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ORIGINAL ARTICLE

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Detection and localization of pectin methylesterase isoforms in pollen tubes of *Nicotiana tabacum* L.

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Abstract Pectin methylesterases (PMEs) were detected in tobacco (*Nicotiana tabacum*) pollen tubes grown in vitro. Seven PME isoforms exhibiting a wide isoelectric-point (pI) range (5.3–9.1) were found in crude extracts of pollen tubes. These isoforms were mainly retrieved in supernatants after low- and high-speed separation of the crude extract. Two isoforms, with pIs 5.5 and 7.3 and molecular weight about 158 kDa, were detected by immunoblotting with anti-flax PME antiserum. Localization of pectins and PME isoforms in pollen tubes was investigated by immunogold labelling with JIM5 monoclonal antibodies and anti-flax PME antiserum, respectively. In germinated pollen grains, two PME isoforms were mainly detected in the exine, Golgi apparatus and secretory vesicles. In pollen tubes the same two PME isoforms were distributed along the outer face of the plasma membrane in the vicinity of the inner layer of the cell wall, in the Golgi and around secretory vesicles. In pollen grains, PME isoforms were, in some cases, mixed with acidic pectins in proximity to the outer surface of the plasma membrane. In pollen tubes the presence of PMEs inside secretory vesicles carrying esterified pectins supports the hypothesis that, during pollen tube growth, PMEs could be transferred by secretory vesicles in a precursor form and be activated at the tip where exocytosis takes place.

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Abbreviations IEF: isoelectric focusing · MAb: monoclonal antibody · pI: isoelectric point · PME: pectin methylesterase · SV: secretory vesicle

Introduction

Pollen tubes grow exclusively at the tip. During the growth process, secretory vesicles (SVs) derived from the Golgi apparatus fuse with the plasma membrane at the tube tip, releasing the membrane and wall precursors (see Derksen et al. 1995, 1999; Li et al. 1997; Taylor and Hepler 1997). The pollen tube wall consists of a primary pecto-cellulosic wall and a secondary callosic wall, which is deposited as an inner sheath behind the growing tip. Pectins appear to be major components of the cell wall at the tube tip (Roggen and Stanley 1969; Li et al. 1994; Yang et al. 1999).

During pollen tube elongation, pectins are deposited at the tip, mainly in their esterified form, and in *Arabidopsis* solely in this form (Lord 2000). De-esterification seems to occur actively behind the growing tip (Li et al. 1994) though this assumption has been challenged recently (Scavetta et al. 1999). De-esterified pectins derived from de-esterification may form egg-box patterns through calcium bridges (Jarvis 1984; McNeil et al. 1984). The pectin network forms a rigid cell wall behind the tip (Derksen 1996; Li et al. 1997). Pectin methylesterases (PMEs) could thus play an important role in dynamic changes in cell wall rigidity/extensibility, related to pollen tube growth.

Plant PMEs have mostly been investigated in fruits (see Bordenave 1995) and occasionally in plant tissues with high growth potential. The growth gradient of mung-bean hypocotyl was correlated with the gradient of methylation of cell-wall pectins and concomitant changes in the composition of PME isoforms (Bordenave

and Goldberg 1994). Another report argued for a direct role of PME in plant cell growth, which was hypothesized as a model for acidic growth (Moustacas et al. 1991). Immunochemical studies revealed that PMEs were located in the cortical tissues (Quentin et al. 1997), associated with acidic pectins and cell membranes, and aligned with the plasma membrane in the flax hypocotyl (Morvan et al. 1998).

By virtue of their tip growth and high, fluctuating growth rate, pollen tubes may be an interesting cell model for studying the nature and mode of action of PME. However, little information is available on PMEs of pollen tubes. Mu et al. (1994) described the partial amino-acid sequence of a putative pectin esterase of *Petunia* pollen. In addition, the pollen-specific genes *NTP303* from tobacco (Weterings et al. 1992) and *Bp 10* from *Brassica* (Albani et al. 1991) showed sequence similarities to pectin esterases. The properties and function of PME in the regulation of pollen tube growth are still unknown.

In this paper we report the detection of some PME isoforms of *Nicotiana tabacum* pollen tubes. PMEs and pectins were localized simultaneously using anti-flax PME antiserum and the anti-homogalacturonan monoclonal antibody (MAb) JIM5. Translocation of PMEs in pollen and pollen tubes is discussed in relation to the biosynthesis of pectins and pollen tube growth.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* L.) pollen was collected from plants grown in the Botanical Garden of the University of Siena, dehydrated and stored at –20 °C. Before germination, pollen was treated according to Moscatelli et al. (1995) and then cultured in BK medium (Brewbaker and Kwack 1963) containing 15% sucrose at 25 °C.

Antibodies

According to the test using defined oligogalacturonides, fully de-esterified nonagalacturonide was recognized by JIM5 (provided by K. Roberts, John Innes Centre, Norwich, UK; Knox et al. 1990; Willats et al. 1999, 2000). In this study the epitopes recognized by JIM5 are named as acidic pectins. The polyclonal anti-serum, anti-pectin methylesterase (anti-PME), was raised against the neutral isoforms (pIs 7.1–7.6), which are relatively rare in flax (*Linum usitatissimum* L.) plantlets, but strongly detected in flax callus and cultivated cells. The antibody cross-reacts with the moderately basic isoform (pI of 8.6), which is strongly detected in flax plantlets (Mareck et al. 1995).

Pollen-tube crude extract

Pollen tubes grown for 3 h were homogenized with a Potter homogenizer (15 strokes) in 2 vol. buffer 1 (see buffers listed below) at 4 °C. Laemmli sample buffer was added to the homogenate which was then boiled for 5 min. It was subsequently centrifuged in a Sorvall SS-34 rotor (20,000 *g*) at 4 °C for 30 min and the resulting supernatant (except the floating material) collected as crude extract (CE).

Pollen tube-fractionation

The buffers used were as follows:

- PEM buffer: 100 mM Pipes (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml Nα-*p*-tosyl-L-arginine methyl ester HCl (TAME), 10 μg/ml leupeptin, 1 μm pepstatin A, 1 μm antipain (protease inhibitors from Sigma), 0.5 mM 1,4-dithio-L-threitol (DTT; Sigma).
- Buffer 1: 15% sucrose in PEM buffer.
- Buffer 2: 0.3 M sucrose in PEM buffer.
- Buffer 3: 0.5 M sucrose in PEM buffer + 1 mM EDTA.
- Buffer 4: 0.3 M sucrose in PEM buffer +1 mM EDTA.

A 100-mg sample of pollen was cultured in the growth medium for 3 h; the pollen tubes were rinsed with 10 ml buffer 1. The pollen tube-suspension was centrifuged at 600 *g* for 15 min at room temperature. The pellet (0.8 ml) was re-suspended in 2 vol. buffer 2 (1.6 ml) and homogenized at 4 °C. The homogenate was centrifuged in a Sorvall SS-34 rotor, at 3,300 *g* and 4 °C for 4 min. The pellet (P1, containing nuclei, generative cells, cell wall fragments) was re-suspended in 1.6 ml final volume of buffer 2. EDTA (1 mM final concentration) was added to the supernatant (S1, containing both the microsomal and soluble fractions). Then S1 (1.2 ml) was loaded onto a 2-ml cushion of buffer 3 and centrifuged in a Sorvall AH-650 rotor at 49,000 *g* at 4 °C for 30 min. The S2 supernatant contained the soluble fraction of the cytoplasm. The P2 pellet (microsomal fraction) was re-suspended in 1.2 ml buffer 4. All samples were frozen in liquid N_2 and stored at –80 °C.

Analysis of PME isoforms by isoelectric focusing (IEF)

PME isoforms contained in extracts obtained during the fractionation of germinated pollen were analysed by IEF. All extracts were incubated with 0.1% (w/v) Triton X-100 for 1 h, clarified at 8,000 *g* for 10 min, then dialysed prior to freeze-drying. Extracts were solubilized in 300 μl water and clarified at 8,000 *g* for 5 min. Samples (20 μl) were loaded on the anode side of a gel and resolved by IEF. Horizontal IEF was performed on ultrathin polyacrylamide slab gels (0.3 mm) with 3.5–10 pH range in 5% (w/v) acrylamide (LKB Pharmacia Sweden) according to the manufacturer's instructions. After IEF separation, PME isoforms were identified on gels by using the 0.4% (w/v) agar-sandwich method (Bertheau et al. 1984). The incubation of sandwiches was performed at 25 °C for 2 h. De-methylated pectins resulting from PME isoform activities were stained with 0.02% (w/v) ruthenium red (Sigma). Immunoblots were carried out on nitrocellulose sheets (0.45 μm pore size; Schleicher & Schuell). Proteins transferred onto membranes were fixed and inactivated in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 10 min. Immunoblots were developed with anti-flax PME antiserum (diluted 1:200) and then with peroxidase-conjugated goat anti-rabbit antibody (diluted 1:200, Sigma), using 4-chloro-1-naphthol (Sigma) as substrate.

Detection of PME activity

PME activity was detected by the potentiometric method described by Gaffe et al. (1994). In brief, 4.95 ml of 0.1% (w/v) citrus pectins (Sigma; degree of esterification 62%), 50 mM NaCl, previously equilibrated at pH 7.5 with NaOH, was mixed with 0.05 ml of each extract. pH was maintained at 7.5 with 0.01 M NaOH. One unit of PME activity represents the amount of enzyme that produces 1 acid eq. s^{-1}. The activity of PME was corrected for the spontaneous de-methylation of citrus pectins at pH 7.5.

SDS-PAGE and western blotting

Protein separation was performed on 4–11% polyacrylamide gradient gels (Laemmli 1970). After electrophoresis, all the gels were

stained with Coomassie Blue R-250. Molecular weight standards were purchased from Bio-Rad. The western blot was performed according to Towbin et al. (1979). Polypeptides were transferred onto 0.45-μm nitrocellulose (Amersham Pharmacia) at 250 mA and 10 °C for 15 h, using a Mighty Small Transphor Tank Unit T 22 (Hoefer Pharmacia Sweden). The anti-PME antiserum was diluted 1:2,500. A goat anti-rabbit peroxidase-conjugated antibody (1:4,000, final dilution) purchased from Cappel was employed as a secondary antibody. Detection of reacted polypeptides was carried out according to the protocol reported in the Amersham ECL kit booklet.

Immunogold labelling

Germinated pollen was chemically fixed, embedded in LR White resin and ultrathin sections of germinated pollen were prepared according to Li et al. (1995). Immunogold labelling was carried out according to Li et al. (1995). The sections were incubated with primary anti-PME antibody (diluted 1:2,000) for single labelling and with JIM5 (diluted 1:5)/anti-PME (diluted 1: 2,000) for double labelling. After washing, the sections were incubated in colloidal-gold goat anti-rat immunoglobulin complex (20 nm) for JIM5 and in colloidal-gold goat anti-rabbit complex (10 nm) for anti-PME (BioCell, Cardiff, UK). Controls consisted of (i) using the preimmune serum instead of the primary antibodies and (ii) reversing the order of the double labelling. The samples were observed with a JEOL jem-100B electron microscope at 80 kV.

Results

IEF separation of PMEs in tobacco pollen tubes

At least seven isoforms of PME were detected by IEF in pollen-tube crude extract, which contained most of the pollen-tube polypeptides. They were characterized by a large pI range: four acidic (pIs of 5.3, 5.5, 5.9 and 6.3), one neutral (pI of 7.3) and two basic proteins (pIs of 8.5 and 9.1; Fig. 1a). All isoforms were mainly associated with supernatant extracts S1 (post-nuclear supernatant, comprising both the microsomal and soluble fractions) and S2 (pollen-tube soluble fraction). By comparison with S1 and S2, weaker activity of the isoforms was detected in P1 (pellet containing nuclei and cell wall debris) and the weakest activity was in P2 (microsomal pellet). Two pollen-tube PME isoforms with pIs of 5.5 and 7.3 were recognized by anti-flax PME antibody (Fig. 1b).

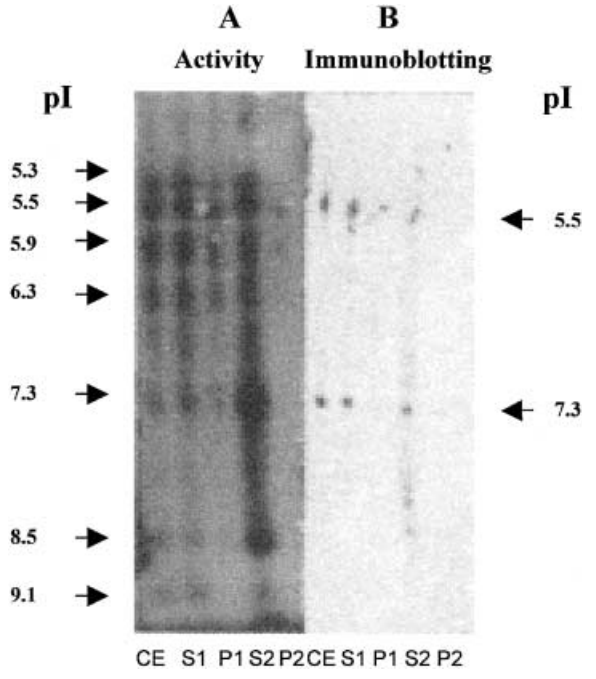

Fig. 1 **a** Activity of PME isoforms after IEF separation of *Nicotiana tabacum* pollen-tube fractions. **b** Pollen-tube PME isoforms detected by anti-flax PME-antibodies at pIs of 5.5 and 7.3. *CE* Crude extract, *S* supernatant, *P* precipitate

Western blot analysis of PME polypeptides

Pollen-tube PME isoforms were further analysed by western blotting after SDS-PAGE. Pollen-tube polypeptides (crude extract) were transferred onto nitrocellulose and immuno-detected with anti-flax PME antibodies. The anti-PME antibody specifically recognized two polypeptides with molecular masses of about 158 kDa (Fig. 2), suggesting correspondence with the pI-5.5 and pI-7.3 isoforms revealed by immunoblotting of IEF-separated PMEs.

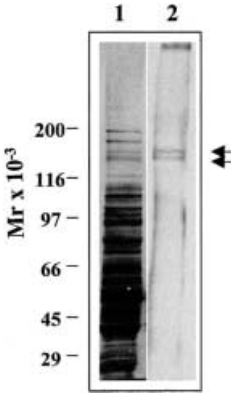

Fig. 2 Detection of immunoreactive PME in *N. tabacum* pollen-tube crude extract containing most of the pollen-tube polypeptides. *Lane 1* Coomassie Blue stained SDS-PAGE, showing total pollen-tube proteins content. *Lane 2* The pollen-tube crude extract probed with the anti-PME antiserum followed by peroxidase detection. Two polypeptides with molecular weights of approximately 160 kDa are prominent (*arrows*)

Subcellular localization of PME isoforms in germinated pollen

In order to detect the cytoplasmic distribution of PME, the anti-flax PME antiserum was used in immunoelectron microscopy on germinated pollen sections. In germinated pollen, PME epitopes were localized in the exine and around the surface of the sculptured outward-facing wall where sporopollenin is usually deposited

(Fig. 3c). Furthermore the anti-PME antiserum recognized epitopes along the outer side of the plasma membrane, towards the intine, the Golgi apparatus and SVs. A few gold particles were also detected in the cytoplasm (Fig. 3c). In pollen tubes, anti-PME gold particles appeared along the outer side of the plasma membrane, the Golgi and inside SVs (Fig. 3d). In control experiments, when the pre-immune serum was used, no labelling was detected in pollen grains (Fig. 3a) or pollen tubes (Fig. 3b).

Co-localization of pectins and PME isoforms in germinated pollen

The co-localization patterns of acidic pectins and PME isoforms in pollen grains and tubes was shown by double immunogold labelling using MAb JIM5 and anti-PME. In pollen grains, the distribution pattern of PME-recognized epitopes (Fig. 4a, b; 10-nm gold particles) was the same as already described in Fig. 3c. The acidic pectins (Fig. 4a; 20-nm gold particles, see arrowheads) were found exclusively in the intine (I). In some cases, PME isoforms were mixed with acidic pectins in proximity to the outer surface of the plasma membrane (Fig. 4a). Immunogold labelling of cross-sections (Fig. 4c) and longitudinal sections (Fig. 4d) of pollen tubes showed that PMEs were localized along the outer face of the plasma membrane in the vicinity of the inner layer of the tube wall (Fig. 4c, d; see arrows), over the Golgi apparatus and around the SVs (Fig. 4c). The acidic pectins were localized in the outer and middle wall layers (Fig. 4c, d; see arrowheads).

No difference was found when the order of the double labelling procedure was reversed.

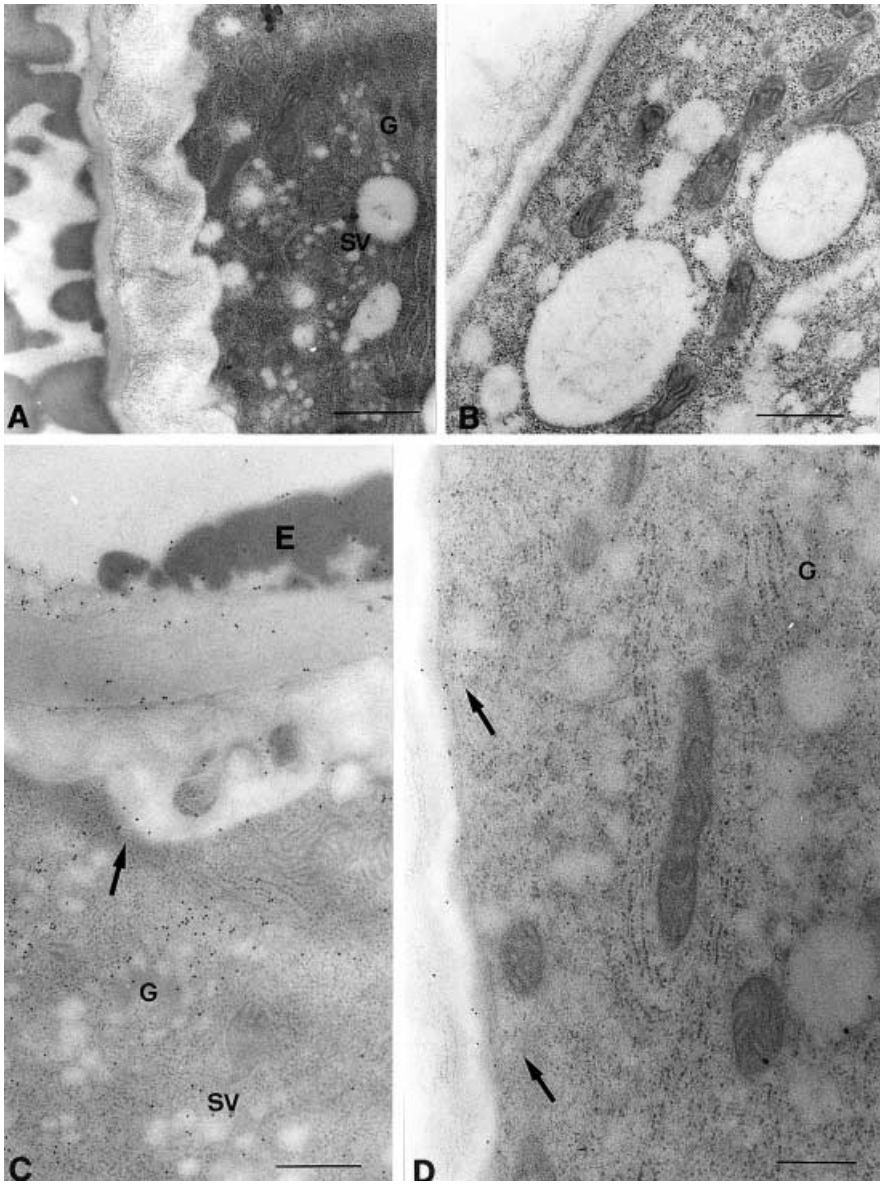

Fig. 3 **a, b** Control sections of *N. tabacum* pollen grain (**a**) and pollen tube (**b**). No immunogold labelling was found. **c, d** Immunogold labelling of germinated *N. tabacum* pollen grain (**c**) and pollen tube (**d**). Anti-flax PME antiserum was employed as the primary antibody. In the pollen grain, label was distributed in the exine (*E*), Golgi apparatus (*G*) and secretory vesicles (*SV*). In the pollen tube label was distributed in the Golgi apparatus (*G*). Gold particles (10 nm) were also present along the plasma membrane in both samples (*arrows*). Bars = 0.5 μm

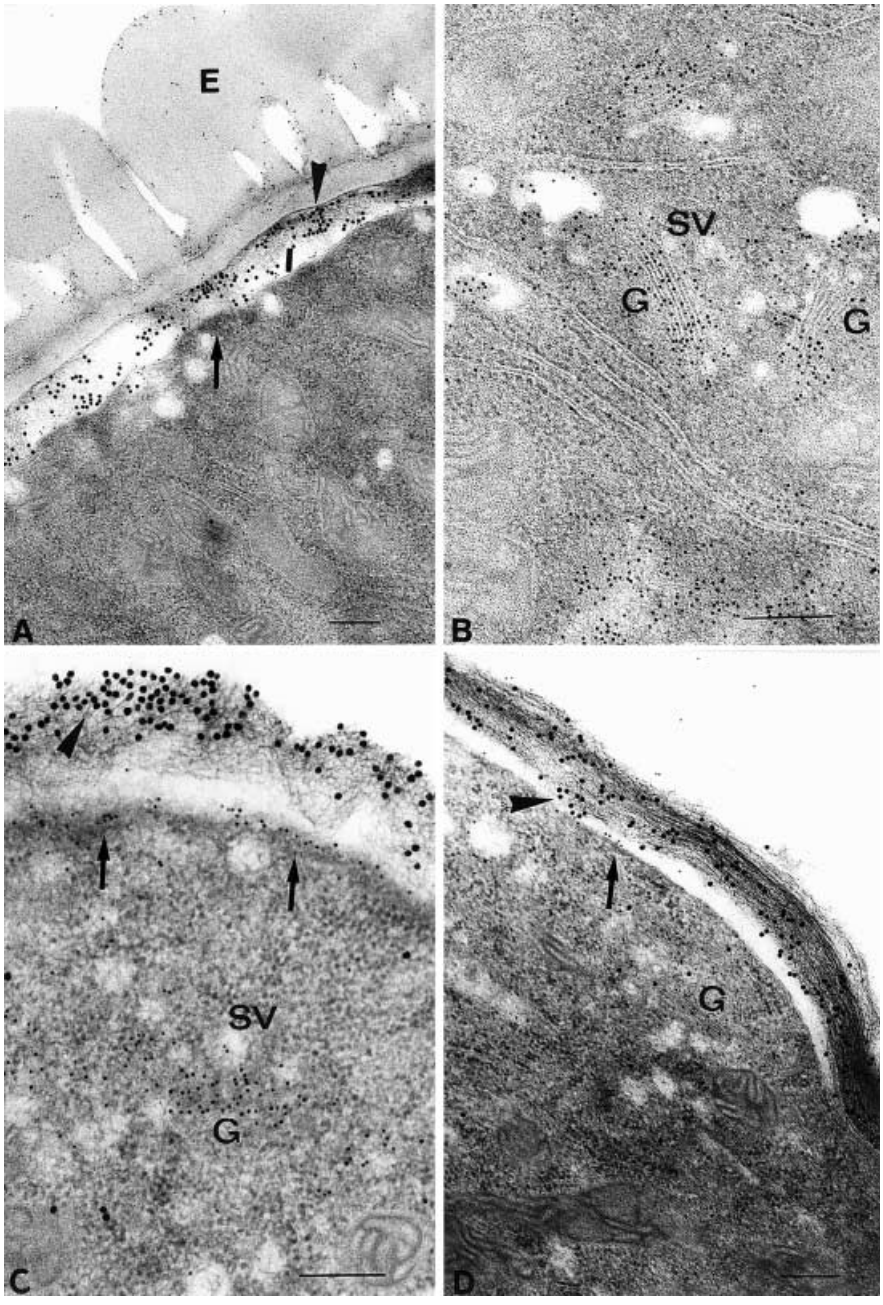

Fig. 4 Double immunogold labelling of *N. tabacum* pollen grain (**a, b**) and pollen tube (**c, d**) with anti-PME (10 nm) and MAb JIM5 (20 nm). In the pollen grain treated with anti-PME, the label was found, as shown in Fig. 3c, around the exine (*E*), Golgi apparatus (*G*), secretory vesicles (*SV*) and along the plasma membrane (*arrow*). Epitopes of JIM5 were exclusively present in the intine (*I*). In both cross-sections (**c**) and longitudinal sections (**d**) of the pollen tube, PME epitopes were located in the Golgi apparatus, around SVs and along the outer face of plasma membrane (*arrows*). In the pollen tube (**c, d**) JIM5 label was detected throughout the pectin wall (*arrowhead*). Bars = 0.2 μm

Discussion

To our knowledge, this is the first report on the detection of PME isoforms and the location of two isoforms in germinated pollen and pollen tubes of tobacco.

Seven isoforms of PME dispersed over a wide pI range, i.e. four acidic (pIs 5.3, 5.5, 5.9 and 6.3), one neutral (pI 7.3) and two basic (pIs 8.5 and 9.1) isoforms, were detected in tobacco pollen tubes. This is a more complex composition than that of fruits, in which one or two moderately basic PME isoforms have been reported (Giovane et al. 1990). It is closer to the PME composition of flax and mung bean, where both neutral and basic isoforms were detected (Bordenave and Goldberg 1994; Gaffe et al.1994). The number of acidic isoforms was particularly varied, e.g. there were few acidic PME isoforms in tobacco hypocotyls and one or two acidic PMEs have been reported in plant pathogens (see Bordenave 1995), but there were as many as four isoforms in tobacco pollen tubes. In general, neutral PME proteins have rarely been reported in fruits, with the exception of a neutral PME isoform in *Actinidia* (Giovane et al. 1990). Neutral PMEs have commonly been detected in vegetative tissues and flowers. For example, two neutral isoforms were described in flax and mung bean hypocotyls, one in stems and leaves of *Medicago sativa* (Markovic and Kohn 1984), and one in poplar cambium (Guglielmino et al. 1997). It is interesting that pollen-tube PMEs also have an isoform with

pI 7.3. Neutral isoforms seem peculiar to plant tissues possessing a high potential for development.

In plants, two action modes of PMEs have been proposed. The first is blockwise de-methylation of pectins, generally by basic isoforms of PME, which results in gelatination of pectin by means of Ca^{2+} bridges. These isoforms are evidently related to maturation phenomena, such as ripening of fruit and autolysis of middle lamella and cell junctions (Gaffe et al. 1994). In pollen tubes, pectin demethylation and gelatination presumably occur in the wall behind the tip region during growth, since esterified pectins were particularly rich in the wall of the tube tip, and diminished in the rest of the tube wall (Li et al. 1994). Apparently, the basic PME isoforms borne by tobacco pollen tubes could contribute to pectin gelatination, strengthening the tube wall.

The second action mode of PMEs is involved in plant growth, which is thought to be the model for acidic growth. PMEs react together with pectin hydrolases, causing random pectin degradation that is not able to induce pectin gelatination. A random mechanism was demonstrated for acidic PMEs of plant pathogens and was also deduced to be carried out by relatively neutral isoforms in the flax hypocotyl, which is characterized by intensive growth (Markovic and Kohn 1984; Bordenave and Goldberg 1994; Bordenave 1995). Acidic PME isoforms, cooperating with pectin hydrolases, were required to degrade the plant cell wall during pathogen invasion. Pollen tubes have an actively fluctuating tip-growth pattern. This suggests that the four acidic PMEs found by us in germinated pollen may function together with pectin hydrolases and/or other enzymes in re-modeling the tip wall during pollen tube elongation.

The antibodies, raised against two neutral isoforms of flax PME, also cross-react with the moderately basic isoforms in flax (Mareck et al. 1995). In pollen tubes, these anti-flax PME antibodies recognized the neutral isoform with pI 7.3, and cross-reacted with another isoform with pI 5.5, one of the four acidic isoforms. This observation that the extremely basic isoforms of PME were not detected by the antibodies is in line with the finding in flax. It is noteworthy that in germinated pollen the PME isoforms were concentrated in the exine, as they may easily be excreted to help the pollen tube elongate and penetrate the stigma. However, in pollen tubes, PME isoforms were not found inside the cell wall, but along the outer side of plasma membrane. Recently, it was reported that specific PMEs may act at different times during plant development with different action patterns on homogalacturonan (Catoire et al. 1998; Liberman et al. 1999; Willats et al. 1999). We propose that the different location patterns of PMEs found in the pollen grain and tube might be related to different action modes of PMEs, which are responsible for dynamic changes in cell wall rigidity/extensibility during pollen germination and tube elongation. This hypothesis, of course, must be evidenced by further study. Meanwhile, there was also a common feature that in both the pollen grain and the pollen tube the PME isoforms were associated with the Golgi apparatus and SVs. This is in line with the finding of membrane localization of PMEs in flax (Alexandre et al. 1997; Morvan et al. 1998). The membrane localization of some PME isoforms could also be confirmed by the discovery of a putative hydrophobic transmembrane domain in the N-terminal extension of *Phaseolus vulgaris* PME (Ebbelhaar et al. 1996). It suggests that during pollen tube elongation PMEs might be retained in the plasma membrane and be released during the need for catalysis of esterified pectin de-methylation that is associated with pectin gelatination.

In plant tissues, pectin synthesis takes place in the Golgi apparatus and methyl-esterification proceeds simultaneously (Levy and Staehelin 1992; Liners and van Cutsem 1992; Zhang and Staehelin 1992; Sherrier and Van de Bosch 1994; Li et al. 1995). The double immunogold labelling pattern revealed that in pollen grains PMEs were either mixed with, or in the vicinity of, acidic pectins. This is consistent with the finding in flax that anti-PME epitopes were associated with all tissue regions rich in acidic pectins, such as the middle lamella and cell junctions (Ebbelhaar et al. 1996). It was proposed that PMEs could be translocated in their inactive precursor forms and become active during/after exocytosis. This hypothesis was supported by the following findings of a molecular biological study of PMEs. Firstly, the N-terminal leader domain of PME sequence in *Arabidopsis* could make the enzyme inactive or more stable during transport (Richard et al. 1994). Moreover, in *Brassica* the removal of this domain would be required for activating PME (Albani et al. 1991). Our results on the presence of PMEs inside SVs (Li et al. 1994, 1995; Hasegawa et al. 1998; Lord 2000) during their translocation towards the tube tip could be the first immunocytochemical support for the hypothesis. This characteristic might be particularly important in pollen tubes where the spatial distribution of the Golgi apparatus and the long-distance transfer of PMEs are especially distinct. The present study, therefore, provides evidence for understanding the crucial role of PMEs in the regulation of pollen tube growth (Derksen et al. 1995, 1999; Li et al. 1997; Taylor and Hepler 1997).

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