

# The Presence of a Heterotrimeric G Protein and Its Role in Signal Transduction of Extracellular Calmodulin in Pollen Germination and Tube Growth

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The role of heterotrimeric G proteins in pollen germination, tube growth, and signal transduction of extracellular calmodulin (CaM) was examined in lily pollen. Two kinds of antibodies raised against animal Gz<sub>α</sub>, one against an internal sequence and the other against its N terminus, cross-reacted with the same 41-kD protein from lily pollen plasma membrane. This 41-kD protein was also specifically ADP ribosylated by pertussis toxin. Microinjection of the membrane-impermeable G protein agonist GTP-γ-S into a pollen tube increased its growth rate, whereas microinjection of the membrane-impermeable G protein antagonist GDP-β-S and the anti-G<sub>α</sub> antibody decreased pollen tube growth. The membrane-permeable G protein agonist cholera toxin stimulated pollen germination and tube growth. Anti-CaM antiserum inhibited pollen germination and tube growth, and this inhibitory effect was completely reversed by cholera toxin. The membrane-permeable heterotrimeric G protein antagonist pertussis toxin completely stopped pollen germination and tube growth. Purified CaM, when added directly to the medium of plasma membrane vesicles, significantly activated GTPase activity in plasma membrane vesicles, and this increase in GTPase activity was completely inhibited by pertussis toxin and the nonhydrolyzable GTP analogs GTP-γ-S and guanylyl-5'-imidodiphosphate. The GTPase activity in plasma membrane vesicles was also stimulated by cholera toxin. These data suggest that heterotrimeric G proteins may be present in the pollen system where they may be involved in the signal transduction of extracellular CaM and in pollen germination and tube growth.

## INTRODUCTION

Heterotrimeric guanine nucleotide binding proteins (G proteins) play a critical role in the signal transduction processes in animals and microbial organisms (Gilman, 1987). Consisting of a plasma membrane receptor, a heterotrimeric G protein, and an effector, they act as intermediaries in transmembrane signal transduction. Receptor-mediated stimulation of G proteins results in the exchange of GTP for GDP bound to the α subunit (termed G<sub>α</sub>), which then dissociates from the βγ complex. The extracellular signal is further transmitted to downstream effectors by the activated G<sub>α</sub> subunit. An endogenous GTPase activity in the G<sub>α</sub> subunit hydrolyzes the bound GTP to GDP, thus desensitizing the protein to its GDP bound state, which favors reassociation with the βγ complex to form the heterotrimeric complex (Gilman, 1987).

A growing body of evidence supports the existence of heterotrimeric G proteins in plant cells. Microsomal and plasma membranes from several plant species have been shown to contain high-affinity GTP binding activities and

polypeptides that can be ADP ribosylated or that cross-react with antiserum raised against the G<sub>α</sub> subunit or G<sub>α</sub> subunit peptides (Ma, 1994). Genomic and cDNA clones encoding a single class of G<sub>α</sub> subunit peptides have been isolated from several plant species (Ma et al., 1990; Ma, 1994; Ishikawa et al., 1995; Kim et al., 1995; Seo et al., 1995; Gotor et al., 1996). There is also some evidence showing the involvement of heterotrimeric G proteins in red and blue light signal transduction (Romero et al., 1991; Neuhaus et al., 1993; Romero and Lam, 1993; Bowler et al., 1994; Calenberg et al., 1998), plant hormone responses (Zaina et al., 1990; Jones et al., 1998), pathogen resistance (Beffa et al., 1995), elicitor-mediated responses (Legendre et al., 1992), and the regulation of K<sup>+</sup> channels in guard cells (Fairley-Grenot and Assmann, 1991; Wu and Assmann, 1994). However, the presence of G proteins and their possible role in the plant pollen system remain largely unknown (Taylor and Hepler, 1997; Trewas and Malhó, 1997).

In recent years, an intracellular messenger Ca<sup>2+</sup> receptor protein, calmodulin (CaM), has also been found extracellularly (Sun et al., 1995a). In plant systems, Biro et al. (1984) first reported the presence of CaM in soluble extracts of oat coleoptile cell walls by using radioimmunoassays. In our laboratory, a series of experiments provided evidence for the

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presence of extracellular CaM. This evidence includes the following: (1) detection and purification of two forms of extracellular CaM from wheat coleoptile cell walls—one is water soluble and the other is ion bound and associated with the cell wall (Ye et al., 1988, 1989); (2) localization of CaM in the cell wall of maize root tips by immunoelectron microscopy (Li et al., 1993); and (3) detection of CaM in the culture medium of suspension-cultured cells from *Angelica dahurica*, carrot, and tobacco (Sun et al., 1994, 1995b). We have found extracellular CaM in all of the plant species that we have examined.

The presence of extracellular CaM suggests that it may play a significant role in the plant kingdom. Recently, further work in our laboratory indicated that CaM could extracellularly stimulate not only the proliferation of several kinds of suspension-cultured plant cells but also cell wall regeneration and protoplast division in, for example, *A. dahurica* (Sun et al., 1994), *Fenistum typhoides*, and *Sataria italica* (Sun et al., 1995b). More recently, we verified that extracellular CaM could induce light-independent expression of the gene encoding the ribulose biphosphate carboxylase small subunit (L. Ma, J. Zhou, S. Zhang, and D. Sun, manuscript in preparation). Most important, we have proven that endogenous extracellular CaM initiates pollen germination and tube growth, whereas exogenous CaM accelerates the above-mentioned processes (Ma and Sun, 1997). Therefore, the transmembrane signaling events following the activation of extracellular CaM are interesting and very important.

In this study, we assessed the role of a heterotrimeric G protein in pollen germination, tube growth, and transmembrane signal transduction of extracellular CaM. By using as modulators of G protein activity bacterial toxins, the guanine nucleotide analogs GTP- $\gamma$ -S, GDP- $\beta$ -S, and guanylyl-5'-imidodiphosphate (GMPPNP), and the anti-G $\alpha$  antibody, we provide evidence that a heterotrimeric G protein is present and involved in pollen germination and tube growth. In addition, according to the effect of exogenous CaM on GTPase

activity, we also obtained evidence suggesting a role for a heterotrimeric G protein in signal transduction of extracellular CaM during pollen germination and tube growth.

## RESULTS

### Immunodetection of a Heterotrimeric G Protein in Pollen

As a first step in testing the role of a heterotrimeric G protein in pollen germination and tube growth, we determined its presence in pollen plasma membranes by using protein gel blot analysis with two types of polyclonal antibodies raised against the internal sequence GTSNSGKSTIVKQMK and the N-terminal sequence RQSSEEKEAARRSRR of animal Gz $\alpha$ . The protocol used for purification of plasma membrane proteins is summarized in Table 1. The data in Table 1 also indicate that the purity of these preparations was sufficient for the following experiments. As shown in Figure 1, the two anti-Gz $\alpha$  antibodies recognized the same 41-kD protein in lily pollen plasma membranes (lanes 3 and 4), whereas no specific labeling at 41 kD was observed with the Gz $\alpha$  preimmune serum (lane 5).

### ADP Ribosylation by Pertussis Toxin

Plant G $\alpha$  subunits contain ADP ribosylation sites for bacterial toxins, such as pertussis toxin (Ma, 1994). To confirm further that the protein cross-reacting with the anti-G $\alpha$  antibodies in our lily plasma membrane preparations represents the G $\alpha$  subunit, we analyzed pertussis toxin-dependent ADP ribosylation. We found that when plasma membrane preparations were subjected to ADP ribosylation in the presence of pertussis toxin, a 41-kD protein, as well as two major proteins of 60 and 30 kD, were ADP ribosylated (Figure 2). In

**Table 1.** Purification of Plasma Membranes from Lily Pollen Protoplasts<sup>a</sup>

Vesicles	Plasma Membrane <sup>b</sup>	Vacuolar Membrane <sup>c</sup>	Mitochondrial Membrane <sup>d</sup>	ER Membrane <sup>e</sup>	Golgi Membrane <sup>f</sup>
U <sub>3</sub> <sup>g</sup>	152.10 ± 17.00	6.60 ± 0.21	13.19 ± 0.11	0.04 ± 0.01	4.38 ± 2.36
U <sub>3</sub> <sup>h</sup>	57.10 ± 18.60	54.90 ± 18.31	286.86 ± 87.81	12.56 ± 1.14	38.88 ± 1.74
LF <sup>i</sup>	14.09 ± 1.88	14.50 ± 3.62	153.74 ± 45.22	15.18 ± 0.21	658.75 ± 28.11

<sup>a</sup>Enzyme activity is given in nanomoles of Pi per minute per milligram of protein or nanomoles of NADP<sup>+</sup> per minute per milligram of protein.

<sup>b</sup>The plasma membrane marker is VO<sub>4</sub><sup>-3</sup> ATPase.

<sup>c</sup>The vacuolar membrane marker is NO<sub>3</sub><sup>-</sup> ATPase.

<sup>d</sup>The mitochondrial membrane marker is N<sub>3</sub><sup>-</sup> ATPase.

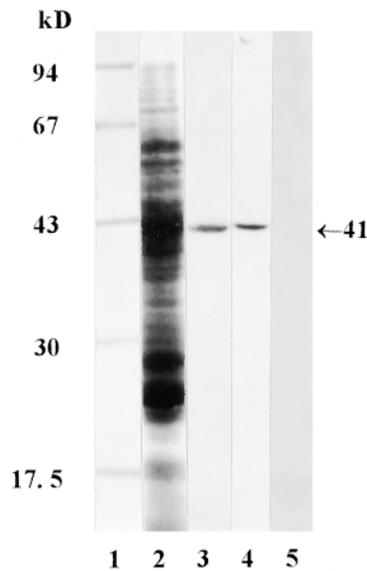
<sup>e</sup>The endoplasmic reticulum (ER) marker is cytochrome c reductase (at pH 7.5). The marker is antimycin A-insensitive NADPH:cytochrome c reductase.

<sup>f</sup>The Golgi membrane marker is UDPase.

<sup>g</sup>Vesicles from the third upper fraction.

<sup>h</sup>Vesicles from the third upper fraction of the first lower fraction.

<sup>i</sup>Vesicles from the third lower fraction of the first lower fraction.



**Figure 1.** Immunoblot Analysis of the Lily Plasma Membrane after SDS-PAGE with Antisera Directed against the Vertebrate  $Gz_{\alpha}$  Subunit.

Lane 1 contains protein molecular mass markers (given in kilodaltons at left) stained with Coomassie Brilliant Blue R 250; lane 2, plasma membrane proteins stained with Coomassie blue; lane 3, protein gel blot of plasma membrane proteins probed with the antibody raised against the  $Gz_{\alpha}$  internal sequence, GTSNSGKSTIVK-QMK; lane 4, protein gel blot of plasma membrane proteins probed with the antibody raised against the  $Gz_{\alpha}$  N-terminal sequence, RQS-SEEKEAARRSRR; and lane 5, protein gel blot of plasma membrane proteins probed with  $Gz_{\alpha}$  preimmune serum. The 41 at right indicates the molecular mass of detected protein in kilodaltons.

Each lane contains the same amount of protein (80  $\mu$ g). The antibodies were diluted 1:1000 for  $Gz_{\alpha}$  internal sequence antibody and 1:500 for  $Gz_{\alpha}$  N-terminal sequence antibody.

the absence of pertussis toxin, the 60- and 30-kD proteins were still covalently labeled (Figure 2, lane 3). This suggests that the 41-kD protein can be covalently labeled specifically by pertussis toxin. In addition, we also observed that ADP ribosylation activity increased when the pertussis toxin concentration was increased from 0 to 400 ng mL<sup>-1</sup> (Figure 2, lanes 3 to 6).

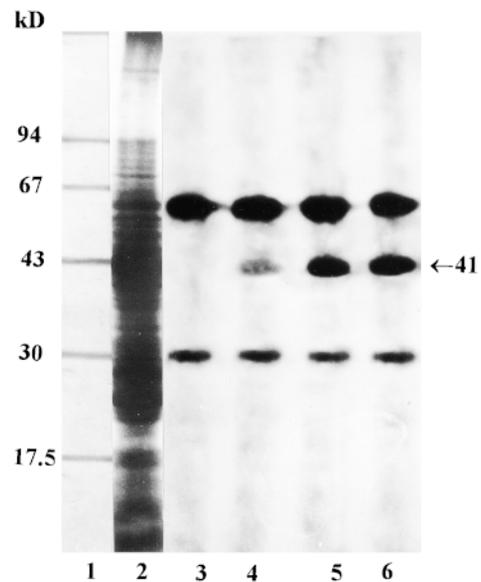
#### Effects of Membrane-Permeable G Protein Modulators on Pollen Germination and Tube Growth

To examine whether a heterotrimeric G protein is involved in pollen germination and tube growth, we studied the effects of cholera and pertussis toxins, which are both membrane-permeable G protein modulators (Ma, 1994), on pollen germination and tube growth. We found that both pollen germi-

nation and tube growth were increased by the G protein agonist cholera toxin (Figure 3), whereas both processes were inhibited by the G protein antagonist pertussis toxin (Figure 4) in a dose-dependent manner. Pollen germination and tube growth were increased >50% by adding 400 ng mL<sup>-1</sup> cholera toxin (Figure 3) and completely stopped by adding 400 ng mL<sup>-1</sup> pertussis toxin (Figure 4). Because pertussis toxin inactivates only the heterotrimeric G protein by uncoupling the receptor from its associated G protein (Simon et al., 1991), the above-mentioned result implicates a heterotrimeric G protein in pollen germination and tube growth.

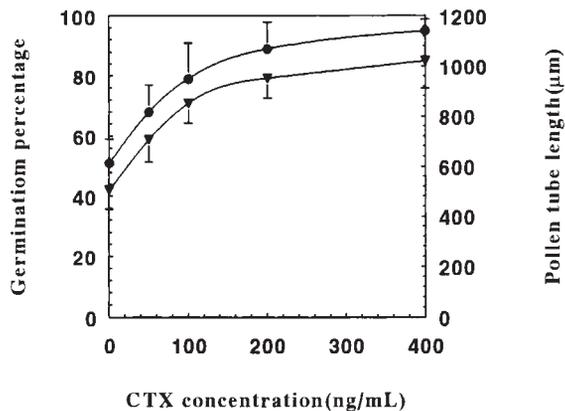
#### Effects of Microinjecting Membrane-Impermeable G Protein Modulators on Pollen Tube Growth

To provide further evidence for the involvement of a heterotrimeric G protein in pollen tube growth, we assessed the effects of the membrane-impermeable G protein modulators GTP- $\gamma$ -S and GDP- $\beta$ -S and the anti- $Gz_{\alpha}$  antibody ( $Gz_{\alpha}$  internal sequence) on pollen tube growth by using a microinjection technique. In contrast to microinjection using Tris-HCl buffer (Figures 5A and 5D), pollen tube growth was increased



**Figure 2.** Pertussis Toxin-Dependent ADP Ribosylation of the Lily Plasma Membrane after SDS-PAGE.

Lane 1 contains protein molecular mass markers (given in kilodaltons at left) stained with Coomassie blue; lane 2, plasma membrane proteins (80  $\mu$ g) stained with Coomassie blue; lane 3, control without pertussis toxin; lane 4, protein incubated with 100 ng mL<sup>-1</sup> pertussis toxin; lane 5, protein incubated with 200 ng mL<sup>-1</sup> pertussis toxin; and lane 6, protein incubated with 400 ng mL<sup>-1</sup> pertussis toxin. Each lane contains 50  $\mu$ g of protein.



**Figure 3.** Effects of Cholera Toxin on Pollen Germination and Tube Growth.

Lily pollen was incubated with the indicated amount of cholera toxin (CTX) that was added to the medium. The percentage of pollen germination (circles) was determined, and pollen tube length (triangles) was measured microscopically at 3 hr after incubation. Data are the mean  $\pm$ SD of five independent experiments.

by microinjection of the G protein agonist GTP- $\gamma$ -S (Figures 5B and 5E) and decreased by microinjection of the G protein antagonist GDP- $\beta$ -S (Figures 5C and 5F). The average growth rate was  $304 \mu\text{m hr}^{-1}$  for the former and  $120 \mu\text{m hr}^{-1}$  for the latter (Table 2). Pollen tube growth also was decreased by microinjecting the anti- $G_{\alpha}$  antibody (Figures 6B and 6D) as compared with microinjecting the preimmune serum (Figures 6A and 6C). The average growth rate was  $226 \mu\text{m hr}^{-1}$  when the preimmune serum was used, and it was  $81 \mu\text{m hr}^{-1}$  when the anti- $G_{\alpha}$  antibody was used (Table 2). Because the anti- $G_{\alpha}$  antibody specifically bound to heterotrimeric G proteins, this result also suggests the involvement of heterotrimeric G proteins in pollen tube growth.

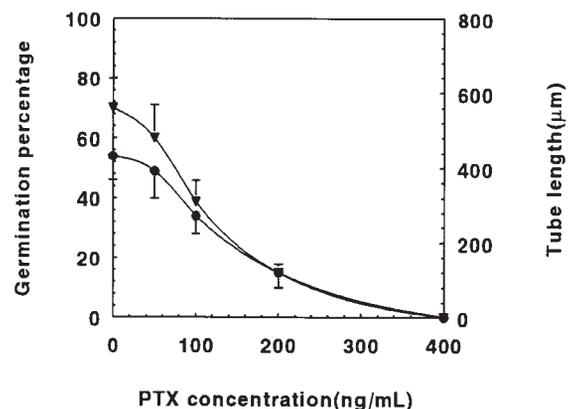
In the microinjection experiments, normal growth rates of individual lily pollen tubes on agarose media varied from 150 to  $300 \mu\text{m hr}^{-1}$  (Figures 5 and 6). Microinjection using Tris-HCl buffer or the preimmune serum reduced the rate of growth to some extent within the first 5 to 10 min after microinjection. Normal growth rates recovered rapidly, and these control microinjections did not stop growth or change cytoplasmic streaming. Because it is difficult to determine whether tube growth cessation was due to injury associated with the microinjection procedure or to inhibition provoked by the addition of GDP- $\beta$ -S or the anti- $G_{\alpha}$  antibody, any tubes that stopped growing completely were not used in calculating the results shown in Figures 5 and 6. However, when GDP- $\beta$ -S and the anti- $G_{\alpha}$  antibody were used for microinjection, the percentages of growth cessation were much higher than those of controls (Table 2). This result suggests that some tube growth might be completely abolished by using GDP- $\beta$ -S and the anti- $G_{\alpha}$  antibody.

### Relationship between Extracellular CaM and the Heterotrimeric G Protein in Pollen Germination and Tube Growth

In a previous report, we verified that endogenous extracellular CaM initiates pollen germination and tube growth and that purified CaM accelerated pollen germination and tube growth when it was added directly to the pollen culture medium (Ma and Sun, 1997). In our current experiments, we show that a heterotrimeric G protein is involved in pollen germination and tube growth. This leads us to speculate that heterotrimeric G proteins might be components in the signal transduction pathway of extracellular CaM. To test this hypothesis, we first assessed the effect of cholera and pertussis toxins on initial pollen germination and tube growth in the presence of extracellular CaM. As shown in Figure 7, the anti-CaM antiserum stopped pollen germination and tube growth (Figure 7, bar b), effects that were completely recovered and accelerated by adding the G protein agonist cholera toxin (Figure 7, bar c). Purified CaM also accelerated pollen germination and tube growth (Figure 7, bar d). The heterotrimeric G protein antagonist pertussis toxin completely inhibited the effect of extracellular CaM (Figure 7, bars e and f). This suggests that heterotrimeric G proteins may act downstream of extracellular CaM in pollen germination and tube growth.

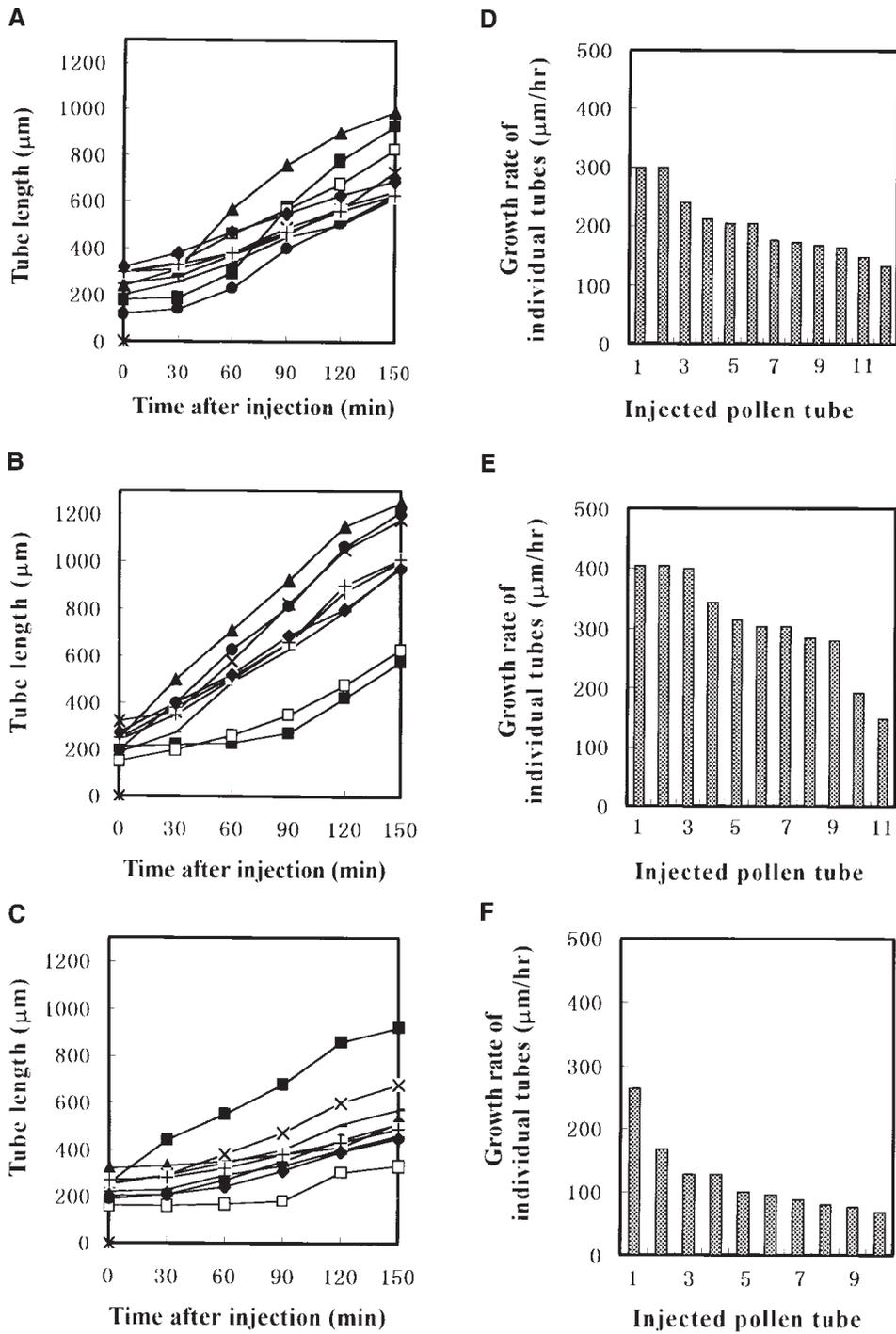
### Effect of Exogenous CaM on GTPase in Pollen Plasma Membranes

To further test this idea, we purified pollen plasma membrane vesicles according to the method of Larsson et al.



**Figure 4.** Effects of Pertussis Toxin on Pollen Germination and Tube Growth.

Lily pollen was incubated with the indicated amount of pertussis toxin (PTX) that was added to the medium. The percentage of pollen germination (circles) was determined, and pollen tube length (triangles) was measured microscopically at 3 hr after incubation. Data are the mean  $\pm$ SD of five independent experiments.



**Figure 5.** Effects of Microinjecting GTP- $\gamma$ -S or GDP- $\beta$ -S on Pollen Tube Growth.

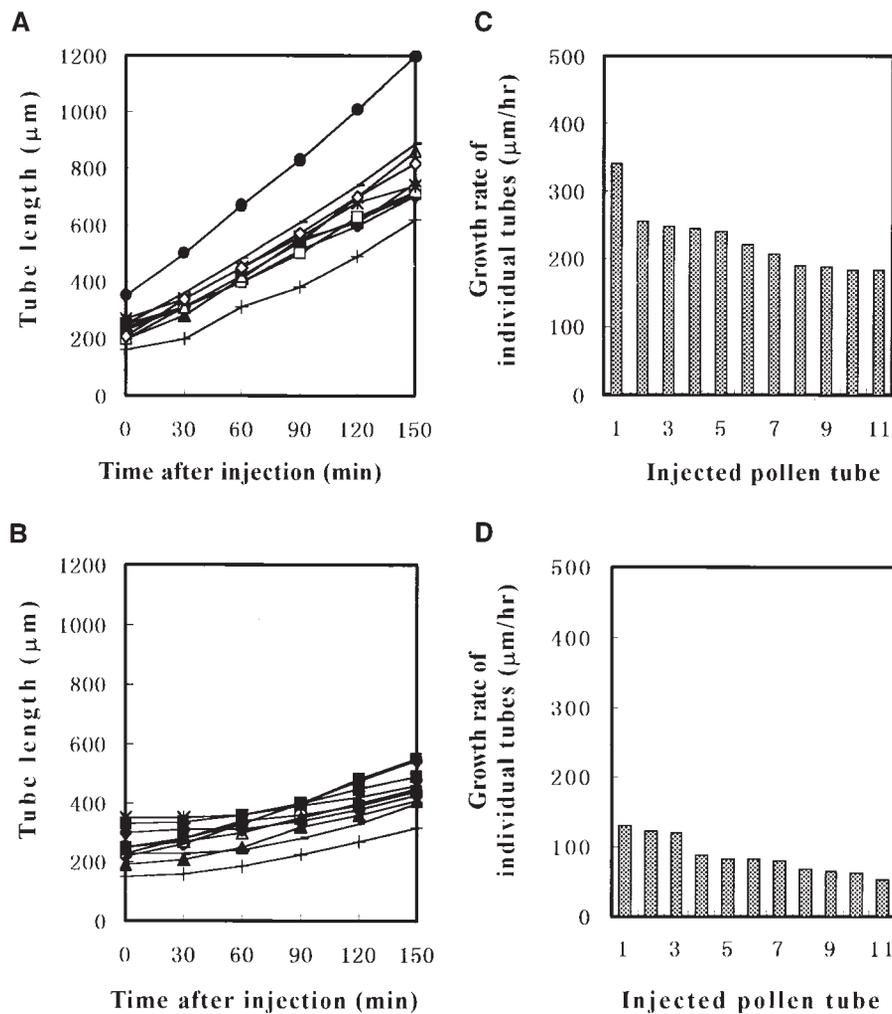
Pollen tubes were cultured on an agarose medium and microinjected with Tris-HCl buffer, GTP- $\gamma$ -S, or GDP- $\beta$ -S, using the procedure described in Methods. Growth rates were measured microscopically at the indicated times after microinjection.

(A) and (D) Controls microinjected with Tris-HCl buffer.

(B) and (E) Microinjection with GTP- $\gamma$ -S.

(C) and (F) Microinjection with GDP- $\beta$ -S.

The x axes in (A) to (C) represent the incubation time after individual pollen tubes were treated. Each tube is indicated with a different symbol. The x axes in (D) to (F) represent the number of individual pollen tubes treated. These data are ordered according to pollen tube growth rate.



**Figure 6.** Effect of Microinjecting the Anti-Gz $\alpha$  Antibody on Pollen Tube Growth.

Pollen tubes were cultured on an agarose medium and microinjected with preimmune serum or the antibody raised against the Gz $\alpha$  internal peptide, using the procedure described in Methods. Growth rates were measured microscopically at different times after microinjection.

(A) and (C) Controls microinjected with preimmune serum.

(B) and (D) Microinjection with the anti-Gz $\alpha$  antibody.

The x axes in (A) and (B) represent the incubation time after individual pollen tubes were treated. Each tube is indicated with a different symbol. The x axes in (C) and (D) represent the number of individual pollen tubes treated. These data are ordered according to pollen tube growth rate.

(1987) (Table 1) to assess whether CaM was able to activate GTPase extracellularly. All of the substrates for GTPase activity were contained inside the vesicles during their preparation, and purified CaM was applied directly to the outside of the vesicles. The data shown in Figure 8 demonstrate that treating purified plasma membrane vesicles with purified CaM activates a GTPase activity in the plasma membrane. This GTPase activity was stimulated ~19-fold by the addition of  $10^{-7}$  M CaM. However, at CaM concentrations

greater or less than  $10^{-7}$  M, less stimulation was observed. GTPase activity was not elicited by the same concentrations of BSA (Figure 8), indicating that the stimulatory effect was not a nonspecific protein effect.

The data presented in Figure 3 show that cholera toxin stimulates pollen germination and tube growth. To further test whether cholera toxin can activate a G protein directly in the pollen system, we measured the effect of cholera toxin on the activity of GTPase in plasma membrane preparations.

**Table 2.** Summary of Microinjection Results with Lily Pollen Tubes

Treatment	Microinjection Concentration	No. of Tubes Treated	Growth Cessation <sup>a</sup>	Average Growth Rate ( $\mu\text{m hr}^{-1}$ )
Tris-HCl	3 mM	17	5 (29)	204
GTP- $\gamma$ -S	3 mM	14	3 (21)	304
GDP- $\beta$ -S	3 mM	28	18 (64)	120
Preimmune serum	1.2 mg mL <sup>-1</sup>	15	4 (26)	226
Anti-Gz $\alpha$ antibody	1.2 mg mL <sup>-1</sup>	21	9 (42)	81

<sup>a</sup>Growth cessation indicates the number of cessation tubes. Numbers within parentheses indicate the percentage of growth cessation.

We found that cholera toxin stimulated GTPase activity in the plasma membrane in a dose-dependent manner. The activity of GTPase was stimulated  $\sim 10$  times by the addition of 400 ng mL<sup>-1</sup> cholera toxin (Figure 9). This result provides additional direct evidence for the presence of a G protein in the pollen plasma membrane.

To confirm that this GTPase activity might be due to a heterotrimeric G protein and not to small G proteins, we used a specific inhibitor of heterotrimeric G proteins, pertussis toxin, to distinguish between activity associated with heterotrimeric G proteins and that associated with small G proteins (Simon et al., 1991). We found that GTPase activity stimulated by purified CaM in plasma membranes was inhibited by pertussis toxin in a dose-dependent manner and that the GTPase activity was completely inhibited by 400 ng mL<sup>-1</sup> pertussis toxin (Figure 10).

Another assay was performed to ascertain whether the enzyme activity determined from the results presented in Figure 10 was specific to GTP. GTP was replaced by two of the nonhydrolyzable GTP analogs, GTP- $\gamma$ -S and GMPPNP. Both are competitive inhibitors of GTPase activity. The data shown in Figure 11 indicate that the two GTP analogs, when used in a 10- and 25-fold excess for GTP- $\gamma$ -S and GMP-PNP, respectively, completely stopped GTPase activity and eliminated exogenous CaM stimulation of GTPase activity in the plasma membrane vesicles (Figure 11). This provides further confirmation that the enzymatic reaction is specific for GTP and that the enzyme activity measured in our experiments is due to a GTPase.

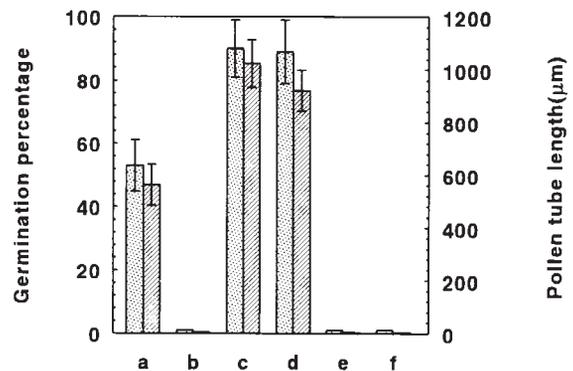
## DISCUSSION

### A Heterotrimeric G Protein Is Present and Functions in Pollen

On the basis of subunit composition and size, G proteins can be classified as heterotrimeric or as small G proteins.

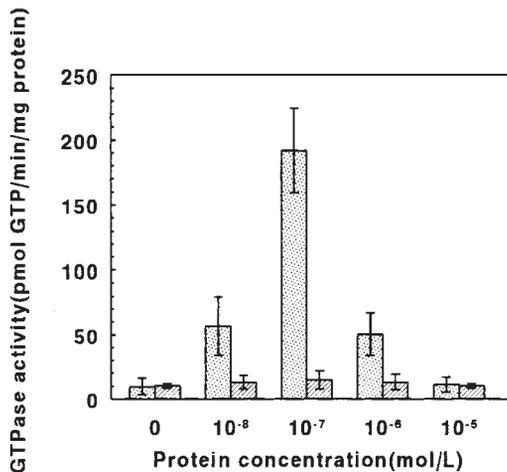
Heterotrimeric G proteins consist of three different subunits: the  $\alpha$  subunit (35 to 45 kD), containing several main functional domains in the general signal transduction pathway; the  $\beta$  subunit (35 to 36 kD); and the  $\gamma$  subunit (8 to 10 kD) (Ma, 1994). Although the presence of a class of small G proteins, Rho, has been shown in plant pollen systems (Lin et al., 1996; Lin and Yang, 1997), the presence and function of heterotrimeric G proteins in plant pollen have not been established (Taylor and Hepler, 1997; Trewavas and Malhó, 1997).

In this study, we present four different kinds of experimental evidence to show that a heterotrimeric G protein(s) is present in plants. First, two anti-G $\alpha$  antibodies raised against the animal Gz $\alpha$  internal sequence or the N-terminal sequence recognize the same 41-kD pollen protein (Figure 1). The apparent molecular mass of the putative G $\alpha$  subunit of the lily pollen plasma membrane is well within the typical molecular mass range (35 to 45 kD) reported for G $\alpha$  subunits of vertebrates and invertebrates and higher plants (Ma, 1994; Pennington, 1994; Gotor et al., 1996). Second, this same 41-kD protein is specifically ADP ribosylated by pertussis toxin (Figure 2). Third, GTPase activity in pollen plasma membranes exhibits general biochemical characteristics of heterotrimeric GTPases (Figures 8 to 11). And fourth, the heterotrimeric G protein-specific antagonist pertussis toxin inhibits pollen germination and tube growth (Figures 4 and 7). These data strongly suggest that heterotrimeric G proteins are present in pollen plasma membranes.



**Figure 7.** Effects of Purified CaM, the Anti-CaM Antiserum, Cholera Toxin, or Pertussis Toxin on Pollen Germination and Tube Growth.

Lily pollen was incubated with the indicated amount of purified CaM, the anti-CaM antiserum, cholera toxin, or pertussis toxin that was added to the medium. The percentage of pollen germination (stippled bars) was determined, and pollen tube length (hatched bars) was measured microscopically at 3 hr after incubation. Bar a is the control; bar b indicates the addition of 200  $\mu\text{g mL}^{-1}$  anti-CaM antiserum; bar c, 200  $\mu\text{g mL}^{-1}$  anti-CaM antiserum and 200 ng mL<sup>-1</sup> cholera toxin; bar d, 10<sup>-7</sup> M CaM; bar e, 400 ng mL<sup>-1</sup> pertussis toxin; and bar f, 10<sup>-7</sup> M CaM and 400 ng mL<sup>-1</sup> pertussis toxin. Data are the mean  $\pm$ SD of four independent experiments.



**Figure 8.** Effect of Purified CaM or BSA on GTPase Activity in Plasma Membrane Vesicles.

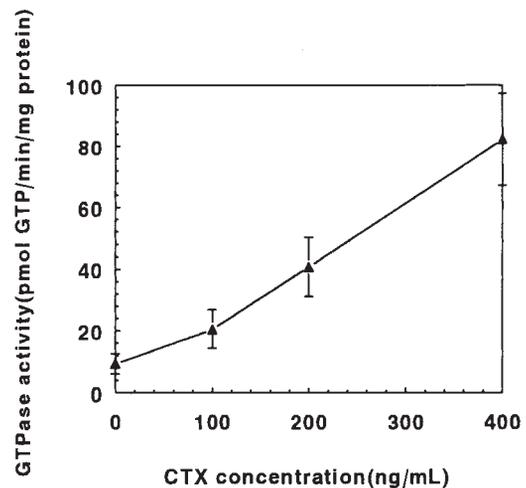
The same amount of plasma membrane vesicles and various concentrations of purified CaM (stippled bars) or BSA (hatched bars) were added directly to the medium containing plasma membrane vesicles. GTPase activity was determined spectrofluorometrically, as described in Methods. Data are the mean  $\pm$ SD of 18 independent experiments.

A common mechanism of signal transduction in animal cells involves the activation of heterotrimeric G proteins that behave as molecular switches that can rapidly change from an active GTP bound form to an inactive GDP bound form (Hepler and Gilman, 1992). In this study, we show that the membrane-permeable G protein agonist cholera toxin stimulates pollen germination and tube growth (Figure 3). Microinjection of the membrane-impermeable G protein agonist GTP- $\gamma$ -S also stimulates pollen tube growth (Figure 5 and Table 2), and microinjection of the membrane-impermeable G protein antagonist GDP- $\beta$ -S decreases pollen tube growth (Figure 5 and Table 2), implicating the involvement of G proteins in pollen germination and tube growth. To distinguish between small G proteins and the heterotrimeric G proteins, we used pertussis toxin and the anti-Gz $_{\alpha}$  antibody, both of which specifically inhibit heterotrimeric G protein activity, in the experiment. The results show that pertussis toxin and the anti-Gz $_{\alpha}$  antibody inhibit pollen germination and/or tube growth when added directly to the culture medium (for the former; Figures 4 and 7) or microinjected into the pollen tube (for the latter; Figure 6 and Table 2). There is a strong correlation among covalently labeled G proteins, GTPase activity, and the biological responses to cholera toxin and pertussis toxin (Figures 2 to 4 and 7 to 11). For example, the extent of pertussis toxin-dependent ADP ribosylation is negatively correlated with GTPase activity and biological response (i.e., pollen germination and tube growth) (Figures 2, 4, 7, 9, and 10). These results provided

direct evidence for heterotrimeric G protein involvement in pollen germination and tube growth.

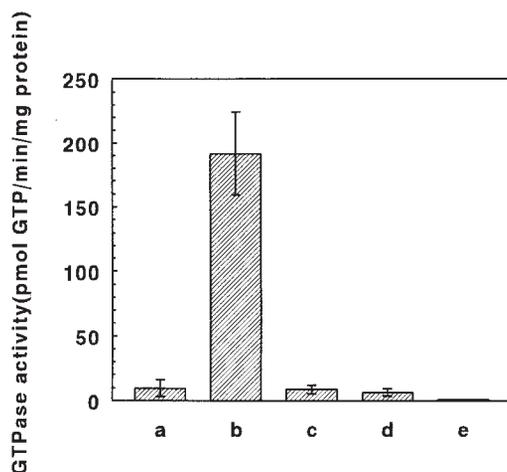
### A Heterotrimeric G Protein Transduces Extracellular CaM Signaling

Polito (1983) has reported that *Pyrus communis* pollen germination and tube growth were enhanced by the addition of purified exogenous CaM and inhibited by CaM antagonists trifluoperazine and chlorpromazine. However, it is difficult to distinguish whether CaM is involved extracellularly and/or intracellularly, because both trifluoperazine and chlorpromazine can penetrate the cell wall and plasma membrane freely. In our previous report, by using the anti-CaM antibody, W7 agarose, and purified CaM, we verified that endogenous extracellular CaM initiates pollen germination and tube growth and that exogenous CaM stimulates the above-mentioned process extracellularly (Ma and Sun, 1997). In this study, we observed that a heterotrimeric G protein is involved in pollen germination and tube growth (Figures 3 to 6). The similarity in the responses of pollen germination and tube growth to cholera toxin and to extracellular CaM raises the question of whether extracellular CaM signals through a heterotrimeric G protein to initiate pollen germination and tube growth. Positive evidence was obtained by introducing pertussis toxin into the pollen cells. We demonstrate that pertussis toxin, which could be expected to uncouple heterotrimeric G protein signaling by uncoupling the receptor



**Figure 9.** Effect of Cholera Toxin on GTPase Activity in Plasma Membrane Vesicles.

The same amount of plasma membrane vesicles and various concentrations of cholera toxin (CTX) were added directly to the medium containing plasma membrane vesicles. GTPase activity was determined spectrofluorometrically, as described in Methods. Data are the mean  $\pm$ SD of four independent experiments.



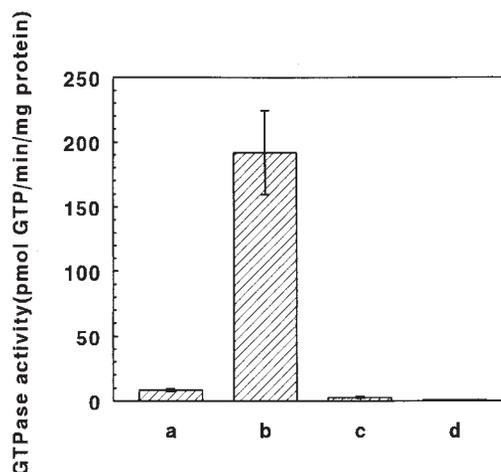
**Figure 10.** Effect of Pertussis Toxin on Purified CaM-Activated GTPase Activity in Plasma Membrane Vesicles.

The same amount of plasma membrane vesicles, various concentrations of pertussis toxin, and 0 or  $10^{-7}$  M purified CaM were added directly to the medium containing plasma membrane vesicles. GTPase activity was determined spectrofluorometrically, as described in Methods. Bar a is the control; bar b indicates the addition of  $10^{-7}$  M purified CaM; bar c,  $10^{-7}$  M CaM and  $100 \text{ ng mL}^{-1}$  pertussis toxin; bar d,  $10^{-7}$  M CaM and  $200 \text{ ng mL}^{-1}$  pertussis toxin; and bar e,  $10^{-7}$  M CaM and  $400 \text{ ng mL}^{-1}$  pertussis toxin. Data are the mean  $\pm$ SD of five independent experiments.

from its associated G protein (Simon et al., 1991), completely eliminated the effects of extracellular CaM on the initiation of pollen germination and tube growth and purified CaM stimulation of pollen germination and tube growth (Figure 7).

To further demonstrate that the heterotrimeric G protein transduces extracellular CaM signal, we prepared plasma membrane vesicles (Table 1). Although it has been reported that  $\text{GP}_{\alpha 1}$  may also be associated with the endoplasmic reticulum, in addition to being associated with the plasma membrane in meristematic cells of Arabidopsis and cauliflower (Weiss et al., 1997), the purity of the plasma membranes used in our experiment was high, and almost no endoplasmic reticulum membrane was present in our membrane vesicle preparations (Table 1). In this system, we verified that purified CaM, when applied directly to the outside of the plasma membrane vesicles, significantly activated the GTPase activity of plasma membranes in a dose-dependent manner (Figure 8). This activation of the GTPase by exogenous CaM was completely inhibited by the heterotrimeric G protein antagonist pertussis toxin (Figure 10). Although there may have been a proportion of inside-out and/or fused vesicles in addition to right-side-out vesicles in our plasma membrane preparations, the GTPase substrate would be on the wrong side in the inside-out vesicles or in the part of the inside-out plasma membrane composed of fused vesicles

and hence would not be available to be hydrolyzed. GTPase activity increased by the addition of purified CaM is measured from the right-side-out plasma membranes. Thus, we believe that the activity of GTPase measured in our experiment is due to right-side-out plasma membranes. CaM functioned extracellularly through its membrane receptor in this case. These results suggest that the heterotrimeric G protein may be involved in extracellular CaM transmembrane signal transduction. However, we cannot exclude the possibility that exogenous CaM can directly activate the G protein by mimicking a positive feedback mechanism of intracellularly activated CaM in other cases (Liu et al., 1997). However, we did not conduct additional experiments on this aspect of G protein activation. We found that extracellular CaM stimulated GTPase activity in a dose-dependent manner (Figure 8). The maximal GTPase activity was obtained at  $10^{-7}$  M CaM. However, at concentrations  $>10^{-7}$  M, the stimulation of GTPase activity did not reach a plateau but decreased completely to basal levels. This result is in agreement with our previous experiments in which extracellular CaM initiated pollen germination and tube growth (Ma and Sun, 1997) and accelerated cell proliferation (Sun et al., 1994), cell wall regeneration, and cell division (Sun et al., 1995b). This phenomenon is common for plant hormones and animal hormones (including some protein or peptide hormones). The above-mentioned result suggests that



**Figure 11.** Effect of GTP- $\gamma$ -S or GMPPNP on Purified CaM-Activated GTPase Activity in Plasma Membrane Vesicles.

GTP- $\gamma$ -S (1 mM) or GMPPNP (2.5 mM) and all inhibitors of GTPase activity, as described in Methods, were added to the plasma membrane vesicles. The same amount of plasma membrane vesicles and 0 or  $10^{-7}$  M purified CaM were added directly to the medium containing plasma membrane vesicles. Bar a is the control; bar b indicates the addition of  $10^{-7}$  M CaM; bar c,  $10^{-7}$  M CaM and 1 mM GTP- $\gamma$ -S; and bar d,  $10^{-7}$  M CaM and 2.5 mM GMPPNP. GTPase activity was determined spectrofluorometrically, as described in Methods. Data are the mean  $\pm$ SD of five independent experiments.

extracellular CaM may act as one of a peptide's primary messengers.

### Possible Mechanisms for Regulation of CaM Function

In the previous studies, we verified that CaM is present extracellularly in all of the plant species that we have examined (Ye et al., 1988, 1989; Li et al., 1993; Sun et al., 1994, 1995b). CaM is also present extracellularly in human body fluids, including blood, saliva, urine, and milk (MacNeil et al., 1984, 1988; Crocker et al., 1988; Remgard et al., 1995; Houston et al., 1997). Moreover, it has been observed that extracellular CaM is the result of positive secretion in both plant and animal systems (Sun et al., 1994, 1995b; Remgard et al., 1995). In addition, we have reported that extracellular CaM has some biological significance in plant cells (Sun et al., 1994, 1995b; Ma and Sun, 1997). Extracellular CaM was also able to stimulate cell proliferation (MacNeil et al., 1984, 1988; Crocker et al., 1988), control the outgrowth of sensory axons (Remgard et al., 1995), and inhibit the release of tumor necrosis factors from monocytes and elastase from neutrophils (Houston et al., 1997) in animal systems.

We proposed that a signal transduction pathway must be present through which extracellular CaM achieves its function. Recently, in a preliminary experiment, by using covalent cross-linking analysis with biotinylated CaM as a molecular probe, we identified a primary CaM binding protein on the outer surface of lily pollen plasma membranes (L. Ma, S. Cui, X. Xu, J. Zhou, and D. Sun, unpublished data). The CaM binding protein was also identified on the outer surface of human monocyte cell plasma membranes, and an affinity-labeling experiment with  $^{125}\text{I}$ -CaM suggested that the above-mentioned CaM binding protein might be a candidate CaM receptor (Houston et al., 1997). Pharmacological experiments have suggested that both a calcium signaling pathway and a phosphoinositide signaling pathway might be involved in the signal transduction pathways of extracellular CaM and that a calmodulin-like domain protein kinase might function downstream of the phosphoinositide signal in pollen (Ma et al., 1998). In this study, we further verified that a heterotrimeric G protein was present in the pollen plasma membrane and might be involved in transmembrane signal transduction of extracellular CaM (Figures 1, 2, 7, 8, and 10). The above-mentioned results suggest that a signal transduction pathway through which extracellular CaM achieves its functions actually might be present in both plant and animal systems.

Because the concentration of extracellular  $\text{Ca}^{2+}$  is higher than that in the cytoplasm, the question of how CaM is regulated extracellularly needs to be addressed, because the exact mechanism is still not known. However, our preliminary data indicate that the  $\text{Ca}^{2+}$ -mediated regulation of extracellular CaM might not be entirely similar to its regulation of intracellular CaM due to the differing environment. The low pH ( $\sim 4.5$ ) of the plant apoplast might be one of the factors that

affect the affinity between  $\text{Ca}^{2+}$  and CaM (Tang et al., 1999). The concentration of calcium for activation of CaM at pH 4.5 is  $>10$  times that at pH 7.4 (Tang et al., 1999). In fact, although total calcium in the extracellular area of the plant cell might be  $\geq 1$  mM, the concentration of free  $\text{Ca}^{2+}$  in vivo is not known. Evans et al. (1991) estimated that it is 10 to 100  $\mu\text{M}$  or more, whereas Trewavas and Gilroy (1991) estimated that it is  $\leq 1$   $\mu\text{M}$ .

In addition, extracellular CaM activity may be regulated not only by calcium. We have verified that CaM binding proteins are present in the extracellular area of *A. dahurica* (Tang et al., 1996) and have localized CaM binding proteins to extracellular areas (Song et al., 1997). Indeed, a 21-kD extracellular CaM binding protein has been purified (Tang et al., 1996); functional analysis with the purified 21-kD CaM binding protein and its antibody has shown that the purified 21-kD protein inhibited proliferation of suspension-cultured *A. dahurica* cells, whereas its antibody stimulated cell proliferation (Mao et al., 1999). These results suggest that extracellular CaM binding proteins may also act as one of the factors regulating the activation and deactivation of extracellular CaM by binding to or disassembling from it. Therefore, it seems that the mechanisms used to regulate extracellular CaM are undoubtedly complicated and require further study.

## METHODS

### Plant Materials

Pollen of *Lilium longiflorum* was used in this study. The pollen grains were collected from freshly opened anthers and stored at  $-74^\circ\text{C}$  for use in each experiment.

### Pollen Culture Conditions

Pollen grains stored at  $-74^\circ\text{C}$  were resuscitated by transfer to  $-20^\circ\text{C}$  for 12 hr and then to  $4^\circ\text{C}$  for another 12 hr. The resuscitated pollen was incubated in the standard medium for germination. The standard medium for pollen germination and tube growth contained 0.44 M sucrose, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{H}_3\text{BO}_3$ , 1 mM  $\text{MgSO}_4$ , and 2 mM citrate-phosphate buffer, pH 5.8. Cholera and pertussis toxins (Calbiochem, La Jolla, CA), calmodulin (CaM), or the anti-CaM antibody were added directly into the medium at various concentrations, as indicated in Figures 3, 4, and 7. For germination, pollen was incubated in small dishes at  $25 \pm 1^\circ\text{C}$  in a saturated atmosphere (100% relative humidity) for 3 hr. The germination percentage and measurements of pollen tube length were determined as described previously (Ma and Sun, 1997).

### Microinjection Procedures

The pollen grains were incubated in the above-described medium for 0.5 to 1 hr and then immediately transferred to a glass plate covered

with a thin layer of 3% low-melting agarose (Sigma) medium. Next, the plate was flooded with a liquid medium of the same composition, as mentioned above, except that agarose was not added. After transfer (1 to 1.5 hr), pollen tubes 150 to 300  $\mu\text{m}$  in length were chosen for microinjection, which was performed on a TMD inverted microscope (Nikon, Tokyo, Japan). Micropipettes for injection were made from borosilicate glass capillaries (model GD-1; Narishige Scientific Instruments, Tokyo, Japan) by using a micropipette puller (model PB-7; Narishige Scientific Instruments). Micropipette tips were backfilled to the shank with injection agents by using a 2-mL plastic injector.

The pipette tip was inserted 50 to  $\sim 100$   $\mu\text{m}$  from the tip of the pollen tubes by using a micromanipulator and a manual pressure injector (models MO-189 and IM-188; Narishige Scientific Instruments). The pipette tip reached no more than 3  $\mu\text{m}$  into the cytoplasm of the pollen tube, and agents were gently loaded into the cytoplasm. The amount of loading agents was estimated to deliver  $\sim 1$  nL of solution into the pollen tube cytoplasm. The volume of injected solution was estimated according to the method of Neuhaus et al. (1993). Five minutes after injection, pipette tips were slowly removed. Pollen tubes that suffered severe mechanical damage or leakage from the cytoplasm were discarded. Microinjected pollen tubes were then incubated at 25°C in a saturated atmosphere (100% relative humidity), and tube length was measured under a Nikon TMD inverted microscope after 0, 30, 60, 90, 120, and 150 min of incubation, respectively.

#### Isolation and Purification of Protoplasts

Protoplast isolation was conducted as described by Tanaka et al. (1987), with some modifications. Isolated pollen grains were directly suspended in the enzyme solution, which contained 1.5% (w/v) cellulase R-10, 1.5% (w/v) pectinase Boerzym M5, 0.5% potassium dextran sulfate (all from Calbiochem), and 0.6 M sucrose in White's modified solution (mineral salts and vitamins) (White, 1963), with the pH adjusted to 5.8. The enzyme treatment was performed at 30°C for  $\sim 3$  hr on a revolving platform shaker operating at 80 rpm. After the enzyme treatment, protoplasts were washed three times with modified White's solution containing 0.6 M sucrose and centrifuged at 500g for 5 min. The final suspension was then layered on to a 15% Percoll (Sigma) solution containing the culture medium for purification.

#### Preparation of Plasma Membrane Vesicles

Plasma membrane vesicles were prepared by sonication and discontinuous centrifugation, essentially according to the method described by Larsson et al. (1987). Briefly, 2 mL of protoplast pellets were suspended in 8 mL of 5 mM phosphate buffer (containing 0.24 M sucrose and 1  $\mu\text{g mL}^{-1}$  proteinase inhibitors [aprotinin, pepstatin A, chymostatin, and leupeptin; Calbiochem]) for protein gel blot analysis or 8 mL of reaction buffer for GTPase activity assay (Gonzalo et al., 1995) and were sonicated with three 30-sec bursts, with a 1-min cooling period between bursts. After the removal of nuclei by centrifugation (10,000g for 30 min at 4°C), the supernatant fraction was recentrifuged at 100,000g for 60 min at 4°C. The pellets were suspended again in 0.25 M sucrose and 5 mM phosphate buffer and centrifuged at 100,000g for 60 min at 4°C. A mixture of dextran T-500 (6.3%) and polyethylene glycol 3350 (6.3%) (two-phase partitioning protocol) was prepared according to Larsson et al. (1987). The 8-g mixture was centrifuged at 500g for 5 min, and the upper fraction

was then removed and added to a new polyethylene glycol 3350 (6.3%) preparation so that the last operation could be conducted a second time. The third upper fraction was centrifuged at 100,000g for 60 min at 4°C. The pellets were harvested and suspended in 2 mL of 0.25 M sucrose, 1 mM  $\text{CaCl}_2$ , 0.2% BSA, and 2 mM Hepes, pH 7.6, and the purified plasma membranes were used immediately for the GTPase activity assay. The purity of plasma membranes isolated by using this procedure was measured as described by Larsson et al. (1987).

#### Electrophoresis and Immunoblot Analysis

One hundred microliters of the purified lily pollen plasma membrane vesicle preparation (1.6 mg  $\text{mL}^{-1}$  protein) was resuspended in 100  $\mu\text{L}$  of protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol, and 0.025% bromophenol blue). The sample was boiled for 3 min and then centrifuged at 10,000g for 10 min. One hundred microliters of supernatant was loaded on a linear gradient SDS-polyacrylamide gel (8 to 15%) and separated by electrophoresis, as described by Laemmli (1970). The protein was transferred to a nitrocellulose membrane (Bio-Rad) and reacted with the anti-G $\alpha$  polyclonal antibody internal sequence (GTSNSGK-STIVKQMK; Calbiochem) and the anti-G $\alpha$  polyclonal antibody N-terminal sequence (RQSSEEKEAARRSRR) (Hendry et al., 1995) diluted 1000- and 500-fold, respectively. The antibodies were detected using an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma).

#### ADP Ribosylation

ADP ribosylation of plasma membrane proteins was performed by the method of Iwasaki et al. (1997), with slight modification. Briefly, the reaction mixture (50  $\mu\text{L}$ ) contained 50 mM sodium phosphate buffer, pH 7.5, 10  $\mu\text{M}$  EDTA, 100  $\mu\text{M}$   $\text{MgCl}_2$ , 100  $\mu\text{M}$  GTP, 10 mM thymidine, 6  $\mu\text{Ci}$  adenylate- $^{32}\text{P}$ -NAD (NEG-023; Du Pont), and 50  $\mu\text{g}$  of plasma membrane proteins. Toxin-catalyzed ADP ribosylation was initiated by the addition of toxin solution that had been incubated with 50 mM DTT at 37°C for 30 min for activation. The ADP ribosylation reaction was stopped by the addition of 10  $\mu\text{L}$  of 10% SDS after incubation at 30°C for 30 min. The mixture was incubated at 70°C for 10 min. The sample was then electrophoresed on a linear gradient SDS-polyacrylamide gel (8 to 15%), as described above, and the gel was dried before x-ray film (Kodak) was exposed to it at  $-75^\circ\text{C}$  for 24 hr.

#### Fluorometric Assay of GTPase Activity

The GTPase activity of heterotrimeric G proteins on the plasma membrane vesicles was measured according to the method of Gonzalo et al. (1995), with some modifications. The reaction buffer used in the sonication step included 8 mM  $\text{MgCl}_2$ , 40 mM KCl, 0.5% BSA, 1 mM EGTA, 0.5 mM EDTA, 0.7 mM 2-mercaptoethanol, 8% (v/v) glycerol, 0.25 M sucrose, 1 mM PEP (Sigma), 100  $\mu\text{M}$  GTP (Sigma), 10 units  $\text{mL}^{-1}$  of pyruvate kinase (Sigma), 15 units  $\text{mL}^{-1}$  of lactate dehydrogenase (Sigma), and 10 mM NADH (Sigma). The reaction buffer was contained inside the purified plasma membrane vesicles that had prepared as described above. The final volume of the fluorometric assay was 2 mL containing 20  $\mu\text{g}$  of plasma membrane proteins. The reaction was conducted at 37°C. The amount of

GTP hydrolyzed was measured by a cascade of reactions coupled by GTPase, pyruvate kinase, and lactate dehydrogenase. Oxidation of NADH into NAD<sup>+</sup> was measured by using a fluorometric assay with a spectrofluorometer (model RF-5000; Shimadzu Corporation, Kyoto, Japan) (excitation at 340 nm and emission at 460 nm). The rate of GTP hydrolysis into GDP could be quantitatively measured according to the decrease of NADH fluorescence emission.

#### Preparation of CaM and the Anti-CaM Antibody

Cauliflower and wheat CaMs were purified by using phenyl-Sepharose 4B affinity chromatography, as described by Biro et al. (1984). The final CaM preparation was homogeneous, as determined by electrophoresis in an SDS-polyacrylamide gel. The anti-CaM antibody from wheat was raised by immunizing rabbits with purified native wheat CaM. The anti-wheat CaM antibody was cross-reacted with various plant CaMs (Li and Sun, 1992), as given in the legend to Figure 7. Protein concentrations were determined as described by Bradford (1976).

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**The Presence of a Heterotrimeric G Protein and Its Role in Signal Transduction of Extracellular Calmodulin in Pollen Germination and Tube Growth**

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