichomes from stages 4 through 6 are consistent with the general role for F-actin in the reinforcement and maintenance of cell pattern in trichome development. Anti-actin antibodies detected actin filaments or fine bundles at each stage of trichome development. After stage 3, many of these bundles are aligned with the local axis of elongation. Similar aligned arrays of fine actin bundles have been observed in a variety of elongating cell types (Heslop-Harrison and others 1986; Jackson and Heath 1993; Parthasarathy 1985). Several groups have proposed that the presence of fine actin bundles, as opposed to heavy bundles of closely associated filaments, is associated with the ability to transport vesicles (Foissner and others 1996; Miller and others 1999; Thimann and others 1992; Waller and Nick 1997; Wang and Nick 1998). The detection of actin-associated vesicle-like structures at all developmental stages and the observation of rapid longitudinal transport of vesicles along the cortex of living stage 6 trichomes (D. Szymanski, unpublished results) are consistent with this idea. It is possible that disruption of actin organization during stages of rapid growth leads to misregulated transport of vesicles that fuse promiscuously at the random positions within the cell. This could explain the variable cell shape defects observed in growing trichomes treated with F-actin-disrupting agents.

Genetics of Trichome Morphogenesis

Microtubule-Dependent Function

The reduced branching and swollen stalk phenotype of the *zwi* trichomes is very similar to the shape

defects of cells that are exposed to microtubule-disrupting agents. Cloning of the *ZWI* gene provided the first evidence for microtubule function during trichome development (Oppenheimer and others 1997). The *ZWI* gene was cloned independently in a screen for calmodulin-binding proteins, and was shown to encode a kinesin-like calmodulin-binding protein (KCBP) (Reddy and others 1996). *ZWI* and *KCBP* are the same gene. Although biochemical, cell biological, and genetic experiments designed to elucidate *ZWI/KCBP* function have been ongoing, a unified description of its role in cell morphogenesis has not emerged. Biochemical analyses have shown that ZWI/KCBP is a minus-end directed microtubule motor protein (Song and others 1997). Both the C-terminal domain, which contains the motor domain, and an N-terminal domain are capable of bundling microtubules *in vitro* (Kao and others 2000). The microtubule-bundling and binding activity is inhibited by Ca^{2+}-calmodulin (Narasimhulu and Reddy 1998; Kao and others 2000). Presumably Ca^{2+}-calmodulin binds the C-terminal calmodulin-binding domain of KCBP/ZWI and blocks the microtubule-binding activity of the motor domain. In terms of *in vivo* function, current data suggest that KCBP/ZWI may affect the organization of microtubules during discrete stages of the cell cycle, particularly during the transition from metaphase to anaphase (Vos and others 2000). Localization of this motor protein to the mitotic spindle during anaphase is consistent with its potential role in cell division (Smirnova and others 1998).

The stalk elongation and branch initiation defect of *zwi* trichomes indicates that *ZWI* is required for trichome cell growth. To date, no localization data for ZWI/KCBP in trichomes or any other interphase cell has been reported. Northern blot data suggest that *ZWI* is expressed at very low levels in leaves (Reddy and others 1996), and the protein may not accumulate to detectable levels. Oppenheimer has taken a genetic approach to understand how *ZWI* regulates trichome morphogenesis. Genetics screens for extragenic suppressors of the *zwi-3* mutant phenotype identified mutations in three distinct loci that partially suppress the branching defect of *zwi-3* mutants (Krishnakumar and Oppenheimer 1999). Subsequent genetic analyses by a number of groups have identified additional genes that are required for normal branch formation (Folkers and others 1997; Krishnakumar and Oppenheimer 1999; Luo and Oppenheimer 1999; Perazza and others 1999). Cloning and characterization of additional genes that affect trichome branching will help clarify the function of microtubules in this process.

The Distorted Mutants and Actin-Dependent Function

A careful morphometric analysis revealed that the strict stage-specific cell shape defects of cytochalasin-D treated trichomes were phenocopied by the "distorted group" mutants (Szymanski and others 1999). The striking similarity of the distorted class of trichome shape mutants to cytochalasin D-treated trichomes suggests that constraint of actin filament-dependent functions such as organelle positioning, directional vesicle transport, exocytosis, endocytosis, and microfilament bundling cause the distorted trichome phenotype. An analysis of actin-dependent growth during trichome development will provide a useful model process to genetically dissect an important growth control pathway (Mathur and others 1999; Szymanski and others 1999). Historically, the *distorted1* and *distorted2* trichome mutants have been used as visual markers for classical genetic mapping experiments for more than 20 years (Feenstra 1978). Six additional mutants in this class were identified by Hülskamp and others (1994). Each distorted mutant displays similar trichome shape defects. For example, both drug-treated and *grarled (grl)* distorted trichomes often exhibit abnormal stalk swelling and expansion along the apical face of the cell, often occurring during the transition to stage 4 (Szymanski and others 1999). Similar stage-specific defects are observed in *crooked (crk)* trichomes (Szymanski 2000). The actin organization of *crk* and *grl* trichomes has been examined in detail (Mathur and others 1999; Szymanski and others 1999). In both cases, altered F-actin organization was coincident with cell shape defects. These results do not prove that *CRK* and *GRL* directly affect actin organization, but demonstrate a close temporal link between misregulated F-actin organization and cell shape defects in the mutants. The actin organization in *alien, klunker, worm,* and *dis1* distorted trichomes also has been examined using GFP-TALIN (Mathur and others 1999). In each case the actin organization of the mutants differs from that of the wild type. Actin immunolocalization in mature *wrm* and *crk* trichomes reveals that the actin cytoskeleton of both mutants is extensively branched, and not surprisingly, the severity of the F-actin organization defects correlates with the severity of cell shape defects (Szymanski 2000).

Future challenges lie in defining the precise relationship between distorted group gene function and actin-dependent morphogenetic events. At this time it is not known which mutations in distorted group genes directly affect the spatial or temporal control of F-actin organization. It is very likely that F-actin

organization, organelle positioning, membrane recycling, and vacuole biogenesis are inter-dependent processes, and distorted mutants may be compromised in any one of them. In any case, these cellular processes are fundamental to plant cell growth, and are not well understood. A genetic analysis of distorted trichome mutants will provide the tools to functionally dissect these important growth control pathways. Molecular and biochemical analysis of the distorted group gene products will lead to a more mechanistic understanding of actin-dependent plant cell growth.

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