Discovery of pollen tube-dependent ovule enlargement morphology phenomenon, a new step in plant reproduction

Ryushiro D. Kasahara\textsuperscript{a,b}, Michitaka Notaguchi\textsuperscript{c,d}, and Yujiro Honma\textsuperscript{a,b}

\textsuperscript{a}PRESTO Kasahara Sakigake Project, Japan Science and Technology Agency (JST), Furo, Chikusa, Nagoya, Aichi, Japan; \textsuperscript{b}Institute of transformative bio-molecules, Nagoya University, Furo, Chikusa, Nagoya, Aichi, Japan; \textsuperscript{c}Graduate School of Science, Nagoya University, Furo, Chikusa, Nagoya, Aichi, Japan; \textsuperscript{d}PRESTO, JST, Furo, Chikusa, Nagoya, Aichi, Japan

ABSTRACT
In animals, when semen is discharged into the uterus, the seminal plasma carries the sperm to the egg. In plants, the function of pollen tube contents (PTC) is analogous to that of the seminal plasma in animals, i.e., carrying sperm cells to the ovules for fertilization. Because the function of the seminal plasma is essential for fertilization in animals, we propose that the function of PTC must be important for plant fertilization. To understand the function of PTC, we examined the transcriptional variation after the release of PTC into the embryo sac. The phenotypic analysis revealed that ovules were enlarged without fertilization when the PTC was released into the ovule, entirely consistent with the transcriptome analysis. We identified a new plant phenomenon, pollen tube-dependent ovule enlargement morphology (POEM) phenomenon that occurs only when the ovule accepts PTC, irrespective of fertilization. POEM is a new phase between the pollen tube guidance and fertilization phases as a reproductive step. Here we established the \textit{in vitro} POEM assay, which effectively measures POEM activity. Using this assay, we identified that a simple dose of plant hormone(s) cannot induce POEM. We also showed that this assay could be a powerful tool for identifying POEM factor(s).

In angiosperms, the pollen tube, upon insertion into ovules, releases its contents containing sperm cells into the embryo sac and completes double fertilization. Recently, we reported that when ovules failed to be fertilized following pollen tube insertion, they expanded and initiated seed coat formation as if they had undergone fertilization.\textsuperscript{1} We termed this phenomenon as pollen tube-dependent ovule enlargement morphology (POEM), and it occurs only when the ovule accepts the pollen tube content (PTC). This was the first report in plants concerning the paternal function of PTC in facilitating the maternal development of the ovule without fertilization.

In animals, when semen is discharged into the uterus, the seminal plasma carries the sperm to the egg.\textsuperscript{2,3} In plants, the function of PTC is analogous to that of the seminal plasma in animals, which is carrying sperm cells to the ovules for fertilization. The seminal vesicle secretory protein 2, which is localized only in the seminal plasma, is required for fertilization in mice.\textsuperscript{4} The function of the seminal plasma is essential for fertilization in animals; therefore, we propose that the function of PTC must be important for plant fertilization. To understand the function of PTC, we examined transcriptional variation after the release of PTC into the embryo sac; the expression profiles of both RNAs were similar. This result was unexpected because early events after pollen tube insertion were considered to be dependent on fertilization, but these events are dependent on PTC instead. Notably, at 24 and 48 HAP, multiple genes associated with cell expansion, cell division, and seed coat formation were upregulated regardless of fertilization. These results suggested that PTC could affect the shape of the ovules. Hence, we checked the phenotype of the ovules. Interestingly, when ovules...
accepted PTC, they expanded without fertilization. This expansion resulted from cell expansion and division of ovules and produced a partial seed coat, which was in coherence with the results of our transcriptome analysis. By using the information from the successful transcriptome analysis, we identified a new plant phenomenon POEM that occurs only when the ovule accepts PTC, irrespective of fertilization. In angiosperms, pollination is the first step toward fertilization. Once pollen reaches the stigma, pollen grains elongate to form pollen tubes and move toward synergid cells found within the female gametophyte. Fertilization occurs when pollen tubes pierce the female gametophyte, terminating their growth, and burst inside the female gametophyte to fertilize 2 sperm cells. POEM phenomenon is a new phase between pollen tube guidance and fertilization phases as a reproductive step because PTC discharge itself could induce POEM.1

Knowing that the trigger for POEM was PTC, we needed to identify compounds that were required for POEM. To identify the compounds, we set up a new in vitro POEM assay (Fig. 1). To ensure that the method was appropriate for assay ing POEM activity, we tested whether POEM is induced by PTC in vitro. We prepared 1 μl droplet of ovule culture medium and sank ovules to the solution. Fig. 1 shows that ovules without crossing stayed the same size over 24 hours with the MYB98::GFP synergid signal on. As we reported previously, when the PTC was released into the female gametophyte, the synergid cells were disrupted at each pollen tube insertion.7,8 However, ovules crossed by gcs1/gcs1 pollen9 induced POEM without fertilization in the 24 hours after pollination. We compared the area of the female gametophyte both in vivo and in vitro. When we compared the area before POEM with that measured after POEM in vivo, ovules after POEM were found to be 2.5 ± 0.8 times (mean ± SD; n = 100 ovules) larger than those before POEM. Conversely, when we compared these areas in vitro, ovules after POEM were found to be 2.3 ± 0.6 times (n = 10 ovules) larger than those before POEM. These results indicated that the sizes of the female gametophyte after POEM are similar in vivo and in vitro, suggesting that the in vitro POEM assay can measure POEM activity. In our previous transcriptome data,1 one auxin synthesis gene, YUCCA6, and gibberellin synthesis genes GA20ox1, GA20ox2, GA3ox1, and GA3ox2 were upregulated by the pollination of WT or gcs1. These suggest that the synthesis of auxins and gibberellins is triggered by PTC somewhere in the harvested sample parts, including ovules and pedicels. Because auxins and gibberellins affect cell division or growth in other tissues, we investigated whether plant hormones can induce POEM. We applied 10 mM, 1 mM, and 0.1 mM 2,4-D or 10 mM, 1 mM, and 0.1 mM GA3 as a single dose, or a mixed dose of 2,4-D and GA3, to the ovule culture medium. No ovule was enlarged by these plant hormones, indicating that applying a plant hormone is not sufficient to induce POEM. These results suggest that a simple dose of plant hormone(s) cannot induce POEM, and compounds in the PTC are essential to induce POEM.

It was also found that PTC was able to initiate central cell/endosperm nuclei division without fertilization when the PTC was released to an autonomous endosperm mutant, mea.13,14 Since 1910, it has been known that in animals, segmentation can be induced by physical stimuli, independent of fertilization, causing some eggs to develop into normal tadpoles.15,16 In plants, we discovered that PTC can increase central cell/endosperm nuclei division without fertilization, suggesting functioning parallel to that in animals in which germ cells divide in response to external stimuli, independent of fertilization. By inducing endosperm nuclei division, PTC facilitates apomixis17 in important crops when POEM phenomenon is combined with autonomous endosperm and embryo mutants. In plants, seed formation without

Figure 1. The ovule enlargement phenomenon by in-vitro POEM assay. (A) Ovules with MYB98::GFP synergid cell marker before pollen tube insertion. These ovules were not enlarged 2 d after incubation because no PTC was released into ovules. MYB98::GFP expression is evidence that the ovules have no PTC. One ovule is lacking the GFP signal because the ovule was damaged in the dissection error. (B) Bright field image of (A). (C) Ovules after pollen tube insertion. These ovules were enlarged 2 d after incubation. No MYB98::GFP expression is evidence that the ovules accept pollen tubes and PTC into the female gametophyte. (D) Bright field image of (C). Bars, 100 μm. The liquid medium for in vitro ovule culture contained the Nitsch basal salt mixture, 5% trehalose dihydrate, 0.05% MES-KOH, and 1 × Gamborg’s vitamin solution.29
fertilization is called apomixis and is valuable for agriculture because the important genetic traits can be easily fixed in apomictic crops, which then propagate without interference from unfavorable environmental conditions. POEM could be categorized as “pseudogamy” defined as any reproductive process requiring pollination but no inheritance from male gametophyte. Although Focke first defined pseudogamy in 1881, as a part of apomixis, its cellular or molecular mechanisms have remained obscure. POEM may be a key to understanding pseudogamy because of concept similarity, particularly regarding pollen and PTC stimuli.

The prominent analogies between the functions of PTC that we have identified, and those of the seminal plasma blur the boundaries between plants and animals, and advanced research may provide additional clues approaching principles of hidden identity in the male fluid. Furthermore, in plants, these functions could have a great potential for producing apomictic crops.

Data and materials availability
All data needed to evaluate the conclusions in the paper are present in the paper. Additional data will be made available by the authors upon request.

Disclosure of potential conflicts of interest
There are no conflicts of interest to declare.

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