Elucidating the molecular mechanics of the final stages of double fertilization

Toshiyuki Mori

Miyagishima Initiative Research Unit, Advanced Science Institute, RIKEN,
2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Summary: Fertilization is one of the most exciting phases in all organisms possessing sex. Understanding fertilization in plants is essential for the resolution of food problems that are dependent on agricultural products. Although plant researchers have had difficulties with analyses of gamete fusion process in angiosperm fertilization for several years, recent reports are showing remarkable findings important for understanding molecular mechanics during double fertilization. In this review, I would like to discuss the nature of angiosperm gamete fusion, based on my recent study findings.

Key words: pollen, male gamete, double fertilization, sexual reproduction

Angiosperm double fertilization

Plants reproduce through fusion of male and female gametes. In flowering plants, fertilization starts with pollination at the top of the pistil (stigma). A pollen grain is composed of two cell types; a large vegetative cell and a pair of sperm cells. While the vegetative cell elongates into a pollen tube after pollination, the sperm cell pair, enclosed in the vegetative cell, migrates in the pollen tube towards an ovule containing the female gametophyte (embryo sac). When the pollen tube reaches the micropyle, its tip is ruptured to release the sperm cell pair into one of the synergid cells, which functions as a pollen tube attractor in the embryo sac. At this time, both sperm cells face the female gametes (egg and central cells). One of sperm cells fuses with the egg cell and the other fuses with the central cell, which develop into an embryo and endosperm, respectively. Although the double fertilization system has been known for more than a century, knowledge of the molecular mechanics during gamete interaction is still poor. In this fertilization scenario, at least three questions must be elucidated, as follows.

Mystery #1: How does the sperm pair move toward the female gamete?

Unlike animal sperm, angiosperm sperm cells are not motile. Amazingly, the sperm pair released from the pollen tube move toward the female gametes while in

the degenerated synergid cell, despite being free of the pollen tube cytoplasm. Several studies have shown that unique actin structures develop in the synergid cell and that each sperm cell is transported on an actin string leading to an egg or a central cell (Russell 1996). If this hypothesis is true, sperm cell migration should be based on the actomyosin system, but isolated sperm cells do not show myosin molecules on their surface (Zhang and Russell 1999). However, the burst pollen tube and degenerated synergid cell have exogenous myosin sources, and the negatively charged sperm cells acquire these myosin molecules for movement (Russell 1996, Zhang and Russell 1999).

Mystery #2: What kinds of mechanisms ensure two fertilization pairs?

Although both sperm cells face the egg and central cells in the degenerated synergid cell, each sperm cell fuses with one of the female gametes. Some studies have reported that sperm cells in the same pair are dimorphic, and that their female target is determined at the time of male gametogenesis (Russell 1983, Russell 1985, Faure et al. 2003). However, this hypothesis is not applicable to all angiosperm species, and the subcellular mechanism ensuring such destined fertilization is unknown. Recent studies using an *Arabidopsis* mutant, whose egg cell is duplicated in the same embryo sac, demonstrated that both sperm cells have the capability to fuse with the egg

cell, resulting in duplicate embryogenesis (Pagnussat et al. 2007, Ingouff et al. 2009). Furthermore, another *Arabidopsis* mutant, *TETRA SPORES (TES)*, shows an interesting fertilization mechanism; a *tes* pollen grain contains four pairs of sperm cells because of a deficiency in microspore segregation, and the pollen tube delivers multiple sperm cell pairs to an embryo sac (Spielman et al. 1997). Interestingly, despite the extra sperm cell pairs, the egg cell fuses with only one sperm cell, while the central cell fuses with the remaining sperm cell of the pair (Scott et al. 2008). Such polyspermy blocking the egg cell has also been shown in a maize *in vitro* fertilization assay (Faure et al. 1994). Taken together, successful double fertilization may be ensured by such an egg cell contribution.

Mystery #3: What promotes gamete fusion?

The nature of gamete fusion is poorly understood in almost all eukaryotes. One of the reasons is that the gamete fusion phenomenon is transient and occurs in a narrow space between male and female gametes. The fertilization mechanism depends on the species, and is based on gamete membrane fusion at the surface of both gametes in some species (e.g., humans and *Chlamydomonas* (Wilson and Snell 1998, Primakoff and Myles 2007)), and on a smaller gamete entering the larger one in others (e.g., insects and *Plasmodium* (Sinden and Croll 1975, Wilson et al. 2006)). Several studies have tried to identify gamete surface factors (Primakoff and Myles 2007), because such factors may provide important clues for understanding gamete recognition and/or gamete fusion. In flowering plants, many previous reports, based on light and electron microscopy, have tried to reveal the moment of gamete fusion (Faure et al. 1993, Mol et al. 1994, Williams and Friedman 2002, Ye et al. 2002). Additionally, biochemical and molecular biological strategies based on isolated gametes have tried to identify male gametic genes (Ueda and Tanaka 1994, 1995a, 1995b, Southworth and Kwiatkowski 1996, Xu et al. 1999, Ueda et al. 2000, Ueda et al. 2005), however, no subcellular or molecular factors related to fertilization have been

reported until recently.

Identification of the first factor critical to angiosperm fertilization

The trumpet lily (*Lilium longiflorum*) is a flowering plant whose pollen generative cells (sperm cell precursors) are efficiently isolatable via pollen protoplasts (Tanaka 1988). Mori et al. (2006) recently succeeded in isolating a novel gene expressed exclusively in the male gamete of *L. longiflorum* pollen. As a result of a differential display assay based on those isolated generative cells, the *GENERATIVE CELL SPECIFIC 1 (GCS1)* gene was identified. Flowering plant GCS1s are all composed of approximately 700 amino acid residues and possess an N-terminal signal sequence and a nearly-C-terminal transmembrane domain followed by a histidine-rich region, suggesting that GCS1 is a novel single-pass transmembrane protein. Interestingly, GCS1 is not flowering-plant specific and putative GCS1 orthologs have been identified in lower plants including algae (e.g., *Chlamydomonas reinhardtii* and *Cyanidioschyzon merolae*), unicellular parasites (e.g., *Plasmodium falciparum* and *Leishmania major*), slime molds (e.g., *Physarum polycephalum* and *Dictyostelium discoideum*), insects (e.g., *Tribolium castaneum* and *Apis mellifera*), and cnidarian species (e.g., *Hydra magnipapillata* and *Nematostella vectensis*) (Mori et al. 2006, von Besser et al. 2006, Hirai et al. 2008, Liu et al. 2008, Steele and Dana 2009). Immunochemical and immunohistological experiments have shown clearly that GCS1 accumulates in the male gamete membrane, implying that it is related to gamete fusion function (Mori et al. 2006). A heterozygous *Arabidopsis* GCS1 knockout line (+/*gcs1*) was characterized to investigate GCS1 function. As a result, *gcs1* pollen was sterile, and *gcs1* sperm cells failed to fuse with the egg and central cells. This strongly suggests that GCS1 is essential for gamete fusion and functions in double fertilization (Mori et al. 2006, von Besser et al. 2006).

Interestingly, GCS1 also functions in the gamete interactions of various phylogenetically distant organisms, and its functional manner is highly conserved. In the rodent malaria parasite *Plasmodium berghei*, two

groups have demonstrated male gamete GCS1 expression and indicated that it is critical for gamete fusion (Hirai et al. 2008, Liu et al. 2008). Liu et al. (2008) also showed that *Chlamydomonas* GCS1 is expressed specifically in the male gamete, and that GCS1 knockout male gametes failed to fuse with the wild type female.

Elucidating the molecular function of GCS1

To date, two types of gamete fusion mechanisms have been elucidated in organisms that reproduce with GCS1. One is gamete fusion based on a merge between male and female mating structures at the gamete fusion site, as seen in *Chlamydomonas* (Liu et al. 2008) (Figure 1). The other is based on the male gamete entering the female gamete, as seen in *Plasmodium* (Hirai et al. 2008, Liu et al. 2008) (Figure 1). In *Chlamydomonas*, the female mating structure (mating tube) penetrates the male structure expressing GCS1, followed by cytoplasmic continuity. Although this situation seems opposite to that of *Plasmodium* fertilization (male entering female), both organisms surely utilize GCS1 as a common factor for fertilization. A detailed study on *Chlamydomonas* GCS1 may have provided some important clues for understanding precise GCS1 function during gamete fusion. In GCS1 knockout *Chlamydomonas*, gamete attachment controlled by FUS1, which is a transmembrane protein from the female mating structure, is not affected, but the gamete membrane fusion never occurs, suggesting that GCS1 functions in membrane fusion or a step immediately before membrane fusion (Misamore et al. 2003, Liu et al. 2008). *Chlamydomonas* GCS1 expression is restricted to the male mating structure, implying that its function only lasts until fusion of the mating structures. In contrast, GCS1 expression in higher plants and *Plasmodium* is distributed throughout the male gametes (Mori et al. 2006, von Besser et al. 2006, Hirai et al. 2008, Liu et al. 2008). In *P. berghei*, the male gamete maintains motility until it completes entering the female gamete, whereas the *gcs1* male gamete never enters, although it recognizes the female gamete (Hirai et al. 2008).

Fig. 1 A schematic model on GCS1 function. In this model, GCS1 molecule is expected to function in gamete membrane tethering.

Furthermore, live imaging has shown that *Arabidopsis* GCS1 expression continues even after the sperm has entered the egg (unpublished data), suggesting that higher plants also use gamete fusion based on male entering female and that the GCS1 function in both organisms probably continues until they complete gamete fusion. Taken together, I present a schematic model of the molecular function of GCS1 in which it serves as a factor tethering male and female gametes (Figure

1). *Chlamydomonas* GCS1 tethers mating structure membranes from both gametes, and gamete membrane fusion follows mating structure fusion. On the other hand, the male gamete of *Plasmodium* and that of higher plants enter the female gamete, adhering to the female gamete membrane with GCS1 molecules. When the male gamete has completely entered the female gamete, membrane fusion occurs. Furthermore, when the *GCS1* gene is disrupted, the male gametes never enter or accept the female gametes.

Acknowledgements

I thank Drs. Miyagishima S., Igawa T., Yanagawa Y. and Inada N. for technical supports to this study. This study is supported by the Special Postdoctoral Researchers Program of Riken, Sumitomo Foundation and Grant-in-Aid for Scientific Research on Innovative Areas (21112008)

References

- Dumas, C., Mogensen, H. L., Rougier, M., Chaboud, A. (2003) Double fertilization in maize: the two male gametes from a pollen grain have the ability to fuse with egg cells. *Plant J* 33:1051–1062
- Faure, J. E., Mogensen, H. L., Dumas, C., Lorz, 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
- Faure, J. E., Digonnet, C., Dumas, C. (1994) An in vitro system for adhesion and fusion of maize gametes. *Science* 263:1598–1600
- Faure, J. E., Rusche, M. L., Thomas, A., Keim, P., Pagnussat, G. C., Yu, H. J., Sundaresan, V. (2007) Cell-fate switch of synergid to egg cell in *Arabidopsis eostre* mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene BLH1. *Plant Cell* 19:3578–3592
- Hirai, M., Arai, M., Mori, T., Miyagishima, S. Y., Kawai, S., Kita, K., Kuroiwa, T., Terenius, O., Matsuoka, H. (2008) Male fertility of malaria parasites is determined by GCS1, a plant-type reproduction factor. *Curr Biol* 18:607–613
- Ingouff, M., Sakata, T., Li, J., Sprunck, S., Dresselhaus, T., Berger F. (2009) The two male gametes share equal ability to fertilize the egg cell in *Arabidopsis thaliana*. *Curr Biol* 19:R19–R20
- Liu, Y., Tewari, R., Ning, J., Blagborough, A. M., Garbom, S., Pei, J., Grishin, N. V., Steele, R. E., Sinden, R. E., Snell, W. J., Billker, O. (2008) The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in *Chlamydomonas* and *Plasmodium* gametes. *Genes Dev* 22:1051–1068
- Misamore, M. J., Gupta, S., Snell, W. J. (2003) The *Chlamydomonas* Fus1 protein is present on the mating type plus fusion organelle and required for a critical membrane adhesion event during fusion with minus gametes. *Mol Biol Cell* 14:2530–2542
- Mori, T., Kuroiwa, H., Higashiyama, T., Kuroiwa, T. (2006) GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. *Nature Cell Biol* 8:64–71
- Mol, R., Matthys-Rochon, E., Dumas, C. (1994) The kinetics of cytological events during double fertilization in *Zea mays* L. *Plant J* 5:197–206
- Primakoff, P., Myles, D. G. (2007) Cell–cell membrane fusion during mammalian fertilization. *FEBS Lett* 581: 2174–2180
- Russell, S. D. (1983) Fertilization in *Plumbago zeylanica*: Gametic fusion and fate of the male cytoplasm. *Am J Bot* 70:416–434
- Russell, S. D. (1985) Preferential fertilization in *Plumbago*: ultrastructural evidence for gamete-level recognition in an angiosperm. *Proc Natl Acad Sci U S A* 82:6129–6132
- Russell, S. D. (1996) Attraction and transport of male gametes for fertilization. *Sex Plant Reprod* 9:337–342
- Scott, R. J., Armstrong, S. J., Doughty, J., Spielman, M. (2008) Double fertilization in *Arabidopsis thaliana* involves a polyspermy block on the egg but not the central cell. *Mol Plant* 1:611–619
- Sinden, R. E., Croll, N. A. (1975) Cytology and kinetics of microgametogenesis and fertilization in *Plasmodium yoelii nigeriensis*. *Parasitology* 70:53–65
- Southworth, D., Kwiatkowski, D. (1996) Arabinogalactan proteins at the cell surface of *Brassica* sperm and *Lilium* sperm and generative cells. *Sex Plant Reprod* 9:269–272
- Spielman, M., Preuss, D., Li, F. L., Browne, W. E., Scott, R. J., Dickinson, H. G. (1997) TETRASPORE is required for male meiotic cytokinesis in *Arabidopsis thaliana*. *Development* 124:2645–2657
- Steele, R. E., Dana, C. E. (2009) Evolutionary history of the HAP2/GCS1 gene and sexual reproduction in metazoans. *PLoS One* 4:e7680
- Tanaka, I. (1988) Isolation of generative cells and their protoplasts from pollen of *Lilium longiflorum*. *Protoplasma* 142:68–73
- Ueda, K., Tanaka, I. (1994) The basic proteins of male gametic nuclei isolated from pollen grains of *Lilium longiflorum*. *Planta* 192:446–452
- Ueda, K., Tanaka, I. (1995a) The appearance of male gamete-specific histones gH2B and gH3 during pollen development in *Lilium longiflorum*. *Dev Biol* 169:210–217
- Ueda, K., Tanaka, I. (1995b) Male gametic nucleus-specific H2B and H3 histones, designated gH2B and gH3, in *Lilium longiflorum*. *Planta* 197:289–295
- Ueda, K., Kinoshita, Y., Xu, Z. J., Ide, N., Ono, M., Akahori, Y., Tanaka, I., Inoue, M. (2000) Unusual core histones specifically expressed in male gametic cells of *Lilium longiflorum*. *Chromosoma* 108:491–500
- Ueda, K., Suzuki, M., Ono, M., Ide, N., Tanaka, I., Inoue,

- M. (2005) Male gametic cell-specific histone gH2A gene of *Lilium longiflorum*: genomic structure and promoter activity in the generative cell. *Plant Mol Biol.* 59:229–238.
- von Besser, K., Frank, A. C., Johnson, M. A., Preuss, D. (2006) *Arabidopsis* HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development* 133:4761–4769
- Wilson, K. L., Fitch, K. R., Bafus, B. T., Wakimoto, B. T. (2006) Sperm plasma membrane breakdown during *Drosophila* fertilization requires sneaky, an acrosomal membrane protein. *Development* 133:4871–4879
- Wilson, N. F., Snell, W. J. (1998) Microvilli and cell-cell fusion during fertilization. *Trends Cell Biol* 8:93–96
- Williams, J. H., Friedman, W. E. (2002) Identification of diploid endosperm in an early angiosperm lineage. *Nature* 415:522–526
- Xu, H., Swoboda, I., Bhalla, P. L., Singh, M. B. (1999) Male gametic cell-specific gene expression in flowering plants. *Proc Natl Acad Sci U S A.* 96:2554–2558
- Ye, X. L., Yeung, E. C., Zee, S. Y. (2002) Sperm movement during double fertilization of a flowering plant, *Phaius tankervilleae*. *Planta.* 215:60–66
- Zhang, Z., Russell, S. D. (1999) Sperm cell surface characteristics of *Plumbago zeylanica* L. in relation to transport in the embryo sac. *Planta* 208:539–544