

# Analysis of gamete membrane dynamics during double fertilization of Arabidopsis

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**Abstract** Angiosperms have a unique sexual reproduction system called “double fertilization.” One sperm cell fertilizes the egg and another sperm cell fertilizes the central cell. To date, plant gamete membrane dynamics during fertilization has been poorly understood. To analyze this unrevealed gamete subcellular behavior, live cell imaging analyses of Arabidopsis double fertilization were performed. We produced female gamete membrane marker lines in which fluorescent proteins conjugated with PIP2a finely visualized egg cell and central cell surfaces. Using those lines together with a sperm cell membrane marker line expressing GCS1-GFP, the double fertilization process was observed. As a result, after gamete fusion, putative

sperm plasma membrane GFP signals were occasionally detected on the egg cell surface adjacent to the central cell. In addition, time-lapse imaging revealed that GCS1-GFP signals entered both the egg cell and the central cell in parallel with the sperm cell movement toward the female gametes during double fertilization. These findings suggested that the gamete fusion process based on membrane dynamics was composed of (1) plasma membrane fusion on male and female gamete surfaces, (2) entry of sperm internal membrane components into the female gametes, and (3) plasmogamy.

**Keywords** Arabidopsis · Double fertilization · GCS1 · Membrane dynamics · Plasma membrane intrinsic protein 2A (PIP2a)

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## Introduction

Angiosperms possess a unique sexual reproduction system called “double fertilization.” Upon pollination, a pollen grain germinates and its pollen tube elongates through the pistil to deliver two immotile sperm cells to an embryo sac that contains two female gametes (the egg cell and the central cell). Once the pollen tube reaches one of the synergid cells in the embryo sac, a pair of sperm cells is discharged from the pollen tube (Sandaklie-Nikolova et al. 2007) to come face to face with the female gametes. One of the sperm cells fertilizes the egg cell to give rise to a zygote that develops into an embryo, whereas the other sperm cell fertilizes the central cell to produce a triploid endosperm that serves as the nurse tissue for embryo development. Thus, the two parallel fertilization events should be controlled precisely for normal seed formation.

To understand in detail the sexual reproduction process in angiosperms, histological observations of non-living cells with light and electron microscopes have been performed and both plasmogamy and karyogamy have been speculated in double fertilization for a long time (Faure 2001; Faure and Dumas 2001). Plant cell observation techniques have shown remarkable improvement with the emergence of fluorescent proteins and high-performance fluorescence microscopes. *Arabidopsis* fluorescent marker lines that visualize specifically the subcellular components of gametes and gametophytic cells with a fluorescent protein have enabled the observation of fertilization in living cells (Berger 2011). Furthermore, a semi in vitro pollen tube guidance assay system was established in *Arabidopsis* (Palanivelu and Preuss 2006; Sandaklie-Nikolova et al. 2007). This assay system has enabled the time-lapse imaging of double fertilization with labeled gametes (Hamamura et al. 2011; Ingouff et al. 2007; Kasahara et al. 2012; Matsushima et al. 2008). Hitherto reported studies of gamete dynamics during double fertilization have focused on gamete cytosol, nuclei or mitochondria (Hamamura et al. 2011; Ingouff et al. 2007; Matsushima et al. 2008).

There are few studies of gamete plasma membrane behavior during gamete fusion in plant (Russell 1980, 1983, 1992) even though the cell surface structure is critical for male and female gamete interactions in order to regulate double fertilization. As syngamy is completed within a short time, it is difficult to observe the behavior of such structures at fertilization. As far as we know, no one has tried to analyze membrane dynamics during gamete fusion in living plant tissues. In this study, *Arabidopsis* female gamete membrane marker lines were produced first to gain an insight into the cell membrane structures during

double fertilization. By using these marker lines in addition to the sperm cell membrane marker line expressing GCS1-GFP (Mori et al. 2010) as the observation tools, sperm membrane behavior in the gamete fusion process was analyzed for the first time.

## Materials and methods

### Plant material

Surface-sterilized *Arabidopsis thaliana* (ecotype Columbia 0) seeds were germinated on agarose plates and the seedlings were grown for 2–3 weeks followed by transfer to soil for acclimatization. Plants were grown at 22 °C under a 16/8 h light/dark cycle.

### Plasmid construction and production of *Arabidopsis* transgenic plants

The synthesized oligonucleotide primers used for plasmid construction are listed in Table S1. *DD45* promoter (ca. 1 kb of the 5' untranslated region of At2g21740) (Steffen et al. 2007) and *FWA* promoter (ca. 2 kb of the 5' untranslated region of At4g25530) (Kinoshita et al. 2004) were amplified by genomic PCR to add a *SalI* site to the 5' terminus and a *BamHI* site to the 3' terminus, respectively. A *BamHI* site and a *KpnI* site were, respectively, added to the 5' and 3' termini of *sGFP* and *TagRFP* by PCR. The open reading frame of *PIP2a* (At3g53420) was amplified by RT-PCR to add a *KpnI* site to the 5' terminus and a *XhoI* site to the 3' terminus. Each PCR product was, respectively, cloned into the pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> vector (Life Technologies Japan, Ltd., Tokyo, Japan) and sequenced. Each of the DNA fragments cloned into the pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> was excised with suitable restriction enzymes and then ligated to pENTR<sup>TM</sup>3C (Life Technologies Japan, Ltd.) to construct “*Promoter::fluorescent protein-PIP2a*” expression cassettes. Each of the expression cassettes was transferred to the destination vector pGWB1 (Nakagawa et al. 2007) with the LR reaction (Gateway; Life Technologies Japan, Ltd.). Each of the produced constructs was introduced into wild type *Arabidopsis* plants. The resulting transformants were selected in kanamycin-containing media.

The sperm nucleus GFP marker line of *+gcs1* background was produced as follows. A genomic HTR10 sequence covering the putative promoter region (ca. 1.2 kb of the 5' untranslated region of At1g19890) (Ingouff et al. 2007) was amplified by genomic PCR and cloned into the upstream site of *sGFP* cDNA conjugated with *NOS* terminator in the pPZP221 binary vector construct,

produced in a previous study (Hirooka et al. 2009). The *pHTR10::HTR10-GFP* construct was introduced into *+gcs1* Arabidopsis plants. The resulting transformants were selected in kanamycin- and gentamycin-containing media.

The GPP-expressing line was derived from a previous study (Mori et al. 2010). The GPP line was crossed with the sperm nucleus RFP marker line (*pHTR10::HTR10-mRFP*) line (a gift from Dr. F. Berger) to obtain the double marker line expressing both sperm nucleus RFP and membrane GFP.

#### Microscopy and image analysis

A confocal laser scanning microscope (CLSM) (FV1000-D; Olympus Corp., Tokyo, Japan) and a two-photon laser scanning microscope (TPLSM) (FV1000-MPE; Olympus Corp.) were used for the acquisition of GFP/RFP and GFP images, respectively. An IR pulse laser that was attached to FV1000-MPE was set to 930 nm for excitation. FluoView software (Olympus Corp.) was used for image acquisition. Differential interference contrast and fluorescence images were captured with an epifluorescence microscope (BX51; Olympus Corp.) using an Olympus DP-72 digital camera and accompanying software (Olympus Corp.). Images were processed with Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA) or ImageJ software (<http://rsbweb.nih.gov/ij/>). Image stacking and production of movie files were performed with ImageJ software.

#### Observation of fluorescent markers during double fertilization

To control the timing of the *in vivo* double fertilization, mature flower buds of female marker lines were emasculated the day before the pollination. The emasculated female marker lines were hand-pollinated with male marker lines. For observation, the ovules were dissected after pollination for 7–8 h because double fertilization was frequently observed at this stage (Faure et al. 2002). When *+gcs1* male marker line was used for pollination, the ovules were dissected after pollination for more than 10 h.

For the time-lapse imaging of the double fertilization, flower buds of the female marker line expressing *pDD45::TagRFP-PIP2a* were emasculated 1 day before the experiment. The male marker line expressing GCS1-GFP (GPP) (Mori et al. 2010) was used for pollination to the emasculated female marker line. *In vitro* fertilization assay was performed as reported by Hamamura et al. (2011) with modification of the culture medium. The culture medium reported by Boavida and McCormick (2007) was used in this study. After the pollen tube reached the micropyle, GFP and RFP images were acquired at 5 min intervals by CLSM.

## Results

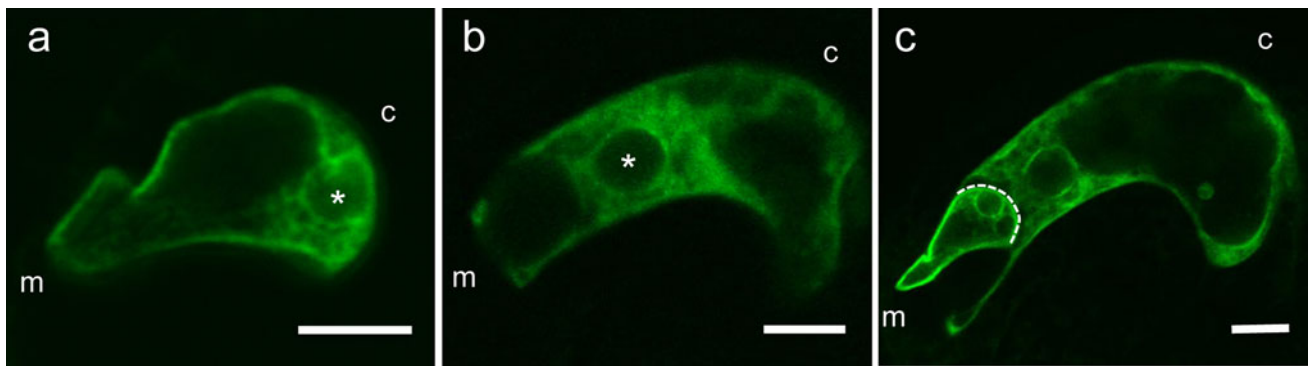
#### Fluorescent marker lines visualizing female gamete plasma membrane

Arabidopsis aquaporin (plasma membrane intrinsic protein 2A; PIP2a), a water channel protein, has been reported to uniformly label the plasma membrane along the surface of the cell (Cutler et al. 2000). To visualize Arabidopsis egg and central cell plasma membranes, *GFP-PIP2a* and *RFP-PIP2a* driven by promoters *DD45* (Steffen et al. 2007) and *FWA* (Kinoshita et al. 2004), which allow egg-cell- and central-cell-specific expression, respectively, were constructed. All the Arabidopsis plants transformed with those constructs were fertile (data not shown).

When the marker lines with GFP-labeled female gamete membrane were observed under TPLSM, the surface of the female gamete was clearly visualized (Fig. 1a–c). The egg cell displayed a rounded shape at the chalazal side and a hook-like shape at the micropylar side (Fig. 1a). The micropylar side of the central cell extended to the egg cell (Fig. 1b, c). The circular empty area within the egg cell and the central cell corresponded to the location of the nucleus (Fig. 1a–c, Fig. S1). The double fluorescent marker line by which both female gamete plasma membranes were visualized with GFP was also obtained by crossing the marker lines where each of the female gamete membranes was visualized with GFP (Fig. 1c). By projecting a three-dimensional configuration (Movie S1), it was confirmed that the chalazal side of the egg cell surface is closest to the micropylar side of the central cell surface.

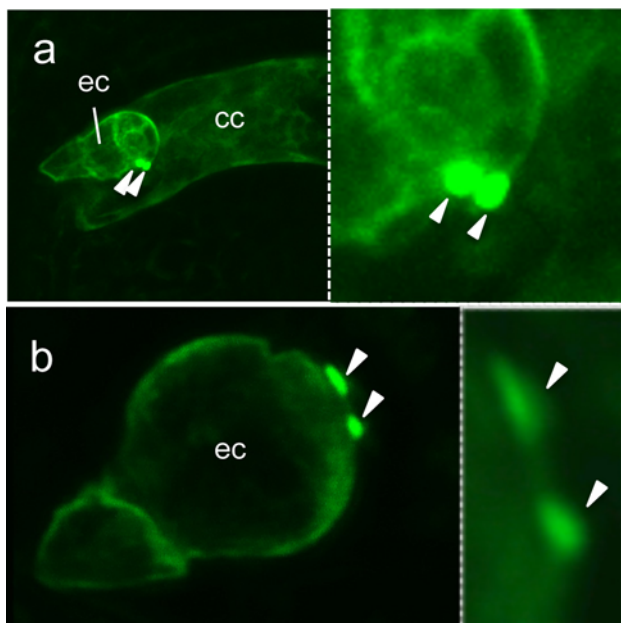
#### Relative locations of male and female gametes before fertilization

GCS1, which is also called HAP2, is a fertilization determinant that is localized on the sperm cell surface (Mori et al. 2006; von Besser et al. 2006). A defect of GCS1 caused sperm cell arrest in the embryo sac, resulting in the inhibition of fertilization in Arabidopsis (Mori et al. 2006). To observe in detail the location of sperm cells after their discharge from the pollen tube, a male fluorescent marker line by which the sperm nuclei were visualized with GFP in a heterozygous Arabidopsis GCS1 mutant (*+gcs1*) was produced. The obtained marker line was applied to the pollination of the female fluorescent marker lines labeling plasma membrane with GFP. GFP signals of sperm nuclei merging with female gamete plasma membranes were distinguishable due to differences in the signal intensity (Fig. 2). The arrested *gcs1* sperm nuclei were confirmed to remain at the boundary of the female gametes as suggested



**Fig. 1** Arabidopsis fluorescent markers visualizing female gamete plasma membrane. **a** Visualized egg cell plasma membrane by expression of *pDD45::sGFP-PIP2a*. **b** Visualized central cell plasma membrane by expression of *pFWA::sGFP-PIP2a*. **c** Double fluorescent marker line visualizing both female gamete plasma membranes

with GFP. *White dot line* represents the region where the egg cell surface is closest to the central cell surface. *m* micropylar side, *c* chalazal side. A single optical slice image obtained by TPLSM is shown. Asterisks mark positions of nuclei. Scale bars 10  $\mu$ m



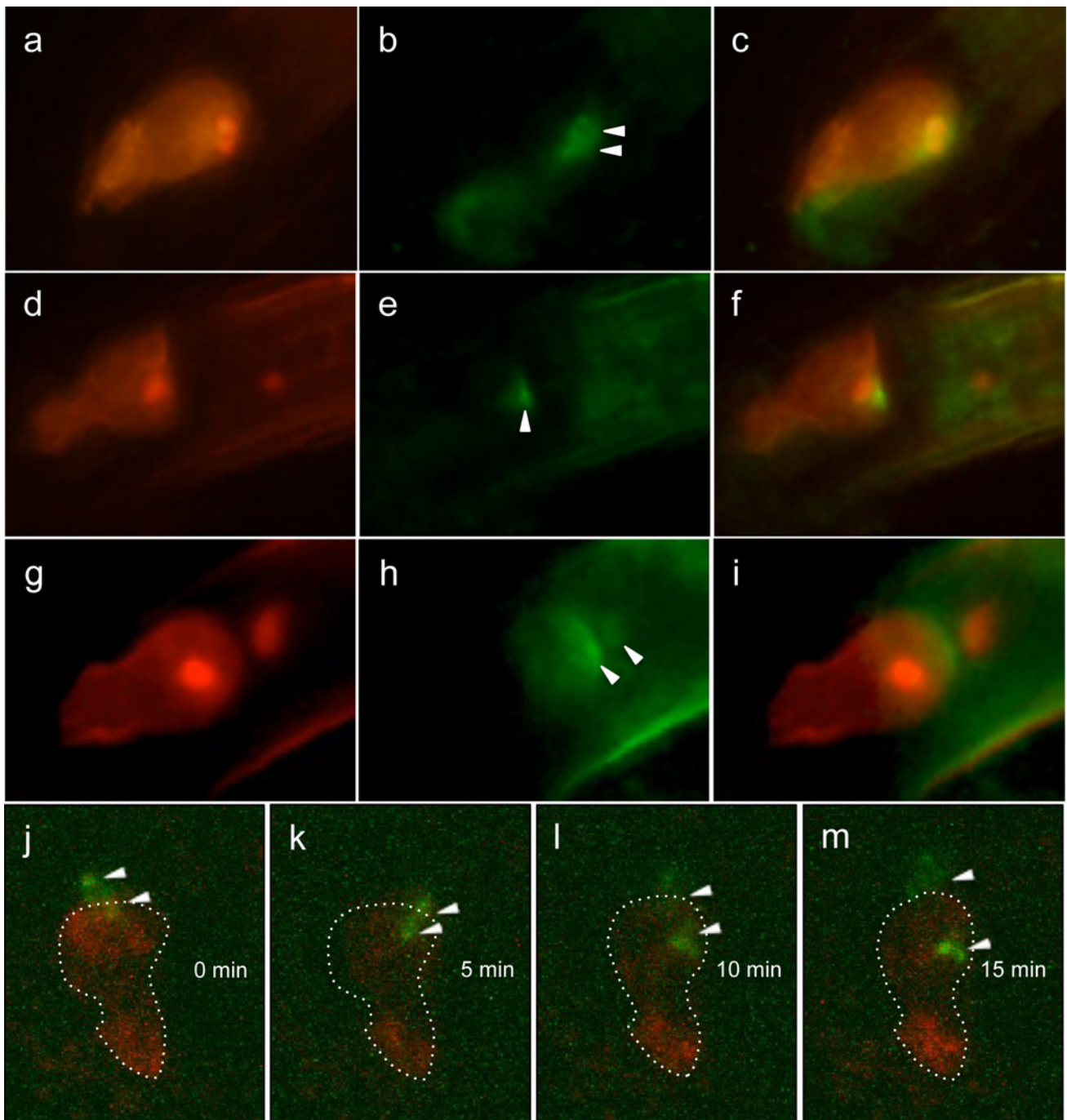
**Fig. 2** Sperm cell locations in an embryo sac before fertilization. **a** Arrested *gcs1* sperm nuclei expressing HTR10-GFP at the boundary of the female gametes (double marker line expressing *pDD45::sGFP-PIP2a* and *pFWA::sGFP-PIP2a*). A single optical slice image was obtained with TPLSM. **b** Arrested *gcs1* sperm nuclei (HTR10-GFP) on the surface of the egg cell expressing GFP-PIP2. A TPLSM stacked image of 30 slices (0.5  $\mu$ m depth interval) is shown. *Right panels* partitioned by a *dashed line* in each figure represent the magnified area where the sperm nuclei are located. *Arrowheads* indicate sperm nuclei. *ec* egg cell, *cc* central cell

in previous studies (Fig. 2a) (Hamamura et al. 2011; Mori et al. 2010). Furthermore, the arrested *gcs1* sperm nuclei on the egg cell surface adjacent to the central cell showed a flattened shape, whereas no remarkable change of the egg cell surface was observed (Fig. 2b).

#### Live cell imaging of sperm cell membrane during double fertilization

After being discharged from a pollen tube, the sperm nuclei remained at the boundary of the female gametes for a while and then resumed movement toward the egg cell or the central cell (Hamamura et al. 2011). The timing of resumption of sperm nucleus movement was considered to be a sign of plasmogamy between male and female gametes in Arabidopsis (Hamamura et al. 2011).

To analyze gamete membrane dynamics during double fertilization, transgenic Arabidopsis expressing GCS1-GFP (GPP) (Mori et al. 2010) was applied as the male gamete membrane marker line for further analysis. GPP specifically labels the sperm cell membrane (plasma membrane and internal membrane components) (Fig. S2). A male double fluorescent marker line expressing both HTR10-mRFP (Ingouff et al. 2007) and GPP, which visualized sperm cell nuclei and membranes, respectively, was also produced and applied to the pollination of the female marker line visualizing the egg cell plasma membrane with RFP (hereafter referred to as EC-RFP-PIP) (Fig. 3a–i). At 7–8 h after the pollination, ovules, where the double fertilization was progressing, were observed. Immediately before the resumption of sperm nucleus movement, bright GFP signals were noted around the sperm nuclei (Fig. 3a–c). After the resumption of sperm nucleus movement, the GFP signals diffused at the boundary of the female gametes and an additional signal was rarely observed around the sperm nucleus within the egg cell or the central cell (Fig. 3d–i). In all cases, an apparent GFP signal was observed at the boundary of the female gametes. In order to obtain time-lapse images of the gamete fusion process, the semi in vitro double fertilization assay was performed with



**Fig. 3** Sperm cell membrane dynamics during double fertilization of Arabidopsis. **a–i** A double male fluorescent marker expressing both HTR10-mRFP and GFP was applied to the pollination of the female marker line EC-RFP-PIP. Sperm cell membrane behavior before (**a–c**) and after (**d–i**) the sperm cell movement. Images were captured with an epifluorescence microscope. **a, d, g** RFP images of the egg cell membrane and the sperm nuclei. Intense RFP signals indicate sperm nuclei labeled with HTR10-mRFP. **b, e, h** GFP images of

sperm membrane. *Arrowheads* indicate the GFP remnants. **c, f, i** Merge of RFP (**a, d, g**) and GFP (**b, e, h**) images. **j–m** Movie clips from Movie S2 indicating sperm cell membrane (GFP) dynamics with the egg cell membrane marker line EC-RFP-PIP during double fertilization. The *dashed line* shows the egg cell shape based on the RFP signal. *Arrowheads* indicate GFP signals. The reference time (0 min) corresponds to the time of release of the sperm cells from the pollen tube

the male marker line expressing GFP and the female marker line EC-RFP-PIP. As a result, the GFP-labeled sperm cells moved in opposite directions after being

discharged from the pollen tube and seemed to gradually diffuse more or less (Fig. 3j–m, Movie S2). In addition, one of the migrating GFP signals merged with the RFP

signal, indicating that the sperm cell membrane components entered the egg cell.

## Discussion

Arabidopsis PIP2a clearly visualized female gamete surfaces

The observation of the gamete fusion process in angiosperms has met with difficulty as the fertilization event occurs in an embryo sac enclosed with thick integumentary tissues. To date, ultrastructural observations of gamete membrane morphology during double fertilization have been conducted in only a few plant species (Russell 1980, 1983, 1992) and no investigations of living cells have been reported so far.

In order to enable the live cell imaging of gamete membrane structures during double fertilization, Arabidopsis gametic marker lines, where PIP2a conjugated with a fluorescent protein was expressed specifically in the female gametes, were produced (Fig. 1). The marker lines clearly showed the shape of the female gametes. In addition, fluorescent signals were detected in the cytoplasm, similar to tobacco protoplast expressing PIP2a-mCherry (Hildreth et al. 2011). Those signals probably reflected the membrane components of cytoplasmic vesicles. The characteristic egg cell figure projected in this study (Movie S1) was similar to the three-dimensional figure based on sequential ultrathin sections (Wang et al. 2010).

In this study, *AtPIP2a* driven by a sperm cell specific *HTR10* promoter (Ingouff et al. 2007) did not clearly visualize the sperm cell membrane because of the low signal intensity for observation (data not shown). It could be due to the level of promoter activity or the limited amount of *AtPIP2a* to localize in the plasma membrane of tiny sperm cells. We therefore utilized a GCS1-GFP marker line (GPP line) for sperm cell membrane observation in further live cell imaging experiments, because GCS1 protein was proven to be localized in the sperm cell membrane components and GPP was found to be functional, in previous studies (Mori et al. 2006, 2010; von Besser et al. 2006).

Gamete morphology before gamete fusion

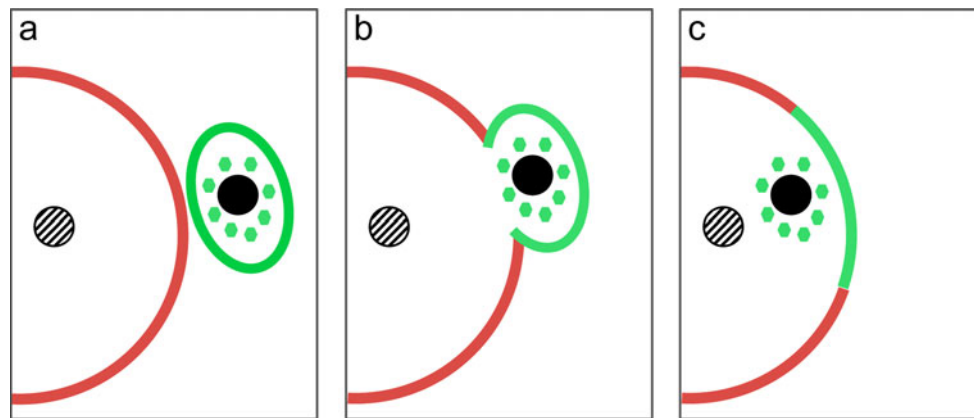
The surface morphology of the female gamete during interaction with the sperm cells before and after fertilization was observed in detail with TPLSM (Fig. 2). The flattened shape of *gcs1* sperm nuclei was rarely observed (Fig. 2b), and it could be an indication that the two sperm cells were appressed to the female gametes, similar to the case of *Plumbago zeylanica* (Russell 1980, 1983, 1992). In addition, no significant changes were observed in the egg

cell facing the *gcs1* sperm cells. In a previous study of Nun orchid, female gametes were found to extend toward sperm cells at the gamete fusion step (Ye et al. 2002). In *Chlamydomonas* fertilization, male and female gametes formed membrane protrusions that enabled them to attach to each other before gamete fusion (Liu et al. 2008). Fusion was arrested in *Chlamydomonas gcs1* male and wild type female gametes, although membrane protrusion mediated attachment occurred (Liu et al. 2008, 2010). In the present study, Arabidopsis gametes did not show such morphological changes before gamete fusion, although the situation was similar to Nun orchid gamete fusion and *Chlamydomonas gcs1* mutant phenotype. This result suggests that morphological changes occurring in the female gamete immediately before gamete fusion are not general, at least in angiosperms. Further analyses of other plant species may yield a general model of gamete morphology in double fertilization.

Arabidopsis gamete fusion process during double fertilization

Electron microscopy of *P. zeylanica* showed that plasma membrane fusion between male and female gametes occurred (Russell 1980, 1983, 1992). On the other hand, it has been reported that the actin-labeled sperm cells of Nun orchid appeared to penetrate female gametes with cytoplasmic mass (Ye et al. 2002). The latter study suggested the possibility that membrane-intact whole sperm cells entered the female gametes, similar to *Drosophila* sperm that entered the egg without membrane fusion (Karr 1991; Wilson et al. 2006). To analyze Arabidopsis gamete membrane behavior during double fertilization, live cell imaging was performed.

From the observations in the present study (Fig. 3d–i), it was suggested that plasma membrane fusion between male and female gametes occurred in Arabidopsis. Similarly, remained signal of the sperm cell membrane was observed on the rice in vitro fertilized zygote (Nakajima et al. 2010; Okamoto 2010). Therefore, GFP signals remaining at the boundary of the female gametes probably reflected the putative gamete membrane fusion site (Fig. 3d–i). In addition, the remaining GFP signals seemed to be somewhat diffused. In *P. zeylanica*, the model that the male and female plasma membranes fused at several sites was proposed based on observations of longitudinal sections (Russell 1980, 1983, 1992). Although whether the remaining diffused GFP signals in Arabidopsis could be an indication of such several membrane fusion events is remained to be judged, plasma membrane fusion between male and female gametes may be a general phenomenon in angiosperms. No remaining GFP signal was detected at the boundary of the female gametes by time-lapse imaging



**Fig. 4** Speculated model of gamete membrane dynamics in Arabidopsis double fertilization. The process from contact of male and female gametes to karyogamy is shown (**a–c**). **a** A male gamete (sperm cell) contacts a female gamete (egg cell or central cell). **b** Fusion of male and female gamete plasma membranes occurs. **c** The

sperm cell cytoplasm enters the female gamete. *Red and green lines* indicate the plasma membranes of female and male gametes, respectively. *Green dots* represent the internal membrane components in the male gamete. The male and female gamete nuclei are, respectively, pictured as a *filled circle* and a *shaded circle*

(Fig. 3j–m, Movie S2), probably because the weak plasma membrane signal could not be captured by the limited focus range of the Z-axis in CLSM.

Apart from the GFP signals at the boundary of the female gametes, additional GFP signals entering both the egg cell and the central cell were observed by time-lapse imaging, suggesting that internal membrane components derived from the sperm cell entered the female gametes. The male internal membrane components could have undergone rapid diffusion or degradation in the fertilized female gametes. Similar to *P. zeylanica*, the process from membrane fusion to the initiation of karyogamy may have taken several minutes in Arabidopsis (Hamamura et al. 2011; Kasahara et al. 2012; Russell 1980, 1983, 1992), and this might have hindered the acquisition of signals of the male internal membrane components in the fertilized female gametes immediately after the gamete membrane fusion.

Liu et al. (2010) have demonstrated that *Chlamydomonas* GCS1 was rapidly degraded in the zygote immediately after gamete fusion, and hypothesized that the degradation of GCS1 prevented polygamy (Liu et al. 2010). On the other hand, in Arabidopsis, GFP signals, namely, intact GCS1 molecules, remained at the putative gamete membrane fusion site until karyogamy, which was characterized by sperm nucleus decondensation (Fig. 3g–i). This suggests that the persistent GCS1 remnant has no effect on normal double fertilization and that polyspermy blocking in double fertilization is not based on rapid GCS1 degradation, unlike the case of *Chlamydomonas*.

Based on the obtained findings, sperm cell membrane dynamics during the double fertilization of Arabidopsis was hypothesized as follows (Fig. 4). After being discharged from the pollen tube, the sperm cells adhere to the

surfaces of female gametes (Fig. 4a). Membrane fusion between one sperm cell and its target female gamete occurs (Fig. 4b). The cytoplasm and nucleus of the sperm cell enter the female gamete (plasmogamy), whereas the sperm cell plasma membrane remains on the surface of the female gamete (Fig. 4c).

The present study provided hitherto undisclosed information regarding gamete membrane dynamics during double fertilization, using live gametic fluorescent marker lines. Membrane behavior in the gamete fusion process was monitored with the male marker line, GPP. Further investigations with other membrane markers and plant species would provide more insight into the gamete membrane dynamics of angiosperms.

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