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Expression of a polygalacturonase enzyme in germinating pollen of Brassica napus

Abstract Penetration of pollen tubes through stigmatic tissues in Brassica napus L. may involve the release of cell wall modifying enzymes from the pollen tube tip. We have examined the expression of a pectin-degrading polygalacturonase (PG) enzyme in unpollinated and early and late pollinated stigmas via immunoblotting and immuno light microscopy using a PG polyclonal antibody. Immunoblotting analysis indicated that PG enzyme was present at low levels in unpollinated stigmas and at high levels in pollinated stigmas. The level of PG did not detectably increase between early and late pollinated stigmas. Immuno light microscopy demonstrated that PG enzyme was present in ungerminated pollen grains, stigmatic papillae and in the tip of pollen tubes growing into the papillar wall. This latter evidence suggests that PG enzyme may play an important role in papillar cell wall penetration during pollination although other interpretations of the role of pollen PG should not be discounted.

Key words Brassica napus Pollen tubes Stigma Pollination Polygalacturonase

Introduction

Pollination in the Brassicaceae involves adhesion of pollen grains to modified stigmatic cells called papillae, hydration of pollen grains and subsequent production of pollen tubes (Dickinson 1995). These latter structures penetrate the stigmatic cuticle and then migrate through the papillar cell walls as they carry the male nuclei towards the embryo sac (Elleman et al. 1988).

The initial step in pistil penetration in many plant pollination processes appears to involve degradation of the stigmatic cuticle (Shayk and Kolattukudy 1977, Hiscock et al 1994). In Brassica napus pollen, the intine has been shown to contain an active cutinase with resemblances to cutinases of a fungal species (Hiscock et al 1994, Kolattukudy et al. 1981). This enzyme, which has been localised to the outer pollen wall in contact with stigmatic papillae (Hiscock et al 1994), may play a key role in cuticular erosion.

In contrast, passage of the pollen tube through the papillar cell wall is less well understood. A number of studies have shown that tube penetration is accompanied by cell wall "expansion" or "loosening" (Elleman et al. 1988, 1992; Elleman and Dickinson 1990, Kandasamy et al. 1994, Dearnaley et al. 1999). These changes to the architecture of the papillar cell wall, which presumably facilitate successful passage of the pollen tube, may be related to the release of wall modifying enzymes from the stigma cell (Elleman and Dickinson 1996) or from the approaching pollen tube (Dearnaley et al. 1999).

The outer papillar wall through which pollen tubes must penetrate appears to be an amorphous matrix of pectin and cellulose (Elleman et al. 1988). Loosening or expansion of this layer presumably involves the release of enzymes such as β-1,4 glucanase and pectinases such as

polygalacturonase (PG), pectic methylesterase and pectic lyase. Potential pistil cell wall degrading enzymes have been observed in a number of plant species. Pollen tubes of maize, tobacco and Oenothera organensis have been shown to contain PG enzymes (Allen and Lonsdale 1993; Dubald et al. 1993; Tebbutt et al. 1994; Brown and Crouch 1990). In Petunia inflata a putative pectin esterase is present in *in vitro* germinated pollen (Mu et al. 1994), while in tomato, pectate lyase has been shown to be present in the tube tip and cytoplasm of *in vitro* germinated pollen (Ursin et al. 1989; Dircks et al. 1996).

In this study we are studying the timing of synthesis and spatial localisation of the pectin-degrading enzyme PG during pollination of Brassica napus. PG enzymes, which degrade pectin by cleaving the polygalacturonate backbone or end chains (see Qiu and Erickson 1996), have a multiplicity of roles in plants. These include organ abscission, fruit ripening and pod and anther dehiscence (reviewed in Hadfield and Bennett, 1998). PG enzymes appear to play an important role in pollination processes as PG mRNA transcripts or protein accumulate to high levels in maturing pollen of a number of plant species (eg. Niogret et al. 1991; John and Petersen 1994; Brown and Crouch, 1990).

We have used a polyclonal antibody generated to a published sequence of a PG enzyme highly expressed in Brassica napus pollen (Robert et al. 1993) to determine the timing of expression of PG enzyme and the spatial localisation of the enzyme in germinating pollen *in vivo*. We have discovered that although the levels of PG enzyme did not detectably rise in germinated pollen, the enzyme was found in the tip of the pollen tube during papillar cell penetration. This latter evidence is consistent with PG playing a role in cell wall modification during pollination in Brassica napus but alternative roles for PG during the pollination process should be considered.

Materials and Methods

Plant material

Plants of Brassica napus cv. Westar were grown in a growth chamber (16 h light, 22°C/8 h dark, 16°C; 60 % relative humidity (R.H.); illuminated by a mix of incandescent and fluorescent sources) for 2-3 months or in a glass house (approximately 9 h light/15 h dark, temperature range 10-25°C).

Stigmas were hand pollinated *in situ* by brushing a dehisced anther over the surface of the tissue so that it formed a complete layer. After pollination, stigmas were detached immediately from pistil tissues and frozen in liquid nitrogen (early pollination) or left for 45 and 90 min (late pollination) before detaching and freezing in liquid nitrogen. Late pollinations were performed at approximately 20-26°C and 50-75% R.H. Unpollinated stigmas were also frozen in liquid nitrogen. Samples were stored at -80°C until ready for processing.

Light microscopy

An initial light microscopy study was carried to determine the stages at which large numbers of pollen grains were either beginning stigmatic penetration or were growing within stigma cell walls. Stigmas were pollinated *in situ* and were then detached immediately or at 45 and 90 min post pollination. Tissues were placed on glass slides and squashed flat with another glass slide. A drop of distilled water and a coverslip were then added and the material was then examined and photographed with a Nikkon E600 upright photomicroscope (Laboratory Supply, Tingalpa, Queensland, Australia).

Production of PG antibody

For peptide synthesis, the sequence RNIRGTSENKDAVKLCKAPGKE was chosen. This sequence is a combination of an internal peptide and the C-terminus of the B. napus cv Westar PG published by Robert et al. (1993). The peptide was custom synthesised by Mimotopes (Clayton, Melbourne, Victoria, Australia) while the antiserum was raised in rabbits by the Institute of Medical and Veterinary Science (Veterinary Services Division, Adelaide, Australia).

Protein separation and immunoblotting

25 stigmas from each time point were ground in microcentrifuge tubes with a plastic grinder for approximately 1 min. After addition of TBS buffer (150 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 8.0) the material was ground again then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and recentrifuged under the same conditions and finally removed to a fresh microcentrifuge tube. After determination of protein

concentrations (Bradford 1976), equal amounts of protein were mixed 1:2 with sample buffer (BioRad, Regents Park, Sydney Australia; plus 5% mercaptoethanol (BDH Chemicals Ltd, Poole, U.K.)) and boiled for 5 min. SDS-PAGE was carried out using a Mini-PROTEAN II electrophoresis system with 10% Tris-HCl acrylamide precast gels (Biorad). Proteins were transferred to nitrocellulose with a Mini-Trans Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked 1 h with 5% milk powder (Nestles, Sydney, Australia) in TBS, washed 3 times with TBST (TBS plus 1% Tween-20) and then incubated with the PG polyclonal antibody (1:500 in TBS, 5% Milk powder) for 2 h at room temperature. After 3 washes in TBST, the membranes were incubated 2 h at room temperature with goat anti-rabbit alkaline phosphatase conjugated secondary antibody (Sigma, Castle Hill, Sydney, Australia; 1:1000 in TBS, 5% milk powder). After washing 3 times in TBS, the antigen-antibody complexes were visualised with a colour reaction kit (Bio-Rad). The post-translational modification web site PSORT (http://psort.nibb.ac.jp/) was used to evaluate molecular weight discrepancies between the predicted PG protein sequence and the major banding pattern on westerns.

Immuno light microscopy

Unpollinated and early and late pollinated stigmas were frozen in Tissue Tek (Miles Laboratories, Napeville, Illinois, USA) onto brass cryoholders with liquid nitrogen. Sections 8-10 μm thickness of stigmas were cut onto poly-l-lysine (Sigma, 0.1% w/v) coated glass slides with an International Equipment Company, Model CTD, Harris Cryotome. After 15 min fixation at 4°C in 4% paraformaldehyde (Sigma) in PBS (phosphate-buffered saline; Roche Molecular Biochemicals, Castle Hill, Sydney, Australia), rinsing 3 times in PBS, the slides were incubated 1 h at room temperature in PG polyclonal antibody (1:50 in PBS/1% BSA (Bovine serum albumin, Sigma). Negative control sections were incubated in buffer without the primary antibody followed by secondary antibody labelling. Following 3 washes in PBS the slides were incubated for 1 h at room temperature in goat anti-rabbit alkaline phosphatase conjugated secondary antibody (Sigma, 1:500 in PBS, 1% BSA) and after washing 3 times in PBS, the antigen-antibody complexes were visualised as described above. Photomicroscopy was carried out as above.

Results

Light Microscopy

When pollen grains are deposited onto the stigmatic surface they are in a dessicated state but immersion in water will cause immediate grain hydration (Fig. 1A). At 45 min post pollination, pollen grains have naturally hydrated and formed pollen tubes which have begun to penetrate into the stigmatic papillar wall (Fig. 1B). By 90 min pollen tubes could be seen passing through the papillar wall (Fig. 1C).

Immunoblotting of PG enzyme in tissues

The PG polyclonal antibody detected minor bands at approximately 74kDa and a major band of approximately 36 kDa in early and late (45 min, 90 min) pollinated stigmas (Fig. 2) which is less than the 42.4 kDa weight estimated for this protein (Robert et al. 1993). This major band was present in unpollinated stigmas but at a much lower level. The level of PG enzyme present did not appear to increase between early and late pollinated stigmas even though by this time pollen grains have germinated and pollen tubes have begun to penetrate through the pistil tissues.

Immuno light microscopy of unpollinated and early and late pollinated stigmas

Immunolabelling with the primary antibody showed that PG enzyme was present in unpollinated stigmas (Fig 3A). Negative controls which omitted the primary antibody showed no labelling in unpollinated stigmas (Fig. 3B). The PG enzyme was present in high concentrations in ungerminated pollen indicated by strong alkaline phosphatase labelling (Fig. 3C) which is substantially higher than the inherant colour of negative control pollen grains (Fig 3D). At 45 min the PG enzyme could be observed in pollen tubes which were beginning to penetrate stigmatic papillae (Fig. 4A). Negative controls of stigmas at this stage showed no labelling (Fig 4B). At 90 min post pollination, pollen tubes migrating through the papillar wall expressed PG protein throughout their cytoplasm and at their tips (Fig. 4C). Negative controls

of pollinated stigmas at this stage showed no labelling (Fig. 4D). Papillae from pollinated stigmas showed positive labelling at all three stages (Figs. 3C, 4A, 4C).

Discussion

Although many reports have documented the expression of cell wall degrading enzymes in germinated pollen (see Introduction) few studies have spatially localised such enzymes to the pollen-pistil interface *in vivo*. Hiscock et al (1994) have shown cutinase is present in the interface between pollen and papillae in B. napus while Dubald et al. (1993) have reported the expression of PG enzymes in *in vivo* germinated maize pollen. Although a previous study by Hong et al. (1997) has documented PG gene expression in germinated pollen in B. napus, details are unclear as to the *in vitro/in vivo* status of this experiment. To our knowledge, therefore, our study may represent the first microscopic localisation of *in vivo* expressed pollen tube PG in a dicotyledonous plant.

The location of the enzyme in pollen tubes in contact with papillar walls suggests that the PG enzyme is involved with the papillar wall modifications observed during pollination in this genus (Elleman and Dickinson 1990; Elleman et al. 1992; Dearnaley et al. 1999). As the enzyme belongs to the exo-PG family (Robert et al. 1993) it possibly degrades the polygalacturonate end chains of the pectin of stigma cell walls. In concert with other, yet to be determined cell wall modifying enzymes released by the pollen tube tip, this wall loosening may provides a means for the tube to migrate towards the ovary.

Hadfield and Bennett (1998) suggest alternative roles for pollen tube PG enzymes other than pistillar cell wall degradation. These authors suggest PG could be simply providing wall precursors for tube growth or that it could be involved with the modification of tube guiding signalling molecules. Alternative explanations for the presence of PG enzyme in B. napus

pollen tubes should thus be kept in mind and more direct analyses on the role of PG during pollination using antisense technology or specific PG inhibitors are required for clarification.

Both Robert et al. (1993) and Hong et al (1997) have previously documented PG gene expression in developing anthers of B. napus and showed that the levels of PG transcript was highest in mature pollen. Our results indicate that the PG *protein* is also present in high concentrations in mature, ungerminated pollen. The fact that the western blots did not detect an increase in protein levels after germination may be attributed to the masking effects of the highly expressed PG protein in pollen grains. Alternatively, it may indicate that PG transcription and translation do not occur at germination. PG enzyme may therefore be similar in expression to pollen cutinase which is presynthesised in pollen probably to allow for rapid deployment at germination (Shayk and Kolattukudy 1977). Prestored pollen PG may also explain why papillar wall loosening has been shown to be insensitive to inhibitors of protein synthesis (Elleman and Dickinson 1996).

The fact that there is expression of PG in pistil tissues is a novel observation, as previous studies of PG gene expression have failed to localise PG to this region (Robert et al. 1993; Hong et al. 1997). Hong and Tucker (2000) have recently shown that PG genes are expressed in the stigmas of tomato while Allen and Lonsdale (1993) have shown PG gene expression in non reproductive tissue in transgenic tobacco. Dubald et al. (1993) have noted the presence of PG in non reproductive tissues of maize and suggested that PG plays a general role in cell wall modification in this species. It is likely the pistillar PG expression we have observed is of this nature although the suggestions of Elleman and Dickinson (1994, 1996) that the stigmatic papillae contain pollination involved wall modifying substances may be pertinent.

The molecular weight discrepancy between the predicted protein sequence and the major band recorded on the westerns suggest that the PG protein undergoes a number of post translational

modifications before its final configuration. Analysis with PSORT software suggests a number of potential modifications including removal of an ER membrane retention signal, N-myristoylation anchor and a vacuolar targeting motif. This latter sequence suggests a possible location of the PG protein in pollen tubes. As the tip of growing B. napus pollen tubes have been shown to contain large populations of vesicles/vacuoles (Dearnaley et al. 1999) it may indicate that PG protein is present in these organelles until exocytotic release. Further studies with immuno electron microscopy would be necessary to confirm this.

One of the reasons for studying cell wall modifying enzyme expression in the Brassicaceae is that we are interested in understanding the cellular basis of self-incompatibility (SI) in this family. Ikeda et al. (1997) have reported that an early step of SI in Brassica involves the activation of aquaporin-like channel protein in stigma cells which may direct water from the pollen-papilla contact zone. Our (Dearnaley et al. 1999) and other studies (Ockendon 1978; Zuberi and Dickinson 1985) have demonstrated that high humidity will enable hydration of self-incompatible pollen grains, highlighting the importance of stigma cell wall penetration as a checkpoint for SI. Brown and Crouch (1990) have shown that PG protein is not present in self-incompatible pollinations in Oenothera. Interestingly, the basis for resistance of B. napus plants to the fungal pathogen, Leptosphaeria maculans appears to involve the production of PG-inhibiting substances which may prevent hyphal penetration of host cell walls (Annis and Goodwin 1997). A follow up study is therefore necessary to determine the behaviour of PG protein in SI pollinations in this species.

Other possible stigma cell degrading enzymes have previously been investigated in Brassica pollen. These include pectic esterases (Albani et al. 1991) and cutinases (Hiscock et al. 1994; Lavithis and Bhalla 1995). This present study demonstrates that PG enzyme may also play an important role in wall modification in pistil tissues in this genus. Although it is likely that

pectin and cutin degradation is a necessary precursor for successful pollinations in this species, degradation of other components of the stigma cell wall must surely be involved in this process. Investigations of cellulase, hemicellulase and other pectin degrading enzymes in pollinated stigmas may reveal more about the cellular control of the stigmatic penetration process.

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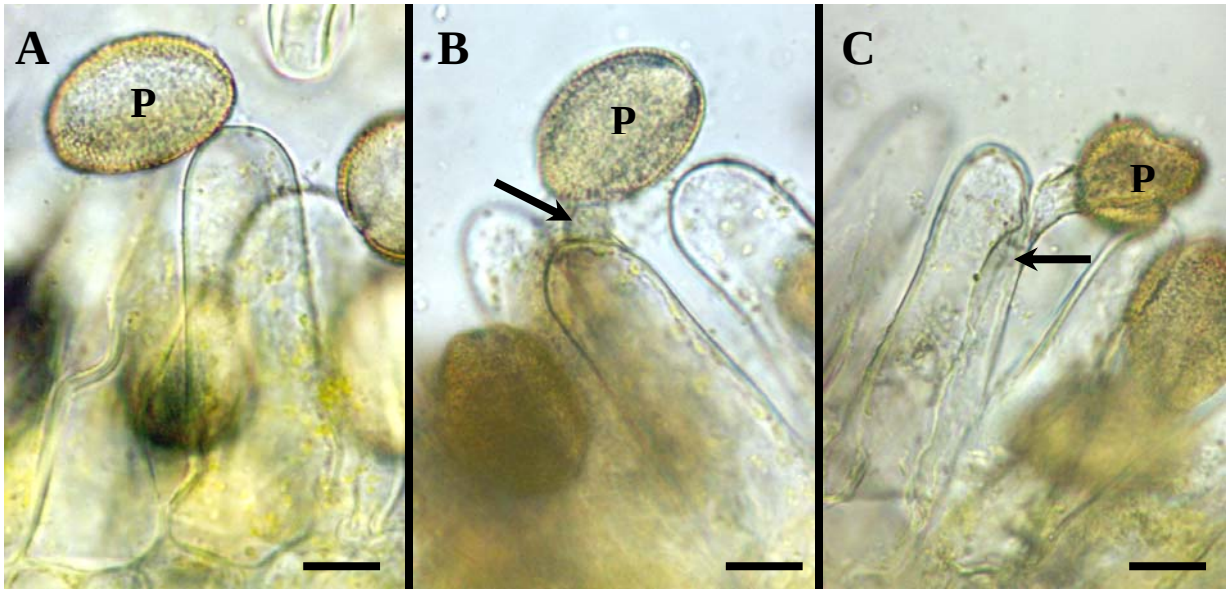


Fig. 1A-C Light photomicrographs of early (at pollination) and late (45 and 90 min post pollination) pollinated stigmatic papillae. **A** Pollen immediately after brushing onto the stigmatic surface. Note the pollen grain appears hydrated as the stigma has been mounted in distilled water. **B** A pollen grain at 45 min post pollination with a tube beginning stigmatic penetration (arrow). **C** A pollen grain at 90 min post pollination with a tube growing within the papillar cell wall (arrow). P = Pollen grain. Scale bars = 13 μm (A, B), 15 μm (C).

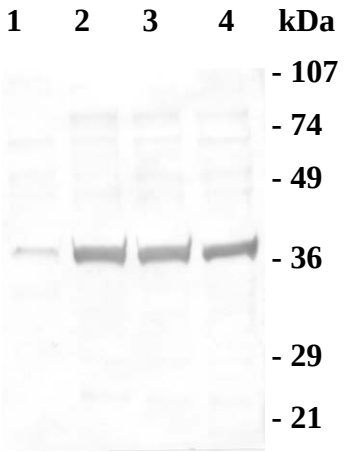
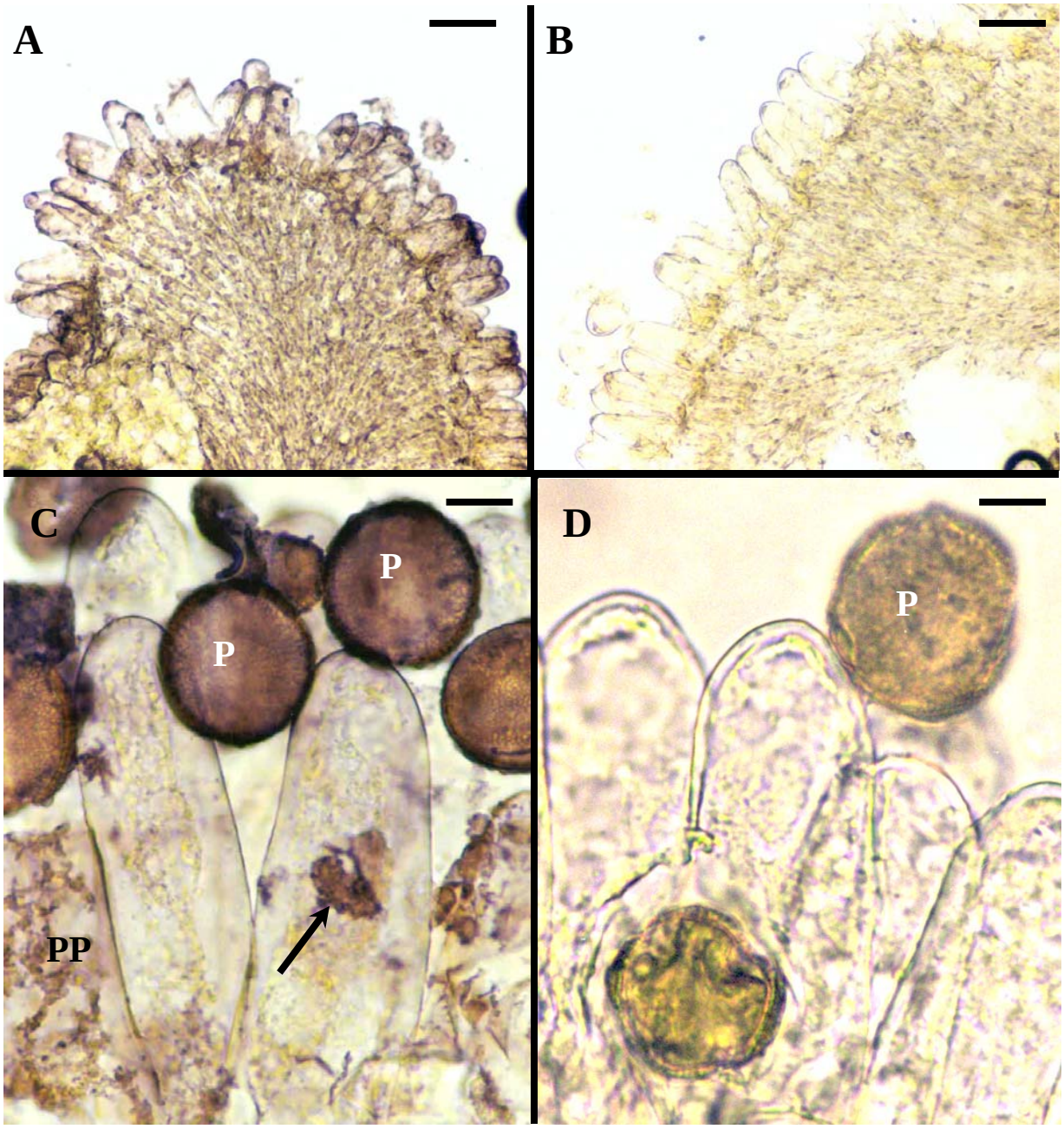


Fig. 2 Western blot of stigmas of *B. napus* probed with PG polyclonal antibody. Lane 1. Unpollinated stigma. Lane 2. Early pollination. Lane 3. Stigma 45 min post pollination. Lane 4. Stigma 90 min post pollination.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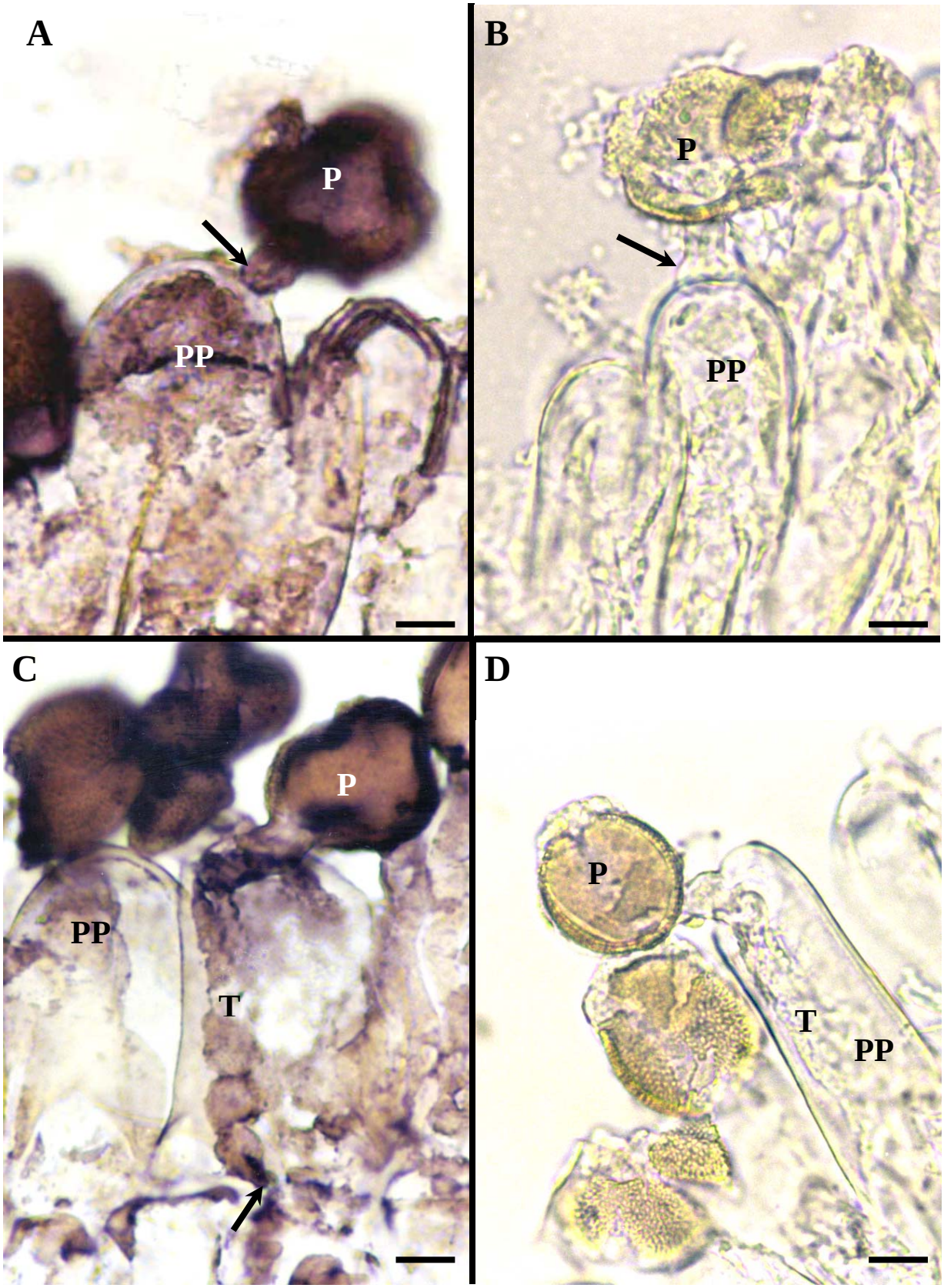
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Fig. 3A-D Light photomicrographs of cryosectioned stigmas immunolabelled with the PG polyclonal antibody. Unpollinated stigmas with (**A**) and without (**B**) PG primary antibody. Ungerminated pollen (P) on papillae with high levels of PG expression (**C**). Note papillae with levels of PG expression (PP). Fragments of heavily staining pollen wall can be seen on papillae (arrow). (**D**) Negative control of stigma with ungerminated pollen (P) showing absence of labelling. Scale bars = 60 μm (A), 52 μm (B), 11 μm (C), 9 μm (D)**.**

Fig. 4A-D Light photomicrographs of cryosectioned stigmas immunolabelled with the PG polyclonal antibody. **A** Late pollination (45 min post pollination) showing high levels of PG protein in pollen grain (P), pollen tube (arrow) and papillae (PP). **B** Negative control of germinated pollen 45 min post pollination showing absence of labelling in pollen grain (P), tube (arrow) and papillae (PP). **C** Pollen tube (at 90 min post pollination) growing within the papillar wall with PG expression in the pollen tube (T) and the tube tip (arrow). Note PG expression in papillae (PP). **D** Negative control of germinated pollen 90 min post pollination showing absence of labelling in pollen grain (P), tube (arrow) and papillae (PP). Scale bars = 6 μm (A), 8 μm (B), 9 μm (C), 10 μm (D)**.**