Plant Biotechnology 33, 39–43 (2016)
DOI: 10.5511/plantbiotechnology.16.0123a

Short Communication

Isolation of gametes from *Brachypodium distachyon*

Takao Matsumura, Takashi Okamoto*

Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan
*E-mail: okamoto-takashi@tmu.ac.jp Tel: +81-426-77-2566 Fax: +81-426-77-2559

Received November 24, 2015; accepted January 23, 2016 (Edited by Y. Hoshino)

Abstract Isolated gametes from maize, rice, Arabidopsis, wheat and tobacco have been used for investigations for mechanisms in reproductive or developmental processes, such as gamete differentiation, gamete fusion, and early zygotic development. In addition, the isolated gametes from maize and rice can also be applied for in vitro fertilization to analyze postfertilization events. In the last decade, *Brachypodium distachyon* (Brachypodium) has emerged as an effective model for wheat, since wheat with its hexaploid nature shows some analytical difficulties. In this study, to take advantages of this new model monocot plant for investigations using isolated gametes, procedures for the isolation of Brachypodium gametes were established as the first step. Ovaries were first harvested from mature and unpollinated Brachypodium flowers. Thereafter, a transverse incision was placed at the bottom region of the ovary, resulting in direct access to the embryo sac, and egg cells, which were released from the dissected ovaries, were isolated. For sperm cell isolation, when pollen grains were immersed in mannitol solution, sperm cells were successfully released from pollen grains.

Key words: Brachypodium, egg cell, gamete, sperm cell.

Fertilization and subsequent events in angiosperms, such as embryogenesis and endosperm development, occur in the embryo sac deeply embedded in ovular tissue (Guignard 1899; Nawaschin 1898; Raghavan 2003; Russell 1992). Since investigations into the molecular mechanisms of fertilization and embryogenesis have been impeded by the difficulties in directly researching the biology of the embedded female gametophyte, zygote and early embryo, studies for these mechanisms have been conducted predominantly through analyses of Arabidopsis mutants or transformants coupled with live imaging (reviewed in Berger 2011; Denninger et al. 2014; Hamamura et al. 2012, 2014; Maruyama et al. 2015). Alternatively, direct analyses using isolated gametes or zygotes are possible, because procedures for isolating viable gametes/zygotes have been established in a wide range of plant species, including monocotyledonous and dicotyledonous plants (reviewed in Kranz 1999 and in Okamoto 2011; Yang et al. 2014). The use of gametes, zygotes or embryos isolated from flowers of maize, wheat, rice, Arabidopsis and tobacco has enabled researchers to successfully identify genes expressed specifically or preferentially in male gametes, female gametes, or early embryos (Abiko et al. 2013b; Anderson et al. 2013; Borges et al. 2008; Márton et al. 2005; Ning et al. 2006; Ohnishi et al. 2011; Russell et al. 2012; Sprunck et al. 2005; Steffen et al. 2007; Wang et al. 2010; Wuest et al. 2010; Yang et al. 2006), since it has been supposed that genes expressing specifically/preferentially in gametes or early embryos function in reproductive or developmental processes such as gamete differentiation, gamete fusion, and early zygotic development. In addition to transcriptome-based analyses, single-cell-type proteomic approaches have widely been employed to determine the functions of specific cells (Dai and Chen 2012), and, recently, proteins expressing in rice gametes were globally identified (Abiko et al. 2013a).

Isolated gametes have also been used with an in vitro fertilization (IVF) system to observe and analyze fertilization and postfertilization processes (reviewed in Wang et al. 2006). Kranz and Lörz (1993) first developed a complete IVF system using maize gametes and electrical fusion, and, to take advantage of the abundant resources stemming from rice research, a rice IVF system was also established by Uchiumi et al. (2007). By the use of these IVF systems, post-fertilization events, such as karyogamy (Faure et al. 1993; Ohnishi et al. 2014), egg activation and zygotic development (Kranz et al. 1995; Nakajima et al. 2010; Sato et al. 2010), paternal chromatin decondensation in zygote nucleus (Scholten et al. 2002), the microtubular architecture in egg cells and zygotes (Hoshino et al. 2004), fertilization-induced/suppressed gene expression (Okamoto et al. 2005), epigenetic resetting in early embryos (Jahnke and Scholten 2009), have been successfully observed and investigated.

Among three major agricultural cereal crops, an IVF system has so far been established in maize and

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; IVF, in vitro fertilization.
This article can be found at http://www.jspcmb.jp/
Published online March 1, 2016

rice (Kranz and Lörz, 1993; Uchiumi et al. 2007). In the case of wheat, although methods of isolation of egg cells, zygotes, and early embryos have been established (Kovacs et al. 1994; Kumlehn et al. 1999; Sprunck et al. 2005), a complete IVF system with wheat gametes has not yet been established. Wheat has the largest genome among the three major agricultural cereals, and the hexaploid nature of the wheat bread genome, consisting of the A, B and D genomes with high sequence similarity, causes problems in functional redundancy when wheat is used as an experimental material (Bhalla 2006; Fu et al. 2007). In the last decade, *Brachypodium distachyon* (Brachypodium) has emerged as an effective model for monocot species (Brkljacic et al. 2011; Opanowicz et al. 2008). Brachypodium has a small stature, short generation time, small genome, the ability to self-pollinate, and is easily grown under simple conditions (Draper et al. 2001). In addition, Brachypodium has been chosen as a model for wheat because it has a more recent common ancestor with wheat than rice or sorghum (Bortiri et al. 2008; International Brachypodium Initiative 2010; Wolny et al. 2011). Therefore, in the present study, we describe a protocol for the isolation of egg cells and sperm cells from unpollinated flowers of Brachypodium as initial step for establishing Brachypodium IVF system.

Seeds of *Brachypodium distachyon* accession Bd21 were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The seeds were first stratified at 4°C for 3 d on moist paper and were germinated in a growth chamber at 25°C under a 16 h/8 h light/dark cycle for 3–7 d. The germinated Brachypodium seedlings were then transferred to soil and grown in a growth chamber at 25°C under a 20 h/4 h light/dark cycle. Ovaries were harvested from unpollinated mature Brachypodium flowers (Figure 1A, Supplementary Figure 1), and transferred in plastic dishes (ϕ 3.5 cm) containing 2 ml of mannitol solution adjusted to 370 mOsmol kg^{-1} H_2O, which corresponds to about 6% mannitol solution. To observe Brachypodium egg cell embeddd in embryo sac, ovules were isolated from the ovaries in the mannitol solution using sharp forceps and a 30G short needle syringe (BD, USA) under a stereoscopic microscope, and observed with an inverted microscope (BX-71, Olympus, Japan). In each ovule, an embryo sac was observed (Figure 1B), and a putative egg cell was clearly visible in an embryo sac (Figure 1C), suggesting that the mature ovaries are suitable materials for egg cell isolation.

Next, to isolate the egg cells, ovaries in mannitol solution (370 mOsmol kg^{-1} H_2O) were first cut transversely with a razor blade at the bottom region (Figure 1A, dotted white line). By gently pushing the basal portion of the lower part of the ovary with a glass needle, a putative egg cell was released from the cut end of the dissected ovary (Figure 1D), since the transverse incision of the ovary provided direct access to the embryo sac. Synergy was hardly released from the the cut end of the dissected ovary. The cells released from the dissected ovaries were transferred into a 1 μl droplet of mannitol solution (370 mOsmol kg^{-1} H_2O) overlaid with mineral oil on a coverslip according to Okamoto (2011), and observed with the inverted microscope. The isolated putative egg cells showed a granular-structured cytoplasm and a developed nucleolus (Figure 1E), being consistent with cellular characteristics of egg cells isolated from rice (Uchiumi et al. 2006) and maize (Kranz et al. 1991; Kranz and Lörz 1993). In addition, vacuoles, ranging in size from 3–10 μm, existed at the peripheral region of the cells (Figure 1E), and these peripheral localization of vacuoles has been reported in isolated egg cells of rice (Uchiumi et al. 2007), maize (Faure et al. 1992) and wheat (Kovacs et al. 1994). These cytological characteristics observed in the isolated cells suggest that the cells released from cut Brachypodium ovaries are egg cells.

Distribution of mitochondria in the putative Brachypodium egg cell was observed, since it has been reported that mitochondria and starch granules are abundantly localized in the granular-structured cytoplasm (Diboll 1968; Faure et al. 1992, 1993; Uchiumi et al. 2006, 2007). To stain mitochondria, the putative Brachypodium egg cells were transferred into 2 μl droplets of mannitol solution (370 mOsmol kg^{-1} H_2O) containing 2 μg/ml MitoTracker Red CMXRos (Molecular Probes, USA) for 40 min at room temperature in darkness. The stained cells were transferred into fresh mannitol droplets twice for washing, and then observed with a BX-71 inverted fluorescence microscope (Olympus) with 520–550-nm excitation and >580-nm emission wavelengths (U-MWIG mirror unit; Olympus). Fluorescent signal from the mitochondrial probe was densely detected around the nucleus (Figure 1F), corresponding to the granular-structured cytoplasm. In contrast, when protoplasts prepared from cultured cells were stained with MitoTracker fluorescent indicator, signals of small foci were uniformly detected in cells (Figure 1G) as described by Uchiumi et al. (2006). The size of mitochondria in putative Brachypodium egg cells appeared to be larger than those in cultured cells, and such large size characters of mitochondria are also known in egg cells of geranium, maize and rice (Faure et al. 1992; Kuroiwa and Kuroiwa 1992; Uchiumi et al. 2006). These observations suggest that the cells isolated from Brachypodium ovaries are reliably identified as egg cells. The size of Brachypodium egg cells is about 30–35 μm in diameter, which is smaller than that in egg cells of rice (40–50 μm in 370 mOsmol kg^{-1} H_2O), maize (60–77 μm in 650 mOsmol kg^{-1} H_2O), wheat (50–70 μm in 620 mOsmol kg^{-1} H_2O) (Kovacs et al. 1994; Kranz et al 1991; Uchiumi et al. 2007). In the present study, egg

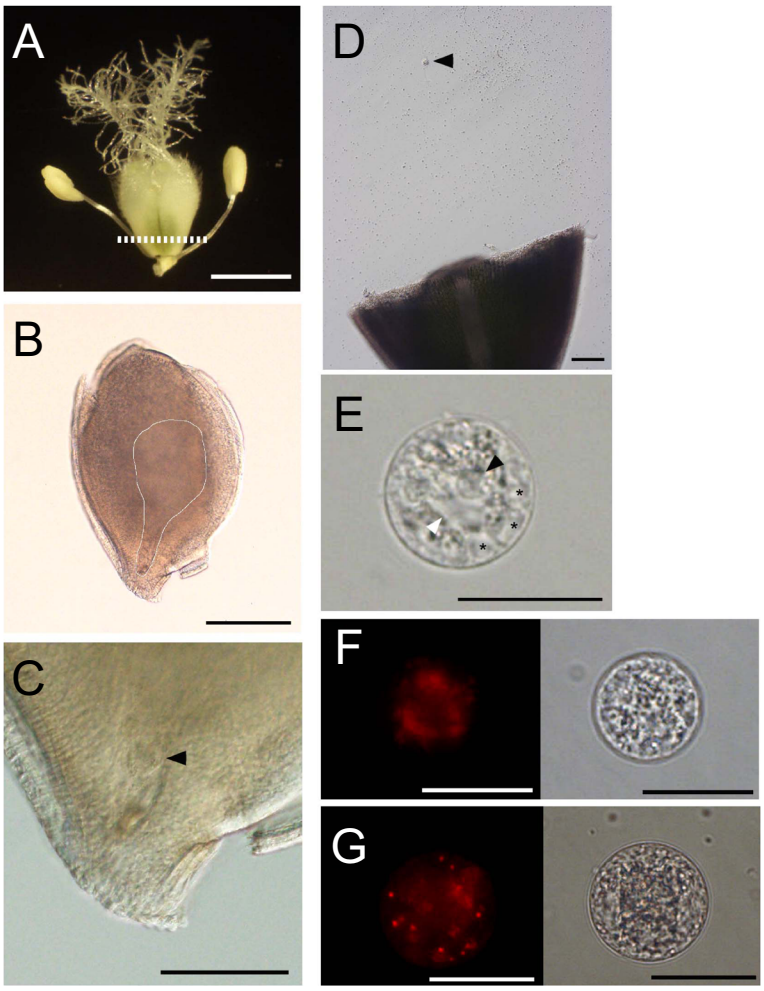


Figure 1. Isolation of Brachypodium egg cells from unpollinated ovaries. (A) A mature ovary with anthers harvested from an unpollinated Brachypodium flower. The dotted white line indicates the incision line on the ovary for egg isolation in panel D. (B) An ovule dissected from an unpollinated ovary. Possible embryo sac is enclosed with white line. (C) An egg cell in the embryo sac. An arrowhead indicates the putative egg cell. (D) An egg cell released from the basal portion of the dissected Brachypodium ovary. An arrowhead indicates the released egg cell. (E) An isolated Brachypodium egg cell. White- and black-colored arrowheads indicate the nucleus and nucleolus, respectively. Asterisks indicate typical vacuoles. (F) Mitochondrial labeling of an isolated Brachypodium egg cell with MitoTracker Red. Left and right panels are fluorescent and bright-field images, respectively. (G) Mitochondrial labeling of a protoplast prepared from cultured rice cells. Left and right panels are fluorescent and bright-field images, respectively. Scale bars = 1 mm in A, 200 μm in B, 100 μm in C and D, and 30 μm in E–G.

cells could be obtained, and, using the protocol, 15–30 egg cells could be isolated by an experimenter in a day.

Anthers harvested from unpollinated mature flowers (Figure 1A, Supplementary Figure 1) were transferred to plastic dishes (ϕ 3.5 cm) filled with 3 ml of 0.1 M sodium phosphate buffer (pH 7.0), containing 0.25 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), 1 mM EDTA and 0.1% Triton X-100, to observe maturation status of pollen grains in the anthers. The anthers were broken with forceps to free the pollen grains, and the released pollen grains were incubated for 1 h at room temperature under darkness. Then, the pollen grains were observed with 360–370 nm excitation and 420–460 nm emission wavelengths (U-MNUA2 mirror unit; Olympus). In a pollen grain, one vegetative and two sperm nuclei were obviously detected (Figure 2A), suggesting that Brachypodium pollen grains were tricellular-type grains, and that these pollen gains are suitable for isolation of sperm cells.

Soaking the pollen grains in a suitable osmotic solution generally results in burst of the grains and in releasing their contents, including sperm cells, into the solution (Kranz et al. 1991; Theunis et al. 1991). Therefore, an osmotic approach to isolate sperm cells from the Brachypodium pollen grains was employed. When the pollen grains were immersed in mannitol solution with the osmolality of 370 mOsmol kg^{-1} H_2O, the grains burst and their contents, including sperm cells, were released from a possible pollen tube pore of the grain after 5–10 min of immersion (Figure 2B). In many cases, a set of two cells was observed (Figure 2B), and these two putative sperm cells appeared to be released from a pollen grain. This releasing pattern of sperm cells is consistent with the Brachypodium pollen grains being

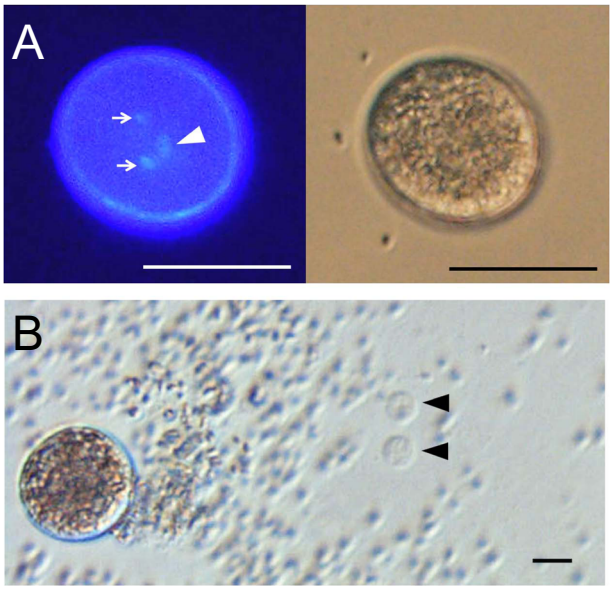


Figure 2. DAPI staining of Brachypodium pollen grains (A), and isolation of sperm cells (B). (A) Fluorescent labeling of nuclear DNA in a pollen grain with DAPI. Left and right panels are fluorescent and bright-field images, respectively. Two arrows and an arrowhead indicate the putative sperm nuclei and vegetative nucleus, respectively. (B) Pollen grain releasing its contents. Arrowheads indicate the released sperm cells. Scale bars = 30 μm in A, and 10 μm in B.

tricellular-type. The size of Brachypodium sperm cells is about 7–9 μm in diameter. Using the isolation procedure, 30–50 sperm cells can be obtained by one experimenter per hour.

Finally, we tested whether the procedure for electrofusion-based IVF with rice gametes is available for the electro-fusion of Brachypodium gametes. According to Uchiumi et al. (2007) and Okamoto (2011), IVF was conducted using 42 pairs of isolated egg and sperm cells. However, gamete fusion was observed in only one pair of gametes, and the remaining 41 pairs of gametes showed no fusion. In addition, a resultant fused gamete, possibly a zygote, did not divide even if the fused gamete was further cultured in N6Z liquid medium, which is used for zygote culture in barley (Kumlehn et al. 1999) and in rice (Uchiumi et al. 2007), for 2 d. The fused production finally degenerated. We are currently attempting to ascertain the optimal conditions for Brachypodium IVF system and for culturing the zygotes produced by IVF. In the present study, we developed a reliable isolation procedure for obtaining Brachypodium gametes, although optimization of parameters for electro-fusion of gametes is essential to establish Brachypodium in vitro fertilization system.

Acknowledgements

We thank Ms. T. Mochizuki (Tokyo Metropolitan University) for isolating Brachypodium egg cells and the RIKEN Bio Resource Center (Tsukuba, Japan) for providing Brachypodium seeds. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants-in-Aid no. 26113715 to T.O.) and the Japan Society for the Promotion of Science (Grant-in-Aid no. 25650083 to T.O.).

References

Abiko M, Furuta K, Yamauchi Y, Fujita C, Taoka M, Isobe T, Okamoto T (2013a) Identification of proteins enriched in rice egg or sperm cells by single-cell proteomics. *PLoS ONE* 8: e69578

Abiko M, Maeda H, Tamura K, Hara-Nishimura I, Okamoto T (2013b) Gene expression profiles in rice gametes and zygotes: Identification of gamete-enriched genes and up- or down-regulated genes in zygotes after fertilization. *J Exp Bot* 64: 1927–1940

Anderson SN, Johnson CS, Jones DS, Conrad LJ, Gou X, Russell SD, Sundaresan V (2013) Transcriptomes of isolated *Oryza sativa* gametes characterized by deep sequencing: Evidence for distinct sex-dependent chromatin and epigenetic states before fertilization. *Plant J* 76: 729–741

Berger F (2011) Imaging fertilization in flowering plants, not so abominable after all. *J Exp Bot* 62: 1651–1658

Bhalla PL (2006) Genetic engineering of wheat: Current challenges and opportunities. *Trends Biotechnol* 24: 305–311

Borges F, Gomes G, Gardner R, Moreno N, McCormick S, Feijó JA, Becker JD (2008) Comparative transcriptomics of *Arabidopsis* sperm cells. *Plant Physiol* 148: 1168–1181

Bortiri E, Coleman-Derr D, Lazo GR, Anderson OD, Gu YQ (2008) The complete chloroplast genome sequence of Brachypodium distachyon: sequence comparison and phylogenetic analysis of eight grass plastomes. *BMC Res Notes* 1: 61

Brkljacic J, Grotewold E, Scholl R, Mockler T, Garvin DF, Vain P, Brutnell T, Sibout R, Bevan M, Budak H, et al. (2011) *Brachypodium* as a model for the grasses: Today and the future. *Plant Physiol* 157: 3–13

Dai S, Chen S (2012) Single-cell-type proteomics: Toward a holistic understanding of plant function. *Mol Cell Proteomics* 11: 1622–1630

Denninger P, Bleckmann A, Lausser A, Vogler F, Ott T, Ehrhardt DW, Frommer WB, Sprunck S, Dresselhaus T, Grossmann G (2014) Male–female communication triggers calcium signatures during fertilization in *Arabidopsis*. *Nat Commun* 5: 4645

Diboll AG (1968) Fine structural development of the megagametophyte of *Zea mays* followings fertilization. *Am J Bot* 55: 787–806

Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P, Hasterok R, Routledge APM (2001) Brachypodium distachyon: A new model system for functional genomics in grasses. *Plant Physiol* 127: 1539–1555

Faure J, Mogensen HL, Kranz E, Digonnet C, Dumas C (1992) Ultrastructural characterization and three-dimensional reconstruction of isolated maize (*Zea mays* L.) egg cell protoplasts. *Protoplasma* 171: 97–103

Faure JE, Mogensen HL, Dumas C, Lörz H, Kranz E (1993) Karyogamy after electrofusion of single egg and sperm cell protoplasts from maize: Cytological evidence and time course. *Plant Cell* 5: 747–755

Fu D, Uauy C, Blechl A, Dubcovsky J (2007) RNA interference for wheat functional gene analysis. *Transgenic Res* 16: 689–701

Guignard ML (1899) Sur les antherozoides et la double copulation sexuelle chez les vegetaux angiosperms. *Rev Gén Bot* 11: 129–135

Hamamura Y, Nagahara S, Higashiyama T (2012) Double fertilization on the move. *Curr Opin Plant Biol* 15: 70–77

Hamamura Y, Nishimaki M, Takeuchi H, Geitmann A, Kurihara D, Higashiyama T (2014) Live imaging of calcium spikes during double fertilization in *Arabidopsis*. *Nat Commun* 5: 4722

Hoshino Y, Scholten S, von Wiegen P, Lörz H, Kranz E (2004) Fertilization induced changes in the microtubular architecture of the maize egg cell and zygote: An immunocytochemical approach adapted to single cells. *Sex Plant Reprod* 17: 89–95

International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass Brachypodium distachyon. *Nature* 463: 763–768

Jahnke S, Scholten S (2009) Epigenetic resetting of a gene imprinted in plant embryos. *Curr Biol* 19: 1677–1681

Kovacs M, Barnabas B, Kranz E (1994) The isolation of viable egg cells of wheat (*Triticum aestivum* L.). *Sex Plant Reprod* 7: 311–312

Kranz E (1999) In vitro fertilization with isolated single gametes. *Methods Mol Biol* 111: 259–267

Kranz E, Bautor J, Lörz H (1991) In vitro fertilization of single, isolated gametes of maize mediated electrofusion. *Sex Plant Reprod* 4: 12–16

Kranz E, Lörz H (1993) In vitro fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *Plant Cell* 5: 739–746

Kranz E, von Wiegen P, Lörz H (1995) Early cytological events after induction of cell division in egg cells and zygote development following in vitro fertilization with angiosperm gametes. *Plant J* 8: 9–23

Kumlehn J, Lörz H, Kranz E (1999) Monitoring individual development of isolated wheat zygotes: A novel approach to study embryogenesis. *Protoplasma* 208: 156–162

Kuroiwa H, Kuroiwa T (1992) Giant mitochondria in the mature egg cell of *Pelargonium zonale*. *Protoplasma* 168: 184–188

Márton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. *Science* 307: 573–576

Maruyama D, Völz R, Takeuchi H, Mori T, Igawa T, Kurihara D, Kawashima T, Ueda M, Itoh M, Umeda M, et al. (2015) Rapid Elimination of the Persistent Synergid through a Cell Fusion Mechanism. *Cell* 161: 907–918

Nakajima K, Uchiumi T, Okamoto T (2010) Positional relationship between the gamete fusion site and the first division plane in the rice zygote. *J Exp Bot* 61: 3101–3105

Nawaschin S (1898) Revision der Befruchtungsvorgange bei Lilium martagon und Fritillaria tenella. *Bull Acad Imp Sci St Pétersbourg* 9: 377–382

Ning J, Peng X-B, Qu L-H, Xin HP, Yan TT, Sun MX (2006) Differential gene expression in egg cells and zygotes suggests that the transcriptome is restructed before the first zygotic division in tobacco. *FEBS Lett* 580: 1747–1752

Ohnishi T, Takanashi H, Mogi M, Takahashi H, Kikuchi S, Yano K, Okamoto T, Fujita M, Kurata N, Tsutsumi N (2011) Distinct gene expression profiles in egg and synergid cells of rice as revealed by cell type-specific microarrays. *Plant Physiol* 155: 881–891

Ohnishi Y, Hoshino R, Okamoto T (2014) Dynamics of male and female chromatin during karyogamy in rice zygotes. *Plant Physiol* 165: 1533–1543

Okamoto T (2011) In vitro fertilization with rice gametes: Production of zygotes and zygote and embryo culture. *Methods Mol Biol* 710: 17–27

Okamoto T, Scholten S, Lörz H, Kranz E (2005) Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation- and PCR-based approach. *Plant Cell Physiol* 46: 332–338

Opanowicz M, Vain P, Draper J, Parker D, Doonan JH (2008) Brachypodium distachyon: Making hay with a wild grass. *Trends Plant Sci* 13: 172–177

Raghavan V (2003) Some reflections on double fertilization, from its discovery to the present. *New Phytol* 159: 565–583

Russell SD (1992) Double fertilization. *Int Rev Cytol* 40: 357–390

Russell SD, Gou X, Wong C, Wang X, Yuan T, Wei X, Bhalla PL, Singh MB (2012) Genomic profiling of rice sperm cell transcripts reveals conserved and distinct elements in the flowering plant male germ lineage. *New Phytol* 195: 560–573

Sato A, Toyooka K, Okamoto T (2010) Asymmetric cell division of rice zygotes located in embryo sac and produced by in vitro fertilization. *Sex Plant Reprod* 23: 211–217

Scholten S, Lörz H, Kranz E (2002) Paternal mRNA and protein synthesis coincides with male chromatin decondensation in maize zygotes. *Plant J* 32: 221–231

Sprunck S, Baumann U, Edwards K, Langridge P, Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (*Triticum aestivum* L.). *Plant J* 41: 660–672

Steffen JG, Kang IH, Macfarlane J, Drews GN (2007) Identification of genes expressed in the Arabidopsis female gametophyte. *Plant J* 51: 281–292

Theunis C, Pierson E, Cresti M (1991) Isolation of male and female gametes in higher plants. *Sex Plant Reprod* 4: 145–154

Uchiumi T, Komatsu S, Koshiba T, Okamoto T (2006) Isolation of gametes and central cells from *Oryza sativa* L. *Sex Plant Reprod* 19: 37–45

Uchiumi T, Uemura I, Okamoto T (2007) Establishment of an in vitro fertilization system in rice (*Oryza sativa* L.). *Planta* 226: 581–589

Wang YY, Kuang A, Russell SD, Tian HQ (2006) In vitro fertilization as a tool for investigating sexual reproduction of angiosperm. *Sex Plant Reprod* 19: 103–115

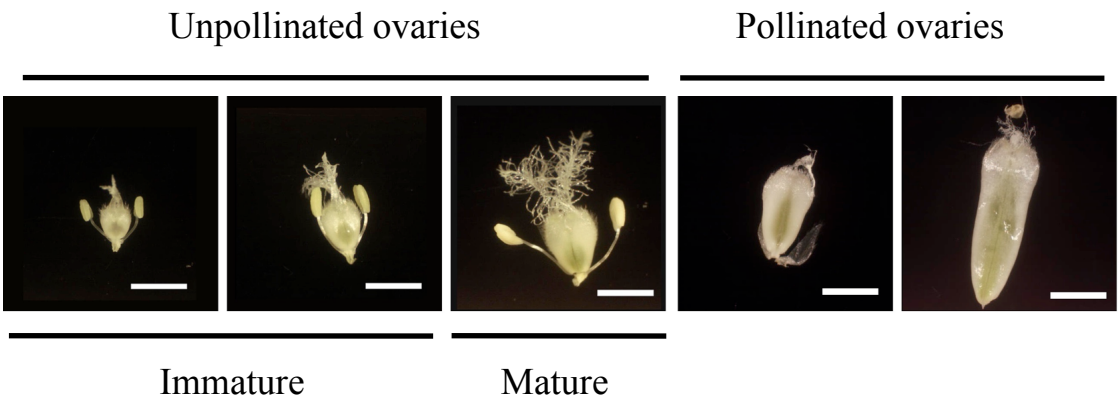
Wang D, Zhang CQ, Hearn DJ, Kang IH, Punwani JA, Skaggs MI, Drews GN, Schumaker KS, Yadegari R (2010) Identification of transcription-factor genes expressed in the Arabidopsis female gametophyte. *BMC Plant Biol* 10: 110

Wolny E, Lesniewska K, Hasterok R, Langdon T (2011) Compact genomes and complex evolution in the genus Brachypodium. *Chromosoma* 120: 199–212

Wuest SE, Vijverberg K, Schmidt A, Weiss M, Gheyselinck J, Lohr M, Wellmer F, Rahnenführer J, von Mering C, Grossniklaus U (2010) *Arabidopsis* female gametophyte gene expression map reveals similarities between plant and animal gametes. *Curr Biol* 20: 506–512

Yang H, Kaur N, Kiriakopolos S, McCormick S (2006) EST generation and analyses towards identifying female gametophyte-specific genes in *Zea mays* L. *Planta* 224: 1004–1014

Yang SJ, Wei DM, Tian HO (2014) Isolation of sperm cells, egg cells, synergids and central cells from *Solanum verbascifolium* L. *J Plant Biochem Biotechnol* 24: 400–407



Supplementary Figure 1. Unpollinated and pollinated Brachypodium ovaries. Unpollinated ovaries at immature and mature stages were presented. Bars = 1 mm.