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Cytological Study of Pollen Tube Growth and Early Seed Development in *Petunia inflata*

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Pollen tube growth from the stigma into the ovule, and the early fruit and seed development following fertilization were examined using fluorescence microscopy, scanning electron microscopy and light microscopy in *Petunia inflata*. After growing intercellularly in the transmitting tract for 24-36 hr, the pollen tubes emerged into the top part of the ovary cavity and grew along the surface of the septum to reach the ovule. It grew around the furnicle and penetrated the micropyle to enter the embryo sac for fertilization. After fertilization, the endosperm nucleus divided first before the embryo, and the cell wall formation occurred following the division, exhibiting the pattern of cellular type of endosperm development. The first division of the zygote did not occur until 3 days after pollination. At 6 days after pollination, the seeds grew considerably and the endosperm has gone through multiple rounds of cell division. High starch formation in the integument, especially around the embryo sac, was also observed.

Key words: pollen tube growth, *Petunia inflata*, seed development, longitudinal section of ovary, microscopy

Plant sexual reproduction depends on intimate interactions between pollen and the pistil. Pollen grains germinate on the stigmatic surface, each extruding a tube that elongates in the stigmatic and the stylar transmitting tissue until it enters the ovule and penetrates the embryo sac. The pollen tube tip bursts inside the embryo sac and releases two sperm cells for fertilization (Dumas and Mogensen, 1993; Mascarenhas, 1993; Hulskamp *et al.*, 1995). The process of double fertilization results in the production of two structures: a diploid embryo and a triploid endosperm. The zygote undergoes a complex series of morphological and cellular changes resulting in the formation of mature embryo (reviewed by West and Harada, 1993). The endosperm is a source of food for the germinating seeds and, in some plants, for the embryo. The endosperm also carries out complex developmental processes which are coordinated with the embryo development (reviewed by Lopes and Larkins, 1993).

Numerous cytological and molecular studies regarding pollen tube growth *in vivo* and *in vitro*, specially on regulation of gene activity during pollen tube growth, cytoskeleton and cell wall structure of pollen tube, and pollen tube-style interactions, have been reported (Rosen, 1971; Sedgley, 1979; Reger *et al.*, 1992; reviewed by Mascarenhas, 1993; Derksen *et al.*, 1995; Janson and Willemse, 1995). In *Petunia*, since the early studies on *in vitro*-pollen tube growth (Maheswari, 1949), only recently research on *in vivo* pollen tube growth has been launched. In *Petunia* and maize, it was shown that flavonols are important for pollen development and pollen tube growth (van der Meer *et al.*, 1992; Shirley, 1996). Flavonol biosynthesis of petunia and other species in Solanaceae family has been investigated and genes involved in the process was reported (Holton *et al.*, 1993; van Eldik *et al.*, 1997). A pollen-expressed gene encoding a putative pectin esterase which may play a role during pollen

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tube growth was also identified in *Petunia inflata* (Mu and Kao, 1994). Additionally, pollen tube growth of *Petunia* has been studied in a different aspect using gametophytic self-incompatibility system, in which the style recognizes self- from nonself-pollen and rejects self-pollen during pollen tube growth (Lee *et al.*, 1994; Huang *et al.*, 1994).

Female gametophyte development, fertilization, and embryo/endosperm development have been intensively studied using cytological and anatomical methods (Lopes and Larkins, 1993; Reiser and Fischer, 1993; West and Harada, 1993; Brown *et al.*, 1994). With recent advances in genetics and molecular biology, underlying cellular mechanisms of those processes have just begun to unfold. In *Petunia*, only a small volume of research has been conducted in this field, including early works of van Went (1970a; 1970b), in which ultrastructure of egg/central cell and fertilized embryo sac were examined in *P. hybrida* using transmission electron microscopy. However, post-fertilization events including early embryo and endosperm development have never been investigated in *Petunia*. In this report, we cytologically examined *in vivo* pollen tube growth and early seed development of *Petunia inflata*, using fluorescence microscopy, scanning electron microscopy and light microscopy.

MATERIALS AND METHODS

Fluorescence Microscopy of Pollen Tubes in the Pistil

The pistils were collected at 12 and 36 hr after pollination, and fixed for 24 hr in a fixing solution composed of absolute ethanol:acetic acid:37% formaldehyde:H_2O (50:5:10:35, v/v). Then the outer ovary wall was removed to expose the ovules. After hydration through graded ethanol series (50% to 0% ethanol) for 30 min each, the tissues were incubated in 10 N NaOH for 2 hr at room temperature and washed with distilled water for 30 min twice. The ovaries were stained in 0.015% toluidine blue (neutral pH) for 15 min, washed in distilled water for 1 hr, and stained in 0.1% aniline blue (in 2% K_3PO_4, pH 10-12) for 1 hr. The whole pistil was then gently squashed onto a microslide in a drop of glycerol and observed under an Axiophot photomicroscope equipped with epifluorescence optics (Zeiss, Thornwood, NY).

Scanning Electron Microscopy

For scanning electron microscopy, the pistil was collected at 24, 36 and 48 hr after pollination, and fixed in a fixing solution composed of absolute ethanol:acetic acid:37% formaldehyde:H2O (50:5:10:35, v/v) for 24 hr. After rinsing with 70% ethanol, the tissues were incubated in 90% ethanol for 30 min, then in absolute ethanol for 30 min twice. After critical point drying in CPD020 Critical Point Dryer (Balzers Union, Balzers, Furstentum Liechtenstein), the outer ovary wall was removed from the ovary to expose the ovules and associated pollen tubes. The samples were gently placed on stubs and coated with 10 nm gold-palladium with Balzers Union sputter coater. Observations were made with a JEOL T300 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Light Microscopy of Longitudinal Sections of Ovary

For light microscopy of longitudinal sections of ovaries at different days after pollination, ovaries were fixed in 1.5% gluteraldehyde and 2.5% paraformaldehyde for 14 hr at room temperature under vacuum. The samples were then washed three times with 0.1 M phosphate buffer (PB), pH 7.4, and fixed in 1% osmium tetroxide in PB for 5 hr at room temperature. After washing in several changes of PB, the tissues were dehydrated with graded ethanol series (50% to 100%) for 30 min each. The ovaries were then stained in 2% uranyl acetate in 100% ethanol at 4°C overnight, washed in 100% ethanol and then 100% propylene oxide, and infiltrated with Spurrs medium (EM Sciences, Gibbstown, USA) and propylene oxide (50%/50%, 75%/25%, 100%, 100% for 8 hr each). The resin was polymerized for 12 hr at 70°C. Thin sections were prepared with LKB III ultramicrotome (LKB-Produkter AB, Bromma, Sweden) and observed on a Austoplan microscope (Leitz, Wetzlar, Germany).

RESULTS

Fluorescence Microscopy of Pollen Tubes in the Pistil

Pollen tube growth *in vivo* from the stigma to the ovule was examined in *Petunia inflata*, using aniline blue staining and fluorescence microscopy as previously described (Smith and McCully, 1978; Muschietti *et al.*, 1994). The process of pollen tube growth began with pollen hydration on top of papillar cells, followed by the subsequent pollen tube germination. The emerged pollen tube penetrated the papillar cell

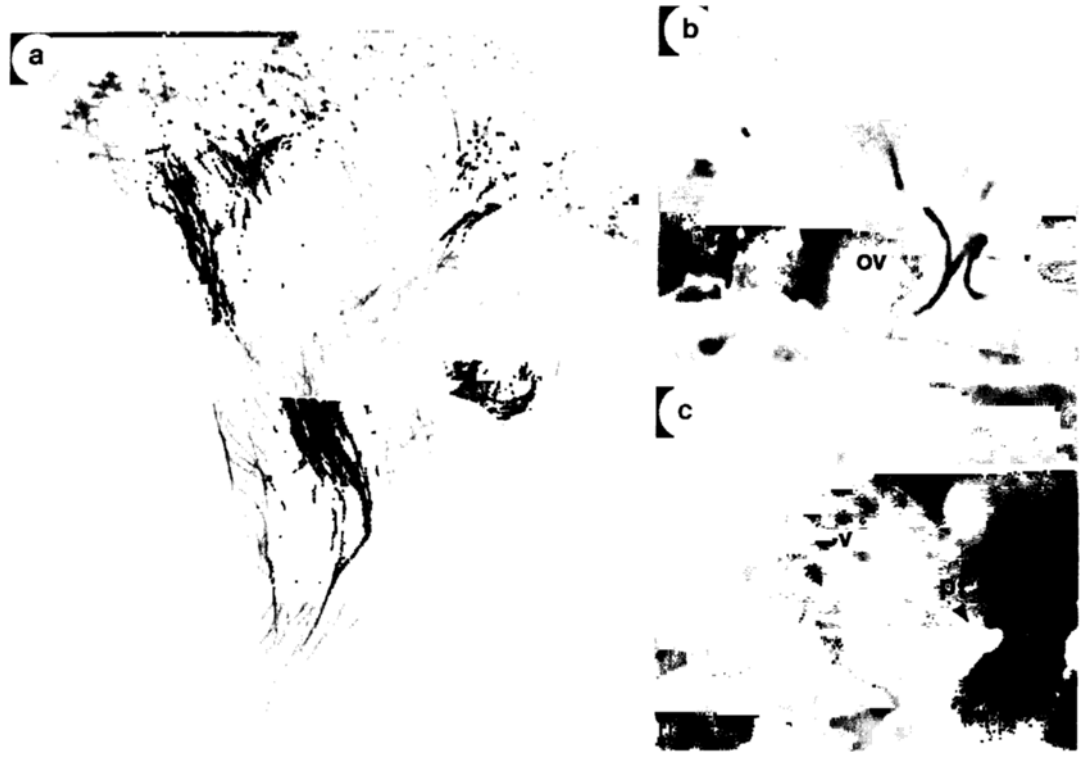

Fig. 1. Fluorescence micrographs of pollen tubes: (a) in the pistil; (b) inside some of the ovules (ov, ovule; pt, pollen tube); (c) entering the micropyle. Arrows shown in (b) and (c) indicate the pollen tubes.

layers (results not shown). At the base of the papillar cell, the pollen tube entered the transmitting tract and commenced intercellular, descending growth in a straight course. At 12 hr after pollination (HAP), the pollen tube grew approximately one third of the style as shown in Fig. 1a. At this stage, callose plugs in the pollen tube wall was not observed. Eventually pollen tube emerged from the transmitting tract onto the surface of the septum, proceeded to a furniculus, and then penetrated the micropyle of the ovule at 36 HAP. Fig. 1b and 1c shows aniline blue staining of the pollen tube penetrated into the micropyle at 36 HAP. At 72 HAP, fluorescence of the pollen tube residues inside the ovule was no longer visible due to the thickening seed coat.

Scanning Electron Microscopy of Pollen Tube Growth in the Ovary

To investigate how pollen tubes emerge onto the surface of septum in the ovary from the stylar transmitting tract and grow to the ovules, ovaries at 24, 36, and 48 HAP were collected and examined by scanning electron microscopy. Outer ovary wall and a number of the ovules were removed to visualize the path of pollen tube growth in the ovary. At 24 HAP, growing pollen tubes did not emerge from the style into the ovary yet, and the surface of the septum was clear (Fig. 2a). At 36 HAP, a bundle of pollen tubes emerged from the transmitting tract into the ovary cavity were clearly visible (Fig. 2b). The pollen tubes grew along the surface of the septum underneath the ovules to reach the closest available ovule. At 48 DAP, the surface of the septum was completely covered by multiple pollen tubes (Fig. 2c). Fig. 2d shows the pattern of pollen tube growth around the ovules at a higher magnification. The growing pollen tubes competed each other for the target and curved back and forth to reach the micropyle of the available ovule (Fig. 2e and 2f).

Study of Early Seed Development Using Light Microscopy

To investigate early seed development of *P. inflata,* longitudinal sections of ovaries at 6 DAP (days after pollination) (Fig. 3a), 3 DAP (Fig. 3c), and 0 DAP (Fig. 3d) were examined under a light microscope. 0 DAP represents mature but unpollinated state of flowers. Ovaries at 6 DAP from flowers pol-

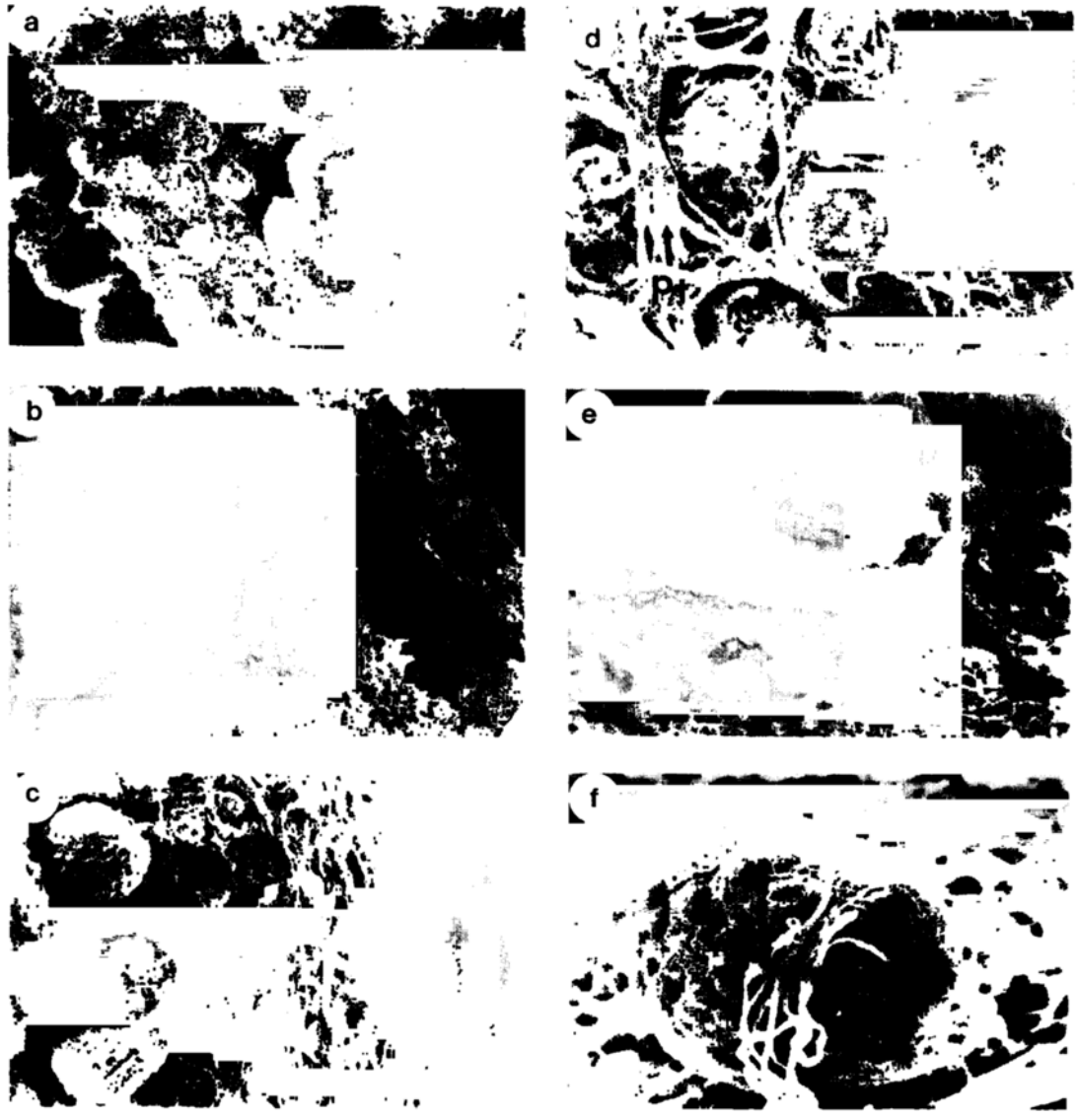

Fig. 2. Scanning electron micrographs of pollen tubes in the ovary. (a) Pollen tubes did not enter the ovary yet (ov, ovary), and the surface of the septum was clear. (b) Pollen tubes emerged from the style into the ovary (pt, pollen tube). (c) The pattern of pollen tube growth on the surface of the septum. (d) The pattern of pollen tube growth on the surface of the septum (at higher magnification). (e) Growing pollen tubes underneath the ovules. (f) Pollen tubes associated with the ovule. The outer ovary wall and some ovules were removed to visualize the associated pollen tubes.

linated with only limited amount of pollen were also sectioned and examined (Fig. 3b). Individually sectioned ovules at 6 DAP (Fig. 3f), 3 DAP (Fig. 3g) and 0 DAP (Fig. 3h) were visualized at higher magnification. In *P. inflata*, an ovary contains approximately 200 ovules which become fully mature by anthesis (Lee *et al.*, 1997). The embryo sac development of *Petunia* follows the pattern of Polygonum type (van Went, 1970a; Lee *et al.*, 1997). In *P. inflata*, the embryo sac consists of an egg apparatus (one egg and two synergids), a central cell containing fused polar nuclei and three degenerating antipodal cells (Lee *et al.*, 1997). By anthesis, the embryo sac, especially the central cell, of *P. inflata* becomes filled with large plastids (Fig. 3h; Lee *et al.*, 1997).

At 36-48 HAP, the synergid of the embryo sac was penetrated by the pollen tube and fertilization soon followed. At 3 DAP, the embryo was still at the zygote stage prior to the first mitosis, whereas the primary endosperm had already divided and the cell wall formation occurred following the division (Fig. 3g). Starch accumulation in the integument,

Fig. 3. Bright field micrographs of longitudinal sections of the ovary. (a) an ovary at 6 DAP; (b) an ovary at 6 DAP, from the flower which was pollinated with limited amount of pollen (f, fertilized ovule); (c) an ovary at 3 DAP; (d) an ovary at 0 DAP (0 DAP represents mature but unpollinated state of flowers); (e) a part of ovary at 6 DAP; (f) an ovule at 6 DAP (en, endosperm nucleus); (g) an ovule at 3 DAP (*zg*, zygote embryo); (h) an ovule at 0 DAP (p, plastids); (i) an aborted ovule at 6 DAP. The scale bars in (a)-(e) indicate 300 micrometer; in (g), 30 micrometer; in (f),(h), and (i), 50 micrometer.

specially near the embryo sac, was also more visible in ovules at 3 DAP (Fig. 3g) than in ovules at 0 DAP (Fig. 3h). In contrast, starch previously accumulated in the embryo sac at 0 DAP was no longer observed at 3 DAP (Fig. 3g). At 6 DAP, the size of the seed became considerably larger and the seed coat thickened. The endosperm went through multiple rounds of cell division (Fig. 3f), while the embryo development progressed to the globular stage (data not shown). High starch formation in the integument, especially around the developing endosperm, was observed. Interestingly, when limited amount of pollen (less than 50 pollen) was applied to the stigma, only ovules located at the top part of the ovary became fertilized to develope into seeds (Fig. 3b). Thus the pollen tube which emerged from the style into the top part of the ovary cavity appeared to enter preferentially the ovule which was closely localized, which is consistent with the observation in *Arabidopsis* (Hulskamp *et al.*, 1995). A naturally aborted ovule which contained degenerating embryo sac and integument was observed at 6 DAP (Fig. 3i).

DISCUSSION

Pollen tube growth is a highly regulated process in which growth phases could be distinguished: growth on the papillar cell, intercellular growth within the transmitting tract, emergence on the surface of the septum, and growth of the pollen tube toward the ovule on the surface of the septum. Once the fertilization takes place, embryo and endosperm development follows to form mature seeds. In this report, *in vivo* pollen tube growth from the stigma to the individual ovule and the following seed development in *Petunia inflata* were investigated using fluorescence microscopy, scanning electron microscopy and light microscopy.

In *P. inflata*, which has the "wet" stigma, pollen hydration and germination occurred indiscriminately, and as a result incompatible pollen could also germinate and start the initial growth on top of the stigma. However their growth subsequently became arrested, and fertilization could not take place (results not shown). In plants with the "dry" stigma, such as *Brassica* and *Arabidopsis*, incompatible pollen could not hydrate and germinate properly on the stigma surface (Reviewed by Nasrallah and Nasrallah, 1993). In *Arabidopsis*, pollen surface lipids are thought to signal the stigma to initiate the pollen hydration process (Preuss *et al.*, 1993).

In *P. inflata*, the pollen tubes grew one third of the style through the transmitting tract at 12 HAP. We did not find callose plugs in the pollen tube wall at the stage, suggesting that the pollen tube growth has not switched from the autonomous growth to the heterotrophic growth (Mulcahy and Mulcahy, 1983). When incompatible pollen tubes grow in the style, gametophytic self-incompatibility interaction in *Petunia* occurs at this stage, which is characterized by arrest of pollen tube growth, irregular callose deposition on tube wall, and tip birst (Lee *et al.*, 1994). In addition, it has been suggested that Pollen Mitosis II, which generates two sperm cells from the generative cell, also occurs at this stage (Rogen, 1971).

Near 24 HAP, the pollen tubes emerged from the transmitting tract into the top part of the ovary cavity and grew along the surface of the septum to reach the ovule. The pollen tubes changed their growth direction to become associated with and eventually enter the ovule. At 36 HAP, the pollen tube penetrated the micropyle of the ovule, and fertilization took place, which resulted in the zygote embryo and triploid endosperm. The directional guide for pollen tubes occurring in the ovary was supported by many studies. Ovary fragments, extracts, and ovules from several species elicited chemotrophic responses from *in vitro*-grown pollen tubes (Reviewed by Mascarenhas, 1993). Furthermore, several *Arabidopsis* mutants defective in embryo sac development showed anomalous pollen tube guidance, in which the mutant ovules could not attract the pollen tubes, and as a result, fertilization could not be achieved (Hulskamp, 1995). These results suggest that intimate interactions between male and female reproductive system are required during *in vivo* pollen tube growth process.

We found that the endosperm development in *P. inflata* follows the pattern of cellular type, in which endosperm wall formation immediately follows the division (Fig. 3g), as other members of Solanaceae family, which include *Solanum nigrum* (Briggs, 1993a; 1993b), *Nicotiana tabacum* (Erdelska, 1985), and *Solanum phureja* (Dnyansagar and Cooper, 1960). Three main modes of early endosperm development has been recognized: nuclear, cellular, and helobial, depending on the development patterns of cellularization (reviewed by Brink and Cooper, 1947; Vijayaraghavan and Prabhakar, 1984). Nuclear type is shown in cereals, in which the primary endosperm nucleus undergoes several rounds of division without cytokinesis to generate a large number of free nuclei organized at the periphery of the central cell. Cytokinesis then initiates, progressing centripetally until the endosperm becomes entirely cellular. In the cellular type of endosperm development, mitosis and cytokinesis occur after the first division of the primary endosperm nucleus and persist throughout the endosperm development. Helobial type is an intermediate and infrequently found mode of the endosperm development.

In *P. inflata*, the endosperm fusion nucleus divided soon after fusion, but the zygote embryo did not carry out the first mitosis until 3 DAP. Thus mitotic activity begins earlier in the primary endosperm nucleus than in zygote, which may indicate an early dependency of the embryo on endosperm development. The same pattern has been observed in other species including french bean (*Phaseolus vulgaris*) (Yeung and Cavey, 1988) and pea (*Pisum sativum*) (Marinos, 1970). However it is not yet known how the interaction is established and regulated.

In this study, using a variety of cytological techniques, we examined pollen tube growth *in vivo* and early seed development in *P. inflata*. It would be important to study cellular mechanisms that underlie the physiological changes in plant reproduction. More detailed understanding of the cell biology, biochemistry, and physiology of the process would shed light on

some of the most basic questions in plant cell biology.

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