Isolation of Gametophytic Cells and Identification of Their Cell-Specific Markers in *Torenia fournieri*, *T. concolor* and *Lindernia micrantha*

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Summary Gametophytic cells in the female embryo sac, the egg, the central and the synergid cells have unique roles in sexual reproduction in higher plants. To investigate their cell-specific features, an efficient method of distinguishing and isolating each gametophytic cell is required. By testing the activities of several cellulase enzymes, we modified our previous cell isolation method and succeeded in isolating each cell from the *Torenia* and *Lindernia* species. We also established genetic markers that show specific expression patterns in each gametophytic cell. These markers, together with the cell isolation method, will be useful for analyzing the function of gametophytic cells.

Key words Egg cell, Central cell, Synergid cell, Protoplast isolation, Gametophytic cell-specific markers, *Torenia*.

Sexual reproduction is a unique feature of higher angiosperms. The embryo sac, which contains 1 egg cell, 1 central cell, 2 synergid cells, and 3 antipodal cells, is the female gametophytic organ required for the process of sexual reproduction in plants (Drews and Yadegari 2002). The egg cell is fertilized by a sperm cell to generate an embryo, while the central cell is fertilized by another sperm cell to generate an endosperm. The synergid cells are required for micropylar pollen tube guidance (Higashiyama *et al.* 2001, Kasahara *et al.* 2005). Given that the embryo sac is deeply embedded in the ovule, knowledge of the genes involved in its development or its function remains limited.

A variety of methods, including osmotic stress, mechanical force, and cellulase enzyme treatments, have been attempted to isolate gametophytic cells from the ovule to analyze their features (Theunis *et al.* 1991, Huang and Russell 1989, Katoh *et al.* 1997, Kovacs *et al.* 1994). Enzyme treatment in particular has been used to generate protoplasts from the female gametophytes of a wide range of plant species including *Ginkgo biloba*, *Salvinia natans*, *Laminaria digitata*, *Petunia hybrida* (Laurain *et al.* 1993, Nakamura and Maeda 1994, Benet *et al.* 1997, Sangthong *et al.* 2009), and *Torenia fournieri* (Mol 1986, Chen *et al.* 2008).

Torenia fournieri is a unique plant species that exhibits protruding embryo sacs (Higashiyama *et al.* 1998). Given this feature, female gametophytic cells are easily accessed when the carpel wall is dissected and the ovules are removed from the ovary (Higashiyama *et al.* 2001, Higashiyama 2002). Previously, we succeeded in generating a cDNA library from 25 synergid protoplasts isolated by cellulase treatment and identified genes specifically expressed in the synergid cells that functioned as pollen tube attractants (Okuda *et al.* 2009). These results suggested that gene expression analysis from isolated cells is a powerful tool to investigate the function of gametophytic

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cells. However, since only 1 egg cell, 1 central cell, and 2 synergid cells exist in an ovule, the total number of obtainable cells from one treatment is limited. Longer enzyme treatment may help isolate a larger number of cells, but may affect the cellular motility and thus the gene expression patterns within the cells (Leduc *et al.* 1995). For these reasons, improving the isolation method for collecting large numbers of cells in a short period of time is required.

Recently, large-scale analyses to identify the genes expressed in specific gametophytic cells have been undertaken using *Oryza sativa* and *Arabidopsis thaliana*. Specifically, intact egg and synergid cells were isolated from the unfertilized ovaries of *O. sativa* by manual manipulation without using enzymes (Ohnishi *et al.* 2010). A differential expression screening between the wild type and *determinant infertile1 (dif1)* mutant, which lacks a functional embryo sac, identified several genes expressed in *Arabidopsis* female gametophytes (Steffen *et al.* 2007). By examining gene expression in *myb98*, which is defective for the synergid cell-specific transcription factor *MYB98*, genes specifically expressed in the synergid cell that functioned under *MYB98* were identified (Punwani *et al.* 2007). For *Arabidopsis*, a laser-associated microdissection method was used to dissect the cells of the mature female gametophyte with minute cross-sections, and genes specifically expressed in each cell type were identified (Wuest *et al.* 2010). Genes that shared similar expression profiles even across monocot and eudicot species were regarded as cell-specific markers common to a wide range of plant species.

Here we compared cellulase enzymes for protoplast isolation activities and report a more efficient method of isolating gametophytic protoplasts from 2 additional plant species, *Torenia concolor* and *Lindernia micrantha*. We also investigated the genes specifically expressed in all types of gametophytic cells. We found that some genes showed cell-type-specific expression in *T. fournieri*, *T. concolor*, and *L. micrantha*, suggesting that these genes would be good markers for the cell types.

Materials and methods

Plant growth conditions

Torenia fournieri cv. Blue & White and *T. concolor* were grown in a plant growth room under stable temperature (25°C) and continuous light conditions. *Lindernia micrantha* was grown in the growth chamber (NK System) under stable temperature (25°C) and cycled light conditions (16 h light and 8 h dark).

Isolation of gametophytic cells

The flowers of *T. fournieri* and *T. concolor* 2 d after blooming were used for isolating cells. Because *L. micrantha* is an auto-pollinated self-compatible species, flowers were emasculated 1 d prior to the experiment.

Isolation of gametophytic cells was conducted as described previously with some modifications (Okuda *et al.* 2009). The cellulase enzyme solution contained 1% (w/v) cellulase, 0.3% macerozyme RS (Yakult), 0.05% pectolyase Y-23 (Yakult), 5 mM CaNO₃, and 0.4 M mannitol. Three types of cellulase, CELF (Worthington), the cellulase from *Aspergillus* (Sigma), and that from *Trichoderma* (Sigma), were used to compare enzyme activities. Aliquots of the cellulase solutions were stored at −80°C until used.

The cellulase enzyme solution was warmed to 28°C just before use. A mature ovary was dissected and the ovules were placed in 300 µl of the cellulase enzyme solution. These were maintained at 28°C for 1 h to generate protoplasts. Cells were distinguished by their morphological features and the quality of the protoplasts was evaluated by observation under an Olympus IX71 inverted microscope. Protoplasts were collected using a PicoPipet (Altair Co., Japan), then rinsed twice with 100 µl of wash solution (5 mM CaNO₃ and 0.4 M mannitol). After washing, they were

collected by brief centrifugation and frozen with liquid N₂ to isolate RNA.

Estimation of enzyme activities for protoplast isolation

To evaluate the enzyme activities for protoplast isolation, first, the ovules were taken from the ovary as described above. Because the protruding embryo sacs might be destroyed during this process, the number of ovules with intact embryo sacs was counted. Next, the ovules were treated with enzyme solution at 28°C. The number of central cells detached from the ovules was counted every 30 min during the treatment. The enzyme activity was defined as the rate of the number of detached central cells *versus* ovules with intact embryo sacs.

DNA staining

Protoplasts were rinsed with wash solution, then stained with 1% SYTO-11 (Invitrogen) in the wash solution for 1 min. Green fluorescence was observed under an Olympus IX71 epifluorescence microscope.

Isolation of RNA from protoplasts and RT-PCR

Total RNA from isolated egg and central and synergid cell protoplasts was extracted using Dynabeads (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScriptIII (Invitrogen) reverse transcriptase, and 250 ng of cDNA was used as a template for each PCR. The sequences of the primers used for RT-PCR were as follows (from 5' to 3'): *DD45*, CAAGTCGTGTAGTAGCGAAG and CACAAAACCCTCGAAGAAC; *SGS3*, GTTTGGCCTCC-TATGGTG and CTCACGATGCCAAAAGTC; *SWN*, CCATAGCGGTAGTCATAAAAG and CTTGTTTTGCAGCTGGAC. *CRP1*, *CRP4*, and *GAPDH* were as previously described (Okuda *et al.* 2009). We used 35 PCR cycles to amplify *CRP1* and *CRP4*, while 50 cycles were run for the other markers.

Results and discussion

We compared the gametophytic cell features between *T. fournieri* and the related species *T. concolor* and *L. micrantha* (Fig. 1). These species show similar floral morphologies to *T. fournieri*

Fig. 1. Flower and ovule morphology of the plant species used in this study. Flowers of *T. fournieri* (A), *T. concolor* (B), and *L. micrantha* (C). Ovules of *T. fournieri* (D), *T. concolor* (E), and *L. micrantha* (F) had protruding embryo sacs (arrowheads). Scale bar shown in F, 100 μ m for (D–F).

(Figs. 1A–C) and exhibit embryo sacs protruding from the ovules (Figs. 1D–F). We attempted to isolate gametophytic cells *via* treatment with cellulase. We previously reported that the synergid cells of *T. fournieri* could be isolated using CELF cellulase (Okuda *et al.* 2009). However, we found that this enzyme was not sufficient for cell isolation from *T. concolor* and *L. micrantha* (Table 1). Moreover, the central cell, located at the innermost position among these 3 types of gametophytic cells, did not detach by cellulase treatment in these 2 species (Table 1, Fig. 2D). Therefore, we attempted to compare the activities of several commercially available cellulases. *Aspergillus* cellulase was ineffective at isolating the central cells of all 3 plant species, even after 2 h of treatment (Figs. 2A, E, Table 1). In contrast, the cellulase from *Trichoderma* worked well (Figs. 2B–C, F, Table 1). The central cells were isolated from more than 40% of *T. fournieri* embryo sacs after 1.5 h of treatment, which is a higher efficiency than that of CELF (Table 1). Even after 1 h of treatment, 7.8% of *T. fournieri* central cells were detached. These results suggest that the cellulase

Table 1. Comparison of cellulase activities for gametophytic cell isolation

Cellulase	Plant species	% of central cells detached from the ovules (central cells/ovules)			
		0.5 h	1.0 h	1.5 h	2.0 h
CELF	<i>T. fournieri</i>	0.0 (0/347)	0.0 (0/347)	0.29 (1/347)	1.2 (4/347)
	<i>T. concolor</i>	0.0 (0/549)	0.0 (0/549)	0.0 (0/549)	0.0 (0/549)
	<i>L. micrantha</i>	0.0 (0/121)	0.0 (0/121)	0.0 (0/121)	0.0 (0/121)
<i>Aspergillus</i>	<i>T. fournieri</i>	0.0 (0/205)	0.0 (0/205)	0.0 (0/205)	0.0 (0/205)
	<i>T. concolor</i>	0.0 (0/290)	0.0 (0/290)	0.0 (0/290)	0.0 (0/290)
	<i>L. micrantha</i>	0.0 (0/301)	0.0 (0/301)	0.0 (0/301)	0.0 (0/301)
<i>Trichoderma</i>	<i>T. fournieri</i>	0.0 (0/462)	7.8 (36/461)	41.9 (83/198)	50.5 (257/509)
	<i>T. concolor</i>	0.0 (0/1252)	1.8 (23/1252)	5.0 (62/1252)	8.5 (106/1252)
	<i>L. micrantha</i>	0.0 (0/125)	0.0 (0/125)	1.6 (2/125)	5.6 (7/125)

Fig. 2. Effects of cellulases for protoplast isolation. Gametophytic cells did not detach from the embryo sac through treatment with the cellulase from *Aspergillus* (A, arrowhead), whereas following treatment with cellulase from *Trichoderma*, the central cell (B, arrowhead), the egg cell (C, arrowhead) and the synergid cells (C, arrows) became protoplasts. Cellulase activities for protoplast isolation by CELF (D), the cellulase from *Aspergillus* (E), and the cellulase from *Trichoderma* (F) were measured after 0.5, 1, 1.5, and 2 h of treatment. The Y-axis represents the isolation rate of the central cell. The black, dark gray, and pale gray bars represent *T. fournieri*, *T. concolor*, and *L. micrantha*, respectively. Scale bar, 20 μm.

from *Trichoderma* can be used to isolate gametophytic cells in *Torenia*.

To isolate and collect the protoplasts of gametophytic cells, we treated ovules with *Trichoderma* cellulase (Fig. 3). The egg, and central and synergid cells of *T. fournieri* exhibited unique morphological features (Figs. 3A–C). The central cell was largest and exhibited 1 large vacuole (Fig. 3B). The egg cell and the synergid cells were similar in size, but numerous cytoplasmic strands and small vacuoles distinguished the egg (Fig. 3A), whereas the synergid cells had 1 large vacuole (Fig. 3C). The isolated protoplasts often appeared fused; thus, we tested whether they were from single cells by staining the cells with the DNA-binding dye SYTO-11,

Fig. 3. Isolated protoplasts from 3 plant species. Protoplasts of the egg cell (A), the central cell (B), and the synergid cells (C) from *T. fournieri*. The same cells were stained with CYTO-11 to visualize DNA (D–F). Nuclei were identified as bright spots. Protoplasts of the egg cell (G), the central cell (H), and the synergid cells (I) from *T. concolor*. Protoplasts of the egg cell (J), the central cell (K) and the synergid cells (L) from *L. micrantha*. Inset in (L), a cluster of protoplasts. One egg cell (arrowhead), 2 synergid cells (arrows), and one central cell (large cell) were detached from the same ovule. Scale bar shown in (A), 20 μ m for (A–L); in the inset of (L), 20 μ m.

which rapidly permeates living protoplasts (Bethke *et al.* 1999). We found that all of the isolated protoplasts had individual nuclei, indicating they were from single cells and that the cells had not fused (Figs. 3D–F). We also isolated protoplasts from *T. concolor* and *L. micrantha* (Figs. 3G–L). In both species, the central cells were large and easily distinguished. To distinguish the egg cell and the synergid cells, the cells were carefully detached and isolated from the cluster of detached gametophytic cells (Fig. 3L, inset), and the cell types were subsequently confirmed by marker gene expression levels (see below).

Next, we isolated mRNA from each type of *T.ournieri* cell. We investigated the expression patterns of the following cell-specific marker genes found in *A. thaliana*: *DD45*, which encodes a cysteine-rich polypeptide and is expressed specifically in the egg cell (Steffen *et al.* 2007); *SGS3* and *SWN*, which encode the SUPPRESSOR OF GENE SILENCING3 protein required for posttranscriptional gene silencing; and a Polycomb group protein SWINGER expressed in the central cell (Mourrain *et al.* 2000, Chanvivattana *et al.* 2004). A putative homolog of *DD45* in *T.ournieri* was specifically found in the cDNA template from the egg cell (Fig. 4, left row). *SGS3* and *SWN* were specifically expressed in the central cell (Fig. 4, middle row), suggesting that these genes could be used as egg cell- and central cell-specific markers in *Torenia*. As previously reported, *CRP1* and *CRP4*, which encode cysteine-rich polypeptides, were specifically expressed in the synergid cell (Fig. 4, right row) (Okuda *et al.* 2009). Next, we tested these markers using the cDNA templates from *T. concolor* and *L. micrantha* (Fig. 4). In both species, the RT-PCR products from these markers were specifically found in each cell type. These results suggest that we successfully isolated protoplasts from each type of cell with no contamination from other gametophytic cells.

Our results indicate that cellulases differ in their cell isolation activities. Given that longer enzyme treatment could harm the cells, while shorter treatment times would more closely mimic natural conditions (Leduc *et al.* 1995), our modified method using the cellulase from *Trichoderma* will be especially useful for the rapid collection of large amounts of high-quality cells and mRNAs for future analysis of gene expression profiles in gametophytic cells. The comparison of gene expression profiles of *Torenia* gametophytic cells with those of eudicot species such as *A. thaliana* (Steffen *et al.* 2007, Wuest *et al.* 2010) and monocot species such as *O. sativa* (Ohnishi *et al.* 2010) will reveal the common and distinct features of plant gametophyte genes.

We showed that putative homologous genes of the *Arabidopsis* gametophytic cell-specific genes, *DD45*, *SGS3*, and *SWN*, were also specifically expressed in the corresponding types of cells in *Torenia* and *Lindernia*. This is the first report of egg cell and central cell markers in these plant

Fig. 4. Cell-specific expression of marker genes. cDNAs generated from an isolated egg cell (EC), central cell (CC), and synergid cell (SY) from *T.ournieri* (left row), *T. concolor* (middle), and *L. micrantha* (right) were used as the templates for RT-PCR. *DD45* was used as an egg cell marker, and *SGS3* and *SWN* were central cell markers. *CRP1* and *CRP4* were synergid cell markers. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) showed ubiquitous expression in all cell types.

species. Together with our previously reported synergid cell-specific genes, *CRP1* and *CRP4*, these will be good markers for gametophytic cells. In addition, this information will be useful for generating constructs that enable us to express or downregulate genes of interest in a gametophyte cell-specific manner.

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References

- Benet, H., Gall, E. A., Asensi, A. and Kloareg, B. 1997. Protoplast regeneration from gametophytes and sporophytes of some species in the order *Laminariales* (Phaeophyceae). *Protoplasma* **199**: 39–48.
- Bethke, P. C., Lonsdale, J. E., Fath, A. and Jones, R. L. 1999. Hormonally regulated programmed cell death in barley aleurone cells. *Plant Cell* **11**: 1033–1046.
- Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y. H., Sung, Z. R. and Goodrich, J. 2004. Interaction of Polycomb-group proteins controlling flowering in *Arabidopsis*. *Development* **131**: 5263–5276.
- Chen, S. H., Ang, Y. H., Liao, J. R., Kuang, A. X. and Tian, H. Q. 2008. Isolation of egg cells and zygotes of *Torenia fournieri* L. and determination of their surface charge. *Zygote* **16**: 179–186.
- Drews, G. N. and Yadegari, R. 2002. Development and function of the angiosperm female gametophyte. *Annu. Rev. Genet.* **36**: 99–124.
- Higashiyama, T. 2002. The synergid cell: Attractor and acceptor of the pollen tube for double fertilization. *J. Plant Res.* **115**: 149–160.
- , Kuroiwa, H., Kawano, S. and Kuroiwa, T. 1998. Guidance *in vitro* of the pollen tube to the naked embryo sac of *Torenia fournieri*. *Plant Cell* **10**: 2019–2032.
- , Yabe, S., Sasaki, N., Nishimura, Y., Miyagishima, S., Kuroiwa, H. and Kuroiwa, T. 2001. Pollen tube attraction by the synergid cell. *Science* **293**: 1480–1483.
- Huang, B. Q. and Russell, S. D. 1989. Isolation of Fixed and Viable Eggs, Central Cells, and Embryo Sacs from Ovules of *Plumbago-Zeylanica*. *Plant Physiol.* **90**: 9–12.
- Kasahara, R. D., Portereiko, M. F., Sandaklie-Nikolova, L., Rabiger, D. S. and Drews, G. N. 2005. MYB98 is required for pollen tube guidance and synergid cell differentiation in *Arabidopsis*. *Plant Cell* **17**: 2981–2992.
- Kato, N., Lorz, H. and Kranz, E. 1997. Isolation of viable egg cells of rape (*Brassica napus* L.). *Zygote* **5**: 31–&.
- Kovacs, M., Barnabas, B. and Kranz, E. 1994. The Isolation of Viable Egg Cells of Wheat (*Triticum-Aestivum* L.). *Sex. Plant Reprod.* **7**: 311–312.
- Laurain, D., Chenieux, J. C. and Tremouillauxguiller, J. 1993. Direct Embryogenesis from Female Haploid Protoplasts of *Ginkgo-Biloba* L., a Medicinal Woody Species. *Plant Cell Rep.* **12**: 656–660.
- Leduc, N., Matthysrochon, E. and Dumas, C. 1995. Deleterious Effect of Minimal Enzymatic Treatments on the Development of Isolated Maize Embryo Sacs in Culture. *Sex. Plant Reprod.* **8**: 313–317.
- Mol, R. 1986. Isolation of Protoplasts from Female Gametophytes of *Torenia-Fournieri*. *Plant Cell Rep.* **5**: 202–206.
- Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A. and Vaucheret, H. 2000. *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**: 533–542.
- Nakamura, M. and Maeda, M. 1994. Isolation and Culture of Protoplasts from Young Sporophytes of *Salvinia-Natans* Aseptically Obtained by Coculture of Female and Male Gametophytes. *Plant Cell Tissue Organ Cult.* **36**: 237–242.
- Ohnishi, T., Takanashi, H., Mogi, M., Takahashi, H., Kikuchi, S., Yano, K., Okamoto, T., Fujita, M., Kurata, N. and Tsutsumi, N. 2010. Distinct Gene Expression Profiles in Egg and Synergid Cells of Rice as Revealed by Cell Type-specific Microarrays. *Plant Physiol.* **155**: 881–891.

- Okuda, S., Tsutsui, H., Shiina, K., Sprunck, S., Takeuchi, H., Yui, R., Kasahara, R. D., Hamamura, Y., Mizukami, A., Susaki, D., Kawano, N., Sakakibara, T., Namiki, S., Itoh, K., Otsuka, K., Matsuzaki, M., Nozaki, H., Kuroiwa, T., Nakano, A., Kanaoka, M. M., Dresselhaus, T., Sasaki, N. and Higashiyama, T. 2009. Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. *Nature* **458**: 357–361.
- Punwani, J. A., Rabiger, D. S. and Drews, G. N. 2007. MYB98 positively regulates a battery of synergid-expressed genes encoding filiform apparatus localized proteins. *Plant Cell*. **19**: 2557–2568.
- Sangthong, R., Chin, D. P., Supaibulwatana, K. and Mii, M. 2009. Gametosomatic hybridization between egg cell protoplast and mesophyll protoplast of *Petunia hybrida*. *Plant Biotechnol. J.* **26**: 377–383.
- Steffen, J. G., Kang, I. H., Macfarlane, J. and Drews, G. N. 2007. Identification of genes expressed in the *Arabidopsis* female gametophyte. *Plant J.* **51**: 281–292.
- Theunis, C. H., Pierson, E. S. and Cresti, M. 1991. Isolation of Male and Female Gametes in Higher-Plants. *Sex. Plant Reprod.* **4**: 145–154.
- Wuest, S. E., Vijverberg, K., Schmidt, A., Weiss, M., Gheyselinck, J., Lohr, M., Wellmer, F., Rahnenfuhrer, J., von Mering, C. and Grossniklaus, U. 2010. *Arabidopsis* Female Gametophyte Gene Expression Map Reveals Similarities between Plant and Animal Gametes. *Curr. Biol.* **20**: 506–512.
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