A Thesis for the Degree of Master of Science

Endosperm-derived hybridization barrier in intergeneric hybrid between *Brassica rapa* and *Raphanus sativus*

배추와 무의 속간교배체에서의 배유 유래 교배 장벽에 관한 연구

FEBRUARY, 2019

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FEBRUARY, 2019

APPROVED AS A QUALIFIED THESIS OF TAEGU KANG
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ABSTRACT

Interspecific or intergeneric hybridization is vital to the evolution of flowering plants. However, interspecific or intergeneric hybridization often faces in hybridization barriers, resulting in seed abortion or sterility of F1 hybrids. In angiosperms, failure of endosperm development is one of the major causes of immediate hybridization barrier. To date, dysregulation of imprinted genes in developing endosperm has been proposed to be explain the endosperm-derived hybridization barrier. A general mechanism of hybridization barrier remains to be demonstrated. x*Brassicoraphanus* is an intergeneric hybrid of Chinese cabbage (*Brassica rapa* L.) and radish (*Raphanus sativus* L.). Newly synthesized x*Brassicoraphanus* F1 hybrids often display seed abortion accompanied with embryo arrest and underdeveloped endosperm. Here I report that intergeneric F1 hybrid between *B. rapa* and *R. sativus* showed endosperm-derived hybridization barrier. RNA-seq was conducted on

intergeneric endosperm to identify imprinted genes related to hybridization barriers. Unexpectedly, the most of transcripts from intergeneric endosperm were maternal reads originated from *B. rapa*. Moreover, SCAR marker analysis revealed that intergeneric hybrid endosperm largely consisted of maternal genome derived from *B. rapa*. Therefore, these results suggested the possibility that the hybridization barrier in intergeneric F1 hybrids of *B. rapa* and *R. sativus* may be involved in the inhibition of fertilization between the central cell and sperm. These results provide further insights into the post-zygotic hybridization barrier between distantly related species in the Brassicaceae family.

Key words: x*Brassicoraphanus*, Brassicaceae, Endosperm, Hybridization barrier, Gene imprinting.

Student number: 2017-22300

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LIST OF ABBREVIATIONS

PRC2	Polycomb repressive complex 2
MEA	MEDEA
FIE	FERTILIZATION INDEPENDENTE ENDOSPERM
FIS2	FERTILIZATION INDEPENDENT SEED2
CDKA;1	CYCLIN-DEPENDENT KINASE A;1
B. rapa	*Brassica rapa*
R. sativus	*Raphanus sativus*
ADM	ADMETOS
FDR	False-discovery rate
MEG	Maternally expressed imprinted gene
PEG	Paternally expressed imprinted gene
SUVH7	SU(VAR)3-9 HOMOLOG 7
EDE1	ENDOSPERM DEFECTIVE 1
GLU	GLAUCE
CAP2	CAPULET 2
CF	Chiifu-401-42
HG	Hagam50

WK	WK10039
KB	KB-68
cv	Cultivar
DAP	Days after pollination
TBA	Tert-butyl alcohol
SCAR	Sequence characterized amplified region
CTAB	Cetyl-trimethylammonium bromide
GEX2	GAMETE EXPRESSED 2
C. rubella	*Capsella rubella*
C. grandiflora	*Capsella grandiflora*
P. zeylanica	*Plumbago zeylanica*
A. thaliana	*Arabidopsis thaliana*
A. lyrata	*Arabidopsis lyrata*

INTRODUCTION

It is postulated that interspecific or intergeneric hybridization in plants plays a vital role for evolution and agriculture. Hybridization between distantly related species often displays seed lethality or hybrid sterility. Hybridization barrier refers to the prevention or limitation of offspring between different species. Hybridization barriers can be divided into prezygotic and postzygotic barriers, depending on the timing of fertilization. Before fertilization prezygotic barriers reduce the frequency of fertilization, as exemplified by the failure of pollen germination and pollen tube growth. Postzygotic barriers are associated with the inviability and sterility of hybrids after fertilization (Rieseberg and Carney, 1998).

During double fertilization, one sperm cell fertilizes the egg cell, leading to the formation of the diploid embryo. The other sperm cell fertilizes the central cell to form the triploid endosperm consisting of two maternal and one paternal genomes. The communication among seed components such as embryo, endosperm, and seed coat is necessary for seed growth and development (Lafon-Placette and Köhler, 2014). Among these components, the endosperm plays a crucial role for the uptake of nutrients as well as signaling of seed development. In angiosperms, endosperm is developed through the syncytial and cellular phases and differentiates into several compartments that have specific functions (Becraft, 2001; Olsen, 2001).

The endosperm initially develops as a syncytium which undergoes cell proliferation without cytokinesis. After a defined period of nuclear division, the endosperm is cellularized and later consumed by the embryo (Li and Berger, 2012). Endosperm cellularization is key to seed development. The failure in cellularization

leads to insufficient nutrient supply, causing embryo arrest and seed abortion (Hehenberger et al., 2012). The timing of endosperm cellularization is modulated by FIS-PRC2-induced repressive histone modification (Grossniklaus et al., 1998; Luo et al., 2000). Mutations in PRC2 components such as *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENTE ENDOSPERM* (*FIE*) and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*), showed autonomous endosperm without fertilization and over-proliferation of endosperm in fertilized mutant seeds (Chaudhury et al., 1997; Grossniklaus et al., 1998). In *Arabidopsis*, mutants in *CYCLIN-DEPENDENT KINASE A;1* (*CDKA;1*) have pollen containing a single sperm cell. This sperm is exclusively fused with the egg cell (Nowack et al., 2006). A positive signal from the fertilized embryo can trigger autonomous endosperm development, involving nuclear division of unfertilized central cell. Endosperm proliferation without fertilization of the central cell is restricted to syncytial phases, followed by seed abortion (Ungru et al., 2008).

Genome dosage imbalance of endosperm is related to the timing of endosperm cellularization (Pennington et al., 2008). Interspecific hybridization barriers with endosperm defects can be bypassed by changing the ploidy level of parental species, implying that endosperm-derived hybridization barriers are involved in dysregulation of imprinted genes (Josefsson et al., 2006). Consistent with theoretical considerations, *ADMETOS* (*ADM*) is a paternally expressed imprinted genes responsible for seed abortion in paternal excess hybrids (Kradolfer et al., 2013). The mutations in the *ADM* gene showed that cellularization of endosperm is partially restored in triploid seeds. A general mechanism for hybridization barriers regulated by imprinted genes remained unsolved.

Chinese cabbage (*Brassica rapa* L.) and radish (*Raphanus sativus* L.) belong to the Brassicaceae family. The intergeneric hybridization between the Brassicaceae family species have been studied (Dolstra, 1982; Lee et al., 2011; Sageret, 1826). Despite many attempts of intergeneric hybridization between *B. rapa* and *R. sativus*, it is generally difficult to obtain genetically stable and fertile intergeneric hybrids. x*Brassicoraphanus*, also known as 'Baemoochae', is a successfully synthesized intergeneric allotetraploid between *B. rapa* and *R. sativus* (Lee et al., 1989; 2002). Newly synthesized x*Brassicoraphanus* hybrids showed seed abortion with embryo arrest and endosperm defect. However, hybrid plants can be obtained by *in vitro* embryo rescue, suggesting that intergeneric hybridization barrier resulted from the failure of endosperm function. Thus, x*Brassicoraphanus* can be an ideal plant material to understand the endosperm-derived hybridization barriers.

Here, hybridization barrier was observed in newly synthesis F1 hybrid seeds of *B. rapa* and *R. sativus*. RNA-seq data from intergeneric hybrid endosperm mostly consisted of maternal reads from *B. rapa*, but not from *R. sativus*. Moreover, only the maternal genome, originated from *B. rapa*, was detected in x*Brassicoraphanus* endosperm, suggesting that intergeneric hybridization barrier prefers exclusive fertilization of egg cell in the embryo sac. In x*Brassicoraphanus*, the embryo was normally formed by fertilization, but endosperm was not. This study can provide opportunities for understanding endosperm-derived hybridization barrier between *B. rapa* and *R. sativus*.

LITERATURE REVIEWS

1. Hybridization barrier

In many crop species, outcrossing is manually conducted to produce hybrid crops that exhibit higher yield and disease resistance. In interspecific or interploidy hybridization, seed lethality or sterile progeny is a frequent problem (Coyne and Orr, 2004). Such phenomenon is called ‘hybridization barrier’. The interspecific and interploidy hybridization plays a vital role in evolution and agriculture, but the underlying mechanism of hybridization barriers remained unclear.

Hybridization barriers can be divided into two types, prezygotic and postzygotic barriers, depending on the timing of occurrence of fertilization. Prezygotic hybridization barrier prevents the sperm cell from fertilizing the egg cell and is associated with failures in pollen germination, pollen tube growth, and the penetration of pollen tube to the ovules. Postzygotic hybridization barrier reduces the viability or reproductive ability potential after successful fertilization associated with seed abortion and sterility of hybrids.

There are several mechanisms to explain the operation of postzygotic hybridization barrier. The Dobzhansky-Muller model proposed that incompatibilities in the hybrid offspring arise as a result of interactions between the genes that diverged in each of the species (Brideau et al., 2006). Alternatively, the “genomic imbalance” model provides an explanation for incompatibilities in hybrids associated with disruption of the balance between maternal and paternal genome dosages (Bikard et al., 2009; Bomblies et al., 2007). Another model is “genome shock”. In hybrids, the change of chromosomal organization or transposable element

activities evokes reconstructing of the genome and epigenome (Kirkbride et al., 2015; Madlung et al., 2002).

In hybrids between different species or different levels of ploidy, endosperm development is a major cause of seed abortion (Brink and Cooper, 1947; Haig and Westoby, 1991). Similar endosperm defects in interspecific and interploidy crosses suggest that a common mechanism induces the hybridization barrier. In many cases, hybrid embryo can be rescued *in virtro*, indicating that the failure of endosperm development appears to be crucial for the hybridization barrier (Rebernig et al., 2015). Endosperm consists of the ratio of two maternal and one paternal genomes (2m:1p), and is regarded as a dosage-sensitive tissue. Parental genome dosage imbalance of endosperm has a profound effect on seed size and seed viability (Gehring and Satyaki, 2017; Lafon-Placette and Köhler, 2016; Scott et al., 1998). Endosperm cellularization is a key transition during seed development (Hehenberger et al., 2012). Maternal genome excess is related to early timing of endosperm cellularization, while paternal genome excess shows delayed cellularization (Pennington et al., 2008; Scott et al., 1998). The effect of parental genome dosage also suggests that genomic imprinting, the phenomenon of parent-of-origin-specific gene expression, may be important for the establishment of hybridization barriers associated with the failure of endosperm development.

A relationship between imprinted genes and seed abortion in interploidy hybrids has been demonstrated (Huang et al., 2017; Kradolfer et al., 2013; Wolff et al., 2015). The mutations in the *ADMETOS* (*ADM*) gene, a paternally expressed imprinted gene (PEG) in endosperm, showed that cellularization of endosperm is partially restored in triploid seeds (Kradolfer et al., 2013). Paternal inheritance of

mutations of three PEGs - *SUVH7*, *PEG2*, and *PEG9* - also partially rescued triploid seed abortion with restoration of proper endosperm cellularization timing (Wolff et al., 2015). Although it is unclear how imprinted genes impact the interploidy hybrid seed abortion, these findings suggest that imprinted genes may play a substantial role in establishing postzygotic hybridization barriers (Lafon-Placette and Köhler, 2015).

2. Seed development

Angiosperms appeared about 200 million years ago and rapidly came to dominate (Doyle, 2012). Double fertilization is a unique feature of flowering plants reproduction. Two haploid sperm cells are delivered through the pollen tube to the female gametophyte. One sperm cell fertilizes the haploid egg cell to form the diploid embryo. Another sperm cell fertilizes the diploid central cell to form the triploid endosperm. Endosperm development that incurs double fertilization has important biological meanings, protection and nourishment of the embryo. The early seed consists of three major components - embryo, endosperm, and integument. These components have specialized roles for seed development.

During embryogenesis in *Arabidopsis*, the zygote shows asymmetric developmental patterns (Goldberg et al., 1994). The zygote divides into a small apical cell and a larger basal cell. The apical cell gives rise to the embryo that specifies the cotyledons, shoot meristem, and hypocotyl region. The basal cell forms a terminally differentiated organ, suspensor, connecting the embryo to the ovule. After a series of apical cell divisions, the globular stage embryo shows radial symmetry (Taiz and Zeiger, 2010). At the heart stage, the shoot apical meristem rapidly divides to form the cotyledon primordia. Further development of cotyledon

occurs at the torpedo stage. Mature seeds undergo dehydration and accumulate storage compounds. Endosperm supporting the developing embryo is closely associated with embryo development.

Endosperm development is divided into syncytial and cellular phases. Initial development entails a nuclear division without cellularization, resulting in the formation of syncytium around the central vacuole. After defined mitotic cycles, endosperm starts to cellularize and differentiate into three distinct parts with specific functions: the micropylar region surrounding the embryo, peripheral region composed of a layer of cell along the seed cavity, and the chalazal region locating at the junction to the maternal vascular system (Berger, 2003; Brown et al., 2003). Cellularization occurs in a wave from the micropylar pole to the chalazal pole (Sørensen et al., 2002). Endosperm cell walls initially developed in a tube-like structure, or alveolus (Olsen, 2004). The developmental transition of endosperm is a crucial step for seed development. Mutation of FIS-PRC2 components displayed endosperm cellularization failure, leading to seed abortion and embryo arrest (Chaudhury et al., 1997; Grossniklaus et al., 1998). The mutation of *ENDOSPERM DEFECTIVE 1* (*EDE1*) is associated with a nuclear proliferation defect during endosperm development and eventually causes embryo arrest as a direct consequence of endosperm cellularization failure (Hehenberger et al., 2012). During endosperm cellularization, the major sink in developing seed shifts from the central vacuole to the embryo with decrese in size of the central vacuole (Morley-Smith et al., 2008). In *fis2* and *ede1* mutants, failures of endosperm cellularization cause the central vacuole to maintain the major sink function, leading to the lack of sucrose transport to developing embryo (Hehenberger et al., 2012). Therefore, the right

timing of endosperm cellularization is crucial for seed development.

The seed coat develops from the maternally derived integuments while protecting the embryo and transferring nutrients to the embryo and endosperm from parental plants. After fertilization, the two layers of outer integument and three layers of inner integument differentiate. In *Arabidopsis*, the innermost layer (endothelium) accumulates proanthocyanidin flavonoid compounds, which later oxidize and develop brown color in the mature seed coat (Debeaujon et al., 2001). Other inner integument layers crush together without differentiation. During seed development, the two layers of outer integument accumulate starch-containing amyloplasts, followed by the production of a thickened wall and secretion of pectinaceous mucilage. Finally, these layers of outer integument die at maturity (Haughn and Chaudhury, 2005).

Embryo development independent of endosperm formation was reported in female gametophytic *glauce* (*glu*) mutants affecting central cell differentiation (Ngo et al., 2007). When *glu* mutants were fertilized with normal pollen, the embryo develops without the fertilization of central cell. Central cell failed to proliferate, arrested at late globular stage, and the *glu* mutant seeds eventually aborte. Similarly, *capulet 2* (*cap2*) mutant seeds showed embryo arrest at the late globular stage (Grini et al., 2002). In *cap2* mutants, the degree of endosperm development is variable from one syncytial division to precocious cellularization. However, embryo can develop to the globular stage. Taken together, these reports demonstrated that the globular stage embryo may serve as a checkpoint for seed development, especially when a defect in endosperm proliferation exists (Nowack et al., 2010).

3. Autonomous endosperm

During seed development, the FIS-PRC2 complex regulates central cell proliferation before and after fertilization (Köhler and Grossniklaus, 2002). Loss of FIS-PRC2 function causes autonomous endosperm development without fertilization and overproliferation of endosperm with fertilization, leading to seed abortion in both cases (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue and Fischer, 1999; Ohad et al., 1996). Unfertilized FIS-class mutant seeds show nuclear divisions of endosperm and the features of early endosperm development with embryo-like structures.

When *cdka;1* mutant pollen containing a single sperm cell is fertilized with wild type plants, a single sperm cell appeared to be exclusively fused with the egg cell (Nowack et al., 2006). Although the central cell is not fertilized, endosperm develops autonomously. Whereas FIS-PRC2 represses the central cell proliferation in the absence of fertilization, the phenotype of *cdka;1* mutant seeds suggests that autonomous endosperm development can be triggered by unknown positive signals from the fertilized embryo.

Autonomous endosperm is observed in both *cdka;1*-fertilized seeds and *FIS* class mutants. When *FIS*-class mutants are fertilized with *cdka;1* pollen, endosperm development is restored without the contribution of paternal genome and the fertilized embryo can develop (Nowack et al., 2006). These reports suggest that endosperm development is regulated by two signaling pathways: FIS-PRC2-dependent regulation of central cell proliferation and the positive signal from the fertilized embryo. Disruption of either two signaling pathways may cause autonomous endosperm formation. However, a mechanism in autonomous

endosperm development remains yet to be understood.

MATERIALS AND METHODS

Plant materials

Chinese cabbage (*B. rapa*) cv. Chiifu-401-42 (CF) and cv. Hagam50 (HG), and radish (*R. sativus*) cv. WK10039 (WK) and cv. KB-68 (KB) were used in this study. The seeds were sterilized and plated on Murashige & Skoog medium (Duchefa, Haarlem, The Netherlands). The plates were placed in the 24°C growth chamber with 16 hours of light and 8 hours of dark. Then, vernalization was conducted at 4°C with 16 hours of light and 8 hours of dark for 4 weeks. After vernalization, plants were moved to soil in pots and grown in a green house. For reciprocal and intergeneric crosses, designated female parents were emasculated and hand-pollinated. Reciprocal crosses of CF and HG and WK and KB were conducted. Intergeneric F1 hybrids were produced in CF pollinated with WK.

Seeds from four female plants were harvested at 14 days after pollination (DAP) to prepare four biological replicates. Endosperm and embryo were collected by hand dissection. Endosperm tissues in embryo were removed using sterile water to avoid tissue contamination. After dissection, samples were immediately frozen in liquid nitrogen prior to DNA and RNA extraction.

Light microscopy

Siliques and seeds after pollination were harvested and fixed in FAA solution (Formaldehyde, acetic acid and alcohol). Samples were dehydrated with an ethyl alcohol series, infiltrated with tert-butyl alcohol (TBA), and embedded in paraplast (Sigma Aldrich, MO, USA) using standard procedures (Berlyn and Miksche, 1976). Eight-micrometer sections were prepared with a microtome

(MICROM Lab., HM 340E, Germany). The sections were affixed to glass slides, deparaffinized and stained for 1 min with Toluidine blue O (Sigma Aldrich, MO, USA). Light microscopy were performed using photomicroscope (Carl Zeiss, Axiophot, Germany). Sections were digitally recorded using a microscope and measurements were performed on captured image using the ZEN 2012 Blue Edition Software (Carl Zeiss, Germany).

RNA extraction for RNA-seq

Total RNA was extracted from 14 DAP endosperm and embryo tissues with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with DNase I treatment following the manufacturer's protocols. Approximately 5 μg of total RNA from endosperm and embryo was used for RNA-seq library construction. mRNA libraries were prepared using TruSeq adapter (Illumina, CA, USA) and sequenced on Illumina NovaSeq with the 101 bp paired end sequencing method at Macrogen (http://dna.macrogen.com).

SNP calling

From unpublished genomic DNA sequencing data of HG and KB, low quality reads were filtered with manual code (Q<20, P<70) and trimmed out using a program Trimmomatic v0.38 (Bolger et al., 2014). The filtered reads were aligned to the reference Chinese cabbage genome (Zhang et al., 2018) and reference radish genome (Jeong et al., 2016) with BWA-MEM (Li, 2013) v0.7.17 by default parameters. SNPs between CF and HG, and WK and KB were discovered with Genome Analysis Toolkit (McKenna, 2010) HaplotypeCaller v3.6-0. The filtering

settings for SNPs were QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, and SOR > 4.0. The SNP calling was performed twice for Base Quality Score Recalibration. Variant filtration was performed with manual script based on read depth.

Identification of imprinted genes

RNA-sequencing reads from embryo and endosperm of each reciprocal crosses were distinguished based on allele-specific SNPs between their parental plants. Low quality reads were filtered with manual code (Q<20, P<70) and trimmed out with Trimmomatic v0.38 (Bolger et al., 2014). Filtered reads were mapped to both parental genomes with tophat v2.0.9 (Trapnell et al., 2009) with no mismatches to calculate the allele-specific read numbers. RNA-seq reads were also aligned to N-masked genome at SNP positions to observed overall expression level of genes, and allele-specific expression levels were used to calculate the ratio of maternal (m) to paternal (p) alleles, 2m:1p and 1m:1p in endosperm and embryo, respectively. The ratios of 2m:1p and 1m:1p expression were set as a null-hypothesis and binominal one-side tests were conducted. Joint P-values were calculated by P = max ($p1$, $p2$)[2], using the two P-values from two reciprocal crosses $p1$ and $p2$. After calculating the false-discovery rate (FDR) as $q = P * n/i$, where n was the overall number of joint P-values and i was the rank of a given P-value. The genes (q-value < 0.01) were selected as maternally expressed imprinted genes (MEGs) and paternally expressed imprinted genes (PEGs), respectively. MEGs and PEGs were defined as displaying 8m:1p and 2m:4p ratio, respectively.

DNA extraction and SCAR marker analysis

Total gDNA extracted from intergeneric endosperm by Cetyl-trimethylammonium bromide (CTAB) extraction method was subjected to SCAR marker analysis. The presence of *B. rapa* and *R. sativus* genome in isolated endosperm and embryo was confirmed by sequence characterized amplified region (SCAR) markers, DG3216 and DG 3217 for *B. rapa* (Br-SCAR) and DG3207 and DG3208 for *R. sativus* (Rs-SCAR) (Table 1). PCR amplification was performed with 35 cycles of 95℃ for 30 sec, 64℃ for 30 sec and 72℃ for 1 min. The PCR products at 23, 27, 31, and 35 cycles respectively were separated on a 1% agarose gel. For duplex PCR, PCR amplification was performed with both Br-SCAR and Rs-SCAR marker primers and confirmed at products at 23, 27, 31, and 35 cycles respectively.

Table 1. List of primers

Name	Sequence (5'→3')	Purpose
DG3207	GCTTTCTGTCCACAGCCAATGCTG	SCAR marker
DG3208	CATACAATGTTACCAGAGTGGGTGCTTTC	SCAR marker
DG3216	GAGCTCATGCATTATGGGAATATGGTTCTG	SCAR marker
DG3217	GAACTACCACCCTCGTGTCCGTTC	SCAR marker

RESULTS

Phenotypic analysis of intergeneric hybrid seeds

Siliques of intergeneric F1 hybrid of *B. rapa* and *R. sativus* were observed under the light microscopy at 14 DAP (Figure 1A). Intergeneric cross resulted in almost underdeveloped and aborted seeds. Three types of seeds were observed in the F1 siliques – swollen, underdeveloped, and aborted seeds (Figure 1B). Swollen seeds were superficially nearly identical to self-pollinated seeds of *B. rapa*. Compared to swollen seeds, developed embryo were not present in underdeveloped seeds. This observation suggests that seed development might take place after fertilization but abruptly arrest at early stages. Aborted seeds were small in size and turned brown and collapsed. Siliques of intraspecific crosses, CF x HG and HG x CF, contained about 70% non-aborted seeds (Table 2). Siliques of intergeneric hybrid of *B. rapa* and *R. sativus* contained 15.7% swollen seeds, 72.5% underdeveloped seeds, and 11.8% aborted seeds. However, reciprocal intergeneric crosses between *R. sativus* and *B. rapa* only produced aborted seeds. It was reported that When *Capsella rubella* was pollinated with *C. grandiflora*, F1 hybrid seeds aborted accompanied with the failure of endosperm cellularization, whereas a reciprocal cross was able to develop seeds with cellularized endosperm (Rebernig et al., 2015). Consistent with the previous reports, seed development of F1 hybrids of *B. rapa* and *R. sativus* revealed a cross direction-dependent.

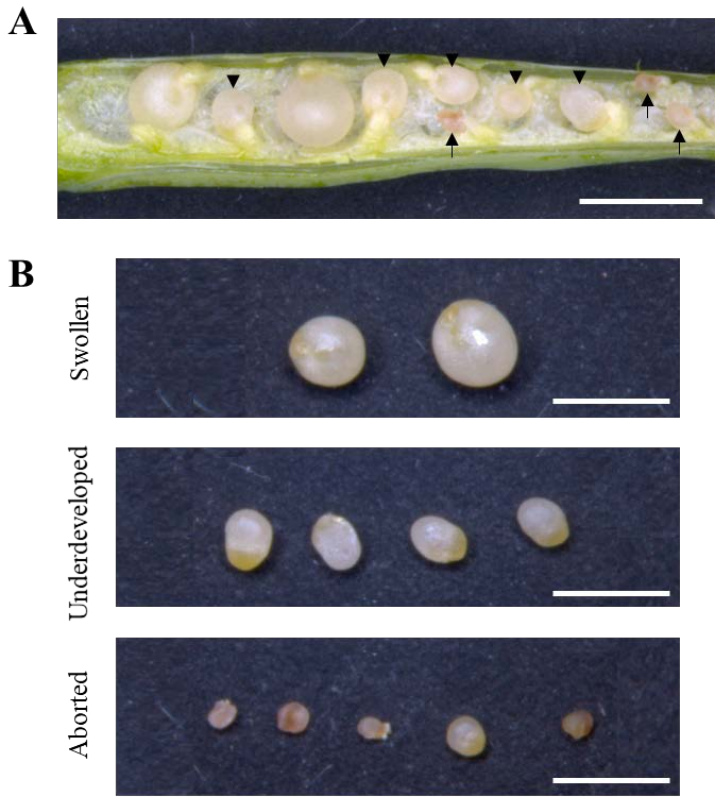

Figure 1. Seed development of intergeneric F1 hybrid between *B. rapa* and *R. sativus*.

(A) Silique resulting from intergeneric hybrids. Black arrows and arrowheads represented aborted and underdeveloping seeds, respectively. Scale bar = 4 mm. **(B)** Types of seed development in intergeneric hybrids. Scale bars = 2mm.

Table 2. Percentage of aborted seeds in intergeneric hybrids

Parental genotype (female x male)	Swollen (%)	Aborted (%)	Underdeveloped (%)	The number of seed (n)
CF x HG	65.7	34.3	-	446
HG x CF	75.4	24.6	-	646
CF x WK	11.8	15.7	72.5	51
WK x CF	0	100	-	39

Embryo and endosperm development in intergeneric hybrids

After double fertilization, endosperm initially develops as syncytium, undergoing nuclear division without cellularization. After the syncytial phase, the endosperm starts to cellularize (Berger, 2012). Normal seed development of *B. rapa* (CF), *R. sativus* (WK), and the intergeneric F1 hybrid was observed at 14 and 19 DAP (Figure 2). The endosperm of *B. rapa* and *R. sativus* became cellularized at 14 DAP (Figure 2A and 2C), whereas the endosperm of intergeneric F1 hybrid was uncellularized (Figure 2B). At 19 DAP, the seeds of *B. rapa* and *R. sativus* contained the bent cotyledon stage embryos with cellularized endosperm (Figure 2D and 2F). As endosperm cellularization proceeded, endosperm nuclei were visible within the cell in *B. rapa* (Figure 3A). Although some F1 hybrid seeds superficially appear normal, the embryo was arrested at the torpedo stage with uncellularized endosperm at 19 DAP (Figure 2E and 3B). Although intergeneric F1 seeds showed uncellularized endosperm, F1 seeds at 14 DAP in all crosses contained early torpedo-stage embryos (Figure 4). These results indicated that an endosperm-derived hybridization block operated in intergeneric hybrids of *B. rapa* and *R. sativus*.

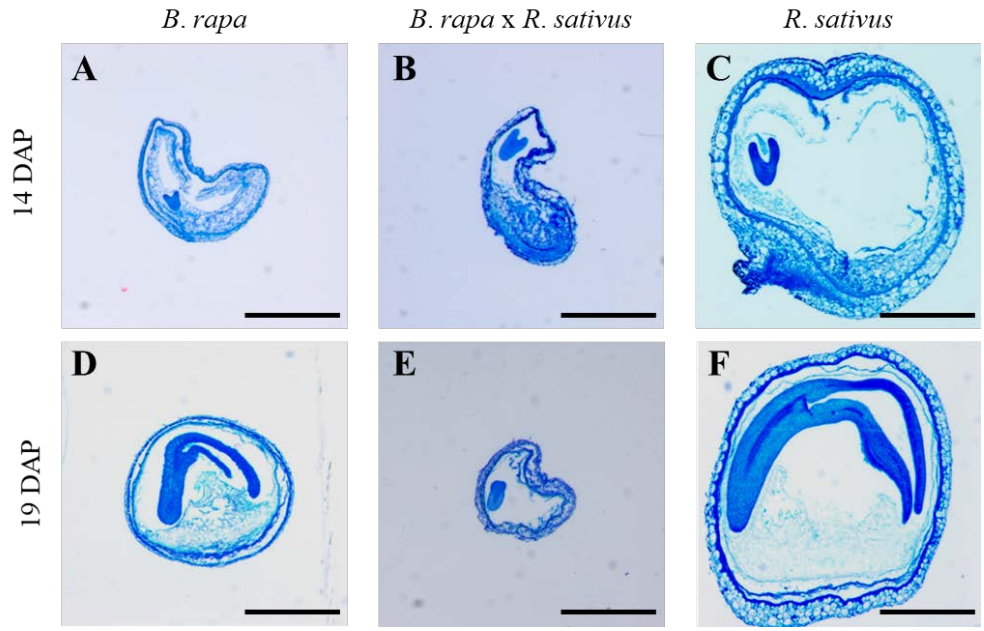

Figure 2. Seeds structure of *B. rapa, R. sativus* and their intergeneric F1 hybrid.

Sections of *B. rapa* (A and D), intergeneric F1 (B and E), and *R. sativus* (C and F) seeds at 14 and 19 DAP. Scale bars = 1 mm.

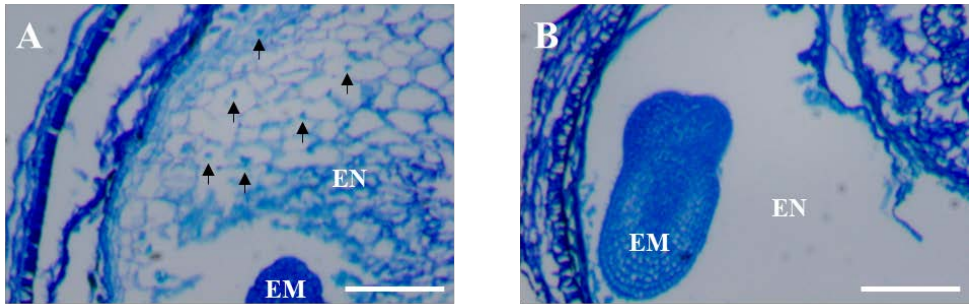

Figure 3. Endosperm development in *B. rapa* and intergeneric F1 hybrid.

Endosperm cellularization in 19 DAP seeds of *B. rapa* and the intergeneric F1 hybrid (A and B). **(A)** Endosperm was cellularized. Black arrows mark cellularized endosperm nuclei. **(B)** Endosperm was uncellularized with arrested embryo at torpedo stage. Scale bars = 0.1 mm. EN = Endosperm; EM = Embryo.

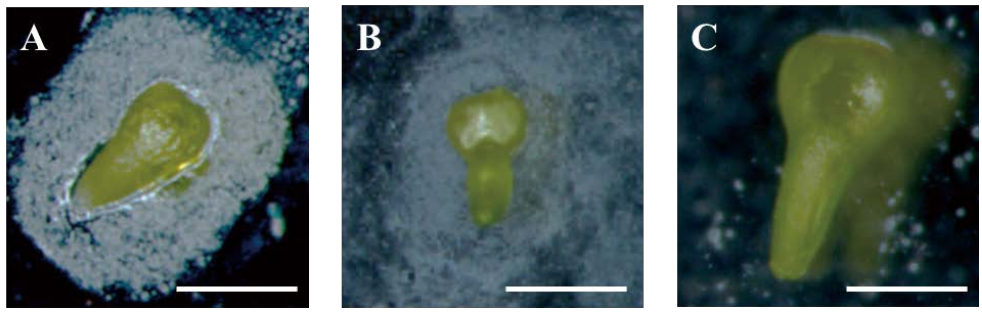

Figure 4. Embryo in *B. rapa* and *R. sativus* and their intergeneric F1 seeds.

Seeds contained early torpedo stage embryos in *B. rapa*, intergeneric hybrid, and *R. sativus* (A, B, and C). Scale bars = 0.5 mm.

Identification of imprinted loci in endosperm and embryo

A relationship between the imprinted genes and a hybridization barrier has been demonstrated (Huang et al., 2017; Kradolfer et al., 2013; Wolff et al., 2015). To analyze intergeneric hybridization barrier in relation to the regulation of imprinted genes, RNA-seq was conducted on endosperm and embryo tissues from *B. rapa* (CF and HG), *R. sativus* (WK and KB), and F1 hybrids between *B. rapa* (CF) and *R. sativus* (WK). Reciprocal crosses between the two cultivars of *B. rapa* (CF and HG) and *R. sativus* (WK and KB) were performed. At the same time, an intergeneric cross between *B. rapa* (CF) and *R. sativus* (WK) was also performed. Developing endosperm and embryo tissues (14 DAP) from all crosses were collected. Total RNA was extracted and then cDNA libraries of four endosperms and four embryos from each cross were constructed, yielding a total of 220 million 101-bp paired-end reads (~8 Gb) each from endosperm and embryo.

In endosperm, a two maternal (m) to one paternal (p) allelic-specific expression ratio 2m:1p is expected. In embryo, 1m:1p allelic-specific expression ratio is expected. The expected ratios in endosperm and embryo sets were subjected to a null hypothesis of gene expression. The list of candidate imprinted genes was obtained with a stringent cut-off standard (for MEGs 8m:1p of the read ratio; for PEGs 2m:4p of the read ratio). The process of detection of imprinted genes is summarized in Figure 5. The ratio of maternal to total expression was used to select imprinted genes in endosperm and embryo (Figure 6). In *B. rapa*, 829 putative imprinted genes in endosperm (823 MEGs and 6 PEGs) and 8 MEGs in embryo were detected. In *R. sativus*, 309 putative imprinted genes in endosperm (241 MEGs and 68 PEGs) but no imprinted genes in the embryo were detected. However, the

proportion of maternal to paternal reads in *B. rapa* endosperm showed mostly maternal allelic-specific expression compared to *R. sativus* endosperm (Figure 6A). This result suggests the possibility that endosperm tissues of *B. rapa* are contaminated with maternal tissues such as seed coat and integument.

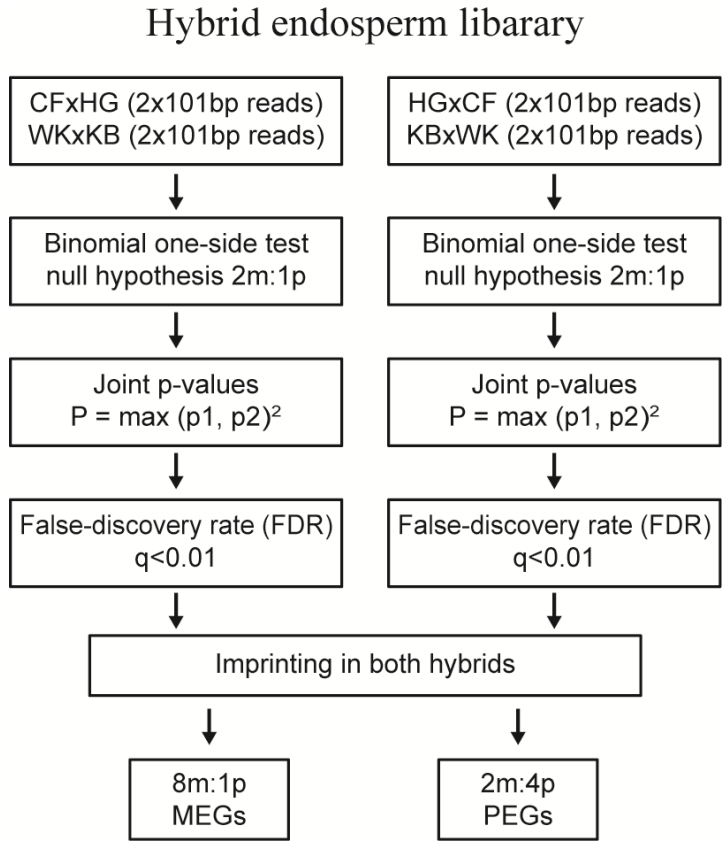

Figure 5. Flowchart for the detection of imprinted genes in *B. rapa* and *R. sativus* endosperm.

Allele-specific SNPs between the parental plants were used to calculate the ratio of maternal to paternal alleles (2m:1p) in endosperm. Binominal one-side tests against the null-hypothesis of 2m:1p expression were conducted. Joint P-values were calculated by $P = \max(p1, p2)^2$. After calculating the false-discovery rate (FDR), the genes (q-value < 0.01) were selected as MEGs and PEGs. MEGs and PEGs were defined as having 8m:1p and 2m:4p ratio, respectively.

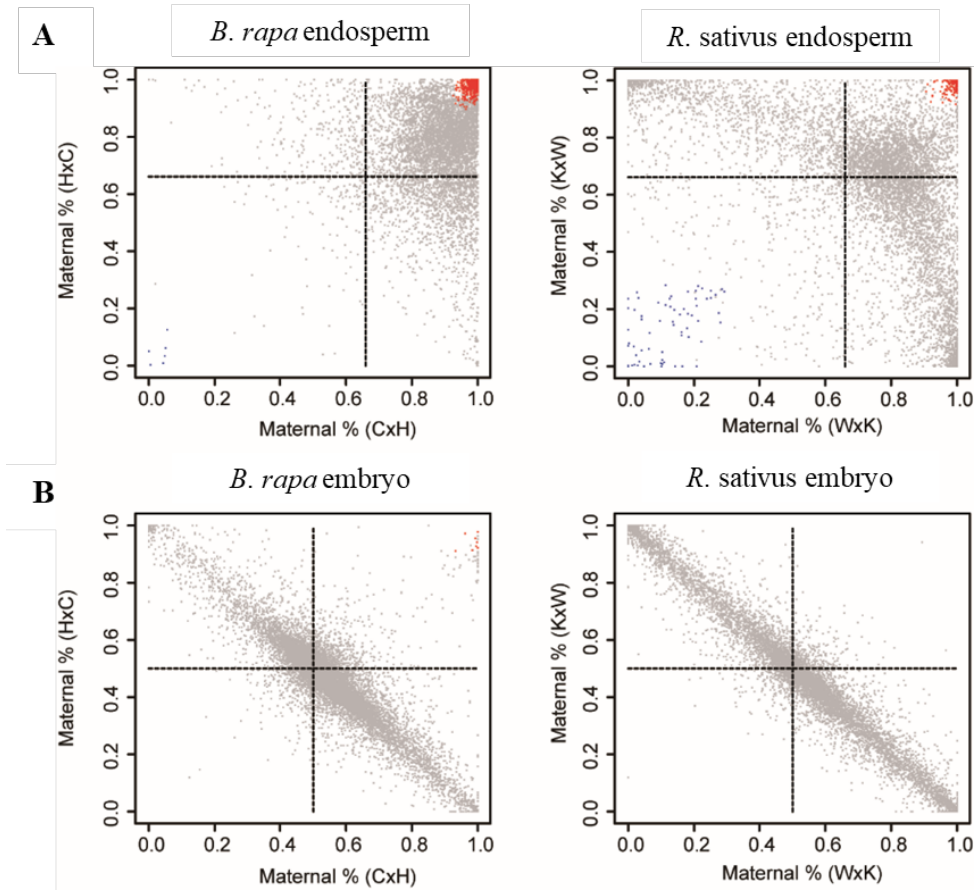

Figure 6. RNA sequencing and identification of imprinted genes in *B. rapa* and *R. sativus*.

(A, B) Scatter plots of the ratio of maternal to total read depths for endosperm **(A)** and embryo **(B)**. The red and blue dots represent MEGs and PEGs, respectively. C = CF; H = HG; W = WK; K = KB.

Double fertilization is disrupted by intergeneric hybridization barrier

While the intergeneric hybrid seeds showed about 1m:1p allelic-specific expression ratios in the embryo, mostly the maternal reads originated from *B. rapa* (>99%) were detected in intergeneric F1 hybrid endosperm (Figure 7). Thus, imprinted genes in intergeneric hybrids cannot be analyzed using this RNA-seq data.

To examine the origin of genome of intergeneric endosperm, Br-SCAR and Rs-SCAR markers were used to specifically amplified *B. rapa* and *R. sativus* sequences, respectively (Figure 8A). The sizes of PCR products were 789 bp for *B. rapa* and 883 bp for *R. sativus*, respectively. A duplex PCR was conducted to assess genome dosage in intergeneric endosperm using the SCAR markers (Figure 8B). While the *B. rapa*-specific bands from intergeneric endosperm were observed at 23 PCR cycles, no *R. sativus*-specific amplification was observed. At least, the *R. sativus* specific PCR products were observed at 31 PCR cycles.

This indicates that the intergeneric F1 hybrid seed contained the endosperm tissue exclusive to the maternal parent, whereas the embryo consisted of zygotic tissues derived from both parents. This also suggests the possibility that fertilization of egg and sperm normally occurs but the central cell in the embryo sac does not fuse with sperm, resulting in diploid endosperm, instead of triploid, presumably by developing autonomous endosperm without the contribution of paternal genome. Such autonomous diploid endosperm is reported in *Arabidopsis* seeds, where *cdka;1* mutant pollen with a single sperm is pollinated with the PRC2 mutant type stigma (Nowack et al., 2007).

This finding strongly suggests that endosperm arrest due to the absence of fertilization of the central cell prevents normal embryo and seed development,

serving as a primary hybridization barrier.

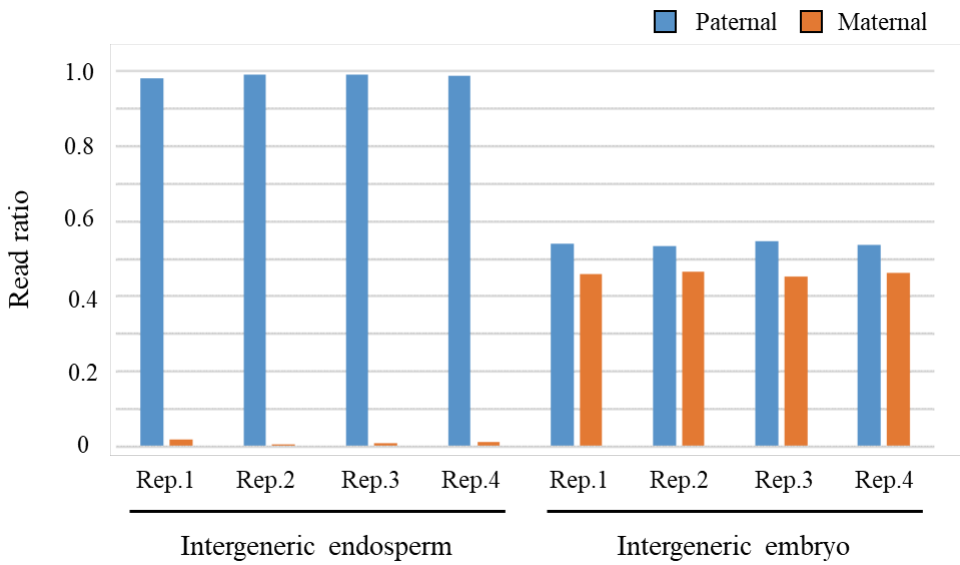

Figure 7. RNA-sequencing read ratio of endosperm and embryo in intergeneric hybrid of *B. rapa* and *R. sativus*.

RNA-seq was conducted on four endosperm and embryo tissues from intergeneric hybrid at 14 DAP. Rep = replicate.

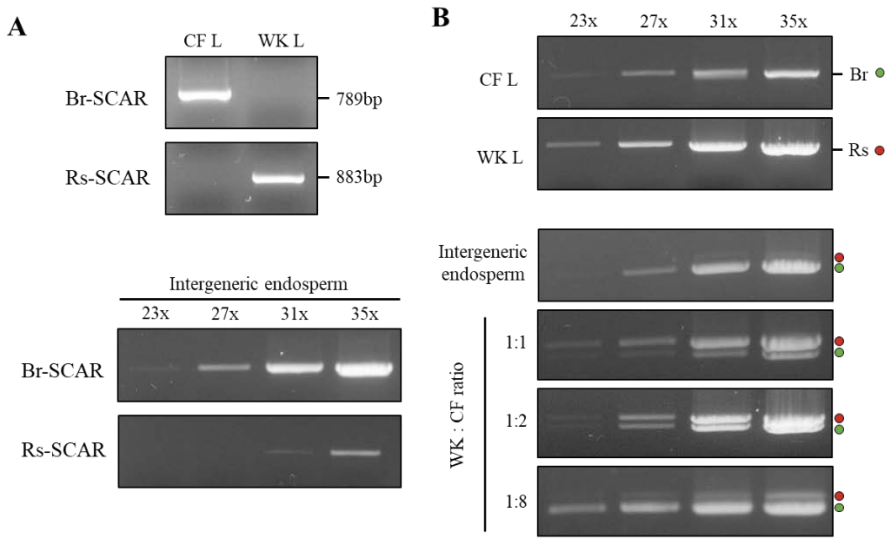

Figure 8. Detection of *B. rapa* and *R. sativus* sequences in intergeneric endosperm by SCAR markers.

(A) SCAR marker analysis of intergeneric endosperm. *B. rapa* and *R. sativus* genomes were amplified with 23, 27, 31, and 35 cycles. L = leaf. **(B)** Duplex PCR with Br-SCAR and Rs-SCAR markers. Green and Red circles indicate PCR products from *B. rapa* and *R. sativus* genome respectively.

Discussion

In interspecific or interploidy hybridization, seed abortion is a frequent problem. Newly synthesized x*Brassicoraphanus* hybrids between *B. rapa* and *R. sativus* showed seed abortion with embryo arrest and endosperm defect. Histological analysis revealed that the intergeneric F1 embryo was arrested at torpedo stage with uncellularized endosperm, suggesting the endosperm-derived hybridization barrier (Figure 2 and 3). To examine the relationship between imprinted genes and hybridization barriers (Huang et al., 2017; Wolff et al., 2015), RNA-seq on endosperm tissue was conducted. RNA transcripts from intergeneric endosperm mostly consist of maternal genome derived from *B. rapa* (Figure 7). In addition, SCAR marker analysis showed that genome of intergeneric endosperm consists of *B. rapa* genome, not *R. sativus* (Figure 8). These results suggest that endosperm-derived hybridization barrier between *B. rapa* and *R. sativus* is likely due to exclusive fertilization of egg cell, not to misregulation of imprinted genes. This finding indicates the possibility that endosperm was autonomously developed without contribution of paternal genome.

Immature F1 embryo from a cross between maternal *B. rapa* and paternal *R. sativus* could be rescued *in vitro*. However, all seeds aborted in reciprocal cross without viable embryos. Such differential hybridization barrier effect was also observed in other plants (Rebernig et al., 2015). When self-pollinating species, *Capsella rubella* pollinated with outcrossing species, *C. grandiflora* produces, hybrid seeds that abort due to the failure of endosperm cellularization. By contrast, reciprocal cross makes small viable seeds with precociously cellularized endosperm

(Rebernig et al., 2015). Based on the "weak inbreeder/strong outbreeder" (WISO) hypothesis, pollen from strong outcrossing species can fertilize ovules of weak self-pollinating species (Brandvain and Haig, 2005). In x*Brassicoraphanus*, however, *B. rapa* with high self-incompatibility is the female suggesting that some other factors may affect hybridization barrier.

In most angiosperms, two sperm cells are isomorphic, except *Plumbago zeylanica*. In *P. zeylanica*, a generative cell was asymmetrically divided, producing two dimorphic sperm cells (Russell, 1985). The central cell was preferentially fertilized with a larger sperm cell containing more mitochondria, and the egg cell was fertilized with a smaller sperm cell. In *Arabidopsis*, such preferential fertilization is not observed, and both isomorphic sperm cells fertilize either an egg cell and the central cell (Hamamura et al., 2011). After sperm cells were delivered to the female gametophyte, how male and female gametes interact each other remains an issue of debate. Using an *in vivo* assay, *Arabidopsis* GAMETE EXPRESSED 2 (GEX2) was shown to be a protein localized to the sperm cell surface, which is required for both sperm-egg cell and sperm-central cell attachments (Mori et al., 2014). The function of GEX2 in hybridization compatibility between *A. thaliana* and *A. lyrata* is crucial, where the filamin-repeat domain of GEX2 might confer hybridization barrier. Molecular divergence in GEX2 suggests that the filamin-repeat domain is rapidly evolving to mediate species-specific gamete attachment (Mori et al., 2014). Further investigation on unfertilized endosperm in intergeneric hybrids will shed light on the mechanism of provide the clue how sperm and the central cell communicate each other.

The fertilized embryo of intergeneric F1 seed between *B. rapa* and *R.*

sativus is likely to promote the central cell proliferation even in the absence of central cell fertilization. In *Arabidopsis*, *cdka;1* mutants displayed exclusive fertilization of egg cell with a single sperm cell, triggering autonomous endosperm development (Nowack et al., 2006). Initiation of central cell proliferation induced by fertilized embryo may indicate the positive signal from a developing embryo. Mutations in the FIS-PRC2 components such as *MEA*, *FIE*, and *FIS2* also cause autonomous endosperm without fertilization and overproliferation of endosperm with fertilization (Chaudhury et al., 1997; Grossniklaus et al., 1998). The function of FIS-PRC2 is the block of central cell proliferation before fertilization. When FIS-class mutants were fertilized with *cdka;1* pollen, endosperm development was restored without the contribution of paternal genome and fertilized embryo developed (Nowack et al., 2007). These findings suggested that early seed development was regulated by two opposite mechanisms, the block of proliferation by PRC2 and a positive signal from the fertilized embryo (Nowack et al., 2006). Consistent with previous reports (Nowack et al., 2006; Nowack et al., 2007), intergeneric hybrids between *B. rapa* and *R. sativus* also developed autonomous endosperm presumably triggered by the fertilized embryo.

Using *cdka;1* mutant pollen, the variations of autonomous endosperm development was reported in different *Arabidopsis* accessions (Ungru et al., 2008). In Sha accession, the central cell contains only a single without nuclear divisions when pollinated with *cdka;1* mutant pollen. Although the central cell did not proliferate in this case, the embryo can reach the globular stage. Some hybrid seeds between *B. rapa* and *R. sativus* contained early torpedo stage embryos (Figure 4B). The embryo developing into the early torpedo stage even with endosperm defects in

intergeneric hybrid seeds is reminiscent of the embryo fertilized with *cdka;1* mutant pollen.

Through RNA-seq analysis, I identified 829 and 309 putative imprinted genes from *B. rapa* and *R. sativus* endosperm. 241 MEGs and 6 PEGs in *R. sativus* endosperm are similar to the number of imprinted genes reported in the previous study in *Arabidopsis* (Gehring et al., 2011). The number of MEGs in *B. rapa* endosperm is larger than that of MEGs in related species. If two maternal alleles and one paternal allele are equivalently expressed, the RNA-seq data from endosperm tissues would theoretically show 2m:1p allelic-specific expression. However, the proportion of maternal to paternal reads in *B. rapa* endosperm showed almost maternal allelic-specific expression (Figure 6). This results can be explained by RNA contamination of maternal tissues, such as seed coat and integument. Although the effect of RNA contamination can be eliminated by filtering of maternal tissue contaminated genes, the possibility of RNA contamination remains a concern (Gehring, 2013).

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초 록

지구상의 많은 식물들은 종간이나 속간 교잡을 통하여 많은 종으로 분화가 되어 왔다. 일반적으로 교배 전 장벽 (Prezygotic hybridization barrier)과 교배 후 장벽 (Postzygotic hybridization barrier)에 의해 새로운 식물체를 얻는 것은 매우 힘들다고 알려져 있다. 특히 교배 후 장벽은 접합자 형성 이후 잡종의 발달과정이나 불임 등으로 생식적 격리가 일어나는 것을 말한다. 속간이질사배체 식물인 '배무채' (x*Brassicoraphanus*)는 배추 (*Brassica rapa* L.)와 무 (*Raphanus sativus* L.) 사이에서 성공적으로 교잡되었다. 하지만, 배배양 (embryo rescue)을 통해서만 새롭게 배무채를 교잡할 수 있고, 이는 배유의 비정상적인 발달이 관련되어 있음을 시사한다. 본 연구에서는 배추와 무 사이의 이속간 교배에 있어서 배유 유래 교배 장벽을 연구하였다. 초기 배추와 무의 교잡 종자의 발달과정 관찰을 통해서 정상적인 배의 발달에 비해, 배유는 정상적으로 발달하지 못함을 확인하였다. 배유 유래 교배 장벽에 있어서 유전자 각인 현상이 관여한다는 최근 연구를 토대로 교잡 종자 배유의 전사체 분석을 하였다. 그 결과 모계의 전사체만을 확인하였다. 또한 SCAR 마커를 이용하여 교잡 종자의 배유에는 모계 유전체만이 존재함을 확인하였다. 정세포 (sperm)가 중심세포 (central cell)와는 수정되지 않고 난세포 (egg cell)와만 수정이 되어도 일시적인 배유 발달이 진행된다고 알려져 있다. 이와 비슷하게 배추와 무의 교잡

종자에서 수정된 배에 의해 일시적으로 미수정된 배유가 발달하는 현상을 보이고, 이후에는 정상적인 종자 발달을 하지 못하는 것으로 생각된다. 따라서 배추와 무의 이속간 교배에서는 배유의 미수정에 따른 교배장벽이 존재하는 것으로 추정된다.

주요어: 배무채, 십자화과, 배유, 교배장벽, 유전자 각인

학번: 2017-22300