Protein phosphatases 1 and 2A and the regulation of calcium uptake and pollen tube development in *Picea wilsonii*

LINGAN KONG,1,2 MAO WANG,2 QINLI WANG,1 XIAOHUA WANG1 and JINXING LIN1,3

1 Key Laboratory of Photosynthesis and Molecular Environment Physiology, Institute of Botany, The Chinese Academy of Sciences, Beijing 100093, China
2 College of Biological Sciences, China Agricultural University, Beijing 100094, China
3 Corresponding author (linjx@ibcas.ac.cn)

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Summary To investigate the roles of protein phosphatases 1 and 2A in the development of pollen tubes of *Picea wilsonii* Mast., pollen grains were cultured in standard medium in the presence and absence of the protein phosphatase inhibitors okadaic acid and calyculin A. At nanomolar concentrations, these compounds blocked pollen tube growth, causing abnormal morphologies of the pollen tubes. Studies with Fluo-3 revealed that the inhibitors reduced the pollen-tube tip-to-base cytoplasmic calcium (Ca\(^{2+}\)) gradient and arrested extracellular Ca\(^{2+}\) uptake. The transmission electron microscope observations indicated that the fusion of paramural bodies with plasma membranes occurred frequently in the tip and sub-tip regions of control pollen tubes, but fusion rarely occurred in inhibitor-treated pollen tubes. Staining with aniline blue showed that callose accumulated in the tip regions of inhibitor-treated pollen tubes. Immunolabeling of pollen tubes revealed that acidic pectin epitopes recognized by the monoclonal antibody JIM5 were present in the tip region and on the flanks of the sub-tip in normal pollen tubes. In inhibitor-treated pollen tubes, these epitopes existed only in the extreme tip region and at higher concentrations than in control pollen tubes. The esterified pectin recognized by JIM7 was located preferentially at the extreme tip region in normal pollen tubes, but at basal sites in inhibitor-treated tubes. Fourier transform infrared (FTIR) analysis further confirmed the changes in acidic and esterified pectin distributions and their relative contents. These results suggest that protein phosphatase 1 or 2A, or both, are involved in the regulation of Ca\(^{2+}\) uptake across the plasma membrane, in exocytic activity and in the biosynthesis of cell wall components, all processes that occur in the tip region of pollen tubes and that control pollen tube development.

Keywords: AcPase, callose, exocytosis, FTIR, immunolabeling, morphology, pectin.

Introduction Protein phosphatases have important roles in the regulation of calcium (Ca\(^{2+}\)) channel activity (Murata et al. 1993) and Ca\(^{2+}\) dynamics (Kleinhaus and Zeman 1994, Sakai and Ambudkar 1996, Allen et al. 1999) and have been implicated in elicitor-induced Ca\(^{2+}\) influx in plant cells (Kauss and Jeblick 1991, Tavernier et al. 1995). Exploitation of protein phosphatase inhibitors (MacKintosh and MacKintosh 1994, Smith and Walker 1996) has greatly aided study of the biological processes controlled by reversible protein phosphorylation.

Among the protein phosphatase inhibitors, okadaic acid (OA) and calyculin A (CalA) have been used to study the roles of protein phosphatases 1 and 2A in many plant cellular processes, particularly those associated with pollen germination and tube development (Smith and Walker 1996, Obermeyer et al. 1998, Foissner et al. 2002). Obermeyer et al. (1998) found that OA and CalA caused multiple effects at the cellular level, including disturbance of pollen tube growth direction and cell wall thickening. Foissner et al. (2002) reported that reversible protein phosphorylation regulated the dynamic organization of the pollen tube cytoskeleton. However, most studies on protein phosphatases 1 and 2A have focused on pollen germination rate and tube growth at the morphological and structural levels (Rundle et al. 1993, Obermeyer et al. 1998), or on alterations in actin and microtubules (Foissner et al. 2002) in various angiosperms. Because no studies have systematically compared the effects of OA and CalA in cell wall components, extracellular Ca\(^{2+}\) uptake and exocytotic activity, the precise function(s) of phosphatases 1 and 2A in pollen tube development remains speculative.

The polarized growth of the pollen tube involves many cellular processes, including subtle regulation of Ca\(^{2+}\) dynamics (Obermeyer and Weisenseel 1991, Feijó et al. 1995), exocytic delivery of material to the extending apex and distinct localized apical vesicle fusion (Steer and Steer 1989, Parton et al. 2003), and biosynthesis and precise organization of the various wall components (Li et al. 1992, 1994, 2002). Therefore, the single cell growing at the tip of the pollen tube provides an excellent model system in which to study the mechanisms that determine growth regulation and polarity (Parton et al. 2003).

Current knowledge of pollen tube biology is derived mainly from angiosperms. Angiosperm pollen tube growth is charac-
terized by a tip-to-base cytoplasmic Ca$^{2+}$ gradient (Pierson et al. 1994, Feijó et al. 1995), a zonal distribution of organelles, an axially organized cytoskeleton (Pierson and Cresti 1992, Derksen et al. 1999a), a reverse fountain-like cytoplasmic streaming pattern and fast autonomous growth in vitro as a result of the secretion of dictyosome-derived vesicles at the tip (Mascarenhas 1993). In gymnosperms, the physiological processes of pollen tube development differ from those in angiosperms in some of these characteristics, including slow pollen germination and pollen tube growth, and the absence of a tip-to-base zonation of organelles (Win et al. 1996, Mogami et al. 1999). Many biochemical processes involved in pollen tube development are much less well studied in gymnosperms than in angiosperm counterparts; for example, there are no available data on cytoplasmic Ca$^{2+}$ dynamics for gymnosperm pollen tubes.

We studied pollen tube development in the gymnosperm Picea wilsonii Mast. in an attempt to elucidate the regulatory roles of protein phosphatases 1 and 2A during pollen tube development. Specifically, we focused on biosynthesis of cell wall components, extracellular Ca$^{2+}$ uptake and exocytotic activity, all of which are assumed to be directly linked with and essential for pollen-tube tip growth.

Materials and methods

Plant materials and pollen tube growth conditions

On April 19, 2003, pollen grains were collected from Picea wilsonii trees growing in the Botanical Garden of the Institute of Botany, Chinese Academy of Sciences and stored at –20 °C. On April 19, 2003, pollen grains were collected from Picea wilsonii trees growing in the Botanical Garden of the Institute of Botany, Chinese Academy of Sciences and stored at –20 °C.

The standard medium for pollen culture contained 0.01% H$_2$BO$_3$, 0.03% Ca(NO$_3$)$_2$, and 12% sucrose in 50 mM citrate-phosphate buffer (pH 5.8). Ten mg of pollen grains was added to 10 ml of standard medium in each Erlenmeyer flask; this density can support a high frequency of pollen germination. All cultures were maintained at 25 °C on a 100-rpm shaker. After 12 h in culture, the pollen (about 50% germinated) was incubated in standard medium containing 30 nM OA or 30 nM CalA (Sigma Calbiochem, La Jolla, CA) dissolved in dimethylsulfoxide (DMSO) for 6 h, or as otherwise indicated. Pollen tubes cultured in standard medium containing the equivalent amount of DMSO were used as controls. The DMSO had no effect on pollen tube growth.

Pollen tube growth determination and morphological observations

The lengths of more than 30 pollen tubes were measured in each of five replicates and mean tube length calculated and plotted against incubation time. A pollen grain was considered to have germinated when tube length was greater than the diameter of the pollen grain. Germinated tubes and the morphology of pollen tubes were viewed with a Zeiss Q500 IW light microscope, and digital images were captured with a Spot II camera (Zeiss, Göttingen, Germany).

Confocal imaging

Pollen tubes were loaded with the Ca$^{2+}$-sensitive fluorescent dye Fluo-3/AM ester (Sigma, St. Louis, MO) as described by Zhang et al. (1998). The stain is highly lipophilic and thus non-disruptively crosses the plasma membrane (Digonnet et al. 1997). Briefly, samples were incubated at 4 °C for 2 h in the dark in culture medium containing 20 µM Fluo-3/AM ester prepared in DMSO. We chose a low-temperature incubation, which had little effect on diffusion of the dye across the plasma membranes, to minimize hydrolysis of Fluo-3/AM ester by extracellular esterases (Zhang et al. 1998). After the 2-h incubation, the pollen tubes were rinsed three times with the corresponding culture medium for 5 min each time and then cultured for 1 h at 25 °C in the dark, allowing the Fluo-3/AM ester to be hydrolyzed by intracellular esterases, releasing membrane-impermeable Fluo-3 into the cytoplasm. The samples were each mounted on a slide with a chamber volume of about 1 mm × 0.1 mm. The samples were excited with a 488-nm argon laser attached to a laser scanning confocal microscope (LSCM; Zeiss, LSM 510 META, Jena, Germany). Emission signals were recorded at 515 nm.

We also used Fluo-3 to determine uptake rates of extracellular Ca$^{2+}$ because its fluorescence signal increases on binding Ca$^{2+}$ (Digonnet et al. 1997). The experiment was carried out as described previously, but with an additional incubation for 10 min in Ca$^{2+}$-free medium before the cells were mounted for confocal microscopic observation. After the first three confocal microscopic pictures (from –20 to 0 s) were taken at 10-s intervals, an aliquot of Ca$^{2+}$ (as Ca$^{2+}$ nitrate, prepared in Ca$^{2+}$-free standard medium) or Ca$^{2+}$ plus the ionophore A23187 was immediately added to each slide, making the final concentration of Ca$^{2+}$ equal to that in the standard medium. Changes in fluorescence intensity were monitored at 10, 30 and 60 s.

Electron microscopy

Pollen tubes were washed three times with standard medium buffered with 100 mM sodium cacodylate (pH 7.2) and fixed for 4 h in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2). After washing with 100 mM sodium cacodylate, the samples were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in an ethanol series, transferred to propylene oxide and finally embedded in Spurr’s epoxy resin. Sections from at least 10 pollen tubes per treatment were cut with an LKB-V ultramicrotome, stained with 2% uranyl acetate (w/v) in 70% methanol (v/v) and 0.5% lead citrate, and observed with a JEM-1230 transmission electron microscope (TEM; JEOL Ltd., Tokyo, Japan) at 80 kV.

Acid phosphatase activity assay

Acid phosphatase (AcPase, EC 3.1.3.2) activity was determined according to Ibrahim et al. (2002) by measuring the release of p-nitrophenol (p-NP) from p-nitrophenyl phosphate (p-NPP). Samples collected from the culture medium after 0, 6, 12, 18, 24 or 30 h of culture were centrifuged at 10,000 g for 30 min at 4 °C. An aliquot of the supernatant was incubated
Germinated pollen tubes were fixed in freshly prepared 3% paraformaldehyde in PME (50 mM Pipes, pH 7.2, 0.5 mM MgCl₂, 1 mM EGTA) for 30 min, rinsed three times for 5 min each with PME buffer, rinsed once in 100 mM phosphate-buffered saline (PBS, pH 7.2), and then incubated with 0.1% decolorized aniline blue prepared with 100 mM PBS (pH 8.2) for about 5 min. The pollen tubes were then immediately mounted on slides and observed in UV light with the aid of a Zeiss Axioplan microscope equipped with a 365–400 nm filter set. Digital images were captured with a Spot II camera (Zeiss, Göttingen, Germany). Bright-field images of the same specimens were also collected. To decrease nonspecific staining by aniline blue, toluidine blue O (0.5% in 0.1 M PBS, pH 7.0) was used after aniline blue staining.

Paraformaldehyde quickly fixes the cell wall components of pollen tubes and maintains the original morphology of the tubes. A preliminary comparison of labeling of paraformaldehyde-fixed pollen tubes versus non-fixed pollen tubes was made to test for possible artifacts of paraformaldehyde fixation. No differences were noted in the localization of callose (or pectins; see following section) or fluorescence intensity in the cell walls between the treatments.

Immunolabeling of pectins

Pollen tubes were fixed in 3% paraformaldehyde in 50 mM PME (pH 7.2) for 30 min, rinsed three times for 5 min each with PME and rinsed once in 100 mM PBS (pH 7.2). Subsequently, the samples were incubated with primary antibody against acidic pectin (JIM5) or esterified pectin (JIM7) at a dilution of 1:5, at room temperature for 2 h. Instead of monoclonal antibodies, the PBS buffer was the negative control. Pollen tubes were then rinsed in 100 mM PBS three times for 10 min each. Samples were then incubated with a second antibody, i.e., fluorescein isothiocyanate (FITC)-labeled sheep anti-rat IgG antiserum (INC ImmunoBiologicals, Irvine, CA), diluted 1:100 with 100 mM PBS (pH 7.2) at room temperature for 2–3 h. After a final rinse series in PBS, the samples were mounted on slides and observed and photographed with the LSCM, with excitation at 488 nm and emission at 522 nm. In addition to confocal epifluorescence imaging of the labeled structures, bright-field images of the same specimens were collected by placing a detector under the condenser and transmitting the signal to the second channel by means of an optical fiber.

Fourier transform infrared microspectroscopy

Pollen tubes were washed with deionized water three times and dried in a single layer on a barium fluoride window (13 mm diameter × 2 mm). The Fourier transform infrared (FTIR) spectra were recorded with a Perkin-Elmer Cetus MAGNA 750 FTIR spectrometer (Nicolet Corp., Tokyo, Japan) equipped with a mercury-cadmium-telluride (MCT) detector. The acquisition parameters were 8 cm⁻¹ resolution and 128 co-added interferograms and were normalized to obtain relative absorbance.

Results

Pollen tube growth and morphology

The pollen germination process required at least 12 h in standard culture medium, by which time about 50% of the pollen grains had germinated. In standard medium, the rate of pollen tube growth was highest between 12 and 24 h of culture (Figure 1). After 36 h of culture in standard medium, pollen tube growth nearly stopped. Both OA and CalA at nanomolar concentrations inhibited pollen tube growth, with CalA generally being more inhibitory than OA at similar concentrations. At a concentration of around 30 nM, both inhibitors exerted about 30% inhibition of tube growth (Figure 2), whereas in the presence of 10 nM OA or 5 nM CalA, about 10% suppression of tube growth occurred (Figure 1). Pollen tubes showed negligible elongation growth when incubated in the presence of 50 nM CalA or 100 nM OA.

Pollen tubes cultured in standard medium were straight and unipolar (Figure 3A), whereas the presence of 30 nM OA or 30 nM CalA gave rise to pollen tubes (about 30%) that grew irregularly, as illustrated in Figures 2B, 2C, and 2D–F. Some pollen tubes swelled at the tip (Figure 3F), bifurcated (Figure 3B) or formed two tubes (Figure 3E), changed over time in growth direction (Figures 3C and 3D), or formed helical tubes (Figure 3D).

Intracellular Ca²⁺ distribution and extracellular Ca²⁺ uptake

Pollen tubes loaded with Fluo-3 showed strong intracellular fluorescence as assessed by confocal microscopy. Normally growing pollen tubes displayed a tip-to-base cytosolic Ca²⁺ gradient (Figure 4A), whereas almost half (~45%) of the pollen tubes treated with 30 nM OA or 30 nM CalA emitted faint fluorescence with a shallow or negligible cytosolic Ca²⁺ gradient from the tip to the base of the tube. In about 35% of the pollen tubes treated with 30 nM OA or 30 nM CalA, the Ca²⁺ gradient had completely dissipated (Figures 4B and 4C).

Fluorescence intensity excited with LSCM at 488 nm was stable within 30 min. It was further noticed that the fluorescence intensity remained constant before the extracellular Ca²⁺ was applied (from ~20 to 0 s) (Figure 4). When Ca²⁺ was added after the first three images (from ~20 to 0 s) had been obtained, the relative fluorescence intensity increased by 113% (from 0 to 30 s) in the apices of control pollen tubes (Figure 4A), whereas the increase was only 24% over the same measurement period in pollen tubes treated with 30 nM OA (Figure 4B). To test whether the fluorescence enhancement was caused by extracellular Ca²⁺ uptake, we scanned another...
set of OA-treated pollen tubes that had been incubated in medium containing Ca$^{2+}$ plus 100 µM A23187 after the first three images (from −20 to 0 s) had been captured, about 122% of the fluorescence enhancement was obtained from 0 to 30 s (Figure 4C). No significant increase in fluorescence was observed from 30 to 60 s in any experiment, even in Figures 4A and Figure 4C. Similar results were obtained with pollen tubes treated with 30 nM CalA.

**Exocytic activity**

During the TEM study, localized paramural body (PB) or single secretory vesicle fusion events that released cell wall materials into the cell wall were frequently detected in the tip and sub-tip regions of pollen tubes cultured in standard medium for 18 h (Figures 5A, 5C and 5D). In the presence of inhibitors, fusion events were rarely observed in pollen tubes (Figures 5B and 5E). No significant changes occurred in cellular organelles, such as the Golgi apparatus, in response to the inhibitor treatments (Figures 5F and 5H). Microtubules were frequently

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**Figure 1.** Effects of protein phosphatase inhibitors on pollen tube growth in *P. wilsonii*. Typical time courses of pollen tube extension growth at different concentrations of okadaic acid (A) and calyculin A (B). Values are means ± SD.

**Figure 2.** Representative fields of *P. wilsonii* pollen tubes are pictured at 18 h after in vitro culture in standard medium or in medium containing inhibitor. (A) Pollen tubes developed normally in standard medium. (B) Pollen tubes cultured for 12 h in standard medium and then treated with 30 nM okadaic acid for 6 h showing morphological abnormalities. (C) Pollen tubes cultured for 12 h in standard medium and then treated with 30 nM calyculin A for 6 h showing morphological abnormalities. Bar = 100 µm.
observed in pollen tubes growing in standard medium (Figure 5D). In pollen tubes cultured in standard medium, the cell wall was smooth in the tip region, whereas it was rough in many pollen tubes growing in inhibitor-treated media (Figure 5G).

A large increase in AcPase activity was monitored in standard culture medium as pollen tubes grew. Compared with AcPase activity determined in standard medium at the same times, AcPase activity decreased by 44, 49 and 44% at 18, 24 and 30 h, respectively, in 30 nM OA-containing medium and the corresponding decreases in medium containing 30 nM CalA were 52, 58 and 56% (Figure 6). When the inhibitors were added to standard medium at the beginning of the culture, no significant variation in AcPase activity was seen during the pollen grain germination period (0–12 h) in either standard or inhibitor-containing media. The AcPase activity that was responsive to the protein phosphatase inhibitors showed an inhibitory pattern similar to that observed for pollen tube growth in the inhibitor-containing media (Figure 1).

**Callose deposition in pollen tube walls**

Fluorescence labeling with decolorized aniline blue demonstrated the presence of callose in the apices and along the entire flanks of control pollen tubes. Cell walls containing callose emitted a yellow-green fluorescence and the fluorescence intensity along the longitudinal axis declined gradually from the tip toward the distal end (Figures 7A and 7B). When the inhibitors were added to standard medium at the beginning of the culture, no significant variation in AcPase activity was seen during the pollen grain germination period (0–12 h) in either standard or inhibitor-containing media. The AcPase activity that was responsive to the protein phosphatase inhibitors showed an inhibitory pattern similar to that observed for pollen tube growth in the inhibitor-containing media (Figure 1).

**Pectin distribution in pollen tubes**

Figures 8 and 9 are representative micrographs of more than 100 pollen tubes from three experiments showing acidic and esterified pectin distributions and relative contents as revealed by LSCM after immunolabeling with JIM5 or JIM7. One of the disadvantages of these images is that the pollen tubes cannot be well focused in all parts because the *P. wilsonii* pollen grains contain two air chambers about three times the diameter of the tubes. Observations by LSCM revealed that the fluorescence signal due to JIM5 labeling indicated a relatively homogeneous distribution of acidic pectin all over the cell wall in control pollen tubes (Figures 8A and 8B). In tubes suppressed by 30 nM OA or 30 nM CalA, the acidic pectin was preferentially located at the extreme tip and its content was increased, as indicated by the more pronounced intensity of fluorescence compared with the control pollen tubes. Very weak or almost no labeling with JIM5 was observed in other parts of the cell wall (Figures 8C–8F). The localization of JIM7 was limited to the growing apex of control pollen tubes (Figures 9A and 9B), whereas a faint fluorescence was emitted at the tip of pollen tubes treated with 30 nM OA or 30 nM CalA, or the content was too low to be immunolabeled by JIM7 (data not shown). In inhibitor-treated pollen tubes, esterified pectin was stained only at the site where the pollen grain stretched the pollen tube (Figures 9C–9F).

**FTIR microspectroscopy**

Figure 10A shows typical FTIR spectra obtained from the tip regions of control and 30 nM OA- or 30 nM CalA-treated pollen tubes; each spectrum represents one of three parallel experiments. In all three treatments, amide-stretching bands of
protein occurred at 1648 and 1540 cm$^{-1}$ (McCann et al. 1994), and saturated esters absorbed at 1744 cm$^{-1}$ (Morikawa et al. 1978, McCann et al. 1994). The carboxylic acid peaks at about 1600 and 1414 cm$^{-1}$ (Morikawa et al. 1978, McCann et al. 1994) were indistinct. To quantitatively distinguish the changes in pectins, difference spectra were generated by digital subtraction of the spectra of the tip regions of 30 nM OA- or 30 nM CalA-treated pollen tubes from the spectra of the tip regions of control pollen tubes. Based on the difference spec-

Figure 4. Effects of protein phosphatase inhibitors on extracellular calcium (Ca$^{2+}$) uptake in pollen tubes of P. wilsonii. Pollen tubes were labeled with Fluo-3 and incubated in Ca$^{2+}$-free standard medium for 10 min before observation. (A) Images of a control pollen tube captured at −20, −10 and 0 s before and 10, 30 and 60 s after Ca$^{2+}$ addition, showing a stability in fluorescence intensity when no Ca$^{2+}$ was added and a rapid temporal rise in fluorescence intensity in tip and sub-tip regions from 0–30 s, but not from 30–60 s after Ca$^{2+}$ was addition. (B) Images of a 30 nM okadaic acid (OA)-treated pollen tube captured at −20, −10 and 0 s before and 10, 30 and 60 s after Ca$^{2+}$ was added, showing a stability in fluorescence intensity when no Ca$^{2+}$ was added and a slow temporal rise in fluorescence intensity in cytoplasm after Ca$^{2+}$ addition. (C) Images of a 30 nM OA-treated pollen tube captured at −20, −10 and 0 s before and 10, 30 and 60 s after Ca$^{2+}$ plus A23187 were added, showing a stability in fluorescence intensity when no Ca$^{2+}$ was added and a rapid temporal rise in fluorescence intensity in the tip region and along the flanks from 0–30 s, but not from 30–60 s after Ca$^{2+}$ addition.

Figure 5. Ultrastructures of P. wilsonii pollen tubes cultured for 18 h. In control pollen tubes, the localized paramural body (PB) or single secretory vesicle (V) fusion events that release cell wall materials into the cell wall (CW) were frequently observed in the tip (A) and sub-tip regions ((C) and (D)); whereas in 30 nM okadaic acid- or 30 nM calyculin A-treated pollen tubes, the fusion events were rarely observed in the tip region (B) and sub-tip region (E). The cellular organelles such as the Golgi apparatus (GL) exhibit no significant alteration between control (F) and inhibitor-treated pollen tubes (H). The cell wall at the apex of many inhibitor-treated pollen tubes became rough (G). White arrows indicate PB fusing with the plasma membrane and releasing cell wall materials. The black arrow indicates a single vesicle fusing with the plasma membrane. White arrowheads indicate microtubule (MT). Bars = 1 µm in (C), (D) and (E); and 0.5 µm in (A), (B), (F), (G) and (H).
tra, a distinct carboxylic acid peak with a positive value appeared at 1600 cm$^{-1}$; another carboxylic acid peak at about 1414 cm$^{-1}$, which was indistinguishable from the C-H absorption band (1400 cm$^{-1}$; Yang and Yen 2002), also exhibited increased absorbance intensity (Figures 10B and 10C), indicating that acidic pectin content increased (Wang et al. 2003). In both difference spectra, the saturated ester peaks around 1744 cm$^{-1}$, representing esterified pectin, emerged as negative values, suggesting that esterified pectin content decreased (Wang et al. 2003).

Discussion

Pollen tube elongation is affected by several factors, including temperature, medium osmolarity, and availability of Ca$^{2+}$ and boron (Obermeyer and Weisenseel 1991, Pierson et al. 1994, Feijó et al. 1995, Wang et al. 2003). Ronne et al. (1991) found that protein phosphatase 2A had dramatic effects on yeast cell growth and bud morphogenesis. By analyzing the glc 7–10 mutation, Andrews and Stark (2000) identified essential functions of yeast protein phosphatase 1 in the regulation of cell wall integrity and polarized growth. Recently, a protein phosphatase 2A gene was isolated from Aspergillus nidulans, demonstrating that protein phosphatase 2A is required for yeast germ tube emergence and hyphal morphogenesis (Kosmidou et al. 2001). We found that the addition of OA and CalA suppressed pollen tube growth and disturbed the orientation of pollen tube growth in P. wilsonii, resulting in highly aberrant morphologies of pollen tubes, leading us to conclude that constitutive dephosphorylation of certain proteins by protein phosphatase 1 or 2A, or both, may function positively to promote cell integrity.

Calcium ion serves as a second messenger in a variety of plant physiological processes. In pollen tubes, the existence of a tip-to-base concentration gradient in cytosolic Ca$^{2+}$ is essential for tip growth (Obermeyer and Weisenseel 1991, Pierson et al. 1994, Malhó et al. 1995, Malhó and Trewavas 1996, Felle and Hepler 1997). However, the factor involved in the regulation of Ca$^{2+}$ channel activity in pollen tube growth has not been identified. Previous studies have shown that protein phosphatases may modulate Ca$^{2+}$ influx in parotid gland acinar and

Figure 6. Effects of protein phosphatase inhibitors on the secretion of AcPases to the extracellular culture medium. As the pollen tube grows, the AcPase activity markedly increased in standard medium, whereas lower activity was found in medium containing 30 nM okadaic acid (OA) or 30 nM (calyculin A) CalA. Values are means ± SD.

Figure 7. Fluorescence images callose distribution in pollen tubes of P. wilsonii cultured for 18 h and stained with decolorized aniline blue. Comparison of the fluorescence images (A), (C) and (E) with the corresponding bright-field images (B), (D) and (F), respectively, shows that fluorescence is emitted from the tip and sub-tip regions of control pollen tubes (A), whereas strong fluorescence is limited to the tip regions of pollen tubes treated with 30 nM okadaic acid (OA) (C) or 30 nM (calyculin A) CalA (E) (indicated by arrows). Bar = 50 µm.
HEK293 cells (Sakai and Ambudkar 1996, Davare et al. 2000) and are associated with the efficacy of the Ca\(^{2+}\) signal in pancreatic beta cells (Sato et al. 1998). In plant cells, protein phosphatases participate in elicitor-induced Ca\(^{2+}\) uptake (Kauss and Jeblick 1991, Tavernier et al. 1995). In pollen tubes, protein phosphatases are considered to be the putative determinants for ion channel activity (Obermeyer et al. 1998). We found that the addition of calcium enhanced the intensity of the fluorescence signal in control pollen tubes within 30 s, whereas only a slight increase was noticed in pollen tubes treated with 30 nM OA or 30 nM CalA. In pollen tubes incubated in solution containing Ca\(^{2+}\) and the Ca\(^{2+}\) transporter A23187, the fluorescence intensity increased by 122%, whereas in all of the pollen tubes studied (Figure 4A–4C), no significant change in fluorescence intensity was observed before Ca\(^{2+}\) or Ca\(^{2+}\) + A23187 was added, indicating that the intracellular fluorescence was due to intracellular Ca\(^{2+}\) ions and that the increased fluorescence intensity was due to Ca\(^{2+}\) uptake. A lower rate of Ca\(^{2+}\) uptake (indicated by the slower increase in fluorescence intensity) was observed in the tip region of inhibitor-treated pollen tubes compared with control pollen tubes, indicating that the retarded Ca\(^{2+}\) entry into the cyto-

Figure 8. Immunolabeling of pollen tubes of *P. wilsonii* with antibody JIM5 coupled to fluorescein isothiocyanate for the epitope of acidic pectin. Comparison of the fluorescence images (A), (C) and (E) with the corresponding bright-field images (B), (D) and (F), respectively, shows that in control pollen tubes (A), strong fluorescence is observed along the whole longitudinal axis, whereas it occurs mainly in the tip region of 30 nM okadaic acid- (C) and 30 nM calyculin A-treated pollen tubes (E) (indicated by white arrowheads). Bar = 50 µm.

Figure 9. Immunolabeling of pollen tubes of *P. wilsonii* with antibody JIM7 coupled to fluorescein isothiocyanate for the epitope of esterified pectin. Comparison of the fluorescence images (A), (C) and (E) with the corresponding bright-field images (B), (D) and (F), respectively, shows that strong fluorescence was observed only in the tip region of control pollen tubes, little to no fluorescence was detected elsewhere (A), but observed only at the sites where the pollen grain stretches over the pollen tube and little to no fluorescence was detected in the tip region of 30 nM okadaic acid- (C) and 30 nM calyculin A-treated pollen tubes (E) (indicated by white arrowheads). Bar = 50 µm.
plasm of pollen tubes might be a consequence of the inhibition of phosphatase 1 or 2A activity, or both.

Exocytosis, a cellular process closely coupled with continued tip growth in pollen tubes, is finely regulated by cytoplasmic Ca$^{2+}$ in maize coleoptile cells (Sutter et al. 2000), lily pollen tubes (Roy et al. 1999), plant secretory cell (Zorec and Tester 1992) and other cells. Our TEM study revealed a much lower frequency of paramural body (PB) and single secretory vesicle fusion events in tip regions of inhibitor-treated pollen tubes, providing direct evidence for a decrease in exocytotic activity in the inhibitor-treated pollen tubes of *P. wilsonii*.

Activity of AcPase has been extensively used to analyze successive steps in the secretory pathway. In *Saccharomyces cerevisiae*, AcPase activity was observed along the secretory pathway and correlated closely with growth sites during budding (Esmon et al. 1981, Hagenauer-Tsapis 1992). In a study of lily pollen tube growth, Ibrahim et al. (2002) noted a conspicuous correlation between secretory pathway activity and AcPase secretion during pollen tube growth and concluded that the secreted AcPase activity might serve as a useful indicator of exocytotic activity. We found that the secreted AcPase activity in media containing OA or CalA was much lower than in standard medium over the same culture period, suggesting that the cellular process leading to exocytosis was disturbed by inhibitors of protein phosphatase 1 or 2A, or both, during pollen tube development of *P. wilsonii*. This result corroborates earlier findings in lily and yeast (Esmon et al. 1981, Ibrahim et al. 2002). Given the link between protein phosphatase 1 or 2A, or both, and exocytotic activity and between phosphatase 1 or 2A, or both, and extracellular Ca$^{2+}$ uptake, our finding suggests that Ca$^{2+}$ homeostasis in the cytoplasm of pollen tubes mediated by phosphatase 1 or 2A, or both, could account for the exocytotic activity that occurs in the tip regions of pollen tubes. An essential role of cytoplasmic Ca$^{2+}$ concentrations in the control of exocytotic activity has been reported previously (Ibrahim et al. 2002, Camacho and Malhó 2003).

Exocytosis is essential to pollen tube growth because the secretory vesicles are packed with inner tube cell wall material that is delivered to the plasma membrane and completely incorporated into the original cell wall (Ibrahim et al. 2002, Camacho and Malhó 2003). The cell wall has a highly complex structure composed of polysaccharides, structural proteins, and various enzymes. The pollen tube wall is unusual in that it consists mainly of callose (β-1,3-glucan, a polysaccharide) and pectins unlike other plant cell walls (Li et al. 1992, 1994, Derksen 1999b). In the apical region of pollen tubes treated with OA or CalA, we observed a heavy deposition of callose, unlike the deposition in control pollen tubes where relatively weak fluorescence was emitted from the tip and sub-tip regions. We speculate that protein phosphatase 1 or 2A, or both, spatially and developmentally mediated the synthesis and distribution of callose, which contributed to the mechanical properties of the cell wall and thereby affected pollen tube growth. This result confirms the view that the massive accumulation of callose in pollen tube tips is an important manifestation of abnormally growing tubes (Wang et al. 2003,
Lalanne et al. 2004) and a common indication of incompatible pollen (Guyon et al. 2004). Pectins are synthesized in the Golgi apparatus, methyl-esterified, modified with side chains and subsequently released into the apoplastic space as highly methylesterified polymers (Micheli 2001). Acidic pectins are produced biochemically from esterified pectins. This dynamic transformation is catalyzed by pectin methylesterases, which are under the regulation of the local pH and Ca\(^{2+}\) concentration (Micheli 2001, Li et al. 2002). Additionally, the acidic pectin can cross-link with Ca\(^{2+}\) through its carboxyl group and form egg-carton patterns to increase the rigidity of the cell wall, whereas esterified pectin is responsible for the elastic behavior of the cell (Jarvis 1984, McNeil et al. 1984). We observed that acidic pectin accumulated, whereas esterified pectin significantly decreased or totally disappeared in the tip region of inhibitor-treated pollen tubes. Based on our immunolabeling study, we propose that conversion of esterified pectin to acidic pectin or the biosynthesis of esterified pectin, or both, are under the control of protein phosphatase 1 or 2A, or both, and that inhibition of these enzymes leads to excessive wall rigidity at the tip of the pollen tube, which may partly account for the inhibition of pollen tube elongation in the presence of OA and CalA. This proposition is strongly supported by the findings of Li et al. (1992, 1994) and Geitmann et al. (1995).

Fourier transform infrared microspectroscopy is a powerful, noninvasive technique based on vibrational spectroscopy that allows quantitative assays of a variety of functional groups (Chen et al. 1998). It is a good method for quick evaluations and for the determination of polysaccharide compositions of samples of pectic origin (Coimbra et al. 1998). Based on FTIR spectroscopy, Pappas et al. (2003) developed a new methodology for the identification of pollen, Wang et al. (2003) demonstrated that boron deficiency causes changes in the concentrations and distributions of acidic pectin, phenolics and saturated esters in the pollen tubes of *P. meyeri* Rehd. et Wils, and Wu et al. (2003) identified the lignin, suberin, cellulose and cell wall proteins in Casparian strips in needles of *Pinus bungeana* Zucc. ex Endl. Our FTIR spectroscopic study showed that the content of carboxylic acid at 1600 cm\(^{-1}\) and 1414 cm\(^{-1}\) representing acidic pectin increased, whereas the absorbance intensity of saturated ester at 1744 cm\(^{-1}\) representing esterified pectin decreased with both treatments and we confirmed these observations by an immunolabeling technique.

Several studies have shown that the synthesis or deposition, or both, of the cell wall components of pollen tubes are under the control of Ca\(^{2+}\) (Eklund and Eliasson 1990, His et al. 2001). Based on the predominant biological roles of Ca\(^{2+}\) homeostasis in modulating esterified acidic pectin transformation (Micheli 2001, Li et al. 2002) and in regulating callose formation (Andrawis et al. 1993, Sivaguru et al. 2000, Kar tusch 2003), we interpreted the changes we observed in callose and pectins as evidence for a disturbance of intracellular Ca\(^{2+}\) homeostasis in inhibitor-treated pollen tubes. In turn, we deduced that protein phosphatase 1 or 2A, or both, likely regulate upstream cytoplasmic Ca\(^{2+}\) dynamics and thus, affect the biosynthesis and distribution of cell wall components as well as cell wall properties.

In conclusion, we obtained several lines of evidence to substantiate the importance of protein phosphatase 1 or 2A, or both, in *P. wilsonii* pollen tube development. We conclude that the dephosphorylation status of certain proteins is essential for the biosynthesis and deposition of cell wall components, Ca\(^{2+}\) dynamics and exocytotic activity and that Ca\(^{2+}\) dynamics play a central role in the phosphatase 1- and 2A-modulated series of events.

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