

# Relocation of a $\text{Ca}^{2+}$ -Dependent Protein Kinase Activity during Pollen Tube Reorientation

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**Pollen tube reorientation is a dynamic cellular event that is crucial for successful fertilization. We have shown previously that pollen tube orientation is regulated by cytosolic free calcium ( $[\text{Ca}^{2+}]_c$ ). In this paper, we studied the activity of a  $\text{Ca}^{2+}$ -dependent protein kinase during reorientation. The kinase activity was assayed in living cells by using confocal ratio imaging of BODIPY FL bisindolylmaleimide. We found that growing pollen tubes exhibited higher protein kinase activity in the apical region, whereas nongrowing cells showed uniform distribution. Modification of growth direction by diffusion of inhibitors/activators from a micropipette showed the spatial redistribution of kinase activity to predict the new growth orientation. Localized increases in  $[\text{Ca}^{2+}]_c$  induced by photolysis of caged  $\text{Ca}^{2+}$  that led to reorientation also increased kinase activity. Molecular and immunological assays suggest that this kinase may show some functional homology with protein kinase C. We suggest that the tip-localized gradient of kinase activity promotes  $\text{Ca}^{2+}$ -mediated exocytosis and may act to regulate  $\text{Ca}^{2+}$  channel activity.**

## INTRODUCTION

Successful fertilization in the angiosperms depends on pollen tubes finding their way to the ovule. Pollen tubes frequently change their direction of growth until they finally reach the micropyle. Previously (Malhó et al., 1994, 1995; Malhó and Trewavas, 1996), we have shown that the distribution of cytosolic free calcium ( $[\text{Ca}^{2+}]_c$ ) in the apical region and  $\text{Ca}^{2+}$  influx by asymmetric activity of  $\text{Ca}^{2+}$  channels play crucial roles in this process. However, the downstream processes involved in signal transduction are not known (Feijó et al., 1995; Trewavas and Malhó, 1997).

Phosphorylation cascades are known to be a major part of signaling pathways in plant cells, particularly those involving  $\text{Ca}^{2+}$  (Trewavas and Malhó, 1997). However, methods for imaging protein kinase in living cells are limited. Olds et al. (1995) used the fluorescent dye NBD-phorbol acetate to visualize the activation of protein kinase C (PKC) in living cells. This dye interacts directly with the protein, and the authors showed that when the cells were incubated in a solution of low-dye concentration, they remained viable and performed their physiological functions; continued protein synthesis and turnover might prevent the inactivation of significant amounts of the protein kinase by interaction with the dye. However, NBD-phorbol acetate is a single-wavelength dye, which makes quantitation difficult because it was designed primarily to label PKC. Despite the importance of this enzyme in

the regulation of animal cell metabolism, direct sequence equivalents in plant cells have yet to be identified. Nevertheless, there is evidence for lipid-activated kinases that might represent functional equivalents (Nanmori et al., 1994; Abo-El-Saad and Wu, 1995; Sokolova et al., 1997; Subramaniam et al., 1997). The loading of fluorescently labeled peptides (syntide-2) recently has been used to map the distribution of a member of the plant  $\text{Ca}^{2+}$ -dependent calmodulin-independent protein kinase (CDPK) family (Ritchie and Gilroy, 1998). This method can be used to target only a few protein kinases and could be made very specific with the appropriate choice of a peptide substrate. However, when this strategy is used, measurements of kinase activity are not possible.

Bisindolylmaleimide is a protein kinase inhibitor with very high selectivity for PKC and other functionally homologous protein kinases (Toullec et al., 1991). It only binds when the protein kinase is catalytically active or when other structural rearrangements remove autoinhibitory regions from the active site. Bisindolylmaleimide has also been shown to inhibit a plant PKC-like protein kinase within the same concentration range used for animal cells, that is, 1  $\mu\text{M}$  (Subramaniam et al., 1997). Furthermore, members of the CDPK family share some functional homologies with PKC (Nanmori et al., 1994; Abo-El-Saad and Wu, 1995), suggesting that bisindolylmaleimide also might target these enzymes with some degree of selectivity. A fluorescent derivative of bisindolylmaleimide has been synthesized recently. The fluorophore of this probe, BODIPY FL, shows a green fluorescence (540 nm)

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upon excitation with light of 514 nm; at high concentrations ( $\geq 500$  nM), it forms excimers, causing a shift of the emission spectrum that can be visualized using long-pass red filters (600 nm; Haugland, 1996). A ratio method to quantify the distribution of bisindolylmaleimide bound to catalytically functional protein kinase can thus be devised. This method enables quantification of protein kinase activity in living cells, namely, pollen tubes undergoing reorientation.

Before BODIPY FL bisindolylmaleimide (BFB) could be used in plants, it was necessary to demonstrate that expected changes in fluorescence ratios of 540/600 nm would accompany the reaction with a plant protein kinase. Currently, the most thoroughly investigated plant kinases are CDPKs. Some are found attached to the plasma membrane (Verhey et al., 1993) and some to the cytoskeleton (Putnam-Evans et al., 1989), and some are soluble (Polya and Micucci, 1985). A protein of this family has been found in pollen tubes (Estruch et al., 1994), thus suggesting its involvement in tip growth. Furthermore, some elements of this family showed functional homologies with PKC (Nanmori et al., 1994; Abo-El-Saad and Wu, 1995). Thus, CDPK was overexpressed in bacteria, and BFB behavior was examined upon modification of protein kinase activity. Imaging of BFB in living pollen tubes was then conducted under different growth conditions and reorientation stimuli.

## RESULTS

### Assessing Pollen Tube Growth in the Presence of BFB

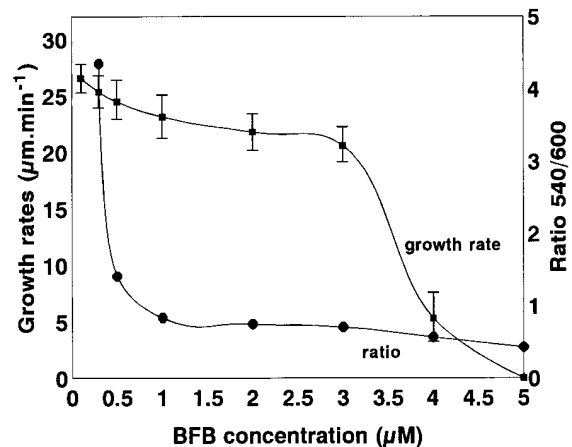
Ratio quantification of protein kinase activity with BFB is dependent on the formation of excimers. Because this necessitates the loading of pollen tubes with a high concentration of the dye, the cytotoxicity of BFB was first assessed. We observed that pollen tubes can grow with no significant decrease in growth rate at concentrations up to 3  $\mu$ M extracellular BFB (Figure 1). Above that value, growth rates decrease abruptly, with total growth inhibition at 5  $\mu$ M. For imaging BFB, we decided to use a final concentration of 500 nM for three main reasons: (1) no strong effect was observed on either growth rates or tube morphology; (2) at this concentration and under the conditions described in Methods, the red emission is within the same order of magnitude as the green one (Figure 1), which is an important parameter for confocal ratio imaging; and (3) BFB may begin inhibiting other protein kinases at concentrations above this value; thus, selectivity would be diminished.

A further complication when using BFB is putative photodamage induced by laser irradiation. Examination of the frequencies of image collection indicated that normal growth and development continued uninterrupted if intervals of 20 sec were maintained between each image. Therefore, shorter time sequences could not be used for data collection. Another problem usually associated with imaging technologies is dye sequestration, namely, in mitochondria (Chen and

Poenie, 1993). Figures 2A to 2C compare the distribution of loaded BODIPY FL and BFB with the vital dye 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>). BFB is distributed throughout the cytoplasm, and particular organelle accumulation does not seem to be present.

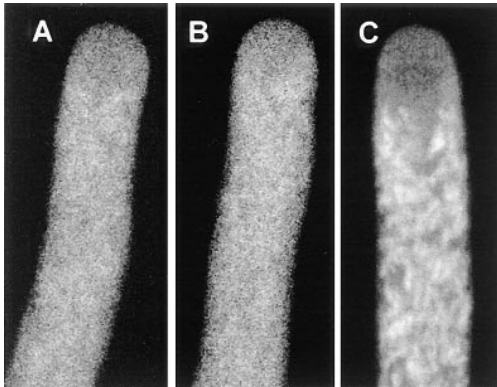
### Imaging of an Apical Ca<sup>2+</sup>-Dependent Protein Kinase Activity in Living Cells

We tested whether BFB would react with and report active protein kinase by overexpressing a plant CDPK in *Escherichia coli* BL21 cells. We observed that BFB reacted with the CDPK by measuring the overall fluorescence emitted at 540 nm by bacterial cells in which CDPK expression was either induced or noninduced (Table 1). After incubating the cells in BFB, we also measured 540/600-nm ratios in the presence of the protein kinase agonists SC-9 (*N*-[6-phenylexyl]-5-chloro-1-naphthalenesulfonamide) or PMA (phorbol 12-myristate 13-acetate); H-7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine, HCl), a kinase antagonist; and CaCl<sub>2</sub>, an enzyme cofactor. The data in Figure 3A show that treatments stimulating protein kinase activity (SC-9, PMA, and Ca<sup>2+</sup>) enhance the 540/600-nm ratio; H7, which inhibits protein kinase activity, decreases the 540/600-nm ratio. These data suggest that BFB is a suitable probe for CDPK as well as for PKC. Furthermore, when a total protein extract was resolved in a polyacrylamide gel (under nonreducing conditions), BFB associated with a single band (Figure 3B). A



**Figure 1.** Correlation Plot of BFB Concentration (Micromolar) versus Pollen Tube Growth Rates (Microns per Minute) and the Emission Ratio of the Green Channel (540 nm) against the Red Channel (>600 nm).

The 540/600 nm ratio was determined by measuring the average pixel intensity in the first 100  $\mu$ m of a pollen tube and converting the obtained value (0 to 255) to a 0 to 2 scale. The given values depend on the technical and optical settings used for image acquisition. Bars represent standard error of the mean.



**Figure 2.** Distribution of Loaded Dyes in Growing Pollen Tubes by Using Confocal Microscopy.

- (A) BODIPY FL.  
 (B) BFB.  
 (C) DiOC<sub>6</sub>.

band of similar size cross-reacted in a protein gel blot with an anti-PKC primary antibody, thus suggesting that the kinase activity detected in this study comes from a Ca<sup>2+</sup>-dependent protein kinase that shares functional homologies with the animal PKC.

To examine the putative distribution of protein kinase in growing pollen tubes, we loaded the dye by incubation in BFB. Intracellular fluorescence emitted at 600 nm did not vary significantly throughout the pollen tube under the conditions tested (see Methods). This is probably due to a lower affinity of the excimers for the targets. Therefore, in areas of higher kinase activity, green emission increased whereas red emission was kept approximately constant. In such cases, a ratio of the two wavelengths (540/600 nm) directly relates with enzyme activity, and changes induced by reorienting treatments could be quantified.

Because BFB is semipermeable to the plasma membrane, the specific dye signal had to be distinguished from the non-bound dye. Therefore, some cells were loaded with BODIPY FL. Because this fluorophore is not known to bind specifically to any cellular molecule, BODIPY-loaded cells then could be regarded as indicative of the distribution of nonbound BFB. The value for the BODIPY FL ratio was used as the zero "marker" for kinase activity (Figure 4A). Only BFB ratio values higher than the zero marker (>0.8 on a 0 to 2 scale) were designated as cytoplasmic areas of protein kinase activity.

#### Activity of the Ca<sup>2+</sup>-Dependent Protein Kinase in the Pollen Tube Apical Region

Under the conditions described above, we used dual emission laser scanning confocal microscopy to image the kinase activity in growing pollen tubes. It was found that

~90% of the growing pollen tubes analyzed ( $n = 49$ ) possessed higher kinase activity in the apical region. Most of the signal was derived from the plasma membrane and the cytosol underneath (Figures 4B to 4D). Within the limits of the spatial resolution of confocal microscopy, we could not determine whether this signal was coming only from the plasma membrane or whether there was a contribution from soluble kinases as well.

To test the specificity of the BFB labeling, we treated growing cells either with the kinase antagonist H-7 dihydrochloride (10  $\mu$ M) or with SC-9 (10  $\mu$ M), a kinase activator. When the cells were treated with H-7 ( $n = 10$ ), growth was arrested within 40 sec, and the tip gradient of kinase activity was abolished (Figure 4B). The average pixel intensity measured in these tubes was similar to the control, suggesting complete inhibition. Because H-7 at this concentration also can inhibit protein kinase A, a second batch of cells was treated with SC-9, which specifically promotes kinase activity in a Ca<sup>2+</sup>-dependent manner (Ito et al., 1986). Growing pollen tubes treated with SC-9 ( $n = 10$ ) showed a marked increase in kinase activity associated with the plasma membrane that, at later stages, could also spread into the subapical region (Figure 4C). Eventually, this treatment led to cell arrest, suggesting a tight relationship between protein phosphorylation and growth. To test the putative Ca<sup>2+</sup> dependency of the protein kinase that was implied by SC-9 enhancement, we repeated the experiment with cells ( $n = 8$ ) growing in a medium with Ca<sup>2+</sup> concentration reduced 10-fold. Under such conditions, the effect of SC-9 was less pronounced (data not shown). However, tubes grew more slowly in the Ca<sup>2+</sup>-poor medium, which could also influence protein kinase activity.

Occasionally, pollen tubes showed bending without any external stimulus ( $n = 5$ ; e.g., Figure 4C, 0 sec). When kinase activity was measured in such cases, asymmetric activity within the apical dome was observed. To further test this hypothesis, we tried to induce asymmetric kinase activities by loading micropipettes with SC-9 and placing these near the tips of growing tubes. As we have shown in previous investigations (Malhó and Trewavas, 1996), the low rates of diffusion from the narrow orifice of the micropipette set up diffusion gradients across the tip. When the tubes were exposed to this stimulus, nine of 12 responded positively by bending toward the activator source and exhibiting an increase in kinase activity, which was most evident in the side closest to the pipette (Figure 4D). Again, long exposures led to tube growth arrest and disruption of the tip gradient (Figure 4D, 200 sec). This experiment was also performed using H-7; however, a considerable decrease in activity values made analysis more difficult.

#### Localized Ca<sup>2+</sup> Increases Reorientation and Stimulates Protein Kinase Activity

Photolysis of caged Ca<sup>2+</sup> at one side of the tube apex results in localized [Ca<sup>2+</sup>]<sub>c</sub> increase and reorientation of the cell

**Table 1.** Typical Fluorescence Values of BFB in Noninduced and CDPK-Induced *E. coli* Cells after Treatment with CaCl<sub>2</sub> or SC-9<sup>a</sup>

Cell Type	CaCl <sub>2</sub> (1 mM)	CaCl <sub>2</sub> (10 mM)	CaCl <sub>2</sub> (50 mM)	SC-9 (0.01 mM)	SC-9 (0.25 mM)	SC-9 (0.5 mM)
Noninduced	5	24	34	22	85	93
Induced	26	51	78	43	191	251

<sup>a</sup> Values are given in a 0 to 255 scale and under the confocal settings described in Methods.

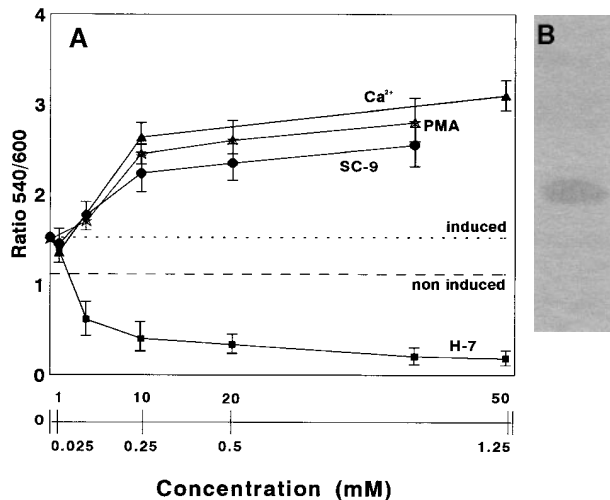
growth axis toward the side of release (Malhó and Trewavas, 1996). To assess kinase activity under such conditions, we repeated these experiments in pollen tubes loaded with BFB ( $n = 8$ ). In agreement with putative Ca<sup>2+</sup> dependence, it was found that caged release at one side of the apical dome was followed by an increase in protein kinase activity; reorientation of the cell growth axis occurred toward the side of release (Figure 5A). This indicates that kinase activity can be modulated not only by extracellular stimuli but also by the intracellular concentration of [Ca<sup>2+</sup>]<sub>c</sub>. Growth arrest, which was usually observed when a high concentration of Ca<sup>2+</sup> was released in the overall apical region, also resulted in a high, although transient increase in kinase activity. Unlike the [Ca<sup>2+</sup>]<sub>c</sub> gradient, which is rapidly dissipated, the protein kinase gradient could be observed up to 30 sec after growth

arrest (Figure 5B). After this period, the gradient totally dissipated and was reestablished only when the cells recovered.

### Modification of Growth Direction by External Inhibitors and Activators

To further test the possible involvement of protein kinase in reorientation, we loaded micropipettes with known protein kinase activators and inhibitors. When these micropipettes were placed near the pollen tube tip, the small amounts of inhibitor/activator diffusing through the orifice (~1 μm in diameter) established a diffusion gradient across the path of the growing pollen tube. This simple technique resulted in unequal modification of protein kinase activity on the two sides of the apical dome (e.g., Figure 4). The effect of a range of molecules known to modify protein kinase activity is shown in Table 2.

It was found that TMB-8, an inhibitor of PKC (Simpson et al., 1984), induced curvature away from the micropipette in ~40% of the tubes. However, TMB-8 also has been shown to block inositol 1,4,5-trisphosphate-dependent Ca<sup>2+</sup> release in plant cells (Schumaker and Sze, 1987). Its inhibitory effects therefore are not specific. The most effective molecules in promoting reorientation were H-7 and bisindolylmaleimide I (away from the micropipette) and SC-9 and PMA (toward the micropipette), emphasizing the importance of a Ca<sup>2+</sup>-dependent protein kinase in this process. Okadaic acid, a molecule that promotes hyperphosphorylation through inhibition of protein phosphatases, had effects similar to SC-9 and PMA, although the percentage of tubes responding to the okadaic acid treatment was lower. In addition, reorientation usually was followed shortly by growth inhibition. The effect of staurosporine also was tested. This PKC antagonist started to induce growth inhibition in concentrations as low as 1 μM but was unable to promote significant reorientation of the tubes.



**Figure 3.** Assessment of BFB Response to Protein Kinase Activity and Binding to Proteins.

(A) Correlation plot of the BFB 540/600-nm ratio in induced bacterial cells exposed to different concentrations of CaCl<sub>2</sub>, SC-9, H-7, and PMA. The dotted line represents the ratio value for induced cells and the dashed line for noninduced cells in simple Luria-Bertani medium. The upper scale is for CaCl<sub>2</sub> (closed triangles) concentrations and the lower scale for PMA (stars), SC-9 (closed circles), and H-7 (closed squares). Bars represent standard error of the mean.

(B) Nondenaturing gel electrophoresis of the protein extract previously incubated with the dye.

## DISCUSSION

### Imaging of the Ca<sup>2+</sup>-Dependent Protein Kinase by Using Fluorescent Techniques

Conventional visualization of protein distribution by immunohistochemical techniques leads to cell death, precluding fur-

ther insights into their possible role *in vivo*. As an alternative, the loading of fluorescently labeled peptide substrates with cross-linking to the protein kinase recently has been shown to be quite effective in mapping protein kinase distribution, albeit with a single-wavelength dye (Ritchie and Gilroy, 1998). The use of specific dyes that allow direct visualization of protein activity therefore may become a very important tool for further studies of signaling pathways. The dye used in this work is based on a selective inhibitor of protein kinases—bisindolylmaleimide—which also has been shown to inhibit a plant  $\text{Ca}^{2+}$ -dependent PKC-type enzyme in potato (Subramaniam et al., 1997). Although plant cells generally are more resistant to organic inhibitors, we found that BFB inhibits pollen tube growth with concentrations of the same order of magnitude as those used to inhibit animal PKC ( $<1 \mu\text{M}$ ). Furthermore, the fluorescence ratio of BFB has been observed to change with treatments that inhibit (H-7) or activate ( $\text{Ca}^{2+}$ , SC-9, or PMA) protein kinase. To determine the usefulness and precision of this fluorescence ratio method, more work is required; nevertheless, our evidence shows that it can be used as a qualitative indicator of activity. However, this method may be valid only for particular systems because it requires a minimum degree of asymmetric activity for it to work.

As a negative control for BFB and a zero marker for the activity of kinase, we used the fluorescence emitted by BODIPY FL under the same experimental conditions. Kinase activity was zeroed to the ratio value obtained from BODIPY FL, thus eliminating the contribution of the nonbound dye. In addition, only ratio values  $\geq 0.8$  (0 to 2 scale) were considered meaningful. Nevertheless, these results should not be regarded as a precise and accurate measurement of protein kinase activity and distribution but only as a qualitative estimate of cellular regions with higher activity.

Our data suggest that growing pollen tubes present a tip-high gradient of kinase activity. The activity is almost restricted to the plasma membrane of the apical region and cytosolic regions underneath. Similar distribution was observed when an immunofluorescence assay was conducted with an anti-PKC antibody (A. Moutinho, A.J. Trewavas, and R. Malhó, unpublished data). Although the results obtained by using this technique should be regarded with caution, it is still noteworthy that the immunodetected protein kinase colocalized with the kinase activity imaged in living cells with BFB. These data on the localization of the kinase protein thus strongly support the BFB experiments, which detected activity. Most of the already characterized plant kinases are activated by micromolar concentrations of  $\text{Ca}^{2+}$  (Roberts and Harmon, 1992; Verhey et al., 1993). Hence, it is likely that the high concentrations of  $[\text{Ca}^{2+}]_c$  in this region promote activation of numerous enzymes, including kinases. The protein kinase gradient could be abolished by treatment with the inhibitor H-7 or enhanced in a  $\text{Ca}^{2+}$ -dependent manner with SC-9. Both treatments eventually lead to an arrest of pollen tube growth, which suggests a tight regulation of the kinase activity.

It is possible that protein kinase activity experiences oscillations, as does the  $[\text{Ca}^{2+}]_c$  gradient (Holdaway-Clarke et al.,

1997; Messerli and Robinson, 1997). Protein kinases have recently been suggested to control  $[\text{Ca}^{2+}]_c$  oscillations (Thomas et al., 1996), and this would explain their confinement to the apical region in pollen tubes. However, the lack of temporal resolution by using our method does not permit us to determine whether this is the case. Further experimentation is currently under way to assess this exciting possibility.

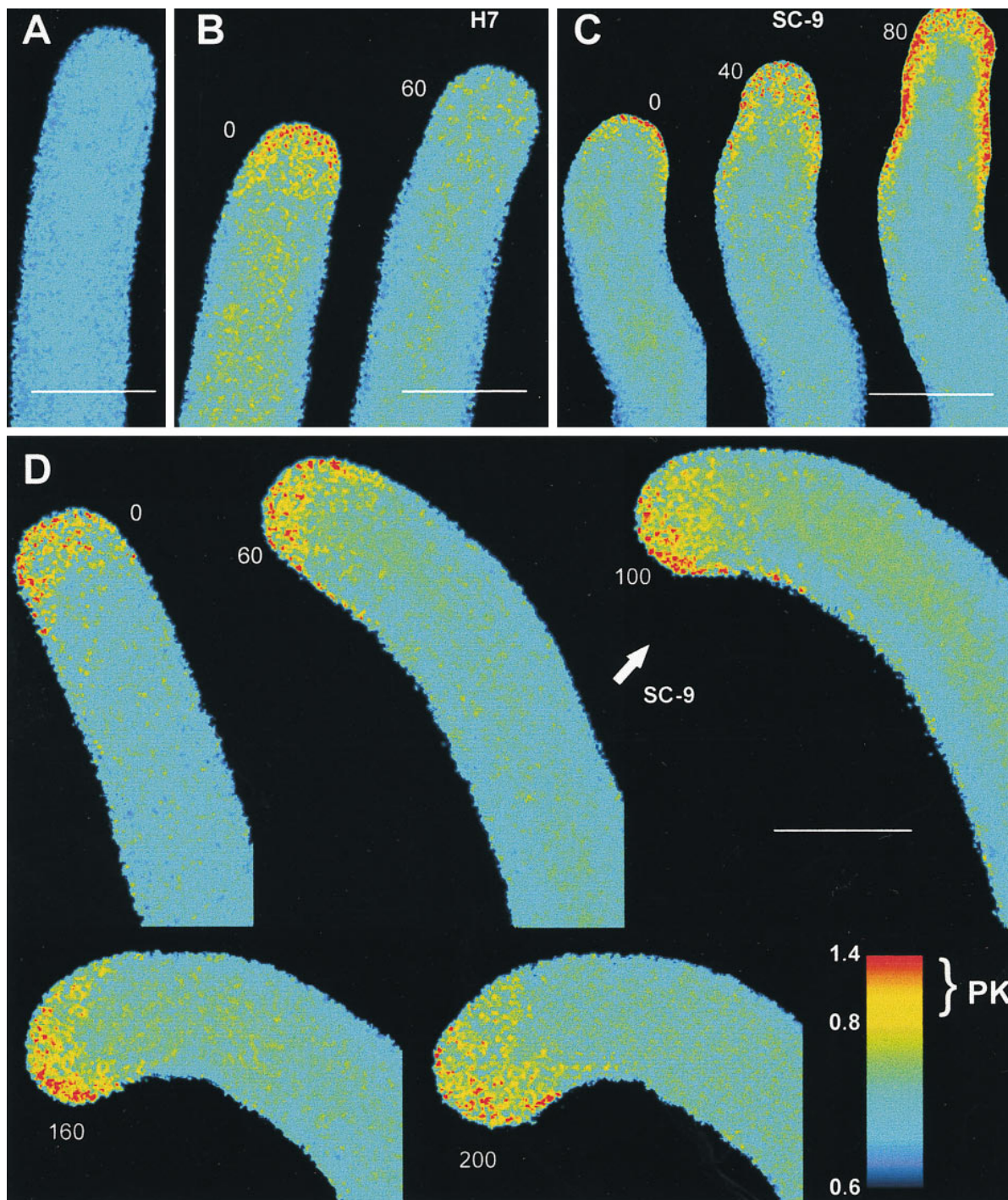
### **Asymmetric Activity of the Apical CDPK Promotes Reorientation**

Experiments with neural crest cells (Nucitelli et al., 1993) have shown that orientation is modified by inhibitors of protein kinases such as H-7. We have also found that H-7 modifies pollen tube orientation. When kinase activity was analyzed in growing reorienting tubes, the data indicated that the side toward which the cell bent showed higher activity. These observations were extended to pollen tubes placed near a micropipette diffusing SC-9, which is a kinase activator. Under these conditions, the bending of the tubes was more pronounced (toward the micropipette) as were the asymmetries recorded for kinase activity. Prolonged treatments resulted invariably in growth arrest, which was accompanied by dissipation of the gradient. Similar effects to SC-9 were observed in treatments with PMA, the most common phorbol activator of animal PKC. The data thus suggest that asymmetric activity of kinases within the growing dome is involved in the reorientation mechanisms. If this asymmetric activity proceeds, reorientation cannot be established at this stage. The caged  $\text{Ca}^{2+}$  experiments suggest that kinase activity may be induced by an increase in  $[\text{Ca}^{2+}]_c$ , but the data gathered with the kinase inhibitors/activators also indicate that an increase in kinase activity per se may trigger reorientation. Possibly, the two phenomena are intrinsically connected and cannot be dissociated.

### **Is a Protein Kinase C-Type Enzyme Present in Pollen Tubes?**

Phosphorylation cascades are known to be part of the signaling aspects of plant development and physiology, for example, cell motility (Linden and Kreimer, 1995). In animal cells, PKC is a vital element in these pathways. It is a known regulator of  $\text{Ca}^{2+}$ -dependent exocytosis (Burgoyne and Morgan, 1993), and it was shown to regulate  $\text{Ca}^{2+}$  influx by modification of channel activity (Petersen and Berridge, 1994).

In recent years, many articles have reported the presence of a wide range of protein kinases in plant tissues, both soluble (Polya and Micucci, 1985; Lu et al., 1996) and associated with the plasma membrane (Verhey et al., 1993). Schaller and co-workers (1992) reported that unlike animal cells, the predominant  $\text{Ca}^{2+}$  and lipid-dependent protein kinase associated with the plasma membrane of oat cells has biochemical properties and amino acid sequence unlike PKC. On the



**Figure 4.** Activity of the Protein Kinase Enzyme in Growing and Reorienting Pollen Tubes.

The times (seconds) at which images were taken are shown adjacent to the tips of the growing tubes. The images are displayed with the color coding shown in (D). Bars = 10  $\mu$ m.

(A) Control. Confocal ratio imaging of BODIPY FL. The 540/600-nm ratio of BODIPY FL in a growing pollen tube revealed a uniform signal all over the tube. This signal was considered to represent zero kinase activity and reflects the distribution of nonbound dye.

(B) Ratio imaging of a pollen tube loaded with BFB before and after the addition of 10  $\mu$ M H-7, an inhibitor of PKC, at  $\sim$ 10 sec. After the addition of H-7, the gradient was disrupted and growth ceased.

(C) Ratio imaging of a pollen tube loaded with BFB before and after the addition of 10  $\mu$ M SC-9, an activator of PKC, at  $\sim$ 10 sec.

(D) Confocal time-course series of images taken after placement of a micropipette containing 1 mM SC-9 near the tip of a pollen tube. The position of the micropipette is indicated by the arrow.

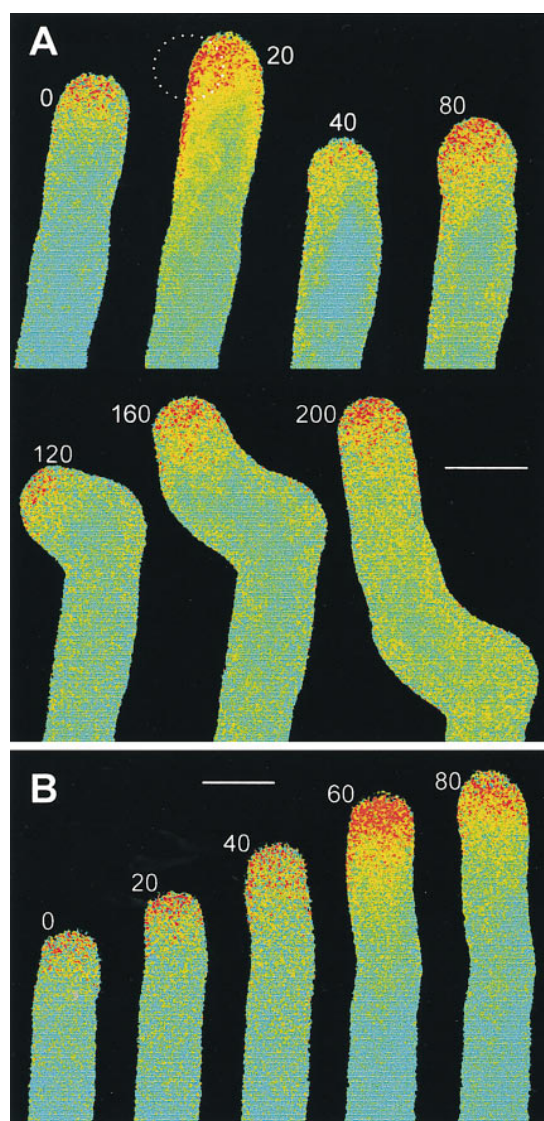
other hand, Van der Hoeven et al. (1996) detected in oat root plasma membranes a protein kinase that is activated by  $\text{Ca}^{2+}$  and *cis*-unsaturated fatty acids, phosphorylating a synthetic peptide with a motif that can be a target for both PKC and CDPK. Elliot and Kokke (1987) also purified a PKC-type enzyme with dependence on micromolar concentrations of free  $\text{Ca}^{2+}$  from seedlings of *Amaranthus tricolor*. The properties of this enzyme include phospholipid activation and reaction with antibodies raised against the regulatory domain of bovine brain PKC. Evidence for putative PKCs also has been obtained by other groups (Nanmori et al., 1994; Abo-El-Saad and Wu, 1995; Sokolova et al., 1997; Subramaniam et al., 1997). Abo-El-Saad and Wu (1995) purified a plasma membrane CDPK of ~58 kD that showed similarities to mammalian PKC, such as phosphorylation dependence on  $\text{Ca}^{2+}$ , inhibition by staurosporine and the PKC inhibitory peptide, no activation upon the addition of calmodulin, and cross-reaction with an anti-PKC antibody in immunoblotting.

In a previous report, we found that the reorientation process was not much affected by extracellular gradients of calmodulin antagonists or activators (Moutinho et al., 1998). Plant kinases associated with the plasma membrane are reported to be calmodulin independent (Schaller et al., 1992), but the current data on their own cannot confirm or dismiss this observation. In a protein gel blot assay performed with our protein extract, an antibody raised against a peptide from the catalytic domain of the animal PKC cross-reacted with a protein of ~50 kD (data not shown). This observation is consistent with reports that indicate a 50-kD species as the catalytic fragment of PKC (Elliot and Kokke, 1987; Subramaniam et al., 1997), and work is currently under way to further characterize this protein. Studies with tomato pollen tubes have shown that two genes encoding receptor-like protein kinases are pollen specific (Muschiatti et al., 1998), and a similar tissue specificity may exist for our protein.

A previous study with pollen found a CDPK to colocalize with F-actin (Putnam-Evans et al., 1989). That suggests that the protein kinase activity measured in the current study corresponds to a different member of this family. Recently, Ritchie and Gilroy (1998) found a member of the CDPK family in barley aleurone that does not cross-react with an antibody raised against the CDPK detected in pollen. However, Putnam-Evans et al. (1989) did not provide data on how the antibody distributes in the apical region. Therefore, a direct comparison with our data cannot be established, and more definitive conclusions require further results. Currently, and until the protein kinase reacting with BFB is purified, any direct similarity with PKC must remain an intriguing possibility.

#### Control of Exocytosis by $\text{Ca}^{2+}$ and Protein Kinase-Directed Growth

In all cases examined, the cellular response to a reorienting stimulus seems to involve changes in  $[\text{Ca}^{2+}]_c$ , and our results suggest a three-step mechanism leading to reorientation: (1)



**Figure 5.** Inducing Reorientation and Higher Kinase Activity with Localized Photolysis of Caged  $\text{Ca}^{2+}$ .

The times (seconds) at which images were taken are shown adjacent to the tips of the growing tubes. The images are displayed with the color coding shown in Figure 4D. Bars = 10  $\mu\text{m}$ .

**(A)** Release of caged  $\text{Ca}^{2+}$  at the left side of the apical region (indicated by a circle) at ~10 sec. This was followed by transient growth arrest, swelling, and formation of a new growth axis toward the side of release.

**(B)** After photolysis in the apical region at ~50 sec, we observed a high increase in kinase activity in this region, which was followed shortly by growth arrest. In this tube, the kinase gradient dissipated totally only ~1 min after photolysis, and the cell did not recover.

localized  $\text{Ca}^{2+}$  channel activity, possibly regulated by protein kinases; (2) localized changes in  $[\text{Ca}^{2+}]_c$ ; and (3) exocytosis.

In animal cells, exocytosis is triggered by high  $\text{Ca}^{2+}$  concentrations at the plasma membrane and is regulated by PKC (Burgoyne and Morgan, 1993). Among the proteins required for exocytosis are annexins, a group of phospholipid-binding proteins already reported in pollen tubes (Blackbourn and Battey, 1993). Trotter et al. (1995) showed that relocation of annexins from the cytosol to the membranes started when  $[\text{Ca}^{2+}]_c$  was elevated to 300 nM; at a concentration of 800 nM, the annexins were no longer detected in the cytosol, reaching a maximum level in the membrane fraction. These  $[\text{Ca}^{2+}]_c$  values are very close to those that we determined for *Agapanthus umbellatus* pollen tubes with the  $\text{Ca}^{2+}$  indicator Indo-1 (Malhó et al., 1994, 1995) for the body of the tube and tip region (180 to 220 and 700 to 1000 nM, respectively). Although highly speculative, in such conditions, annexins would be associated mainly with the plasma membrane in the apical region, whereas they would be located in the cytosol in regions farther back. Trotter et al. (1995) also showed that annexins bind to PKC, and Kissmehl et al. (1996) demonstrated that reversible protein phosphorylation is essential in controlling exocytosis. In addition, a Rho GTPase concentrated in the cortical region of the pollen tube apex is involved in tip growth (Lin et al., 1996; Lin and Yang, 1997). Rho proteins (GTP binding proteins) are known to be protein kinase regulators (Nagata and Hall, 1996) and to be involved in the maintenance of cell wall integrity (Drgonová et al., 1996). All of this evidence points to a tight regulation of exocytosis (and possibly deposition of new wall material) by the activity of protein kinases and their dependence on the local concentration of  $[\text{Ca}^{2+}]_c$ . Another possible function for the

apical CDPK could be the inhibition of  $\text{H}^+$ -ATPases (Lino et al., 1998).  $\text{H}^+$ -ATPases were reported to be present in the apical region of pollen tubes (Feijó et al., 1992) but to be inactive during growth (Feijó et al., 1995). Van der Hoeven et al. (1996) have found that  $\text{H}^+$  extrusion activity of oat coleoptiles is inhibited by PKC inhibitors that also decrease the protein kinase activity in plasma membranes vesicles.

In previous reports, we suggested that localized changes in the  $[\text{Ca}^{2+}]_c$  tip-focused gradient should alter the influx of  $\text{Ca}^{2+}$  in specific domains of the dome, in turn modifying vesicle fusion. Based on recent reports of actin distribution in living pollen tubes (Miller et al., 1996), we now extend these suggestions and propose that Golgi vesicles in the extreme apical region are not actually "delivered" to the plasma membrane but have random movements and fuse with the plasma membrane on contact. Asymmetric fusion would depend only on the conditions existing on the plasma membrane (activity of channels and concentration of ions, phospholipids, and proteins) without necessarily involving a cytoskeletal rearrangement. This would occur at a later stage as a result of the interactions of actin with phosphoinositides, actin-binding proteins, and protein kinases.

Our results on the activity of the  $\text{Ca}^{2+}$ -dependent protein kinase also are supported by data obtained in other cell systems. Petersen and Berridge (1994) observed that a low-level activation of PKC could potentiate  $\text{Ca}^{2+}$  influx, apparently by blocking the inactivation of the  $\text{Ca}^{2+}$  influx. It also explains the continued elevation in  $[\text{Ca}^{2+}]_c$  observed when caged  $\text{Ca}^{2+}$  was released at one side of the apex (Malhó and Trewavas, 1996). High levels of protein kinase activity could inhibit the influx and act as a negative regulator mechanism. After initial  $\text{Ca}^{2+}$  influx, the signal transduction path-

**Table 2.** Effect of External Gradients Determined by Placing a Micropipette near the Tip of Pollen Tubes to Reorient the Growth Axis

Agent <sup>a</sup>	Decrease in Growth Rates	Reorientation <sup>b</sup> Toward (T)/Away (A)	Growth Arrest	Arrest, Swelling, Random Curvature
TMB-8				
1 mM ( <i>n</i> = 35)	24	3 (T)/13 (A)	4	7
H-7				
2.75 mM ( <i>n</i> = 25)	23	23 (A)	2	0
Bisindolylmaleimide I				
100 $\mu\text{M}$ ( <i>n</i> = 20)	15	12 (A)	0	5
SC-9				
0.5 mM ( <i>n</i> = 25)	18	15 (T)	5	2
Okadaic acid				
300 $\mu\text{M}$ ( <i>n</i> = 25)	22	11 (T)	0	3
Staurosporine				
10 $\mu\text{M}$ ( <i>n</i> = 20)	14	6 (T)/4 (A)	0	6
PMA				
100 $\mu\text{M}$ ( <i>n</i> = 20)	20	15 (T)	0	0

<sup>a</sup> The concentration of the chemical refers to its value inside the micropipette.

<sup>b</sup> The micropipette was removed after initiation of the response to prevent further toxic effects. Reorientation was considered only if it occurred at the beginning of treatment and without total growth arrest (even if transient).



way involved in reorientation possibly includes further  $\text{Ca}^{2+}$  release in the shank of the pollen tube from inositol 1,4,5-trisphosphate-dependent stores (Malhó, 1998). Exactly which signal is transduced still needs to be determined.

## METHODS

### Plant Material

Pollen of *Agapanthus umbellatus* was harvested and stored, and pollen tubes were grown in vitro, as described previously (Malhó et al., 1994; Malhó and Trewavas, 1996).

### Ratio Imaging of the $\text{Ca}^{2+}$ -Dependent Protein Kinase

Pollen tubes growing on the same focal plane were exposed for 5 to 15 min to 500 nM BODIPY FL bisindolylmaleimide (BFB) (Molecular Probes, Eugene, OR) in the dark at room temperature. The BFB solution was freshly prepared in growth medium at pH 6.0 from a stock solution of 300  $\mu\text{M}$  in DMSO. The effect of BFB on tube growth rates and morphology was analyzed according to the criteria and procedure described previously (Malhó et al., 1994).

Fluorescence was analyzed with an MRC-600 (Bio-Rad Microscience Ltd., Hemel Hempstead, Herts, UK) laser scanning confocal microscope equipped with a 25-mW argon laser. Optical sections  $\sim 8 \mu\text{m}$  thick were collected in the F1 mode (1 sec per frame) with 3% laser intensity and the A1/A2 filter block (excitation, 514 nm; barrier filter 1 of 525 to 555 nm; barrier filter 2 of  $>600$  nm). All images were collected with a zoom factor of  $\times 3.0$  by using a Nikon  $\times 60$  plan apo objective (NA of 0.95) (Nikon UK Ltd., Telford, Shropshire, UK). The time interval between image acquisition was 20 sec. Occasionally, time sequences at 10-sec intervals were collected; however, in these cases, photoirradiation caused the pollen tubes to stop growing after  $\sim 2$  min. Fluorescence ratios (540/600 nm) were obtained with TCSM software (Bio-Rad). As a control for nonspecific dye distribution, cells were loaded with equal concentration of BODIPY FL, and the ratio values were considered as zero kinase activity. BFB ratios equal to or less than BODIPY FL values were considered nonspecific and were not taken into account.

### Loading and Photoactivation of Caged $\text{Ca}^{2+}$

$\text{Ca}^{2+}$  values were artificially manipulated by photoactivating caged  $\text{Ca}^{2+}$  (Calbiochem, Nottingham, UK) in different cytological regions of the pollen tube by using an XF-10 flash photolysis system (Hi-Tech Scientific, Salisbury, UK). This system allows photolysis in circular areas of  $\sim 80$  to  $95 \mu\text{m}^2$  ( $\sim 10$  to  $11 \mu\text{m}$  in diameter), and detailed analysis of its characteristics (resolution, efficiency, and limitations) can be found in Malhó and Trewavas (1996).

Caged  $\text{Ca}^{2+}$  was microinjected (100 to 500  $\mu\text{M}$ ) using mild ionophoresis (described in Malhó and Trewavas, 1996), and tip growth and morphology were recorded before and after photoactivation. The concentration of  $\text{Ca}^{2+}$  released was estimated by the caged fluorescein method (Malhó and Trewavas, 1996). Details of the experimental procedure and criteria used to establish the success of microinjection can be found in Malhó et al. (1994). Numerical data presentation follows the criteria defined in Malhó and Trewavas (1996).

### Plant Protein Extraction and Gel Electrophoresis

Pollen tubes with an approximate size of 400 to 600  $\mu\text{m}$  collected with a 100- $\mu\text{m}$  mesh were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM EGTA, 1 mM mercaptoethanol, and 10  $\mu\text{M}$  leupeptin) and thoroughly sonicated. The sample was centrifuged at 5000 rpm for 10 min at  $4^\circ\text{C}$  and further concentrated with Centricon tubes (Amicon, Beverly, MA). After incubation for 1 hr with 0.7 mM BFB, the total protein extract was resolved in a polyacrylamide gel with an SE 250 Gel Electrophoresis Unit (Hoefer Pharmacia, Lisbon, Portugal). The gel was run under nondenaturing conditions to maintain protein activity. In a similar set of experiments, proteins were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R 250.

### Protein Expression and Purification of a Plant CDPK

A plasmid containing CDPK $\alpha$  as a fusion protein with glutathione S-transferase (kindly provided by A. Harmon, University of Florida, Gainesville) was introduced in *Escherichia coli* BL21(DE3) pLysL under the control of the *lacZ*-inducible promoter. Cells were grown overnight at  $37^\circ\text{C}$  in Luria-Bertani broth with 100  $\mu\text{g}/\text{mL}$  ampicillin to an  $\text{OD}_{600}$  of 0.5, cooled to room temperature, diluted with fresh medium (1 to 20 inoculum), and induced for 3 hr at room temperature with shaking by adding isopropyl  $\beta$ -D-thiogalactosidase to a final concentration of 0.5 mM. After induction, cells were harvested by centrifugation at 5000 rpm for 15 min at  $4^\circ\text{C}$ , resuspended in a minimal amount of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20  $\mu\text{g mL}^{-1}$  aprotinin, and 10  $\mu\text{g mL}^{-1}$  leupeptin), and sonicated, and the supernatant was recovered by centrifugation for 5 min at 13,000 rpm at  $4^\circ\text{C}$ .

The purification was performed in glutathione agarose columns (Sigma). The columns were packed with agarose beads and washed extensively with washing buffer (50 mM Tris, pH 8.0, and 150 mM NaCl). After loading, the column was washed thoroughly and finally eluted with elution buffer (50 mM Tris, pH 8.0, and 10 mM reduced glutathione). All steps were monitored by SDS-PAGE.

### CDPK Imaging in *E. coli*

Induced CDPK-expressing *E. coli* cells were concentrated by centrifugation and resuspended in an assay medium containing Luria-Bertani broth, BFB (40  $\mu\text{M}$ ), and when appropriate, the correct dilution of an inhibitor or activator. The final concentration of inhibitors and activators is shown in Figure 1. The bacterial cells were then permeabilized by brief sonication in a water bath and centrifuged for 5 min at 13 rpm, and the pellet was washed twice with Luria-Bertani broth. After resuspension in 50  $\mu\text{L}$  of Luria-Bertani broth, cells were imaged by confocal microscopy, and the 540/600-nm ratios were calculated as described above.

### Effect of External Gradients

External gradients were imposed by diffusion of stock solutions (made in growth medium) from a pressure micropipette placed near the tip of the growing tubes (Malhó and Trewavas, 1996).

The effect of the following compounds on pollen tube growth reorientation was tested: TMB-8 (1 mM; Sigma), H-7 dihydrochloride (2.75 mM; Calbiochem, Lisbon, Portugal), bisindolylmaleimide I (100  $\mu\text{M}$ ; Calbiochem), SC-9 (0.5 mM; Calbiochem), okadaic acid (300  $\mu\text{M}$ ;

Calbiochem), staurosporine (10  $\mu$ M; Calbiochem), and PMA (100  $\mu$ M; Sigma). Growth reorientation was defined as a change in the growth axis  $>5^\circ$ , either to the left or right (Malhó et al., 1994).

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#### REFERENCES

- Abo-El-Saad, M., and Wu, R.** (1995). A rice membrane calcium-dependent protein kinase is induced by gibberellin. *Plant Physiol.* **108**, 787–793.
- Blackbourn, H.D., and Battey, N.H.** (1993). Annexin-mediated secretory vesicle aggregation in plants. *Physiol. Plant.* **89**, 27–32.
- Burgoyne, R.D., and Morgan, A.** (1993). Regulated exocytosis. *Biochem. J.* **293**, 305–316.
- Chen, C.-S., and Poenie, M.** (1993). New fluorescent probes for protein kinase C. Synthesis, characterization and application. *J. Biol. Chem.* **268**, 15812–15822.
- Drgonová, J., Drgon, T., Tanaka, K., Kollar, R., Chen, G.C., Ford, R.A., Chan, C.S.M., Takai, Y., and Cabib, E.** (1996). Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* **272**, 277–279.
- Elliot, D.C., and Kokke, Y.S.** (1987). Partial purification and properties of a protein kinase C type enzyme from plants. *Phytochemistry* **26**, 2929–2935.
- Estruch, J.J., Kadwell, S., Merlin, E., and Crossland, L.** (1994). Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. *Proc. Natl. Acad. Sci. USA* **91**, 8837–8841.
- Feijó, J.A., Malhó, R., and Pais, M.S.** (1992). A cytochemical study on the role of ATPases during pollen germination in *Agapanthus umbellatus* L'Her. *Sex. Plant Reprod.* **5**, 138–145.
- Feijó, J.A., Malhó, R., and Obermeyer, G.** (1995). Electrical currents, ion channels and ion pumps during germination and growth of pollen tubes. *Protoplasma* **187**, 155–167.
- Haugland, R.** (1996). *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed. (Eugene, OR: Molecular Probes Inc.).
- Holdaway-Clarke, T.L., Feijó, J.A., Hackett, G.R., Kunkel, J.G., and Hepler, P.K.** (1997). Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *Plant Cell* **9**, 1999–2010.
- Ito, M., Tanaka, T., Inagaki, M., Nakanishi, K., and Hidaka, H.** (1986). *N*-(6-Phenylhexyl)-5-chloro-1-naphthalenesulfonamide, a novel activator of protein kinase C. *Biochemistry* **25**, 4179–4184.
- Kissmehl, R., Treptau, T., Hofer, H.W., and Plattner, H.** (1996). Protein phosphatase and kinase activities possibly involved in exocytosis regulation in *Paramecium tetraurelia*. *Biochem. J.* **317**, 65–76.
- Lin, Y., and Yang, Z.** (1997). Inhibition of pollen tube elongation by microinjected anti-Rop1Ps antibodies suggests a crucial role for Rho-type GTPases in the control of tip growth. *Plant Cell* **9**, 1647–1659.
- Lin, Y., Wang, Y., Zhu, J.-k., and Yang, Z.** (1996). Localization of a Rho GTPase implies a role in tip growth and movement of the generative cell in pollen tubes. *Plant Cell* **8**, 293–303.
- Linden, L., and Kreimer, G.** (1995). Calcium modulates rapid protein phosphorylation/dephosphorylation in isolated eyespot apparatuses of the green alga *Spermatozopsis similis*. *Planta* **197**, 343–351.
- Lino, B., Baizabal-Aguirre, V.M., and de la Vara, L.E.G.** (1998). The plasma-membrane H<sup>+</sup>-ATPase from red beet root is inhibited by a calcium-dependent phosphorylation. *Planta* **204**, 352–359.
- Lu, Y.-Y., Hidaka, H., and Feldman, L.J.** (1996). Characterization of a calcium/calmodulin-dependent protein kinase homologue from maize roots showing light-regulated gravitropism. *Planta* **199**, 18–24.
- Malhó, R.** (1998). Role of 1,4,5-inositol triphosphate-induced Ca<sup>2+</sup> release in pollen tube orientation. *Sex. Plant Reprod.*, in press.
- Malhó, R., and Trewavas, A.J.** (1996). Localized apical increases of cytosolic free calcium control pollen tube orientation. *Plant Cell* **8**, 1935–1949.
- Malhó, R., Read, N.D., Pais, M.S., and Trewavas, A.J.** (1994). Role of cytosolic free calcium in the reorientation of pollen tube growth. *Plant J.* **5**, 331–341.
- Malhó, R., Read, N.D., Trewavas, A.J., and Pais, M.S.** (1995). Calcium channel activity during pollen tube growth and reorientation. *Plant Cell* **7**, 1173–1184.
- Messerli, M., and Robinson, K.R.** (1997). Tip localized Ca<sup>2+</sup> pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum*. *J. Cell Sci.* **110**, 1269–1278.
- Miller, D.D., Lancelle, S.A., and Hepler, P.K.** (1996). Actin microfilaments do not form a dense meshwork in *Lilium longiflorum* pollen tube tips. *Protoplasma* **195**, 123–132.
- Moutinho, A., Love, J., Trewavas, A.J., and Malhó, R.** (1998). Distribution of calmodulin protein and mRNA in growing pollen tubes. *Sex. Plant Reprod.*, in press.
- Muschietti, J., Eyal, Y., and McCormick, S.** (1998). Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2. *Plant Cell* **10**, 319–330.
- Nagata, K.-i., and Hall, A.** (1996). The Rho GTPase regulates protein kinase activity. *Bioessays* **18**, 529–531.
- Nanmori, T., Taguchi, W., Kinugasa, M., Oji, Y., Sahara, S., Fukami, Y., and Kikkawa, U.** (1994). Purification and characterization of protein kinase C from a higher plant, *Brassica campestris* L. *Biochem. Biophys. Res. Commun.* **203**, 311–318.
- Nucitelli, R., Smart, T., and Ferguson, J.** (1993). Protein kinases are required for embryonic neural crest cell galvanotaxis. *Cell Motil. Cytoskeleton* **24**, 54–66.
- Olds, J.L., Favit, A., Nelson, T., Ascoli, G., Gerstein, A., Cameron, M., Cameron, L., Lester, D.S., Rakow, T., De Barry, J., Yoshioka, T., Freyberg, Z., Baru, J., and Alkon, D.L.** (1995). Imaging protein kinase C activation in living sea urchin eggs after fertilization. *Dev. Biol.* **172**, 675–682.

- Petersen, C.C.H., and Berridge, M.J.** (1994). The regulation of capacitative calcium entry by calcium and protein kinase C in *Xenopus* oocytes. *J. Biol. Chem.* **269**, 32246–32253.
- Polya, G.M., and Micucci, V.** (1985). Interaction of wheat germ  $\text{Ca}^{2+}$ -dependent protein kinases with calmodulin antagonists and polyamines. *Plant Physiol.* **79**, 968–972.
- Putnam-Evans, C., Harmon, A.C., Palevitz, B.A., Fechheimer, M., and Cormier, M.J.** (1989). Calcium-dependent protein kinase is localized with F-actin in plant cells. *Cell Motil. Cytoskeleton* **12**, 12–22.
- Ritchie, S., and Gilroy, S.** (1998). Calcium-dependent protein phosphorylation may mediate the gibberellic acid response in barley aleurone. *Plant Physiol.* **116**, 765–776.
- Roberts, D.M., and Harmon, A.C.** (1992). Calcium-modulated proteins: Targets of intracellular calcium signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 375–414.
- Schaller, G.E., Harmon, A.C., and Sussman, M.R.** (1992). Characterization of a calcium- and lipid-dependent protein kinase associated with the oat plasma membrane. *Biochemistry* **31**, 1721–1727.
- Schumaker, K.S., and Sze, H.** (1987). Inositol 1,4,5-trisphosphate releases  $\text{Ca}^{2+}$  from vacuolar membrane vesicles of oat roots. *J. Biol. Chem.* **262**, 3944–3946.
- Simpson, A.W.M., Hallam, T.J., and Rink, T.J.** (1984). TMB-8 inhibits secretion evoked by phorbol ester at basal cytoplasmic free calcium in quin-2 loaded platelets much more effectively than it inhibits thrombin-induced calcium mobilization. *FEBS Lett.* **176**, 139–143.
- Sokolova, M., Prufer, D., Tacke, E., and Rohde, W.** (1997). The potato leafroll virus 17K movement protein is phosphorylated by a membrane-associated protein kinase from potato with biochemical features of protein kinase C. *FEBS Lett.* **400**, 201–205.
- Subramaniam, R., Després, C., and Brisson, N.** (1997). A functional homolog of mammalian protein kinase C participates in the elicitor induced defense response in potato. *Plant Cell* **9**, 653–664.
- Thomas, A.P., Bird, G.S.J., Hajnoczky, G., Robb-Gaspers, L.D., and Putney, J.W., Jr.** (1996). Spatial and temporal aspects of cellular calcium signaling. *FASEB J.* **10**, 1505–1517.
- Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilobsky, J.** (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**, 15771–15781.
- Trewavas, A.J., and Malhó, R.** (1997). Signal perception and transduction: The origin of the phenotype. *Plant Cell* **9**, 1181–1195.
- Trotter, P.J., Orchard, M.A., and Walker, J.H.** (1995).  $\text{Ca}^{2+}$  concentration during binding determines the manner in which annexin V binds to membranes. *Biochem. J.* **308**, 591–598.
- Van der Hoeven, P.C.J., Siderius, M., Korthout, H.A.A.J., Drabkin, A.V., and De Boer, A.H.** (1996). A calcium and free fatty acid-modulated protein kinase as putative effector of the fusicocin 14-3-3 receptor. *Plant Physiol.* **111**, 857–865.
- Verhey, S.D., Gaiser, J.C., and Lomax, T.L.** (1993). Protein kinases in zucchini. Characterization of calcium-requiring plasma membrane kinases. *Plant Physiol.* **103**, 413–419.