The Role of Microtubules in Guard Cell Function[1]

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Guard cells are able to sense a multitude of environmental signals and appropriately adjust the stomatal pore to regulate gas exchange in and out of the leaf. The role of the microtubule cytoskeleton during these stomatal movements has been debated. To help resolve this debate, in vivo stomatal aperture assays with different microtubule inhibitors were performed. We observed that guard cells expressing the microtubule-binding green fluorescent fusion protein (green fluorescent protein::microtubule binding domain) fail to open for all major environmental triggers of stomatal opening. Furthermore, guard cells treated with the anti-microtubule drugs, propyzamide, oryzalin, and trifluralin also failed to open under the same environmental conditions. The inhibitory conditions caused by green fluorescent protein::microtubule binding domain and these anti-microtubule drugs could be reversed using the proton pump activator, fusicoccin. Therefore, we conclude that microtubules are involved in an upstream event prior to the ionic fluxes leading to stomatal opening. In a mechanistic manner, evidence is presented to implicate a microtubule-associated protein in this putative microtubule-based signal transduction event.

Guard cells regulate gas exchange in and out of the leaf by precisely controlling the size of their stomatal aperture. These dynamic changes in aperture size (i.e. stomatal movements) occur in response to environmental and endogenous stimuli that may involve auxins, light, CO_2, and humidity (for review, see Assmann, 1993). In a specific manner, blue and red light induce stomatal opening by activating a plasma membrane H^+ ATPase (Shimazaki et al., 1986; Serrano et al., 1988). Activation of the proton pump leads to an influx of K^+, due to an opening of the voltage-gated K^+ channels and consequently, water enters the cell via osmosis. This increase in turgor pressure causes the opening of the stomatal aperture. Although these general physiological events are well documented, the precise signaling events required for proton pump activation are less understood.

The molecular intricacies of stomatal opening are widely under investigation; however, how guard cells transduce the signal from perception to stomatal opening remains unclear. One area of interest involves the role of microtubules. These proteinaceous, dynamic polymers (Mitchison and Kirschner, 1984) have been implicated in guard cell development (Palevitz and Hepler, 1974; Palevitz, 1982; Apostolakos and Galatis, 1998, 1999). Like other diffusely growing plant cells, microtubules in the cortex of developing guard cells guide the deposition of cellulose microfibrils (Giddings and Staehelin, 1991). However, unlike other plant cells, the cortical microtubules in guard cells remain well organized after differentiation is complete. This suggests that microtubules have an additional role in guard cells beyond cellular morphogenesis. Some possibilities include an involvement in the opening or closing of the stomata and/or the transduction of the signal from perception to proton pump activation. In other eukaryotes, microtubules have been implicated in cell signaling (Leiber et al., 1993; Bershadsky et al., 1996), but little direct evidence exists for their involvement in signal transduction in plants. Nonetheless, plant microtubules are likely regulated by calcium (Fisher et al., 1996; O'Brien et al., 1997), a ubiquitous cell-signaling molecule in plants (Poovaiah and Reddy, 1987; Gilroy et al., 1993). Calcium also regulates stomatal opening and closure (Blatt, 1999), therefore, an interplay between calcium, microtubules, and stomatal movements is possible.

Anti-microtubule drugs, such as colchicine, propyzamide, and oryzalin have been used to investigate if microtubules regulate stomatal opening. These compounds depolymerize the microtubule cytoskeleton by preventing the addition of tubulin dimers to dynamic microtubules (Morejohn, 1991). Inhibition of dimer addition results in the eventual loss of microtubules in a treated cell, as a function of the microtubules dynamic state (i.e. highly dynamic microtubules are more sensitive than stable microtubules). Microtubule-stabilizing drugs (e.g. taxol) have also been used. Current research assessing guard cell function after these drug treatments have produced conflicting results. Assmann and Baskin (1998) reported that colchicine-treated cells were able to open upon white light induction, which was interpreted as showing that microtubules are not required for guard cell function. In contrast, Fukuda et al. (1998) reported that propyzamide-treated guard cells failed to open in response to white light, suggesting that microtubules are required for guard cell opening. These

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discrepancies have prompted us to investigate the role of microtubules in stomatal opening.

To gain insight into the role of microtubules in guard cell function we have taken an approach that involves the transient expression of a synthetic, microtubule-binding protein that expresses a dominant-negative phenotype. In previous work we found that guard cells would readily express a transgene, comprised of elongation factor 1α and green fluorescent protein (EF-1α::GFP). These cells not only had fluorescently labeled microtubules, but were crippled in their ability to open in response to white light (Moore and Cyr, 2000). One confounding factor of this previous study was that this multifunctional protein (Durso and Cyr, 1994) was overexpressed, thereby making it difficult to know if the observed negative phenotype was the result of microtubule inhibition or another function (e.g. an alteration in protein translation activity). Here we present data obtained using an alternative gene whose product binds to and affects the function of microtubules. It is important to note that this gene has no obvious homolog in plants and therefore, predictable confounding effects are minimized. In conjunction, the affects of chemical inhibitors on stomatal opening were re-examined. The results suggest that microtubules are necessary for stomatal opening. In a specific manner, they are involved in the events prior to the ionic fluxes that lead to stomatal opening. The data is consistent with a model by which a signaling molecule associates with microtubules to affect stomatal opening.

RESULTS

Stomatal Guard Cell Opening in Excised Leaves Can Be Inhibited by the Expression of the Microtubule-Binding Gene, GFP::Microtubule-Binding Domain (MBD)

The MBD of the mammalian microtubule-associated protein (MAP4) fused to GFP has been created as described (Marc et al., 1998; Fig. 1). This construct has been used as a microtubule reporter gene because it binds to microtubules, thereby affording in vivo visualization of microtubules in plant cells. At low expression levels microtubule dynamics and orientation appear normal (Granger and Cyr, 2000), however, at high expression levels abnormal phenotypes can occur (Marc et al., 1998). Hence this gene can be used to disrupt normal microtubule function.

Stomatal cell opening was investigated in leaves that expressed high levels of GFP::MBD. Biolistically transformed guard cells expressing GFP::MBD displayed distinct microtubule arrays that were characteristic of this cell type (Fig. 1), however, upon exposure to 2 h of white light, the transformed guard cells failed to open ($P > 0.05$; Fig. 2). This data suggests that the binding of GFP::MBD to microtubules prevents stomatal opening in excised leaves after white light stimulation.

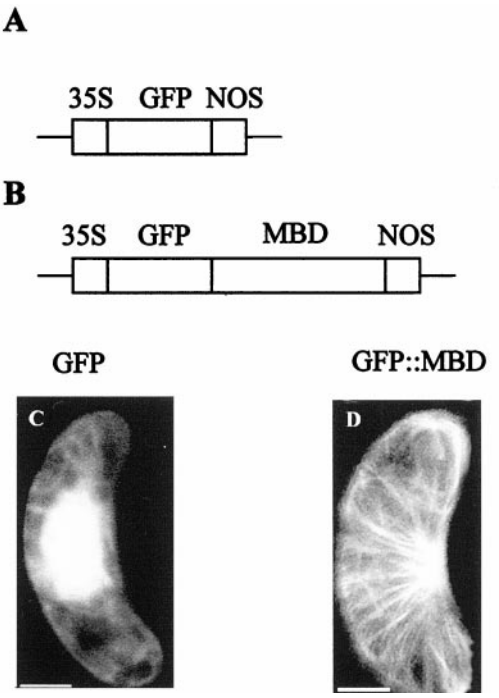

Figure 1. Gene constructs and expression patterns. Stomatal guard cells were biolistically transformed with GFP (A) or GFP::MBD genes (B). Both constructs were inserted between a 35S promoter and a NOS termination sequence and inserted into the pUC 18 plasmid. Confocal images of guard cells transformed with only GFP display cytoplasmic fluorescence (C). However, guard cells transformed with GFP::MBD display localized fluorescence on the microtubules (D). Scale = 10 μm.

To confirm that the GFP component of the chimeric gene is not responsible for the inhibition, guard cells were transformed with GFP alone. Unlike the GFP::MBD construct, these transformed guard cells displayed a cytoplasmic fluorescence (Fig. 1) and opened normally upon white light induction ($P < 0.05$; Fig. 2).

The finding that guard cells in excised leaves transformed with GFP::MBD did not open after white light induction provides evidence for the involvement of microtubules in stomatal opening. To more precisely study the putative role of microtubules in stomatal opening, the major environmental stimuli that trigger opening in guard cells (i.e. blue, white, and red light, and low ambient CO_2) were similarly tested. Epidermal peels were used to eliminate any possible confounding influences from nonepidermal tissue and to determine whether guard cells expressing GFP::MBD could respond to any or all of these signals.

GFP::MBD Inhibits a Signal Common to All Major Environmental Cues That Stimulate Stomatal Opening

Guard cells in excised leaves were transformed with GFP::MBD and epidermal peels were made from the transformed leaves. Each group of peels was

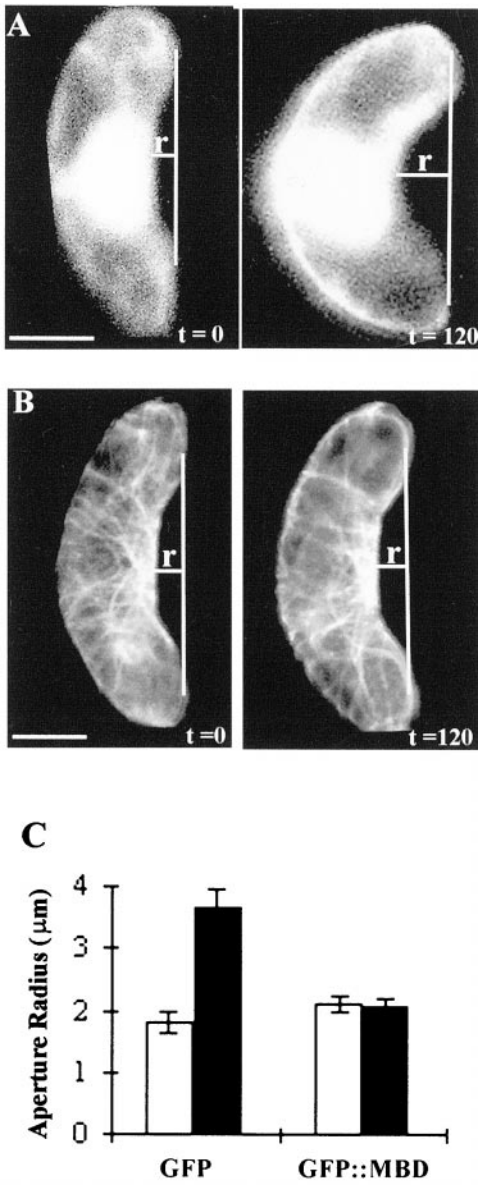

Figure 2. Effect of GFP::MBD on stomatal opening in intact leaves. Confocal images of a guard cell expressing GFP shows normal stomatal opening after 120 min of white light (A). In contrast, a guard cell expressing GFP::MBD fails to open after 120 min of white light stimuli (B). The aperture radius (r) was used to measure stomatal opening as described in "Materials and Methods." C, A bar graph of the average radial aperture size prior to (white bars) and after light stimuli (black bars) in excised leaves for GFP and GFP::MBD expressing cells (±SE). Stomatal guard cells expressing GFP::MBD fail to open ($P > 0.05$); however, guard cells expressing only GFP open normally ($P < 0.05$). Average aperture radius was calculated from a total of 62 measurements taken from three replicate experiments. Scale bars = 10 μm.

subjected to different environmental stimuli (i.e. white, blue, and red light, and low ambient CO_2). As with intact leaves, the guard cells in epidermal peels that expressed GFP::MBD failed to open in white light. The average stomatal aperture size of GFP::MBD-transformed guard cells subjected to 2 h of white light induction did not significantly increase ($P > 0.05$) as compared with its average aperture size in the dark. In a similar manner, guard cells expressing this construct failed to open with blue light, red light, and low ambient CO_2 (P values > 0.05).

As a control, the same set of experiments described above were performed with GFP-transformed guard cells. The average aperture size of these cells significantly increased for each of the respective treatments ($P < 0.05$; Figs. 2 and 3). Therefore, GFP alone did not inhibit the opening of guard cells and is consistent with the idea that the MBD domain inhibited stomatal opening. As an additional control, fluorescein diacetate was used to confirm that GFP::MBD-expressing cells were alive. This vital stain was able to enter and remain in these guard cells, thereby confirming that GFP::MBD-transformed cells were alive (data not shown).

The Anti-Microtubule Drugs Propyzamide, Oryzalin, and Trifluralin All Prevent Stomatal Opening As Ascertained Using Brightfield Microscopy, However Colchicine Does Not

Propyzamide, colchicine, trifluralin, and oryzalin were used to disrupt microtubules in guard cells (non-transformed guard cells were used to replicate previous experiments using these drugs). These results show that propyzamide, oryzalin, and trifluralin disrupted normal stomatal function. Guard cells treated with 20 μM propyzamide opened signifi-

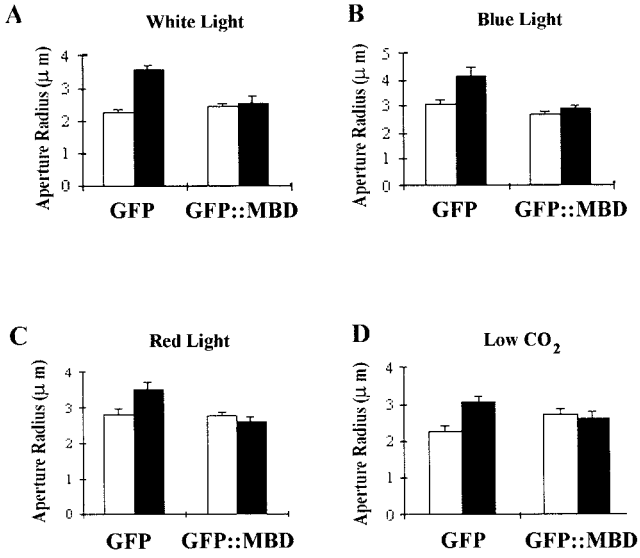

Figure 3. Effect of GFP::MBD on stomatal opening in epidermal peels. Guard cells expressing GFP::MBD in epidermal peels fail to open in white light (A), blue light (B), red light (C), and low ambient CO_2 (D; $P > 0.05$ for all stimuli). Control cells expressing GFP all open under the same conditions. All measurements were taken prior to (white bars) and after (black bars) 2 h of the respective stimuli (±SE). Low CO_2 induction was performed in a tightly sealed chamber in the dark. Average aperture radius was calculated from a total of 67 measurements taken from three replicate experiments.

cantly less than untreated guard cells ($P < 0.05$; Fig. 4) and a opening was completely inhibited with oryzalin- (10 μM) and trifluralin- (10 μM) treated guard cells. However, guard cells treated with colchicine (1 mM) opened after 2 h of white light treatment ($P < 0.05$). Control solutions containing 0.1% (v/v) dimethyl sulfoxide (DMSO) and ethanol alone had no effect on stomatal aperture size.

Guard Cells Transformed with GFP::MBD or Treated with Anti-Microtubule Drugs Are Able to Open with Fusicoccin

Fusicoccin induces guard cell opening through activation of the proton pump (Assmann and Schwartz, 1992) and perhaps by acting on K^+ channels (Clint and Blatt, 1989). Thus this fungal toxin bypasses the guard cells transduction machinery and directly stimulates the ionic events leading to opening. Aperture measurements indicated that GFP::MBD-transformed guard cells were able to open with fusicoccin ($P < 0.05$; Fig. 5). In addition, aperture measurements demonstrated that guard cells treated with propyzamide, oryzalin, and trifluralin were also able to open.

Guard Cells with Stabilized Microtubules Open in Response to White Light

The GFP::MBD gene, when expressed at high levels, predictably has two effects on microtubules; it stabilizes them, and it displaces endogenous MAPs. To separate these effects and thereby determine the cause of guard cell inhibition, taxol, a drug known to stabilize plant microtubules (Wymer et al., 1996) was used. Taxol (5, 10, and 15 μM) was added during pre-incubation in the dark and throughout white light induction. Aperture sizes of the GFP-transformed guard cells after white light induction significantly increased for all concentrations ($P < 0.05$), thus revealing that GFP-transformed guard cells with stabilized microtubules can open in response to white light ($P < 0.05$; Fig. 6). A control solution of 0.1% (v/v) ethanol alone had no effect on stomatal aperture size.

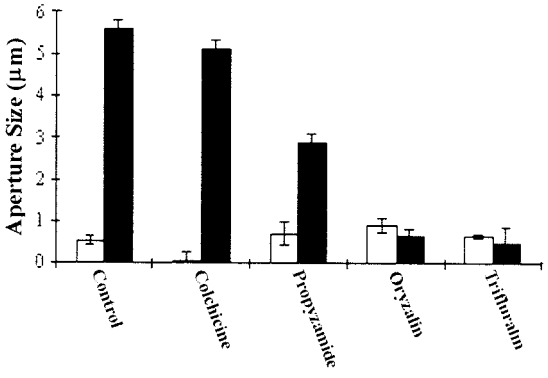

Figure 4. Most anti-microtubule herbicides inhibit stomatal opening. Brightfield microscopy with non-transformed epidermal peels shows that propyzamide (20 μM), oryzalin (10 μM), and trifluralin (10 μM) disrupt normal stomatal function, whereas colchicine (1 mM) does not. Propyzamide-treated guard cells have stomatal apertures that are significantly less than the control after white light stimuli ($P < 0.05$), whereas oryzalin and trifluralin completely inhibited opening. The average aperture size of guard cells treated with colchicine and untreated guard cells after stimulation is not significantly different ($P > 0.05$). White bars represent the average aperture size prior to treatment and black bars represent the average aperture size after treatment (±SE). Average aperture size was calculated from a total of 60 measurements taken from three replicate experiments.

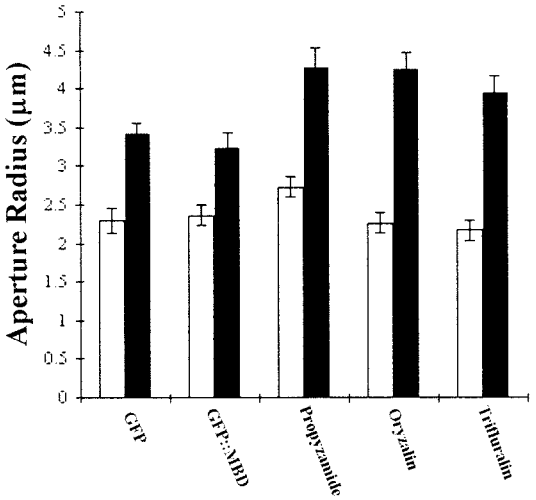

Figure 5. Effect of fusicoccin on stomatal opening. Guard cells expressing GFP::MBD and propyzamide- (20 μM), oryzalin- (10 μM), and trifluralin- (10 μM) treated guard cells can be opened with fusicoccin ($P < 0.05$). Guard cells under these conditions are unable to open with white light stimuli in the absence of fusicoccin; however, activation of the proton pump by fusicoccin (10 μM) induced opening. Measurements were taken prior to (white bars) and after (black bars) 2 h of fusicoccin incubation in the dark (±SE). Average aperture radius was calculated from a total of 69 measurements taken from three replicate experiments.

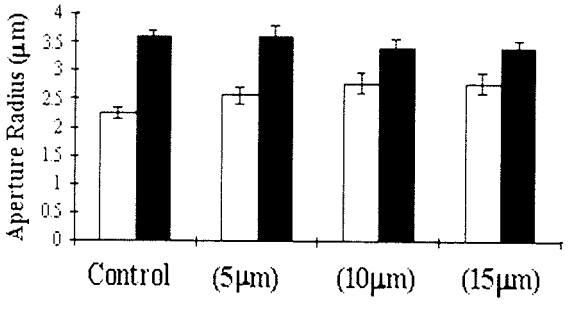

Figure 6. GFP-expressing guard cells treated with the microtubule-stabilizing drug, taxol, are able to open after 2 h of white light stimuli. There were no significant differences between the amounts of stomatal opening for the various taxol concentrations ($P > 0.05$). Taxol was added to the pre-incubation solution and incubation solutions bathing the epidermal peels. Measurements were taken prior to (white bars) and after (black bars) taxol treatment (±SE). Average aperture radius was calculated from a total of 60 measurements taken from three replicate experiments.

DISCUSSION

Microtubules Are Required for Stomatal Opening

The microtubule cytoskeleton is required for guard cell morphogenesis although additional roles in stomatal function have been debated. Guard cells maintain their unique radial microtubule array after morphogenesis, suggesting that microtubules are required for stomatal function. Our results support the hypothesis that microtubules are required for stomatal opening, however, prior investigations have led to conflicting results. Fukuda et al. (1998) concluded that microtubules are involved in stomatal opening, using the antimicrotubule drug propyzamide, whereas Assmann and Baskin (1998) concluded they are not involved using colchicine. It could be argued that one or both of these drugs are unable to enter the guard cell; however, both antimicrotubule drugs are able to disrupt the microtubule cytoskeleton in stomates based upon immunolocalization results (Assmann and Baskin, 1998; Fukuda et al., 1998). One explanation for this discrepancy could be experimental artifact, though similar results were obtained in our laboratory when these experiments were repeated. In an alternate manner, the different drugs used by the two groups (i.e. propyzamide and colchicine) had differential effects on microtubules. This warrants consideration because colchicine can be a poor inhibitor in plants due to its low binding affinity to plant tubulin. For example, colchicine has a K_d of 9.7×10^3 M^{-1} for rose tubulin, whereas in neuronal tubulin its K_d is 2.47×10^6 M^{-1} (Morejohn, 1991). Due to its relatively low affinity for plant tubulin, colchicine may not completely inhibit microtubule formation in stomates, but may require a longer exposure time. Gastelier-Couot and Louguet (1992) demonstrated that extended treatment with colchicine could inhibit stomatal opening. Furthermore, the results presented here indicate that colchicine is the only anti-microtubule agent that did not disrupt normal stomatal function. However, we cannot completely rule out the possibility that colchicine is the only drug that specifically targets microtubules, whereas oryzalin, trifluralin, and propyzamide all have cryptic non-microtubule secondary effects that result in stomatal closure. Therefore, an alternative approach was taken to determine if microtubules are required for stomatal opening.

The chimeric protein, GFP::MBD, has previously been used to study microtubule organization (Marc et al., 1998; Granger and Cyr, 2000) in vivo. Data obtained in this study revealed that this microtubule-binding protein, when expressed at high levels, inhibits stomatal opening in transformed guard cells, in whole leaves, and in epidermal peels for all environmental cues of stomatal opening tested (i.e. blue, red, and white light, and low ambient CO_2). Because aberrant microtubule phenotypes are associated with high levels of expression with this construct (Marc et al., 1998), it is reasonable to assume that this chimeric protein inhibits stomatal opening by disrupting normal microtubule function. This data is consistent with a role for microtubules in stomatal opening and suggests that the environmental cues tested all triggered a similar, ubiquitous, microtubule-dependent mechanism for stomatal opening.

Because this chimeric gene product can only be localized to the microtubule array (Fig. 1), this technique provides a more precise method for disrupting normal microtubule function. However, an alternative hypothesis is that GFP::MBD has another, non-microtubule target that inhibits stomatal opening. We feel this is not the case because work with another chimeric microtubule-binding protein derived from the MBD of EF-1α has similar effects on white light-induced stomatal opening (Moore and Cyr, 2000). Because GFP::MBD and chimeric EF-1α share little amino acid homology, the possibility that both produce the same phenotype by affecting the identical, cryptic non-microtubule target is remote. Thus the results with GFP::MBD, taken in conjunction with the data using the anti-microtubule drugs, provide compelling evidence for the utilization of microtubules in stomatal opening.

Microtubules Are Required Upstream to the Ionic Events Leading to Stomatal Opening

The finding that microtubules are required for stomatal opening prompted us to further characterize their function. Experiments with fusicoccin revealed that the disturbance of the microtubule array with GFP::MBD, propyzamide, oryzalin, and trifluralin did not inhibit fusicoccin-stimulated stomatal opening. Because fusicoccin activates the proton pump (Assmann and Schwartz, 1992) and has been suggested to act upon K^+ channels (Clint and Blatt, 1989), we conclude that microtubules are required somewhere upstream to the ionic events (i.e. H^+ efflux and K^+ influx) that lead to stomatal opening. One possibility is that microtubules are participating in the signal transduction events leading to the ionic fluxes. The mechanistic details of these signaling events are not well characterized, although many common eukaryotic signals such as calcium (Leckie et al., 1998), calmodulin (Shimazaki et al., 1992), phospholipase D (Jacob et al., 1999), G-proteins (Fairleygrenot and Assmann, 1991; Kelly et al., 1995), and cAMP (Jin and Wu, 1999) have been discovered in guard cells. It is likely that these signaling molecules play similar roles in guard cells and that microtubules, in some manner, are involved in the signaling process.

Participation of the microtubule cytoskeleton in plant signal transduction has not been well documented. However, in other eukaryotic cells, G-protein β-subunits are closely associated with the microtubules and they may play a role in microtubule assembly (Wu et al., 1998). In addition, disruption of the

microtubule array inhibits cyclin-dependent kinases and either activates or inactivates many protein kinases (Wang et al., 1999). Although little direct evidence exists for a role for microtubules in plant signal transduction, plant microtubules can be regulated by calcium and calmodulin (Allan and Hepler, 1989; Cyr, 1991; Fisher et al., 1996) and by protein kinases (Mizuno, 1994). In addition, the disruption of microtubules with anti-microtubule agents increases calcium channel activity (Thion et al., 1998), further suggesting a role for microtubules in signal transduction.

One problem that arises when working with drugs that affect major cellular processes is the possibility of secondary effects. In the most extreme case, a drug may severely cripple a cell and the effect observed is simply a prelude to cell death. Here it could be argued that the anti-microtubule agents, including GFP::MBD, have severely damaged the guard cells and that they now lack the ability to open under any condition. However, cells were viable as judged by fluorescein diacetate. Moreover, these treated cells could open after the application of fusicoccin, indicating the basic machinery involved in opening remains unaffected by the anti-microtubule agents. Therefore, we conclude that the effect of these treatments was specific to microtubules and not the result of a general depression in metabolic competency.

Is a MAP Involved in Stomatal Opening?

The data presented herein suggests that microtubules participate in the events prior to proton pump activation. Their inherent ability to reorient and their interaction with other proteins suggests two mechanistic possibilities for their involvement. The taxol data presented in this paper, and that presented by Fukuda et al. (1998) show that guard cells are able to open when their microtubules have been stabilized. Therefore, it is unlikely that microtubule dynamicity per se is critical for stomatal opening. In an alternate manner, stomatal opening may require the binding of a MAP to a microtubule. Little data exists to implicate MAPs in cellular signaling events in plants; however, there is evidence for MAP regulation by calcium (Bender and Rebhun, 1986; Moore et al., 1998), a key player in guard cell function. Our GFP::MBD data is consistent with a microtubule-MAP interaction. This chimeric protein decorates the microtubule (Marc et al., 1998), and likely inhibits stomatal opening by displacing endogenous MAPs that are critical to stomatal opening. Hence, GFP::MBD might be outcompeting endogenous MAPs through steric hindrance or by saturating critical microtubule-binding sites. Different MAPs can compete for the same microtubule-binding sites, which in turn, may play a role in their regulation (Hagiwara et al., 1994; Trinczek et al., 1999). In this case GFP::MBD could be negatively regulating a MAP critical to the cellular signaling events necessary for stomatal opening. Although this hypothesis remains speculative, a MAP-microtubule interaction for guard cell function remains a possibility.

MATERIALS AND METHODS

Plant Material

Plants of fava bean (*Vicia faba*) were grown in chambers under a 10-h light and 14-h dark regime as described in Assmann and Baskin (1998). Leaves for all experiments were fully grown and came from 3- to 4-week-old plants. Epidermal peels were made from the abaxial side of these leaves for all experiments.

Fluorescent Constructs

Two GFP-containing genes were used in this study. The first contained a synthetic S65T GFP gene by itself that was codon optimized and generously donated by J. Sheen (Department of Molecular Biology, Massachusetts General Hospital, Boston). This gene was cloned into a modified pUC 18 plasmid containing a 35S promoter with a NOS termination sequence. The second was a GFP::MBD construct that was created as previously described (Marc et al., 1998), in which the MBD of mammalian protein MAP4 was fused to the carboxy terminus of GFP using recombinant PCR. The resulting in-frame chimeric gene was 1,985 bp and was similarly cloned into the modified pUC 18 plasmid.

Biolistic Transformation of Fava Bean Leaf Epidermal Cells

Biolistic transformation of leaf epidermal cells with GFP and GFP::MBD constructs was done as previously described (Marc et al., 1998). In brief, 3 μg of plasmid DNA was mixed with 1 mg of 1-micron gold particles (Bio-Rad, Richmond, CA) in a 25-μL aqueous solution. Spermidine (0. 1 M) and $CaCl_2$ (2. 5 M) was added to the solution and the resulting suspension was mixed and sonicated. Subsequent centrifugation and washing with ethanol yielded DNA-coated gold particles, which were resuspended in ethanol and loaded onto plastic carrier discs. The DNA-loaded gold particles were fired into the abaxial side of the leaves at 1,350 psi in a vacuum using the particle delivery system-1000/He (Bio-Rad) and incubated overnight in the dark.

Confocal Microscopy

Transformed guard cells of the abaxial epidermis were located with a laser scanning confocal microscope (LSM model 410, Zeiss, Thornwood, NY) equipped with a 150-W xenon epifluorescent illuminator. The 488-nm line of an argon laser was used as an excitation source and images were recorded with a 488/543 dual dichroic excitation mirror with a 510

to 540 nm emission filter. Images were taken of intact leaves and epidermal peels (zoom 6.5 and 8-s scan time) with a 40× Achroplan water immersion objective (NA 0.8, Zeiss). The focal plane of the fluorescing guard cell was selected to give the greatest clarity of the stomatal aperture.

Stomatal Assays with Transformed Guard Cells

For stomatal assays with intact GFP::MBD- and GFP-transformed leaves, the leaves were first placed in pre-incubation solution {1 mM $CaCl_2$ and 10 mM MES [2-(*N*-morpholino)-ethanesulfonic acid], pH 6.1} abaxial side up in the dark for 30 min to close all stomates. The leaves were then transferred to incubation solution (30 mM KCl, 0. 1 mM $CaCl_2$, and 10 mM MES, pH 6.1) and put under white light for 2 h to induce opening. Images of guard cells expressing the constructs were taken after pre-incubation in the dark to obtain closed measurements and after incubation under white light to measure opening. Stomatal assays with epidermal peels made from GFP::MBD- and GFP-transformed leaves followed the same protocol. However, to isolate blue and red light effects, a blue or red light filter was placed over the peels for the respective treatments (the passage of only blue light and only red light was confirmed using a spectrophotometer). In addition, stomatal assays of GFP::MBD-transformed epidermal peels in low ambient CO_2 were done for 2 h in the dark. A low ambient CO_2 environment was created using air passed through the CO_2-absorbing substance, soda lime (Sigma, St. Louis). This air was then rehydrated and blown into the incubation solution bathing the peel, as well as on top of the epidermal peel. A CO_2 meter confirmed that the concentration of ambient CO_2 inside the chamber was reduced (480 μL/L prior to CO_2 treatment and 52 $\pm$ 2 μL/L during treatment).

For experiments with fusicoccin, epidermal peels from GFP::MBD- and GFP-transformed leaves and drug-treated peels were placed in pre-incubation solution and imaged as described above. The peels were transferred to incubation solution containing fusicoccin (10 μM), then returned to the dark for 2 h, and then imaged again. As a control, all experiments (i.e. stomatal assays under white light, blue light, red light, low ambient CO_2, and fusicoccin) were performed with leaves or epidermal peels expressing GFP by itself under identical conditions as the respective treatment.

Each experiment was performed at least three times with an average of 20 stomatal aperture measurements per trial. The mean aperture size was calculated using the measurements from all replicates, (i.e. each mean data point represents about 60 measurements). Aperture measurements were obtained from digitized images (Image Pro Plus, Media Cybernetics, MD) by measuring the radius of the aperture of fluorescing guard cells. The aperture radius was determined by first drawing a line connecting the points of contact between the fluorescing guard cell and its sister guard cell. Next the aperture radius was measured by drawing a perpendicular line from the innermost portion of the aperture that bisects the original line (Fig. 2). This method was validated using brightfield microscopy (data not shown). The averages of these measurements prior to and after induction were calculated and tested for statistical significance using the Student's *t* test.

In all experiments, the same leaves or peels used to obtain dark (closed) measurements were used to obtain light (open) measurements. We found that the radial assay was a sensitive measure, especially in the closed state, although daily variation was noted as to the degree of closure.

Stomatal Assays with Anti-Microtubule Drugs

The anti-microtubule agents were propyzamide (20 μM), oryzalin (10 μM), trifluralin (10 μM), and colchicine (1 mM). Each of these drugs was added at the indicated concentrations to the pre-incubation solution, as well as the incubation solutions for the respective treatments. Images were taken after pre-incubation in the dark and after incubation under 2 h of white light. A similar protocol was used when doing stomatal aperture measurements with taxol (5, 10, and 15 μM) using fluorescence microscopy. These non-transformed epidermal peels were treated with taxol during pre-incubation in the dark and in incubation solutions in the light. Each treatment was repeated at least three times and data were analyzed as previously mentioned.

Chemicals

Propyzamide (20 mM) and oryzalin (10 mM) stock solutions were prepared in DMSO and diluted with water to the appropriate working solution. Trifluralin and taxol were prepared as a 1-mM solution in ethanol and diluted to the appropriate working solution with water. Fusicoccin (1 mM) and colchicine (100 mM) were prepared as aqueous solutions. Control solutions contained identical concentrations of DMSO or ethanol alone.

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