

expression provides a plausible mechanism for varying the size and number of segments among the different ventral appendages.

Finally, these results raise the possibility that there may be a relationship between the developmental ground state appendage defined here and the ancestral leg (evolutionary ground state) that predates the arthropods. Although all arthropods have highly segmented legs, it is likely that the predecessor to the arthropods had simpler, unsegmented legs (28, 29). Because the developmental ground state appendage is a simple leglike appendage, it is plausible that at some time during evolution, legs developed without Hox or *hth* inputs. If nonarthropod phyla are surveyed for Hox and *hth* expression patterns, it may be possible to find an extant example of such an appendage.

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13. Because *Distal-less* (*Dll*) is a selector gene for all ventral appendages [N. Gorfinkiel, G. Morata, I. Guerrero, *Genes Dev.* **11**, 2259 (1997)], it is not required for differences in appendage identity. Similarly, *spinless* (*ss*) is required for both distal antennal and distal leg fates, where it is required for tarsal segmentation [D. M. Duncan, E. A. Burgess, I. Duncan, *Genes Dev.* **12**, 1290 (1998)]. Thus, *ss* and *Dll* are required for the ground state, and their activities are apparently modified by the identity selector genes.
14. Clones were generated by flp-mediated mitotic recombination: *Antp*⁻ clones: *y w; FRT82B Antp^{NS+RC3}/TM6B*; *Antp*⁻ *hth*⁻ clones: *y w; FRT82B Scr^{C1} Antp^{NS+RC3} hth^{P2}/TM2*; and *hth*⁻ clones: *y w; FRT82B hth^{P2}/TM2*. Males of these genotypes were crossed to *y hs-FLP; FRT82B hs-CD2, y+ M/TM2* females, and the progeny were heat-shocked for 30 min at 37°C, at 0 to 24, 24 to 48, 48 to 72, or 72 to 96 hours after egg laying (AEL). The Minute technique [G. Morata, P. Ripoll, *Dev. Biol.* **42**, 211 (1975)] was used to produce large clones. T1 legs require two Hox genes, *Scr* and *Antp*, whereas T2 legs only require *Antp* (5). Thus, *Scr^{C1} Antp^{NS+RC3} hth^{P2}* clones generated the two-segment appendage in both T1 and T2. Clones induced 0 to 24 or 24 to 48 AEL resulted in ground state appendages in the thorax. Clones induced 0 to 24, 24 to 48, 48 to 72, or 72 to 96 hours AEL resulted in ground state appendages in the head. Two-segment appendages were observed in all three legs at similar frequencies in *hth*⁻ clones.
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21. About 20% of *Antp*⁻ T1 or T3 legs had fusions between the femur and tibia; the remainder appeared wild type. The low penetrance of this phenotype may be due to partial redundancy with *Scr* and *Ubx*. Where fusions were observed, the leg segments were also ~50% smaller than their normal size. About 20 *Antp*⁻ T1 or T3 leg discs were stained with an antibody to Hth, and no evidence for derepression was observed.
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27. Imaginal discs were dissected from third instar larvae or early pupae and prepared for confocal microscopy with standard methods. The antibodies used were mouse antibody to CD2 (Serotec), guinea pig antibody to Hth (9), rat antibody to Ser (23), guinea pig

- antibody to Dl (22), rabbit antibody to β-galactosidase (Cappell), mouse antibody to Dac (17), and mouse antibody to Dll (28). Fluorescein isothiocyanate, Texas Red, or Cy5-conjugated secondary antibodies were used. To identify the mutant tissue, we heat shocked larvae or pupae for 30 min at 37°C to induce CD2 expression; they were then recovered for 30 min at room temperature just before dissection. Expression of *odd*, *fng*, and *ff* was accomplished by examining *lacZ* expression from the enhancer traps *odd-lacZ* (*odd*⁰¹⁸⁶³), *fng-lacZ* (23), and *ff-lacZ* (26).
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Pollen Tube Attraction by the Synergid Cell

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In flowering plants, guidance of the pollen tube to the embryo sac (the haploid female gametophyte) is critical for successful fertilization. The target embryo sac may attract the pollen tube as the final step of guidance in the pistil. We show by laser cell ablation that two synergid cells adjacent to the egg cell attract the pollen tube. A single synergid cell was sufficient to generate an attraction signal, and two cells enhanced it. After fertilization, the embryo sac no longer attracts the pollen tube, despite the persistence of one synergid cell. This cessation of attraction might be involved in blocking polyspermy.

During fertilization in flowering plants, the pollen tube (the male gametophyte) grows to the embryo sac (the female gametophyte) in the pistil and delivers immotile male gametes. As with axons in the developing nervous system, directional growth of the pollen tube cell is controlled by complex interactions with the female reproductive system along its path (1–4). Lipids on the stigma (5, 6), arabinogalactan proteins in the style (7–9), and adhesion of the pollen tube to surrounding tissues (10) all appear to participate in this control. The female sporophytic tissues alone cannot guide the pollen tube to the embryo sac within the ovule; guidance by the target embryo sac itself is required (11–13). In *Torenia fournieri*, the pollen tube is at-

tracted directly to the embryo sac in vitro (14), suggesting that the embryo sac produces a diffusible signal. It has been proposed that it is the synergid cell adjacent to the egg cell that attracts the pollen tube, because of its location, appearance, and histochemical properties (15–17). Here we used the *T. fournieri* in vitro system to try to identify the cell responsible for the attraction of the pollen tube to the embryo sac.

Torenia fournieri has a naked embryo sac that protrudes from the micropyle of the ovule (18). In general, the embryo sacs of flowering plants are enclosed within thick layers of ovular tissues. When pollen tubes that grew through a cut style were cocultivated with ovules (Fig. 1A), some of the pollen tubes were attracted to the micropylar end of the embryo sac (Fig. 1, B and C) (14). Pollen tubes barely collide with the micropylar end of the embryo sac before attraction takes place (14). Continuous observation of the pollen tube after arrival but before entrance into the embryo sac showed that the tip

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of the growing tube always remained directed toward the micropylar end of the sac, suggesting that the tube is attracted to this region of the sac (Fig. 1D). The micropylar end corresponds to the entrance site into the embryo sac in *T. fournieri* (18, 19) and in other flowering plants (15).

Four gametophytic cells are close to the micropylar end of the embryo sac: the egg cell, two synergid cells, and the central cell (Fig. 2A). These cells generally form a female germ unit, which is defined as the minimum number of cells required to accept the contents of the pollen tube and to accomplish double fertilization (20). Wide variation has been reported in the number and size of the antipodal cells at the opposite pole (21, 22). In *T. fournieri*, three antipodal cells are degenerating in the mature embryo sac (Fig. 2A) (23, 24). Young embryo sacs with seven immature gametophytic cells do not attract any pollen tube (25), consistent with the observation in *magatama* (*maa*) mutants of *Arabidopsis* (13), in which the development of the embryo sac is delayed.

To investigate the contribution of each gametophytic cell to pollen tube attraction, we performed laser ablation. Gametophytic cells of *T. fournieri* were relatively resistant to an ultraviolet (UV) laser beam, as compared with the sporophytic cells of the ovule. When the nucleus or cytoplasm with its many mitochondria was irradiated by a laser beam, attraction of the pollen tube was not impaired as long as the cell was viable. We found that the plasma membrane was relatively weak in gametophytic cells, and ablation of the targeted cell alone could be performed successfully (Fig. 2, B through D) (26).

There are a few hundreds ovules in a culture, in which about 35% of embryo sacs are not damaged during excision of the flower and are complete with four intact gametophytic cells. Each gametophytic cell of the complete embryo sac was ablated by a UV laser beam under an inverted microscope at the start of cultivation, and the manipulated embryo sacs were then monitored for 24 hours (Table 1) (27). Gametophytic cells can be readily distinguished from each other by their morphological characteristics (18). In our system, which was improved by adding 15% polyethylene glycol 4000 to the medium (19), almost all complete embryo sacs attracted a pollen tube [98%, $n = 49$ sacs (Table 1)]. This frequency was not impaired when 20 to 30 surrounding ovular cells were ablated (100%, $n = 30$), suggesting that these cells were not necessary for generating the attraction signal.

When the egg cell or the central cell was ablated, attraction was also not affected (94%, $n = 37$; 100%, $n = 10$, respectively). These two cells are the female germ cells of the flowering plant, which develop into the

embryo and endosperm, respectively, through double fertilization (15). Our results indicate that the egg cell and the central cell are not directly responsible for attracting a pollen tube. The central cell, however, may play an

indirect role in attracting the pollen tube. When the central cell was killed by the laser beam, frequently all of the gametophytic cells in the embryo sac broke down (in 140 out of 206 experiments; eliminated from the results

Fig. 1. Attraction of the pollen tube to the naked embryo sac of ovules in vitro. (A) Low-magnification view of a culture observed by dark-field microscopy at 8 hours after the start of cultivation. Pollen tubes growing semi-in vitro through a cut style are cocultivated with ovules in a dish. (B) An ovule with a protruding embryo sac. (C) Sequential images of the attraction of pollen tubes to the embryo sac shown in (B). Two pollen tubes are attracted to the micropylar end of an embryo sac. The time after the start of cultivation is indicated at the bottom right of each panel. (D) Continuous observation of a pollen tube attracted to the embryo sac. The time after the start of video recording (from 10 hours after the start of cultivation) is indicated at the upper right of each panel. Arrowheads and arrows indicate the micropylar end of the embryo sac and the tip of the pollen tube, respectively. Scale bars, 300 μm in (A); 50 μm in (B) and (C); and 30 μm in (D).

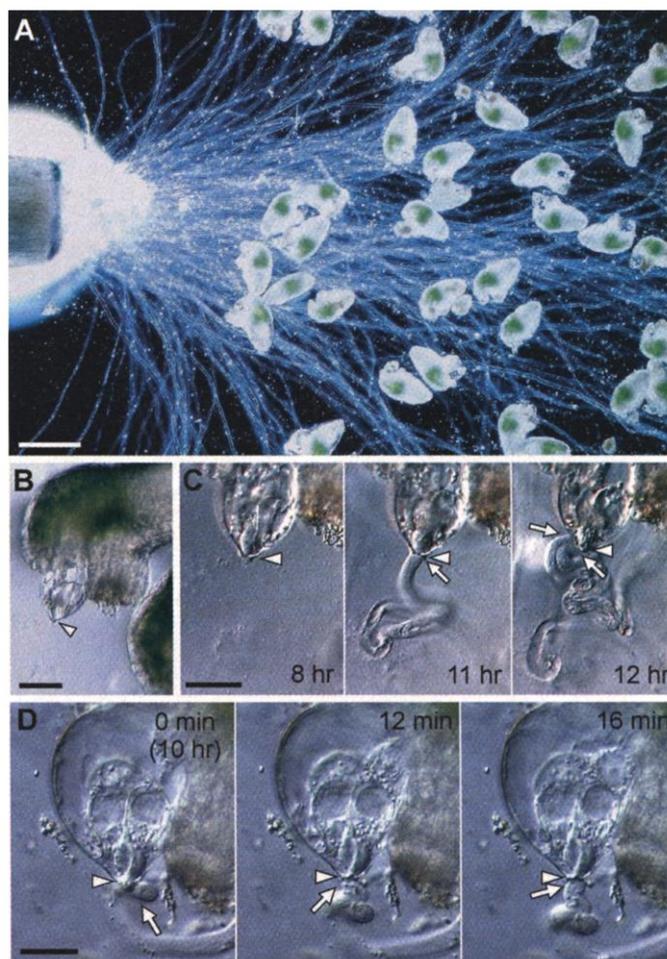


Table 1. Types of embryo sacs attracting pollen tubes after laser cell ablation. Complete embryo sacs were operated on by laser at the start of cultivation and were monitored until 24 hours later to determine whether pollen tubes were attracted. The number of embryo sacs observed was accumulated from many independent experiments.

Type of embryo sac	Existence of each cell				Frequency of attraction (%)
	EC	CC	SY	SY	
Complete	+	+	+	+	48/49 (98%)
Complete, with killed ovular cells	+	+	+	+	30/30 (100%)
One cell killed	-	+	+	+	35/37 (94%)
	+	-	+	+	10/10 (100%)
	+	+	-	+	35/49 (71%)
Two cells killed	-	-	+	+	13/14 (93%)
	-	+	-	+	11/18 (61%)
	+	-	-	+	10/14 (71%)
Three cells killed	+	+	-	-	0/77 (0%)
	-	-	-	+	5/8 (63%)
	+	-	-	-	0/20 (0%)
	-	+	-	-	0/18 (0%)
All four cells killed	-	-	-	-	0/79 (0%)

reported in Table 1), and all attraction ceased. This breakdown was avoided by culturing ovules in a hypertonic medium containing 12% sucrose (although growth of the pollen tube was impaired under such conditions). Because female gametophytic cells of flowering plants are not entirely covered by a cell wall (15, 16), we suggest that the large central cell may support the entire embryo sac with its turgor pressure.

When one synergid cell was ablated, the attraction frequency decreased (71%, $n = 37$) but did not cease completely. Next, we ablated two gametophytic cells (Table 1). When both the egg cell and the central cell were ablated, the attraction frequency did not change [93%, $n = 14$ (Fig. 2E)]. However, combinations that included the ablation of one synergid cell decreased the frequency (61%, $n = 18$ for ablation of the egg cell and a synergid cell; 71%, $n = 14$ for ablation of the central cell and a synergid cell). When two synergid cells were ablated, we found that no embryo sac attracted a pollen tube [0%, $n = 77$ (Fig. 2F)]. Thus, at least one synergid cell is necessary for pollen tube attraction.

When we left a single gametophytic cell in the embryo sac by ablating three gametophytic cells (Table 1), the synergid cell attracted pollen tubes [63%, $n = 8$ (Fig. 2G)]; thus, a single synergid cell appears sufficient to generate an attraction signal. A single egg cell or central cell did not attract a pollen tube (0%, $n = 20$; 0%, $n = 18$, respectively). When all four gametophytic cells were ablated, pollen tube attraction ceased completely (0%, $n = 79$). These results of laser ablation indicate that one synergid cell is necessary and sufficient to attract the pollen tube.

It remains unclear whether one or both synergid cells attract the pollen tube. It has been suggested that the two synergid cells differ physiologically (15–17). Generally, only one synergid cell degenerates as a result of accepting the contents of the pollen tube. The receptive synergid cell often shows signs of degeneration before pollen tube arrival. In the *in vitro* *T. foeneri* system, only one synergid cell breaks down when the pollen tube begins to discharge its contents into the embryo sac (19). In the above experiments, we showed that the frequency of attraction decreased when one synergid cell was ablated by a laser beam (Table 1). This suggests two possibilities: The ability to attract pollen tubes is lowered because of a decrease in the source of the attraction signal, or only one of the two synergid cells attracts the pollen tube. In the former case, the effective range of attraction should be shortened, as mathematically calculated for axon guidance (2, 28); the probability that a pollen tube enters the effective range would then be decreased. In the latter case, attraction of the pollen tube

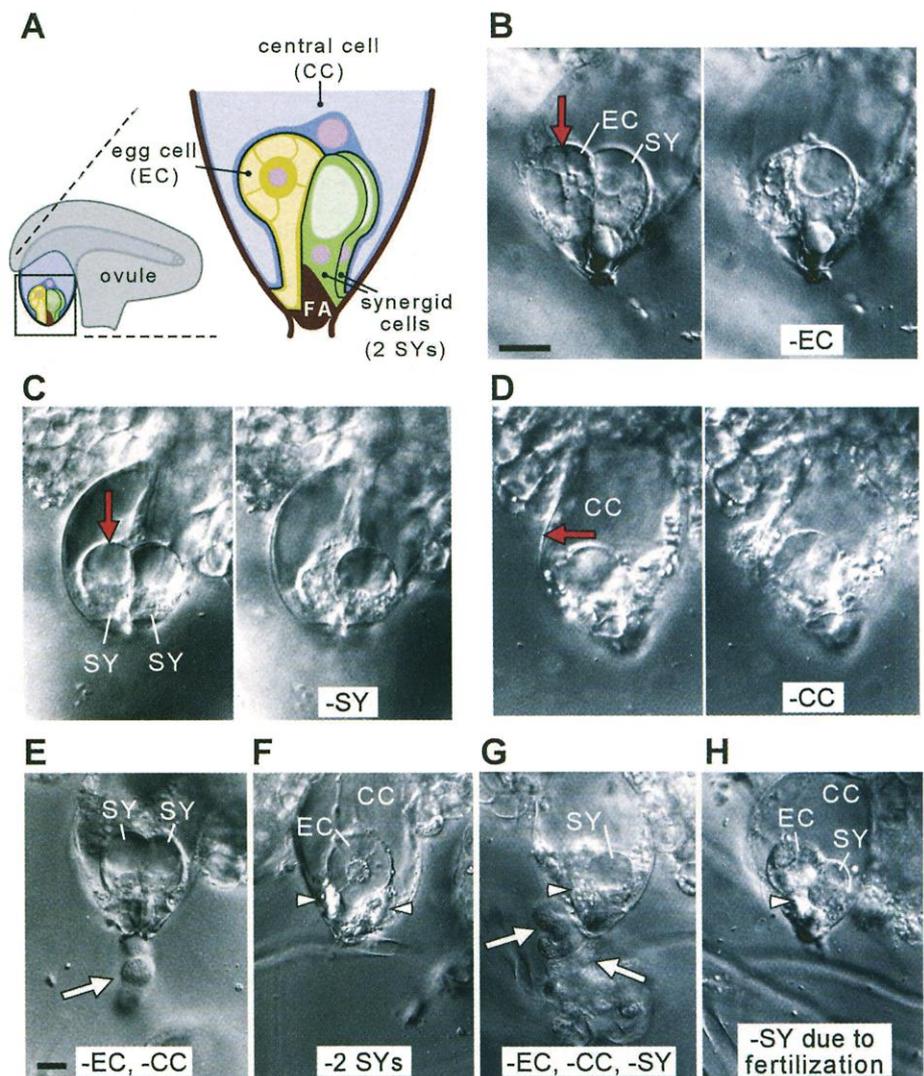


Fig. 2. Laser ablation of each gametophytic cell in the embryo sac and its influence on pollen tube attraction. (A) Schematic representation of the ovule (left) and the embryo sac (right) of *T. foeneri*. The embryo sac contains the egg cell (EC), two synergid cells (2 SYs), and the central cell (CC). The filiform apparatus (FA) is the structure formed by the thickened cell walls of two synergid cells. (B through D) Embryo sacs before and after laser ablation of the EC (B), one of two SYs (C), and the CC. (D). Because of the narrow focal plane of differential interference microscopy, some gametophytic cells in the embryo sac are out of focus. Red arrows indicate the region irradiated by a narrow but intense UV laser beam. (E through H) Pollen tube attraction in the different kinds of ablated embryo sac. Embryo sacs with disrupted EC and CC (E); 2 SYs (F); EC, CC, and SY (G); and SY due to fertilization (H) are shown. In (H), the pollen tube that fertilized *in vivo* is broken at the micropylar end of the embryo sac. Pollen tubes are attracted in (E) and (G) (indicated by arrows), but not in (F) and (H), where pollen tubes are passing near the micropylar end of the embryo sac. Arrowheads indicate SYs disrupted by laser beam [(F) and (G)] and by fertilization (H). Scale bar in (B), 20 μm for (B) through (D); scale bar in (E), 10 μm for (E) through (H).

should cease only when the synergid cell responsible for the attraction is ablated. To discriminate between these possibilities, we next investigated the frequency of attraction after changing the number of pollen tubes in the culture (Table 2), in an attempt to increase the likelihood that a pollen tube would enter the effective range of the attraction.

One flower was usually used for each culture; one pollinated style was cocultivated with ovules excised from the same flower. In this condition, most complete embryo sacs

attracted pollen tubes normally (98%, $n = 49$), whereas the embryo sac with an ablated synergid cell attracted pollen tubes less effectively (71%, $n = 49$). We increased the number of pollen tubes by adding another pollinated style to the culture and found that most embryo sacs with an ablated synergid cell attracted pollen tubes (96%, $n = 24$). Embryo sacs with ablated two synergid cells attracted no pollen tube, despite the increase in the number of pollen tubes (0%, $n = 31$), suggesting complete loss of the attracting activ-

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Table 2. Change in the frequency of attraction resulting from the number of pollen tubes and fertilization. The number of pollen tubes was controlled by the number of styles and anther loculi used. A few hundred pollen tubes grew through one style. The SY was disrupted by laser ablation. Embryo sacs in which one synergid cell degenerated with fertilization or was disrupted mechanically during excision were also monitored.

Number of pollinated styles	Type of embryo sac	Frequency of attraction (%)
One style	Complete	48/49 (98%)
	–1 SY	35/49 (71%)
	–2 SYs	0/79 (0%)
Two styles	Complete	62/65 (95%)
	–1 SY	23/24 (96%)
	–2 SYs	0/31 (0%)
One style with 1/8 pollen	Complete	20/28 (71%)
	–1 SY	8/23 (35%)
	–2 SYs	0/21 (0%)
Two styles	–1 SY due to fertilization	0/68 (0%)
	–1 SY during excision	28/30 (93%)

ity. On the other hand, when the number of pollen tubes was decreased, the frequency of pollen tube attraction decreased not only for embryo sacs with an ablated synergid cell (35%, $n = 23$) but also for complete embryo sacs (71%, $n = 28$). These results indicate that the attraction frequency depends on the number of pollen tubes and, moreover, that all embryo sacs with an ablated synergid cell had the ability to attract a pollen tube, although the attraction was less than that of the complete embryo sacs. This suggests that both synergid cells can attract pollen tubes [the probability that only the synergid cell not responsible for the attraction was consistently ablated is below $(1/2)^{24} \approx 10^{-7}$], although it may be that pollen tubes are more effectively attracted by the existence of two synergid cells in the embryo sac.

Embryo sacs with an ablated synergid cell were able to attract pollen tubes. This raised the question of whether the one synergid cell remaining after fertilization could attract pollen tubes. We previously showed that a fertilized embryo sac did not attract a pollen tube (14). However, we could find no difference between fertilized embryo sacs and embryo sacs in which one synergid cell was disrupted during cultivation, due to the persistent low frequency of attraction in culture. Because most embryo sacs with one ablated synergid cell could attract a pollen tube when two pollinated styles were used in our culture conditions [96% (Table 2)], we cultivated fertilized ovules using two pollinated styles (Table 2) (29). No pollen tubes were attracted to fertilized embryo sacs [0%, $n = 68$ (Fig. 2H)], despite the persistence of a synergid cell in the sac. This is not due to the sensitivity of the pollen tube, because the pollen tube is capable of reacting to the attraction signal in the presence of a fertilized embryo sac (14). In contrast, embryo sacs with a synergid cell that was mechanically disrupted during excision attracted pollen tubes (93%, $n = 30$), as in the case of laser ablation.

Combined, these results showed that the loss of one synergid cell was not the reason for the cessation of the attraction of the pollen tube to a fertilized embryo sac and that a more active mechanism to halt the attraction exists. One embryo sac or ovule usually accepts only one pollen tube (13, 21, 30), and multiple pollen tubes in one embryo sac can sometimes cause polyspermy (21). The cessation of pollen tube attraction to the fertilized embryo sac might be involved in preventing polyspermy in flowering plants at the pollen tube guidance step.

Attraction of the pollen tube by the synergid cell is likely to occur universally in flowering plants, because the synergid cell is present in almost all flowering plants and always appears similar and active in secretory function (15–17). Although there are some plants such as *Plumbago zeylanica* that do not possess any synergid cells, the egg cell of these plants seems to carry out the function of the synergid cell, because some characteristic structure of the synergid cell, such as the filiform apparatus, is observed in the egg cell (31). We propose that in the pistil, the pollen tube approaches the ovule by successive directional controls from the female reproductive system and reaches the effective range of attraction by synergid cells, which appears to be a few hundred micrometers at most (2, 13, 14). A combination of a genetic approach with the characterization of the chemical properties of the attraction signal using the *in vitro* *T. fournieri* system may lead to the identification of such a directional cue derived from the synergid cell.

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- A PALM laser interface system (PALM, Bernried, Germany) equipped with an N_2 laser (wavelength, 337 nm) was used for laser ablation of cells. The intensity of the laser beam was adjusted so that it was possible to make a hole in the coverslip, as previously described for laser ablation in *Caenorhabditis elegans*. The laser beam was focused at the edge (the plasma membrane) of gametophytic cells. In most cases, irradiation of the chalazal edge of the egg or synergid cell caused rupture of their plasma membrane but not of the neighboring central cell. In the case of disruption of the central cell, irradiation of the plasma membrane nearest to the objective lens was the most effective. Disruption of the central cell was monitored by observation of cessation of either the cytoplasmic streaming or the Brownian movement of organelles. Laser pulses (13 pulses per second) for up to several seconds were sufficient for disruption, and longer irradiation caused later degeneration of the entire sac. After laser ablation at room temperature for about 1 hour, the culture dish (D210402; Matsunami Glass, Osaka, Japan) was kept at 30°C in the dark.
- To monitor embryo sacs after laser cell ablation, a stereoscopic microscope image of the whole culture was video-captured at the start of cultivation and printed out immediately after processing with Adobe Photoshop. The result of laser ablation of each embryo sac was recorded on the printed paper. This "map" of the culture was used to find treated embryo sacs and to confirm that only the targeted cell was specifically disrupted before arrival of the pollen tube.
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