Disruption of Actin Filaments by Latrunculin B Affects Cell Wall Construction in *Picea meyeri* Pollen Tube by Disturbing Vesicle Trafficking

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The involvement of actin filaments (AFs) in vesicle trafficking, cell wall construction and tip growth was investigated during pollen tube development of Picea meyeri. Pollen germination and tube elongation were inhibited in a dose-dependent manner by the latrunculin B (LatB) treatment. The fine AFs were broken down into disorganized fragments showing a tendency to aggregate. FM4-64 labeling revealed that the dynamic balance of vesicle trafficking was perturbed due to F-actin disruption and the fountain-like cytoplasmic pattern changed into disorganized Brownian movement. The configuration and/or distribution of cell wall components, such as pectins, callose and cellulose, as well as arabinogalactan proteins changed in obvious ways after the LatB application. Fourier transform infrared (FTIR) analysis further established significant changes in the chemical composition of the wall material. Our results indicate that depolymerization of AFs affects the distribution and configuration of cell wall components in Picea meyeri pollen tube by disturbing vesicle trafficking.

Keywords: Actin filaments — Cell wall — *Picea meyeri* — Pollen tube growth — Vesicle trafficking.

Abbreviations: AF, actin filament; DIC, differential interference contrast; DMSO, dimethylsulfoxide; FTIR, Fourier transform infrared; LatB, latrunculin B; LSCM, laser scanning confocal scanning microscopy; TEM, transmission electron microscopy.

Introduction

Pollen tubes are highly elongated cells whose polarized growth permits them to accomplish invasive growth within female gametophytic tissues to deliver the sperm cells for fertilization. This process is relatively fast, and dependent on an intact actin cytoskeleton (Taylor and Hepler 1997, Staiger 2000, Hepler et al. 2001, Vidali et al. 2001). Because the pollen tube grows rapidly and for long distances, there must be an equilibrium among intracellular vesicle trafficking, the dynamic construction of the existing cell wall and the continuous addition of new cell wall material (Taylor and Hepler 1997, Geitmann 1999). The construction and composition of the cell wall and the configuration of the building components are important features that regulate both pollen tube growth and the physiology of the fertilization process.

Pollen tubes are well-established model systems for investigating polarized tip growth when actin filaments (AFs) are the fundamental elements involved in tip extension (Staiger 2000, Hepler et al. 2001). Previous reports have indicated that AFs could be important guiding elements for cytoplasmic streaming and continuous transport of cell wall precursors to the tube tip (Vidali et al. 2001). Disruption of AFs with an actin polymerization inhibitor perturbs actin distribution and vesicle trafficking, and ultimately causes the accumulation of vesicles at the tip (Geitmann et al. 1996, Anderhag et al. 2000). Several cytoskeletal inhibitors (such as cytochalasins B and D, oryzalin, etc.) have been used to investigate the spatial distribution of the cytoskeleton and the regulation of cytoskeleton-dependent organellar movement in relation to elongation of gymnosperm pollen tube (Anderhag et al. 2000, Justus et al. 2004). Nevertheless, the mechanistic understanding of diverse roles of AFs in the tip growth is still unknown. Microtubules, the other important cytoskeletal component, have been assumed to control the positioning of organelles and influence the streaming direction by mediating AF organization (Anderhag et al. 2000, Justus et al. 2004), but their exact functions in tip growth remain largely unclear.

Besides F-actin, the cell wall is another critical structural element essential for the tip growth. The pollen tube wall, which is the product of continuous secretion, exhibits gradients in molecular composition as well as in mechanical properties (Geitmann 1999, Lennon and

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Lord 2000). The elongation of pollen tubes requires the coordination of complex mechanisms, which involve the polarized transport of vesicles along AFs and deposition of new wall/membrane material at the growing tips. Pectins, callose, cellulose and constitutively expressed glycoproteins are the main construction components whose distributions and amounts will eventually determine the mechanical properties and the polarized growth of the pollen tube (Ferguson et al. 1998, Parre and Geitmann 2005b). However, the complex relationship between the organization of AFs and the construction of the pollen tube wall from different components is still unclear. Only a few investigations on the construction of the pollen tube wall have been reported (Derksen et al. 1999, Lennon and Lord 2000), and no study has examined the relationship between these processes in gymnosperms.

Pollen tube development of conifers differs from that of their counterparts of angiosperms in various ways, such as an extended period of growth, a relatively slow growth rate and an extremely delayed gametogenesis (Fernando et al. 2005, Lazzaro et al. 2005). These differences represent a major evolutionary divergence in the male gametophyte development of flowing plants, and are of prime importance for more in-depth investigation (Lazzaro et al. 2003, Justus et al. 2004). In this study, we tested the hypothesis that AF organization in conifer pollen tubes is essential for the elaborately directed deposition and distribution of pollen tube wall components. Pollen grains were grown in vitro in germination media containing increasing concentrations of the potent actin polymerization inhibitor latrunculin B (LatB). Germination as well as tube length was subsequently examined, and the obtained data were evaluated statistically. The effects of different concentrations of LatB on the organization of AFs and the distribution of pectins, callose, cellulose and arabinogalactan proteins (AGPs) in *Picea meyeri* pollen tubes were investigated.

Results

Pollen tube growth and morphological observations

Pollen started to germinate after a 12h culture in standard medium and reached its maximum germination percentage of 90% after 30h. The highest growth rate was observed between 12 and 24 h after germination initiation in controls. After 30 h, growth nearly stopped. Pollen germination and tube growth were inhibited in a dosedependent manner after the LatB application (Fig. 1). LatB at a concentration of 10 nM had an evident effect on the maximum germination percentage (Fig. 1A). In the presence of 10 nM LatB, the average growth rate of the pollen tubes was $8.2 \,\mu m \, h^{-1}$. When the LatB concentration was increased to 20 nM, the average growth rate dropped to $3.6 \,\mu\text{m}\,\text{h}^{-1}$ in comparison with $13.2 \,\mu\text{m}\,\text{h}^{-1}$ in the control pollen tubes after germination (Fig. 1B). When incubated in the presence of 30 nM LatB, we found that pollen germination was almost entirely blocked. The pharmacological study of LatB at nanomolar concentrations showed that it inhibited the elongation of *P. meyeri* pollen tubes.

Inhibitory effect of LatB on actin polymerization and organization

The organization of AFs was examined under laser scanning confocal microscopy (LSCM) by tetramethylrhodamine isothiocyanate (TRITC)-phalloidin labeling (Fig. 2). In the control cells, AFs were distributed throughout the whole pollen tube in a net axial array



Fig. 1 Effect of LatB on pollen germination and tube growth in *Picea meyeri*. Pollen germination and tube elongation were inhibited in a dose-dependent manner. (A) Typical serial course of pollen germination in the presence of different concentrations of LatB. Pollen germination was inhibited by LatB at nanomolar concentrations in a dose-dependent manner and was half-maximally inhibited at 20 nM. (B) Typical time course of mean pollen tube length in the presence of different concentrations of LatB. Pollen was also inhibited by LatB treatment in a dose-dependent manner, and almost completely inhibited by 30 nM LatB. Values are means ± SD.





Fig. 2 Distribution of F-actin and microtubules in pollen tubes of *Picea meyeri* after LatB treatment. (A) A pollen tube cultured in the standard medium for 20 h showing F-actin bundles distributed throughout the whole pollen tube in a net axial array mainly parallel to the direction of elongation, but only disorganized F-actin filaments could be found at the tip. (B) A pollen tube cultured in the presence of 10 nM LatB for 20 h. Obvious twisted actin cables can be observed in the cortical region of the pollen tube. Some filaments throughout the grain and tube began to break. (C) A pollen tube cultured in medium containing 15 nM LatB for 20 h showing severe disruption of actin filaments. The short fragments of actin filaments were scattered throughout the whole tube. Some actin fragments tended to aggregate into clusters in the tube subapex. (D) A pollen tube cultured in the presence of 20 nM LatB for 20 h. The microfilament network was completely disrupted. (E) Microtubules were found throughout the tube primarily in the cell cortex as a dense network in the control cell. (F) A pollen tube cultured in the medium containing 15 nM LatB for 20 h showing and polymerization. (A–D) The bar represents 50 µm. (E, F) The bar represents 20 µm. All figures were projected along the *z*-axis from 20–30 optical serial sections.

mainly parallel to the direction of elongation. AFs formed a continuous network initiated from the grain to the tube, but only disorganized fragments could be found in the elongating tip (Fig. 2A). We did not detect a distinguishable border between these two polymeric forms of AFs. AFs were also disrupted in a dose-dependent manner by LatB. Obvious twisted actin cables were observed in the pollen tubes cultured in medium containing 10 nM LatB. Some of the filaments throughout the grain and tube began to break (Fig. 2B). The depolymerization of F-actin was more severe with the 15 nM LatB treatment (Fig. 2C). At 20 nM LatB, we found that the fine organization of the AFs was completely disrupted and that short disorganized actin fragments were scattered along the whole length of the pollen tube instead of the axial-oriented AFs being mainly parallel to the long axis (Fig. 2D). In order to show that the cytological changes were directly due to inhibition of AF polymerization, changes in the distribution/configuration of AFs and cell wall components were examined after increasing the osmoticum and treatment with brefeldin A (Supplementary Figs. S1, S2). The results from the two control experiments demonstrated that the changes in cell wall construction after LatB treatment resulted directly from AF disorganization.

FM4-64 staining for vesicle trafficking dynamics

The uptake of FM4-64 into *P. meyeri* pollen tubes followed a strict time course (Fig. 3A). Fluorescent staining associated with the plasma membrane was immediately detected after the dye application, while the subsequent dye internalization could only be discerned after about 7 min. Bright near-spherical structures could be observed in the apical and subapical region when dye uptake began. The characteristic FM4-64 staining became apparent



Fig. 3 FM4-64 uptake time course in *Picea meyeri* pollen tube. (A) Characteristic pattern of FM4-64 staining in a normally growing pollen tube of *P. meyeri*. Uptake of FM4-64 into the pollen tube followed a strict time course and dye uptake took place along the apex and the subapical region (as indicated by asterisks; arrows indicate the direction of dye distribution). The corresponding bright field image shows the exact cytoplasmic organization of the pollen tube. (B) FM4-64 staining of a 10 nM LatB-treated pollen tube. FM4-64 internalization took place almost along the whole length of the tube. Dye uptake followed an irregular sequence and finally reached saturated status after about 9 min. Dye fluorescence was distributed unevenly and showed no characteristic pattern in the end. (C) FM4-64 staining of a 20 nM LatB-treated pollen tube, showing evenly distributed fluorescence. Dye uptake was detected along the apex and the subapical region (as indicated by asterisks), but was greatly decreased in number and intensity. No distinct direction of dye redistribution was observed during the uptake course. (D) At approximately 15 min after dye loading, the dye uptake reached saturated status in control cells. A reverse V-like staining pattern appeared. (E) At approximately 8 min after dye loading, the dye uptake reached saturated status in 20 nM LatB-treated cells. The dye was evenly distributed in the whole tube in the end. The bar represents 50 µm.

and reached a stable state as a reverse V-like staining pattern in the cytoplasm after 10–15 min (Fig. 3D).

In contrast, the FM4-64 labeling of LatB-treated pollen tubes produced a completely distinct staining pattern in the cytoplasm (Fig. 3B, C). Few bright near-spherical structures were detected in the extreme tip when dye uptake began, and most of the dye uptake events took place in the subapical region. Finally, it reached saturated status after approximately 10 min (Fig. 3E). The whole pollen tube was strongly stained by FM4-64 by the end of treatment with 20 nM LatB (Fig. 3C). Interestingly, the uptake of FM4-64 required less time in the LatB-treated cells than in the controls, indicating that there might be some remarkable variations in cell wall construction or the mode of dye internalization. In addition, the morphology and distribution of organelles (e.g. mitochondria, endoplasm reticulum) were also affected, and ultrastructural observation revealed that vesicles of larger size were frequently observed in the cytoplasm, and mitochondria showed an obvious tendency to aggregate (Fig. 4).

Effects of LatB treatment on pectin distribution

In pollen tubes grown in standard medium, the localization of JIM5-reactive pectins indicated a uniform distribution of de-esterified pectins in the subapical parts

along the tube wall with depleted tips (Fig. 5A), whereas the localization of JIM7-reactive esterified pectins was limited to the very tips of the growing pollen tubes (Fig. 5C). Pollen tubes cultured in medium containing 20 nM LatB showed completely different pectin distributions. Esterified pectins were localized only in the basal sites, while de-esterified pectins were localized along the whole surface of the tube as a layer in a uniform pattern (Fig. 5B, D). No remarkable differences in fluorescence intensity were found between the two lines.

Changes in callose and cellulose deposition after LatB treatment

In control cells, we found that callose was uniformly distributed along the tube shank and that only very faint fluorescence could be visualized in the apical region of pollen tubes (Fig. 6A). Callose was randomly deposited on the tube wall instead of being evenly distributed upon the application of 10 nM LatB (Fig. 6B). After the 20 nM LatB treatment, the callose content dramatically increased in the tip region, and intense callose fluorescence could only be found at the extreme tip (Fig. 6C). In controls, calcofluor labeling indicated that cellulose was present throughout the tube wall, including the elongating tip (Fig. 6D). There was no preference for deposition of cellulose from the basal part



Fig. 4 Organelle zonation is not maintained in the gymnosperm pollen tube. LatB treatment induced redistribution of organelles and vesicles. (A) A pollen tube cultured in standard medium, showing a nearly uniform distribution of mitochondria behind the apical region (bar = 50 μ m). (B) A pollen tube cultured in the presence of 20 nM LatB, showing an irregular distribution of mitochondria and greatly reduced fluorescence. Arrows show that mitochondria tended to accumulate in the cytoplasm (bar = 50 μ m). (C) Mitochondria were mostly round in shape, and distributed uniformly at the tip region in the control cells (bar = 4 μ m). (D) Mitochondria in a normal pollen tube, showing intact outer membranes and compact cristae (bar = 1 μ m). (E) Mitochondria and rough endoplasmic reticulum in control pollen tubes, showing normal morphology and numerous ribosomes associated with rough endoplasmic reticulum (bar = 1 μ m). (F) LatB induced the redistribution of organelles. Mitochondria are no longer distributed evenly and show a tendency to accumulate. Arrows show numerous mitochondria accumulated in the cytoplasm (bar = 4 μ m). (G) Mitochondria in a 20 nM LatB-treated pollen tube, showing that some mitochondria underwent slight morphological alteration and vesicles of large size were frequently observed in the cytoplasm (bar = 0.6 μ m). (H) The amount of rough endoplasmic reticulum is reduced in 20 nM LatB-treated pollen tubes, and vesicles of large size were frequently observed in the cytoplasm (bar = 1 μ m).

to the growing tip. Pollen tubes treated with 10 nM LatB showed an irregular deposition of cellulose along the shank of the tube (Fig. 6E). The calcofluor fluorescence was difficult to distinguish in the swelled region of the wall. When the LatB concentration was increased to 20 nM, the pollen tubes displayed even less cellulose than the control cells, especially at the extreme apex, as suggested by the fluorescence intensity (Fig. 6F). The cellulose layer could only be ambiguously discerned in the extreme apex, indicating a slight amount of cellulose deposition in this tube region. A large proportion of treated pollen tubes burst at their tips during the course of observation.

Effects of LatB application on distribution of AGPs

Pollen tubes of *P. meyeri* that were grown in standard medium showed a characteristic ring-like pattern with

remarkable periodicity of AGP deposition along the whole length, as revealed by immunolocalization with the LM2 antibodies (Fig. 7A), and these regular ring-like structures were mainly perpendicular to the long axis of the pollen tube. In contrast, pollen tubes cultured in the presence of 20 nM LatB showed a completely different distribution of AGPs. The characteristic feature of ring-like deposition disappeared, and fluorescence was either only found randomly deposited on the cell wall (Fig. 7B) or accumulated in the basal region of the pollen tubes (Fig. 7C).

FTIR analysis of wall components of pollen tube

As previously reported, in the mid-infrared (IR) spectrum, the saturated ester peak (representative of esterifed pectins) occurred at $1,740 \text{ cm}^{-1}$ (Morikawa et al. 1978);



Fig. 5 Fluorescent immunolabeling of pollen tube pectins. (A) JIM5 labeling of pollen tubes cultured for 20 h in standard medium, showing that strong fluorescence occurred along the entire pollen tube wall except the tip region. (B) JIM5 labeling of pollen tubes cultured for 20 h in the presence of 20 nM LatB, showing that fluorescence occurred all along the pollen tube wall. (C) JIM7 labeling of pollen tubes cultured for 20 h in standard medium. The esterified pectins localized in the extreme tip region of the pollen tube. (D) JIM7 labeling of pollen tubes incubated for 20 h in medium containing 20 nM LatB. The esterified pectins were confined to the basal part of the pollen tube wall. Corresponding bright field images are shown at a reduced size. The bar represents 50 μm.

amide I-and amide II-stretching bands of proteins absorbed at 1,650 and 1,550 cm⁻¹ (Jurgen and Lukas 1999); carboxylic acid groups absorbed at 1,600 and 1,414 cm⁻¹ (representative of de-esterifed pectins) (Morikawa et al. 1978); some carbohydrates (polysaccharides) appeared between 1,200 and 900 cm⁻¹ (McCann et al. 1992); and bands from cellulose absorbed at 1,165, 1,072 and 1,056 cm⁻¹ (McCann et al. 1993). Highly reproducible Fourier transform infrared (FTIR) spectra showed a marked decrease in the proportion of esterified pectins and no obvious changes in de-esterified pectins after treatment with LatB (Fig. 8). In the presence of 20 nM LatB, the protein peaks at 1,650 and 1,550 cm⁻¹ together with the cellulose peaks at 1,160 and 1,080 cm⁻¹ all decreased, while the peak stretches at 980 to 800 cm⁻¹ substantially increased (Fig. 8B).

Discussion

Polarized growth is finely regulated by an interplay between various forces that complement and/or counteract each other (Geitmann 1999, Feijo et al. 2004). This balance is based on several features on the inside of the cell: omnipresent turgor pressure and propulsive forces from the assembly of cytoskeletal components (Geitmann 1999). On the outside of the cell, the cell wall is the primary structure that withstands the internal forces (Somerville et al. 2004). The wall must be strong enough to withstand the internal turgor, but sufficiently plastic to allow the incorporation of wall precursors to support polarized growth (Taylor and Hepler 1997). This mechanically dual characteristic is mainly achieved by the mixture of polysaccharides and proteins.

Actin polymerization inhibitors have been used in studies focused on physiological roles of AFs in plants (Baluška et al. 2001, Samaj et al. 2002). Among them, LatB has often been used to show that pollen tube development depends on the AFs (Gibbon et al. 1999, Chen et al. 2006) and has been reported specifically to alter the actin-monomer subunit interface to prevent polymerization (Morton et al. 2000). In the present study, our results showed marked disruption of AFs in a dose-dependent manner after the application of LatB. Accumulating evidence has suggested that an intensive interaction may exist between AFs and microtubules. The role of microtubules in angiosperm pollen tube growth remains controversial. Microtubule disruption alters organelle zonation in the tube (Taylor and Hepler 1997) and inhibits elongation in some studies (He et al. 1996), while having no effects in others (Astrom et al. 1995). In gymnosperm species, Justus et al. (2004) found that microtubules control the positioning of organelles into and within the tip and influence the direction of streaming by mediating microfilament organization in Picea abies. Our results demonstrated that the distribution of microtubules did not obviously change while AFs were completely disrupted after treatment with 15 nM LatB, indicating that LatB did not influence the normal distribution of microtubules and that the changes in cell wall components resulted directly from AF disruption.

We reported for the first time that FM4-64 staining of a gymnosperm pollen tube showed a nearly reverse V pattern in the cytoplasm. Since organelle zonation is not maintained in conifer pollen tubes, this staining pattern is a direct reflection of the distribution of cytoplasmic components (including vesicles and different organelles) maintained by vesicle trafficking. FM-dyes enter the cell primarily by endocytic vesicles and are distributed to different organelle membranes via vesicle trafficking (Parton et al. 2001, Bolte et al. 2004). Given that the ultimate distribution pattern of FM4-64 was quite different from that of angiosperms (Parton et al. 2001), we may conclude that the cytoplasmic streaming in gymnosperms was hydrodynamically different. The direction of this streaming was opposite to that of angiosperms and could be termed 'fountain-like streaming', which is consistent with the results from pollen tubes of *P. abies* (Justus et al. 2004). Time-lapse images of the LatB-treated pollen tube showed an even distribution of FM4-64 fluorescence in the cytoplasm, which indicated that well-regulated vesicle trafficking was apparently perturbed due to AF disruption. Interestingly, the dye internalization was faster in the LatB-treated cells, which indicated the occurrence





Fig. 6 Effect of LatB on callose and cellulose deposition in pollen tube walls. (A) Pollen tubes cultured in the standard medium showing evenly distributed fluorescence along the tube shank when stained with decolored aniline blue. The fluorescence in the tip region was difficult to detect. (B) Pollen tubes cultured in the medium containing 10 nM LatB showing random deposition of callose in the tube wall. (C) Pollen tubes cultured in the medium containing 20 nM LatB showing strong fluorescence in the tip region, indicating enhanced callose synthesis and deposition in that region. (D) Cellulose was present throughout the tube wall in the control pollen tube including the elongating tip. No remarkable preference for deposition was seen from the basal part to the growing tip. (E) Pollen tubes treated with 10 nM LatB showing irregular deposition of cellulose along the shank of the tube. The calcofluor fluorescence was difficult to distinguish in the region of swelling on the wall. (F) Pollen tubes showing less cellulose than in control cells, especially in the extreme apex. The cellulose layer could only be ambiguously discerned in the extreme apex. Treated pollen tubes frequently burst in the tip region during the course of observation. The bar represents 50 µm; corresponding bright field images are shown at a reduced size; asterisks indicate enhanced deposition of callose and decreased deposition of cellulose.

of changes in wall modeling and/or removal of apical AF restriction on endocytosis. Recently, a cortical fringe of AFs was supposed to be helpful to restrict the endocytosis machinery at the tip region (Lovy-Wheeler et al. 2005). Though no such feature was detected during our investigations, we still found that apical AFs were essential for the regulation of the endocytosis machinery since the disruption of apical AFs by 10 nM LatB greatly accelerated the internalization of FM4-64. Considering that FM dyes internalize primarily by endocytic activity, we concluded that dye uptake was still active in the apical and subapical region after LatB treatment. The correlation between the cytoskeletal apparatus and endocytosis still requires intensive investigation.

Plant cell walls are dynamic structures that are mostly composed of polysaccharides with high molecular weights and highly glycosylated proteins (Somerville et al. 2004). The pollen tube wall must actively respond to stimuli posed by the application of inhibitor (Geitmann 1999, Geitmann and Parre 2004), and the tube must reflect the changes in the distributions and amounts of wall components. It has been documented that osmotic treatment directly reorganizes the actin filaments and regulates cell shape in plant cells, mammalian cells and yeast cells (Slaninova et al. 2000, Komis et al. 2002) while brefeldin A has also been proven to be a specific drug perturbing the vesicle trafficking between Golgi stacks and endoplasm reticulum without obvious effects on the cytoskeleton (Satiat-Jeunemaitre et al. 1996). The results from the control experiments demonstrated that the changes in cell wall construction after LatB treatment were closely related to AF disruption, showing that the cytological changes occurring in cell wall components after inhibitor treatment were a direct result of the inhibitory effects of LatB on AFs.

Pectins at the extreme apex are secreted as esterified pectin residues, which become increasingly de-esterified through the activity of pectin methylesterase in pollen tubes (Geitmann 1999). The resulting carboxyl groups are targets



Fig. 7 Effects of LatB on AGP distribution in the pollen tube wall. (A) Pollen tubes incubated in standard medium showing a characteristic ring-like pattern with remarkable periodicity of AGPs along the whole length after staining with LM2 antibodies; the ring-like structure was mainly perpendicular to the long axis of the pollen tube. (B and C) A pollen tube cultured in the presence of 20 nM LatB showing a completely different distribution of AGPs by LM2 labeling: the characteristic ring-like depositions disappeared; the fluorescence could either only be found in the basal region of pollen tubes (Fig. 6B) or randomly deposited in the cell wall (Fig. 6C). Corresponding bright field images are shown at a reduced size; arrows indicate deposition of AGPs in the cell wall. The bar represents 50 μm.

for cross-linking by Ca²⁺, creating a gradient in the cell wall composition from esterified to de-esterified pectins that correlates with an increase in rigidity and a decrease in extensibility (Taylor and Hepler 1997, O'Neill et al. 2004). We found that de-esterified pectins accumulated in the tip, while esterified pectins markedly decreased or were totally absent from the tip region of LatB-treated pollen tubes. Evidence is accumulating that the dynamic balance between the two forms of pectin has a profound effect on the extension capacity of the tube wall (Stepka et al. 2000, Parre and Geitmann 2005b). In addition, both types of pectins are sensitive to the AF disruption as they are transported either via the Golgi apparatus-based (esterified pectins) constitutive secretion (Geitmann et al. 1996) or via endosomal recycling (de-esterified pectins) pathways (Baluška al. 2002). Given the correlations among AFs, et



Fig. 8 Fourier transform infrared microspectroscopic (FTIR) spectra obtained from the apical region of *Picea myeri* pollen tubes cultured for 20 h. (A) FTIR spectra obtained from the tip region of pollen tubes cultured in standard medium (Control) or in medium containing 20 nM LatB revealed that LatB treatment induced displacements of the peaks or changes in absorbance. (B) The difference spectra generated by digital subtraction of the spectra of control tube walls from the spectra of LatB-treated tube walls, showing that the content of proteins greatly decreased while an increase in polysaccharide was obvious in the apical region after LatB treatment.

vesicle trafficking and the variation in pectin transport and distribution, our results further confirmed that AFs are essential for the targeted transport and fusion of secretary vesicles to the tip, and thus regulate the extensibility and rigidity of plant cell walls.

Callose is normally produced in the growing angiosperm pollen tube in order to minimize the cytoplasmic volume (Parre and Geitmann 2005a). However, these callose plugs are not a consistent feature in gymnosperm pollen tubes (Yatomi et al. 2002). The enhanced synthesis and deposition of callose has been reported to be a well-characterized response to wounding or stress in plant cells and to be induced by a variety of stimuli, notably an increase in cytosolic Ca²⁺ concentration (Bhuja et al. 2004). Treatment with LatB affected the synthesis and deposition pattern of callose in the pollen tube wall, especially in the extreme tip. From the location of these callose deposits, we postulated that treatment with LatB triggered the stimuli-responsive mechanism and eventually activated the callose synthase located at the extreme tip. Previous studies have documented that a disorganized cellulose synthase enzyme complex in the plasma membrane may be capable of catalyzing the synthesis of both cellulose and callose (Delmer 1987, Somerville 2006). Since AF breakdown induced differential expression of certain proteins that are involved in precursor synthesis and cell wall modeling (Chen et al. 2006), we can conclude that pollen tubes under unfavorable conditions might employ the highly compacted material via an increase of callose to act as part of the responsive machinery in the tip region.

Calcofluor labeling suggests that cellulose is detected at the extreme tip of the pollen tubes, confirming the presence of cellulose in cell walls at the tips of Pinus sylvestris and P. abies pollen tubes (Derksen et al. 1999, Lazzaro et al. 2003). This result differs completely from that of angiosperms, in which cellulose microfibrils are absent from walls until 5-15 µm back from the tips of Nicotiana tabacum pollen tubes (Ferguson et al. 1998). Conifer pollen tubes grow almost two orders of magnitude more slowly than angiosperms. The average growth rate for P. abies pollen tubes in vitro is $20 \,\mu\text{m}\,\text{h}^{-1}$ (Anderhag et al. 2000), while it is $5-25 \,\mu\mathrm{m}\,\mathrm{min}^{-1}$ in *Lilium longiflorum* (Parton et al. 2001). This slow growth rate might allow for the insertion and activation of cellulose synthase complexes within the tip region, while in angiosperms the insertion of the synthase complex occurred outside of the growing tip (Ferguson et al. 1998, Lazzaro et al. 2003). In addition, cellulose deposition in some specific area on the wall (especially the swelling site) decreased after LatB treatment compared with control cells. The fine AFs were disrupted after LatB treatment, which would directly perturb the polarized transport of macromolecular complexes such as cellulose synthase to the extreme tip; the synthesis of cellulose there was consequently blocked. According to previous studies, another possibility is that the disruption of F-actin alters the conformation of cellulose synthase, the enzyme which catalyzes the synthesis of $1,4-\beta$ -glucans, and catalyzes the biosynthesis of callose (Delmer 1987, Somerville 2006). It might be hypothesized that under natural conditions, the synthesis of $1,4-\beta$ -D-glucans (cellulose) normally predominates, but treatment with LatB may trigger the activity transition and promote the change from 1,4- β -D-glucan synthase activity to 1,3- β -D-glucan synthase activity.

We report here for the first time that AGPs are deposited in a characteristic ring-like feature in pollen tubes of gymnosperms, which had been previously hypothesized to be characteristic for several plant species with a solid-type style (*N. tabacum, Petunia hybrida*, etc.) (Li et al. 1992). The ring-like pattern of AGP deposition should be an adaptive mechanism that plants employ to reinforce the mechanical strength of the wall architecture and would enable the pollen tube to grow through the stylar intercellular spaces toward the ovule. AFs have been reported to be essential for the targeting of vesicles to the growing tip, so it is expected that the distribution of AGPs changes dramatically when the continuous trafficking of vesicles is reorganized or perturbed due to the application of LatB.

FTIR analysis demonstrated that treatment with 20 nM LatB triggered the displacement of peaks or changes in spectral absorbance. The differential spectra (Fig. 8B) showed that the amide-stretching bands (1,650 and $1,550 \,\mathrm{cm}^{-1}$) and bands from cellulose (1,160 and $1,080 \,\mathrm{cm}^{-1}$) decreased, while the bands in the range of 980 to 800 cm⁻¹ increased after the LatB treatment in the cell wall from the tip region (McCann et al. 1993, Chen et al. 1997, Jurgen and Lukas 1999), and esterified pectins also decreased $(1,740 \text{ cm}^{-1})$. These results suggested that the content of protein and cellulose both decreased, while some other polysaccharides increased after LatB application. This is in accordance with our results on the labeling of AGPs and cellulose in the tip region. Considering that the enhanced deposition of callose occurred in the tip region of LatB-treated pollen tubes while other polysaccharide components in the cell wall have been assigned to specific peaks, we speculated that the increase in the peak at $980-800 \,\mathrm{cm}^{-1}$ might have been caused by the enhanced synthesis and deposition of callose in the apical region.

In summary, our results clearly showed that LatB perturbed the pollen tube development by disrupting the AF network in a dose-dependent manner. LatB treatment directly triggered the disorganization of vesicle trafficking, and the cell wall underwent prominent changes in the composition and distribution of components. The changes in the cell wall composition and growth arrest could clearly be attributed not only to transformation of esterified pectins to de-esterified pectins but also to the enhanced deposition of cellulose in the tip region. Based on these results, we conclude that the depolymerization of AFs results in obvious changes in cell wall construction, which give rise to the morphological abnormalities and growth arrest of the pollen tube.

Materials and Methods

Plant materials

Pollen cones were collected in the Botanical Garden of the Institute of Botany, the Chinese Academy of Sciences, on April 15, 2005. Dried pollen grains were stored at -20° C until use. For germination, pollen grains were kept at room temperature for 30 min, and then suspended in the germination medium containing

12% sucrose, 0.01% H₃BO₃ and 0.01% Ca(NO₃)₂ at pH 6.8. Various concentrations of LatB (Sigma, St Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO) were added to the germination medium at the beginning of the culture period. Moreover, the control was also cultured in the presence of DMSO; all working concentrations of DMSO were <1%, a level necessary to dissolve LatB and which was shown to have no effect on pollen germination and tube growth. Pollen cultures were incubated on a shaker (120 r.p.m.) at 27°C in the dark. All of the following experiments were carried out at 20 h after incubation unless otherwise noted.

Pollen tube growth determination and morphological observation

Pollen grains were considered to be germinated when the tube length was longer than the diameter of the grain. About 60 pollen tubes were measured in each of the three replicates for tube length, and the mean value was plotted against the incubation period. Germinated tubes and the morphology of pollen tubes were documented under a Zeiss Axioskop 40 microscope, and digital images were captured using a Spot Π camera (Diagnostic Instruments Inc.).

Fluorescence labeling of F-actin

Labeling of F-actin was performed as previously described (Chen et al. 2006). After washes, the samples were mounted in 50% glycerol and examined using a Zeiss LSM 510 META LSCM with a rhodamine filter set (with excitation at 514 nm). All images were projected along the z-axis. According to the inhibitory effects of LatB on AFs, treatment stages were set for the rest of the experiments.

FM4-64 staining to analyze vesicle trafficking in the tube apex

Loading of cells with FM4-64 dye was generally achieved by direct application to the growing pollen tubes as described previously with slight modifications (Parton et al. 2001). Fluorescence from FM4-64 staining was detected using LSCM. The samples were excited at 514 nm with a 25 mW argon ion laser, achieved by means of neutral density filters, with a nearly closed pinhole and the gain adjusted to below a level of 7.00. Serial optical sections were performed every 20s for about 60 images 2–3 min after dye application until the fluorescence reached saturation at the end, and the images were processed with LSM 5 software.

TEM analysis of pollen tube ultrastructure and mitochondria staining

Transmission electron microscopy (TEM) analysis was carried out according to Wang et al. (2005) with slight modifications. Mitotracker Red dye was purchased from Molecular Probes (Eugene, OR, USA). The mitochondria were stained with 500 nM Mito-tracker Red by direct addition of dye solutions to the culture medium, and then washed with fresh medium after 5 min of incubation. Pollen tubes were mounted and photographed by LSCM as described above (excitation was at 488 nm and emission at 522 nm).

Immunolabeling of pollen tubes for pectins

Immunolabeling of pectins in the pollen tube wall was carried out following the procedures described by Derksen (1999) with slight modification. Pollen tubes were mounted and photographed by LSCM (excitation was at 488 nm and emission at 522 nm). Controls were prepared by omitting the primary antibody.

Localization of callose in the pollen tube wall

Pollen tubes were collected and fixed with 3% (w/v) paraformaldehyde in PME buffer for 30 min, rinsed three times in PME buffer and once in phosphate-buffered saline (PBS, pH 7.2), then incubated with 0.1% decolorized aniline blue prepared with 0.15 M K₂HPO₄ (pH 8.2) for approximately 5 min. The stained samples were immediately transferred to glass slides and examined with differential interference contrast (DIC) followed by epifluorescence (ultraviolet excitation), then photographed in a Zeiss Axioskop 40 microscope (excitation filter BP395-440, chromatic beam splitter FT460, barrier filter LP 470).

Localization of cellulose in the pollen tube wall

The localization of cellulose was examined by calcofluor staining as previously described (Lazzaro et al. 2003). Samples were collected, then incubated in 1 mg ml^{-1} calcofluor (in 70% sorbitol solution) in the dark for 45 min, mounted and examined under a Zeiss Axioskop 40 microscope (excitation filter BP365, chromatic beam splitter FT395, barrier filter LP 420), and digital images of epifluorescence and DIC were immediately captured using a Spot Π camera (Diagnostic Instruments Inc.)

Fluorescent immunolabeling of AGPs by LM2

Immunolabeling of AGPs on the pollen tubes wall was carried out as described (Li et al. 1992). The experimental procedure was the same as that of pectin immunolabeling except that LM2 monoclonal antibodies were used to localize AGPs.

FTIR analysis of the pollen tube wall

Samples were collected and washed three times with deionized water, then dried in a layer on a barium fluoride window (13 mm diameter $\times 2$ mm). Spectra were obtained from the tip regions of pollen tubes with a MAGNA 750 FTIR spectrometer (Nicolet Corporation, Tokyo, Japan) equipped with a mercury–cadmium–telluride detector. Spectra were obtained at a resolution of 8 cm⁻¹, with 128 co-added interferograms, and normalized to obtain relative absorbance as described (Wang et al. 2005).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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