Protoplasma (2008) 233: 39–49
DOI 10.1007/s00709-008-0310-2

PROTOPLASMA
Printed in Austria

The block of intracellular calcium release affects the pollen tube development of *Picea wilsonii* by changing the deposition of cell wall components

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Received 4 January 2008; Accepted 23 February 2008; Published online 26 August 2008
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Summary. Two potent drugs, neomycin and TMB-8, which can block intracellular calcium release, were used to investigate their influence on pollen tube growth and cell wall deposition in *Picea wilsonii*. Apart from inhibiting pollen germination and pollen tube growth, the two drugs largely influenced tube morphology. The drugs not only obviously disturbed the generation and maintenance of the tip-localized Ca^{2+} gradient but also led to a heavy accumulation of callose at the tip region of *P. wilsonii* pollen tubes. Fourier transform infrared (FTIR) spectroscopy analysis showed that the deposition of cell wall components, such as carboxylic acid, pectins, and other polysaccharides, in pollen tubes was changed by the two drugs. The results obtained from immunolabeling with different pectin and arabinogalactan protein antibodies agreed well with the FTIR results and further demonstrated that the generation and maintenance of the gradient of cross-linked pectins, as well as the proportional distribution of arabinogalactan proteins in tube cell walls, are essential for pollen tube growth. These results strongly suggest that intracellular calcium release mediates the processes of pollen germination and pollen tube growth in *P. wilsonii* and its inhibition can lead to abnormal growth by disturbing the deposition of cell wall components in pollen tube tips.

Keywords: Cell wall; Intracellular calcium release; *Picea wilsonii*; Pollen tube.

Introduction

The pollen tube has the essential task of delivering the sperm nuclei to the ovules in the sexual reproduction of higher plants. As a typical tip-growing system, its polarized growth involves a variety of essential cellular processes, including the exocytic delivery of materials to the extending apex and distinct localized apical vesicle fusion (Cheung et al. 2002, Parton et al. 2003), the biosynthesis and precise organization of the various wall components (Li et al. 2002), and subtle regulation of calcium dynamics (Feijo et al. 1995). Therefore, pollen tubes of various species have served as model systems for studying tip growth (Cai et al. 1996), intracellular transport (Geitmann et al. 1996), and the dynamics of secretion (Ibrahim et al. 2002) as well as signal transduction (Kaothien et al. 2002). There are many characteristics of pollen tube growth which differ between angiosperms and gymnosperms (Taylor and Hepler 1997, Derksen et al. 1999b, Lazzaro et al. 2005, Wang et al. 2005). In general, germination and growth of gymnosperm pollen proceed slowly, and the tubes tend to ramify and lack a tip-to-base zonation of organelles (de Win et al. 1996, Mogami et al. 1999, Wang et al. 2005). The gap between pollination and fertilization is also much longer in gymnosperms than that in angiosperms (Pettitt 1985). There is a significant body of evidence showing that the synthesis of polysaccharides and proteins (Fidlerova et al. 2001, Li et al. 2002), as well as the tip-focused cytoplasmic calcium gradient (Franklin-Tong 1999, Coelho and Malhó 2006), are essential for pollen tube development. However, most of this knowledge is based on studies of angiosperm species and little information from coniferous species is available. Thus, more attention should be paid to the mechanisms underlying pollen tube growth in gymnosperms.

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The presence of a tip-focused Ca^{2+} gradient is a general characteristic of polarized cell growth in all kingdoms, from bacteria to animals. There is evidence to support the hypothesis that the generation and maintenance of the Ca^{2+} gradient during growth in plants and fungi is closely related to the phosphoinositide signaling in root hairs (Felle and Hepler 1997), fungal tips (Silverman-Gavrila and Lew 2002), and pollen tubes (Franklin-Tong et al. 1996, Franklin-Tong 1999, Lazzaro et al. 2005, Monteiro et al. 2005). In addition, the inositol 1,4,5-trisphosphate (IP_3)-stimulated release of Ca^{2+} from the cytoplasm has been suggested to be very important for tip growth of plant cells (Franklin-Tong et al. 1996, Felle and Hepler 1997, Silverman-Gavrila and Lew 2002, Monteiro et al. 2005). Neomycin and 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) are two potent drugs for inhibiting intracellular Ca^{2+} release (Brummell and Maclachlan 1989, Franklin-Tong et al. 1996, Silverman-Gavrila and Lew 2002). TMB-8 inhibits the IP_3-activated large conductance Ca^{2+} channel as an intracellular antagonist of calcium, while neomycin blocks the tip-localized calcium gradient as a specific inhibitor of the activity of phospholipase C (Silverman-Gavrila and Lew 2002). On the basis of the specific inhibitory effects of these drugs, some results have been obtained which clarify the mechanisms regulating polarized ion gradients and fluxes, turnover of cytoskeletal elements, and exocytosis and endocytosis of membrane vesicles (Franklin-Tong et al. 1996, Franklin-Tong 1999, Hepler et al. 2001, Silverman-Gavrila and Lew 2002, Monteiro et al. 2005).

The cell wall is one of the key structural players regulating plant cell growth. In general, plant cell growth is driven by internal turgor pressure and restricted by the ability of the cell wall to extend under this pressure. The equilibrium between turgor and cell wall determines the rate of tip growth in cells exhibiting polar elongation. In the pollen tube, polarized growth requires the synthesis and deposition of a cell wall that is rigid enough to withstand substantial internal turgor pressure, yet flexible enough to permit the cell to grow (Hepler et al. 2001). Therefore, the production of the cell wall, its composition, and the configuration of its components are important features regulating pollen tube growth (Taylor and Hepler 1997; Hepler et al. 2001; Parre and Geitmann 2005a, b). It has been found that the wall components at the extreme apex of pollen tubes are secreted largely as esterified pectin residues and the resulting carboxyl groups are targets for cross-linking by Ca^{2+}. The gradient in cell wall composition from esterified to de-esterified pectins is correlated with an increase in the degree of cell wall rigidity or a decrease in extensibility (Geitmann 1999). Cellulose and callose are also present, but both are usually several micrometers behind the tip, suggesting that they are not taking part in the extension process per se (Heslop-Harrison 1987). There have been several investigations of the development of pollen tubes in conifers (Pettitt 1985; Derksen et al. 1999a; Lazzaro et al. 2003, 2005; Wang et al. 2003; Justus et al. 2004; Hao et al. 2005; Chen et al. 2006; Sheng et al. 2006; Wang et al. 2006), although the relation between intracellular Ca^{2+} release and cell wall deposition remained to be investigated.

Here, we present a study of the deposition of cell wall components during pollen tube growth of a gymnosperm species, *Picea wilsonii*, under inhibition of intracellular Ca^{2+} release. Two potent drugs, neomycin and TMB-8, were used to elucidate the role of the Ca^{2+} release system in the regulation of pollen tube development with emphasis on the deposition of cell wall components, such as callose, pectin, and other polysaccharides.

Material and methods

Plant materials and pollen tube growth conditions

Pollen grains were collected from *Picea wilsonii* (Mast) trees growing in the Botanical Garden of the Institute of Botany, Chinese Academy of Sciences on 19 April 2003 and stored at −20 °C. Pollen was germinated and grown in a basal medium containing 0.01% H_3BO_3, 0.03% $Ca(NO_3)_2$, 12% sucrose, and citrate-phosphate buffer (pH 5.8). The density of pollen grains was 10 mg per Erlenmeyer flask containing 10 ml of liquid medium, and this density can support a high frequency of pollen germination. All cultures were maintained at 25 °C on a shaker (100 rpm). After 12 h in culture, the germinated pollens (about 70% showed tube emergence and 50% were considered to be germinated) were treated with 0, 1.0, 5.0, 10.0, 20.0, 50.0, and 100.0 μM neomycin or 0, 10, 50, 100, 150, 200, and 250 μM TMB-8 (obtained from Sigma and dissolved in distilled water) for 18 h. Pollen tubes cultured in standard medium were used as a control. Pollen tubes treated with inhibitors for 8 h were used for the analysis of pollen tube growth rates and cytochemistry. For the germination rate assay, the inhibitors were added to the medium at the start of culture and the data were collected after 18 h.

Pollen tube growth determination and morphological observation

More than 200 pollen tubes from three independent experiments were measured for mean tube growth rates and a total of at least 1000 grains were used for calculating the germination rates. Pollen grains were considered germinated only when the tube length was longer than the diameter of the pollen grain. Germinated tubes and their morphology were viewed under a Zeiss Q500 IW light microscope and digital images were captured with a Spot Π camera (Diagnostic Instruments Inc.).

Confocal laser scanning microscopy imaging

Cytosolic Ca^{2+} was measured with the Ca^{2+}-sensitive fluorescent dye Fluo 3-AM ester (4-(6-acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4′-methyl-2,2′-(ethylenedioxy)dianiline-N,N,N′,N′-tetraacetic acid tetrakis (acetomethyl) ester; Sigma, St. Louis, Mo., U.S.A.), which entered the

cells via a nondisruptive route (Digonnet et al. 1997). Pollen tubes were incubated at 0–4 °C for 2 h in the dark in standard culture medium with a final concentration of 20 μM Fluo 3-AM ester added from a 1 mM stock solution prepared in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the incubation solution was approximately 0.1% (v/v). After rinsing three times in phosphate-buffered saline (PBS) (pH 7.2) and incubating in medium containing TMB-8 or neomycin for 1 h at 25 °C in the dark, the samples were examined using a Zeiss LSM 510 META confocal laser scanning microscope (CLSM) with an Alexa 488 filter set (the samples were excited at 488 nm with a 40 mW argon laser and emission signals were collected at 515 nm). Optical serial sections were collected at 1 μm intervals at a thickness of 0.5 μm. All images were projected along the z-axis.

Fourier transform infrared microspectroscopy

Pollen tubes were fully washed six times with deionized water and then dried in a layer on a barium fluoride window (13 mm diameter, 2 mm thickness). Fourier transform infrared (FTIR) spectra were recorded with a PerkinElmer Cetus Magna 750 FTIR spectrometer (Nicolet Corp., Tokyo, Japan) equipped with a mercury-cadmium-telluride detector, and a PerkinElmer Cetus microscope interfaced with a personal computer. An area of approximately 100 by 100 μm was selected for FTIR analysis. The acquisition parameters were 8 cm^{-1} resolution and 128 co-added interferograms, normalized to obtain relative absorbance.

Aniline blue staining of callose

Pollen tubes fixed in 3% freshly prepared formaldehyde in 0.1 M PBS buffer (pH 7.2) for 30 min were incubated with 0.01% decolorized aniline blue in 0.1 M PBS buffer (pH 8.2) for approximately 5 min, and immediately mounted and photographed with the laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Federal Republic of Germany). The samples were excited at 488 nm with a 25 mW argon ion laser, and emitted at 515 nm. To decrease unspecific staining by aniline blue, toluidine blue O (0.5% in 0.1 M PBS buffer, pH 7.0) was used after aniline blue staining.

Immunolabeling of pectins and arabinogalactan proteins

Pollen tubes were fixed in 3% freshly prepared paraformaldehyde in PME buffer (50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.2, 1 mM EGTA, 0.5 mM $MgCl_2$) for 2 h, and rinsed three times for 5 min each with PME buffer and once in PBS (pH 7.2). Subsequently, the samples were incubated with the following primary antibodies: JIM5 and JIM7 monoclonal antibodies diluted 1 : 20 (w/v), LM2, LM5, LM6, and LM7 monoclonal antibodies diluted 1 : 10 (w/v), and RGII polyclonal antibody diluted 1 : 50 (w/v). All primary antibodies were diluted in PBS supplemented with 1% (w/v) bovine serum albumin, and sections were incubated for 2 h at room temperature. After rinsing in PBS three times (5 min each), samples were incubated for 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-rat immunoglobulin G (ICN, ImmunoBiologicals, Irvine, Calif., U.S.A.) diluted 1 : 100 (w/v) in PBS containing 1% (w/v) bovine serum albumin. After a further rinse series in PBS, the samples were mounted on slides and observed and photographed with the laser scanning confocal microscope (LSM 510 Meta, Zeiss) with excitation at 488 nm and emission at 522 nm.

Results

Pollen tube growth and morphology

The germination process of *P. wilsonii* pollen grains needed at least 12 h, after which about 50% of the pollen had germinated. The highest growth rate was between 12 and 24 h of culture in controls, and after 36 h the growth had nearly stopped. As shown in Fig. 1, the two drugs, neomycin and TMB-8, inhibited the pollen germination rate and the effect increased with the concentration of the drugs (Fig. 1A, C). The pollen tube growth rate was also inhibited by the two drugs (Fig. 1B, D). The inhibitory effects of neomycin were stronger than those of TMB-8, especially at higher concentrations. This was also observed during kinetic analysis of pollen tube growth in the presence of drugs (Fig. 1E). It was further noticed that neomycin exerted a moderate inhibition of tube growth at a concentration of around 5 μM, while TMB-8 showed the same effect at 150 μM. In addition, the germination process was more sensitive to the drugs than pollen tube growth. When incubated in the presence of 10 μM neomycin or TMB-8, only about 6.3 or 16.2% of pollen germinated, respectively, whereas the pollen tube growth rate was about 33.1 or 83.9% of those of the respective controls.

Pollen tubes of *P. wilsonii* cultured in standard medium showed straight and smooth polar growth (Fig. 2A, I). In the presence of 10 or 20 μM neomycin, pollen tubes were shorter (Fig. 2B, C) and many of them (about 30%) exhibited obvious abnormalities, including wavy shapes (Fig. 2B), loss of the clear zone at tube tips (Fig. 2C), and even branching and bipolar growth (Fig. 2H). Similarly, treatment with 100 or 200 μM TMB-8 also led to abnormalities in pollen tube growth patterns (Fig. 2D, E). Many germinated pollen grains (about 40%) developed two pollen tubes (Fig. 2G) and showed wavy pollen tube shapes (Fig. 2D, E) and/or swollen pollen tube tips (Fig. 2F).

Intracellular Ca^{2+} distribution

The Ca^{2+}-sensitive fluorescent dye Fluo 3-AM ester is highly lipophilic, thus it easily crosses the plasma membrane by a nondisruptive route. As shown in Fig. 3, pollen tubes loaded with Fluo 3 showed strong intracellular fluorescence under confocal microscopy. Normally growing pollen tubes of *P. wilsonii* displayed a typical tip-focused cytosolic free Ca^{2+} gradient (Fig. 3A, B). In contrast, about 40% of pollen tubes treated with neomycin or TMB-8 emitted faint fluorescence with a relatively low cytosolic Ca^{2+} gradient from the tip to the base of the tube. Moreover, these effects were more pronounced at higher concentrations of both inhibitors (Fig. 3C–J). In the majority (about 60%) of inhibitor-treated tubes, the Ca^{2+} gradient was completely dissipated (data not shown).

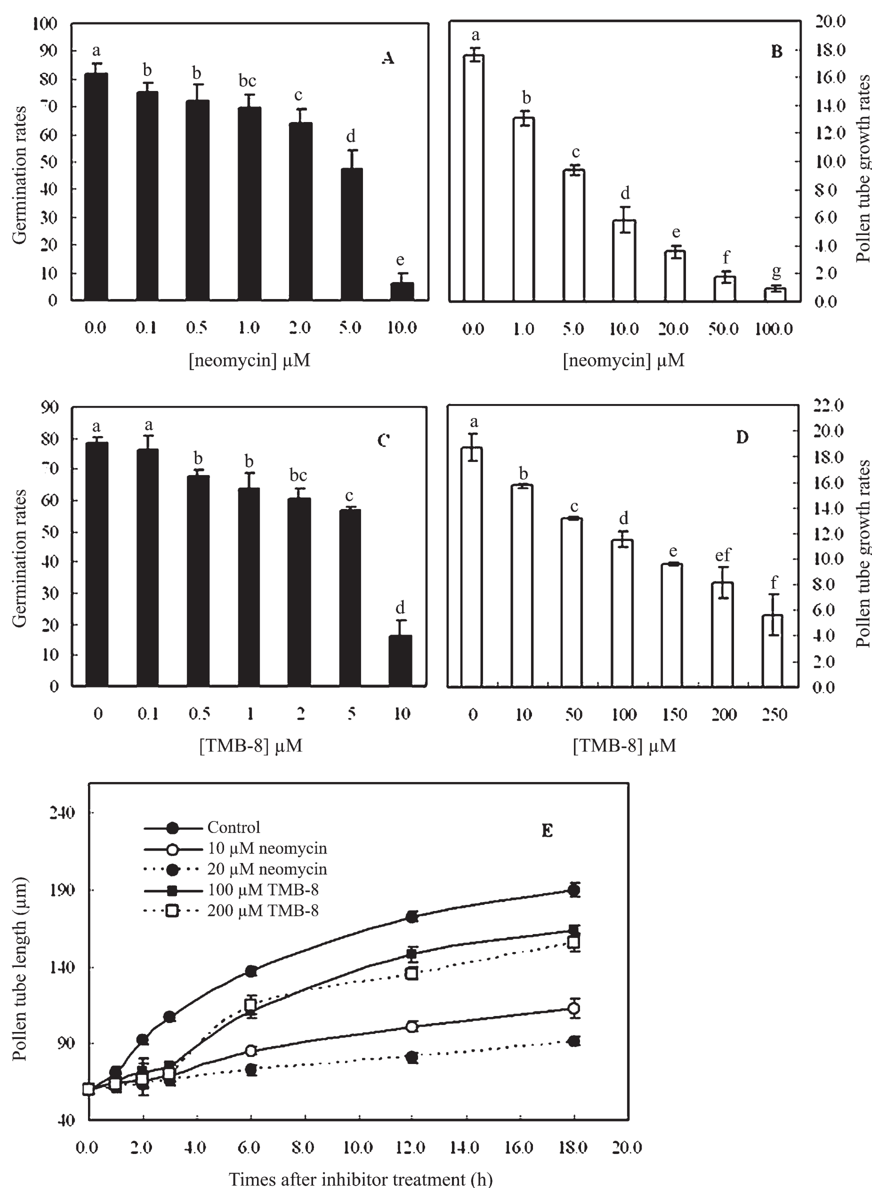

Fig. 1. Effects of neomycin (**A** and **B**) and TMB-8 (**C** and **D**) on pollen germination and pollen tube growth in *P. wilsonii*, and the typical time courses of pollen tube extension growth at different concentrations of neomycin and TMB-8 (**E**). The values are means with standard deviations. More than 200 pollen tubes from three independent experiments were measured to determine the tube growth rates, and a total of at least 1000 grains were used to calculate the germination rates. One-way analysis of variance was used for comparisons between the means. Bars with different letters are significantly different at $P \leq 0.05$

FTIR microspectroscopy

The typical FTIR spectra obtained from the tip region of control and 10 μM neomycin- or 100 μM TMB-8-treated pollen tubes are shown in Fig. 4A. Each spectrum represents a mean value from three independent experiments. Amide-stretching bands of proteins occurred at 1650 and 1550 cm^{-1} (McCann et al. 1994), saturated esters absorbed at 1740 cm^{-1} (McCann et al. 1994), and polysaccharide absorbed at 1200–900 cm^{-1} (Hori and Sugiyama 2003). The lignin and carboxylic acid peaks around 1462, 1425, and 1244 cm^{-1} (Pandey and Pitman 2003) and the cellulose and hemicellulose peaks around 1375 and 1157 cm^{-1} (Pandey and Pitman 2003) were distinctly visible in the control spectrum. However, it was difficult to quantitatively determine the changes in pectins upon treatment with the inhibitors from the original FTIR spectra. To distinguish these changes, difference spectra were generated by digital subtraction of the spectra of the tip regions of 10 μM neomycin- or 100 μM TMB-8-treated pollen tubes from that of control pollen tubes. In the difference spectra for the 10 μM neomycin-treated tubes (Fig. 4B), a distinct pectin peak with a positive value appeared at 1600 cm^{-1} (McCann et al. 1994, Wang et al. 2003), whereas in the difference spectra for the 100 μM TMB-8-treated tubes

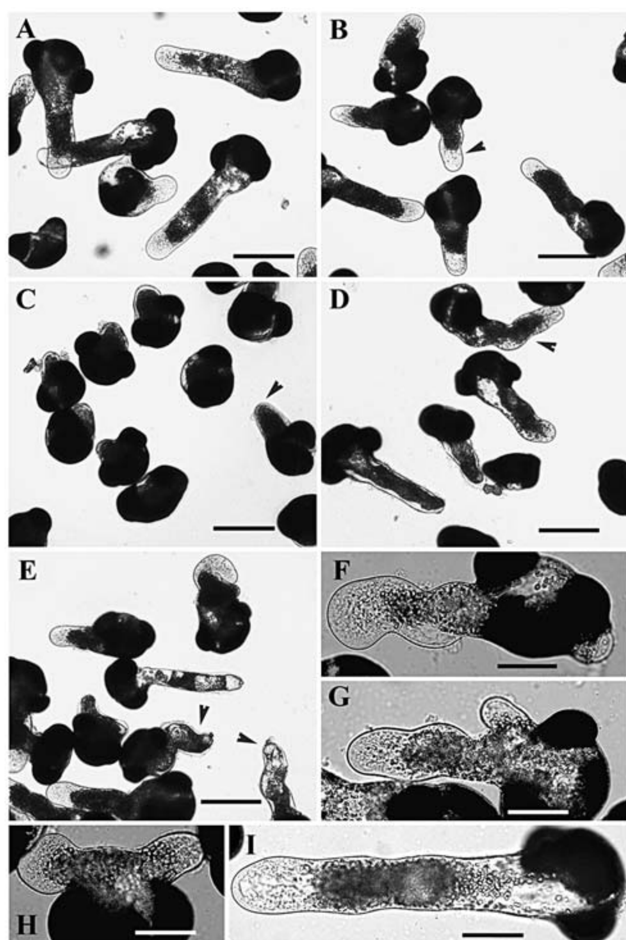

Fig. 2 A–I. Effect of inhibitors of the phosphoinositide signaling pathway on the morphology of *P. wilsonii* pollen tubes after 18 h culture. **A** and **I** Pollen tubes grown in standard medium. **B** Pollen tubes exposed to 10 μM neomycin. **C** and **H** Pollen tubes exposed to 20 μM neomycin. **D** Pollen tubes exposed to 100 μM TMB-8. **E**, **F**, and **G** Pollen tubes exposed to 200 μM TMB-8. Bars: A–E, 100 μm; F–I, 50 μm

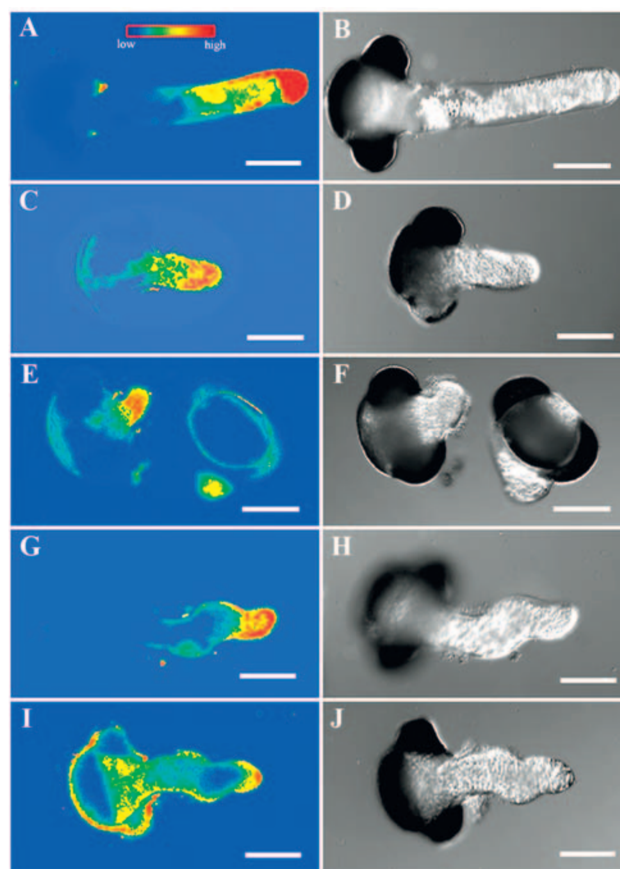

Fig. 3 A–J. Tip-high calcium gradient and effects of neomycin and TMB-8 on it in *P. wilsonii* pollen tubes. Comparison of the fluorescence images with the corresponding bright-field images shows that normally growing pollen tubes displayed a typical tip-focused cytosolic free Ca^{2+} gradient (**A** and **B**), whereas neomycin (**C**–**F**) or TMB-8 (**G**–**J**) disturbed the gradient and the inhibitory effects were increased at higher concentrations of inhibitors. **A** and **B** Controls; **C** and **D** 10 μM neomycin; **E** and **F** 20 μM neomycin; **G** and **H** 100 μM TMB-8; **I** and **J** 200 μM TMB-8. Bars: 50 μm

(Fig. 4 C), a distinct pectin peak with a positive value appeared at 1010 cm^{-1} (McCann et al. 1994, Hori and Sugiyama 2003).

Callose deposition in pollen tube walls

Fluorescence labeling with aniline blue revealed callose distribution at the apex and along the first half behind the tip in control pollen tubes of *P. wilsonii* (Fig. 5). The fluorescence intensity along the longitudinal axis declined gradually from the tip toward the distal end in the tubes cultured in standard medium, but little accumulation of callose was found at the tips of tubes (Fig. 5 A, B). In contrast, in pollen tubes treated with 10 μM neomycin (Fig. 5 C, D) or 100 μM TMB-8 (Fig. 5 E, F), strong fluorescence intensity was present at the tip regions, showing heavy accumulation of callose.

Pectin and AGP distribution in pollen tubes

Fluorescence labeling with JIM5 antibody showed a relatively homogeneous distribution of acidic pectins all over the walls of pollen tubes of *P. wilsonii* (Fig. 6 A–F). The pattern of fluorescence intensity along the longitudinal axis declined gradually from the distal end to the tube tip in both control and drug-treated tubes (Fig. 6 B, C), but the overall intensity of fluorescence was much higher in drug-treated tubes compared with the control (Fig. 6A), especially at distal end regions. Sometimes one of the branched tube tips showed reduced labeling (Fig. 6 C). In contrast, fluorescence associated with JIM7 showing the esterified pectin distribution was pronounced at the very tip region of pollen tubes cultured in standard medium (Fig. 6 G), but in pollen tubes treated with the two drugs,

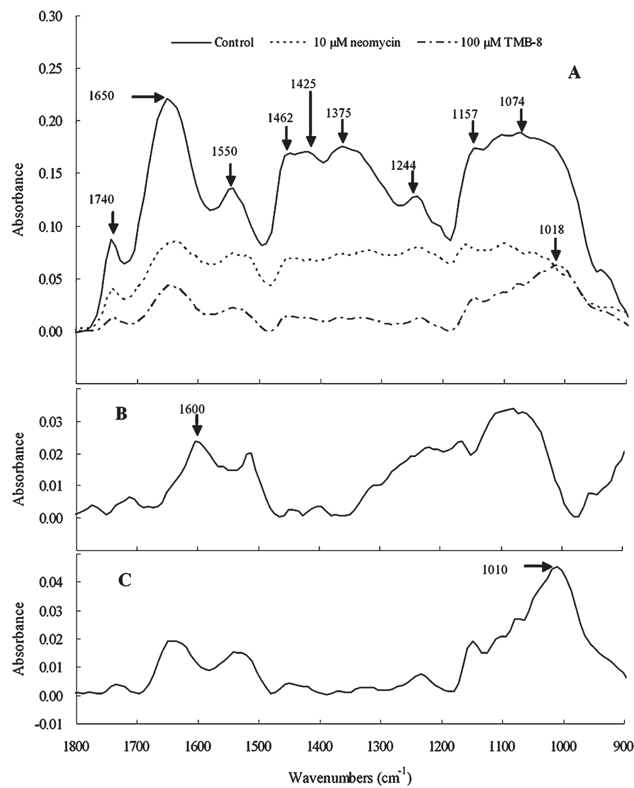

Fig. 4 A–C. FTIR spectra obtained from the tip regions of *P. wilsonii* pollen tubes. **A** FTIR spectra obtained from the tip regions of control and inhibitor-treated pollen tubes. **B** Difference spectra generated by digital subtraction of 10 μM neomycin-treated spectra from the control spectrum. **C** Difference spectra generated by digital subtraction of 100 μM TMB-8-treated spectra from the control spectrum

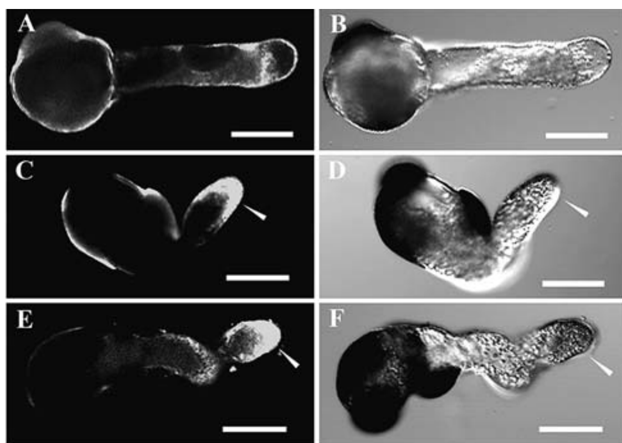

Fig. 5 A–F. Fluorescence images of pollen tubes of *P. wilsonii* cultured for 18 h and stained with decolorized aniline blue, showing callose distribution. Comparison of the fluorescence images (**A**, **C**, and **E**) with the corresponding bright-field images (**B**, **D**, and **F**), respectively, shows that the fluorescence intensity along the longitudinal axis declined gradually from the tip toward the distal end in the tubes cultured in standard medium and little accumulation of callose was found in the tips of tubes (**A**), whereas in pollen tubes treated with 10 μM neomycin (**C**) or 100 μM TMB-8 (**E**), strong fluorescence intensity was predominantly present in the tip regions, showing heavy accumulation of callose (indicated by arrowheads). Bars: 50 μm

neomycin and TMB-8, strong fluorescence was observed mainly in the walls near the distal end (Fig. 6H, I). Similarly, fluorescence labeling associated with the arabinogalactan protein (AGP) epitope LM2 showed distribution all along the cell walls of pollen tubes cultured in standard medium (Fig. 7A) but was observed only in the walls near the distal end regions of pollen tubes treated with either drug (Fig. 6B, C). The pectin antibody LM6, which recognizes (1→5)-α-L-arabinan, a structural feature of the side chains of pectins (Willats et al. 1998), showed distribution patterns in the control and inhibitor-treated pollen tubes (Fig. 7M–R) similar to those described for LM2. Finally, pollen tubes labeling with LM5, a neoglycoprotein antibody which recognizes a linear tetrasaccharide in (1→4)-β-D-galactans of pectin polysaccharides (Jones et al. 1997), showed fluorescence only in the walls of very distal end regions in control and drug-treated tubes (Fig. 7G–L).

Discussion

There are a number of factors, including temperature, medium osmolarity, ion gradients and fluxes, membrane trafficking, and phosphoinositide and other signaling components, involved in pollen tube development (Feijo et al. 1995, Taylor and Hepler 1997, Franklin-Tong 1999, Hepler et al. 2001, Monteiro et al. 2005). In general, the generation and maintenance of a tip-focused gradient of cytosolic Ca^{2+} are fundamental for pollen tube growth, and a functional phosphoinositide signal-transducing system involving IP_3-stimulated intracellular Ca^{2+} release plays an essential role in both these processes (Franklin-Tong et al. 1996, Franklin-Tong 1999, Hepler et al. 2001, Monteiro et al. 2005). Due to its ease of use, the Ca^{2+}-sensitive fluorescent dye Fluo 3-AM ester method has been widely used to evaluate the cytosolic free calcium gradient in plants (Digonnet et al. 1997, Zhang et al. 1998, Kong et al. 2006), even though the esterified versions of Ca^{2+} dyes are known to produce significant artifacts because this kind of dye is easily decomposed by esterases in the cell walls during the loading process. As in angiosperm species, a tip-localized high Ca^{2+} gradient was observed in pollen tubes of *P. wilsonii*. The two drugs neomycin and TMB-8, which block intracellular Ca^{2+} release in the polarized growth of plants by inhibiting tip-localized phospholipase C activity and the IP_3-activated large conductance Ca^{2+} channel, respectively (Franklin-Tong et al. 1996, Hepler et al. 2001, Silverman-Gavrila and Lew 2002), strongly influenced pollen tube development and maintenance of the Ca^{2+} gradient. The in-

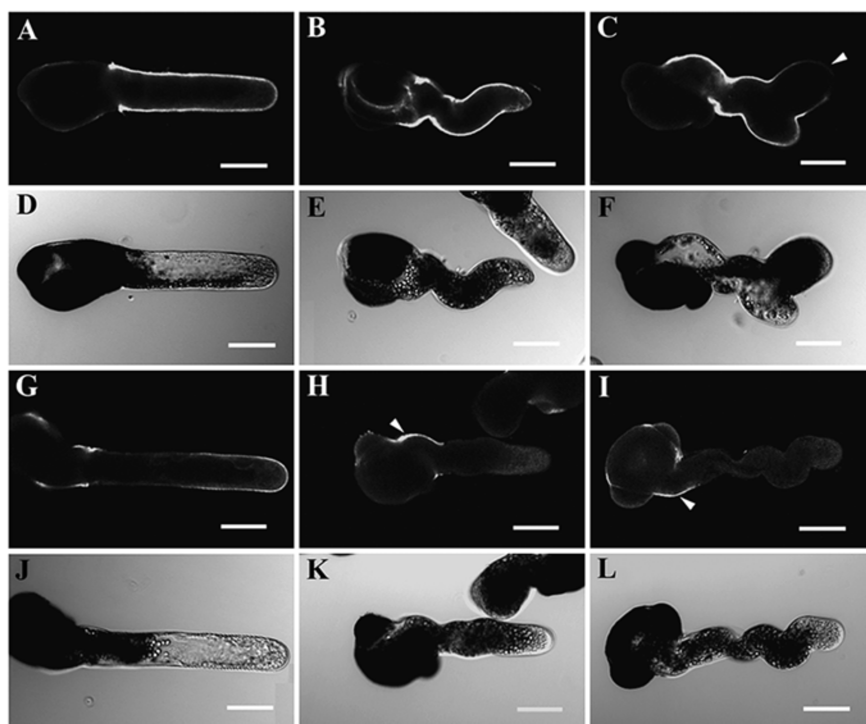

Fig. 6 A–L. Immunolabeling of pollen tubes of *P. wilsonii* with JIM5 and JIM7, which recognize pectin epitopes. **A–C** Labeling with JIM5 with the corresponding bright-field images (**D–F**), showing the distribution of acidic pectins all over the walls of pollen tubes. The fluorescence intensity along the longitudinal axis declined gradually from the distal end to the tube tip, and the tubes treated with 10 μM neomycin (**B** and **E**) or 100 μM TMB-8 (**C** and **F**) showed greater fluorescence intensity compared with the controls (**A** and **D**). **G–I** Labeling with JIM7 with the corresponding bright-field images (**J–L**), showing the methyl-esterified pectins in the walls of the very tip of pollen tubes under control conditions (**G** and **J**), but mainly in the walls near the distal end regions (indicated by arrowheads) in 10 μM neomycin- (**H** and **K**) or 100 μM TMB-8-treated (**I** and **L**) tubes. Bars: 50 μm

hibitory effects, including the decreased germination and tube growth rates, the abnormal tube morphogenesis, and the weakened tip-high Ca^{2+} gradient, which have been found in most angiosperm pollen tubes under inhibitor treatment or stressed conditions (Li et al. 1999, Lalanne et al. 2004), were also observed in *P. wilsonii* pollen tubes. In our previous study, we observed a similar phenomenon and external Ca^{2+} addition did not disturb the tip-localized Ca^{2+} gradient in the pollen tubes of *P. wilsonii* (Kong et al. 2006). These results indicate that the intracellular Ca^{2+} release is important for the pollen tube growth of *P. wilsonii* and its inhibition can lead to disordered development of tubes, presumably by affecting the free Ca^{2+} gradient of tube tips.

The cell wall has a highly complex structure, composed of polysaccharides, structural proteins, and enzymes. Unlike other higher-plant cell walls, the cell wall of pollen tubes consists mainly of callose (β-1,3-glucan, a polysaccharide), pectins, and a small amount of cellulose, and their continued incorporation during extension is quite important for tube growth (for review, see Taylor and Hepler 1997). As a powerful, noninvasive technique for the quantitative assessment of a variety of functional groups, FTIR analysis has been widely used for the quick evaluation of the chemical composition of cell walls in plants (Chen et al. 1998, Coimbra et al. 1998, Hori and Sugiyama 2003). On the basis of FTIR spectroscopy, Pappas et al. (2003) developed a new methodology for the identification of pollen. Wang et al. (2003) revealed that boron deficiency caused changes in the concentrations and distributions of acidic pectin, phenolics, and saturated esters in the pollen tube of *Picea meyeri*. In the present study, we found that the distinct peaks assigned to saturated esters (the peak at 1740 cm^{-1}), proteins (the peaks at 1650 and 1550 cm^{-1}), lignin and carboxylic acid (the peaks at 1642, 1425, and 1244 cm^{-1}), cellulose and hemicellulose (the peaks around 1375 and 1157 cm^{-1}), and other polysaccharides (the peaks at 1200–900 cm^{-1}) were present in the control pollen tube spectrum, but the peaks assigned to lignin and carboxylic acid, cellulose, and polysaccharides were relatively faint in spectra of pollen tubes treated with either drug. Distinct peaks assigned to pectins could be observed in the difference spectra obtained by digital subtraction of the spectra of pollen tubes treated with either drug from that of control pollen tubes, indicating that there are less pectins in drug-treated pollen tube tips compared with controls. These data suggest that the block of intracellular Ca^{2+} release significantly influences the deposition of cell wall components, such as carboxylic acid, pectins, and cellulose, into pollen tube cell walls of *P. wilsonii*.

As an important polysaccharide component, callose is well known as a permeation barrier and leak sealant in plant cells. In pollen tubes, it has been reported that the

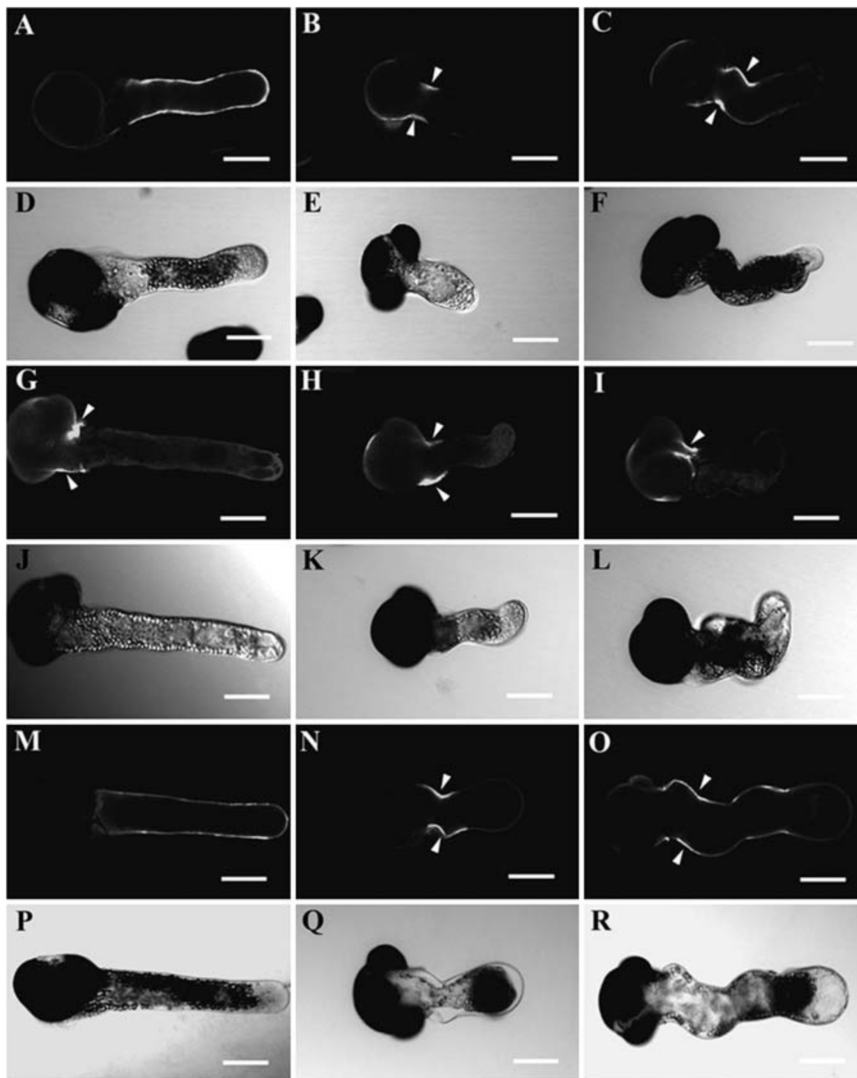

Fig. 7 A–R. Immunolabeling of pollen tubes of *P. wilsonii* with LM2, LM5, and LM6, which recognize epitopes of AGPs and pectins. **A–C** Labeling with LM2 with the corresponding bright-field images (**D–F**), showing the distribution of an AGP. Bright immunofluorescence was present throughout the walls of pollen tubes grown under control conditions (**A** and **D**), whereas bright immunofluorescence was only present in the distal end region (indicated by arrowheads) and had vanished from the tip region of pollen tubes treated with 10 μM neomycin (**B** and **E**) or 100 μM TMB-8 (**C** and **F**). **G–I** Labeling with LM5 with the corresponding bright-field images (**J–L**), a neoglycoprotein antibody, showing the distribution of (1→4)-β-D-galactans of pectins only in the walls of very distal end regions (indicated by arrowheads) in control (**G** and **J**) and 10 μM neomycin- (**H** and **K**) or 100 μM TMB-8-treated (**I** and **L**) tubes. **M–O** Labeling with LM6 with the corresponding bright-field images (**P–R**), another pectin antibody, showing the distribution of (1→5)-α-L-arabinan of pectins in pollen tubes. Immunofluorescence was also present throughout the walls of control pollen tubes (**M** and **P**) but only in the distal end region (indicated by arrowheads) and not in the tip region of 10 μM neomycin- (**N** and **Q**) or 100 μM TMB-8-treated (**O** and **R**) tubes. Bars: 50 μm

massive accumulation of callose in the 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). The accumulation of callose in plant cells might also be a drug-induced response which can increase the resistance of pollen tubes to tension and compression stress (Parre and Geitmann 2005a). In *Solanum chacoense* pollen tubes, callose is mainly distributed in the distal parts and is completely absent from the tube tips (Parre and Geitmann 2005b). For *Pinus sylvestris*, Derksen et al. (1999a) found that callose is present in the tip and the younger parts of pollen tubes and then ultimately disappears from the tubes. In the present work, however, we found that callose declined gradually along the longitudinal axis from the tip toward the distal end in the *P. wilsonii* pollen tubes. This is consistent with findings from our previous study (Kong et al. 2006). The differentce in the distribution of callose in pollen tubes between conifer species and angiosperms is an interesting phenomenon that may provide some information related to the low tube growth rate in conifers, since callose occurs in exceptionally large amounts in pollen and has a mechanical role in growing plant cells (Parre and Geitmann 2005a). In the present study, we also found that a heavy deposition of callose presented in the apical region of tubes treated with two Ca^{2+} release inhibitors, indicating that the synthesis and distribution of callose is likely to be spatially and developmentally mediated by a Ca^{2+} signaling system. The block of intracellular Ca^{2+} release can significantly influence the tube growth by disturbing the metabolism of callose in the tips of *P. wilsonii* pollen tubes.

Pectins are the main cell wall component of the growing pollen tubes and they are synthesized in the Golgi, methyl-

esterified and modified with side chains, and subsequently released into the apoplastic space as highly methyl-esterified polymers (Micheli 2001, Parre and Geitmann 2005b). There are different kinds of pectins in plant cell walls (Ridley et al. 2001) and the recycling of cell wall pectins is essential for their proper assembly (Baluška et al. 2002, Yu et al. 2002, Šamaj et al. 2004). Acidic pectins (JIM5-reactive pectins) are biochemically produced from esterified pectins (JIM7-reactive pectins). This dynamic transformation is catalyzed by pectin methylesterases which are regulated by the local pH and Ca^{2+} concentration (Micheli 2001, Li et al. 2002). Additionally, acidic pectins can cross-link through their carboxyl group and form egg-carton patterns to increase the rigidity of the cell wall, while esterified pectins are responsible for the elastic behavior of the cell (Franklin-Tong 1999, Ridley et al. 2001, Baluška et al. 2002). In *S. chacoense* pollen tubes, Parre and Geitmann (2005b) found that the total pectin concentration was higher at the apex than at the distal regions of pollen tubes. The amount of acidic pectin was almost the same at the apex and the distal regions, while the amount of methyl-esterified pectin was obviously higher at the apex regions. In the present study, using immunolabeling with JIM5 and JIM7, we found that the amount of acidic pectins seemed to decline gradually along the longitudinal axis from the distal end toward the tube tip, whereas that of methyl-esterified pectins was higher in the tube tip than in the distal end region in *P. wilsonii* pollen tubes grown under control conditions. This is consistent with the finding that the gradient in cell wall composition from apical esterified to distal de-esterified pectins, which correlates with an increase in the degree of cell wall rigidity and a decrease in viscoelasticity, is involved in pollen tube growth and architecture (Parre and Geitmann 2005b). However, for *Pinus sylvestris* pollen tubes, Derksen et al. (1999a) found that esterified pectins are only present in the tip and younger parts of tubes and acid pectins can be detected both in the tip and tubes. Here, compared to the respective controls, the distribution of acidic pectins was not changed in pollen tubes treated with the two inhibitors of the intracellular Ca^{2+} release pathway, whereas methyl-esterified pectins were present in large amounts in the distal end region but had vanished from the tip region of the inhibitor-treated pollen tubes, indicating that the intracellular Ca^{2+} release system mediates the generation and maintenance of the pectin gradient and its inhibition influences the transportation of pectins from the cytoplasm to the cell walls of pollen tube tips.

The results obtained from immunolabeling with another pectin antibody, LM6, also confirmed that the generation and maintenance of the pectin gradient depends on the intracellular Ca^{2+} release system. Moreover, a similar gradient was observed with the LM2 antibody, which recognizes an AGP epitope. AGPs, a type of cell wall glycoprotein, are important contributors to the formation and structure of the complex extracellular matrix and to the cell as whole. Given their extraordinary polysaccharide content (sometimes up to 95% of the protein molecular mass) and their adhesive properties, AGPs are also candidate molecules for providing nutrients, adhesive support, or lubrication for the pollen tube growth process and may mediate specificity in cell–cell interaction (Lord 2000, Hepler et al. 2001). It has been found that the transmitting tissue-specific AGPs can display a gradient of increasing glycosylation along the length of the style, coincident with the direction of pollen tube elongation, and therefore, they are believed to promote pollen tube growth by serving as a nutrient and attracting pollen tubes by providing chemical and physical guidance cues within the style (Hepler et al. 2001). In the present study, we found that both LM2 and LM6 labeling presented bright fluorescence along the walls of pollen tubes grown under control conditions, whereas this was present only in the distal end region and had vanished from the tip region of pollen tubes grown under drug treatment conditions. In a previous study, two AGP epitopes, including LM2, were localized, implying an important role in the tip growth of maize root hairs (Šamaj et al. 1999). These results indicate that a proportional distribution of AGPs and pectins in cell walls is necessary for the normal growth of pollen tubes in *P. wilsonii* and that the blocking of the intracellular Ca^{2+} release pathway can disrupt the development of pollen tube walls by influencing the transport of AGPs from the endomembrane system represented by the endoplasmic reticulum and Golgi apparatus (Šamaj et al. 2000) to the cell walls of tube tips. This implies that the incorporation of cell wall constituents might be affected by blocking the intracellular Ca^{2+} release pathway. The previous findings of Brummell and Maclachlan (1989) support our conclusion. Using pea seedlings, they found that TMB-8 can abolish about 60% of the total incorporation of glucose into the cell walls in the presence of auxin, and thus they suggested that TMB-8 can inhibit cell wall formation and that a Ca^{2+} signaling system is involved in the promotive actions of auxin on cell wall synthesis and long-term growth.

In contrast, using immunolabeling with LM5, a neoglycoprotein antibody which labels a linear tetrasaccharide in $(1\rightarrow4)$-β-D-galactans of pectin polysaccharides (Baluška et al. 2002), we found that this type of pectin might be not

essential for pollen tube growth in *P. wilsonii*. This is consistent with the concept that differences exist in the pectic epitopes in different cell and tissue types (Ridley et al. 2001). Results obtained from pollen tubes labeled with two other pectic polysaccharide antibodies, LM7 and RGII, which label the non-blockwise de-esterified homogalacturonan domain and the rhamnogalacturonan II dimer cross-linked with boron, respectively (Baluška et al. 2002), also support this hypothesis, since no immunofluorescence in the walls of tubes was observed in both inhibitor-treated and control tubes (data not shown). Although the (1→4)-β-D-galactans and non-blockwise de-esterified homogalacturonan and RGII cross-linked pectins are predominantly located in the cell walls of maize or wheat roots (Baluška et al. 2002, Yu et al. 2002) and play a role in the development and regulation of many plant cells and tissues (Ridley et al. 2001), data reported here suggest that they might not be involved in the pollen tube growth of *P. wilsonii*.

In summary, the intracellular Ca^{2+} release system mediates pollen germination and pollen tube growth in *P. wilsonii*, and its inhibition can lead to abnormal growth by disturbing the tip-focused Ca^{2+} gradient and changing the deposition of cell wall components at pollen tube tips. The generation and maintenance of the gradient in cell wall composition from apical esterified to distal de-esterified pectins, as well as the secretion of AGP cross-linked pectins from the cytoplasm to the cell wall spaces, are essential for *P. wilsonii* pollen tube growth. The differential expression of pectin epitopes may also play an important role in the tube development.

Acknowledgments

This work was supported by grants from the Ministry of Science and Technology of China (2006CB910600) and a key grant from the National Natural Science Foundation of China (30570100), and by grants from the EU Research Training Network TIPNET (project HPRN-CT-2002-00265) obtained from Brussels, Belgium, and from the Deutsches Zentrum für Luft- und Raumfahrt (DLR, Bonn, Federal Republic of Germany). J.Š. and F.B. are supported by the Grant Agencies APVT (grant nr. APVT-51-002302) and Vega (grant nr. 2031), Bratislava, Slovakia. We are very grateful to Shi-Fu Weng, Peking University, for technical assistance with FTIR microspectroscopy.

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