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Endosperm development and seed viability in *Arabidopsis* and x*Brassicoraphanus*

애기장대와 배무채에서의 배유발달과

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ABSTRACT

Interspecific or intergeneric hybridization provides a crucial source of evolution in nature. However, such evolutionary driving forces are prevented by hybridization barriers, among which postzygotic barriers involve hybrid inviability such as abnormal embryo/endosperm development and hybrid sterility. x*Brassicoraphanus*, a newly synthesized hybrid between a chinese cabbage (*Brassica rapa*) and a radish (*Raphanus sativus*), displays seed abortion which is a common type of postzygotic barriers. To examine developmental defects in the hybrid, we examined seed development of x*Brassicoraphanus* and several *Arabidopsis* mutants defective in endosperm development, *medea* (*mea*), *demeter* (*dme*), *a-type cyclin dependent kinase* (*cdka;1*), and *f-box-like 17* (*fbl17*). The histological analysis of x*Bassicoraphanus* seeds revealed that a newly synthesized hybrid was successfully fertilized but exhibited an endosperm cellularization failure which is similar to

endosperm defects in *Arabidopsis* mutants *dme* and *mea*. In order to overcome seed inviability caused by abnormal endosperm, we performed the *in vitro* culture of embryos surrounded by aberrant endosperm and successfully rescued viable plantlets. This work suggests that a developmental defect of endosperm may contribute to the postzygotic hybridization barrier to prevent a cross between unrelated species.

Key words: x*Brassicoraphanus*, Endosperm, Hybridization barrier, Abnormal endosperm, *in vitro* culture.

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LIST OF ABBREVIATIONS

DME	DEMETER
MEA	MEDEA
FIS	FERTILIZATION INDEPENDENT SEED
PRC2	Polycomb repressive complex 2
CDKs	Cyclin-depedent kinases
CDKA;1	A-type CDK
FBL17	F-box-Like 17
SCF	SKP1–Cullin1–F-box protein
SCF^{FBL17}	SCF E3 ubiquitin ligase complex
B. rapa	*Brassica rapa*
R. sativus	*Raphanus sativus*
C. rubella	*Capsella rubella*
C. grandiflora	*Capsella grandiflora*
MEGs	Maternally expressed genes
PEGs	Paternally expressed genes
FWA	*FLOWERING WAGENINGEN*
BDMIs	Bateson–Dobzhansky–Muller incompatibilities

WISO	Weak inbreeder/strong outbreeder
EDE1	*ENDOSPERM DEFECTIVE 1*
JAS	*JASON*
PHE1	*PHERES 1*
ADM	*ADMETOS*
FIS-PRC2	FIS-polycomb repressive complex 2
FIE	*FERTILIZATION INDEPENDENT ENDOSPERM*
CF	Chiifu-401-42
HG	Hagam50
WK	WK10039
TDW	Triple distilled water
Col	Columbia
Ler	Landsberg *erecta*
MS	Murashige and Skoog medium
TBA	Tert-butyl alcohol
DIC	Differential interference contrast
SSLP	Simple sequence length polymorphism
gDNA	Genomic DNA
DAP	Days after pollination

CTAB	Cetyl-trimethylammonium bromide
RT-PCR	Reverse transcription-PCR
cDNA	Complementary DNA
CDS	Coding DNA sequence
UBC9	*Ubiquitin 9*
ICM	*In vitro* culture medium
CV	Central vacuole
EM	Embryo
ISC	Inner seed coat
MCE	Micropylar endosperm
PEN	Peripheral endosperm
SNP	Single nucleotide polymorphism
EN	Endosperm

INTRODUCTION

Interspecific or intergeneric hybridization has been a valuable source for evolution and plant breeding. However, such driving forces are prevented by hybridization barriers, which reduce the opportunity of propagation or cause infertile offspring between two different species. Depending on the timing of fertilization events, hybridization barriers are divided into prezygotic and postzygotic barriers. Prezygotic barriers block fertilization between an egg cell in the embryo sac and a sperm cell from a pollen grain. Postzygotic barriers occur when a zygote is generated, inducing low viability of hybrids or sterile progeny from the crosses. In interspecific or intergeneric crosses, the major factor of postzygotic hybridization barrier is abnormal development of reproductive tissues such as endosperm and embryo (Teng et al., 2006).

During double fertilization in angiosperms, one haploid sperm cell fuses with a haploid egg cell to generate the diploid embryo, and the other haploid sperm cell fertilizes a diploid central cell to make the triploid endosperm which provides nutrients to the embryo (Berger et al., 2008). Endosperm develops from an initial syncytial phase through the later cellular phase. At the syncytial phase, endosperm undergoes thousands of free-nuclear divisions without cytokinesis, resulting in the single-celled syncytium (Boisnard-Lorig et al., 2001; Olsen, 2004). At the cellular phase, the syncytium differentiates micropylar, peripheral, and chalazal regions and

undergoes cellularization except at the chalazal pole, which leads to the organization of the endosperm cell wall (Brown et al., 1999).

Endosperm is a dosage-sensitive tissue containing two maternal and one paternal genomes. Due to parental dosage imbalance, endosperm may develop precociously with a decreased number of nuclear cells leading to seed abortion or proliferate excessively with delayed cellularization along with an arrested embryo (Scott et al., 1998; Bushell et al., 2003). Thus, synchronization between endosperm and embryo development is closely associated with seed viability (Lafon-Placette and Kohler, 2014). For endosperm development, epigenetic mechanisms such as DNA methylation and histone modification are important (Lafon-Placette and Kohler, 2016). In the central cell, DEMETER (DME) DNA glycosylase that excises 5-methylcytosine from DNA activates the maternal allele of *MEDEA* (*MEA*), which is a component of the FERTILIZATION INDEPENDENT SEED (FIS)-polycomb repressive complex 2 (PRC2) (Gehring et al., 2006). The FIS-PRC2 regulates early endosperm development and cellularization, and its loss of function causes abnormal endosperm and seed abortion (Chaudhury et al., 1997; Grossniklaus et al., 1998).

Endosperm development also requires a functional male gametophyte produced by precise cell divisions during pollen mitosis, which is essential for the production of two haploid sperm. Cell divisions are regulated by cyclin-dependent kinases (CDKs), and only the A-type CDK

(CDKA;1) exists in *Arabidopsis* (Hemerly et al., 2000). In *cdka;1* mutants, the pollen contains only one sperm cell which exclusively fertilizes an egg cell, showing autonomous endosperm triggered by positive signals from the embryo (Nowack et al., 2006). Moreover, F-box-Like 17 (FBL 17), a component of an SKP1–Cullin1–F-box protein (SCF) E3 ubiquitin ligase complex (SCF^{FBL17}), targets CDK inhibitors, and the *fbl17* mutant displays a similar phenotype to the *cdka;1* mutant (Gusti et al., 2009).

Despite a variety of barriers in synthesizing new intergeneric hybrids, a genetically stable intergeneric allotetraploid between Chinese cabbage (*Brassica rapa*) and radish (*Raphanus sativus*), called 'Baemoochae' (x*Brassicoraphanus*), was successfully generated (Lee et al., 1989; Lee et al., 2011). However, the newly synthesized x*Brassicoraphanus* shows seed lethality and can survive only when the fertilized immature ovule is cultured *in vitro*. Considering that a defect in endosperm development is a major cause of hybridization barrier in intergeneric crosses (Dinu et al., 2004; Roy et al., 2011), the newly synthesized hybrid may have endosperm defects and such endosperm-based barriers can be overcome by *in vitro* culture.

In this study, the seed development in the newly synthesized intergeneric hybrids between *B. rapa* and *R. sativus* was carefully examined and compared with *Arabidopsis* mutants that have a defect in endosperm development such as *mea*, *dme*, *cdka;1*, and *fbl17*. It was shown that the newly synthesized hybrid generally underwent a failure in endosperm

cellularization and the embryo eventually arrested. The presence of both maternal (*B. rapa*) and paternal (*R. sativus*) DNA in newly synthesized x*Brassicoraphanus* endosperm indicates that a developmental defect is post-zygotic after normal double fertilization. Expression analysis also revealed that both genomes equally contribute to transcription in the x*Brassicoraphanus* endosperm. In an attempt to rescue seed abortion accompanied with abnormal endosperm development, the *in vitro* culture was conducted in *Arabidopsis* seeds of heterozygous *dme* and *mea* mutants. Successful recovery of homozygous *dme* mutant plantlet indicates that the embryo arrest and seed abortion is mainly caused by abnormal endosperm development and that the in vitro culture is a promising technique to rescue the arrested embryo within the mutant seed with a defect in endosperm.

LITERATURE REVIEWS

1. Hybridization barriers

Interspecific or intergeneric hybrids have great values in many useful traits such as disease resistance and high yield. Interspecific or intergeneric hybridization is evolutionary important for angiosperm species diversity (Soltis and Soltis, 2009; Jiao et al., 2011). Although there have been numerous attempts to generate interspecific or intergeneric hybrids, most failed to produce viable seeds in various crops. Such phenomena in which hybrid individuals of different species fail to produce progenies or fertile offspring are called the 'hybridization barrier'. However, the mechanism of hybridization barrier remains elusive, despite its significant agricultural value.

Hybridization barriers are divided into prezygotic and postzygotic barriers according to the timing of fertilization. Prezygotic barriers prevent an egg cell from being fertilized with a sperm cell in geographical, temporal, mechanical, and gametic manners. The habitat separation of ancestral species may affect the adaptation of offspring to different environments (Wiens, 2004). *Ceanothus cuneatus* and *Ceanothus roderickii* which live in different habitats cross infrequently even though they are genetically similar (Burge et al., 2013). The temporal isolation of flowering asynchrony prevents hybridization between closely related crops (Ohigashi et al., 2014). For example, when the flowering time of *Anthoxanthum odoratum* was changed

to the boundaries between two groups with different flowering times, gene flow by pollen was substantially restricted (Silvertown et al., 2005). Mechanical isolation in angiosperms is also associated with insect pollinators (Grant, 1994). The flowers of *Salvia apiana* and *S. mellifera* that differ in size and conformation are pollinated by different bees, which greatly reduces the chance to hybridize each other (Grant and Grant, 1964). The pistil-pollen relationship might be responsible for incompatibility based on co-evolution of floral organs (Hogenboom, 1975). Gametic isolation is manifest at different stages of pollination such as pollen germination on the stigma, pollen tube growth inside the style, penetration of pollen tube into the embryo sac, and zygote fertility.

Postzygotic barriers occur after the zygote is formed but often accompany with low viability or sterile progeny. Asymmetric postzygotic isolation is common in reciprocal interspecific crosses displaying hybrid sterility/inviability and presumably caused by nuclear-cytoplasmic interactions (Turelli and Moyle, 2007; Tiffin et al., 2001). The mechanism of chromosome rearrangement is considered as one of the postzygotic reproductive isolation factors in plants (Stebbins, 1950; Rieseberg et al., 1999). The different chromosome structure and composition between the progenitors of hybrids should bring about the failure of meiotic chromosomes to precisely segregate or crossover during meiosis often leading to chromosomal deletions, duplications, inversions, and rearrangements.

Histological studies demonstrate that developmental failure of endosperm confers another critical impact on the hybrid seed lethality (Brink and Cooper, 1947; Roy et al., 2011).

Interspecific or intergeneric hybridization barriers might have similar mechanisms to the interploidy hybridization barrier. Two major similarities include endosperm defects and the direction of a cross (Sansome et al., 1942; Sukno et al., 1999; Dinu et al., 2004; Rebernig et al., 2015). The study of interspecific hybrids of *Capsella rubella* and *C. grandiflora* suggested that *C. rubella* is a self-fertilizing species that has diverged from an outcrossing species *C. gradiflora* species while losing self-incompatibility (Foxe et al., 2009). The cross between *C. rubella* and *C. grandiflora* results in inviable enlarged seeds with a defect in endosperm cellularization, whereas the reciprocal cross produces small seeds with precocious endosperm (Rebernig et al., 2015). Non-reciprocal endosperm defects in interspecific hybrids also occur in monkey flower *Mimulus guttatus* and *M. tilingii* (Garner et al., 2016).

Interploidy hybridization barriers are common phenomena caused by hybridization between two different individuals of different ploidy levels. In the case of paternal excess, the seed becomes small with premature endosperm, but in the opposite case of maternal excess, the seed shows delayed endosperm cellularization with enlarged seed size. This concept that differential ploidy levels of the parents lead to seed lethality is called a ‘triploid block’ (Marks, 1966). The triploid block accompanies endosperm

defects resulting from unbalanced chromosome numbers (Kohler et al., 2009). Parental dosage imbalance induces a failure of synchronization between endosperm and embryo development that eventually causes embryo arrest (Lafon-placette and Kohler, 2016).

Endosperm is a dosage-sensitive tissue constituted with the ratio of two maternal and one paternal genomes (2m: 1p) and its development is regulated by imprinted genes. Genomic imprinting is a prominent epigenetic process that allows genes to be expressed in a parent-origin-specific manner. According to the gene expression pattern in the parent, imprinted genes are classified into two groups – maternally expressed genes (MEGs) and paternally expressed genes (PEGs). In *Arabidopsis*, *DEMETER* (*DME*) that encodes a 5-methylcytosine DNA glycosylase is expressed in the central cell and induces *MEA* expression by removing DNA methylation of maternally imprinted genes (Gehring et al., 2006). The *MEA*, *FLOWERING WAGENINGEN (FWA)* and *FIS2* genes are maternally expressed whereas the paternal alleles are silenced by DNA methylation in fertilized endosperm (Kiyosue et al., 1999; Luo et al., 2000).

According to the Bateson–Dobzhansky–Muller incompatibility hypothesis (BDMIs), the major cause of postzygotic hybridization barrier is the negative epistatic interactions in the hybrid (Seehausen et al., 2014). The weak inbreeder/strong outbreeder (WISO) hypothesis predicts an outcrossing pollen donor parent can fertilize an inbreeding egg donor and proceed normal

seed development but the inbreeding pollen donor cannot, suggesting the unilateral incompatibility (Brandvain and Haig, 2005). The kin selection theory suggests that individual values are measured by the range of shared alleles and determine the possibility of reproductive success (Haig, 1987). In addition, a dosage imbalance between the parents negatively affects endosperm development and ultimately leads to embryo arrest in hybrid seeds (Lafon-Placette and Kohler, 2016).

2. Seed development

In *Arabidopsis*, a seed is a product of double fertilization process. Double fertilization starts with attachment of pollen grains to stigma. Next, pollen tube germinates through the style to the ovary and releases two sperm cells in the megagametophyte. One sperm cell fertilizes the egg cell to form the zygote which will develop into the embryo, and the other combines with the two polar nuclei of the large central cell to produce the endosperm.

After fertilization, a single-celled zygote undergoes embryogenesis to be transformed into more complex individual. During early embryogenesis, the apical-basal axis of polarity is established, followed by an asymmetric differentiation producing basal and apical cells (Mansfield and Briarty, 1991). The basal cell develops into the suspensor which connects the embryo and maternal vascular system. The apical cell undergoes a series of divisions to generate the globular embryo, and after rapid cell divisions at the top of the globular embryo, heart stage embryo is formed (Aida et al., 1999; Bowman and Eshed, 2000). In torpedo stage, cell elongation along the axis occurs and cotyledon further develops. In mature stage, the seed is dehydrated and enters dormancy.

Fertilized endosperm undergoes syncytial phase and cellular phase. In the syncytial phase, early endosperm called coenocytic endosperm goes through free-nuclear division without cytokinesis, generating thousands of nuclei within the single-celled syncytium (Brink and Cooper, 1947; Boisnard-

Lorig et al., 2001; Olsen, 2004). The nuclear division in early syncytial endosperm is faster than that of embryo, probably due to the absence of cytoplasm synthesis (Bennett et al., 1975). In cellular phase, radial systems of nuclear-based microtubules cellularize the multinucleate syncytium. The cell wall is organized between adjacent nuclear cytoplasmic domains (Brown et al., 1999). The cellularization process starts as a wave in micropylar region, progressing through peripheral and chalazal regions. The micropylar endosperm cellularization is completed around the embryo, and the peripheral endosperm is sequentially cellularized in peripheral layer. The chalazal endosperm remains syncytial until the late stage of mature seed (Olsen, 2004). In seed development, the developmental stages of embryo and endosperm are closely associated and crucial for seed viability (Lafon-Placette and Kohler, 2014).

3. Abnormal endosperm development

Endosperm is important for seed viability and hybridization process by providing nutrients and producing network signals among seed components. Cellularization failure of endosperm induces the central vacuole as a sink and it leads to nutrition insufficiency for the embryo (Lafon-Placette and Kohler, 2014). There are several mechanisms causing aberrant endosperm. Abnormal endosperm development is classified into three types; abnormal endosperm development after central cell fertilization, autonomous endosperm without fertilization of an egg cell, and autonomous endosperm with the fertilized embryo.

First, the abnormal endosperm development after double fertilization is observed in hybrids with an unbalanced parental dosage demonstrated in some *Arabidopsis* mutants (Scott et al., 1998; Adams et al., 2000). Abnormal endosperm development with an arrested embryo is a widespread phenomenon in interspecific or interploidy crosses. For example, when the seed of \a pollen donors results in uncellularized endosperm and the reciprocal hybrids have precociously cellularized endosperm (Lafon-Placette et al., 2017). The endosperm in *Arabidopsis* mutants defective for *ENDOSPERM DEFECTIVE 1* (*EDE1*), which is a microtubule-associated protein, fails to cellularize, while containing a reduced number of nuclei with the embryo being arrested at early heart stage (Pignocchi et al., 2009). The *Arabidopsis* mutant *jason* (*jas*) fails to accomplish first division restitution

and produces unreduced 2n pollen (De Storme and Geelen, 2011). The *jason* mutant shows enlarged seeds and sometimes aborted seeds with increased *PHERES 1* (*PHE1*) expression (Erilova et al., 2009). The *Arabidopsis* seeds which have an increased dosage of PEG gene *ADMETOS* (*ADM*) display a developmental arrest establishing the hybridization barrier, but the *jas adm* double mutant with the triploid embryo shows normal seed development because of the balanced parental dosage (Kradolfer et al., 2013). A similar mechanism also exists in PEG mutants (Wolff et al., 2015).

Second, endosperm development without pollination can occur when endosperm-regulating factors are disrupted or excessive auxin present in the endosperm. The FIS-Polycomb Repressive Complex 2 (FIS-PRC2), a chromatin-remodeling complex, contains *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*; WD40 protein), *FIS2* (zinc finger protein), and *MEA* (SET domain). The FIS-PRC2 complex regulates not only central cell proliferation but also cellularization of endosperm, and the loss of FIS-PRC2 function causes autonomous endosperm development including failure of cellularization (Kiyosue et al., 1999; Ohad et al., 1999; Luo et al., 2000;). In addition, *Arabidopsis* seeds with ectopic auxin treatment and auxin-overexpressing transgenic lines exhibit autonomous endosperm proliferation (Figueiredo et al., 2015).

Lastly, abnormalities of pollen cell division can induce a fertilized egg cell with autonomous endosperm. In *cdka;1* mutant pollen, the generative

nucleus generated from male meiosis failed to progress through the second mitosis, resulting in one vegetative cell and only one generative-like cell (Nowack et al., 2006). The generative-like cell fertilizes an egg cell and a positive signal from the fertilized embryo initiates endosperm proliferation (Nowack et al., 2006). However, the cross between the *mea* mutant and the *cdka1* mutant could rescue seed abortion phenotypes by synchronization between an embryo and endosperm development (Nowack et al., 2007). Furthermore, FBL17 protein is a component of an SCF^{FBL17} complex and represses CDK inhibitors, subsequently, the similar phenotype is also observed in the *fbl17* mutant (Gusti et al., 2009).

MATERIALS AND METHODS

Plant materials and growth conditions

Chinese cabbage (*B. rapa*) cv. Chiifu-401-42 (CF) and cv. Hagam50 (HG), and radish (*R. sativus*) cv. Wonkyo10039 (WK) were used to investigate the intergeneric hybridization barrier. The seeds were sterilized with the 50% bleach solution containing 0.02% Triton X-100, followed by ten washes with triple distilled water (TDW). The seeds were planted on half-strength Murashige and Skoog medium (MS) (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, the plants were vernalized 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 intraspecific and intergeneric crosses, flowers of designated female parents were hand-pollinated. Intraspecific cross of CF and HG was conducted. Intergeneric F1 hybrids were produced in CF pollinated with WK. Endosperm and embryo after 14 DAP were collected in ice by hand dissection and stored in mixture of 0.3M sorbitol and 5mM MES monohydrate. Isolated embryos were washed using sterile water to avoid endosperm tissue contamination. After dissection, samples were immediately frozen in liquid nitrogen prior to DNA and RNA extraction.

Arabidopsis ecotypes Columbia (Col) and Landsberg *erecta* (L*er*) were used as wild type. *Arabidopsis* mutant lines were *dme-2* (Choi et al.,

2002) and *mea* (GABI_546B04), *fbl17* (GABI_170E02), *cdka;1* (SALK_106809) derived from Col accession. Seeds were sterilized in 70% ethanol for 5 min and vernalized at 4°C for 3 days. Sterile seeds were sown on half-strength MS medium and transplanted to soil in pots at 23°C with 16 hours of light and 8 hours of dark. For crosses, flowers of maternal plants were emasculated and pollinated by hand. The mutant plants were examined by genotyping with primers DG3525 and DG3526 for *dme-2*, DG3659 and DG3660 for *mea*, DG3903 and DG3904 for *fbl17*, and DG3648 and DG3649 for *cdka;1*.

Microscopy and histology

Siliques of CF crossed with WK were harvested and fixed in FAA solution (formaldehyde:alcohol:acetic acid, 2:10:1, v/v/v; Berlyn and Miksche, 1976). Samples were dehydrated through 50, 70, 85, 95, and 100% ethanol series, followed by dehydration in 50%, 75% and 100% tert-butyl alcohol (TBA) series, infiltrated in 50%, and 100% mineral oil, and embedded in paraplast (Sigma-Aldrich, St Louis, MO, USA) as described by Johansen (1940). Eight-micrometer sections were prepared with a microtome (Thermo Fisher Scientific, HM 325 Rotary Microtome, USA). The sections were affixed to glass slides, deparaffinized and stained for 30 sec with Toluidine blue O (Sigma Aldrich, MO, USA). Light microscopy was performed using a Zeiss AxioSkop 2 microscope. Images were recorded with an Axiocam 506

color digital camera.

Developing *Arabidopsis* seeds were isolated from individual siliques at different stages of development. For clearing of ovules and seeds, the whole pistils/siliques were fixed with ethanol:acetic acid (9:1, v/v) at 4°C, washed in 70% ethanol (Figueiredo et al., 2015), and incubated for 1 hour in Hoyer's solution (chloralhydrate:TDW:glycerol, 8:2:1, v/v/v; Tian et al., 2014). The ovules/seeds were observed under differential interference contrast (DIC) optics using an Axio Imager A1 microscope. The images were recorded using an AxioCam MRn. The images were acquired, analysed and exported using AxioVision Rel. 4.8.

Arabidopsis siliques at different stages were accessed for light microscopy. The Samples were analyzed with a Zeiss AxioSkop 2 microscope. Images were recorded with an Axiocam 506 color digital camera.

DNA extraction and simple sequence length polymorphism (SSLP) analysis

Genomic DNA (gDNA) was extracted from leaves as control, embryos, and endosperm after 14 days after pollination (DAP) using cetyl-trimethylammonium bromide (CTAB) extraction method. The presence of *B. rapa* and *R. sativus* genome in isolated endosperm and embryo was confirmed by SSLP markers, DG3590 and DG3591 for *B. rapa* and DG3259 and DG3260 for *R. sativus* (Table 1). The gDNA of embryo and endosperm was

extracted from a single seed of F1 hybrid between *B. rapa* and *R. sativus* and diluted to 10 ng/ μl and 100 ng/ μl, respectively. 1 μl of the diluted gDNA was used for SSLP analysis. PCR amplification was performed with 35 cycles of 95℃ for 30 sec, 62℃ for 30 sec and 72℃ for 1 min 20 sec using T100™ Thermal Cycler. The PCR products were analyzed on 0.8% agarose gel.

RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from 16 DAP endosperm and embryo tissues using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with DNase I treatment following the manufacturer's protocols. For the complementary DNA (cDNA) synthesis, GoScript™ Reverse Transcriptase was used according to manufacturer's instructions (Promega, Madison, WI, USA). Following cDNA synthesis, the reaction was diluted to 100 ng L^{-1}.

The coding DNA sequence (CDS) of the *Arabidopsis Ubiquitin 9* (*UBC9*) gene (At4G27960) was used to query homologous *Brassica* and *Raphanus* sequences. The primers for UBC9 were designed using *B. rapa* ortholog (BraA08g019230.3C; available on Brassica database http://brassicadb.org) and *R. sativus* ortholog (Rs425570; available on Raphanus database http://radish-genome.org). For RT-PCR primers, DG3907 and DG3908 for *B. rapa*, and DG3909 and DG3910 for *R. sativus* were used (Table 1). PCR products were amplified with 30 cycles of 95℃ for 1min, 62℃ for 30 sec and 72℃ for 40 sec using T100™ Thermal Cycler. The PCR products were

analyzed on 0.8% agarose gel.

***In vitro* culture**

For ovule culture, *Arabidopsis* siliques of different stages were selected and sterilized by a short dip in 70% ethanol. The siliques were allowed to dry and placed onto double adhesive tape that was sterilized. Under a dissecting scope, the siliques were cut open along the replum with needles and the ovules were carefully transferred onto the *in vitro* culture medium (ICM) in the 23°C growth chamber under dim light (Sauer and Friml, 2004). ICM contained 5% sucrose, half-strength MS salts and 0.3% phytagel (Sigma, St Louis, MO, USA), and pH was adjusted with KOH to 5.9. After autoclaving at 121°C for 10 min, the medium was allowed to cool down to 50°C, then 25 ml L^{-1} of a 16 g L^{-1} aqueous stock solution of glutamine (final concentration 400 mg L^{-1}) was filter-sterilized into the medium. After germination, the ovules were transferred from ICM to half-strength MS medium and placed in the 23°C growth chamber with 16 hours of light and 8 hours of dark.

Table 1. List of primers

Name	Sequence (5′→3′)	Purpose
DG3590	ACACATATTGGACCAGCCCC	Br-SSLP
DG3591	AGCTCAGACAACTAGTTAAGCC	Br-SSLP
DG3259	ACCACATCCAGAACTCATTCAC	Rs-SSLP
DG3260	GCTTCGGCGTAAAACTCAAC	Rs-SSLP
DG3907	GAAGGATCCTCCTACTTCCTGC	Br-UBC9
DG3908	CGTACTTTTGGGTCCAGGTCCTT	Br-UBC9
DG3909	GAAGGATCCTCCTACTTCTTGT	Rs-UBC9
DG3910	GTACTTTTGGGTCCAGGTCCGA	Rs-UBC9
DG3525	CACTGATTGTGATGTTCCAC	Genotyping
DG3526	TTGACCATCATACTCATTGCTG	Genotyping
DG3659	CCGAGTCTAGATCCGTAAGCA	Genotyping
DG3660	CAAATTAAATCTCATGGATCTTCCG	Genotyping
DG3903	ATATTGACCATCATACTCATTGC	Genotyping
DG3904	ATCACAGATGTTCAAGGGATTACC	Genotyping
DG3648	GCGTGGACCGCTTGCTGCAACTCTCTCAGG	Genotyping
DG3649	CCAGATTCTCCGTGGAATTGCG	Genotyping

RESULTS

The seed of x*Brassicoraphanus* shows a failure of endosperm cellularization resulting in seed abortion.

A fertilized seed requires synchronization of endosperm and embryo development to become a mature seed (Lafon-Placette and Kohler, 2014). Endosperm develops through syncytial and cellular phases, while being differentiated into micropylar, pheriphral, and chalazal domains (Olsen, 2004). The abortion rate of self-pollinated *B. rapa* (CF) seeds was 36.03% and that of an intergeneric F1 hybrid between *B. rapa* (CF) and *R. sativus* (WK) was 70.20% (Table 2). The seeds of self-pollinated CF and the intergeneric F1 hybrid between CF and WK were examined from 8 to 20 DAP. In the seed of *B. rapa* CF, the inner seed coat gradually became thinner over time and the central vacuole enlarged, whereas the inner seed coat of the intergeneric F1 seed remained thick restricting the expansion of internal space (Figure 1). The endosperm of *B. rapa* CF seeds started to cellularize from the micropylar domain surrounding the heart-stage embryo at 12 DAP (Figure 1B). At 14 DAP, the early torpedo embryo of self-fertilized *B. rapa* CF seed was observed and the endosperm differentiated to form micropylar, pheriphral, and chalazal regions (Figure 1C). After 16 DAP, synchronous periclinal division of alveolar nuclei occurred and cell wall deposition took place for the completion of endosperm cellularization (Figures 1D and 1E). By contrast,

in the intergeneric F1 seeds of CF and WK, the endosperm rarely developed until 12 DAP (Figures 1F and 1G) and failed to form alveoli nuclei with retarded embryo development at the heart stage (Figures 1H and 1I). At 20 DAP, the torpedo-stage embryo of F1 of CF and WK was surrounded by the thick inner seed coat and the endosperm remained underdeveloped (Figure 1J). These results indicated that although fertilization normally occurred, intergeneric hybridization between *B. rapa* and *R. sativus* was postzygotically prevented by disrupted endosperm development.

Table 2. Proportion of aborted seeds in *B. rapa* and x*Brassicoraphanus*

Parental genotype (female × male)	Number of swollen seeds (%)	Number of aborted seeds (%)	Number of siliques	n
CF (selfed)	87 (63.97%)	49 (36.03%)	9	136
CF × WK	135 (29.80%)	318 (70.20%)	34	453

n = total number

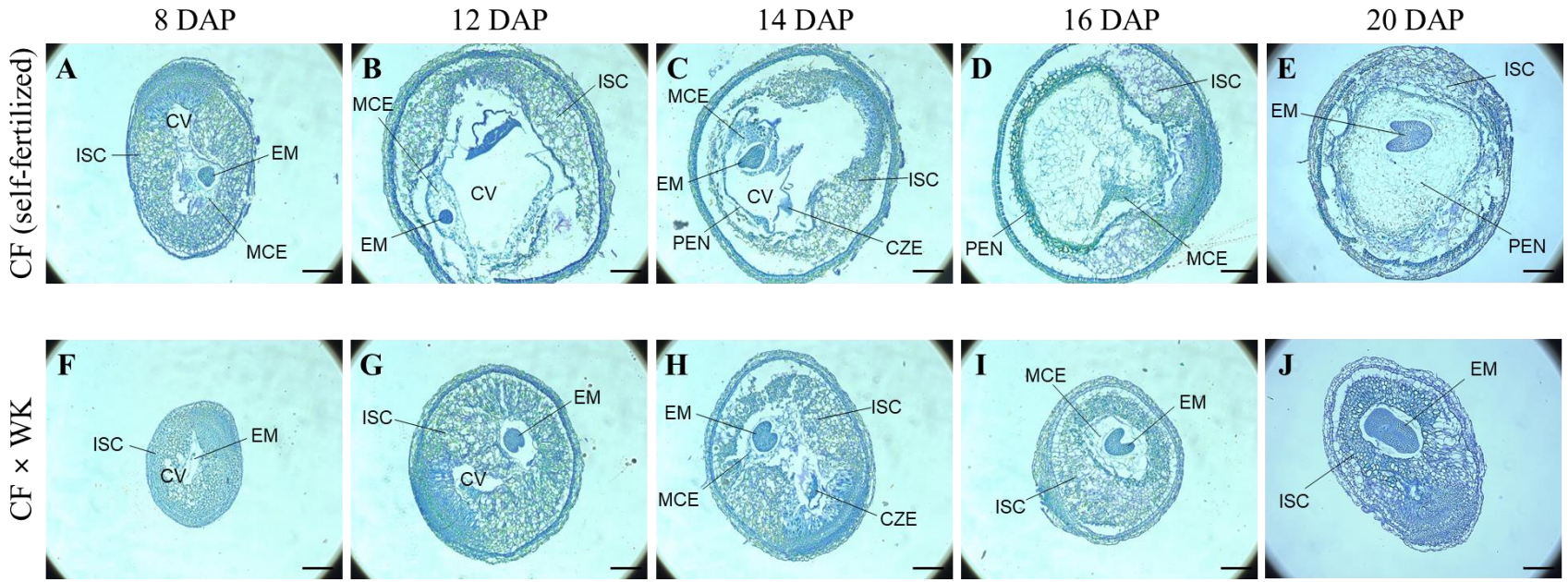

Figure 1. Seed development of *B. rapa* after self-pollination or outcrossing with *R. sativus.*

(A-E) Sections of self-pollinated *B. rapa* seeds at 8 **(A)**, 12 **(B)**, 14 **(C)**,16 **(D)**, and 20 **(E)** DAP. **(F-J)** Intergeneric F1 seeds between *B. rapa* and *R. sativus* at 8 **(F)**, 12 **(G)**, 14 **(H)**, 16 **(I)**, and 20 **(J)** DAP. CV, central vacuole; EM, embryo; ISC, inner seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Scale bar = 200 μm.

Seed development in *Arabidopsis dme−2/+*, *mea/+*, *cdka;1/+*, and *fbl17/+* mutants defective in endosperm development.

Angiosperms require double fertilization for seed development. In *Arabidopsis*, from 1 to 4 DAP, the seed contains a globular embryo and syncytial endosperm, and initiates endosperm cellularization at the heart embryo stage at 6 DAP (Orozco-Arroyo et al., 2015). After 6 DAP, the embryo goes through a transition from the torpedo stage to the mature embryo and the endosperm becomes completely cellularized (Le et al. 2010; Lafon-Placette and Kohler 2014). Normal seed development was observed in Col accessions from 2 to 13 DAP (Figures 2A and 3A). By contrast, the endosperm of *dme-2/+* mutant developed normally until 6 DAP displaying the free-nuclear division of endosperm, but a failure of endosperm cellularization led to seed lethality (Figure 2B). A similar phenotype was observed in *mea* mutants, and the seed was eventually aborted at 13 DAP (Figure 2C). In the aborting seeds of heterozygous *fbl17/+* and *cdka;1/+* mutants, the autonomous endosperm rarely proliferated, and the embryos were arrested at globular stage at 4 DAP and eventually aborted at 6 DAP (Figures 3B and 3C). These results suggest that endosperm development is coordinated with embryo development.

The seeds of heterozygous *Arabidopsis* mutants that showed endosperm defects were observed. All *Arabidopsis* siliques harbored transparent seeds up to 4 DAP (Figure 4). In wild-type Col, the color of seeds

gradually turned green and eventually became bright brown (Figure 4A). The seeds of all heterozygous mutants in this study exhibited roughly equal numbers of normal and aborted seeds, respectively, whereas most seeds from the wild-type plants are viable (Table 3). Since 8 DAP, approximately half of seeds from heterozygous *dme-2*/+ and *mea*/+ mutants turned brown and aborted (Figure 4B and 4C). The self-pollinated heterozygous *fbl17* and *cdka;1* mutants defective in the cell cycle of pollen development produced a half of small seeds that never matured and the other half of normal seeds (Figure 4D and 4E). Retarded cellularization of endosperm or autonomous endosperm development led to aborted embryo, indicating that synchronization of developmental stages between embryo and endosperm is important for seed viability. Thus, the timing of seed abortion might be closely associated with endosperm development.

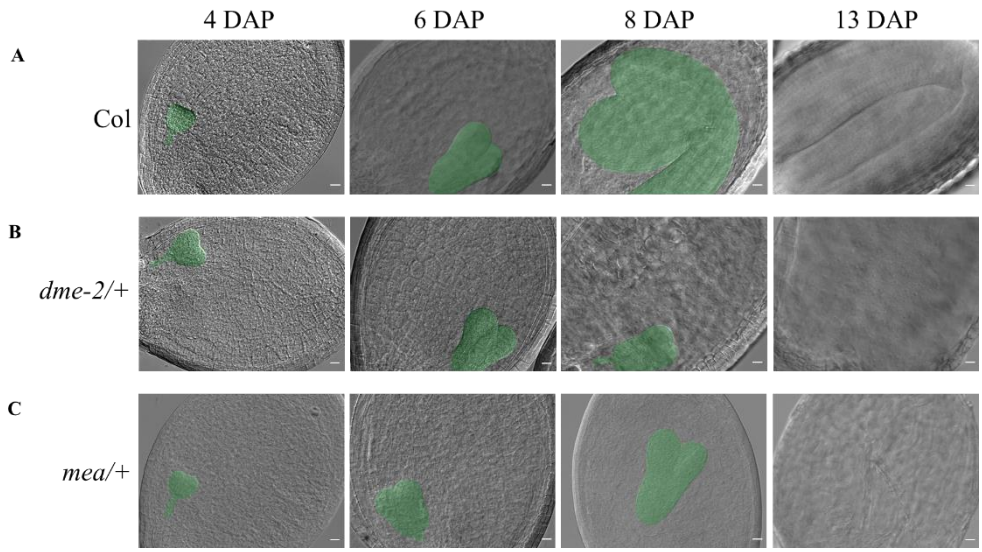

Figure 2. Seed development in heterozygous *dme-2/+* and *mea/+* mutants. **(A-C)** The seeds in self-pollinated *Arabidopsis* Col **(A)** and heterozygous *dme-2/+* **(B)** and *mea/+* **(C)** mutant plants at 4, 6, 8, and 13 DAP observed by Nomarski microscopy. Green color represents an embryo. Scale bar = 20 μm.

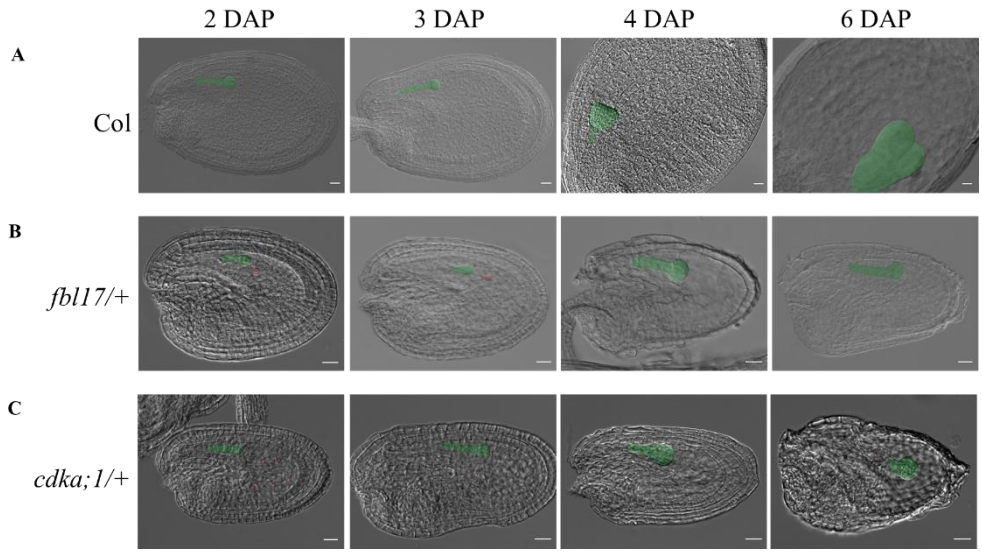

Figure 3. The seeds of heterozygous *fbl17/+* and *cdka;1/+* mutants.

(A-C) Seed structures in self-pollinated Col **(A)**, *fbl17/+* **(B)**, and *cdka;1/+* **(C)** mutant plants at 2, 3, 4, and 6 DAP analyzed using DIC microscopy. Green color represents an embryo. Scale bar = 20 μm.

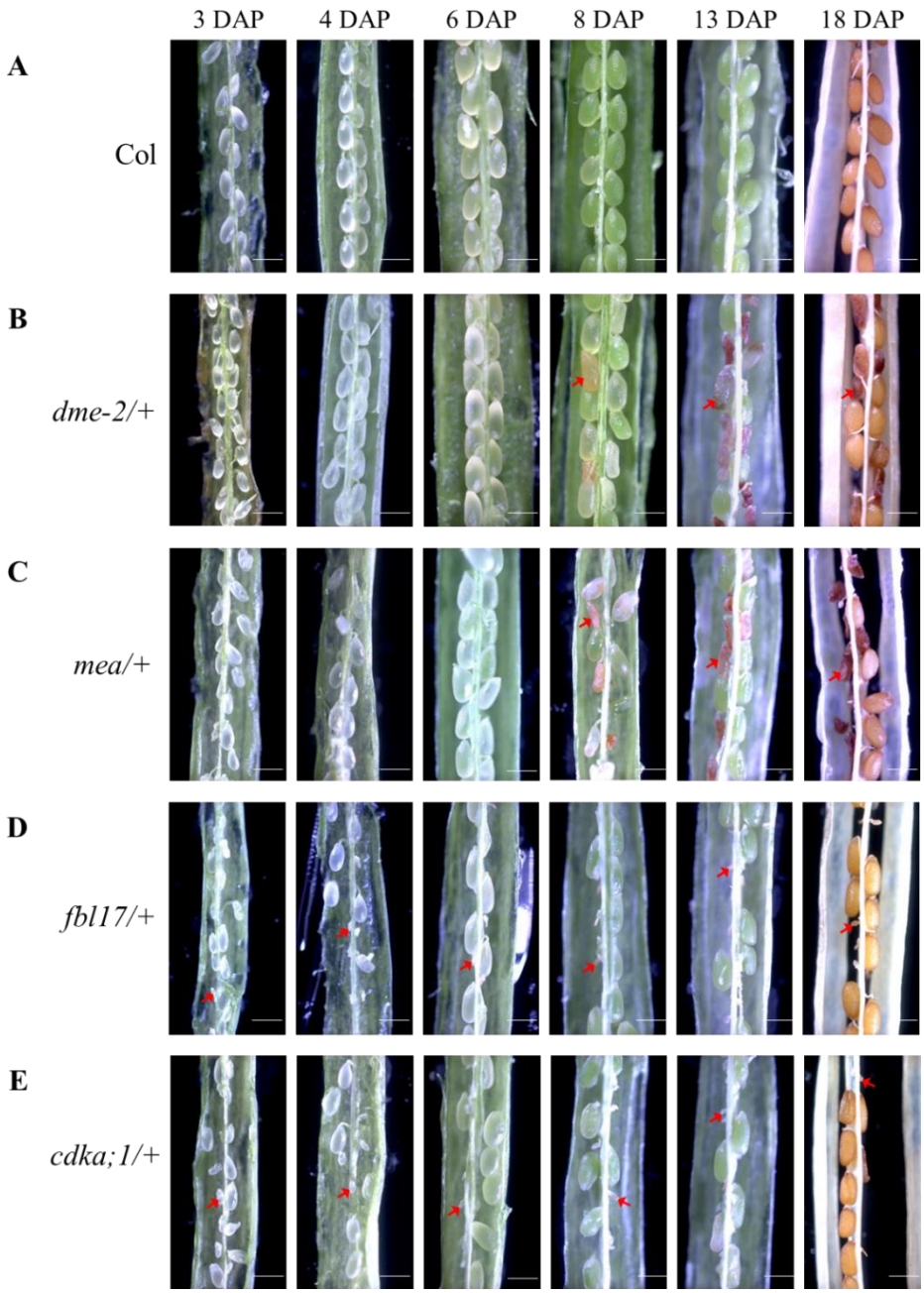

Figure 4. Siliques of *Arabidopsis* heterozygous mutants defective in endosperm development.

Arabidopsis siliques of wild-type Col **(A)**, and self-pollinated heterozygous *dme-2/+* **(B)**, *mea/+* **(C)**, *fbl17/+* **(D)**, and *cdka;1/+* **(E)** at 3, 4, 6, 8, 13, and 18 DAP observed using light microscopy. Red arrow represents aborted seeds. Scale bar = 500 μm.

Table 3. Seed abortion in *Arabidopsis* mutants

Parental genotype	Number of normal seeds (%)	Number of aborted seeds (%)	Number of siliques	n
Col (selfed)	363 (99.18%)	3 (0.82%)	9	366
Ler (selfed)	320 (98.16%)	6 (1.84%)	7	326
dme-2/+ (selfed)	178 (51.74%)	166 (48.26%)	8	344
mea/+ (selfed)	145 (53.70%)	125 (46.30%)	7	270
fbl17-1/+ (selfed)	182 (50.98%)	175 (49.02%)	7	357
cdka;1/+ (selfed)	131 (49.06%)	136 (50.94%)	7	267

n = total number

The newly synthesized x*Brassicoraphanus* undergoes double fertilization.

To examine the genetic composition of endosperm of the intergeneric F1 seeds, the embryo and endosperm of intergeneric hybrid seeds of *B. rapa* and *R. sativus* were dissected after 14 DAP. At 14 DAP, the hybrid seeds contained uncellularized endosperm with the embryo arrested at heart stage and the seed coat in yellow-green color (Figure 5). Almost all the underdeveloped seeds were small in size with dark brown color (Figure 5A).

The genotype of the intergeneric endosperm was analyzed with the SSLP marker. The SSLP markers were designed to specifically target single nucleotide polymorphism (SNP) regions of the *B. rapa* or *R. sativus* sequences at the intergenic regions. The Br-SSLP marker only amplified the *B. rapa* sequence while the Rs-SSLP marker only amplified the *R. sativus* sequence (Figure 6A). The pooled endosperm and embryo from intergeneric F1 hybrid seeds exhibited both *B. rapa* and *R. sativus* specific bands, but only the *Raphanus*-specific band was amplified from the endosperm (Figure 6A). A total of 15 seeds F1 of CF and WK were observed to examine whether autonomous endosperm development occurred without the contribution of paternal WK genome. Both CF and WK sequences were amplified in all embryos by Br-SSLP and Rs-SSLP markers (Figure 6B). All endosperm samples also produced the *Brassica*-specific bands and 13 out of 15 samples produced *Raphanus*-specific bands (Figure 6C). These results indicated that the ovules of *B. rapa* were successfully fertilized with *R. sativus* pollen, albeit

two endosperm samples failed to produce WK-specific fragments by PCR probably due to insufficient genomic DNA in reaction, and therefore suggest that seed abortion of intergeneric F1 hybrid of CF and WK is caused by some other factors rather than the absence of fertilization between CF central cell and WK sperm initiating autonomous endosperm formation.

The cDNA from CF and HG leaves was PCR-amplified with the Br-UBC9 primer pairs and that of WK leaves amplified with the Rs-UBC9 primers (Figure 7). The embryo and endosperm of F1 hybrid of CF and HG only showed *Brassica*-specific mRNA expression. The embryo from intergeneric seeds of CF and WK expressed both *Brassica*- and *Raphanus*-specific ubiquitin conjugating enzyme UBC9, whereas the endosperm expressed only *Brassica*-specific UBC9 (Figure 7), indicating that the embryo is a zygote but the endosperm might form autonomously without fertilization by *R. sativus* WK pollen.

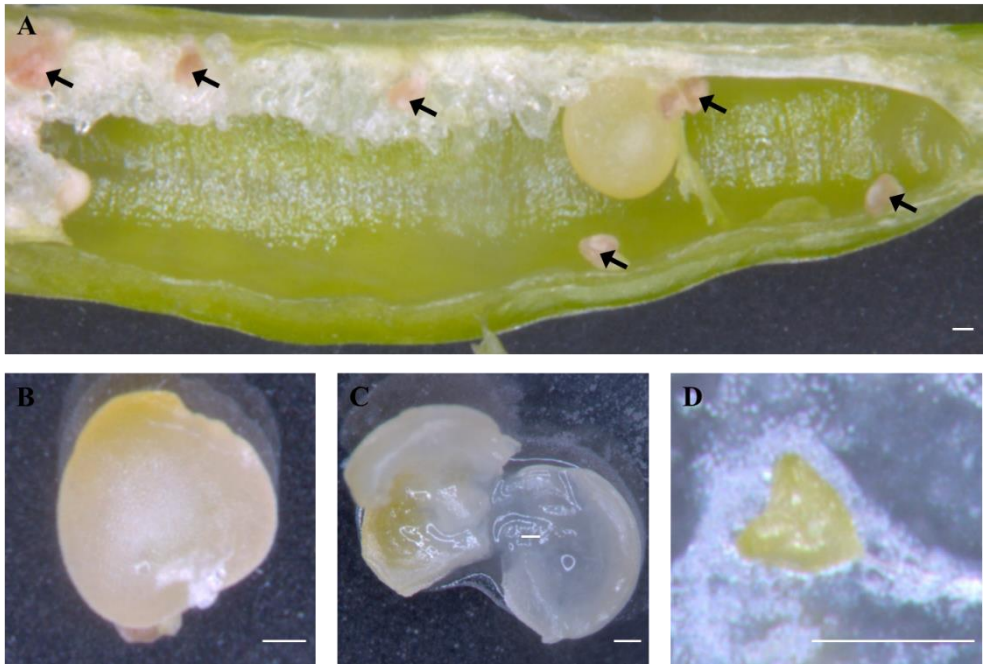

Figure 5. Intergeneric *B. rapa* seeds of 14 days after crossing with *R. sativus.*

(A) Silique from intergeneric hybridization between *B. rapa* and *R. sativus*. Black arrow represents aborted seeds. **(B)** A swollen seed with yellow-green color. **(C)** Seed coat and uncellularized endosperm. **(D)** An embryo at heart stage. Scale bar = 200 μm.

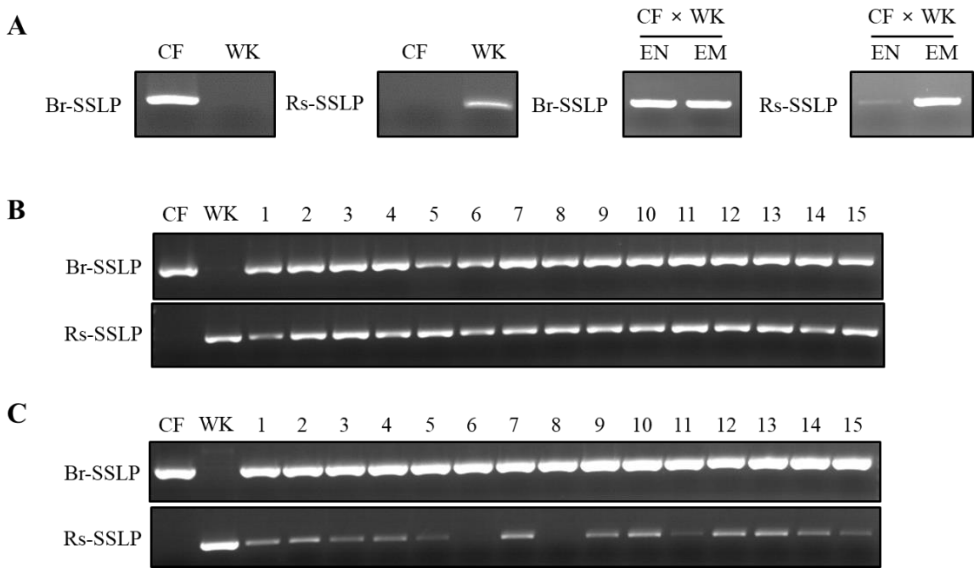

Figure 6. Detection of *B. rapa* and *R. sativus* sequences in endosperm of x*Brassicoraphanus* by SSLP markers.

(A) Amplification of Br-SSLP and Rs-SSLP sequences in CF and WK leaves, and in pooled embryo and endosperm tissues from an intergeneric F1 hybrid of *B. rapa* CF and *R. sativus* WK. **(B)** SSLP analysis on the embryos isolated from single seeds of F1 hybrid individuals. **(C)** SSLP analysis on the endosperm isolated from single seeds of F1 hybrid individuals. EM, embryo; EN, endosperm.

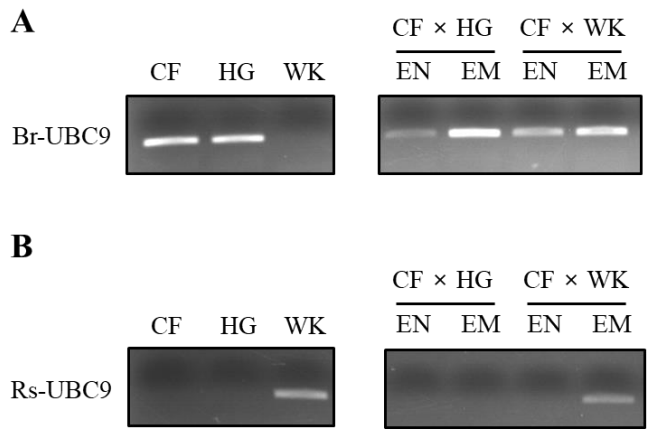

Figure 7. Expression of UBC9 in endosperm of intraspecific and intergeneric F1 hybrid seeds.

(A) *Brassica*-specific RT-PCR analysis on intraspecific F1 (CF x HG) and intergeneric F1 (CF x WK) seeds. **(B)** *Raphanus*-specific RT-PCR analysis on intraspecific (CF x HG) and intergeneric(CF x WK) F1 seeds.

Seed lethality caused by endosperm defects can be overcome by *in vitro* culture.

The *Arabidopsis* Col was reciprocally crossed with Ler as a control, and the *dme-2*/+ and *mea*/+ mutants were pollinated with wild-type Ler and the wild-type Ler was pollinated with the *fbl17*/+ and *cdka;1*/+ mutants which produce abnormal pollen to examine the viability of the seeds with abnormal endosperm and to investigate whether aborting seeds can be rescued by the *in vitro* culture. The fertilized ovules were isolated and *in vitro* cultured at 3, 4, 6 and 8 DAP. For wild-type seeds, the efficiency of the embryo rescue technique was greatly influenced by the developmental stage, in which the survival rate of older ovules was significantly higher than that of younger ovules (Table 4). Interestingly, the survival rate of the seeds with a *dme-2* or *mea* mutant allele was the highest at 4 DAP, whereas the embryos with a *fbl17* or *cdka;1* mutant allele were rarely rescued, especially with 0% of the survival rate at 8 DAP (Table 4). This result indicates that the efficiency of the *in vitro* culture is correlated with the developmental stage of embryo and endosperm and that the stages for efficient rescue are varied for mutation types.

Based on the results that the seeds with abnormal endosperm can survive by the *in vitro* culture, the heterozygous *dme-2*/+ mutant was self-pollinated to produce a homozygous *dme-2*/- mutant plant. Out of 59 seeds, three seeds (5.08%) were viable, and only one seed (1.69%) was identified as a homozygous *dme-2* mutant (Figure 8A). At early vegetative stages, the

homozygous *dme-2* mutant plant produced two cotyledons but failed to generate a sufficient number of rosette leaves (Figure 8D). The size of rosette leaves of *dme-2* mutant was much smaller than the wild-type. Root development also appeared to be defective in a *dme-2* mutant because the root did not strongly hold and support the above-ground shoots. During transition from vegetative to reproductive growth, a *dme-2* mutant produced a few inflorescence, the diameter of which was conspicuously smaller than the wild-type. Cauline leaves emerging from the inflorescence were also smaller than the wild-type. The flowers of homozygous *dme-2* mutant were smaller in size but anatomically similar to wild-type flowers consisting of four sepals, four petals, six stamens, and two carpels fused to from a pistil (Figure 8). The homozygous *dme-2* mutant produced seemingly normal pollen similar to wild-type flowers. In a homozygous *dme-2* mutant, smaller siliques were formed containing only aborted seeds with much less swollen seeds (10.09 per silique, total = 9 siliques) than the wild-type (40.67 per silique; Figure 8C and Table 3). This result suggested that *DME* is important not only for endosperm and seed development after fertilization but also for development of vegetative organs such as rosette leaves, roots, and inflorescence stems.

Overall, the *in vitro* culture was able to rescue the mutant seeds defective in endosperm development. This suggested not only that the embryo develops normally and its development is tightly associated with endosperm

development but also that seed abortion caused by endosperm defects can be overcome by rescuing the embryo arrested at early stages.

Table 4. Embryo rescue in *Arabidopsis* mutant crossed with wild-type

Parental genotype (female×male)	3 DAP				4 DAP				6 DAP				8 DAP			
	Number of plants with WT allele (%)	Number of plants with mutant allele (%)	Number of Aborted seeds (%)	n	Number of plants with WT allele (%)	Number of plants with mutant allele (%)	Number of Aborted seeds (%)	n	Number of plants with WT allele (%)	Number of plants with mutant allele (%)	Number of Aborted seeds (%)	n	Number of plants with WT allele (%)	Number of plants with mutant allele (%)	Number of Aborted seeds (%)	n
Col × Ler	1 (9.09%)	N/A	10 (90.91%)	11	3 (25.00%)	N/A	9 (75.00%)	12	7 (15.56%)	N/A	38 (84.44%)	45	20 (74.07%)	N/A	7 (25.93%)	27
Ler × Col	8 (9.88%)	N/A	73 (90.12%)	81	17 (42.50%)	N/A	23 (57.50%)	40	36 (50.00%)	N/A	36 (50.00%)	72	41 (64.06%)	N/A	23 (35.94%)	64
dme-2/+ × Ler	12 (16.44%)	6 (8.22%)	55 (75.34%)	73	8 (9.20%)	15 (17.24%)	64 (73.56%)	87	21 (40.38%)	5 (9.62%)	26 (50.00%)	52	37 (54.41%)	5 (7.35%)	26 (38.24%)	68
mea/+ × Ler	1 (1.22%)	2 (2.44%)	79 (96.34%)	82	2 (3.03%)	7 (10.61%)	57 (86.36%)	66	12 (22.22%)	3 (5.56%)	39 (72.22%)	54	26 (50.98 %)	2 (3.92%)	23 (45.10%)	51
Ler × *fbl17*/+	17 (21.25%)	1 (1.25%)	62 (77.50%)	80	17 (25.37%)	3 (4.48%)	47 (70.15%)	67	20 (25.97%)	1 (1.30%)	56 (72.73%)	77	18 (27.69%)	0 (00.00%)	47 (72.31%)	65
Ler × *cdka;1*/+	3 (3.66%)	3 (3.66%)	76 (92.68%)	82	19 (25.33%)	7 (9.33%)	49 (65.33%)	75	N/A	N/A	N/A	N/A	27 (29.35%)	7 (7.61%)	58 (63.04%)	92

n = total number.

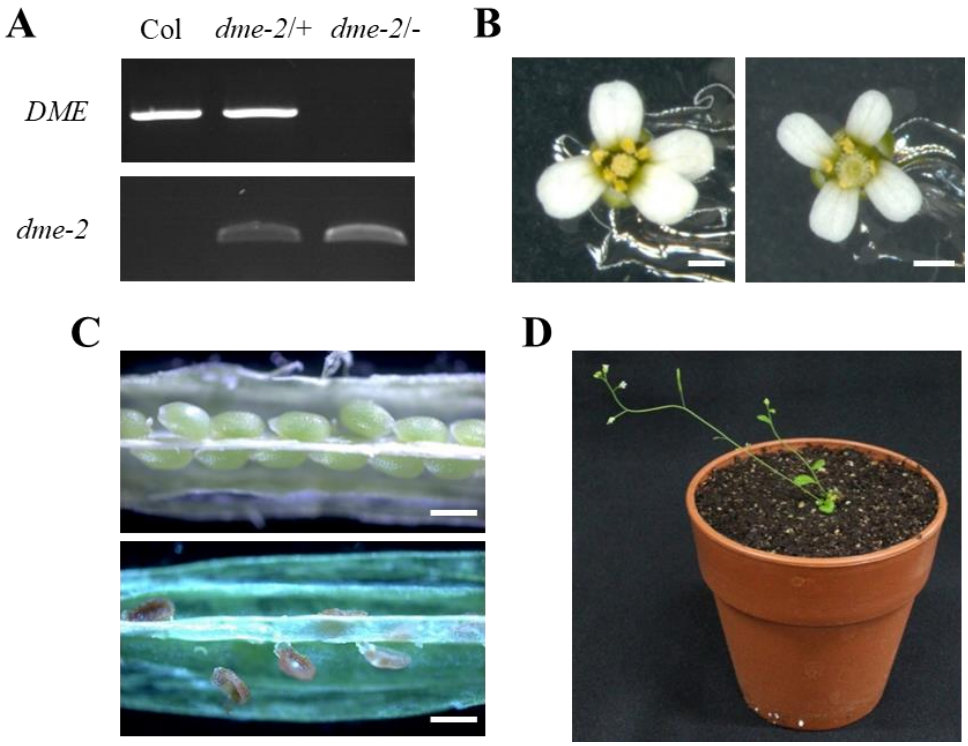

Figure 8. Abnormal development of homozygous *dme-2*/- mutant.
(A) Genotypes of *Arabidopsis* Col, heterozygous *dme-2*/+ mutant, and homozygous *dme-2*/- mutant. **(B)** Flowers in wild-type (left) and homozygous *dme-2*/- mutant (right). **(C)** Siliques in wild-type (top) and homozygous *dme-2*/- mutant (bottom). **(D)** Whole plant of homozygous *dme-2*/- mutant. Scale bar = 500 μm.

Discussion

The intergeneric F1 hybrid seeds of x*Brassicoraphanus* crossed between *B. rapa* and *R. sativus* undergo seed abortion, and thus viable F1 individuals of x*Brassicoraphanus* can only be produced by *in vitro* culture (Lee et al., 1989). Histological analysis showed that the intergeneric F1 seeds between *B. rapa* and *R. sativus* failed to produce peripheral endosperm with a small central vacuole and a thick inner seed coat, resulting in seed abortion (Figure 1). These results indicated that newly synthesized F1 hybrid between *B. rapa* and *R. sativus* suffers from endosperm-based hybridization barriers which often bring about autonomous endosperm or uncellularized endosperm.

Comparison of endosperm defects of x*Brassicoraphanus* with those of *Arabidopsis* mutants suggested that the intergeneric F1 hybrid seeds have cellularization defects rather than autonomous endosperm development. *DME* encodes a DNA demethylase that excises 5-methylcytosine from DNA and activates *MEA* (Gehring et al., 2006). MEA is a component of FIS-PRC2 which modifies chromatin structure and regulates endosperm cellularization (Kiyosue et al., 1999; Ohad et al., 1999; Luo et al., 2000). Loss of function mutations in *DME* or FIS-PRC2 cause central cell overproliferation without fertilization and failure of endosperm cellularization (Kiyosue et al., 1999; Ohad et al., 1999; Luo et al., 2000; Gehring et al., 2006). In *Arabidopsis cdka;1* and *fbl17* mutant, the sperm cell exclusively fertilizes the embryo and

by a positive signal from the embryo, the endosperm develops autonomously without the paternal genome (Nowack et al., 2006; Gusti et al., 2009). The *dme-2* and *mea* mutant seeds showed the free-nuclear divisions of the endosperm, but a failure of endosperm cellularization and the retarded embryo growth at an early torpedo stage led to seed abortion (Figure 2B and 2C) while the endosperm of *fbl17* and *cdka;1* mutants rarely proliferated without fertilization and did not differentiate to the distinct endosperm regions (Figure 3B and 3C). Although the endosperm of the intergeneric F1 hybrid between *B. rapa* and *R. sativus* was differentiated into CZE, MCE, and PEN, the developmental stage of the PEN did not progress to the cellular phase, remaining a syncytium, and the embryos were retarded at from an early to late torpedo stages (Figure 1). This suggested that endosperm defects in intergeneric hybrid may result from a problem related to the function of FIS-PRC2 which regulates early endosperm proliferation and endosperm cellularization.

To examine whether the autonomous endosperm can be established in newly synthesized x*Brassicoraphanus*, the species-specific SSLP and RT-PCR analysis were conducted. Most of endosperm and all embryos of intergeneric F1 seeds had both paternal and maternal genomes, suggesting that the egg cell and the central cell of *B. rapa* were successfully fertilized with the sperm cells of *R. sativus* (Figure 6). Although endosperm of intergeneric F1 seeds contained paternal genome, cDNA of F1 hybrid was not

amplified by Rs-specific marker (Figure 7B). Previous studies reported that some paternally inherited alleles were not transcriptionally active during early seed development and became expressed at later stages in *Arabidopsis* (Vielle-Calzada et al., 2000). In the intergeneric F1 hybrid seeds, paternal genome might be also silenced during early stages of endosperm development.

Unlike the mutants of other DME family members that do not display prominent developmental defects (Penterman et al., 2007), *dme* homozygous mutants were lethal mainly due to seed abortion (Figure 8C). However, it is largely unknown whether the *dme* mutant embryo is functional and has a capacity to drive the growth and development of mutant offspring after germination. Seed abortion of *dme* mutants is caused by hypermethylation and silencing of several imprinted genes such as *MEA* and *FIS2*, which are maternally expressed in a parent-of-origin-dependent manner in fertilized endosperm (Luo et al., 2000; Choi et al., 2002; Gehring et al., 2006). Despite abnormal endosperm development, the seeds carrying a maternal allele of *dme* or *mea* could be rescued by the *in vitro* culture (Table 4). This suggests that the *dme* mutant embryo is still viable and functional, and that the lethal phenotype is largely determined by the endosperm defect. This also implies that defective endosperm may serve as a reproductive barrier to prevent the development of the embryo. The homozygous *dme-2* mutant displayed retarded growth of root, reduced numbers of rosette leaves, and smaller siliques with aborted seeds compared to wild-type (Figure 8B and 8D). Based

on the previous report that *DME* is expressed in shoot apical meristem, leaf primordia, and root apical meristem (Park et al., 2017), loss of function mutation of *DME* would result in abnormal vegetative and development patterning. The study on the homozygous *dme-2* mutant will provide a valuable insight into the mechanism by which DME-mediated DNA demethylation regulates other cellular processes in vegetative development as well as genomic imprinting in endosperm.

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초 록

자연계에서는 종간이나 속간 교잡을 통해 종의 합성이 이루어지고 진화적으로 다양한 종이 분화되어왔다. 하지만 종·속간 교잡시 교배 장벽이 존재하여 새로운 교잡종이 합성되기 어려운데, 교배 장벽 중 수정 후 교배 장벽(postzygotic hybridization barrier)은 수정 후 배유나 배의 발달에 문제가 있어 교잡체 자체가 죽거나, 만들어진 교잡체가 불임인 경우를 말한다. 배추(*Brassica rapa*)와 무(*Raphanus sativus*)의 교잡종으로 새로 합성된 배무채(x*Brassicoraphanus*)는 수정 후 교배 장벽의 흔한 현상인 종자의 치사를 보인다. 신배무채 종자의 배유에서 발달적 결함을 찾기 위해, 본 연구에서는 배유 발달에 결함이 있는 애기장대 *DME*, *MEA*, *CDKA;1*, *FBL17*의 돌연변이체 종자와 신 배무채의 표현형을 비교하였다. 그 결과 신배무채의 배유는 성공적으로 수정은 되나 세포화(cellularization) 과정에 문제가 있는 애기장대 *DME*와 *MEA* 돌연변이체의 배유와 비슷한 표현형을 가지는 것으로 판단되었다. 이러한 비정상적 배유 발달로 인한 종자 치사를 극복하기 위해, 본 실험에서는 비정상적 배유를 가진 종자를 기내배양(*in vitro* culture)하여 정상적인 식물체로 발달할

수 있음을 확인하였고 이를 통해 유전적으로 거리가 먼 종들 사이에서 나타나는 수정 후 교배 장벽이 극복될 수 있음을 시사한다.

주요어: 배무채, 배유, 교배장벽, 비정상 배유, 기내배양

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