Live Imaging and Laser Disruption Reveal the Dynamics and Cell–Cell Communication During Torenia fournieri Female Gametophyte Development

Daichi Susaki¹, Hidenori Takeuchi¹, Hiroki Tsutsui¹, Daisuke Kurihara¹,² and Tetsuya Higashiyama¹,²,³,*

¹Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8602 Japan
²JST ERATO Higashiyama Live-Holonics Project, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8602 Japan
³Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8602 Japan

*Corresponding author: E-mail, higashi@bio.nagoya-u.ac.jp; Fax, +81-52-747-6405.

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The female gametophytes of many flowering plants contain one egg cell, one central cell, two synergid cells and three antipodal cells with respective morphological characteristics and functions. These cells are formed by cellularization of a multinuclear female gametophyte. However, the dynamics and mechanisms of female gametophyte development remain largely unknown due to the lack of a system to visualize directly and manipulate female gametophytes in living material. Here, we established an in vitro ovule culture system to examine female gametophyte development in *Torenia fournieri*, a unique plant species with a protruding female gametophyte. The four-nucleate female gametophyte became eight nucleate by the final (third) mitosis and successively cellularized and matured to attract a female gametophyte. The four-nucleate female gametophyte became eight nucleate by the final (third) mitosis and successively cellularized and matured to attract a pollen tube. The duration of final mitosis was 28 ± 6.5 min, and cellularization was completed in 54 ± 20 min after the end of the third mitosis. Fusion of polar nuclei in the central cell occurred in 13.1 ± 1.1 h, and onset of expression of *LURE2*, a pollen tube attractant gene, was visualized by a green fluorescent protein reporter 10.7 ± 2.3 h after cellularization. Laser disruption analysis demonstrated that the egg and central cells were required for synergid cells to acquire the pollen tube attraction function. Moreover, aberrant nuclear positioning and down-regulation of *LURE2* were observed in one of the two synergid cells after disrupting an immature egg cell, suggesting that cell specification was affected. Our system provides insights into the precise dynamics and mechanisms of female gametophyte development in *T. fournieri*.

**Keywords:** Female gametophyte • Live-cell imaging • Megagametogenesis • *Torenia fournieri*.

**Abbreviations:** Ato, atropos; CCD, charge-coupled device; CCG, central cell guidance; clo, clotho; FG, female gametophyte; GFP, green fluorescent protein; liss, lachesis; tdTomato, tandem dimer Tomato.

**Introduction**

Cell–cell communication is essential for cell specification and differentiation during plant development and reproduction (Van Norman et al. 2011). In plant reproduction, cell–cell communication is critical not only for male–female interactions (Suzuki 2009, Higashiyama 2010) but also for reproductive cell development (Grossniklaus 2011). Female gametophyte development enables study of how individual cell fates are specified in plants (Sprunck and Groß-Hardt 2011, Drews and Koltunow 2011). Most flowering plants (~70%) have the Polygonum-type female gametophyte, which contains seven cells of four distinct types: two synergid cells, one egg cell, one central cell and three antipodal cells (Fig. 1A; Yadegari and Drews 2004). One diploid megaspore mother cell undergoes meiosis and produces four haploid megaspores. One megaspore becomes a functional megaspore and undergoes three rounds of mitosis without cytokinesis, resulting in the formation of an eight-nucleate syncytium. After cellularization, the seven-celled female gametophyte is formed. Two nuclei in the central cell, which are called polar nuclei, fuse to form the secondary nucleus.

Each cell of a female gametophyte has distinct functions for double fertilization and distinct cell polarity in its morphology (Yadegari and Drews 2004, Sprunck and Groß-Hardt 2011). A nucleus is located at the chalazal end of the egg cell in many species, and a vacuole(s) occupies the micropylar end. In contrast, synergid cells have the opposite polarity (Christensen et al. 1997). The two synergid cells play essential roles in pollen tube attraction (Higashiyama et al. 2001, Kasahara et al. 2005) and reception (Escobar-Restrepo et al. 2007, Capron et al. 2008, Kessler et al. 2010, Ngo et al. 2014). Synergid cells secrete attractant peptides, including the LURE peptides of *Torenia* (Okuda et al. 2009, Goto et al. 2011, Kanaoka et al. 2011) and Arabidopsis (Takeuchi and Higashiyama 2012). In maize, ZmA1 attractant peptide is expressed in the egg apparatus (egg and synergid cells) and is required for pollen tube guidance (Márton et al. 2005, Márton et al. 2012). In contrast, the egg and central cells give rise to the embryo and endosperm, respectively, after fertilization.

Some *Arabidopsis thaliana* mutants are defective in the functions and specifications of the female gametophyte cells. MYB98, which encodes R2R3-MYB protein as a transcription factor, is required for differentiation of synergid cells, and the
myb98 mutant is defective in pollen tube guidance (Kasahara et al. 2005). GAMETE EXPRESSED 3 (Alandete-Saez et al. 2008), which encodes a plasma membrane-localized protein and is expressed in the male gametophyte and in the egg cell of the female gametophyte, and CENTRAL CELL GUIDANCE (CCG; Chen et al. 2007), which encodes a putative transcriptional regulator with unclear biochemical function in the central cell, are not expressed in the synergid cell. However, these mutants also show a defect in pollen tube guidance. As the ccg mutant is unlikely to produce LURE peptides (Takeuchi and Higashiyama 2012), cell–cell communication between the gametic cells and the synergid cell must be required for pollen tube guidance.

Moreover, this communication may play a role in the female gametophyte cell fate specification. Genes expressed in the egg cell, LACHESIS (LIS), which encodes A. thaliana homolog of the yeast pre-mRNA splicing factor (Groß-Hardt et al. 2007, Völz et al. 2012), and ZmEAL1 of maize, which encodes an EA1 box peptide (Krohn et al. 2012), prevent the synergid cell and antipodal cells, respectively, from acquiring germ cell fates. The results of several mutant analyses suggest that nuclear positions are important for the cell fate specification during female gametophyte development (Sprunck and Groß-Hardt 2011). Mutants with aberrant cell fates, such as lis (Groß-Hardt et al. 2007, Völz et al. 2012), eostre (Pagnussat et al. 2007), clotho/gametophyte factor1, atropos (clo/gfa1, ato; Moll et al. 2008) and oiwa (Martin et al. 2013), have mislocated nuclei in the female gametophyte. Thus, the behavior of each female gametophyte nucleus appears to play a role in cell specification that is critical for achieving double fertilization. However, direct observation of the development of the female gametophyte is difficult because of the embedded structure deep in the ovule. Current mutant analyses with fixed samples lack real-time temporal information; therefore, how cells communicate during female gametophyte development remains largely unknown.

Torenia fournieri is a unique plant species with a protruding female gametophyte (Fig. 1B; Higashiyama et al. 1997). The attraction of the pollen tube to the protruding female gametophyte has been examined directly by light microscopy and laser disruption (Higashiyama et al. 1998, Higashiyama et al. 2001). If female gametophyte development occurred in cultivated Torenia ovules, the precise dynamics of nuclear behavior and cellularization, as well as intercellular gametophytic communication for cell specification and function, could be examined by laser disruption. In this study, we established an in vitro culture system to investigate T. fournieri female gametophyte development. We examined cell–cell communication during pollen tube attraction using laser disruption of cells or their nuclei in immature female gametophytes after cellularization. We report that gametophytic cell specification and function require intercellular communication during female gametophyte development.

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**Fig. 1** Development of the *Torenia fournieri* female gametophyte. (A, B) Schematic of development of a *Polygonum*-type female gametophyte (A) and a *T. fournieri* ovule with a protruding female gametophyte (B). (C) Female gametophyte in the pistil from 2 d before to the day of anthesis. Ovules at various stages were excised from these buds or flowers and observed. The number of nuclei in the female gametophyte before protrusion of the ovule was unclear. The female gametophyte undergoes a final round of mitosis, cellularization and migration of polar nuclei from each pole at the FG5 stage. Two polar nuclei fuse at the FG6 stage. Arrows show micropylar nuclei at the late FG4 stage. The female gametophyte undergoes a final round of mitosis, cellularization and migration of polar nuclei from each pole at the FG5 stage. Two polar nuclei fuse at the FG6 stage. Arrows show micropylar nuclei at the FG4 stage.

EC, egg cell; SY, synergid cell; CC, central cell; AC, antipodal cell; FG, female gametophyte; OV, ovule; MC, megaspore cell; MP, micropyle; PN, polar nucleus; SYN, synergid cell nucleus; SN, secondary nucleus. Scale bar = 20 μm.
Results and Discussion

*T. fournieri* female gametophyte development

We observed ovules excised from ovaries of flowers at different stages, namely from 2 d before to the day of anthesis, to examine *T. fournieri* female gametophyte development (Fig. 1C). As described in the Materials and Methods, flower buds 2 d before anthesis had 0.7–0.8 cm styles including the stigma. We followed Polygonum-type female gametophyte development in *T. fournieri* using differential interference (Nomarski) microscopy, which was in part reported by Imre and Kristóf (1999), to isolate various female gametophyte stages as protoplasts. The micropylar end of developing female gametophytes began to emerge from the micropyle of the ovule at the early four-nucleate stage (FG4) under our culture conditions (Fig. 1C, before protrusion and early FG4). Two micropylar nuclei were observed at the micropylar pole of the protruding female gametophyte. No positional difference was observed between the two nuclei along the micropylar–chalazal (basal–apical) axis of the female gametophyte at the early FG4 stage. The two micropylar nuclei lay along a line orthogonal to the micropylar–chalazal axis (Fig. 1C, FG4 late). Cellularization was completed following the third mitosis at the FG5 stage (Fig. 1C, FG5 early), and the polar nuclei migrated from each pole and met at the middle of the female gametophyte (Fig. 1C, FG5 late). The egg and synergid cells began to show different polarities soon after cellularization was complete; the egg nucleus was in the chalazal part, the synergid nucleus was in the micropylar part and a large vacuole occupied the chalazal part of the synergid cell. The polar nuclei of the central cell fused to form a secondary nucleus at the FG6 stage (Fig. 1C, FG6). At this stage, ovules are matured.

There are approximately 500 ovules in a *T. fournieri* ovary (Higashiyama et al. 1997). Ovules are in various stages, even when excised from the same bud. The *T. fournieri* female gametophyte appeared to take approximately 2 d from the early FG4 stage to the FG6 stage under our conditions. Two days before anthesis, 87 ± 6.3% ovules showed a protruding female gametophyte, of which 61 ± 6.6% were still in the FG4 stage (n = 3 buds). On the day of anthesis, 100% of the ovules were at the FG6 stage (n = 4 flowers).

Female gametophyte development in vitro

We established a method to culture developing *T. fournieri* ovules so that we could investigate female gametophyte development in real time. At first, most of the female gametophytes from immature ovules 2 d before anthesis burst in the culture medium during development using the culture method described by Higashiyama et al. (2006). Imre and Kristóf (1999) reported that osmotic pressure in the *T. fournieri* female gametophyte changes significantly during development. FG4-stage female gametophytes have the highest osmotic pressure (Imre and Kristóf 1999). Because the osmotic pressure was higher than that of the mature female gametophyte, we added 5% (w/v) trehalose to the medium, which increased the frequency of ovules developing without bursting from 10% (n = 26) to 50% (n = 16) and enabled us to visualize female gametophyte development directly (Fig. 2; Supplementary Movie S1). In several plants, trehalose is used for in vitro culture medium, as it increases the viability of cultivated material including Torenia plants (Yamaguchi et al. 2011).

Fig. 2 and Supplementary Movie S1 show time-lapse images of female gametophyte development in a cultured ovule. The female gametophyte began to emerge from the micropyle at the early FG4 stage and continued to elongate (Supplementary Movie S1). The female gametophyte is shown at the early FG4 stage (Fig. 2; 14:15); it then proceeded to the late FG4 stage in the next few hours; two micropylar nuclei lay along the micropylar–chalazal axis (Fig. 2; 18:30). Cytoplasmic streaming stopped completely in the female gametophyte during the third mitosis, at about 19 h of culture (Supplementary Movie S1). Divided nuclei were observed at the micropylar end of the female gametophyte, and cellularization proceeded (Fig. 2; 19:30). The egg and synergid cells began to show different polarities, as noted in Fig. 1C, and a polar nucleus in the central cell migrated gradually in the chalazal direction (Fig. 2; 28:30). The polar nucleus met with the other polar nucleus from the chalazal pole at the middle of the central cell (Fig. 2; 34:00) and fused to form the secondary nucleus (Fig. 2; 35:30). These observations were consistent with those that occurred under the in vivo conditions described in Fig. 1, suggesting that female gametophytes can develop normally in vitro.

We investigated the dynamics of female gametophyte development more precisely with a shorter time-lapse imaging interval (1 min) using this in vitro system (Fig. 3A, B; Supplementary Movies S2, S3). The most micropylar-located nucleus at the late FG4 stage gave rise to two synergid cell nuclei (Fig. 3A, top panels). Another nucleus gave rise to an egg cell nucleus and a polar nucleus of the central cell (Fig. 3A, bottom panels). Such cell (nucleus) lineages have generally been reported by observing fixed material sections (Huang and Russell 1992, Christensen et al. 1997, Sprunck and Groß-Hardt 2011) but were confirmed here for the first time by continuous observations. The duration of the third mitosis was 28 ± 6.5 min (n = 4) from disappearance to reappearance of the micropylar nuclei nucleolus. Cellularization began just after mitosis (Fig. 3B; Supplementary Movies S2, S3). The duration of cellularization was 54 ± 20 min (n = 4). The cell membrane between the central cell and other micropylar cells appeared to originate between sister nuclei, namely the polar nucleus and the egg cell nucleus (Fig. 3B, 1:44), which expanded to separate the central cell from the other cells (Fig. 3B, 1:52 and 2:18). The cell membrane between non-sister nuclei of the egg and synergid cells also formed at the same time (Fig. 3B, 1:52). These observations are consistent with classical observations of fixed material (Huang and Russell 1992), and the dynamics were first revealed in our study.

Moreover, we observed the onset of LURE2 expression using a *Tf* LURE2p::GFP transformant (Fig. 3C; Supplementary Movie S4). Nuclei of the ovule have been visualized by...
RPSSAp::H2B-tdTomato (Maruyama et al. 2013). Green fluorescent protein (GFP) expression under the LURE2 promoter began to be observed at 10.7 ± 2.3 h (n = 12) in both synergid cells after completion of cellularization (cell membrane formation between the central cell and other micropylar cells) (Fig. 3C, 17:20). This result suggests that LURE2 expression begins after cellularization of the female gametophyte and during synergid cell differentiation. Consistently, pollen tubes were not attracted to female gametophytes before cellularization when ovules were cultured 1 d before anthesis for semi-in vitro pollen tube attraction experiments (Higashiyama et al. 2006) (n = 6 experiments).

In the central cell, fusion of polar nuclei occurred 13.1 ± 1.1 h (n = 12) after the end of cellularization. The polar nuclei met and fused at the middle of the central cell in all cases we observed (n = 12).

**The immature egg and central cells are required for acquisition of the pollen tube attraction function by synergid cells**

We tested pollen tube attraction ability in order to examine whether the female gametophyte developed normally in the culture medium (Fig. 4A). We added two pollinated styles to each culture 28 h into the immature ovule culture, when female gametophytes were in the FG6 stage. The egg and two synergid cells were clearly distinguished by their characteristic cell polarities (Fig. 4B, 24 h). After a further 12 h of culture, pollen tubes were attracted to the micropylar end of the female gametophyte (Fig. 4B, 40 h; 68%, n = 157 ovules). Coiling behavior of the pollen tube toward the ovules (Fig. 4B, 40 h) indicated pollen tube attraction (Higashiyama et al. 1998). Some female gametophytes received a pollen tube (Fig. 4B, 40 h), as reported previously using mature ovules (Higashiyama et al. 1998). This result suggests that the synergid cell not only acquired the pollen tube attraction function normally but also that of pollen tube reception in vitro (Kessler and Grossniklaus 2011, Wolf and Höfte 2014).

We next performed laser disruption of immature gametophytic cells to analyze cell–cell communication during synergid cell differentiation for pollen tube attraction using the system shown in Fig. 4 (Fig. 5). We disrupted target cells or ovule nuclei at FG5 soon after cellularization by irradiation with a UV laser. The laser power and pulses were always constant. We then observed pollen tube attraction 40 h later; the remaining egg and synergid cells appeared sufficiently elongated. As noted above, 68% of female gametophytes with the complete set of gametophytic cells attracted pollen tubes without irradiation by the UV laser (Fig. 5B; n = 157). Pollen tube attraction was not affected when several sporophytic funicular cells close to the female gametophyte were disrupted (73%, n = 22). Disruption of one of the two synergid cells also did not...
significantly impair pollen tube attraction (Fig. 5; 60%, n = 15), which was consistent with a previous result in mature ovules with two pollinated styles (increased number of pollen tubes; Higashiyama et al. 2001). However, the frequency of pollen tube attraction decreased significantly when the egg cell was disrupted (Fig. 5; 19%, n = 47; Fisher’s exact test, P < 0.01). Disrupting one of the two polar nuclei in the central cell also significantly reduced pollen tube attraction (25%, n = 28; Fisher’s exact test, P < 0.05). The polar nucleus was targeted because disrupting the central cell resulted in disruption of the entire female gametophyte in our system.

In previous laser disruption studies using mature ovules, disrupting only the synergid cell affected pollen tube attraction, disruption of two synergid cells completely impaired pollen tube attraction, whereas disruption of the egg or central cell did not affect pollen tube attraction (Higashiyama et al. 2001). To confirm these results by using the modified media containing trehalose, we disrupted mature female gametophyte cells after FG6. As shown in Fig. 5C, disruption of two mature synergid cells, but not the mature egg and central cells, impaired pollen tube attraction in the modified media. Considering the results of immature and mature ovule disruption, two immature female gametes, namely the egg and central cells, are required for acquisition of the pollen tube attraction function by the synergid cell during female gametophyte development. Consistently, CCG of the central cell is required for pollen tube attraction and production of AtLURE1 peptides in the A. thaliana synergid cell (Chen et al. 2007, Takeuchi and Higashiyama 2012). CCG may be necessary for the onset of pollen tube attraction by the synergid cell through cell–cell communication during female gametophyte development. Following sufficient differentiation of the synergid cell, it attracts a pollen tube independently of the egg and central cells, at least in T. fournieri (Higashiyama et al. 2001).

Differentiation of one of the two synergid cells is affected by disruption of an immature egg cell

Characteristic alterations were observed in the morphology of one of the two synergid cells when immature egg cells were subjected to laser disruption (Fig. 6A). After disrupting an immature egg cell, the nucleus of one of the two synergid cells changed position from the micropylar end to the chalazal end. This position of the nucleus within the cell was similar to that of the egg cell (Figs. 1, 2). The nucleus of the other synergid cell always remained at the micropylar end. Fig. 6B shows the relative position of nuclei in the synergid and egg cells compared with that in the other synergid cell. The nucleus of the egg cell was relatively at the chalazal end compared with that of the synergid cell without laser irradiation (Fig. 6B; SY/SY and EC/SY, w/o disruption). The nuclear position of one of the two synergid cells changed significantly when an immature egg cell...
was disrupted (Fig. 6B; SY*/SY, EC disruption). Linear discriminant analysis (Massart et al. 1988) suggested that the nuclear position of one of the two synergid cells was at the chalazal end in 40% of the ovules (n = 30).

The synergid cell marker TjLURE2p::GFP was used to determine whether synergid cell properties were lost in this synergid cell with an aberrant nucleus position (Fig. 6C; Supplementary Movie S5). As described above, the GFP signal was detected in both synergid cells after cellularization without UV laser irradiation (Fig. 3C; Supplementary Movie S4). When immature egg cells were disrupted in TjLURE2p::GFP ovules, the nucleus moved to the chalazal end in one of the synergid cells in six cases out of 38. The GFP signal began to be detected similarly in both synergid cells after laser disruption of an immature egg cell (Fig. 6C, 12:40 and 19:00). However, the signal intensity decreased gradually in one of the cells (Fig. 6C, 31:20 and 43:40). Although the nucleus remained at the micropylar end in cells abundantly expressing TjLURE2p::GFP, the nucleus migrated towards the chalazal side in the cell with decreased GFP expression (Fig. 6C). The GFP expression level decreased in all synergid cells in which the nucleus clearly moved to the chalazal end (n = 6), compared with that in the other synergid cell. Taken together, these results suggest that cell fate specification was affected in one of the two synergid cells, which might partly explain the impaired pollen tube attraction. The time of change of the nucleus position in the synergid cell (Fig. 6C; Supplementary Movie S4; 17:20) suggested that such a duration was required for the alteration of the cell fate specification in the synergid cell after laser disruption.

Recent studies in Arabidopsis and maize propose that the egg cell has a central role in cell fate maintenance in the female gametophyte via cell–cell communication (Groß-Hardt et al. 2007, Krohn et al. 2012). Synergid cell fate in eostre and oiw mutants switches to that of the egg cell (Pagnussat et al. 2007, Martin et al. 2013). In addition, lis, clo and ato mutants convert cell fate from that of the synergid cell to that of the egg cell and from that of the antipodal cell to that of the central cell (Groß-Hardt et al. 2007, Moll et al. 2008). Although LIS is expressed in both the egg and central cells, only down-regulation of LIS in the egg cell affects cell fate maintenance in all gametophytic cells (Völz et al. 2012). Interestingly, genetic ablation of the egg cell using the toxin barnase induces an egg cell marker in one of the two synergid cells (Lawit et al. 2013). Moreover, down-regulating ZmAAL1, which encodes a peptide secreted by the egg cell, induces acquisition of central cell fate in antipodal cells (Krohn et al. 2012). Disrupting the egg cell affected cell fate maintenance of the synergid cell in wild-type Torenia plants. Identification of markers for each female gametophytic cell is now in progress to investigate Torenia development further using live-cell imaging (Kawano et al. 2011). Cell type-specific transcriptomics in the reproductive tissue is a powerful approach to identify cell-specific markers (e.g. Wuest et al. 2010, Osaka et al. 2013, Kubo et al. 2013, Palovaara et al. 2013).
In this study, the dynamics of female gametophyte development were studied in *T. fournieri* using living material (Fig. 7). The localization and division plane of micropylar nuclei appeared to be precisely controlled just before and during the third mitosis (Figs. 2, 3; Supplementary Movies S1, S2, S3). Cytoplasmic streaming stopped completely in the female gametophyte before laser irradiation after laser irradiation 24 hr 40 hr

![Fig. 5](image-url) The immature egg and central cells are required for synergid cells to acquire the pollen tube attraction function. (A) A synergid cell, a polar nucleus of a central cell or an egg cell from an immature female gametophyte is disrupted by a UV laser at FG5 soon after cellularization. Female gametophytes are observed before and after disruption, and 24 and 40 h later with co-cultivated pollen tubes. Lightning bolts indicate the time of UV laser irradiation. Insets show enlarged images of the target cells or nuclei. Arrowheads indicate the point of laser irradiation. (B, C) The frequencies of pollen tube attraction to the female gametophyte with each immature cell, nuclear disruption (B) or mature ovule cell disruption (C). *n*, total number of trials. Single and double asterisks indicate significant differences using Fisher’s exact test (*P* < 0.05; **P** < 0.01). w/o, no laser irradiation, SP, sporophytic cell. Scale bar = 20 μm.

**Conclusion**

In this study, the dynamics of female gametophyte development were studied in *T. fournieri* using living material (Fig. 7).
Fig. 6 One of two synergid cells shows aberrant behavior after disruption of the immature egg cell. (A) A female gametophyte with or without a disrupted immature egg cell before and after 24 h of cultivation. (B) Relative position of nuclei between egg and synergid cells or two synergid cells with or without disrupted egg cells. Numbers on the y-axis indicate the ratio of the distance from the micropylar end to the nuclei between two cells. (C) Time-lapse series of the female gametophyte after disrupting an immature egg cell. Two synergid cells showed GFP signals at the start of cultivation. Equivalent GFP signals are detected 12 h 40 min later. The GFP signals in the left synergid cell decreased gradually 31 h 20 min later. The nucleus in the left cell moves towards the middle of the cell. SY*, synergid cell with aberrant position of the nucleus. Scale bars = 20 μm.
gametophyte during the third mitosis (28 ± 6.5 min). Cellularization of the female gametophyte started at the end of the mitosis, possibly initiated between sister nuclei, and was completed in 54 ± 20 min. Fusion of the two polar nuclei in the central cell (Supplementary Movie S1) and onset of LURE2 marker gene expression in the synergid cell (Supplementary Movie S4) were visualized by live-cell imaging after cellularization.

Synergid cell differentiation depended on the egg and central cells (Figs. 5, 6). Mature synergid cells attracted pollen tubes in a cell-autonomous manner (Higashiyama et al. 2001; Supplementary Fig. S1). However, laser disruption of the egg cell or the polar nucleus of the central cell severely inhibited pollen tube attraction (Fig. 5). Synergid cell differentiation was also impaired by disrupting an immature egg cell (Fig. 6). Interestingly, one of the two synergid cells showed aberrant polarity of the nucleus and reduced LURE2 gene expression (Supplementary Movie S5). The system developed in this study facilitated investigation of female gametophyte development by live-cell analysis.

Materials and Methods

Plant materials and growth conditions

*Torenia fournieri* cv. ‘Blue and White’ was used for the laser disruption and pollen tube guidance experiments (Takeuchi and Higashiyama 2012). Immature ovules 2 d before anthesis were cultured in solid medium (2006), which was modified by adding 5% (w/v) trehalose. Ovules were excised from buds at various stages. Flower buds at approximately 2 d before anthesis were used to cultivate immature ovules with a protruding FG female gametophyte and had 0.7–0.8 cm styles including the stigma. The peduncle tissue began to become erect, allowing the bud to face upwards, and the pistil style tissue in the buds began to elongate rapidly under stigma. The peduncle tissue began to become erect, allowing the bud to face upwards, and the pistil style tissue in the buds began to elongate rapidly under our culture conditions 2 d before anthesis.

Vector construction

The pPZP211 binary vector (Hajdukiewicz et al. 1994) was modified by inserting the nopaline synthase terminator (NosT), which was amplified with the primers 5'-GGGGGTACCTAGAGCTCGATCGTTCAAACA-3' and 3'-ACCCCCGGGAATTATTTGGTTAATTAACCAAAT-5', using the SacI and blunt-ended EcoRI sites, and then GFP was used with a linker sequence and was amplified from pGWB4 (Nakagawa et al. 2007) with the primers 5'-GTAACTAGTTCTCCGGGAAAGGGTGGGCG-3' and 3'-TAGGAGCTCTACTCGAGATTGGTACCCTTGTACAGC-5', using the Smal and SacI sites, and designated as pPZP211G. The TjLURE2 promoter (Takeuchi and Higashiyama 2012) was amplified from *T. fournieri* genomic DNA with the primers 5'-GGCTTCAGAGTGACAGTG AAGATGCGATG-3' and 3'-ACCCGGGAATTATTTGGTTAATTAACCAAAT-5', and ligated using the pPZP211G Xbol and Smal sites, resulting in pPZP211-TjLURE2p::GFP.

Plant transformation

We used *T. fournieri* cv. ‘Blue and White’ for TjLURE2p::GFP and ‘Crown violet’ for *RPSSAp::H2B-tdTomato* (Maruyama et al. 2013) transformation. The construct was introduced into *T. fournieri* using the leaf disc method, as described previously (Aida and Shibata 1995, Takeuchi and Higashiyama 2012). Appropriately transformed T1 plants were screened based on GFP or tdTomato (tandem dimer Tomato) expression.

In vitro ovule cultivation and pollen tube attraction assay

Immature ovules 2 d before anthesis were cultured in solid medium (Higashiyama et al. 2006), which was modified by adding 5% (w/v) trehalose. To observe pollen tube attraction, two cut styles from hand-pollinated pistils were added to a dish with cultivation medium 28 h after beginning the immature ovule cultivation. We inspected the ovules at 40 h to determine whether pollen tubes had been attracted.

Time-lapse imaging of a developing female gametophyte

We used three microscope systems for live imaging of a developing female gametophyte, as follows.

We used an inverted fluorescence microscope (IX-71: Olympus) equipped with a three-charge-coupled device (CCD) digital camera (C7780, Hamamatsu Photonics Ltd.) or a CCD camera (DP73; Olympus). Time-lapse images were acquired using a ×40 objective lens (ULPlanFl 40X, WD = 2.7–4 mm, NA = 0.60; Olympus) or a ×60 oil-immersion objective lens (UPlanSApo X60, WD = 0.15 mm, NA = 1.35; Olympus).

We used the PALM CombiSystem (Zeiss) equipped with a CCD camera (AxioCam ICC1; Zeiss) with the above microscope system for laser disruption. Images were acquired using a ×40 objective lens (LD Plan-NEOLUAR, WD = 3.9 mm, NA = 0.60; Zeiss). The laser irradiation conditions are described below.

We used an inverted confocal microscope system with a stable incubation chamber (CV1000; Yokogawa Electric) equipped with 488 nm LD lasers (Yokogawa Electric), and an EMCCD camera (ImagEM 1K C9100-14 or ImagEM C9100-13; Hamamatsu Photonics) to observe the fluorescence markers. Time-lapse images were acquired with a ×40 objective lens (UPLSAPO 40X, WD = 0.18 mm, NA = 0.95; Olympus). We used a band-pass filters of 520/35 nm for GFP and 617/73 nm for tdTomato.
Laser disruption of female gametophyte cells or nuclei
An Nd:YAG laser (355 nm; Sigma Koki) and 6FT5355-50 solid-state laser (355 nm; PALM MicroBeam, Zeiss) were used to laser disrupt cells or nuclei, as described previously (Higashiyama et al. 2006). The laser beam was focused at the edge of targeted cell or the nuclear membrane of immature ovules.

Image processing
Image processing was carried out essentially as described in Hamamura et al. (2014). In brief, all images were adjusted for brightness and contrast using Adobe Photoshop CS6 (Adobe Systems, Inc.). The ImageJ software (http://rsbweb.nih.gov/ij/index.html) was used to edit the images and movies.

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Disclosures
The authors have no conflicts of interest to declare.

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Supplementary data
Supplementary data are available at PCP online.

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