Molecular Plant Advance Access published March 7, 2012

Molecular Plant • Pages 1–10, 2012 RESEARCH ARTICLE

Microtubules Are Essential for Guard-Cell Function in *Vicia* and *Arabidopsis*

William Eisinger[a,b], David Ehrhardt[b] and Winslow Briggs[b,1]

a Department of Biology, Santa Clara University, Santa Clara, CA 95053, USA
b Department of Plant Biology, Carnegie Institution for Science, Palo Alto, CA 94305, USA

ABSTRACT Radially arranged cortical microtubules are a prominent feature of guard cells. Guard cells expressing GFP-tubulin showed consistent changes in the appearance of microtubules when stomata opened or closed. Guard cells showed fewer microtubule structures as stomata closed, whether induced by transfer to darkness, ABA, hydrogen peroxide, or sodium hydrogen carbonate. Guard cells kept in the dark (closed stomata) showed increases in microtubule structures and stomatal aperture on light treatment. GFP-EB1, marking microtubule growing plus ends, showed no change in number of plus ends or velocity of assembly on stomatal closure. Since the number of growing plus ends and the rate of plus-end growth did not change when microtubule structure numbers declined, microtubule instability and/or rearrangement must be responsible for the apparent loss of microtubules. Guard cells with closed stomata showed more cytosolic GFP-fluorescence than those with open stomata as cortical microtubules became disassembled, although with a large net loss in total fluorescence. Microtubule-targeted drugs blocked guard-cell function in *Vicia* and *Arabidopsis*. Oryzalin disrupted guard-cell microtubules and prevented stomatal opening and taxol stabilized guard-cell microtubules and delayed stomatal closure. Gas exchange measurements indicated that the transgenes for fluorescent-labeled proteins did not disrupt normal stomatal function. These dynamic changes in guard-cell microtubules combined with our inhibitor studies provide evidence for an active role of microtubules in guard-cell function.

Key words: cytoskeleton dynamics; fluorescence imaging; guard cells; microtubules.

INTRODUCTION

Guard cells regulate leaf gas exchange by altering their shape and thereby regulate stomatal aperture. Precise regulation of leaf gas exchange is essential for plants, since it directly affects photosynthesis, transpiration, xylem translocation, and plant water potential. A great deal is known about light regulation of guard-cell function and the mechanism of stomatal opening. Red light is required to provide photosynthetically derived energy for stomatal opening (Ogawa et al., 1978; Ogawa, 1981; Zeiger and Field, 1982) and blue light triggers the opening mechanism through photoactivation of the phototropins (Kinoshita et al., 2001). The well-studied mechanism of stomatal opening involves blue-light activation of a plasma membrane proton ATPase that powers solute uptake. Subsequent increase in guard-cell solute concentration results in osmotic uptake of water and resultant cell expansion (Blatt, 2000). Owing to the unique structure of guard-cell walls, this increase in cell volume results in stomatal opening. The plant hormone abscisic acid (ABA) plays an important role in stomatal closure by inducing release of accumulated solute from guard cells (Blatt, 2000). Mutant plants insensitive to ABA (*abi 1–1*) display a wilty phenotype because of impaired stomatal closure.

The cytoskeleton may play an important role in stomatal closure (see review by Galatis and Apostolakos, 2004). Actin cytoskeleton reorganization has been linked to ABA-induced stomatal closure (Eun and Lee, 1997). In addition, actin antagonists alter the normal stomatal response to ABA (Hwang et al., 2000). Several studies also concern the possible role of microtubules in stomatal opening and closing. Guard cells are unique in that their microtubules are radially arranged. This unusual arrangement begins in early development and continues with guard-cell maturation (Galatis and Apostolakos, 2004). It is likely responsible for the radial pattern of cell-wall cellulose

[1] To whom correspondence should be addressed. E-mail briggs@stanford.edu, tel. (650) 739-4207, fax 650–325–6857.

doi: 10.1093/mp/sss002
Received 6 October 2011; accepted 1 January 2012

microfibrils that contributes to the asymmetric cell expansion that provides the cell shape needed for the opening mechanism.

However, the role of microtubules in the function of mature guard cells is unclear. Early studies using immunofluorescence gave contradictory results (see review by Galatis and Apostolakos, 2004). For example, Fukuda et al. (1998) reported that, in darkness, *Vicia faba* guard cells had few and poorly organized microtubules. However, guard-cell microtubule numbers and radial order increased during light-induced stomatal opening. Taxol treatment (both 10 and 40 μM) of guard cells, which stabilizes cortical microtubules, retained microtubule number and radial order and delayed stomatal closure in the dark. Propyzamide (50 μM), which disrupts microtubules, hastened closure in the dark. Fukuda et al. proposed that radially arranged guard-cell microtubules control diurnal stomatal movement. By contrast, Assmann and Baskin (1998) reported that guard cells with opened and closed stomata in epidermal peels of *Vicia faba* showed a similar pattern of radially arranged microtubules as visualized in cryofixed thin sections treated with anti-tubulin antibody. They also reported that neither colchicine (1.0 mM) nor taxol (20 μM) treatment of guard cells in epidermal peels affected stomatal response to light and dark. These authors concluded that microtubules are not required for guard-cell function.

In more recent studies using confocal microscopy and plants genetically modified to express fluorescent-labeled microtubules, Marcus et al. (2001) reported that guard cells treated with the anti-microtubule drugs (propyzamide, oryzalin, and trifluralin) did not open their stomata in response to environmental conditions normally conducive to stomatal opening. In addition, they used transient (biolistic) transformation of *Vicia faba* with a green fluorescent-labeled microtubule-associated protein (GFP::MBD (MAP4)) to visualize radially arranged guard-cell microtubules. These transformed guard cells failed to open their stomata in response to white light. Nevertheless, based on their results with anti-microtubule drugs, they concluded that microtubules play an important role in guard-cell function. They also reported that inhibition of stomatal opening caused by GFP-MAP4 label or anti-microtubule drugs could be overcome by treatment with the proton-pump activator, fusicoccin. Considering these results, Marcus et al. (2001) suggested that microtubules are involved in an upstream event in the signal-transduction pathway for stomatal opening.

Lahav et al. (2004), using immunofluorescence in guard cells of *Commelina communis*, reported that light treatment resulted in more ordered and densely bundled microtubules. They reported that although blue (20 μmol protons m^{-2} s^{-1}) or white (200 μmol protons m^{-2} s^{-1}) light induced similar changes, red light (20 μmol protons m^{-2} s^{-1}) did not affect microtubule order or density. Treating guard cells with fusicoccin (2 μM) resulted in stomatal opening without changes in microtubule order or number. In the same study, these authors also observed living *Arabidopsis thaliana* plants genetically modified to express GFP fused to α-tubulin (TUA6). In the dark, about half of the guard cells showed a diffuse pattern of GFP label suggestive of unpolymerized tubulin in the cytosol. Light treatment (white, 200 μmol protons m^{-2} s^{-1}), which induced stomatal opening, resulted in more organized microtubules in guard cells and less of the diffuse label. In addition, the authors reported that, upon light treatment, microtubules in adjacent pavement cells (subsidiary) became perpendicularly oriented with respect to guard-cell microtubules. Finally, they observed that light-induced reorganization of guard-cell microtubules preceded increases in stomata aperture. Lahav et al. (2004) concluded that guard-cell microtubules have a role in the daily cycles of stomata opening and closing.

Here, we re-examine the effects of microtubule drugs on guard-cell function in *Arabidopsis*, and use live cell imaging to evaluate quantitatively whether guard-cell function is related to changes in microtubule number and organization. As a control, we validate the use of fusion proteins as probes for the cytoskeleton in guard cells by measuring changes in stomatal conductance of individual leaves and transpiration in whole plants to assess the potential impact of various transgenes on guard-cell function.

RESULTS

Microtubule Stability and Guard-Cell Function: Microtubule Counts and Order

To probe the role of microtubules in guard-cell function, we used confocal microscopy to image microtubule organization in *Arabidopsis* guard cells expressing GFP-TUA5 (Figure 1). Stomatal aperture was evaluated by brief visualization using bright-field illumination (see 'Methods'). Guard cells from open stomata in z-projected images showed more radially arranged microtubule structures (Figure 1B) than guard cells with closed stomata (Figure 1C). We use the term 'structure' to refer both to single microtubules and to co-linear bundles of microtubules that cannot be resolved by optical microscopy. If we count resolved microtubule structures in randomly chosen guard cells from light-grown leaves of greenhouse plants, we find a strong correlation between numbers of these structures and stomatal aperture (R^2 = 0.9566, Figure 1A). On this basis, fewer than 20 microtubule structures were recognizable in z-projections of guard-cell confocal images when no stomatal aperture was visible. Guard cells with open stomata (2 μm) averaged over 50 resolved microtubule structures. When guard cells with open stomata were placed in the dark, stomatal aperture and the number of resolvable microtubule structures both declined by 50% within 30 min (Figure 2). Regression of the curves revealed them to be near linear (R^2 = 0.9647 and 0.8667, respectively). Microtubules in surrounding pavement cells were not radially arranged and did not appear to change when stomata closed.

Quantitative Changes in Fluorescence

As an additional means of quantitation, we measured GFP fluorescence associated with cortical microtubules and with

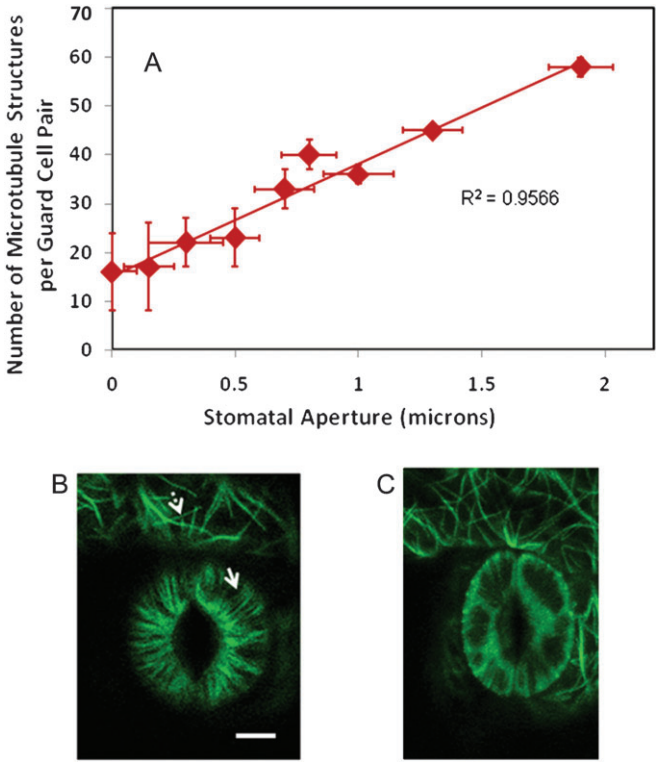

Figure 1. Correlation between Guard Cell Microtubule Structure Number and Stomatal Aperture.

The number of resolvable guard-cell microtubule structures is positively correlated with stomatal aperture for randomly selected greenhouse-grown plants **(A)** (n = 18). Error bars are standard deviations. Bar = 10 microns. Confocal image of GFP:tubulin-labeled guard-cell pair with open stoma **(B)** or closed stoma **(C)**. Microtubules (fluorescent images) in guard cells are radially arranged (solid arrow) but fluorescent images in surrounding pavement cells are more random (dashed arrow).

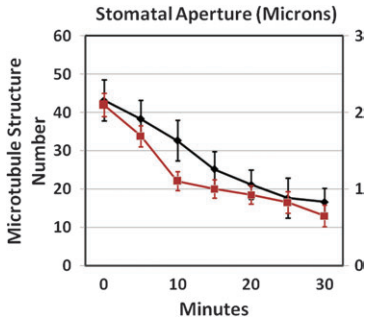

Figure 2. Guard-Cell Microtubule Structure Number and Stomatal Aperture Decline in Parallel with Stomatal Closing.

When leaves from light-grown (greenhouse) plants are placed in the dark there is a parallel decline in number of microtubule structures (diamonds) and stomatal aperture (squares). n = 6. Error bars are standard deviations.

the cytosol of guard cells and adjacent pavement cells from light-treated (Figure 3, T0) leaves and from leaves held in the dark for 40 min (Figure 3, T40, see 'Methods' and Supplemental

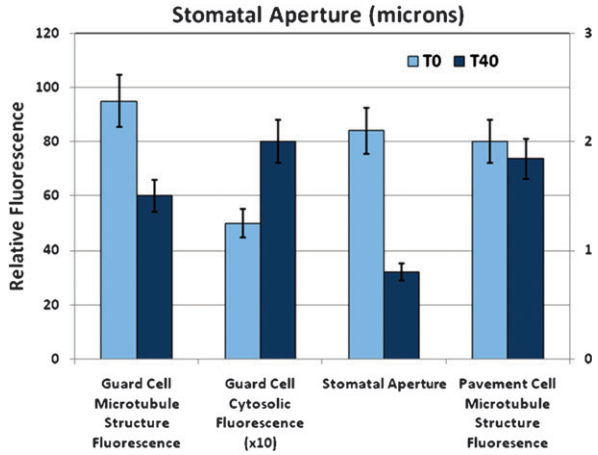

Figure 3. Guard Cell Microtubule Structure and Cytosolic Fluorescence Changes on Stomatal Closing.

Total GFP-tubulin fluorescence in guard cells declined over 40 min in darkness with stomatal closure but cytosolic fluorescence increased. Total fluorescence in adjacent pavement cells did not change significantly. T0, transfer to darkness; T40, values after 40 min in darkness (n = 20). Error bars are standard deviations.

Figure 1). Total guard-cell fluorescence declined about 35% and stomatal aperture declined about 40% during the 40-min dark period. However, guard-cell cytosolic fluorescence increased by about 60%, presumably as microtubule depolymerization resulted in more cytosolic tubulin dimers. Note that cytosolic fluorescence intensity is about an order of magnitude less than typical microtubule fluorescence intensity. Thus, there is a major overall loss of fluorescent tubulin. By contrast, total fluorescence in adjacent pavement cells did not change significantly during the 40-min dark period.

Evidence of cytosolic GFP-fluorescence in guard cells with closed stomata can be seen in Figure 4. We imaged guard cells from plants expressing GFP-tubulin at 4-s intervals for a total of 5 min. Guard cells with closed stomata showed 'blobs' of fluorescence moving rapidly through their cytosol (Figure 4 and Supplemental Video 1); such dynamic and bright cytosolic fluorescence was not observed in guard cells of open stomata (Supplemental Video 2).

Light-Induced Opening

Guard cells from greenhouse-grown leaves stimulated to open their stomata in response to 3-h light showed significantly more microtubule structures than equivalent dark controls (Figure 5). After 3 h of continuous light, guard cells averaged about 50 recognizable structures per cell and a stomatal aperture of about 2 μm. Guard cells in darkness for 12 h averaged fewer than 20 structures and stomatal apertures less than 0.3 μm. After 1-h white light treatment (100 μmol photons m^{-2} s^{-1}) guard cells averaged about 50 structures per cell and stomatal apertures of about 1.5 μm. Hence, 1 h of white light was nearly sufficient to restore microtubule structure numbers and stomatal aperture to the values

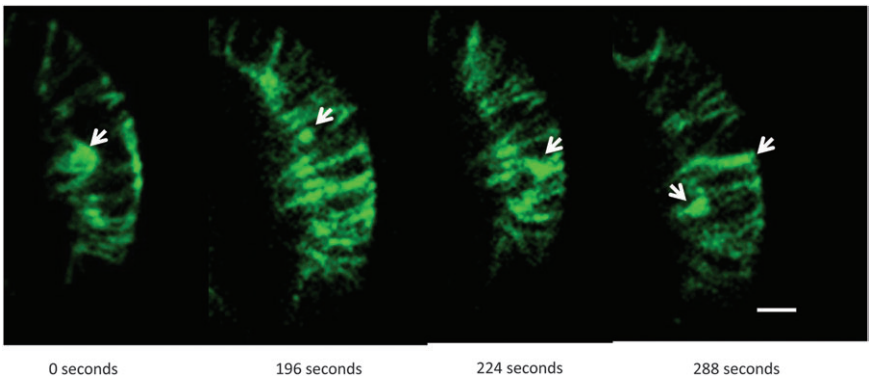

Figure 4. GFP-Tubulin in the Cytosol of Guard Cell from Closed Stomata.

Guard cells with closed stoma show many irregularly shaped fluorescent structures in the cytosol. The four images track the changes in these structures over time in a single guard cell. White arrows indicate areas of cytosolic fluorescence that change dramatically with time. Bar = 10 microns.

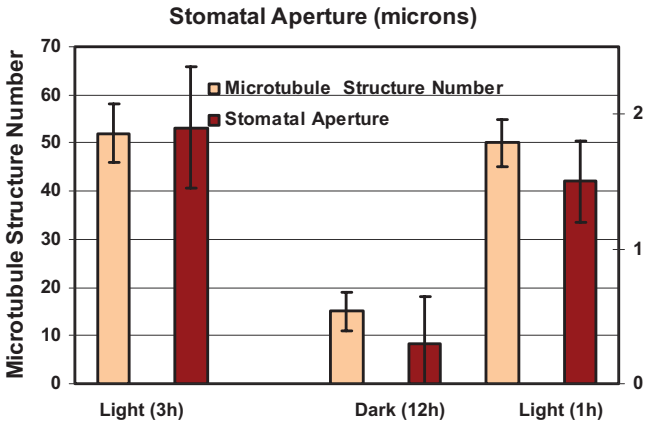

Figure 5. Changes in Guard Cell Microtubule Structure Number on Stomatal Closing in Darkness and Opening in Light.

Numbers of microtubule structures in guard cells and stomatal aperture both decline significantly in darkness following transfer to darkness from the greenhouse, but are restored with subsequent white light treatment (1 h, 100 μmol photons m^{-2} s^{-1}). The dark leaf samples were photographed at the end of the dark period then illuminated and used as the 1-h light samples (n = 6). Error bars are standard deviations. Bar = 10 microns.

obtained with continuous light. To determine whether continuous white light of that fluence rate caused bleaching of the GFP, we kept a leaf in continuous white light and imaged a single guard-cell pair at 10-min intervals for 110 min. The results (Supplemental Figure 2) showed no signs of bleaching. However, when we attempted to obtain images more frequently, we began to detect some bleaching, precluding obtaining a high-resolution time course for stomatal opening in the light.

Chemically Induced Closure

Abscisic acid (ABA) treatment (10 μM for 1 h) of greenhouse-grown leaves with open stomata reduced resolved microtubule structures by more than 50% and induced stomatal closure (Figure 6). However, ABA treatment (10 μM for 1 h) did not appear to affect microtubule structures in adjacent pavement cells (Figure 6). Treatment with hydrogen peroxide (0.1 mM for 1 h), an intermediate in ABA signal transduction (Desikan et al., 2004), also reduced the numbers of recognizable microtubule structures in guard cells and decreased stomatal aperture (Supplemental Figure 3A). Similar results were obtained with MAP4-GFP-expressing guard cells (Supplemental Figure 4). Likewise, sodium hydrogen carbonate (1.2 mM for 1 h) reduced guard-cell microtubule structure number (Supplemental Figure 3B).

Microtubule Assembly

Several possible explanations could account for the decline in resolved microtubule structures in guard cells as stomata close. There could be fewer microtubule assembly sites, slower rates of assembly, or faster rates of microtubule disassembly. In addition, bundling of existing microtubules might result in fewer resolved structures and, if so, such changes in structure number could be incorrectly interpreted as changes in total microtubule number. Finally, it was possible that we might not be able to detect single microtubules efficiently under our imaging conditions but only detect them when they came in associations of two or more.

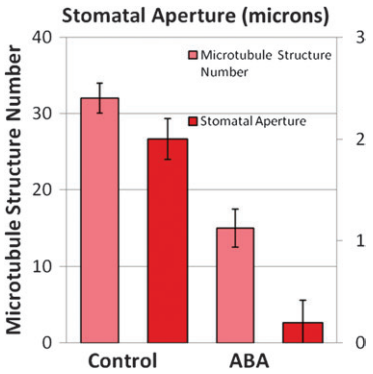

Figure 6. Decline in Guard Cell Microtubule Structure Number and Stomatal Aperture with ABA Treatment.

Numbers of microtubule structures in guard cells (n = 8) and stomatal apertures (n = 8) from greenhouse plants both decline with ABA treatment. Control pavement cells averaged 19 ± 2 structures; ABA pavement cells averaged 18 ± 2 structures. Error bars are standard deviations. Bar = 10 microns.

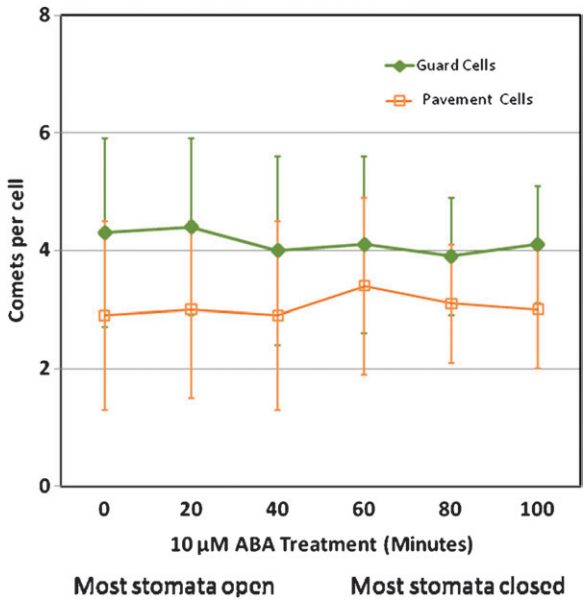

Figure 7. No Change in the Number of Microtubule Growing Ends during Stomatal Closure.

The number of GFP-EB1 comets per cell in guard cells and adjacent pavement cells remained constant as ABA treatment of greenhouse plants induced stomatal closure (n = 20). Error bars are standard deviations.

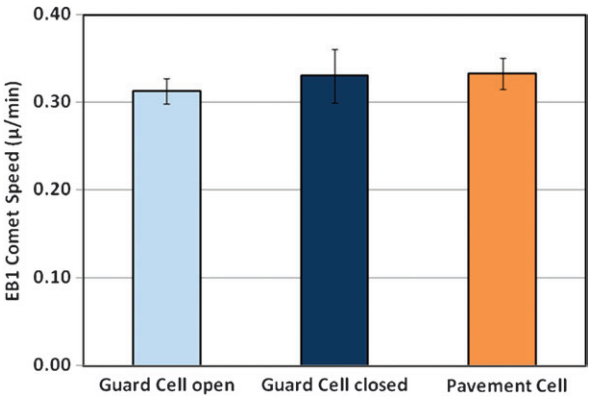

Figure 8. No Change in the Rate of Movement of Microtubule Growing Ends during Stomatal Closure.

The rate of GFP-EB1 comet movement is not significantly different for guard cells with open or closed stomata or for adjacent pavement cells (n = 80). Error bars are standard deviations.

To assess the rate of assembly, we followed End-Binding-Protein 1 (EB1) over time in guard cells from open and closed stomata as well as in adjacent pavement cells. EB1 is localized to the plus (growing) ends of microtubules, visualized as a dynamic 'comet' of GFP-labeled EB1 expressed in live cells (see Supplemental Video 3). The number of these comets in guard cells and adjacent pavement cells was unchanged by ABA treatment of greenhouse-grown leaves (10 µM ABA for 100 min; Figure 7), although this ABA treatment caused stomatal closure and reduced the number of microtubule structures that we could detect (Figure 6).

To determine whether the apparent reduction in microtubule structure number observable could be at least partially accounted for by a slowing down of microtubule assembly, we tracked and calculated the velocity of EB1 comets (related to the rate of microtubule assembly) using IMARIS software. This technique allowed us to track over 50 growing microtubule plus ends per guard cell (Figure 8). No difference in plus-end growth velocity was measured among guard cells with open or closed stomata or in adjacent pavement cells. The results eliminated the possibility that the changes we were seeing in microtubule structures on stomatal opening and closing were even partially the result of changes in the rate of microtubule assembly. We therefore suggest that the changes we observe are caused by changes in the rate of microtubule disassembly.

Effects of Microtubule Stabilization and Disruption on Stomatal Function

All of the results above indicate a close relationship between changes in stomatal aperture and changes in microtubule organization. However, they provide no evidence for any functional relationship between the observed microtubule changes and the mechanisms regulating stomatal aperture. Hence, to investigate the possible role of microtubules in guard-cell function, we performed tests with microtubule stabilizing and destabilizing drugs on both *Vicia faba* and *Arabidopsis* guard cells expressing GFP-TUA5. Treatment of leaf tissue of both species with oryzalin (0.1 mM), which disrupts microtubules, blocked light-induced stomatal opening, but treatment with taxol (20 µM), which stabilizes microtubules, had no significant effect on opening (Figure 9). Inset 9A shows an orylazin-treated guard-cell pair in *Arabidopsis* tissue with closed stoma and few, if any, recognizable GFP-labeled microtubules (visualized by confocal microscopy). Inset 9B shows a taxol-treated pair

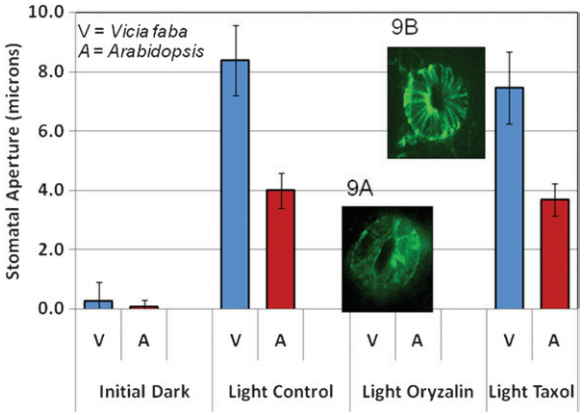

Figure 9. Oryzalin Blocks Stomatal Opening in the Light.

Oryzalin, but not taxol, blocked light-induced stomatal opening in Vicia and Arabidopsis. Epidermal peels of Vicia leaves and fragments of Arabidopsis leaves were held in darkness for 1 h to induce stomatal closing followed by 1 h of darkness in inhibitor solution. These leaf materials were then exposed to 100 μmol photons $m^{-2}s^{-1}$ white light for 1 h. Oryzalin disrupted guard cell fluorescent images (Inset 9A) and prevented stomatal opening but taxol-treated guard cells had normal fluorescent structures (Inset 9B) and opened the guard cell fully. The experiment was repeated 4 times with similar results. A minimum of 50 stomata was measured for each treatment in each experiment. Results from a typical experiment are shown.

with open stoma and prominent radially arranged microtubules. Conversely, taxol, but not orylazin, delayed stomatal closure in darkness (Figure 10). Inset 10A shows orylazin-treated guard cells with closed stoma and few, if any, detectable microtubules. Inset 10B shows taxol-treated guard cells with an open stoma and normal-appearing fluorescent structures. Thus, when the microtubules are either stabilized or disrupted, stomatal action is severely compromised or eliminated. The results indicate that microtubules play a functional role in environmental or hormonal regulation of stomatal aperture.

Transpiration and Stomatal Conductance

Working with plants expressing fluorescent proteins, some of which are overexpressed, we were concerned that the genetic modifications might affect guard-cell function. To address this issue, we compared light-induced transpiration and stomatal conductance in fluorescent protein-expressing plants with wild-type plants using both a LiCor 6400 gas-exchange system fitted with an *Arabidopsis* leaf chamber and a modified Qubit gas-exchange system.

At the start of an experiment using the LiCor system, we gave the plants a 30-min dark period sufficient to close the stomata and stabilize the system. We then gave the leaf in the chamber 30 min of light (100 μmol photons m^{-2} s^{-1}) followed by 30-min darkness. From the LiCor measurements, we determined that single leaves from all transgenic and control plants showed equivalent increases in stomatal conductance with continuous light during the 30-min period of illumination and conductance declined similarly when the lights were turned off (Figure 11).

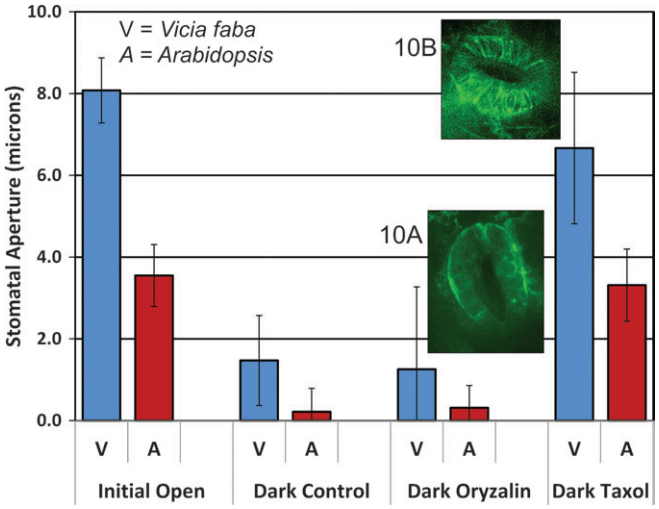

Figure 10. Taxol Delays Stomatal Closing in the Dark.

Taxol, but not oryzalin, blocked stomatal closure in darkness in Vicia and Arabidopsis.

Epidermal peels of Vicia leaves and fragments of Arabidopsis leaves were held in the light (100 μmol photons $m^{-2}s^{-1}$) for 1 h to induce stomatal opening followed by 1 h of light in inhibitor solution. Then these leaf materials were placed in darkness for 1 hour. Oryzalin disrupted guard-cell fluorescent images (Inset 10A) but allowed stomatal closing whereas taxol stabilized microtubule structure despite darkness (Inset 10B) and prevented stomatal closing. The experiment was repeated 4 times with similar results. A minimum of 50 stomata was measured for each treatment in each experiment. Results from a typical experiment are shown.

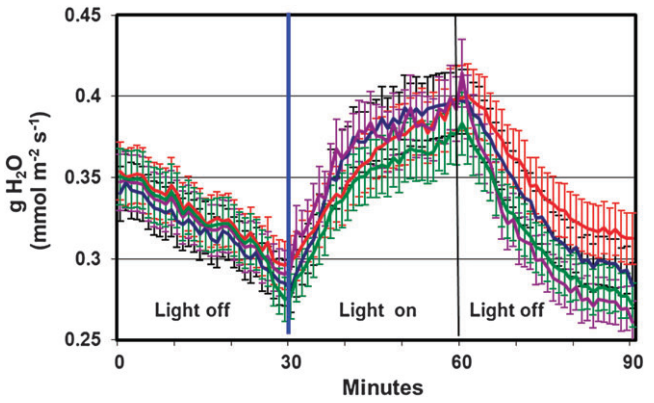

Figure 11. Transgenic Seedlings Show Normal Conductance Changes in Response to Light and Darkness.

Plants genetically modified to express fluorescent proteins show a normal light-induced increase in stomatal conductance (g) and a subsequent decrease in darkness. Whole-leaf conductance was measured using a LiCor 6400 gas exchange system. Black trace, wild type; purple trace, GFP-EB1; green trace, GFP-MAP4; red trace, GFP-TUB. n = 6. Error bars are standard deviations.

For transpiration measurements of whole *Arabidopsis* plants with the Qubit system, four plants from the greenhouse were placed in four identical chambers and allowed to accommodate to darkness for 30 min. Each chamber was then illuminated by a red and a blue LED light source (each approximately

100 μmol photons m^{-2} s^{-1}) for 30 min. Lights were turned off and the chambers were kept in darkness for an additional 30 min. At the end of the initial 30-min dark period, transpiration rates were arbitrarily set to zero for all chambers. Light induced similar rates of transpiration in all plants and transpiration continued to increase throughout the illumination period (Supplemental Figure 5). All plants showed a similar decline in transpiration during the subsequent 30-min dark period regardless of genetic status. There was no significant difference in the transpiration increase among whole plants or individual leaves during illumination and little significant difference during a subsequent dark period regardless of genetic status. Hence, the fluorescent-labeled proteins transformed into the *Arabidopsis* plants had little or no effect on light-regulated stomatal opening or stomatal closure in darkness as measured by either method.

DISCUSSION

Guard cells facilitate photosynthetic gas exchange and regulate transpiration and xylem transport. Plants have evolved a number of sophisticated environmental sensors to optimize the balance between the need for gas exchange and the need to conserve water. These environmental sensors are fairly well understood, as are the osmotic-based stoma opening/closing mechanisms (Schroeder et al., 1987). However, the signal-transduction systems that link these sensors to the osmotic mechanisms remain unclear.

Microtubules are a prominent feature of guard cells and their radial arrangement is unique. However, controversy has surrounded their proposed role in guard-cell stomatal function (Galatis and Apostolakos, 2004). Our results generally reinforce reports by Lahav et al. (2004) but provide quantitative information from several new sources to support a positive correlation between guard-cell microtubule structure number and ordering and stomatal aperture. There are recognizable differences between guard cells labeled with GFP-tubulin when their stomata are open or closed (insets in Figure 1). We found a positive correlation between detected microtubule structure number and stomatal aperture (Figure 1). In addition, we found a parallel decline in structure number and stomatal aperture when plants were transferred to darkness (Figure 2). Likewise, structure number and stomatal aperture were restored in leaves that had been kept in the dark by a 1-h light treatment (Figure 3). Regardless of light and dark conditions, fluorescent images in adjacent pavement cells appeared to be unaltered (Figure 1). Overall, we see a positive correlation between microtubule structure numbers and physiological state that is unique to guard-cell microtubules.

Our results are consistent with those of Fukuda et al. (1998), who reported that guard cells in *Vicia faba* lost microtubule numbers and order in the dark, but regained microtubule numbers and order in the light. In addition, we confirmed their finding that taxol stabilized microtubules and delayed closure while drugs that disrupt microtubules lead to closure. Likewise, Lahav et al. (2004) reported light-induced microtubule rearrangements in *Commelina communis* and *Arabidopsis* guard cells. However, Assmann and Baskin (1998), using epidermal peels of *Vicia faba*, did not see changes in guard-cell microtubules when stomata opened or closed. They also reported that treatment of epidermal peels with colchicine (1 mM) to destabilize microtubules or taxol (20 μM) to stabilize them did not prevent either stomatal opening or closing. They concluded that microtubules are not invariably required for guard-cell function. However, our results (Figures 9 and 10) are consistent with those reported by Marcus et al. (2001) and Fukuda et al. (1998). Perhaps the older tissue-preparation techniques used in the Assmann paper could account for why their results are so different from those from other laboratories.

Total GFP-tubulin fluorescence associated with the cell cortex is greater in guard cells with open rather than closed stomata (Figure 3). As signal density in assembled microtubule structures is about 10-fold higher than in the cytosol, the majority of signal at the cell cortex, where most microtubules reside in mature guard cells, is from polymerized tubulin. Greater cortical fluorescence represents a net increase in polymerized tubulin, since mere bundling of existing microtubules should not change total fluorescence. Increases in assembled tubulin would mean tubulin monomers, abundant in the cytosol of guard cells with closed stomata, would decrease, as microtubules are assembled when stomata open (possible appearance of new signal by *de novo* protein synthesis would be delayed by the time needed for transcription and GFP chromophore formation). This hypothesis is supported by quantitative fluorescence data; as cortical fluorescence decreased, cytosolic fluorescence as measured in subcortical focal planes increased (Figures 3; see Supplemental Figure 1). Synthesis of new GFP-tubulin monomers might be inhibited when tubulin monomers are abundant in the cytosol (closed stomata) and hence total fluorescence would be lower than in open stomata. Our observation of more cytosolic fluorescence in guard cells with closed stomata reinforces the report by Lahav et al. (2004) that more diffuse GFP-label appeared in the cytosol of guard cells under dark conditions. In addition, we observed rapidly moving, generally poorly defined fluorescent shapes in the cytosol of guard cells with closed stomata (Figure 4, and Supplemental Videos 1 and 2). Note that the total gain in cytosolic fluorescence on stomatal closure in no way matched the loss of fluorescence from assembled microtubules. These results would be explained by significant monomer degradation during closure, dramatically decreased synthesis, or both.

The generally radially arranged microtubule structures correlated very well with stomatal aperture and microtubule structure numbers declined in the dark, as did stomatal apertures (Figure 5). Both structure number and stomatal aperture recovered quickly in the light (Figure 5). Stomatal closure induced by a variety of factors (darkness, ABA (Figure 6), H_2O_2, and sodium hydrogen carbonate (Supplemental Figure 3)) was always accompanied by a decline in the number of detectable structures. None of these factors significantly affected microtubule

structure numbers in adjacent pavement cells; guard-cell microtubules were uniquely sensitive to these factors. Our structure counts are probably biased towards bundled microtubules. These brighter structures are more clearly defined and easier to recognize than the dimmer ones. In addition, in guard cells with closed stomata, cytosolic fluorescence from GFP-labeled tubulin monomers could well obscure the weak signal from single microtubules. However, the latter is probably not a major factor, since GFP-tubulin structures seen in images of MAP4-GFP-labeled guard cells (Supplemental Figure 2) show similar differences in recognizable structures. MAP4-GFP associates with the microtubule lattice, with little free label remaining in the cytosol compared to that observed with GFP labeled tubulin (D.W. Ehrhardt, unpublished observations).

The amount of microtubule polymer will be determined by the balance of the rate of new microtubule initiation and rate of polymer growth, against the rate of polymer depolymerization (which may be aided by severing). In higher plants, the picture is complicated because interphase microtubules are released from their nucleation complexes and have two dynamic ends (Shaw et al., 2003; Nakamura et al., 2010). It would be logical to assume that microtubule numbers decline in guard cells because of a decrease in nucleation rate and/or rate of assembly at the plus end. However, during stomatal closure, the number of growing plus ends identified by EB1 localization (Figure 7) and rate of plus-end growth (Figure 8) did not change when guard cells closed their stomata. Therefore, we propose that stomatal closure is associated with an increase in microtubule disassembly. This hypothesis is consistent with increases in cytosolic fluorescence (Lahav et al., 2004, and Figures 3 and 4) when stomata close. However, we are only able to monitor assembly at the plus ends using EB1. We have no direct information about disassembly at the plus ends or assembly or disassembly at the minus ends.

In the accompanying paper (Eisinger et al., 2012), we again address the question as to whether there is a real decline in the number of microtubules on stomatal closure and the reverse on opening. Our findings indicate that what appears to be a gain of structures is largely an increase in the microtubule association that results in brighter and more readily detectable structures. An increase in the total amount of polymerized tubulin has only a minor effect. The converse is true for stomatal closing. Given the extremely close correlation between the number of observable structures and stomatal aperture, the microtubule changes must be tightly controlled.

Introduction of fluorescent labels into *Arabidopsis* plants has allowed for detailed studies of guard-cell microtubules *in situ* (Granger and Cyr, 2000; Ueda et al., 1999; Dhonukshe and Gadella, 2003; Shaw et al., 2003). However, such genetic manipulations could compromise guard-cell function. Here, we observed no significant difference in light-induced increase in stomatal conductance (individual leaves) or transpiration (whole plants of our genetically modified plants compared with wild-type and, at most, a nominal effect on closure in darkness) (Figure 11 and Supplemental Figure 5).

Microtubules in guard cells are highly ordered, dynamic structures that undergo predictable changes as stomata open and close. We used a number of different techniques to quantify the correlation between guard-cell microtubules and guard-cell function. In every case, there was a positive correlation between data consistent with increased numbers of resolvable microtubule structures and ordering, and increased stomatal aperture. The apparent decline in guard-cell microtubule numbers on stomatal closure appears to be the result of accelerated disassembly of microtubules rather than a decrease in rate of microtubule assembly. Our results are all consistent with our microtubule drug studies: disassembly leads to closure (Figure 9, orylazin) and microtubule stability delays closure (Figure 10, taxol). All factors tested that affected stomatal aperture also affected guard-cell microtubules. Guard-cell microtubules differ from microtubules in adjacent pavement cells in their radial orientation and sensitivity to factors that affect stomatal aperture. We propose that microtubules have an essential role in guard-cell function. Our results with inhibitors confirm a close functional relationship between microtubule changes and changes in stomatal aperture.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana and *Vicia faba* plants were greenhouse grown relying on natural light supplemented with artificial lights (75 μmol photons m^{-2} s^{-1}) to ensure at minimum a 16-h photoperiod. Temperature was modulated using heaters and evaporational cooling systems to maintain approximately 22°C day and 20°C night. For all of the experiments with *A. thaliana*, we used mature leaves from plants 4–6 weeks old. For *V. faba*, we used mature leaves from plants 6–8 weeks old.

Fusion Proteins

A. thaliana tubulin was visualized using 35S::GFP-TUA/pBS (Col-0 background) supplied by Takashi Hashimoto, Nara Institute of Science and Technology, Nara, Japan. MAP4 was visualized using 35S::GFP-MAP4 (*Landsberg erecta* background, Granger and Cyr, 2000). EB1 localization was visualized with 35S::EB1-GFP, Col-O background (Mathur et al., 2003).

Microscopy

All images were acquired with a Leica DM IRE2 inverted fluorescence microscope equipped with a Leica 63× n.a. = 1.3 glycerin immersion objective lens and a Yokogawa CSU-10 spinning-disk confocal head (Paradez et al., 2006). GFP was excited at 488 nm with an argon ion laser delivering approximately 3 mW of power at the end of the fiber feeding into the confocal head. Emission filtering was performed with a bandpass filter (525/50, Chroma Technology, Rockingham, VT), and images acquired with a Roper Cascade 512b EMCCD camera. Typical integration

times were 600–800 ms. Instrumentation was controlled by Metamorph software (Molecular Devices, Sunnyvale, CA).

Measuring Stomatal Apertures

Epidermal peels were prepared from *V. faba* leaves and small leaf fragments were prepared from *A. thaliana* leaves. These leaf materials were photographed through a light microscope and stomatal apertures were quantified using the measurement tools in ImageJ software.

Microtubule Inhibitor Studies

The leaf materials were incubated in abscisic acid (ABA, 10 μM) for 1 h. Both taxol (paclitaxel, Calbiochem, San Diego, CA) and orylazin (Roedel-deHaën, Seelze, Germany) incubation solutions contained 10 mM MES buffer (pH 6.1) and 0.2% DMSO. *Vicia* epidermal peels and *Arabidopsis* leaf fragments were incubated in 0.1 mM orylazin, 20 μM taxol, or a 0.2% DMSO/MES buffer control for 1 h.

Recognition of Open Stomata

Using the confocal microscope, it is difficult to determine whether stomata are actually closed, since the thick walls of guard cells adjacent to the pores are not visible in fluorescent images. Guard-cell pairs were briefly viewed with white light in the confocal microscope to differentiate closed stomata clearly from open stomata. If no light was seen penetrating between the two guard cells, the stoma was considered closed. If light was seen, the stoma was considered open.

EB1-GFP Velocities

EB1-GFP comet speed was determined from analysis of confocal image stacks using IMARIS tracking software (Bitplane, Zurich). We used a particle diameter of six pixels and threshold value of 10. For tracking, we used 'autoregressive motion' with a track duration threshold of five frames. We excluded particle tracks that displayed non-linear motion.

Measurement of Fluorescence

GFP-tubulin fluorescence of guard-cell microtubules and cytosol was measured from confocal image stacks using ImageJ. A sub-stack of the five top-most sections at the upper cell cortex and a sub-stack of five planes in the middle of the cell were projected to create brightest-point volumes that included cortical microtubules or cytosol, respectively (Supplemental Figure 3). The upper sections showed well-defined microtubule structures. The middle sections showed diffuse fluorescence that increased in intensity as stomata closed. An eight-pixel-wide line was drawn across the projected images of both the upper sections and middle sections, and Path Profile used to measure fluorescence intensity. After subtraction of background, the resultant curve was integrated to give total guard-cell and cytosolic fluorescence.

Transpiration

Whole-plant transpiration rates were measured with a modified Qubit photosynthesis system (model CO650). *Arabidopsis* plants in 5.5 × 5 × 6-cm plastic pots were placed in 7 × 7 × 11-cm clear plastic chambers (500-ml capacity). Air was drawn into each chamber through two 7-mm holes 2 cm from the base. Air was drawn out of the chamber through two 7-mm holes 2.5 cm from the top of the chamber. Air flow was driven by a Qubit gas pump (model G100) and flow rate was set to 1 L h^{-1} using a Qubit flow meter (model F1000). Humidity was measured using a Qubit humidity/temperature sensor (model s161) that uses a Honeywell HIH-3610 Integrated Circuitry Humidity Sensor. Input from four identical chambers was simultaneously processed using Logger Pro software (supplied with the photosynthesis system). Relative humidity data were converted to transpiration data using the Template for Calculating Photosynthetic and Transpirational Parameters provided by Qubit Systems.

Stomatal conductance of individual rosette leaves was measured using a Li-Cor 6400 portable photosynthesis system fitted with a Li-Cor *Arabidopsis* chamber (1-cm-diameter aperture). The following parameters were used: air flow, 250 μmol s^{-1}; CO_2, 350 μmol mol^{-1}; and block temperature 20°C.

Fusion Proteins

A. thaliana tubulin was visualized using 35S::GFP-TUA/pBS (Col-0 background) supplied by Takashi Hashimoto, Nara Institute of Science and Technology, Nara, Japan. MAP4 was visualized using 35S::GFP-MAP4 (*Landsberg erecta* background, Granger et al., 1998). EB1 localization was visualized with 35S::EB1-GFP Col-O background (Mathur et al., 2003).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

FUNDING

This work was supported by NSF grant 0843617 and funds from the Carnegie Institution for Science. The authors are grateful for this support.

ACKNOWLEDGMENTS

We wish to thank Dr Joseph Berry (Carnegie Department of Global Ecology) and Graham Dow (Department of Biology, Stanford University) for assistance with transpiration studies. No conflict of interest declared.

REFERENCES

Assmann, S.M., and Baskin, T.I. (1998). The function of guard cells does not require an intact array of cortical microtubules. J. Exp. Bot. **49,** 163–170.

Blatt, M.R. (2000). Cellular signaling and volume control in stomatal movements in plants. Ann. Rev. Cell Dev. **16,** 221–241.

Desikan, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T., and Neill, S.J. (2004). ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J. Exp. Bot. **55,** 205–212.

Dhonukshe, P., and Gadella, T.W., Jr (2003). Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein-CLIP170 microtubuole plus-end labeling. Plant Cell. **15,** 597–611.

Eisinger, W., Kirik, V., Lewis, C., Ehrhardt, D., and Briggs, W. R. (2012). Quantitative changes in microtubule distribution correlate with guard cell function in Arabidopsis. Mol. Plant. **5,** in press.

Eun, S.-O., and Lee, Y. (1997). Actin filaments of guard cells are reorganized in response to light and abscisic acid. Plant Physiol. **115,** 1491–1498.

Fukuda, M., Hasezawa, S., Asai, N., Nakajima, N., and Kondo, N. (1998). Dynamic organization of microtubules in guard cells of *Vicia faba* L. with diurnal cycle. Plant Cell Physiol. **39,** 80–86.

Galatis, B., and Apostolakos, P. (2004). The role of cytoskeleton in the morphogenesis and function of stomatal complexes. New Phytologist. **161,** 613–639.

Granger, C.I., and Cyr, R.J. (2000). Microtubule reorganization in Tobacco BY-2 cells stably expressing GFP-MBD. Planta. **210,** 502–509.

Hwang, J.-U., Eun, S.-O., and Lee, Y. (2000). Structure and function of actin filaments in mature guard cells. In Actin:A Dynamic Framework for Multiple Plant Cell Functions, Staiger,C.J., Baluska, F., Volkmann, D., Barlow, P.W., eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 427–436.

Kinoshita, T., Doi, M., Suegtsugu, N., Kagawa, T., Wada, M., and Shimazaki, K.-I. (2001). Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature. **414,** 656–660.

Lahav, M., Abu-Abied, M., Belausov, E., Schwartz, A., and Sadot, E. (2004). Microtubules of guard cells are light sensitive. Plant Cell Physiol. **45,** 573–582.

Marcus, A.I., Moore, R.C., and Cyr, R.J. (2001). The role of microtubules in guard cell function. Plant Physiol. **125,** 387–395.

Mathur, J., Mathur, N., Kernebeck, B., Srinivas, B.P., and Hülskamp, M. (2003). A novel localization pattern for an EB1-like protein links microtubule dynamics to endomembrane organization. Current Biol. **13,** 1991–1997.

Nakamura, M., Ehrhardt, D.W., and Hashimoto, T. (2010). Microtubule and katanin-dependent dynamics of microtubule nucleation complexes in the acentrosomal *Arabidopsis* cortical array. Nature Cell Biol. **12,** 1064–1070.

Ogawa, T. (1981). Blue light response of stomata with starch-containing (*Vicia faba*) and starch-deficient (*Allium cepa*) guard cells under background illumination with red light. Plant Sci. Lett. **22,** 103–108.

Ogawa, T., Ishikawa, H., Shimada, K., and Shibata, K. (1978). Synergistic action of red and blue light and action spectra for malate formation in guard cells of *Vicia faba* L. Planta. **142,** 61–65.

Paradez, A.R., Somerville, C.R., and Ehrhardt, D.W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. Science. **312,** 1491–1495.

Schroeder, J.I., Raschke, K., and Neher, E. (1987). Voltage dependence of K^+ channels in guard cell protoplasts. Proc. Natl Acad. Sci. U S A. **84,** 4108–4112.

Shaw, S.L., Kamyar, R., and Ehrhardt, D.W. (2003). Sustained microtubule treadmilling in *Arabidopsis* cortical arrays. Science published online 24 April, doi: 10.1126/science.1083529.

Ueda, K., Matsuyama, T., and Hashimoto, T. (1999). Visualization of microtubules in living cells of transgenic *Arabidopsis thaliana*. Protoplasma. **206,** 201–206.

Zeiger, E., and Field, C. (1982). Photocontrol of the functional coupling between photosynthesis and stomatal conductance in the intact leaf: blue light and PAR-dependent photosystems in guard cells. Plant Physiol. **70,** 370–375.