Master's Thesis of Hyunjin Yoo

Optimized methods for the isolation of *Arabidopsis* egg cell, synergid cell and developing embryos

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ABSTRACT

Optimized pipeline of sampling specific Egg cell and Synergid cell and Early *Arabidopsis* embryo

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Arabidopsis female gametophyte, so called embryo sac, contains seven cells with eight haploid nuclei. An egg cell fertilized with a sperm cell generates a diploid zygote which becomes an embryo while a diploid central cell fertilized with the other sperm cell forms an endosperm. Synergid cell is known to play an important role for pollen tube entry so that the two sperm cells can be successfully delivered into the ovules for the double fertilization. After fertilization an embryo develops within the seeds along with several distinctive stages; globular, heart, torpedo, bending torpedo and mature green stage. Except central cells, epigenetic states of the female gametophytic cells are still largely unknown mainly due to the technical difficulties of isolation of that specific cells that are deeply buried within the several layers of the other maternal tissues. To explore the epigenetic state, isolation of pure egg cells and synergid cells is prerequisite for the construction of DNA methylome. Therefore, here I present the optimized conditions for the isolation of egg cells and synergid cells using cell-specific markers. This method involves in degradation of the cell walls and making protoplasts from the ovules to take out the egg cell and synergid cell within the embryo sac. Then, those cells can be picked up using microcapillary under

the microscope. For the developing embryos, the sample enrichments are optimized depending on the embryo developmental stages for the methylome construction. The methods I developed will help to understand the epigenetic states and the cell fate between egg cell and synergid cell that are originated from the same mitotic products of the megaspore. My optimized method for the developing embryos, especially for the earlier embryos, will also help to broaden our knowledge for the epigenetic reprogramming during plant reproduction.

Keywords : DNA Methylome, *Arabidopsis*, Small amount,
Early embryo, Egg cell, synergid cell, Central cell,
Double fertilization

Student Number : 2016-20391

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I. Introduction

1. DNA methylation in *Arabidopsis* and embryogenesis

Double fertilization is critical trait of flowering plants, also known as angiosperm. During double fertilization, one sperm cell fertilizes an egg cell to form a diploid zygote, which gives rise to the embryo, while the other sperm cell and the diploid central cell fuse to form a triploid endosperm, which is the tissue providing the nutrition to the developing embryo during embryogenesis. Although the evolutionary origins of endosperm are unclear, the development of endosperm has been thoroughly investigated in plants, particularly in the *Arabidopsis thaliana*. Both endosperm and embryo have distinct traits, but they show interactive developmental process. After fertilization the diploid zygote divides and differentiates into an embryo and, the triploid endosperm initially undergoes several nuclear divisions without cell division until the 8th mitotic cycle, forming the syncytium (Park et al, 2020).

Epigenetic is a study of heritable but reversible phenotype changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself and its inheritance. Unlike other various popular model organisms, *Arabidopsis* has retained a complex methylation system that contributes to gene and transposon silencing, imprinting, and genome stability and many of the findings are applicable to other organisms (Gehring et al, 2007). Generally, 5-methylcytosine(5mC) is associated with transcriptional silencing.

In *Arabidopsis* genome, it contains methylation at 24% of CG sites, 6.7% of CHG and 1.7% of CHH (Cokus et al.,2008) and DNA methylation has a tendency to prefer being located at repetitive DNA sequences. This preference of methylation in repetitive DNA sequences suggests that one of methylation's main functions which is to silence that transcription of transposable elements (TEs) (Zilberman ,2004; Ghering et al, 2007). In *Arabidopsis*, transposons are generally methylated

throughout their length at cytosines in all sequence contexts, although distinct patterns do emerge at individual Loci (Lippman et al.,2003; Lippman et al.,2004; Zhang et al.,2006; Zilberman et al., 2007). In all organisms, it had adopted mechanisms to keep TEs silent, including RNA-based chromatin silencing, histone modifications, DNA methylation (Slotkin and Martienssen,2007).

In the methylation mechanism, DNA methyltransferase is an enzyme that donates methyl group to the carbon 5 position of cytosine and all known cytosine 5-methyltransferase belong to a single family with several subfamilies (Gehring et al, 2007). In *Arabidopsis*, there are main subfamilies of DNA methyltransferases: CG maintenance methyltransferases that have a function in maintaining 5-mC of CG methylation context, and the *de novo* methyltransferases that have to construct a new 5mC in CHH context since there is no complementary 5-mC to be used as a template for maintaining the methylation (Ghering et al, 2008).

In *Arabidopsis*, METHYLTRANSERAE1 (MET1) is the CG maintenance methyltransferase in *Arabidopsis* which is similarly to Dnmt1, the orthologous mammalian maintenance methyltransferase (Finnegan and Kovac, 2000). CMT3 (CHROMOMETHYLASE3) is another methyltransferase unique to plants containing a chromodomain which makes it possible to bind to methylated lysines in histone tails (Henikoff et al., 1998). CMT3 maintains methylation in the CHG sequence context. DRMs (DOMAINS REARANGED METHYLTRANSFERASES) were identified as de novo methyltransferases based on homology to the mammalian de novo methyltransferases Dnmt3a and Dnmt3b (Cao and Jacobsen, 2002). DRM2 seems to be the only functional enzyme in *Arabidopsis* (Jeddeloh, Stokes and Richards 1999).

TEs invade genomes and increase in copy number, with strong potential for damaging the host. There is conserved adopted mechanisms in all organisms, to keep TEs silent, including RNA-based chromatin silencing, histone modifications, DNA methylation, or a combination thereof (Slotkin and Martienssen,

2007).

Interestingly, DNA methylation landscape is not only cell-type specific but also dynamic and its global reprogramming is seen in mammals (Zeng and Chen, 2019), which occurs during gametogenesis and embryo development. On the other hand, seed plants had been thought to not likely have such reprogramming since the known fidelity of maintenance of DNA methylation over multiple generations particularly on CpG context (Hofmeister et al., 2017; Picard et al., 2017). However, recent temporal methylome data show global and gradual increase of CHH (H is A or C or T) methylation during embryo development (Bouyer et al., 2017; Kawakatsu et al., 2017; Papareddy et al., 2020). Those data suggest plant specific dynamic change of methlyome during embryo development (Fig. 1).

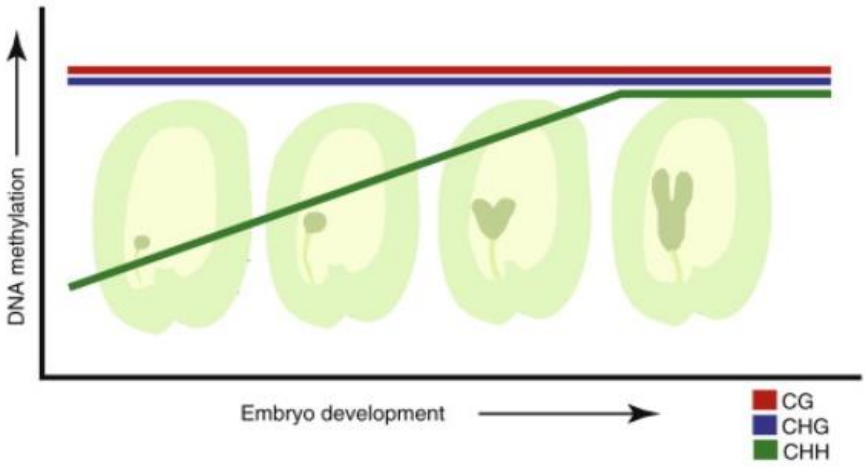


Figure 2. DNA methylation dynamics through embryo development

(Calarcol et al., 2012).

Methylome data show global and gradual increase of CHH (H is A or C or T) methylation during embryo development

In flowering plants, embryogenesis is initiated by fertilization of the egg cell by one of the two male gametes. During the fertilization in the angiosperm, a pollen tube grows into one of the female gametophyte's two synergid cells and a sperm-egg fusion that forms the embryo, and a sperm-central cell fusion that forms the nutritive endosperm. DNA methylation regulates gene expression in the egg cell, central cell, embryo, endosperm, and even other plant tissues. This seems to be critical for seed development because mutations in genes involved in demethylation such as dme mutants results in seed abortion in *Arabidopsis thaliana*(Choi et al., 2002) Synergid cell is reported to be required for pollen tube growth and release of pollen tube ceontests(willemse, 1984). Recent studies have addressed that synergid cell's function or reproductive role in the *Arabidopsis* or other angiosperms (Li et al., 2009). In the *Arabidopsis thaliana*, recent studies reported that epigenetic inheritance from mother to daughter plants is critical for transferring the genetic information during reproduction (Claude Becker et al, 2011). These are many studies which addressed about where and, how the MET1, DRM2, CMT3 and DME are expressed during reproduction(Jullien et al., 2012)(Fig. 2).

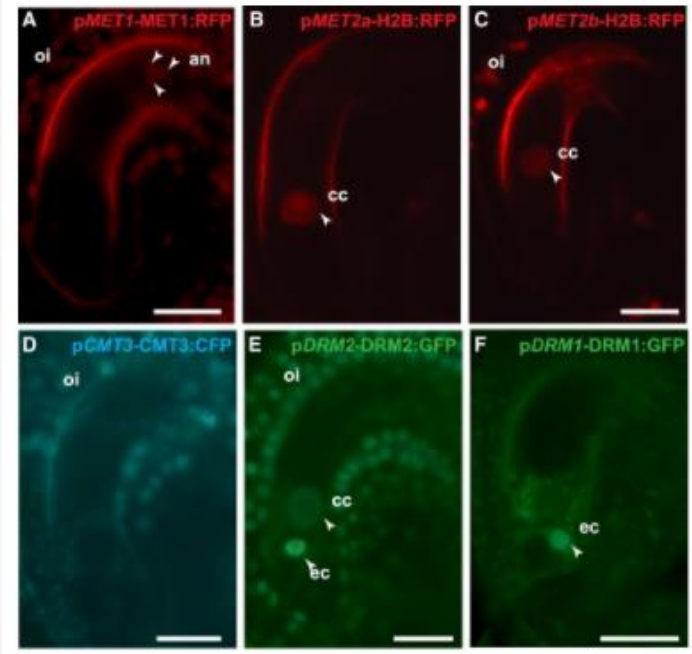


Figure 2. Expression of Reporters for DNA Methylatrnasferases in *Arabidopsis* Mature ovules(Uullen et al, 2012)

(A) pMET1-MET RFP expression is detected in the nucleus of antipodal cells(an) and the ovule integumetns(oi).

(B) pMET2a-H2B:RFP expression in the central cell nucleus (cc)

(C) pMET2b-H2B:RFP expression in the central cell nucleus (cc) and the other layers of the integuments (oi).

(D) pCMT3-CMT3:CFP expression is detected in the nucleus of the ovule integuments (oi).

(E) pDRM2-DRM:GFP expression is detected in the central cell(cc), the egg cell (ec), and in the nucleus of the ovule integumetns

(F) pDRM1-DRM:GFP expression is detected in the egg cell (ec)

Early embryogenesis in *Arabidopsis thaliana* is differentiated by a predictable pattern during cell divisions (Bowman and Mansfield. 1994). First, zygote divides asymmetrically into two parts, which has a small apical cell and a large basal cell (Goldberg et al.,1994; Scheres and Benfey,1999). The apical cell and the basal cell develop into embryo proper and the suspensor, respectively, after the several rounds of divisions. The embryo displays representative pattern of development as globular, heart, torpedo, and walking stick stages, mature green stages as cell division proceeds (Goldberg et al.,1994).

Since DNA methylation is frequently associated with gene regulation. TE silencing and genome integrity, tremendous efforts have been put into obtaining epigenetic states of specific tissues and cells. Accordingly, methods of detection of the methyl cytosine have been accelerated recently. Bisulfite sequencing let us know whether the cytosine base in the contexts is methylated or not because bisulfite treatment of DNA converts cytosine to uracil by hydrolytic deamination while methyl-cytosine remains unaffected. PCR amplification of bisulfite treated DNA leads to the conversion of uracil to thymine (CT conversion) by DNA polymerases (Frommer et al., 1992). The bisulfite sequencing technique develops fast and now it is widely used for obtaining methylome in whole genome level as well as in a single cell level(Chatterjee et al.,2012;Clark et al, 2017;Karemaker and Vermeulen, 2018; Krueger et al.,2012;Li et al., 2011; Smallwood et al., 2014).

In spite of recent advances in the understanding of methylome pattern changes in whole seeds (Bouyer et al., 2017;Kawakatsu et al., 2017;Lin et al., 2017), methylation dynamics during embryo development in *Arabidopsis* are poorly understood. To learn how the DNA methylation change affects developmental shift from vegetative stage to reproductive stage and from gamete cells to embryo development, isolation of the cells of interests followed by enrichment of the purity isolated cells are the critical prerequisite to fulfill the purpose. However, structural complexity and the small size of the *Arabiopsis* genome compared with that of animal model organisms make, difficult and challenging to isolate

haploid cells in the embryo sac. Except for the cell (Plark et al., 2016), methylome data of the egg cell and synergid cell have not been addressed until today mainly because obtainable number of that particular cell is restricted and they are deeply buried in the maternal tissues. Therefore, delicate sampling skills for taking out those specific cells from the ovules are absolutely required. Here, I have strategies for isolating pure egg cells and synergid cells using protoplasting of ovules for taking out the haploid cells inside the embryo sac and subsequent manual picking of the specific cells with fluorescent makers. I discovered and set up the optimized protocols for sampling pure egg cell and synergid cell.

Recently, Papareddy et al. examined methylome dynamics at CHH context using dissected embryos after fertilization (Papareddy et al., 2020). However, their methodology is labor-and time-consuming as it requires purified DNA from more than 50 embryos in all stages as an input for mehtylome construction. In order to reduce the amount of this input, I found and optimized conditions necessary for embryo isolation and degradation of early embryo cell walls to allow their use as inputs for methylome construction. From globular to torpedo stage embryos, I discovered that as few as five raw globular embryos are sufficient as input material for methylome construction without the need for DNA purification. I believe my methods for isolating less amounts of the specific haploid cells and the diploid developing embryos will help to understand the future research using the cells such as cell fate and differentiation.

II. Materials and methods

1. Plant Material and Growth Conditions

Arabidopsis thaliana, Col-gl (Columbia-glabrous) ecotype was used for early embryo, and Col-0 (Columbia-0) ecotype for egg cell, synergid cell isolation. DD45:GFP, DD2:GFP transgenic lines are used for egg cell isolation, synergid cell isolation, respectively. Plants were grown on soil in an environmentally controlled room at 22°C under long photoperiods (16 hours of light/ 8 hours of dark) with cool white fluorescent light (100 umole/m2/s).

2. Emasculation

Plants with healthy inflorescences are one of the most critical factors for the successful sampling. Fully matured anthors were removed using the scissors or forceps (Fine Science Tools, Inox Fine Forceps, Dumont #5). Specifically single healthy inflorescences usually have 2-3 floral buds at the ideal stage. Using forceps, I removed all of the floral organ except the pistil per each bud. Generally, 30-50 emasculations per experiment were performed and emasculate plants were then incubated in an environmentally-controlled room or growth chamber at 22°C with 60% humidity under long-day photoperiods (16h light, 8h dark) for 24 hours to pistils fully stay healthy.

3. Pollination

Pollination was done after 24 hours of emasculation (Park et al., 2016), Fully matured stamens were picked from open flowers with tweezers and pollen was rubbed onto the emasculated stigma for fertilization, under a dissection microscope. And then, pollinated plants were incubated in the growth room or artificially controlled growth chamber until they reached a suitable stage for sampling; 4 days after pollination (DAP4) for globular, DAP5 for heart, DAP7 for torpedo, DAP9 for bending torpedo, and DAP12 for mature green stage embryos (Fig. 3).

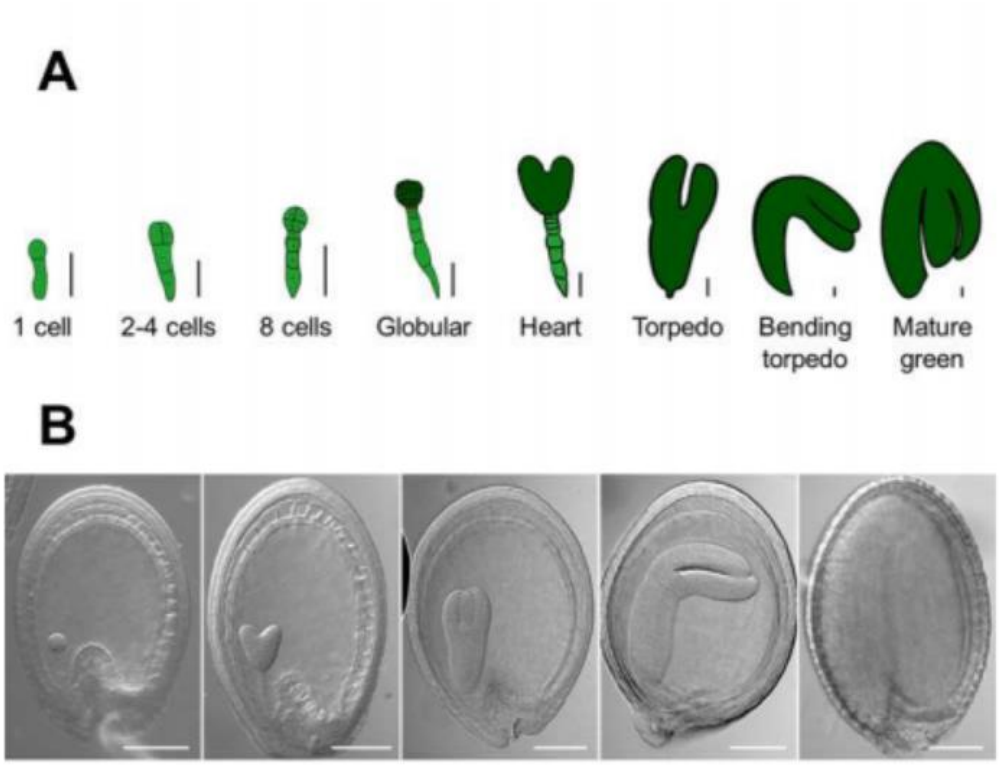


Figure 3. The developmental stages of *Arabidopsis* embryos

(A) The developmental stages of *Arabidopsis* thaliana embryos. Vertical scale bar, 50um.

(B) Differential interference contrast (DIC) images of embryos within seeds used in this study. From left to right: globular, heart, torpedo, bending torpedo and mature green stage. Scale bar, 100um.

4. Preparation of the GFP expression lines for specific egg cell and synergid cell

For using isolation specific egg cell and synergid cell, specific cell-type GFP expression lines are required. Since DD2 and DD45 promoter activities were observed in the synergid cell and in the egg cell, respectively (Joshus G. Steffen et al, 2007). DD2::NTF transgenic lines and DD45::NTF lines were used for isolation of synergid and egg cell, NTF (Nuclear Trgeting Fusion) domain was fused to GFP (Deal and Henikoff, 2010) The NTF consists of three parts: the WPP domain of *Arabidopsis* RNA GTPase activating protein 1 (RanGAP1, locus At3g63110; amino acids 1-111, inclusive), which is necessary and sufficient for the association with the nuclear envelope in plants (Rose and Meier, 2001), green fluorescent protein (GFP) for visualization, and the biotin ligase recognition peptide (BLRP), which serves as a substrate for the E.coli biotin liagse BirA. Thus, expression of BirA and the NTF in the same cell type produces biotin-labeled nuclei exclusively in those cells. I first sow DD2::NTF and DD45::NTF lines on the MS basta plate for selection and then, I confirmed their expression under the upright microscope (Fig 4)(Fig 5).

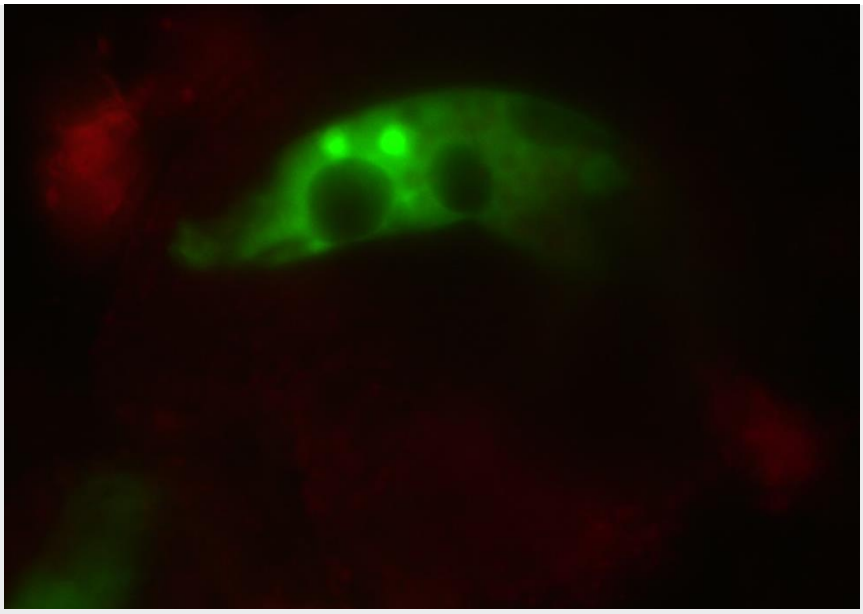
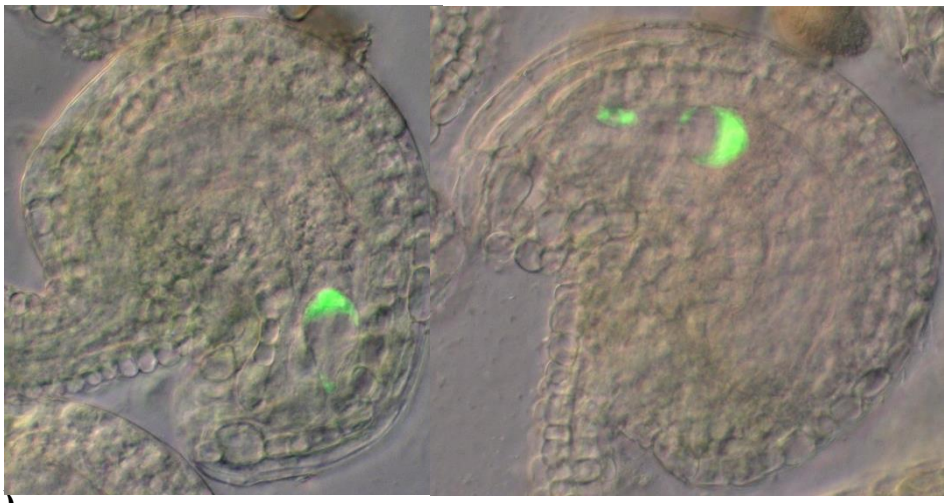


Figure 4. DD2::NTF transgenic line expression check.

After protoplast the ovules in the enzyme solution, synergid cell comes out from the ovule. The synergid cell is 30-45um size.

Its' size is 30-45um.



(A)

(B)

line	detection	specificity*
1	11	A
3	8	A
5		A
6	10	A
8	14	A
9		A
10		A
11		A
13		C
15	13	A

Figure 5. DD45::NTF transgenic line expression in the egg cells.

(A) GFP expression was confirmed in egg cell

(B) The table shows GFP detection ratio and the specificity of the egg cell in each siliques of the DD45::NTF GFP transgenic lines.

5. Ovule protoplasting and the isolation of egg cell and synergid cell manually

Firstly, I emasculate the selected DD45::NTF , DD2::NTF T2 transgenic lines which express specifically in the egg cells and synergid cell, respectively. 24 hours after emasculation, emasculated pistils were dissected (at least 15-20 siliques) and then ovules were scrapped into the glass dish with enzyme solution for protoplast. The ovules were incubated for 1 hour-2 hours within the enzyme solution (20mM MES; 4-morpholineethanesulfonic acid, PH5.7, 1.25% cellulose R10, 0.1% pectolyase, 1% hemicellulose, 2.3% pectinase, 0.4M mannitol and 20Mm kcl)(Fig. 6) (Fig. 7). The enzyme solution

was first preheated at 70°C for 5min to resolubilize any crystals. This solution allowed to cool to room temperature and enzymes added after by incubation of the solution at 55°C for 30min, enhancing enzyme solubility and to inactivate DNase and proteases. 10mM CaCl2 was then added finally and filter sterilized by syringing through a 0.45um membrane. I picked the egg cell, and synergid cell using GFP markers under the inverted fluorescent imager (ZOE fluorescent cell imager, BIORAD:1450031) using a manual micro-pipetting device (Nepagene:MPP-200B). Isolate egg cells and synergid cells were subsequently washed; five or six droplets of Mannitol buffer(~10 ul each) were placed on the lass slide, and egg cells and synergid cells were moved between these droplets. This washing step was repeated until all debris, accumulated during dissection process, was removed. For manual isolation, the lid of a flat capped PCR tube was cut (WATSON, Cat NO. 137-211C). Pure egg cells and synergid cells were transferred onto this cap in 5-10 ul Mannitol buffer. Sample purity was confirmed under the microscope, before the PCR tube was reassembled with the lid containing the purified egg cells, synergid cells. The PCR tube was centrifuged for 30 seconds to 1minute and stored at the -80°C. Using 30,50um sizes of micro glass pipettes were used according to the egg cell, synergid cell size (NEPAGENE:1-GT30S-6,30um,1-GT50S-6,50um).

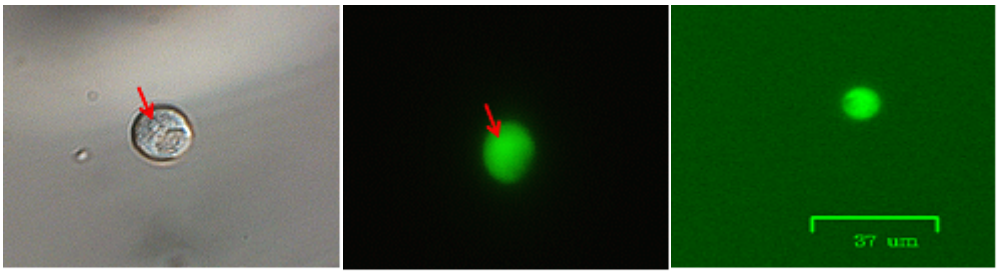


Figure 6. Synergid cell : DD2::NTF T2 transgenic line cell GFP expression check.

After protoplast the ovules in the enzyme solution, synergids cell comes out from the ovule.

Its' size is 30-45um. (arrow : nuclei)

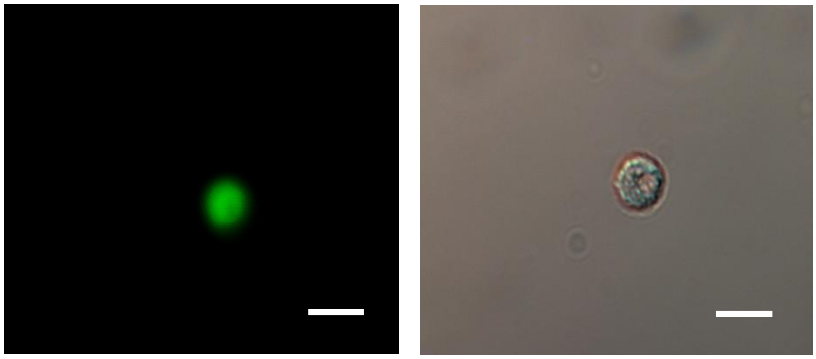


Figure 7. Egg cell ; DD45::NTF T2 transgenic line cell GFP expression check

After protoplast the ovules in the enzyme solution, egg cell comes out from the ovule.

Its' size is 30-45um.

6. Preparation of globular stage embryos

DAP4 seeds were collected together for globular embryo preparation in 1.5mL tubes (seeds from 3-5 siliques per tube) with 50-100 ul of isolation buffer (1x TE buffer; 10Mm Tris-HCl (PH 8.0), 1mM EDTA (PH 8.0)). After brief centrifugation, seeds were ground gently with a pestle which allows intact globular embryos to be released from the seeds.

7. Preparation of heart stage embryos

At the DAP5, DAP5 siliques were dissected in 50ul isolation buffer for heart stage embryo preparation. I modified the dissection method from the previous study (Xiang et al., 2011), with incisions made

horizontally at the upper part of the seed, and then vertical cuts were made at the chalazal side, to avoid embryo damage (Fig. 8). During dissection of the seeds, since heart stage embryos appear transparent, reflected light from the dissection microscope was used to visualize embryos, which were released using tweezers. Finally, released embryos were transferred to a new droplet of isolation buffer using a pipet for enrichment.

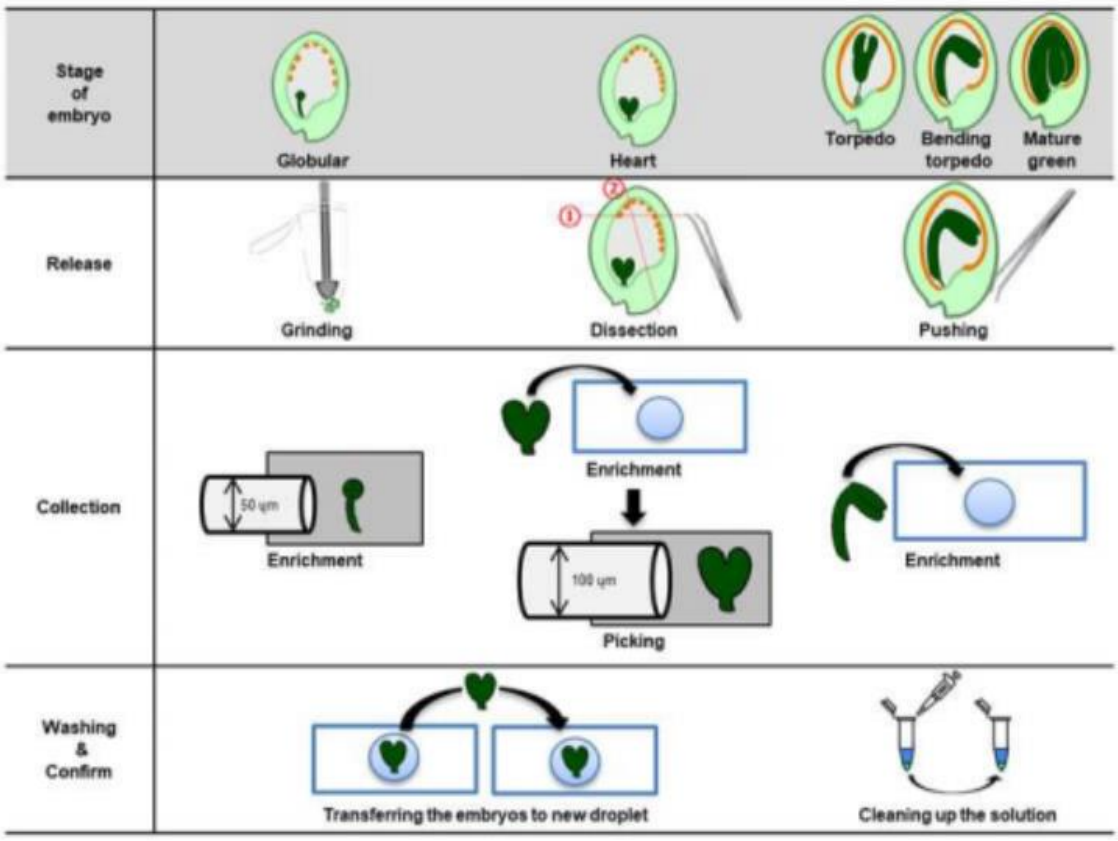


Figure 8. A summary of sampling methods according to developmental stage

There are 3 optimized steps (release, collection, and washing and confirm) for each embryo stage. The heart stage embryo was dissected for sampling first horizontally then vertically. The diameters of S shaped glass micropipettes were various. 50 um and 100 um used for globular and heart stage embryo

each.

8. Collection of the globular and heart stage embryos

Samples in 10-20ul isolation buffer were moved onto glass slides for isolation of embryos using a manual micro-pipetting device (Nepagene; MPP-200B Micro Pick and Place Manipulator System B without Microscope) under an inverted fluorescent imager (ZOE fluorescent cell imager, BIORAD : 1450031)(Fig. 9). Unlike sampling globular stage embryos, for heart stage embryos, gentle tapping of the microcapillary was used to position the axis of the embryos towards the microcapillary allowing access to the embryo for suctioning (Fig. 8). This skill needs especially for heart stage embryo sampling because its size is much bigger than globular stage and the shapes are various in few ways. Isolated globular and heart stage embryos were subsequently washed; five or six droplets of isolation buffer (~10ul each) were placed on the glass slide and embryos were moved between these droplets (Fig. 8). This washing process was repeated until all debris, accumulated during dissection process, was removed at all. For manual isolation, the lid of a flat capped PCR tube was cut (WATSON, Cat No. 137-211C). Pure embryos were moved onto this cap in 5-10 ul of isolation buffer. Before the PCR tube was reassembled with the lid, sample purity was confirmed under the microscope. The PCR tubes were centrifuged for 30 seconds to 1 minute briefly and stored at the -80°C directly. Various diameter sizes of micro glass pipette were depends on the embryo type and size; 50 um for globular stage embryo and 100 um for heart stage embryo. (Fig. 9)(NEPAGENE: 1-GT50S-6, 50 um; 1-GT100S-6, 100um)

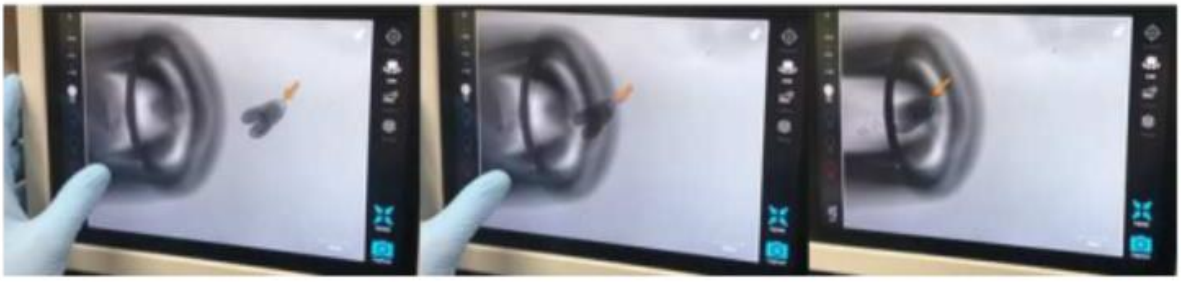


Figure 9. The isolation of released embryo using micro-glass pipette

Isolation of a late heart to early torpedo embryo (orange arrow) using a micro pipetting device under an inverted fluorescent imager.

9. Preparation and isolation of torpedo to mature green stage embryos

At the stage of torpedo to mature green stage embryos sizes are bigger than before and the shape is little more complicated than before stage embryos. Sample preparation was conducted using DAP7, DAP9 and DAP12 seeds for isolation of torpedo, bending torpedo and mature green stage embryo, respectively. At these DAP7, DAP9, and DAP12 stages, embryos are more easily detectable due to their greener color and larger size compared with globular and heart stage embryos. Due to these traits, sampling can be done on slide glass. Seeds were stabbed and pushed using tweezers to release the embryos, leaving seed coat and endosperm clumps behind. And then, the released embryos were collected in 20 ul of isolation buffer. A sufficient number of embryos were transferred to 1.5mL tubes and washed in isolation buffer. After several washes, purified embryos were transferred to a new 1.5mL tube and stored at -80°C(Fig. 7)., respectively. The washing step was conducted repeatedly until embryos are purified.

10. Cell wall degradation test in the globular-heart stage embryos

Before re-freezing in liquid nitrogen, samples were thawed at room temperature and incubated at 95°C for 30 seconds in a water bath. This freeze-thaw cycle was repeated at least five times. Samples were centrifuged briefly ,30s -1min and vortexed for 1 minute for globular embryos or 5 minutes for the heart embryos, respectively. For torpedo embryos, vortex time was extended and sample degradation could be seen by visually. After degradation, M-digestion buffer (2X, Zymo: D5021-9) and 1 ul of Proteinase K (Zymo: D3001-2-5) were added to the PCR tube lids (up to 20ul total) and the samples were centrifuged and vortexed briefly at the final step. Samples were incubated at 50°C for 30 minutes, and vortexed as described above for each embryo stage. When the further brief centrifugation is done, samples were ready for CT conversion, which is the first step in the construction of bisulfite sequencing library.

III. Results and discussion

1. Isolation of the egg cell and the synergid cell

I selected the DD45 and DD2 promoters for the isolation egg cells and synergid cells by visualizing GFP expression. My strategy of sampling was isolation of the egg cell and synergid cell as pure as possible. At least 15 pistils are required for the sampling. 24hours later after emasculation, dissected ovules were put together in the glass petri dish within the enzyme solution and the ovules were incubated. At least 1hour-2hours the room temperature, At the final step after manual picking of the egg and synergid cell by microglass pipette(Fig. 10), cells were washed several times till all debris were removed as described at the Materials and methods part. All cleared samples stored at the -80°C.

The two samples were then subsequently tested for bisulfite sequencing by my colleague, Park. Each sample has 50 cells and sequenced QC (Quality Check) results are fine. But there was mapping efficiency problem in both samples. Mapping efficiency is determined by the number of uniquely mapped reads divided by the total number of reads. Consequentially, the mapping efficiency of both samples was very low that I could not get methylome information from both samples. The mapping efficiency result of two samples of egg cell was 1%, 2% each. And the mapping efficiency result of two samples of synergid cell was 0.1%, 0.3% each (Fig.11).

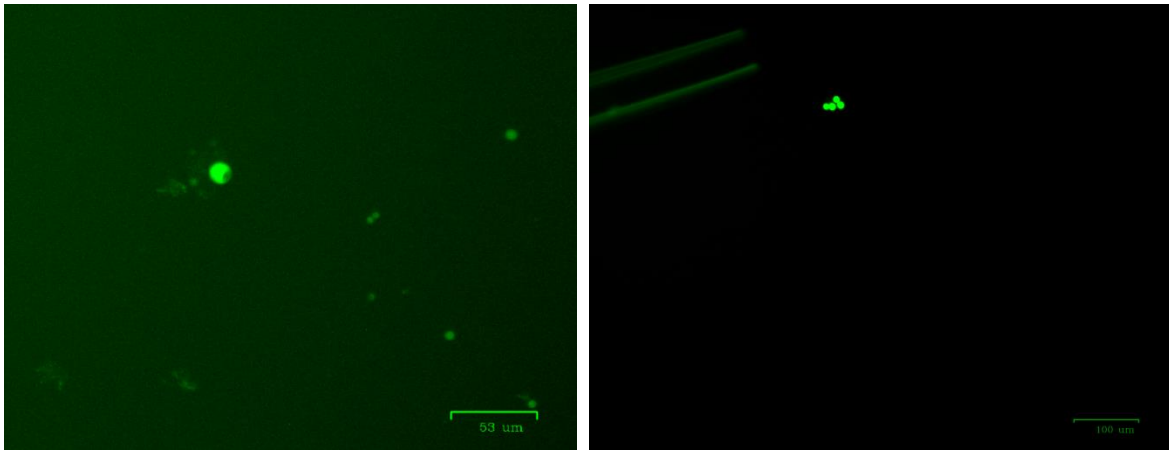
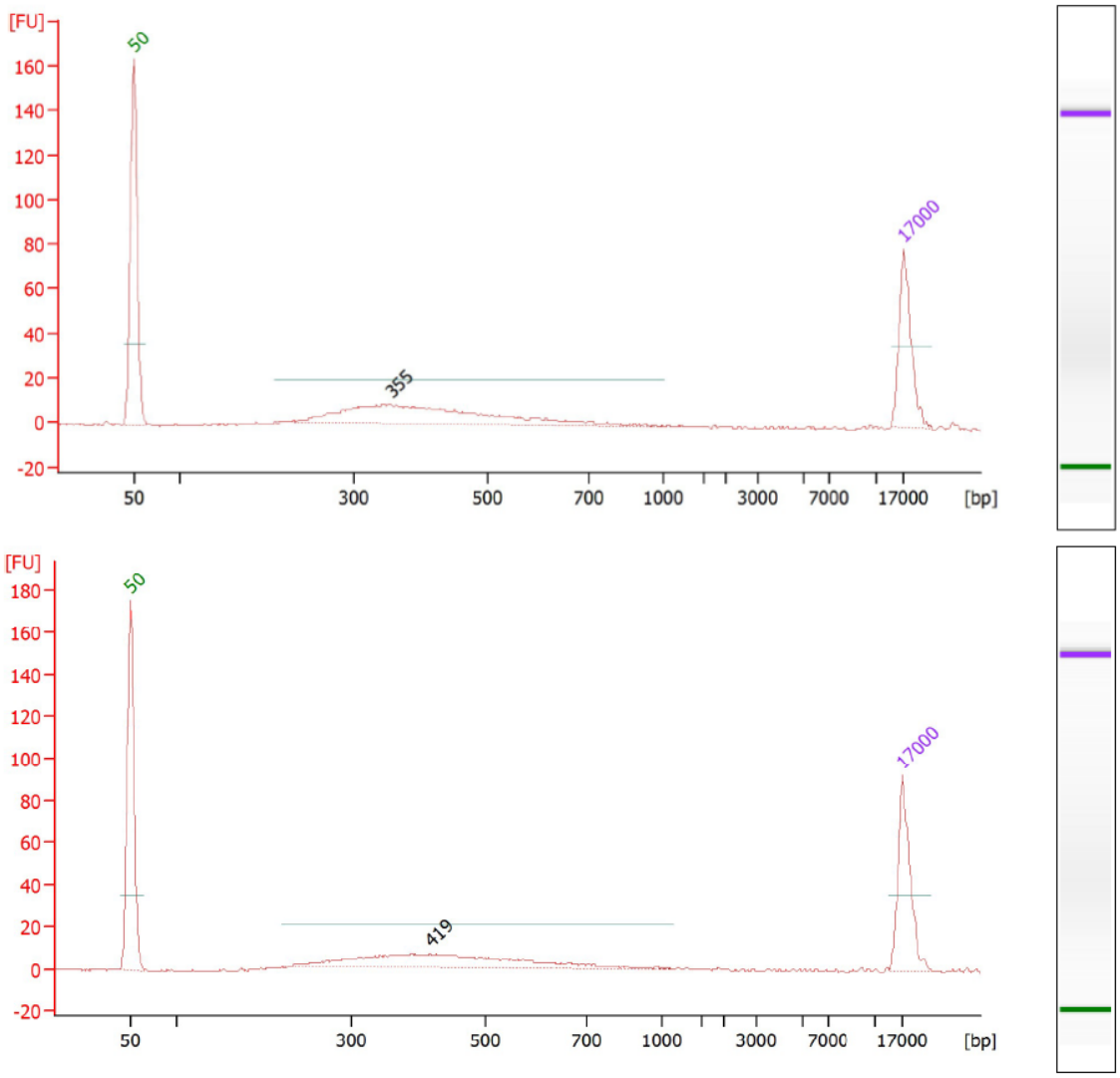
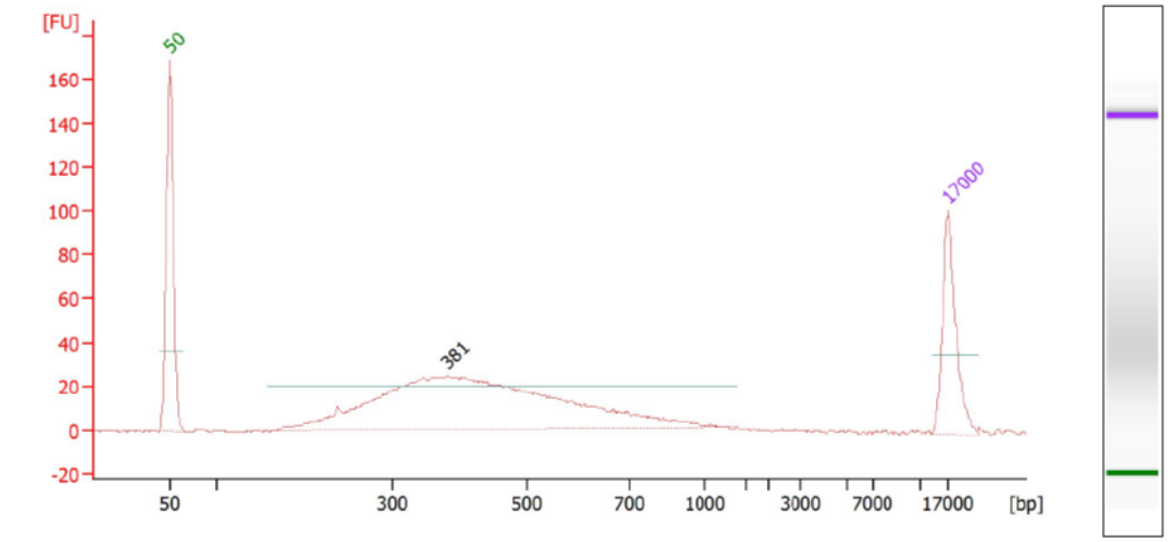


Figure 10. Isolation synergid cells under the microscope.

Pick the synergid cell with the micro glass pipette. Right figure shows that several egg cells collected at the final step of isolation process.

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160
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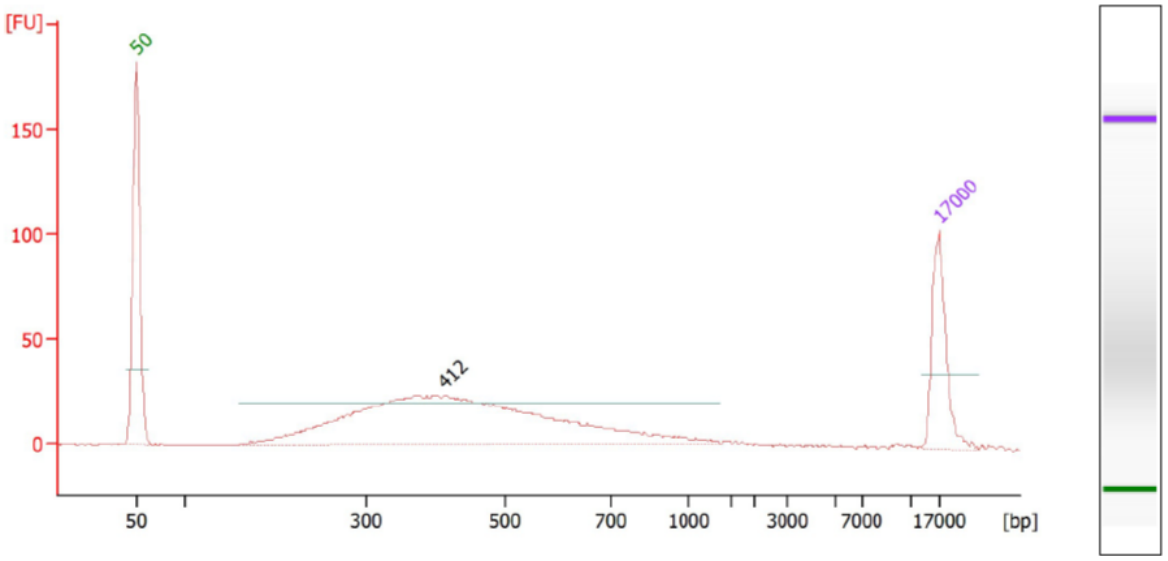
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Library Name	Library Code	Volume (µL)	Fragment Size (bp)	Concentration (ng/µL)	Molar Concentration (nmol/L)	Conclusion*	Remarks
ECA (5.3.19)	CHKPEI85219050297	18	355	8.23	89.60	Failed	Peak tailing
SY (5.3.19)	CHKPEI85219050298	18	381	21.32	267.08	Failed	Peak tailing
ECA (9.3.19)	CHKPEI85219050299	18	419	5.82	71.14	Failed	Peak tailing
SY (9.3.19)	CHKPEI85219050300	18	412	19.25	232.64	Failed	Peak tailing

Figure 11. Sequencing the sampled egg cell and synergid cell.

There are 2 libraries of egg cells and synergid samples of which the mapping efficiency were not satisfied. Results are not successed. The table dictates above graphs information. (Upper 2 graphs; Egg cell samples each, Down 2 graphs; Synergid cell samples each)

2. Periodically manual sampling the specific synergid cell

During isolation, GFP fluorescence was visible through out when I observed the sample. Tracking the GFP signal through all over the samples, and I was able to observe the fluorescence of synergid cell from the micropylar end of the ovule. Through repeated sampling process, I had successfully isolated synergid cell (Table. 1). The number of samples per experimental trial was different at the time, but the purity of all samples was good enough to do the further experiments for sure. The purity of synergid cell was obtained using a fluorescence ZOE imager microscope as GFP signal. The total number of synergid cells isolated was upto 200 by myself. I found that 200 synergid cell number can manage to ceonsturct the methylome library. The number of synergid cell required for the construction of

methylome is based on the data in central cell of the female gametophyte in which the library was successfully generated from 100 diploid central cells (Park et al., 2016).

	Synergid cell isolation						BSseq
Label	Date	Synergid cell	Total	cell purity	content Pe	Detail	ample pooling
	Total	100	100	100%	100%	total volume(ul) (sample + 1X lysis buffer)	
sy1	########	2	2	100%	100%	6.5	2
sy2	########	14	14	100%	100%	11.4	1
sy3	########	2	2	100%	100%	4	2
sy4	########	4	4	100%	100%	6	2
sy5	########	2	2	100%	100%	8	2
sy5	########	2	2	100%	100%	8	2
sy7	########	6	6	100%	100%	13	2
sy8	########	9	9	100%	100%	11.5	1
sy9	########	2	2	100%	100%	4	2
5_23	########	2	2	100%	100%	4	2
No name	########	4	4	100%	100%	9	2
5_30	########	1	1	100%	100%	7	2
6_28	########	5	5	100%	100%	6.5	2
7_5	########	10	10	100%	100%	11	1
7_11	########	5	5	100%	100%	14.5	2
7_13	########	9	9	100%	100%	22.9	2
7_17	########	3	3	100%	100%	8.4	2
8_20	########	8	8	100%	100%	12	1
8_29	########	8	8	100%	100%	14.2	1
8_31	########	4	4	100%	100%	8	1
9_13	########	5	5	100%	100%	7	2
10_10	########	1	1	100%	100%	4	2
10_30	########	1	1	100%	100%	4	2

Table 1. The process of sampling the synergid cell periodically.

The number of sampled per experiment would be various because it depends hardly on the condition of plants. Through these repeated sampling process, sampled 200 synergid cells.

3. Early globular-to torpedo-stage embryos can be used as inputs for the construction of methylomes without DNA purification

Epigenetic patterns in early globular to heart embryos in particular, change more rapidly than late-

stage embryos (Laux et al.,2004). During embryogenesis, enlightened changes to epigenetic patterns become epigenetic patterns become established. However, studies into the epigenetic states in *Arabidopsis* have mainly focused on late stage embryos or seeds (Bouyer et al., 2017; Kawakatsu et al., 2017), mainly because of the relatively ease with which methylome libraries in these stages are generated. However, to understand the comprehensive DNA methylation pattern progress during embryogenesis, the development of methods tailored to each developmental stage is required. While methods for the construction of methylome libraries from single mammalian cells have been developed (Clark et al., 2017; Smallwood et al., 2014), these methods cannot be applied directly to *Arabidopsis* without extensive optimization due to the relatively small genome size of this model organism. In addition, early embryos are technically far more difficult to isolate than late-stage embryos as they are transparent, consisting of a small number of cells that are buried deep within seeds. Therefore, I first optimized the isolation method of each stage of embryos to avoid contamination for providing as an input to generate methylome libraries with 5-20X coverage.

First, I applied seed dissection to release early embryos. Because of the small size of embryo and the strong connection between suspensor and seed coat, globular embryos were difficult to release. Therefore, I adopted the previously described seed grinding method (Raissig et al., 2013), which allowed me to collect a number of globular embryos sufficient for the subsequent processes. This method similarly allowed for the release of earlier-stage embryos from before DAP4 (Fig. 12). However, embryos without intact suspensors were released as well (Fig. 12).

Bigger heart stage embryos can be easily detected under a dissection microscope. These were dissected from seeds in isolation buffer using a protocol modified from previously described (Raissig et al., 2013). However, this method produced debris, which resulted in contamination. Therefore, after dissection the released embryos were purified and enriched. Embryos in the globular stage were taken

out of the seeds with gentle pestle grinding, without breaking the embryo (detailed in the Matrials and Methods). Purified globular and heart embryos were enriched using different sized microcapillaries.

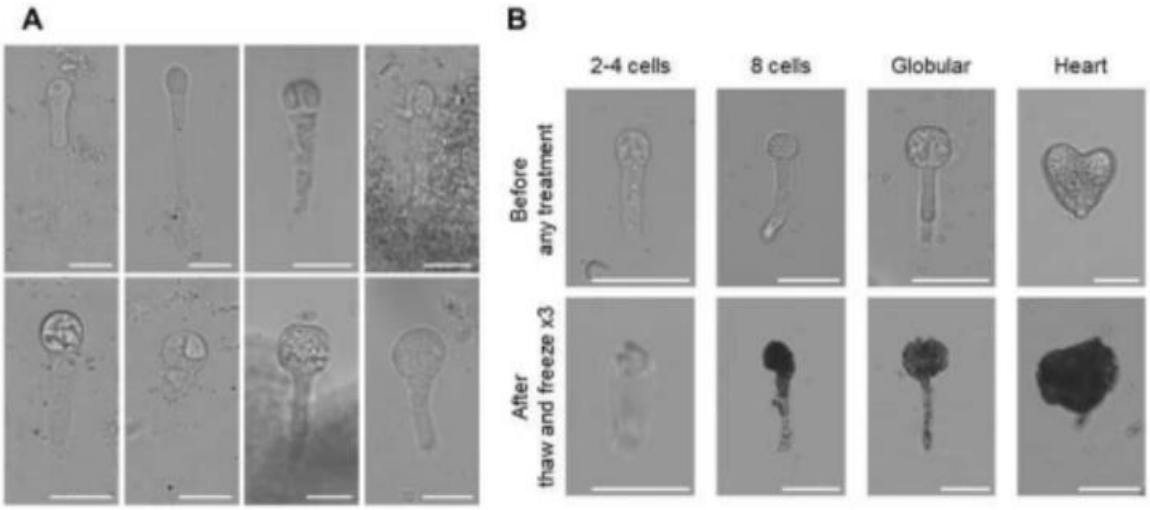

Figure 12. Released embryos from intact seeds.

(A) The release of globular embryos from seeds by gentle grinding. Embryos consisting of 1-4 cells are shown in the upper panel. Scale bar, 25 um.

(B) Before and after 3 cycles of freeze-thaw without vortexing. Cell wall degradation is observed in the lower panel. Scale bar, 50um.

4. Pestle grinding and DNA purification in bending-torpedo stage embryos can be used as inputs for the construction of methylomes without DNA purification

Sampling of the embryos from the bending-torpedo stage required the freeze-thaw cycle method

described above cannot be applied to. These stage embryos, which have a greater cell number than globular-to torpedo-stage embryos with rigid cell walls, were not degraded by freeze-thawing only. For these reasons, DNA purification is required for embryos in this stage. The previously described DNA purification method (Allen et al., 2006), could be examined whether these relatively small embryos (roughly 0.5 mm in diameter) compared to other plant tissues such as leaves could be ground with a pestle. Under a microscope, consistent degradation of embryos from bending torpedo stage to mature green stage was observed, but not of the torpedo embryos. A general DNA purification method (Allen et al., 2006) with 10 bending torpedo embryos from DAP9 was confirmed to be sufficient for methylome construction.

IV. Discussion

Recent techniques have been extensively developed for obtaining global gene expression profiles and epigenetic data, including DNA methylation at whole genome level and single-cell level (Chatterjee et al., 2012; Clark et al., 2017; Karemaker et al., 2018; Kruger et al., 2012; Li et al., 211; Rich-Griffin et al., 2020; Smallwood et al., 2014; Stuart and Satija, 2019). There are still challenges remained in profiling of *Arabidopsis*, even though these descriptive advances have been of great benefit, cell- or tissue- type specific epigenome profiling in *Arabiodpsis*. This is mainly because it has smaller genome size than mammals. Furthermore, the structural complexity and variation of each tissue type has made data interpretation difficult, especially if the input sample is a mixture of cell types. For example, there are three parts in the seeds: seed coat, embryo, and endosperm. Accordingly, DNA methylation patterns differ mainly between the tissue types. For instance, the DNA methylation patterns with endosperm

more hypomethylated than the embryos which demonstrated that endosperm DNA demethylation is initiated from the Central cell(Hsieh et al., 2009; Ibarra et al., 2012, Park et al., 2016).

In the *Arabidopsis*, reproduction is a critical process, especially the epigenetic changes influence not only the development of the seed but also transferring the genome to their daughter generation. Recent study addressed that in the male and female gametophyte, DME acts to demethylate DNA genome-wide (Choi et al., 2002; Ibarra et al., 2012; Kim et al., 2008). But so far, except central cell, epigenetic states of other haploid cells of the female gametophyte cell, egg cell and synergid cell were not clarified yet. To date, genome-wide DNA methylation levels of the later stages of the embryo and the endosperm have published (Gehring et al., 2009; Hsieh et al., 2009; Ibarra et al., 2012). Genome-wide transcriptional profiles of the female gamete cells have been generated by using Laser-Capture Micro-dissection techniques (LCM) (Schmidt et al., 2012; Wuest et al., 2010). But LCM is a treatment which is not perfectly clear and the samples are likely contaminated with other debris. To understand the correct epigenetic states in the haploid cells before fertilization as well as early embryos after fertilization, it is critical to purify the cells of interest without contamination.

For getting the specific epigenomic profile information of the egg cells, synergid cells and early-late stage embryos, here I introduced an optimized sampling protocols of each sample. I have developed effective methods for collecting egg and synergid cells released from the ovules as well as for developing embryos in a developmental stage-dependent manner. In early embryo sampling steps, it is followed by manual embryo picking with micro glass pipettes (Fig. 8 and 9).

The methods I provided here lead to success for making methylation libraries by small amount of embryos. This protocol enables the researchers to make methylome from the smallest amounts of inputs as far as I know until today. The methods presented here have the advantage that each pipeline protocol can be applied to other tissues from plants, or even to other species, since this method provides

information on what to consider for each stage, especially sampling.

Here, I presented the optimized sampling process of the egg cell, synergid cell specifically from the *Arabidopsis thaliana*, which was aim for the construction of each cell methylomes. To aid the cell specific isolation of the egg cell and synergid cell, I have developed effective methods for release of these cells synergid cell from ovules and followed by manual picking with micro glass pipettes.

Seed plants have comprehensive and versatile DNA methylation systems and patterns of methylation are tightly regulated. Therefore, the development of methods to study these patterns to support understanding of genome heterogeneity and the methylome are of considerable importance.

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