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Guidance in Vitro of the Pollen Tube to the Naked Embryo Sac of *Torenia fournieri*

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The precise guidance of the pollen tube to the embryo sac is critical to the successful sexual reproduction of flowering plants. We demonstrate here the guidance of the pollen tube to the embryo sac in vitro by using the naked embryo sac of *Torenia fournieri*, which protrudes from the micropyle of the ovule. We developed a medium for culture of both the ovule and the pollen tube of *T. fournieri* and cocultivated them in a thin layer of solid medium. Although pollen tubes that had germinated in vitro passed naked embryo sacs, some pollen tubes that grew semi–in vitro through a cut style arrived precisely at the site of entry into the embryo sac, namely, the filiform apparatus of the synergids. When pollen tubes were unable to enter the embryo sac, they continuously grew toward the same filiform apparatus, forming narrow coils. Pollen tubes selectively arrived at complete, unfertilized embryo sacs but did not arrive at those of heat-treated ovules or those with disrupted synergids. These results convincingly demonstrate that pollen tubes are specifically attracted to the region of the filiform apparatus of living synergids in vitro.

INTRODUCTION

During the fertilization of flowering plants, the pollen tube grows directionally inside the pistil and delivers the immotile male gametes to the embryo sac. Pollen tubes never enter the sporophytic cells of the pistil; instead, they grow through the extracellular matrix of the female sporophytic tissues. A unique feature of the directional growth of the pollen tube is that it is controlled by female sporophytic tissues along its path. The architecture, chemical properties, chemoattractants, substrate adhesion molecules, and intercellular communications of these tissues all appear to participate in such control (Mascarenhas and Machlis, 1962b; Heslop-Harrison, 1987; Lord and Sanders, 1992; Wilhelmi and Preuss, 1997).

Lipids within the stigmatic exudate of tobacco are essential for the directional growth of the pollen tube, enabling it to penetrate the stigma (Wolters-Arts et al., 1998). It has been proposed that these lipids control the flow of water to the pollen. In the stylar matrix of tobacco, transmitting tissue–specific (TTS) proteins, which belong to the arabinogalactan protein family, are incorporated into the walls of pollen tubes and facilitate the growth of pollen tubes toward the ovary (Cheung et al., 1995; Wu et al., 1995). Two genes of Arabidopsis, *POLLEN–PISTIL INTERACTION2* (*POP2*) and *POP3*, are expressed in both male and female sporophytic tissues and mediate the last phase of guidance of the pollen tube within the ovary (Wilhelmi and Preuss, 1996). A heterogeneous micropylar exudate is present in the micropyle of the ovule of *Paspalum* spp (Chao, 1971, 1977), *Ornithogalum caudatum* (Tilton, 1980), and sugar beet (Bruun and Olesen, 1989; Olesen and Bruun, 1990). It has been proposed that this exudate originates from nucellar and/or integumentary cells and facilitates the final growth of the pollen tube.

These successive controls exerted by female sporophytic tissues might potentially define the path along which the pollen tube grows to reach the embryo sac. Although the embryo sac is the target of the pollen tube in the pistil, it has never been shown whether the pollen tube is specifically attracted to the embryo sac in the absence of female sporophytic tissues along the pathway. Thus, the mechanism of targeting of the pollen tube remains unclear and has been a topic of considerable discussion for many years (Heslop-Harrison and Heslop-Harrison, 1986).

Genetic studies in Arabidopsis have shown that the target haploid embryo sac governs the final guidance of the pollen tube. For example, Hülskamp et al. (1995) analyzed the growth path of pollen tubes in some mutants of Arabidopsis that were defective in ovule development. Sporophytic mutations in the ovule tissue affected development of the embryo sac, and one mutant line, 54D12, had a variable phenotype with respect to the development of the embryo sac. In each flower of the 54D12 line, pollen tubes were guided mainly to ovules with normal embryo sacs, rarely to ovules with incomplete embryo sacs, and never to those that lacked embryo sacs. These observations suggested that the final phases of guidance of the pollen tube might be

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governed by the embryo sac or by the sporophytic tissues adjacent to the embryo sac that also control development of the embryo sac.

Ray et al. (1997) distinguished between these two possibilities by using a line of Arabidopsis that was heterozygous for a chromosomal reciprocal translocation: the semisterile line TL-1. In line TL-1, all diploid sporophytic cells are genotypically normal, whereas half of the haploid gametophytes are aborted because of duplication and deficiency of chromosomal arms during meiosis. In each TL-1 flower, pollen tubes are guided to ovules containing normal embryo sacs but not to the ovules containing degenerated embryo sacs. These observations suggest that the embryo sac itself governs the guidance of the pollen tube. However, as noted by Ray et al. (1997), it is also possible that a signal derived from the embryo sac might indirectly cause changes in the surface properties of the surrounding sporophytic cells among which the pollen tubes make their way.

Structural analyses of embryo sacs by light and electron microscopy support the possibility of chemotropic guidance by the embryo sac. The two synergids in the embryo sac, one of which receives the pollen tube, seem to be active in secretory functions, and they are believed to secrete some chemotropic substance that attracts the pollen tube (Huang and Russell, 1992a; Russell, 1992). The synergids of general angiosperms have a so-called filiform apparatus at their micropylar ends (Huang and Russell, 1992a). The filiform apparatus consists of cell walls of the two synergids that resemble the walls of transfer cells (Gunning and Pate, 1969). Resembling transfer cells, synergids are typically rich in mitochondria. These mitochondria have well-developed cristae and tend to be concentrated near the filiform apparatus (Huang and Russell, 1992a, 1994; Murgia et al., 1993). The activity of adenosine triphosphatase has been detected in the synergids of *Saintpaulia ionantha*, suggesting that the synergids secrete some molecules from the embryo sac (Mogensen, 1981). Similarly, the micropylar exudate of *Gasteria verrucosa* was proposed to originate primarily from the filiform apparatus of the synergids (Franssen-Verheijen and Willemse, 1993). A high concentration of calcium has also been reported in the synergids of several plants (Chaubal and Reger, 1990, 1992; Huang and Russell, 1992b; Tirlapur et al., 1993). These calcium molecules could potentially attract the pollen tube (Mascarenhas and Machlis, 1962a, 1964; Reger et al., 1992; Malhó and Trewavas, 1996). Despite many structural analyses, no researchers have reported either direct evidence of chemotropism to the synergid or a system in vitro for observing the directional growth of pollen tubes toward synergids.

To investigate whether the pollen tube is specifically attracted to the embryo sac in vitro, one must remove the sporophytic tissues around the embryo sac that might define the path of the pollen tube to the embryo sac. In most angiosperms, the embryo sac is located inside the ovule and is covered with thick layers of the integument and the nucellus. These sporophytic tissues of the ovule cannot be readily removed, and difficult techniques are required for isolation of embryo sacs. However, the embryo sacs of some species of *Philadelphus*, *Thesium*, *Galium*, *Utricularia*, *Vandellia*, and *Torenia* protrude from the micropyle of the ovule (Maheshwari, 1950).

In *Torenia fournieri*, the naked embryo sac grows through the micropyle and along the funiculus to the placenta (Balicka-Iwanowska, 1899; Guilford and Fisk, 1952; Tiwari, 1982). The egg cell, the two synergids, and approximately half of the central cell are located outside the ovule in this species. In the ovary of *T. fournieri*, the surface of the placenta is covered with mucilaginous material, and the micropylar portion of the naked embryo sac is immersed within it (Balicka-Iwanowska, 1899; Guilford and Fisk, 1952; Van der Pluijm, 1964; Tiwari, 1982). Pollen tubes grow through the mucilaginous material on the surface of the placenta and enter the naked embryo sac through the region of the filiform apparatus of the synergids (Van der Pluijm, 1964; Higashiyama et al., 1997). When ovules of *T. fournieri* are simply excised from the placenta, naked embryo sacs are directly exposed to the medium (Erdelská, 1974; Higashiyama et al., 1997). If the naked embryo sac itself was the source of some directional signal to attract the pollen tube, then the pollen tube should also be attracted to the filiform apparatus of exposed embryo sacs.

In this study, we developed a method for the cocultivation of ovules and pollen tubes of *T. fournieri*, which we used to examine the attraction of the pollen tube to the naked embryo sac. With this system, we were able to observe the growth of pollen tubes directly toward the filiform apparatus of the synergids, thereby providing evidence that pollen tubes are specifically attracted to the embryo sac in vitro. Moreover, the specific conditions that are required to support accurate pollen tube guidance offer insight into the guidance mechanism.

RESULTS

Structural Features of the Ovule and the Naked Embryo Sac of *T. fournieri*

Figure 1A shows a living ovule of *T. fournieri* that was excised from the placenta. The integument of the ovule is thin, and the embryo sac protrudes from the micropyle of the ovule. The egg cell, the two synergids, and approximately half of the central cell are contained in the extramicropylar region of the embryo sac. The egg cell and the two synergids usually cannot be observed together in the same focal plane. Figure 1B shows a high-magnification view of a living naked embryo sac, with the focus on the two symmetrical synergids. The cell walls of the two synergids comprise a well-developed filiform apparatus at their micropylar ends. The pollen tube enters the embryo sac between the two synergids through the region of the filiform apparatus. The wall of the embryo sac is ruptured at the region of the fili-

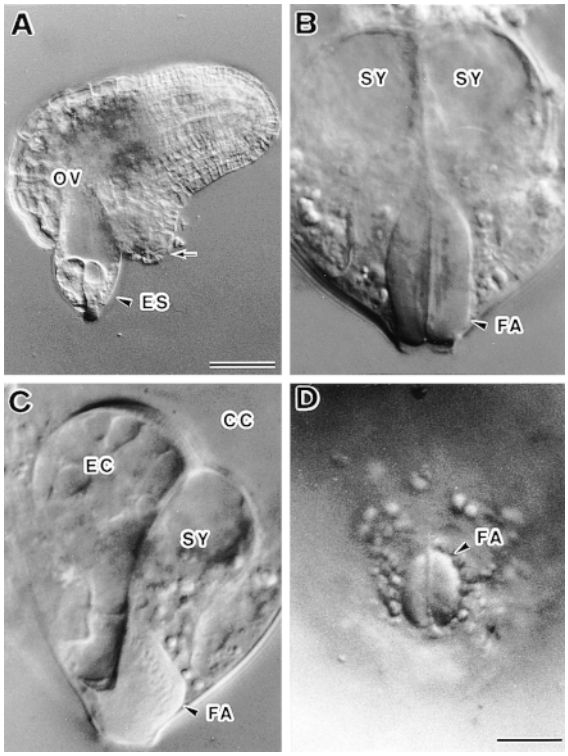

Figure 1. The Living Ovule and Naked Embryo Sac of *T. fournieri.*

(A) An ovule excised from the placenta. An arrow indicates the cut end of a funiculus.
(B) A naked embryo sac with the focus on the two synergids.
(C) A naked embryo sac with the focus on the egg cell and one of the two synergids.
(D) A naked embryo sac observed horizontally from the micropylar end. CC, central cell; EC, egg cell; ES, embryo sac; FA, filiform apparatus; OV, ovule; SY, synergid. Bar in **(A)** = 50 μm; bar in **(D)** = 10 μm for **(B)** to **(D)**.

form apparatus; thus, the filiform apparatus of the two synergids is exposed directly to the medium in vitro. Figure 1C shows a naked embryo sac, with the focus on the egg cell and one of the two synergids. The filiform apparatus of the synergid occupies the micropylar end of the embryo sac, and the base of the egg cell does not face the end of the embryo sac. This arrangement is more apparent when a naked embryo sac is observed horizontally from the micropylar end (Figure 1D).

Preparation of a Medium for Cocultivation of Ovules and Pollen Tubes

We previously used the medium developed by Nitsch (1951) for cultivation of ovules of *T. fournieri* (Higashiyama et al., 1997). Nitsch's medium also supported the growth of pollen tubes of *T. fournieri*; however, growth was poor. To cocultivate ovules and pollen tubes of *T. fournieri*, we modified Nitsch's medium so that it would support the growth of pollen tubes (Table 1). We examined the effects of various factors on the growth of pollen tubes in a preliminary investigation (data not shown). Calcium, borate, and sugar were required for the growth of pollen tubes of *T. fournieri*, as is the case for most angiosperms. Among the various components of Nitsch's medium, H_2SO_4 neutralized with KOH inhibited the growth of pollen tubes. To promote the growth of pollen tubes, we eliminated H_2SO_4 from Nitsch's medium and increased the amount of H_3BO_3 from 0.5 to 10 mg/L (Table 1). Other modifications were made to improve the viability and survival of explants. The original concentration of sucrose, 5.0% (w/v), appeared to be the optimum concentration not only for culture of the naked embryo sac but also for culture of the pollen tube.

Figure 2 shows pollen tubes of *T. fournieri* during growth in the original (Figures 2A and 2C) and the modified (Figures 2B and 2D) versions of Nitsch's medium. In each version of Nitsch's medium, pollen tubes were cultured under two different sets of conditions: Figures 2A and 2B show pollen tubes that had germinated on the medium (in vitro), and Figures 2C and 2D show pollen tubes that had emerged from the cut ends of pollinated styles and continued to grow in the medium (semi–in vitro). Compared with those in Nitsch's original medium (Figures 2A and 2C), many pollen tubes grew longer in the modified version (Figures 2B and 2D). Callose plugs were also deposited normally in the modified version. The growth of pollen tubes differed between the two culture conditions, even in the same medium. Compared

Table 1. Modification of Nitsch's Medium for Culture of Naked Embryo Sacs and Pollen Tubes of *T. fournieri*

Component	Nitsch's Medium[a] (mg/L)	Modified Medium (mg/L)
NH_4NO_3	—[b]	80
KNO_3	125	125
$Ca(NO_3)_2{\cdot}4H_2O$	500	500
$MgSO_4{\cdot}7H_2O$	125	125
KH_2PO_4	125	125
$FeC_6O_5H_7{\cdot}5H_2O$	10	—
H_2SO_4[c]	0.5 mL/L	—
$MnSO_4{\cdot}4H_2O$	3	3
$ZnSO_4{\cdot}7H_2O$	0.5	0.5
H_3BO_3	0.5	10
$CuSO_4{\cdot}5H_2O$	0.025	0.025
$Na_2MoO_4{\cdot}2H_2O$	0.025	0.025
Sucrose	50,000	50,000
Casein	—	500

[a] Nitsch (1951).
[b] Dashes indicate the absence of the component.
[c] Specific gravity = 1.83.

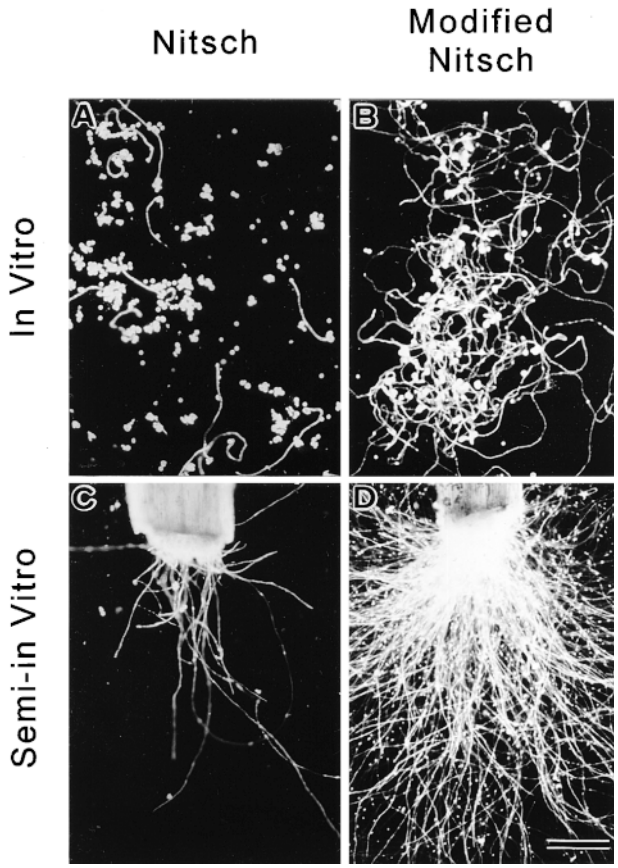

Figure 2. Dark-Field Images of Pollen Tubes before and after Modification of Nitsch's Medium.

(A) Pollen tubes germinated on Nitsch's medium.
(B) Pollen tubes germinated on modified Nitsch's medium.
(C) Pollen tubes growing in Nitsch's medium through a cut style.
(D) Pollen tubes growing in modified Nitsch's medium through a cut style.
Bar in **(D)** = 0.5 mm for **(A)** to **(D)**.

with pollen tubes in vitro (Figures 2A and 2B), pollen tubes under semi–in vitro conditions elongated in straight lines, at higher growth rates, and with smaller diameters (Figures 2C and 2D). Moreover, accidental bursting of pollen tubes occurred less frequently, and pollen tubes became longer under semi–in vitro conditions. Both culture conditions were tested for cocultivation with ovules.

Growth of the Pollen Tube toward the Micropylar End of the Naked Embryo Sac

To investigate whether the pollen tube is directly attracted to the naked embryo sac in vitro, we cocultivated ovules and pollen tubes of *T. fournieri.* We had to use a liquid medium for excision of the tender, fragile ovules from ovaries. However, pollen tubes did not grow toward naked embryo sacs in the liquid medium. Therefore, we attempted cocultivation in solid medium prepared with ultra-low gelling temperature agarose. Ovules were excised from the placenta in a liquid medium that contained ultra-low gelling temperature agarose and cultured after the medium had cooled and solidified. In each experiment, ovules were excised from one ovary.

For cocultivation with pollen tubes, pollen grains were first dusted on the medium in which ovules were being cultivated (in vitro; Figure 3A). To count the number of pollen tubes correctly, we dusted pollen grains at the center of crowds of ovules. On the thin layer of medium solidified with ultra-low gelling temperature agarose, the pollen had a low germination frequency (30%; $n = 3000$ pollen grains; Figure 3B). Over the course of 10 experiments, a total of 1930 pollen tubes were monitored. The pollen tubes elongated around ovules, but no pollen tubes reached the micropylar end of naked embryo sacs (Table 2).

Next, we cocultivated ovules with a pollinated style (semi–in vitro; Figure 3C). Pollen tubes began to emerge ~6 hr after pollination from the cut end of a style 15 mm in length, and they continued to grow in the solid medium (Figure 3D). Even when cultured in the thin layer of medium used for in vitro conditions, the growth of pollen tubes was well maintained semi–in vitro, as mentioned above (Figure 2). When cultured with ovules, pollen tubes tended to meander (Figure 3D), whereas pollen tubes cultured without ovules grew straight (Figure 2D). Under these culture conditions specifically, some pollen tubes were observed to grow directly toward the micropylar end of the naked embryo sac without associating with any sporophytic cells of the ovule (Figures 3E through 3G). Moreover, pollen tubes grew precisely toward the central region of the filiform apparatus between the two synergids (Figures 3H and 3I). This site corresponds to the site of entry of the pollen tube into the naked embryo sac for the succeeding double fertilization. Over the course of 21 experiments, we monitored 6804 pollen tubes. Among these, 273 (4.0%) were found to reach the micropylar end of the naked embryo sac within 24 hr after the start of cultivation (Table 2). Wide variation was observed from experiment to experiment, with averages of between 1.2 and 13.3% of pollen tubes reaching the micropylar end.

A total of 8190 ovules were monitored during the same experiments. Of these, 210 (2.6%) were in contact with pollen tubes (Table 2). The number of pollen tubes reaching embryo sacs (a total of 292) was larger than the number of embryo sacs in contact with pollen tubes (a total of 210) because several pollen tubes sometimes grew toward a single embryo sac, as shown in Figure 3G.

On average, 5 ± 4 pollen tubes had passed within 100 μm of an embryo sac when a pollen tube reached that embryo sac ($n = 45$). Meandering pollen tubes tended to reach embryo sacs, whereas pollen tubes growing in straight lines at the bottom of the medium passed embryo sacs. Although

the density of pollen tubes was higher near the cut end of the style, pollen tubes tended to grow in straight lines in this area. Consequently, the arrival of pollen tubes at the micropylar end of naked embryo sacs was observed at equal frequencies throughout each culture. The number of pollen tubes in each culture ranged from 177 to 564 and the number of ovules from 262 to 572. At least within these ranges, we observed no correlation between the number of pollen tubes that arrived at embryo sacs and the number of pollen tubes or embryo sacs (R^2 = 0.069 and 0.004, respectively). Arrival of the pollen tube did, however, seem to increase when two pollinated styles were present in each culture and to decrease when a few pollen grains were deposited on a stigma (data not shown). High densities of pollen tubes not only hampered accurate counts of their numbers but also interfered with observations.

To investigate whether the arrival of pollen tubes at the micropylar end of naked embryo sacs was the result of accidental collision, we cocultivated heat-treated ovules and pollen tubes (Table 2). When ovules were excised from an ovary that had been heat treated at 90°C for 5 min, all ovular sporophytic and gametophytic cells were dead, but the profile of the ovules remained. During 11 heat treatment experiments, we monitored 2992 pollen tubes. Pollen tubes elongated normally around heat-treated ovules, as in the case of cocultivation with intact ovules. The pollen tubes tended to meander also. However, only one pollen tube (0.03%) reached the micropylar end of a naked embryo sac. Thus, accidental collision of pollen tubes with this small target seemed to occur very rarely.

Subsequently, intact and heat-treated ovules excised from each ovary were mixed and cocultivated by scattering them uniformly throughout a culture (Table 2). Heat-treated ovules were readily distinguished from intact ones by their degraded cellular components and brownish chloroplasts within the integument. Over the course of five experiments, 1475 pollen tubes were monitored. Of these, 60 (4.1%) selectively reached the micropylar end of the naked embryo sac of intact ovules (Table 2). No pollen tubes reached the micropylar end of the naked embryo sac of heat-treated ovules. Frequencies of pollen tubes arriving at embryo sacs (4.1%) and ovules coming into contact with pollen tubes (3.0%) were similar to those cultured without heat-treated ovules (4.0 and 2.6%, respectively; Table 2). These observations indicate that some fraction of pollen tubes selectively grew toward intact ovules and precisely reached the micropylar end of naked embryo sacs.

Behavior of Pollen Tubes Growing toward the Naked Embryo Sac

We next observed in detail the behavior of pollen tubes growing toward the naked embryo sac. Figure 4 shows a sequential series of images of pollen tubes growing toward a naked embryo sac. In Figure 4, two pollen tubes reached the same filiform apparatus in succession. The first pollen tube (PT1) grew at 8.1 μm/min and reached the filiform apparatus in the frame labeled 6 min. PT1 was unable to enter the embryo sac, and the tip of the pollen tube slipped off the filiform apparatus. PT1 did not, however, leave this naked embryo sac. PT1 continued to grow toward the filiform apparatus, forming a narrow coil, as observed in frames labeled 9 min to 18 min (Figure 4).

The second pollen tube (PT2) grew at 7.0 μm/min and reached the filiform apparatus in the frame labeled 18 min (Figure 4). Tips of both PT1 and PT2 precisely reached the filiform apparatus upon first contact with the naked embryo sac. Pollen tubes adjusted their directions toward the filiform apparatus during their growth in the medium and never collided with other regions of the naked embryo sac before reaching the filiform apparatus. Such behavior is illustrated in Figures 3E through 3H, and it appeared to be common to pollen tubes when they reached the naked embryo sac. The tip of PT2 was held on the surface of the filiform apparatus and pushed its tube back in the frame labeled 22 min. Such behavior is more evident in Figure 3I; the coiling part of the tube had been pushed back. The tips of both PT1 and PT2 did not adhere to the filiform apparatus and sometimes slipped off the filiform apparatus because of accumulated physical pressure. Both PT1 and PT2 continued to grow toward this filiform apparatus, with considerable coiling, but they did not enter the embryo sac.

Figure 5 shows the variations in the fate of pollen tubes after their arrival at the naked embryo sac. Seventy-eight percent of such pollen tubes (213 of 273) did not enter the embryo sac, and most of them coiled considerably, as shown in Figures 5A and 5B. This coiling was due to the continuous growth of the pollen tube toward the filiform apparatus, as shown in Figure 4. Pollen tubes did not depart from the region of the filiform apparatus. Thus, the coiling behavior of pollen tubes indicates that the pollen tube was continuously attracted to the region of the filiform apparatus. When pollen tubes were cocultivated with heat-treated ovules or other parts of explants, such as leaf disks or stem segments, no pollen tubes formed such a narrow coil and grew toward a definite target continuously (data not shown). When a pollen tube did collide with the micropylar end of the naked embryo sac of a heat-treated ovule (Table 2), the tube changed its direction of growth and left the ovule. Among the pollen tubes that failed to enter the embryo sac, some tubes ruptured and ceased to grow before coiling considerably.

Twenty percent of pollen tubes (54 of 273) appeared to enter the naked embryo sac through the region of the filiform apparatus and to discharge their contents toward one of the two synergids (Figure 5C). One of the synergids had completely degenerated and was filled with opaque contents of the pollen tube, as also occurs in vivo (Higashiyama et al., 1997). Most of the tubes entered the embryo sac without much coiling. We failed to observe and record the discharge process in this culture condition. It remains unclear whether double fertilization was completed in these embryo

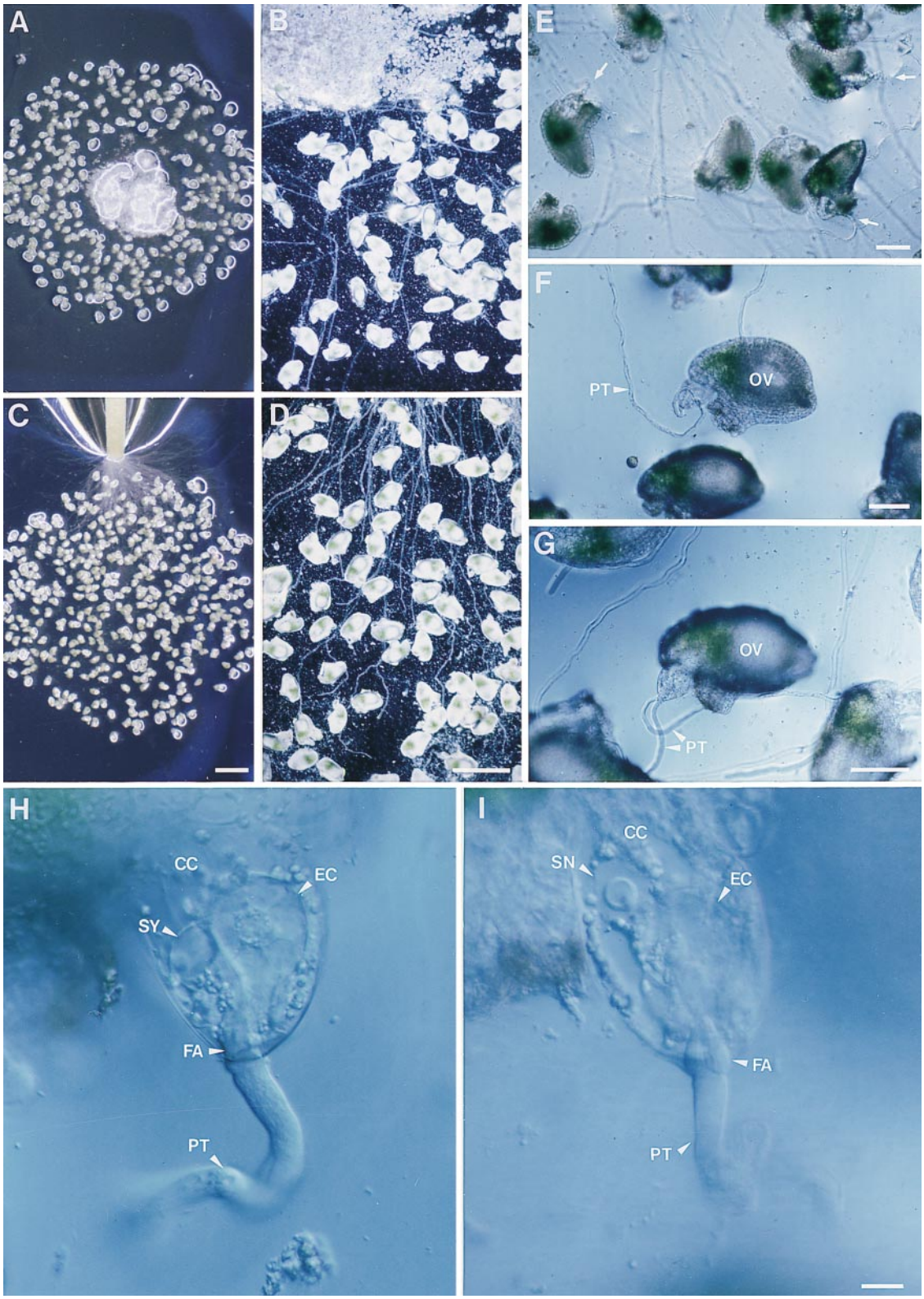

Figure 3. Growth of the Pollen Tube toward the Micropylar End of the Naked Embryo Sac during Cocultivation of Ovules and Pollen Tubes.

sacs. The other 2% of pollen tubes (six of 273) showed unusual behavior: they entered the naked embryo sac but did not cease to grow within the sac (Figure 5D).

As shown in Figure 6, the arrival of the pollen tube was evident mainly from 10 to 20 hr after the start of cultivation, and the coiling of pollen tubes gradually became extensive. Arrival of pollen tubes was most frequently observed in cultures between 14 and 16 hr after the start of cultivation, namely, ~10 hr after the emergence of pollen tubes from the cut end of the style. Discharge of the contents of the pollen tube into the embryo sac was most frequently observed between 12 and 14 hr after the start of cultivation, earlier than the peak of the arrival of tubes.

Condition of the Embryo Sac for Attraction of Pollen Tubes

The coiling behavior of pollen tubes suggests that pollen tubes were attracted to the region of the filiform apparatus of naked embryo sacs. These embryo sacs that were in contact with pollen tubes appeared to be always viable and complete (Figures 3E to 3I), whereas there were many disrupted and incomplete embryo sacs in the vicinity. We next investigated the correlation between the condition of embryo sacs and the arrival of pollen tubes (Table 3).

At the extramicropylar region of the embryo sac, four gametophytic cells, namely, the egg cell, the two synergids, and the central cell, are observed (Figure 1). When ovules were excised from an unpollinated flower, as in the above-described experiments, there were five types of embryo sac at the start of cultivation: wholly disrupted; damaged with only the central cell remaining; with the central cell and the egg cell remaining; with the central cell, the egg cell, and one of the two synergids remaining; and complete. The relative levels of each of five types of embryo sac were 38.0 ± 11.0%, 0.8 ± 0.8%, 23.9 ± 8.1%, 8.7 ± 4.0%, and 28.7 ± 10.8%, respectively, at the start of cultivation (n = 21 experiments). There were no embryo sacs in which the egg cell had been broken but the synergids remained intact. The synergids appeared to be very fragile.

During the cultivation of ovules, the viability of gametophytic cells within the naked embryo sac decreased gradually. Fourteen hours after the start of cultivation, when the arrival of pollen tubes was most conspicuous (Figure 6), 74.7 ± 11.3% of naked embryo sacs were wholly disrupted (Table 3). There were only 8.8 ± 7.3% complete embryo sacs. However, among 203 pollen tubes, 197 (97%) selectively arrived at these complete embryo sacs. There also seemed to be a slight correlation between the number of tubes arriving and the relative number of complete embryo sacs (R^2 = 0.32). Pollen tubes were rarely associated with embryo sacs in which one or both synergids had been broken. Four of 203 (2%) pollen tubes were associated with the micropylar ends of embryo sacs of which one synergid had been disrupted, and two of 203 (1%) pollen tubes were associated with those of which both synergids had been disrupted. The synergids of such embryo sacs might, however, have been broken after the arrival of the pollen tube but before the subsequent observation (i.e., within 2 hr). We also observed some pollen tubes moving away from embryo sacs in which synergids had been disrupted after the arrival of the pollen tube.

A sixth type of embryo sac (1.5 ± 1.8%) was observed 14 hr after the start of cultivation. These embryo sacs appeared to have received the contents of a pollen tube, as shown in Figure 5C. No more pollen tubes reached an embryo sac that had received a pollen tube (Table 3). Thus, pollen tubes appeared not to be attracted to embryo sacs that had received a pollen tube. An embryo sac that had succeeded in accepting a pollen tube was always complete before the pollen tube discharged its contents.

Very few embryo sacs received pollen tubes in each culture: only 1.5 ± 1.8% at 14 hr after cultivation (Table 3). To confirm whether pollen tubes are not attracted to an embryo sac that has already received a pollen tube, we next used ovules excised from pollinated flowers. Within 10 hr after pollination, ovules that locate at the apical region of each

Figure 3. (continued).

(A) Ovules cultured with pollen grains.
(B) Dark-field image of ovules and pollen tubes cultured as shown in **(A)**. Pollen tubes were grown in vitro.
(C) Ovules cultured with a pollinated style.
(D) Dark-field image of ovules and pollen tubes cultured as given in **(C)**. Pollen tubes were grown semi–in vitro.
(E) Arrival of pollen tubes at the micropylar end of naked embryo sacs. Three pollen tubes have reached the micropylar end of three naked embryo sacs (arrows). All of the pollen tubes shown here and in **(F)** to **(I)** were cultured semi–in vitro, as given in **(C)**.
(F) A pollen tube that has reached the micropylar end of a naked embryo sac in an ovule.
(G) Two pollen tubes that have reached one naked embryo sac at the same time.
(H) A pollen tube in contact with the filiform apparatus of the synergid. The egg cell and one of the two synergids are in focus.
(I) A pollen tube in contact with the central region of the filiform apparatus between two synergids.
CC, central cell; EC, egg cell; FA, filiform apparatus; OV, ovule; PT, pollen tube; SN, secondary nucleus; SY, synergid. Bar in **(C)** = 1 mm for **(A)** and **(C)**; bar in **(D)** = 0.5 mm for **(B)** and **(D)**; bars in **(E)** to **(G)** = 100 μm; bar in **(I)** = 10 μm for **(H)** and **(I)**.

ovary receive a pollen tube, and most of them are fertilized (Higashiyama et al., 1997). Ovules were excised from pollinated flowers 10 hr after pollination, and they were cocultivated with pollen tubes (Table 3). Fourteen hours after the start of cocultivation, 6.1 ± 3.7% of embryo sacs (n = 11 experiments) were unfertilized and remained complete, whereas 6.3 ± 5.7% of embryo sacs had received a pollen tube and had viable gametophytic cells, namely, the central cell, the egg cell, and the persistent synergid. Among 53 pollen tubes scored, all selectively arrived at complete, unfertilized embryo sacs and not at those that had received a pollen tube (Table 3). This result suggests that pollen tubes are not attracted to an embryo sac that has already received a pollen tube. Embryo sacs that had received a pollen tube in the ovary were readily distinguished from those that had received a pollen tube in vitro by the broken and shrunken structure of the pollen tube; all 6.3 ± 5.7% embryo sacs in this course of experiments had received a pollen tube in the ovary.

Although pollination causes some physiological changes in the embryo sac of *T. fournieri* before the arrival of the pollen tube (Higashiyama et al., 1997), no apparent differences in the capacity for attraction and the efficiency of acceptance of pollen tubes were observed in vitro between pollinated and unpollinated ovules. Under our culture conditions, it seemed more difficult to maintain the viability of naked embryo sacs that had been excised from pollinated flowers than the viability of those from unpollinated flowers.

DISCUSSION

In this study, we showed that in *T. fournieri*, pollen tubes arrived at the micropylar end of the naked embryo sac when cultured with ovules under semi–in vitro conditions. Pollen tubes seldom collided with the micropylar end of the naked embryo sac of heat-treated ovules, and they selectively arrived at the sac of intact ovules (Table 2). Moreover, pollen tubes selectively arrived at the complete, unfertilized embryo sac (Table 3). The frequency with which ovules contacted pollen tubes was only 2.6% with respect to the total number of ovules (Table 2). However, only 8.8% of ovules contained a complete, unfertilized embryo sac at 14 hr after the start of cultivation (Table 3), when the arrival of pollen tubes was most frequently observed (Figure 6). Thus, among these ovules that contained a complete, unfertilized embryo sac, ~30% were in contact with pollen tubes. These results indicate that the arrival of pollen tubes did not reflect the accidental collision of growing pollen tubes with naked embryo sacs. It is suggested that pollen tubes were selectively guided to the complete, unfertilized embryo sac of ovules in vitro.

The guidance was remarkably precise, as we observed in vivo. The micropylar end of the naked embryo sac is occupied by the filiform apparatus of the synergids; the short axis of the oval surface of the filiform apparatus is only ~10 μm in length (Figure 1D). Each pollen tube, which was ~9 μm in diameter, was precisely guided to the central region of this small target between the two synergids (Figure 3I). This site corresponds to the site of entry of the pollen tube into the embryo sac in *T. fournieri* (Van der Pluijm, 1964; Higashiyama et al., 1997) and also in cotton (Jensen and Fisher, 1968) and soybean (Dute et al., 1989; Folsom and Cass, 1992).

Moreover, when pollen tubes were unable to enter the embryo sac, they continued to grow toward the filiform apparatus, slipping on the surface of the filiform apparatus and forming narrow coils (Figures 4 and 5). They never departed from their respective target embryo sacs as long as the sacs remained complete. This coiling behavior of the pollen tube toward the filiform apparatus is indicative of pollen tubes that are specifically attracted to this region of the embryo sac within ovule tissues. It has been extremely difficult to demonstrate guidance of the pollen tube in vitro in a con-

Table 2. Frequency of the Arrival of Pollen Tubes at the Micropylar End of the Naked Embryo Sac of Ovules[a]

Culture Conditions		No. of Pollen Tubes		No. of Ovules		
Pollen Tubes	Ovules	Total	Reached Embryo Sacs[b]	Total	Contact with Pollen Tubes[c]	No. of Experiments
In vitro	Intact	1930	0 (0%)	3790	0 (0%)	10
Semi–in vitro	Intact	6804	273 (4.0%)	8190	210 (2.6%)	21
Semi–in vitro	Heat treated[d]	2992	1 (0.03%)	3476	1 (0.03%)	11
Semi–in vitro	Mixed (intact/heat treated)	1475	60 (4.1%)	1520/1450	45 (3.0%)/0 (0%)	5

[a] Frequency was investigated 24 hr after the start of cultivation.
[b] The numbers within parentheses give the relative frequency of pollen tubes reaching the micropylar end of naked embryo sacs with respect to the total number of pollen tubes.
[c] The numbers within parentheses give the relative frequency of ovules making contact with pollen tubes with respect to the total number of ovules.
[d] Whole ovaries were heat treated at 90°C for 5 min before the excision of ovules.

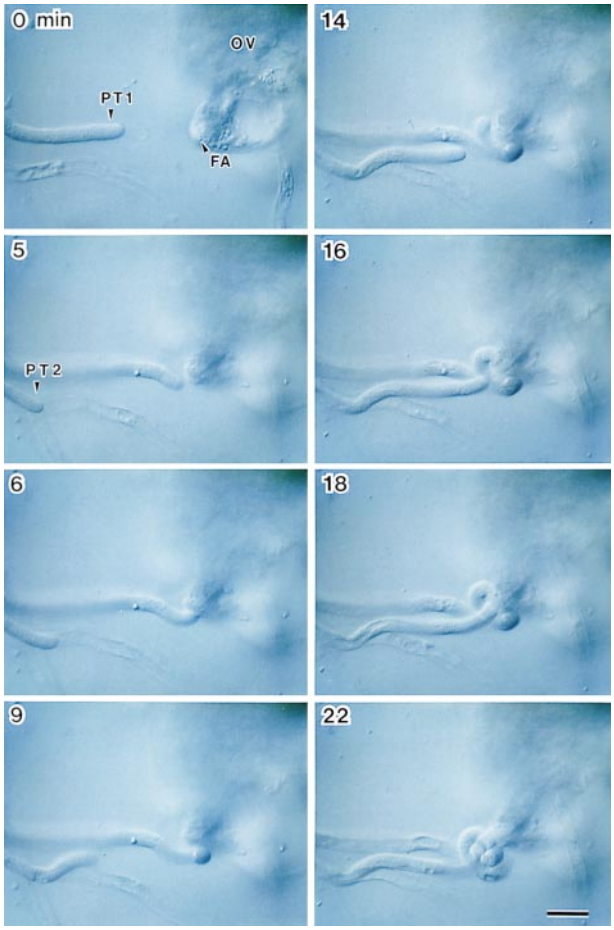

Figure 4. A Sequential Series of Images of Two Pollen Tubes Growing toward the Filiform Apparatus of the Synergids.

The passage of time is indicated at upper left in minutes. FA, filiform apparatus; OV, ovule; PT1, the first pollen tube; PT2, the second pollen tube. Bar = 30 μm.

vincing manner (Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987). However, our results provide ample evidence that genuine guidance of the pollen tube to the embryo sac has been achieved in vitro.

Pollen has a high capacity to support its own activity during germination and growth of the tube (Vasil, 1987). In our system, pollen tubes that had germinated on the medium elongated around ovules (Figure 3B). However, these pollen tubes were never guided to the embryo sac (Table 2). Germination of the pollen on the stigma and passage of the tube through the cut style were essential for guidance (Table 2). Thus, these female sporophytic tissues must contribute to the guidance capability of the pollen tube.

The extracellular matrix of the style tissue is enriched with secretory materials, such as free sugars, polysaccharides, amino acids, glycoproteins, and glycolipids, that might support the growth of pollen tubes. TTS proteins of tobacco, which belong to the arabinogalactan protein family, are incorporated into the walls of pollen tubes and promote their growth (Cheung et al., 1995; Wu et al., 1995). After pollen tubes emerged from the cut end of the style, the physiological condition of the pollen tubes appeared to be maintained even in the medium; compared with pollen tubes germinating in vitro, pollen tubes under semi–in vitro conditions elongated in straight lines, at higher growth rates, and with smaller diameters. This type of support for growth from female sporophytic tissues might be necessary for the pollen

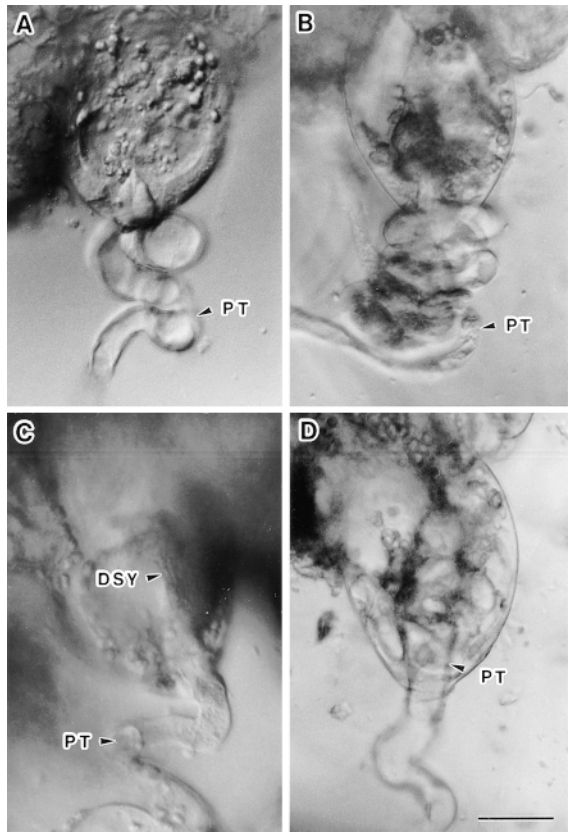

Figure 5. Variations in the Fate of a Pollen Tube after Its Arrival at the Filiform Apparatus in Vitro.

(A) A coiled pollen tube that failed to enter the embryo sac but grew continuously toward the filiform apparatus.
(B) A pollen tube coiled to a greater extent than that shown in **(A)**.
(C) A pollen tube that succeeded in discharging its contents into the naked embryo sac.
(D) A pollen tube that entered the embryo sac but did not cease to grow.
DSY, degenerated synergid; PT, pollen tube. Bar in **(D)** = 20 μm for **(A)** to **(D)**.

tube to acquire the ability to respond correctly to the directional signal to the embryo sac. Cheung et al. (1995) also used a semi–in vitro system of tobacco and successfully demonstrated that TTS proteins attract pollen tubes in vitro.

In several plant species, mature seeds can be obtained in vitro without the style tissue when pollen grains are directly dusted over ovules that have been cultivated with the placenta tissue (test tube fertilization; Kanta et al., 1962; Kanta and Maheshwari, 1963; Shehata and Zenkteler, 1991). In *G. verrucosa*, pollen tubes that have germinated on the medium penetrate the micropyle of ovules in vitro and precisely reach the synergid within ovule tissues (Plyushch et al., 1995). The embryo sac of these plants resides inside the ovule and is covered with thick layers of ovular sporophytic tissues. Unlike the guidance to the exposed embryo sac of *T. fournieri* in vitro, guidance to such an embryo sac within ovule tissues might be contributed by sporophytic tissues around the embryo sac. Some ovules of *T. fournieri* are also fertilized without the style tissue when pollen grains are directly introduced inside the excised ovary (Shehata and Zenkteler, 1991).

In our system, only a small fraction of pollen tubes was guided to the embryo sac (Table 2). It might be possible to improve the frequency with which pollen tubes are guided by increasing dependence on the female reproductive system. For example, the surfaces of the placenta and the naked embryo sac of *T. fournieri* are covered with mucilaginous material (Balicka-Iwanowska, 1899; Guilford and Fisk, 1952; Van der Pluijm, 1964), which might originate from sporophytic tissues (Tiwari, 1982). The addition of this mucilaginous material might make pollen tubes more responsive to the directional signal and result in an increase in the frequency of guidance. It might also be possible to investigate whether extracts of the style tissue substitute for the stigma and the style in germinating pollen grains. Working to improve the present system may tell us a great deal about what is required for high-efficiency guidance of the pollen tube.

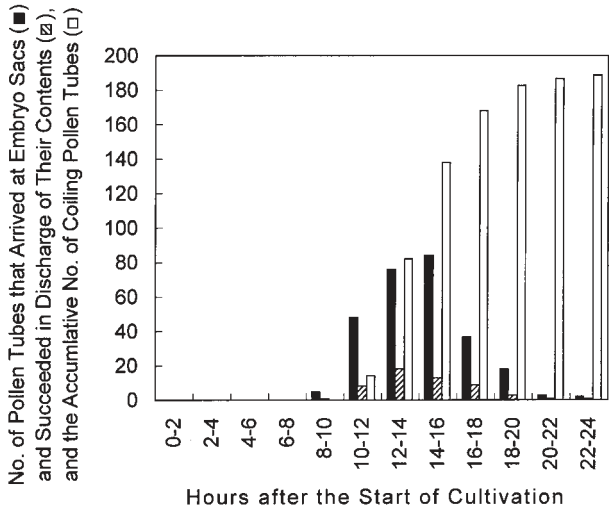

Figure 6. Time Course of the Guidance Process in Vitro.

Shown are time courses for the arrival of pollen tubes at the filiform apparatus (black bars), the discharge of the contents of pollen tubes into the naked embryo sac (striped bars), and the accumulation of coiling pollen tubes that failed to enter the naked embryo sac (white bars). Pollen tubes began to emerge from the cut end of the style at 6 hr after the start of cultivation.

The mechanism(s) that underlies targeting of the pollen tube has been a topic of considerable discussion for many years (Heslop-Harrison and Heslop-Harrison, 1986). Ray et al. (1997) showed genetically that the final stage of guidance was governed by the haploid embryo sac in Arabidopsis. The possibility that the embryo sac contributes to pollen-tube guidance is also supported in *T. fournieri* by the strict correlation between guidance and the physiological condition of the embryo sac (Table 3). Ray et al. (1997) proposed two types of control for guidance by the embryo sac: one is that the embryo sac directly secretes some signaling molecules, and the other is that the signal derived from the embryo sac indirectly causes the change in the surface properties of the surrounding sporophytic cells. In *T. fournieri*, pollen tubes were attracted directly to exposed embryo sacs in vitro, without any need for contact with sporophytic tissues around the embryo sac. It is likely that the living embryo sac emits some directional signal to attract pollen tubes, especially through the region of the filiform apparatus between the synergids. Our observations in *T. fournieri* support the former hypothesis, direct control, but do not discount the possibility that the two types of control operate together in the pistil.

As determined from the small number of pollen tubes that were attracted to each embryo sac and the ability of multiple pollen tubes to grow toward the same embryo sac, the signaling activity for guidance seemed to be effective over a relatively short distance. This signal does not appear to be effective when the embryo sac has already received a pollen tube (Table 3). This mechanism might be involved in the efficient targeting of the pollen tube to an unfertilized embryo sac in *T. fournieri*. It is most likely that the directional signal to the embryo sac at the final stage of guidance is provided by the target embryo sac itself, but we cannot completely exclude the possibility that ovular sporophytic cells might participate in this process because we cultivated whole ovules. Enzymatic isolation of both the embryo sac and individual gametophytic cell is possible in *T. fournieri* (Mól, 1986), as in other plants (Theunis et al., 1991). Experiments with isolated embryo sacs and isolated gametophytic cells might provide more insight into the origin of pollen tube–attracting activity. Culture conditions determined in this study should provide a basis for such experiments in *T. fournieri* and also in other plants.

In general, synergids appear to have active secretory functions at the ultrastructural level, and it has been pro-

Table 3. Correlation between Conditions of the Naked Embryo Sac and the Arrival of Pollen Tubes

	Condition of the Naked Embryo Sac[a]					
	Wholly Disrupted	CC Remaining	CC and EC Remaining	CC, EC, and 1 SY Remaining	Complete	Receiving PT
Ovules from unpollinated flowers[b]						
Frequency (%)[c]	74.7 ± 11.3	1.1 ± 1.0	11.2 ± 5.1	2.6 ± 1.4	8.8 ± 7.3	1.5 ± 1.8
Number of arrivals[d]	0 (0%)	0 (0%)	2 (1%)	4 (2%)	197 (97%)	0 (0%)
Ovules from pollinated flowers[e]						
Frequency (%)[c]	79.2 ± 9.8	0.5 ± 0.8	5.7 ± 1.5	1.8 ± 1.3	6.1 ± 3.5	6.7 ± 6.0
Number of arrivals[d]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	53 (100%)	0 (0%)

[a] Conditions of the naked embryo sac are classified into six types based on the survival of the four gametophytic cells, namely, the egg cell (EC), the two synergids (SYs), and the central cell (CC). An embryo sac in which one synergid had received a pollen tube and degenerated but other gametophytic cells remained intact was scored as the embryo sac receiving the pollen tube (PT).

[b] Ovules were excised from unpollinated flowers.

[c] Frequencies of each type of embryo sac were investigated 14 hr after the start of cultivation by monitoring 100 embryo sacs in each culture. Values are means ±SD of values from 21 experiments (ovules from unpollinated flowers) and from 11 experiments (ovules from pollinated flowers).

[d] The condition of embryo sacs was scored within 2 hr of the arrival of the pollen tube at the naked embryo sac. The numbers within parentheses give the relative frequency of the arrival of pollen tubes to each type of embryo sac with respect to the total number of arrivals (203 among ovules from unpollinated flowers and 53 among ovules from pollinated flowers).

[e] Ovules were excised from pollinated flowers 10 hr after pollination. Some ovules had already received a pollen tube in the ovary (Higashiyama et al., 1997).

posed that they secrete some chemoattractant through the filiform apparatus (Huang and Russell, 1992a; Russell, 1992). In *T. fournieri*, pollen tubes were always attracted to the region of the filiform apparatus of the synergids in vitro (Figure 3). Pollen tubes appeared not to be attracted to an embryo sac of which synergids had been disrupted (Table 3). Although further investigations are necessary to confirm the origin and the nature of pollen tube–attracting activity, living synergids might play an important role in attracting the pollen tube. Moreover, pollen tubes were attracted to the embryo sac in solid medium but not in liquid medium (data not shown). An adequate gradient in the concentration of the chemoattractants is considered to be crucial for chemotropic guidance of the pollen tube in vitro (Reger et al., 1992). Thus, it can be speculated that secretion and diffusion of attractants are adequately balanced in solid medium. For many years, many researchers have attempted but failed to identify chemoattractants derived from ovules (Reger et al., 1992). Our study should provide significant impetus to the search for such a chemoattractant.

The interactions between the pollen tube and the embryo sac are critical for the successful fertilization of flowering plants (Russell, 1996). This study has emphasized the necessity for precise gametophytic interactions. It is clear that strictly controlled physiological conditions of both gametophytes are required for precise pollen tube guidance in *T. fournieri.* After arrival at the embryo sac, pollen tubes must enter the sac and discharge their contents appropriately. The coiling behavior of the pollen tube suggests that some gametophytic interaction for the entrance of the pollen tube into the embryo sac has been impaired, although guidance has been accomplished. Similar overgrowth of the pollen tube within the embryo sac, as shown in Figure 5D, is observed after incompatible interspecific crosses in Rhododendron (Williams et al., 1986). The system established in this study might allow future physiological and molecular studies of gametophytic interactions between the pollen tube and the living embryo sac.

Establishment of this system also opens the way for analyses of double fertilization in vitro. We determined previously the precise time course of the entire process of double fertilization in *T. fournieri* by monitoring large numbers of living embryo sacs (Higashiyama et al., 1997). We realized at the same time that it was necessary to observe the entire series of fertilization events continuously if we were to identify the mechanisms of dynamic processes, such as the discharge of the contents of the pollen tube into the embryo sac and the transport of the two sperm cells to their target female gametes. Systems for in vitro fertilization of flowering plants with isolated gametes have been reported (Dumas and Faure, 1995; Kranz et al., 1998). However, there are no fertilization systems, to our knowledge, that involve the naked or isolated embryo sac and the pollen tube. In our system, some pollen tubes appeared to succeed in discharging their contents into the naked embryo sac (Figure 5C). Double fertilization might also occur in such embryo sacs, because we sometimes observed early embryogenesis and endospermgenesis, as was also observed in vivo (Higashiyama et al., 1997).

METHODS

Torenia fournieri was grown in a regulated chamber at 25°C with a 16-hr photoperiod (~150 μmol m^{-2} sec^{-1}). Flowers with freshly opened stigmas were hand-pollinated with their own pollen. Ovules and naked embryo sacs were observed as described previously (Higashiyama et al., 1997).

The medium described by Nitsch (1951) was modified for growth of *T. fournieri* pollen tubes. H_2SO_4 and $FeC_6O_5H_7 \cdot 5H_2O$ were removed, and the concentration of H_3BO_3 was increased to 10 mg/L. In addition, NH_4NO_3 at 80 mg/L and casein hydrolysate at 500 mg/L were included (pH 5.8, without adjustment). The effects of such modification on the growth of pollen tubes were examined on medium solidified with 0.15% Gellan gum (Wako Pure Chemical Industries Ltd., Osaka, Japan) and observed by dark-field microscopy, as described by Matsunaga et al. (1997).

After Gellan gum had been added at 0.3%, the modified version of Nitsch's medium was autoclaved at 120°C for 20 min. Then, 20-mL aliquots were solidified in 9-cm Petri dishes that had been modified for differential interference microscopy: squares of 30 × 40 mm^2 of the top and bottom of each dish were cut out and replaced by cover slips of 40 × 50 mm^2. Medium containing 1.5% ultra-low gelling temperature agarose (agarose type IX-A; Sigma) was also prepared and autoclaved at 120°C for 20 min.

A 2 × 2-cm square of medium on the lower cover slip was removed, and 200 μL of liquid medium that contained 1.5% ultra-low gelling temperature agarose was poured into the space. An ovary was harvested from a flower just after pollination, sterilized by 70% (v/v) ethanol, and immersed in the medium. When pollen tubes were cultured semi–in vitro, we also excised a 15-mm-long style from the pollinated flower. For experiments using heat-treated ovules, we harvested whole ovaries into a liquid medium and heat-treated them at 90°C for 5 min. Ovules were excised from each ovary and scattered uniformly under a stereoscopic microscope with forceps and a needle (SZ-PT; Olympus Optical Co., Tokyo, Japan). The excised style was placed with the cut end facing the crowd of ovules, at a distance of ~1 mm. The Petri dish was sealed and cooled at 4°C for 3 min to solidify the ultra-low gelling temperature agarose. The medium did not solidify completely, and the dish was tipped over to let liquid flow out of the square well. When pollen tubes were cultured in vitro, we dusted pollen grains at the center of crowds of ovules. Ovules and pollen tubes were cultured at 30°C in continuous darkness.

Ovules and pollen tubes cultured in such Petri dishes were observed under an inverted differential interference microscope (IMT-2; Olympus Optical Co.). Dark-field microscopy was performed as described by Matsunaga et al. (1997). The sequential image of growth of pollen tubes toward the naked embryo sac (Figure 5) was videorecorded in color with a chilled CCD camera (C5810; Hamamatsu Photonics Ltd., Hamamatsu, Japan).

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