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Evaluation of fertilization state by tracing sperm nuclear morphology in Arabidopsis double fertilization

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TITLE:

Evaluation of Fertilization State by Tracing Sperm Nuclear Morphology in Arabidopsis Double Fertilization

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KEYWORDS:

developmental biology, fertilization, gamete, flowering plant, fluorescence microscope, ovule, pollination

SUMMARY:

We demonstrate a method to determine successful or failed fertilization on the basis of sperm nuclear morphology in *Arabidopsis* double fertilization using an epifluorescence microscope.

ABSTRACT:

Flowering plants have a unique sexual reproduction system called 'double fertilization,' in which each of the sperm cells precisely fuses with an egg cell or a central cell. Thus, two independent fertilization events take place almost simultaneously. The fertilized egg cell and central cell develop into zygote and endosperm, respectively. Therefore, precise control of double fertilization is essential for the ensuing seed development. Double fertilization occurs in the female gametophyte (embryo sac), which is deeply hidden and covered with thick ovule and ovary tissues. This pistil tissue construction makes observation and analysis of double fertilization quite difficult and has created the present situation in which many questions regarding the mechanism of double fertilization remain unanswered. For the functional evaluation of a potential candidate for fertilization regulator, phenotypic analysis of fertilization is important. To judge the completion of fertilization in *Arabidopsis thaliana*, the shapes of fluorescence signals labeling sperm nuclei are used as indicators. A sperm cell that fails to fertilize is indicated by a condensed fluorescence signal outside of the female gametes, whereas a sperm cell that successfully fertilizes is indicated by a decondensed signal due to karyogamy with the female gametes' nucleus. The method described here provides a tool to determine successful or failed fertilization under in vivo conditions.

INTRODUCTION:

Flowering plants produce seeds through double fertilization, a process that is directly controlled by interactions between proteins localized on gamete plasma membrane[1,2]. Flowering plant male

gametes, a pair of sperm cells, develop in pollen. A pollen tube that grows after pollination delivers a pair of sperm cells to female gametes, an egg cell and a central cell, which develop in an embryo sac. After the male and female gametes meet, proteins on the gamete surface promote recognition, attachment, and fusion to complete double fertilization. In previous studies, the male gamete membrane proteins GENERATIVE CELL SPECIFIC 1 (GCS1)/HAPLESS2 (HAP2)[3,4] and GAMETE EXPRESSED 2 (GEX2)[5] were identified as fertilization regulators involved in gamete fusion and attachment, respectively. We recently identified a male gamete-specific membrane protein, DUF679 DOMAIN MEMBRANE PROTEIN 9 (DMP9), as a fertilization regulator involved in gamete interaction. We found that a decrease of *DMP9* expression results in significant inhibition of egg cell fertilization during double fertilization in *A. thaliana*[6].

As double fertilization occurs in an embryo sac, which is embedded in an ovule that is further wrapped with ovary tissue, it is difficult to observe and analyze the states of double fertilization processes. For this reason, there are still many unclear points that hinder a complete understanding of the whole mechanism of double fertilization control. The establishment of observation techniques to trace the behavior of gametes during double fertilization under in vivo conditions is indispensable for the functional analysis of potential candidates for fertilization regulators. Recent studies have yielded marker lines where gamete subcellular structures are labeled with fluorescent proteins. In this article, we demonstrate a simple and quick protocol for observing double fertilization that has occurred in an embryo sac derived from artificially pollinated pistils. Using sperm cell nucleus marker line HTR10-mRFP[7], the fertilization state of each female gamete can be discriminated on the basis of sperm nuclear signal morphology. Our protocol focusing on such a morphological change of the sperm nuclei at fertilization can efficiently obtain a sufficient amount of data for statistical proof. A *DMP9*-knockdown line with HTR10-mRFP background (*DMP9*$^{KD/HTR10\text{-}mRFP}$) was used as male plants to show a single fertilization pattern. The protocol is also suitable for the functional analysis of other fertilization regulators.

PROTOCOL:

1. Artificial pollination

NOTE: Before starting the process, a pair of No. 5 forceps is required.

1.1. Grow *A. thaliana* (Col-0) at 22 °C under a 16-h light/8-h dark cycle in a growth chamber.

NOTE: Cut and remove the first developed flower stalk with scissors to promote development of axillary buds. Vigorously growing plants (2-3 weeks after cutting of the first stalk; plant height about 25 cm) are suitable for analysis.

1.2. To emasculate, remove sepal (**Figure 1B**), petal (**Figure 1C**), and stamen (**Figure 1D**) of flower buds at stage 11[8] (**Figure 1A**) using No. 5 forceps. Bud with bits of petals seen at the top is best.

NOTE: Use a suitable female gamete marker line. In this protocol, we used a wild type plant as the female parent.

1.3. Fifteen to eighteen hours after emasculation, take the stamen of a *DMP9*$^{KD/HTR10\text{-}mRFP}$ flower at stage 13[8] by pinching the filament with forceps.

1.4. To pollinate, gently pat the stigma of an emasculated pistil several times with a dehiscent anther.

2. Preparation of ovule for observation

NOTE: The following items are required: a slide glass with double-sided tape attached, No. 5 forceps, a 27 G injection needle, and a dissecting microscope.

2.1. Seven to eight hours after pollination (HAP), collect the pistil and place it on the double-sided tape, then press gently with forceps to fix the pistil on the tape (**Figure 2A,A'**).

NOTE: Most ovules in a pistil receive at least one pollen tube 10 HAP[9]. If both or any one of the sperm cells from the first pollen tube fail to fertilize, a second pollen tube would be attracted by the ovule due to the fertilization recovery system[10]. To analyze the sperm nuclei morphology from the first pollen tube, it is recommended to complete ovule preparation by 10 HAP at the latest.

2.2. Cut off the upper and lower ends of the ovary using an injection needle under a dissecting microscope (**Figure 2B,B'**).

2.3. Slit the ovary wall along both sides of the replum (**Figure 2C,C'**) by moving the tip of the injection needle.

NOTE: Insert the injection needle shallowly to prevent ovule separation from the septum.

2.4. Evert the ovary wall by using the injection needle (**Figure 2D,D'**).

2.5. Pinch the base of the septum to which ovules are connected, and lift it up carefully with forceps (**Figure 2E**).

2.6. Transfer the ovules into a drop of water on a slide glass, and gently cover with a cover glass for observation under a fluorescence microscope (**Figure 2E,E'**).

3. Microscopy

NOTE: In this protocol, we used an epifluorescence microscope equipped with a fluorescence filter cube (see **Table of Materials**), a digital camera, and the accompanying software.

3.1. Acquire images of ovules containing sperm nuclei labeled with mRFP using a 20x or 40x objective lens and the equipped digital camera.

3.2. Confirm the number of mRFP-labeled sperm nuclei in an embryo sac.

NOTE: Ovules containing two mRFP-labeled sperm nuclei can be included in the population size for statistical analysis.

3.3. Confirm the shape and position of each mRFP-labeled sperm nucleus in an embryo sac.

NOTE: Immediately after being released from a pollen tube, a pair of condensed mRFP-labeled sperm nuclei are localized between the egg and the central cell. A decondensed mRFP-labeled sperm nucleus detected at the side of chalazal end indicates central cell fertilization, for instance. By using a suitable female gamete membrane marker line, as shown in **Supplementary Figure 1**, whether or not the sperm cell is undergoing plasmogamy (after membrane fusion but before karyogamy) can be monitored clearly.

REPRESENTATIVE RESULTS:

Ovules from a pistil pollinated with *DMP9*$^{KD/HTR10\text{-}mRFP}$ were collected at 7-8 HAP and observed. Most ovules contained two decondensed mRFP-labeled sperm nuclei at the egg cell (micropylar side) and central cell (charazal end side) nucleus positions, respectively (**Figure 3A**), indicating successful double fertilization. In addition, ovules containing a decondensed mRFP-labeled sperm nucleus at the central cell nucleus plus a condensed mRFP-labeled sperm nucleus outside the egg cell (**Figure 3B**) were observed, indicating single fertilization. In the case of failed double fertilization, two condensed mRFP-labeled sperm nuclei were observed at the boundary of the egg cell and the central cell (**Figure 3C**), as in *gcs1* mutant sperm cells (**Figure 3D**)[3,4].

In the analysis using *DMP9*$^{KD/HTR10\text{-}mRFP}$ pollen, ovules containing a single condensed mRFP-labeled sperm nucleus (**Figure 4A**) or three or more mRFP-labeled sperm nuclei (**Figure 4B**) were rarely observed.

FIGURE AND TABLE LEGENDS:

Figure 1: Flower buds suitable for emasculation. (**A**) A flower bud at stage 11, showing bits of the petals at the top. (**B**–**D**) A flower bud in which was removed the sepals (**B**) and petals (**C**) and that was then emasculated completely (**D**). The anther dehiscence has not occurred yet. Scale bar: 1 mm.

Figure 2: Flow of ovule sample preparation. The procedures are shown by illustrations (**A**–**E**) and photographs (**A'**–**E'**). (**A**, **A'**) A pistil is placed on double-sided tape attached to a slide glass, and its upper and lower ends are cut off with an injection needle. (**B**, **B'**) The ovary wall is incised along both sides of the replum. (**C**, **C'**) Both sides of the ovary walls are opened, everted, and pressed onto the tape for fixing. (**D**, **D'**) The septum where ovules are connected in arrays is exposed. (**E**, **E'**) One end of the septum is pinched and lifted up with forceps. The septum with connecting ovules is transferred to a drop of water on a slide glass.

Figure 3: Fertilization phenotypes judged by sperm nuclear signal morphology in ovule. (**A**) Successful double fertilization indicated by two decondensed mRFP-labeled sperm nuclei (arrowheads). (**B**) Single fertilization by *DMP9*$^{KD/HTR10\text{-}mRFP}$ sperm cells indicated by one decondensed mRFP-labeled sperm nucleus (arrowhead) and one condensed mRFP-labeled sperm nucleus (arrow). (**C**) Failed double fertilization by *DMP9*$^{KD/HTR10\text{-}mRFP}$ sperm cells indicated by two condensed mRFP-labeled sperm nuclei (arrows). (**D**) An example of failed double fertilization by *gcs1* $^{HTR10\text{-}mRFP}$ sperm cells. A marker line where the egg cell nucleus (EN) is labeled with RFP was used. Two condensed mRFP-labeled sperm nuclei (arrows) are arrested without fertilization at 18 HAP. (**E**) Schematic illustration of an ovule. EC = egg cell, CC = central cell, SC = synergid cells, ES = embryo sac, MP = micropyle, CHZ = chalazal end. Scale bars: 20 µm.

Figure 4: Other potential fertilization phenotypes. Ovules from a pistil pollinated with *DMP9*$^{KD/HTR10\text{-}mRFP}$ rarely contain a single condensed mRFP-labeled sperm nucleus (**A**), or three or more condensed mRFP-labeled sperm nuclei (**B**) (arrows). Scale bars: 20 µm.

Supplementary Figure 1: Condensed sperm nuclei at plasmogamy during fertilization, judged by combination with a female plasma membrane marker line. *pFWA::GFP-PIP* where the central cell plasma membrane is visualized was used as female parent (GFP). GFP-PIP also labels endo membranes[12]. The ovule contains two condensed mRFP-labeled sperm nuclei (arrows; RFP). The RFP signal at charazal end side (arrow) is shown in the central cell (Merge), indicating that the sperm nucleus is in plasmogamy (after gamete membrane fusion, but before karyogamy). The panel on the right is a magnification of the area surrounded by dashed lines in the merged image. A blank area marked by an asterisk corresponds to the position of the central cell nucleus. The dashed line indicates the outline of the central cell facing the egg cell. Scale bar: 20 µm.

DISCUSSION:

HTR10-mRFP labels paternal chromatin (i.e., visualizes sperm cell nuclei), and the dynamics in double fertilization have been reported[7]. Immediately after release from a pollen tube, HTR10-mRFP-labeled sperm nuclei are still condensed. However, each of the sperm nuclei is decondensed upon merging with a fertilized female gamete nucleus at karyogamy three to four hours after gamete membrane fusion[7]. Unfertilized sperm cells remain condensed, as shown in an embryo sac in which *gcs1* sperm cells are arrested without fertilization (**Figure 3D**). Usually, when the fertile HTR10-mRFP is used as the pollen parent, 57.9% ± 17.8% (mean ± s.d.; n = 16 pistils) of ovules in a pistil at 7-8 HAP contain two mRFP-labeled sperm nuclei signals, and almost all of the signals (97.2%) are decondensed, reflecting successful double fertilization (a similar signal pattern is shown in **Figure 3A**). In the case of *DMP9*$^{KD/HTR10\text{-}mRFP}$, ovules containing two mRFP-labeled sperm nuclei were observed at a similar frequency to that of *HTR10-mRFP*, but 17.6% of them showed single fertilization[6]. We adopted the HTR10-mRFP dynamics for observation of a large number of ovules under in vivo conditions using an epifluorescence microscope. The protocol is simple and quick, which enables the collection of sufficient data for statistical proof. Using this protocol as the first instance, the significance of single fertilization of the central cell by *DMP9*$^{KD/HTR10\text{-}mRFP}$ sperm cells was proved[6].

Based on the morphological differences of HTR10-mRFP-labeled sperm nuclei derived from one pollen tube, the fertilization patterns are classified into three phenotypes: (1) successful double fertilization, which is characterized by two decondensed HTR10-mRFP-labeled sperm nuclei (**Figure 3A**); (2) single fertilization, which features one condensed HTR10-mRFP-labeled sperm nucleus and one decondensed HTR10-mRFP-labeled sperm nucleus (**Figure 3B**); and (3) failed double fertilization, which has two condensed HTR10-mRFP-labeled sperm nuclei (**Figure 3C**).

Other potential causes of phenotypes (2) and (3) should be considered. It has been reported that sperm cells respectively begin plasmogamy with an egg cell or central cell several minutes after being released into an embryo sac under semi-in vitro culture conditions[11]. In addition, a time lag of a few minutes exists between the first and second fertilizations, although there is no preference for the order of fertilization of the egg cell and the central cell[11]. Therefore, a pair of condensed and decondensed sperm nuclei in phenotype (2) might indicate a time lag between the fertilizations instead of single fertilization. In order to confirm the occurrence of single fertilization at a significant frequency in phenotype (2), a number of samples sufficient for statistical analysis should be examined. Phenotype (3), which has two condensed sperm nuclei (**Figure 3C**), could reflect a period of immobility of sperm cells prior to plasma membrane fusion[12] or the period between plasmogamy and karyogamy. Therefore, two condensed sperm nuclei at 7-8 HAP do not fully reflect 'failure' of double fertilization, and it is necessary to observe the ovules at a later time after pollination to assess the success or failure of double fertilization. In this regard, ovule observation at 16-18 HAP is recommended, because most of the ovules would have completed double fertilization with sperm cells delivered by the first pollen tube, and HTR10-mRFP signals of unfertilized sperm nuclei would remain until the next day of pollination, as shown in **Figure 3D**.

A pattern of single condensed HTR10-mRFP-labeled sperm nucleus (**Figure 4A**) was rarely detected, which was likely due to the quick disappearance of another paternal HTR10-mRFP signal in the fertilized female gamete as a result of single fertilization. In this analysis, three or more HTR10-mRFP-labeled sperm nuclei were also detected as a rare case (**Figure 4B**), due to second pollen tube acceptance by the fertilization recovery system[10]. In this protocol, these cases were excluded from the data, because tracing the behaviors of two sperm cells derived from one pollen tube is critical for accurate assessment.

Since the polarity for the positions of the female gametes in an embryo sac is well regulated (i.e., the egg cell and the central cell differentiate at the micropylar and the chalazal side, respectively), which female gamete is fertilizing can be judged by the relative positions of the mRFP-labeled sperm nuclei. However, there is a limitation in distinguishing between unfertilized and plasmogamy states, because both states are indicated by the condensed sperm nuclei signal. To evaluate precisely whether plasmogamy after gamete membrane fusion has occurred or not when the sperm nuclei are condensed, it is required to use female gamete membrane marker lines, as reported by Takahashi et al. (2018)[6]. For example, when a *pFWA::GFP-PIP* plant[12] in which the central cell plasma membrane was visualized was used as a female parent, it is clear that a sperm cell fused with the central cell was in plasmogamy (**Supplementary Figure 1**).

In summary, our protocol described here can be used for statistical assessment of success or failure of fertilization in each female gamete. Although the employment of the female plasma membrane marker is required to judge the plasmogamy, our method is useful for functional analysis of the fertilization regulator.

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DISCLOSURES:

The authors have nothing to disclose.

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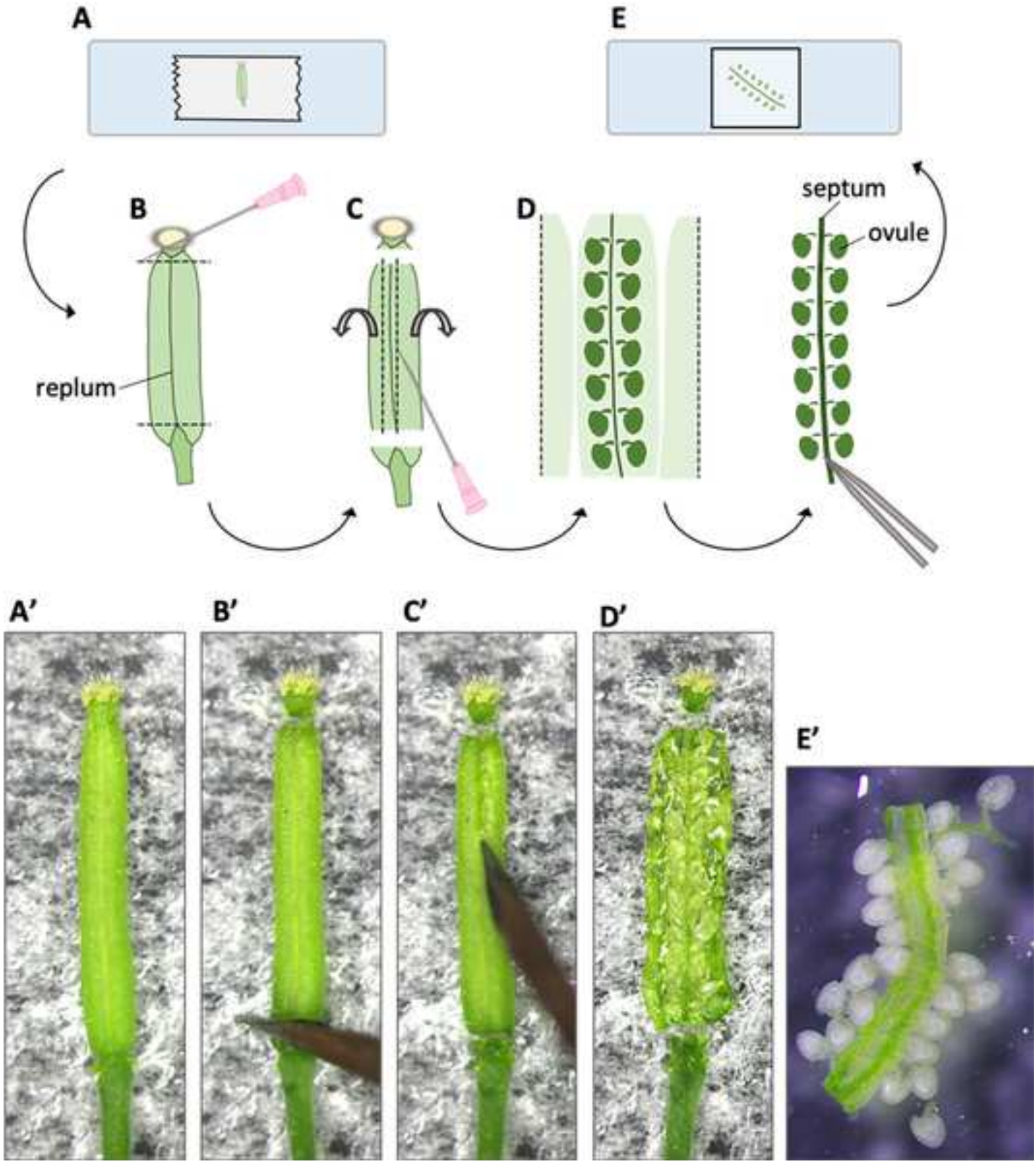
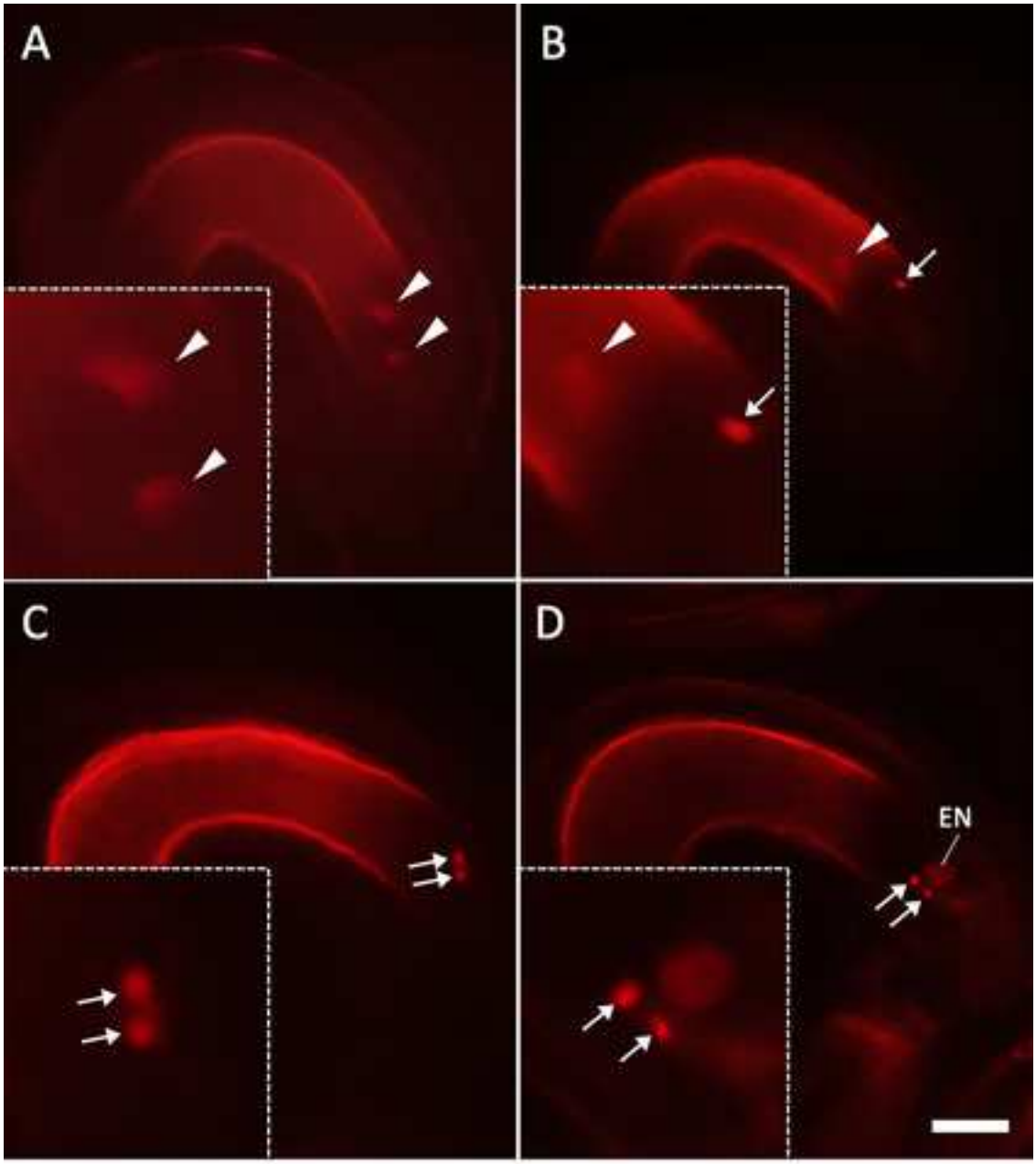
Figure1

Figure2





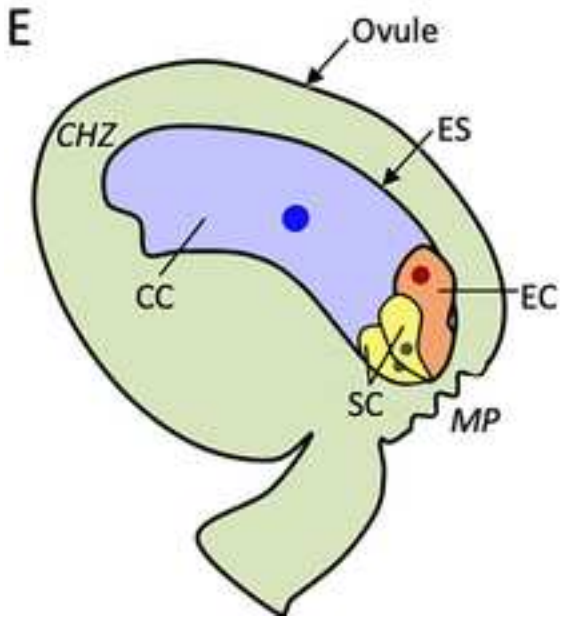
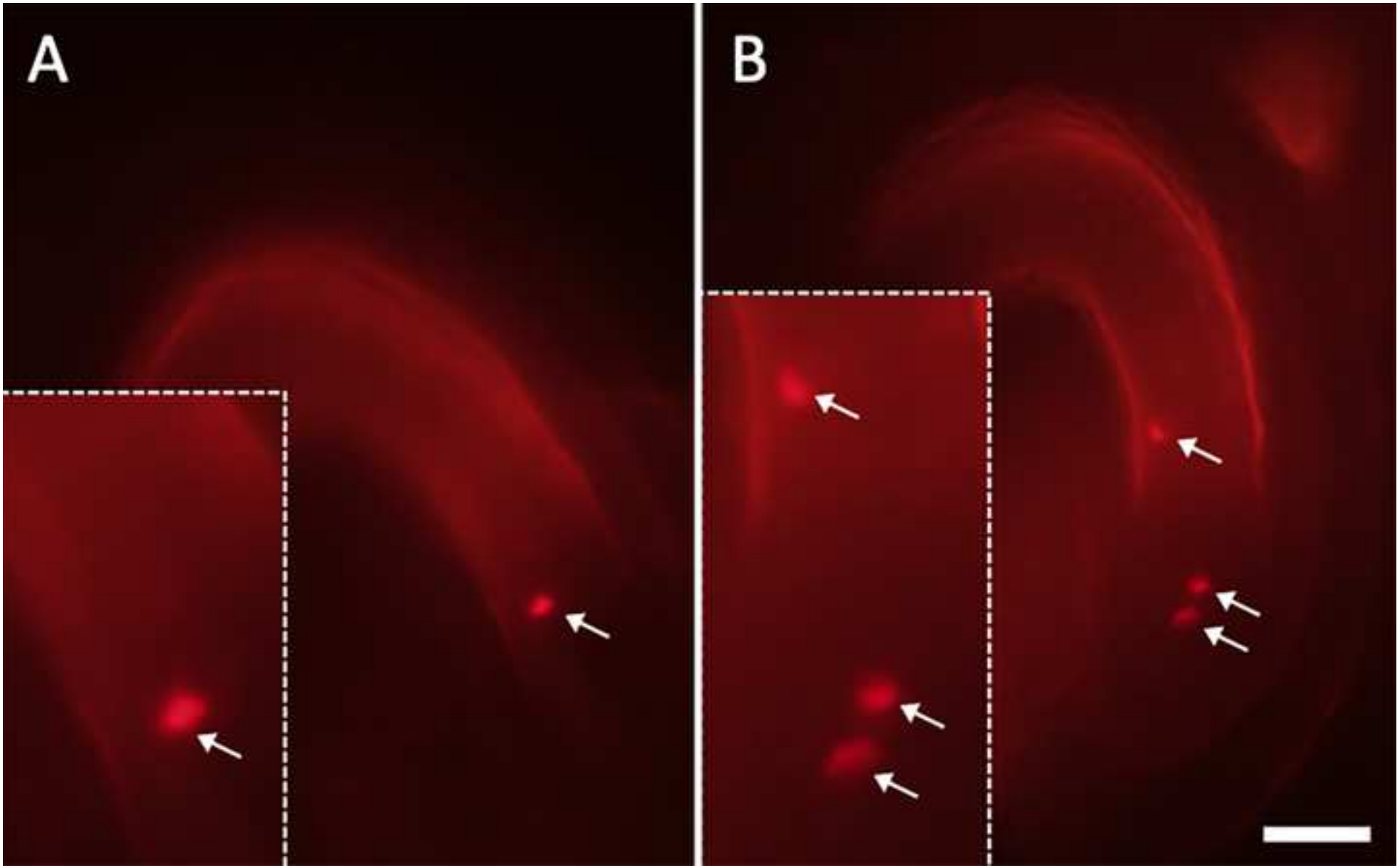


Figure4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BX51	Olympus		Epifluorescence microscope
Cover glass	Matsunami glass	C018181	
DMP9 $^{KD/HTR10\text{-}mRFP}$			*Arabidopsis thaliana* , *HTR10-mRFP* background Takahashi et al. (2018)[6]
Double-sided tape	Nichiban	NW-15S	15 mm width
DP72	Olympus		Degital camera
Forceps	Vigor		Any No. 5 forceps are available
Growth chamber	Nihonika	LPH-411PFQDT-SP	
HTR10-mRFP			*Arabidopsis thaliana* , ecotype Columbia-0 (Col-0) background Ingouff et al. (2007)[7]
Injection needle	Terumo	NN-2719S	27 gauge
Slide glass	Matsunami glass	S9443	
SZX9	Olympus		Dissecting microscope
U-MRFPHQ	Olympus		Fluorescence Filter Cube (Excitation: BP535-555, Emission: BA570-625, Dichromatic mirror:DM565)
UPlanFL N 40x	Olympus		Objective lens (NA 1.3), oil-immersion
UPlanSApo 20x	Olympus		Objective lens (NA0.75), dry

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Dear Editor,

We would like to express our thanks for the useful comments by the editor and the reviewers. We revised the manuscript following the comments. We would be grateful if you accept our revised version for publication.

Response to the editor and the reviewers

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- The revised manuscript was checked by a proofreading service.

Protocol:

1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. Where do the plants come from? There is nothing in the Table of Materials.
2. 3.1: What excitation/emission wavelengths are used here?

- The information was added in the revised table.

Discussion:

1. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:

a) Any modifications and troubleshooting of the technique

b) Any limitations of the technique

c) The significance with respect to existing methods

- We revised construction and added some descriptions to cover the above points.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

- We checked and added some items in the revised table.

Reviewer #1:

Please make it clear which ecotype as the wild type was used in this protocol, since pollen tube growth rate and pistil length vary a lot among different ecotypes.

- The ecotype was indicated in both text and table in the revised version.

Reviewer #2:

-The authors used HTR10-mRFP marker lines to evaluate fertilization state, but is it possible to analyze for no fluorescence marker lines with chemical dyes like DAPI or something?

- We have not tried staining for observation of gametes, but probably it is difficult to distinguish gamete signals in an embryo sac using an epifluorescence microscope. I think signals from cells surrounding an embryo sac would hinder to find the gamete signals. However, it may be possible under the confocal laser scanning or two-photon microscopy. Although we didn't mention this in the manuscript, we appreciated the reviewer's question.

-p1, line 22, "using a fluorescence microscope"

-p2, 1. artificial pollination, the step by step illustration of this procedure like figure 1 would be better to understand for the readers. Especially, the photo of "Bud with a bit of petals seen at the top" is useful. In addition, "bits of petals".

-p3, line 130, "using a 20x or 40x objective lens"

-p5, line 185, "condensed sperm nuclei"

-In Fig.2-3, if all scale bars have same magnification, please indicate only one image.

- Thank you very much for correcting our mistakes. We revised all of them. In addition, photos of flower buds were added as figure 1 in the revised manuscript.

-In Fig.2, it is difficult to distinguish the difference between Fig. 2A and Fig. 2B. It is better to line up a more enlarged image like supplementary figure 1 rather than the whole image of the ovule.

- We added each enlarged part as an inset in Figure 3 and Figure 4 in the revised manuscript.

-In supplementary fig. 1, this GFP image does not like plasma membranes. Why GFP signals are detected central cell nucleus in pFWA::GFP-PIP?

- GFP-PIP also localizes to the endomembranes such as nuclear membrane, in addition to plasma membrane labeling the outline of the cell clearly (Igawa et al., 2013). We added a description in the legend of the figure.

Reviewer #3:

Major Concerns:

Methodology is not novel

- Although the dynamics of HTR10-mRFP have been reported, we first adopted that morphological feature for assessment of fertilization state. Moreover, by observation using an epifluorescence microscope, enough data for statistical analysis can be obtained quickly. Actually, we utilized this analysis method and the biased single fertilization caused by the knock-down of *DMP9* was proven. In this regard, we added descriptions in the revised manuscript.

Minor Concerns:

Not full details of microscopy methods

- Information of a filter cube for RFP was added in the protocol.

Reviewer #4:

The authors describe a method to study double fertilization in Arabidopsis in vivo. They pollinated wild type ovules with pollen of DMP9 knock down plants carrying HTR10-mRFP reporter construct.

The authors described how to pollinate the pistil and how to prepared the sample to be analysed however they do not mention how many ovules they have analysed .

Live cell imagining of the pollination and fertilization, using HTR10-mRFP reporter construct, was already described in other manuscripts (i.e Hamamura et al., 2011). Takahaschi et al 2018 had already used pollen of DMP9 knock down plants carrying HTR10-mRFP reporter construct to pollinated wild type pistil to study DMP9 function during pollination.

I think that the authors have to add the efficiency of the protocol they have described. How many ovules were fertilized? How many pistils have they analysed?

- The frequency of the ovules progressing double fertilization was added in the text.

Reviewer #5:

Major Concerns:

The fading nature of this marker, combined with strong red auto-fluorescence of the embryo sac and some occurrences of polytubey and/or post-fertilization pollen tube arrivals, makes it difficult to determine whether fertilization has occurred or not. To my opinion, in order to prove that fertilization has actually occurred (or not), it is always necessary to include female marker too!

- It has been reported that the second pollen attraction starts after 10 HAP (Kasahara et al., reference 9). Therefore, we set the observation timing at 7-8 HAP. In spite of that, more than 3 sperm nuclei in an ovule are observed in a rare case, so that we suggested not to employ such ovules as data.

I do agree that the suggested protocol could be useful to some extant during pre-screens for fertilization abnormalities, that in any case would need further confirmations using female markers too.

- The difference in the shape of the mRNA-labeled sperm nucleus between before and after the karyogamy is apparent. At least, karyogamy means the success of fertilization. We adopted this feature for the analysis method. As the reviewer commented, our protocol allows quick screening of fertilization abnormality, in addition to obtaining a number of the samples for statistical proof. We understand the importance of the use of female marker line to add information which steps during fertilization the analyzed factor is involved in, as another result of different analysis. By summarizing these data obtained by each analysis, the function of fertilization regulator is concluded. Therefore, we believe our protocol is useful for assessment of fertilization in each female gamete.

The presented data lacks completely any kind of essential quantification ! for example : the percentage of the different fertilization states presented in Fig 2 and 3 is missing. The author's nice 2018 Development paper is mentioned (in which proper quantification was done). I understand that the presented data is not taken from that paper, if I'm wrong, same data should not be published twice ! Moreover, the HTR10 fluorescent intensity before/after fertilization is lacking, the area of the HTR10 signal was not done, the nucleus HTR10 signal intensity and shape in the final stage of pollen tube arrival is missing... etc. A general scheme / cartoon of the sperm's

nucleus shape during each one of these stages (late pollen tube arrival / before / after fertilization) is required.

- We have been told “This is a novel publication and the text for the manuscript has to be novel, however, we do not require novel results, only representative results” by an editor of JoVE. We understand that detailed insight about HTR10 dynamics during the reproduction process is also important, but it should be published as another analytical paper.

Supplemental File (Figures, Permissions, etc.)

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