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Release of an acid phosphatase activity during lily pollen tube growth involves components of the secretory pathway

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Summary. An acid phosphatase (acPAse) activity was released during germination and tube growth of pollen of *Lilium longiflorum* Thunb. By inhibiting components of the secretory pathway, the export of the acPase activity was affected and tube growth stopped. Brefeldin A (1 μM) and cytochalasin D (1 μM), which block the production and transport of secretory vesicles, respectively, inhibited the acPase secretion. The Ca^{2+} channel blocker gadolinium (100 μM Gd^{3+}) also inhibited acPase secretion and tube growth, whereas 3 mM caffeine, another Ca^{2+} uptake inhibitor, stimulated the acPase release, while tube growth was inhibited. The Yariv reagent (β-D-glucosyl)$_3$ Yariv phenylglycoside stopped tube growth by binding to arabinogalactan proteins of the tube tip cell wall but did not affect acPase secretion. A strong correlation between tube growth and acPase release was detected. The secreted acPase activity had a pH optimum at pH 5.5, a K_M of 0.4 mM for *p*-nitrophenyl phosphate, and was inhibited by zinc, molybdate, phosphate, and fluoride ions, but not by tartrate. In electrophoresis gels the main acPase activity was detected at 32 kDa. The conspicuous correlation between activity of the secretory pathway and acPase secretion during tube elongation strongly indicates an important role of the acPase during pollen tube growth and the secreted acPase activity may serve as a useful marker enzyme assay for secretory activity in pollen tubes

Keywords: Acid phosphatase; Exocytosis; *Lilium longiflorum*; Pollen; Secretion; Tip growth

Abbreviations: acPase acid phosphatase; *p*NPP *para*-nitrophenyl phosphate; Yariv-Glc (β-D-glucosyl)$_3$ Yariv phenylglycoside; Yariv-Gal (β-D-galactosyl)$_3$ Yariv phenylglycoside.

Introduction

Elongation of pollen tubes in common with several plant- and fungal-cell types is restricted to the extreme tip of the tube. This special growth pattern, tip growth, is reflected in the organisation of the cytoplasm (Rosen et al. 1964, Lancelle and Hepler 1992, Derksen et al. 1995). As the tube elongates, new cell wall and plasma membrane material has to be delivered to the tip region. Therefore, the tip is filled with secretory vesicles derived from dictyosomes located at the end of the tip region. The vesicles are transported from the dictyosome to the tip by cytoskeletal elements, mainly actin filaments (Heslop-Harrison and Heslop-Harrison 1989, Cai et al. 1996). Whether the vesicles move into the tip by Brownian motion or are guided by actin filaments still is not clear (de Win et al. 1998), but when they reach the plasma membrane of the extreme tip, they will eventually fuse and release their contents, e.g., sugars and enzymes, for cell wall synthesis (for reviews, see Battey and Blackbourn 1993, Bassham and Raikhel 1996, Thiel and Battey 1998). Thus, the rate of vesicle fusion, or exocytosis, is a pivotal parameter determining the growth rate of pollen tubes (Picton and Steer 1983, 1985; O'Driscoll et al. 1993; Parton et al. 2001). Despite its importance for tube growth, detailed knowledge on secretion during pollen tube growth is still poor due to difficulties in monitoring the secretory activity, and so far, molecular information about vesicle targetting, trafficking, and fusion is mainly based on comparison with other cell types, e.g., yeast and animal cells (see Sanderfoot and Raikhel 1999, Hepler et al. 2001 and references therein).

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In plant cells, exocytosis could be observed by several different methods (Battey et al. 1996). The most sensitive one is to measure the electrical capacitance of the plasma membrane which increases almost stepwise as individual secretory vesicles fuse with the plasma membrane (Thiel et al. 1998). But this technique requires the removal of the cell wall to obtain a protoplast accessible to a patch clamp electrode, an experimental disadvantage resulting in the loss of cell polarity. Alternatively, exocytosis was measured by monitoring the release of secretory products in plant-cell cultures (Sticher et al. 1981, Conrad et al. 1982, Kunze et al. 1998), aleuron protoplasts (Jones et al. 1998), or maize coleoptiles (Schindler et al. 1994). In *Saccharomyces cerevisiae* the secretion of an acid phosphatase activity (acPase, EC 3.1.3.2) correlated with the growth sites during budding and was found along the secretory pathway (Field and Schekman 1980, Esmon et al. 1981).

In pollen a secretion of proteins has been observed during germination and tube growth (Speranza and Calzoni 1989), and an acPase activity was detected in pollen grains which increased during germination (Haeckel 1951, Sharma 1982). Additionally, an acPase activity was detected histochemically in elements of the secretory pathway, e.g., endoplasmic reticulum (ER), secretory vesicles, the plasma membrane, and the cell wall (Lin et al. 1977).

Available evidence indicates that an acPase is released during pollen tube growth via the secretory pathway. To test this hypothesis we measured the release of an acPase activity while inhibiting cellular processes of the secretory pathway with well-documented inhibitors. We demonstrated a strong correlation between acPase secretion and tube growth, supporting the hypothesis and suggesting that the acPase is a useful marker enzyme for secretory activity in pollen tubes.

Material and methods

Plant material and pollen culture

Mature pollen grains were collected from dehydrated anthers of flowers of *Lilium longiflorum* Thunb. and used immediately for experiments. Lily plants were purchased at local flower shops. Pollen grains of one anther were resuspended in germination medium (10% sucrose, 1 mM KCl, 0.1 mM $CaCl_2$, 1.6 mM H_3BO_3 and pH adjusted with Tris or morpholineethanesulfonic acid [MES] to 5.6) and washed 5 times to remove any cell-surface-bound acid phosphatase activity. Pollen grains were finally resuspended in 12 ml of germination medium and incubated at room temperature in a petri dish. The average density of pollen grains in the germination medium was approximately 17,000/ml. After 90 min the volume of the pollen suspension was divided into equal aliquots and each aliquot was gently mixed with 6 ml of germination medium (controls) or 6 ml of medium containing a compound affecting secretion. Pollen grains were allowed to settle at the bottom of the petri dish and triplicate samples of 200 µl were taken every 20 min from the surface of the pollen culture to determine the acPase activity. Samples were immediately frozen and stored for less than 3 days at –20 °C before further analysis.

The percentage of germinated pollen grains and the mean tube length were also determined every 20 min by counting at least 200 pollen grains and by taking images to measure the mean tube length, respectively. All experiments were repeated three times.

Acid phosphatase assay

The activity of the acPase was measured according to Pfeiffer (1996) by measuring the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl phosphate (*p*NPP). Samples of 200 µl were incubated with 200 µl of reaction buffer containing 40 mM MES-Tris, pH 5.5, 5 mM *p*NPP, and 10 mM $MgCl_2$, for 45 min at 30 °C. The reaction was stopped by the addition of 800 µl of 400 mM borate buffer, pH 9.8, and the concentration of *p*NP was determined at 405 nm wavelength (U3000; Hitachi, Tokyo, Japan). All assays were performed as triplicates.

For biochemical characterisation the acPase activity assay was adapted to microtiter plates (Rainbow scanner; Tecan, Grödig, Austria) with a 5 times less volume for the acPase assay. The samples were adjusted to the same protein content. The protein concentration was determined according to Lowry et al. (1951) with bovine serum albumin for calibration.

Preparation of a secreted acPase fraction

Lily pollen grains of 25 flowers were collected, resuspended in 50 ml of germination medium, and gently shaken for 1 min. Pollen grains were washed five times by subsequently pelleting the grains, resuspending in fresh medium, and shaking for 1 min to remove cell-wall-bound acPase. After this washing procedure the pollen suspension was pipetted into petri dishes for germination. Acid phosphatase secretion was monitored by sampling aliquots every 20 min and analysing them for acPase activity. After 4 h the pollen grains and tubes were pelleted and aliquots of the supernatant containing the secreted acPase activity were frozen and stored at –70 °C.

Chemicals

Stock solutions of all substances were prepared in water, dimethylsulfoxide or ethanol. The solvent concentrations used did not affect the growth of pollen tubes or the acPase assay. Brefeldin A, cytochalasin D, $GdCl_3$, and caffeine were obtained from Sigma (Vienna, Austria). The Yariv reagents $(\beta\text{-D-glucosyl})_3$ Yariv phenylglycoside (Yariv-Glc) and $(\beta\text{-D-galactosyl})_3$ Yariv phenylglycoside (Yariv-Gal) were synthesised according to Yariv et al. (1962) with phloroglucinol and *p*-aminophenyl-D-glycopyranoside precursors. The purity of the synthesised products was determined spectrophotometrically and by high-pressure liquid chromatography.

All other chemicals (analytical grade) were obtained from Sigma, Merck, Pierce, Bio-Rad, or Fluka.

Electrophoresis and in-gel activity detection

Aliquots of the secreted acPase fraction (15 µg of protein) were incubated in polyacrylamide gel electrophoresis sample buffer omitting sodium dodecyl sulfate and incubated for 10 min at 48 °C. A

pseudo nondenaturing polyacrylamide gradient (7.5–16%) gel electrophoresis (no sodium dodecyl sulfate in sample buffer and gel) was performed with these samples and gels were blotted onto polyvinylidene difluoride membranes according to standard protocols. Both steps were carried out at 4 °C. The activity of acPases was detected in the gels or on the polyvinylidene difluoride membrane by washing the gels or membranes four times for 10 min at 4 °C with ABS buffer (20 mM Na-acetate, pH 5, 0.8% [w/v] NaCl, 0.02% [w/v] KCl) and staining for acPase activity according to Lefebvre et al. (1990) with a staining solution (100 mM Na_2 acetate, pH 5.8, 0.02% [w/v] Fast Garnet GBC (Sigma), 0.02% [w/v] α-naphthyl acid phosphate, 5 mM $CaCl_2$). Washing the gels or the membranes in 15% methanol and 7% acetic acid stopped the reaction.

Results

After 4 h, 64% ± 8% of the pollen grains had germinated and the mean tube length was 1200 ± 170 µm (n = 45) with the described in vitro culture conditions and germination medium. A very high initial activity of acPase was detected but could be removed by washing the pollen grains at least 5 times with germination medium. This acPase activity was probably bound to the cell surface of mature pollen grains but decreased rapidly during extensive washing (Fig. 1 A). When the washed pollen grains were incubated in germination medium, the acPase activity increased as pollen grains started to germinate and pollen tubes elongated. In Fig. 1 B a typical example (average of 3 independent experiments) of almost parallel increases in acPase activity, percentage of germinated pollen grains, and mean tube length is shown. To analyse the relation of germination and tube growth with the increase in acPase activity, the data of 8 experiments were plotted in a three-dimensional graph (Fig. 1 C). At each time point, namely, every 20 ± 5 min, the percentage of germinated pollen grains, the mean tube length, and the acPase activity were measured. A correlation between germination and acPase activity as well as between tube length and acPase activity could be noticed, indicating that an acPase is exported during pollen germination and tube growth.

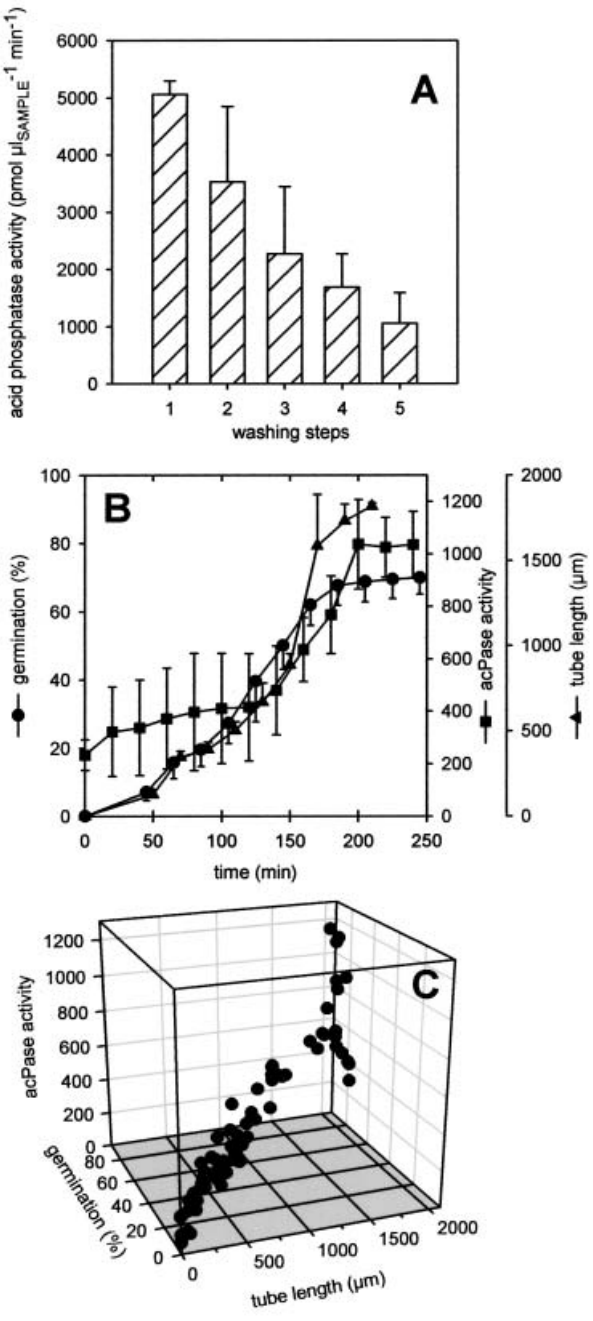

Fig. 1 A–C. Acid phosphatase activity measured in the supernatant of a suspension of pollen grains of *L. longiflorum* during pollen germination and tube growth. **A** An acPase activity associated with the pollen grain surface declines with the number of washing steps. Pollen grains were washed with germination medium. **B** Increase in acPase activity during germination and tube growth. Washed pollen grains were incubated in germination medium to start germination. The mean tube length, germination frequency, and acPase activity were measured every 20 min (n = 3). **C** The acPase activity plotted against germination frequency and tube length which were determined at almost the same time points (Δt ≅ 5 min) showing a strong correlation between the three parameters (n = 8). AcPase activity is given in pmol per µl of sample per min

Biochemical characterisation

To characterise the properties of the secreted acPase activity, biochemical parameters (K_M, pH optimum, and values of 50% inhibitory dose of putative inhibitors) were determined (Fig. 2 and Table 1). The secreted acPase showed a K_M of 0.38 mM for *p*NPP (Fig. 2 A). The K_M values were determined from a Hanes plot with the data of Fig. 2 A. The acPase activity showed an optimum at pH 5.5 (Fig. 2 B). Addition-

ally, an alkaline phosphatase activity could be measured between pH 7.5 and 8.0 but showed much less activity than the acPase. Molybdate (0.15 mM) decreased the secreted acPase activity by 80% (Fig. 2 C). The concentration that inhibited 50% of the initial activity was determined for a variety of substances known to inhibit acPases, namely, molybdate, phosphate, fluoride, and Zn^{2+} (Table 1). K^+ or Cl^- were used as the counter ions and KCl (up to 50 mM) itself had no effect on acPase activity (data not shown). By using an in-gel activity stain a protein band with a molecular mass of 32 kDa was detected (Fig. 2 D), suggesting that the activity is caused by a single protein. Immunoblotting using a commercially available antibody against a potato acPase (Rockland, Gilbertsville, Pa., U.S.A.) was not suitable and resulted in over 20 major protein bands that were recognised by the primary antibody.

Table 1. Inhibition of the secreted acPase activity by various components[a]

Inhibitor	50% inhibitory dose (mM)	Max. inhibition (%)
Molybdate	0.035	80
Phosphate	0.32	56
Fluoride	0.26	70
Tartrate		0
Zinc	0.08	56

[a] Data represent mean values from 3 experiments. All inhibitors were added as K^+ or Cl^- salts

Physiological characterisation of acPase secretion

To investigate the cellular mechanisms that might be responsible for the export of the acPase activity, specific inhibitors, all inhibiting processes known to be involved in secretion, were applied to the germinating pollen grains. The effective dose of the used inhibitors (brefeldin A, caffeine, Gd^{3+}, cytochalasin D, and Yariv reagents) was determined in previous studies (unpubl. data) and finally optimised to inhibit mainly the elongation of lily pollen tubes. The inhibitors were added to the pollen culture after 90 min and all three parameters, acPase activity, germination frequency, and tube length, were determined every 20 min. An example of the effects of brefeldin A is given in Fig. 3, showing the average of 3 experiments. The addition of 1 µM brefeldin A inhibited the export of acPase activity (Fig. 3 A) and tube elongation (Fig. 3 C) immediately, whereas the germination of pollen grains (Fig. 3 B) was less affected.

Relative inhibition and stimulation of acPase activity, germination and tube growth have been summarised in Fig. 4. All data have been obtained from pollen suspensions incubated for 4 h. The putative Ca^{2+} uptake antagonist caffeine (3 mM) (Pierson et al. 1996, Roy et al. 1999) inhibited germination and tube

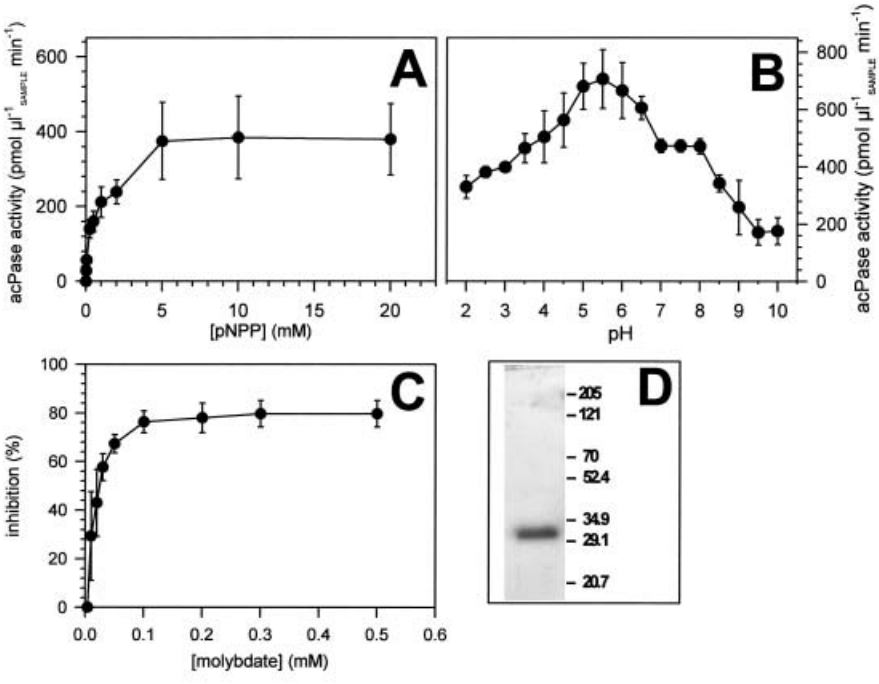

Fig. 2 A–D. Biochemical characteristics of the acPase activity secreted during pollen tube growth. In all experiments 100 µg of protein per reaction were used. **A** Substrate dependence of acPase acitivities giving a K_M of 0.38 mM for the secreted acPase. **B** pH dependence of the secreted acPase showing an optimum at pH 5.5. **C** Inhibition of the acPase activities by the typical acPase inhibitor molybdate, added as K^+ molybdate. The value of the 50% inhibitory dose is 35 µM for the secreted acPase. Data are means with standard deviations from 3 experiments. **D** An in-gel stain of the acPase activity. In the molecular-mass range of 5–220 kDa one major protein band at 32 kDa was detected. 15 µg of protein per lane

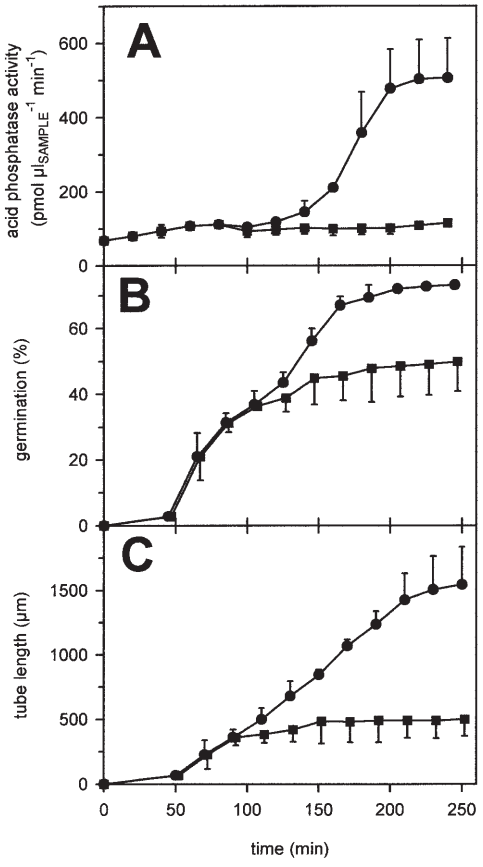

Fig. 3. A typical inhibitor experiment showing the effects of brefeldin A on acPase activity (**A**), germination frequency (**B**), and mean tube length (**C**). Pollen grains were incubated in germination medium and after 90 min brefeldin A (1 µM) was added to one half of the pollen culture (■), whereas the other half served as a control (●). All three parameters were determined every 20 min. The data points represent means with standard deviations of 3 independent experiments

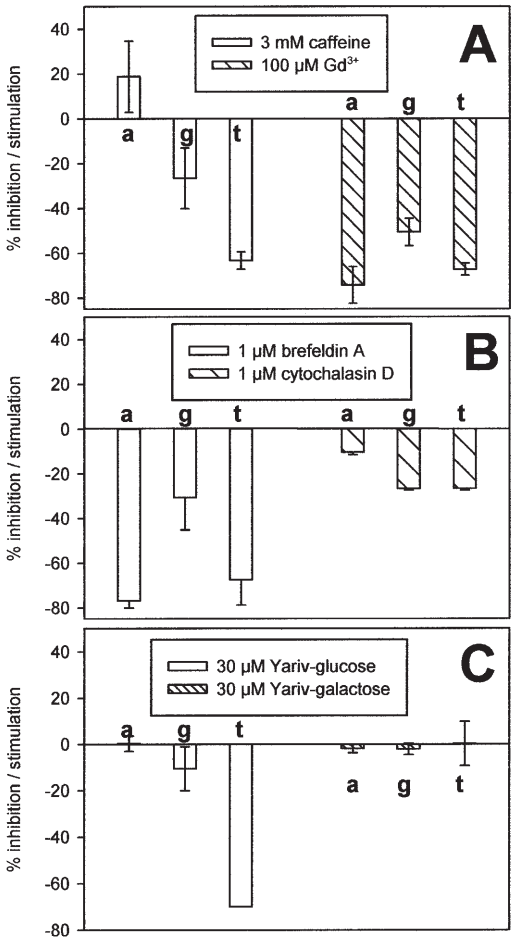

Fig. 4 A–C. Summary of the relative effects of various components on acPase activity (*a*), germination frequency (*g*), and mean tube length (*t*). Positive values represent stimulation, whereas negative values reflect the inhibition of any of the three parameters (n = 3). **A** Effects of the Ca^{2+} channel antagonists caffeine (3 mM) and $GdCl_3$ (100 µM). **B** Disturbing the vesicle production by 1 µM brefeldin A and the vesicle transport by 1 µM cytochalasin D resulted in a decrease of all three parameters. **C** Yariv-Glc (30 µM) inhibited tube growth but did not affect the secretion of the acPase activity. The inactive Yariv-Gal (30 µM) had no effect

growth, whereas the export of acPase activity was stimulated (Fig. 4 A). The addition of the well-known Ca^{2+} channel blocker gadolinium (100 µM $GdCl_3$) (Malhó et al. 1995, Messerli and Robinson 1997) did inhibit all three parameters, indicating a different mode of action between caffeine and Gd^{3+}.

The addition of brefeldin A (1 µM) and cytochalasin D (1 µM), affecting the production (Rutten and Knuiman 1993, Schindler et al. 1994, Satiat-Jeunemaitre et al. 1996, Ueda et al. 1996) and the transport (Heslop-Harrison and Heslop-Harrison 1989, Tang et al. 1989, Cai et al. 1996) of secretory vesicles, respectively, also inhibited acPase secretion, germination, and tube growth (Fig. 4 B). Note that blocking the vesicle production resulted in a much stronger inhibition of acPase export than blocking the transport of the vesicles.

Recently, the effects of Yariv phenylglycosides (30 µM) on pollen tube growth have been reported and an inhibition of tube elongation was observed, whereas fusion of secretory vesicles still occurred resulting in a thickened cell wall at the tube tip (Roy et al. 1998, 1999). As shown in Fig. 4 C tube elongation was strongly inhibited by Yariv-Glc, whereas export of the acPase activity was not affected. The inactive Yariv-Gal showed no effects on tube elongation, germination, and acPase activity.

Discussion

In pollen grains of *Lilium longiflorum* an acPase activity has been detected in two fractions: an activity associated with the cell surface (cell-wall-bound) and another that is released or secreted during tube growth. The secretion of acPase activity could be measured after the cell-wall-bound acPase activity was reduced to a minimum by extensive washing of the pollen grains prior to germination.

The secreted lily pollen acPase showed a pH optimum at pH 5.5, a K_M of 0.4 mM for *p*NPP and the enzyme was inhibited by zinc, molybdate, phosphate, and fluoride ions but not by tartrate ions. The molecular mass of the protein showing an acPase activity was 32 kDa. Acid phosphatases with almost similar biochemical properties have been isolated and characterised in many different plant organs and species, e.g., barley roots (Panara et al. 1990), tomato leaves (Tanaka et al. 1990), lupin seeds (Olczak et al. 1997), red kidney beans (Cashikar et al. 1997), and soybean suspension cultures (LeBansky et al. 1992). As acid phosphatases are a very heterogeneous family of enzymes, the characterised acPase of lily pollen cannot yet be classified according to its biochemical properties alone. Due to its resistance to tartrate the lily pollen acPase may be classified as a purple acid phosphatase; but so far, reverse transcriptase-polymerase chain reaction using lily pollen mRNA and primer for an *Arabidopsis thaliana* purple acPase (del Pozo et al. 1999) has produced no amplification products (our unpubl. results).

Physiological characterisation of acPase secretion

The acPase activity in the germination medium increased with germination time, showing strong correlation between external acPase activity and pollen grain germination or tube growth, suggesting that an acPase is secreted during pollen tube growth (Figs. 1 and 4). In various pollen species a release of proteins into the surrounding medium has been observed during in vitro germination and pollen tube growth (Stanley and Linskens 1965, Kamboj et al. 1984), and in apple pollen the protein secretion could be decreased when tube elongation was inhibited by monensin or cytochalasin B (Speranza and Calzoni 1989, 1992), indicating a connection between protein secretion and tube growth. A similar relation between protein secretion, especially the export of an acPase, and growth has been observed in other growing plant tissue, e.g., growth of roots (Dodd et al. 1987) and elongation of maize coleoptiles (Pfeiffer 1996). In *Saccharomyces cerevisiae* growth was accompanied by a local secretion of acPase at the growing site of the cell (Field and Schekman 1980) and the acPase activity was found in all organelles involved in the secretory pathway, including Golgi apparatus and secretory vesicles (Harsay and Bretscher 1995). The presence of an acPase in the secretory pathway has also been demonstrated for pollen tubes (Lin et al. 1977) and the increase in acPase activity in the germination medium observed in the present study suggests that the acPase is released via this secretory pathway, maybe together with the cell wall material, during germination and pollen tube growth. The secretion of the acPase may simply mirror the release of proteins during tube growth in general, but so far, the acPase activity is one of the first characterised in detail and may thus serve as a marker enzyme for exocytotic activity in pollen.

If the secretion indeed is tightly related to tube growth, modulations of tube growth will in turn affect the acPase secretion and vice versa. By using compounds influencing the secretory pathway in lily pollen (Figs. 3 and 4), we observed a change in acPase secretion. Actually, acPase secretion ceased after the inhibition of particular secretory steps, like production and transport of secretory vesicles, which are known to be inhibited by brefeldin A and cytochalasin D, respectively (Heslop-Harrison and Heslop-Harrison 1989, Tang et al. 1989, Rutten and Knuiman 1993, Schindler et al. 1994, Cai et al. 1996, Ueda et al. 1996). Similarly, electron microscopy of pollen tubes of *Nicotiana tabacum* showed that the intracellular transport of pectin-containing vesicles was inhibited upon addition of brefeldin A or cytochalasin D while tube growth was ceased (Geitmann et al. 1996).

Another important parameter in the secretory pathway is the tip-localised Ca^{2+} gradient facilitating the local fusion of secretory vesicles with the tube tip plasma membrane (Zorec and Thiel 1992, Hepler 1997). Lanthanides like La^{3+} and Gd^{3+} block the Ca^{2+} influx at the tube tip and decrease the $[Ca^{2+}]_{cyt}$ in the tip region thus inhibiting tube growth (Obermeyer and Weisenseel 1991, Malhó et al. 1995, Messerli and Robinson 1997). The acPase secretion is inhibited by Gd^{3+}, as $[Ca^{2+}]_{cyt}$ of the tube tip is decreased. However, caffeine, which also blocks tip-localised Ca^{2+} influx (Pierson et al. 1996, Roy et al. 1999), did not inhibit acPase secretion. In contrast, acPase activity increased

upon caffeine treatment, indicating a mode of action different from that of the lanthanides.

Yariv-Glc binds to arabinogalactan proteins and thereby destabilises the newly built cell wall, consequently stopping the elongation of lily pollen tubes (Roy et al. 1998). An increase in the $[Ca^{2+}]_{cyt}$ in the apical 100 µm of the tube has been observed, which was followed by large accumulations of cell wall material at the sites of increased $[Ca^{2+}]_{cyt}$, e.g., at the tube tip and its flanks, indicating that exocytosis is not inhibited in Yariv reagent-treated pollen tubes (Roy et al. 1999). In the present study (Fig. 4C), Yariv-Glc inhibited tube growth by 70% but did not affect acPase secretion; consistent with secretion via exocytosis.

In conclusion, our data provide a coherent picture to support the hypothesis that in lily pollen tube tips the acPase is secreted by the Ca^{2+}-dependent fusion of Golgi-derived secretory vesicles with the plasma membrane. The export of the acPase correlates well with the elongation of pollen tubes, suggesting that the acPase is probably secreted together with the cell wall material at the tube tip from where it diffuses into the medium. The function of the secreted acPase during tube growth remains unknown; however, its activity provides a valuable marker assay of secretory activity in pollen tubes as well as in other plant cells.

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