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Intergeneric Allotetraploid x*Brassicoraphanus* Fruits Display a Compound Phenotype Derived from the Parents *Brassica rapa* and *Raphanus sativus*

속간이질사배체 배무채에서 배추와 무로부터 유래된 복합표현형 과실에 관한 연구

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ABSTRACT

Hybridization and polyploidization occur in many flowering plants. In particular, hybridization between different species or genera may produce novel plant species with neomorphic phenotype. Although hybrids from interspecific or intergeneric crosses are generally hard to obtain. x*Brassicoraphanus*, also known as 'Baemoochae', was successfully produced as a newly synthesized intergeneric allotetraploid between Chinese cabbage (*Brassica rapa* L.) and radish (*Raphanus sativus* L.). One of the distinct characteristics is a compound morphology of the x*Brassicoraphanus* pistil and silique. The pistil and silique of x*Brassicoraphanus* display discrete but mixed features of both Chinese cabbage and radish. In this study, we examined the reproductive development of x*Brassicoraphanus* focusing on the pistil and silique formation and dehiscence. x*Brassicoraphanus* produces the flowers whose organ structures are similar in shape to the parents, but with a

silique formation intermediate between Chinese cabbage and radish silique. For example, distal segment of the x*Brassicoraphanus* silique resembles that of radish. These segments are indehiscent after maturation. However, the shape of proximal segment of x*Brassicaraphanus* fruit coincides with that of Chinese cabbage's silique. Proximal segment form dehiscence zone of valves through the replum, allowing the segments to separate. Interestingly, an unusual transverse cleft appears at the junction of proximal and distal segment, forming a joint region structure. To investigate duplicate gene alteration in fruit development, we isolated homologs of major genes in the fruit development genetic pathway of *Arabidopsis*. We explained fruit development by comparing gene expression levels between x*Brassicoraphanus* and its parents. This study is an example to show how altered expression of duplicate genes after genome hybridization makes neomorphic phenotype.

Key Words: Intergeneric hybrid, Polyploid, x*Brassicoraphanus*, Fruit development, Heteroarthrocarpy, Dehiscence, Duplicated gene.

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LIST OF ABBREVIATIONS

B. rapa	*Brassica rapa*
R. sativus	*Raphanus sativus*
ETT	ETTIN
ARF	Auxin Response Factor
bHLH	basic Helix-Loop-Helix
SPT	SPATULA
STY1, 2	STYLISH 1,2
KAN1, 2	KANADI 1, 2
CRC	CRABSCLAW
ANT	AINTEGUMENTA
LUG	LEUNIG
FUL	FRUITFULL
MADS	MCM1, AGAMOUS, DEFICIENS, SRF
SHP1, 2	SHATTERPROOF 1, 2
IND	INDEHISCENT
RPL	REPLUMLESS
WGD	Whole Genome Duplication
TEs	Transposable Elements

cv	Cultivar
FAA	Formaldehyde-Acetic acid-Alcohol
TBA	Tert-Butyl Alcohol
SEM	Scanning Electron Microscopy
RNA-seq	RNA sequencing
dist	Distal segment
prox	Proximal segment
VM	Valve margin
R	Replum
S	Septum
ena	Endocarp *a*
enb	Endocarp *b*
ll	Lignified layer
sl	Separation layer
J	Joint region
XC	Exocarp
MC	Mesocarp
OW	Ovary wall
FPKM	Fragments per kilobase million

Br. A	*Brassica rapa* A genome
Rs. R	*Raphanus sativus* R genome
xB. A, R	x*Brassicoraphanus* A, R genome

INTRODUCTION

The fruit which bears seeds is one of the final products of reproduction and the important organ for successful progression to the next generation. Fruits are also the final objects of the cultivation in many cases. That is why fruit morphologies and features are of great interest to many plant scientists. For example, dehiscence (the spontaneous opening of fruits at maturity), or indehiscence of fruits is an important characteristic, which has been selected in history of human cultivation.

Fruits in the *Brassicaceae* family are diverse in shape, size, and structure (Rollins, 1993; Koch et al., 2003, Appel and Al-Shehbaz, 2003). Heteroarthrocarpic fruits are common in the *Brassicaceae* family and often bisected by a joint structure that abscise or not at maturity. Only the ovary wall of the proximal segment differentiates into a valve with the distal portion of the valve margin forming a joint (Hall et al., 2006). This unique fruit feature generally appears in the tribe *Brassicaceae* with segmentation along the longitudinal axis (Rollins, 1993; Hall et al., 2006). Fruits of *Brassicaceae* plants such as Chinese cabbage (*Brassica rapa*), turnip (*B. rapa*), rapeseed (*B. napus*), and radish (*Raphanus sativus*) are economically important organs because dehiscence or seed dispersal is related to harvest yield and profit (Spence et al., 1996).

The intergeneric hybridization between *Brassicaceae* plants has been reported since 1820' (Sageret, 1826; Karpechenko, 1924; 1927; Mcnaught, 1973; Dolstra, 1982; Lee et el., 2011). There were many attempts to generate an intergeneric hybrid between *Brassica* and *Raphanus*, but hybrids from interspecific

or intergeneric crosses were generally hard to obtain. x*Brassicoraphanus*, as known as 'Baemoochae', was successfully synthesized as an intergeneric allotetraploid from a cross between *B. rapa* and *R. sativus* (Lee et al ., 1989; Hong et al., 1995; Lee et al., 2002).

x*Brassicoraphanus* shows many intermediate phenotypes between *B. rapa* and *R. sativus* for leaf shape, hypocotyl length, and the fruit shape. Especially, the shape of fruit displays mixed features of both parents. For example, the lower half of the x*Brassicoraphanus* fruit is reminiscent of the Chinese cabbage silique, while the upper half resembles that of radish. One of the interesting features is a joint region structure, which is transversely split at the junction of upper and lower parts of x*Brassicoraphanus* silique. Heteroarthrocarphic fruit shapes have been sparsely reported in the hybrid between *Brassica* and *Raphanus* (Dolstra, 1982; Karpechenko, 1924; 1927; Mcnaught, 1973; Oost, 1984; Sageret, 1826).

To my best knowledge, the microscopic morphology of the heteroarthrocarpic fruit in intergeneric hybrids has yet been reported even though a few studies reported heteroarthrocarpic fruits with features blended from the parents in case of interspecific and intergeneric crosses. Furthermore, developmental processes of heteroarthrocarpic fruits are largely unknown.

In this study, to examine the heteroarthrocarpic features of intergeneric hybrid, fruits of *B. rapa*, *R. sativus*, and x*Brassicoraphanus* were examined with help of light and electron microscopy. Expressions of fruit development-related genes were analyzed with the transcriptiom data obtained from RNA-seq. This work will provide not only the basis for elucidating the developmental mechanisms of heteroarthrocarpic fruits but also a good example of neomorphism generated

after allopolyploidization. Additionally, this study will be an example showing how expression of duplicate genes is altered after genome hybridization.

LITERATURE REVIEWS

1. Fruit development

The fruit is a matured ovary after fertilization, which forms a complex structure. Fruit development or patterning is related to pod or seed shattering in plant. In crop plants, such as oilseed rape, a seed loss is substantially associated with the harvest yield and revenue, and spreading of seeds can be environmental contamination (Spence et al., 1996; Ferrándiz, 2002).

Most carpels and fruits have similar shapes including *Arabidopsis thaliana* in the *Brassicaceae* family. The carpel has two fused cylinders forming the gynoecium. This carpel is a pre–fertilization structure that develops into the fruit (Dinneny et al., 2004). The external structure of the fruit is composed of three major regions: the valves, the replum, and the valve margins. It has two valves which are connected to the replum. The valves are the seedpod walls that play an important role in seeds protection. The valve margins form at the junction between the valves and the replum. When the fruit matured and dried, dehiscence occurs at the valve margins while the valves are detached from the replum. The valve margins have a separation layer and a lignified layer. As the fruit matures, pod shattering takes place in the separation layer in conjunction with lignification of the lignified layer. This section is called a dehiscence zone which is important for pod shattering and seed spreading (Liljren et al., 2004; Dong et al., 2015).

There are three groups of genes involved in the gynoecium and fruit development. These categories include (1) apical to basal axis of gynoecium development, (2) mediolateral axis of gynoecium development, and (3) post-

fertilization fruit development (Dinney and Yanofsky, 2004).

The gynoecium before fertilization is divided into apical, medial, and basal region. At the apical region of the gynoecium, the stigma develops, on top of which the pollen adheres and germinates. Also, the style guides the growing pollen tube to the ovules (Johnson and Preuss, 2004). The medial region develops the ovary with numerous ovules inside. Gynophore is located at the basal region.

The plant hormone, auxin plays an important role in the differentiation of the apical-basal region of the gynoecium (Nemhauser et al., 2000). A high level of auxin is proposed to promote stigma and style development, while the suitable auxin level in the medial region promotes ovary development, and in the basal region, a low level of auxin promotes gynophore development (Benkova et al., 2003). This gradient of auxin alters development of the three domains in the gynoecium. *ETTIN* (*ETT*), as Auxin Response Factor (ARF), acts to mediate a response of auxin level in the ovary regulating the size of the medial region of gynoecium (Sessions and Zambryski, 1995). Development of the apical region of gynoecium is affected by *SPATULA* (*SPT*), a member of the bHLH (basic helix-loop-helix) family of transcription factor, which is necessary for development of stigma and style (Alvarez and Smyth, 1999; Heisler et al., 2001). Formation of apical-basal region is regulated by *ETT* that represses *SPT* expression (Alvarez and Smyth, 1998; Heisler et al., 2001). Furthermore, *STYLISH 1* and *2* (*STY1*, *2*) promote style development. *STY* encodes a SHI-like zinc-finger transcription factor (Kuusk et al., 2002).

A bilateral symmetric structure from the fusion of two carpels is observed in the transverse section of the gynoecium. This feature emerges very early in gynoecium development (Bowman, 1993; Bowman et al., 1999). A number of

genes are important for the formation of mediolateral organs. *KANADI 1* and *2* (*KAN1*, *2*) repress the expansion of internal-medial tissue development into the lateral region of the gynoecium. *KAN* mutants showed ectopic development of style and ovules in place of the replum (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). *CRABS CLAW* (*CRC*) has a similar function, cooperatively working with *KAN* genes. *KAN1* is expressed at the external side of replum (Kerstetter et al., 2001), and *CRC* promotes development of lateral regions of the gynoecium, repressing the expansion of internal-medial tissue (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Eshed et al., 1999). In addition, *AINTEGUMENTA* (*ANT*) and *LEUNIG* (*LUG*) genes have important roles in lateral organ development. *ANT* promotes growth of lateral organs in a meristematic state (Elliott et al., 1996; Klucher et al., 1996; Krizek, 1999; Mizukami and Fischer, 2000). *LUG* plays a role in gynoecium development, especially for carpel fusion near the style and the apical region structure of valves (Liu and Meyerowitz, 1995; Conner and Liu, 2000). These genes work together, playing a role for development of organ in the medial region such as replum, style, and septum (Liu et al., 2000).

Many genes are involved in post-fertilization fruit development. *FRUITFULL* (*FUL*), a MADS-box transcription factor, is expressed in a central dome of the gynoecium during early flower development. Later, *FUL* promotes lignification of the enb layer but negatively regulates expression of the valve margin identity genes in the valve (Gu et al., 1998; Ferrándiz et al., 2000). *SHATTERPROOF1* and *2* (*SHP1*, *2*) are another MADS-box genes that are first expressed in all medial tissues including the valve margin, replum, septum and ovules. As the gynoecium matures, *SHP* is no longer expressed in the replum. *SHP* promotes the valve margin development and lignification of the enb layer (Ferrándiz et al., 2000; Liljegren et al., 2000).

As a member of the bHLH transcription factors, *ALCATRAZ* (*ALC*) play a crucial part in controlling dehiscence. *ALC* controls the development of separation layer at the valve margin. In *alc* mutants, a lignified layer is well-developed but separation layer tissues are absent (Rajani and Sundaresan, 2001). *INDEHISCENT* (*IND*) encodes a member of an atypical class of bHLH transcription factors and is critical for valve margin development. *IND* is only expressed at the valve margin region, promoting the development of lignified layer and separation layer (Liljegren et al., 2004).

The valve margin development is controlled by several factors. *FUL* is suppressed in the valve for the valve margin development, and at the same time, its expression is limited in the replum. *REPLUMLESS* (*RPL*) is a member of the BEL subfamily of homeodomain transcription factors, and is expressed in the replum and septum in mature gynoecium. *RPL* promotes replum development by repressing expression of valve margin genes (Roeder et al., 2003).

Fruit development and dehiscence genes have an intricate genetic network. After fruit maturation, dehiscence occurs at the valve margin. Valve margin development is controlled by overlapping functions of *SHP1*, *2*, *ALC*, and *IND*. *FUL* is involved in the valve development, while suppressing valve margin identity gene expressions in the valve. Moreover, *RPL* promotes replum development, preventing expansion of the valve margin. Fruit shape and development are controlled by complex regulation of several genes.

2. Polyploidy

Polyploid is containing more than two paired chromosomes in a cell and organisms. Most eukaryotes have two sets of chromosomes, one set of that inherited from each parent (Lutz, 1907). However, polyploidy means that change in whole set of chromosome number. For this reason, polyploidy refer to whole genome duplication (WGD) (Comai, 2005; Otto, 2007).

Polyploidy is a common phenomenon in some animals and many plants but is particularly widespread flowering plants (angiosperms). Approximately 30-70% of present plant species are polyploid and more than 70% of extant flowering plants are undergoing poliploidization (Ramsey and Schemske, 1998; Brochmann et al., 2004). In all eukaryotes genome, polyploidy is an evolutionary important feature and factor. (Chen, 2007; Jiao et al., 2011; Leitch and Leitch, 2008). Many genome researches showed that an ancient WGD event occurred early in angiosperm evolution and most species were derived from polyploid ancestors (Cui et al., 2006; Soltis et al., 2008). In addition, many polyploid organisms are well-adapted to their environments (Van de Peer and Meyer, 2005). Two rounds of WGDs happened before divergences of extant angiosperms are indicated that occurred diversification of important genes and pathways of plant development, and survival of dominant plant on the earth (Van de peer et al., 2009; Jiao et al., 2011).

Polyploidy is divided into autopolyploidy and allopolyploidy based on the origins and composition of chromosomes (Stebbins, 1950; Chen, 2007). An autopolyploidy is duplication of a single same genome within single individual or population of crossed between different plants within a species. And an allopolyploidy is combination of two or more divergent genomes between two

distantly different species (Soltis and Soltis, 2009). Therefore allopolyploids contain diploid set of each parental chromosomes which are homologous and homeologous chromosomes (Comai et al., 2000). In ancient, autopolyploidy is anticipated to occur more frequently than alloplolyploidy, and intraspecific pairing is predicted to be more than interspecific pairing (Stebbins, 1971). But allopolyploids are more appeared than autopolyploids in nature (Hegarty and Hiscock, 2008; Schatlowski and Kohler, 2012).

Orthologous and homeologous genes in polyploids have different fate by several mechanisms. First, the most orthologous or homeologous genes are coexpressed in a nucleus. If the level of gene expressionin in polyploids is additive, the expression levels have the midparent values (1+1= 2). The values of nonadditive gene expression are larger or smaller than 2 (2< 1+1 <2) (Chen, 2007). Second, when two genomes occur merging and poyiploidization, some duplicate genes are mutated, diverged, or lost. Consequently, most organisms had been paralogous genes through the evolution (Lynch and Conery, 2000). Third, epigenetic modifications may change gene expression and networks (Song and Chen, 2015). This mechanism appears differently in many diverse polyploids. For example, sequence elimination, chromosomal translocation and transposition (Song et al., 1995; Feldman et al., 1997; Tate et al., 2006). One of the factors is an activation of transposable elements (TEs). In particular, the TEs regulate nonadditive gene expression in allopolyploids (McClintock, 1984, Yoo et al., 2014).

The evolutionary significance of polyploidy defies description. Polyploidy is important genetic source for environmental adaptation (Crow and Wagner 2006). Also, Polyploidy may decrease extinction of species and increase chances to survive from the extreme situation (Fawcett et al., 2009).

3. Fruit morphology in *Brassicaceae*

Brassicaceae is a medium-sized family consisting of approximately over 330 genera and 3,700 species (Warwick et al., 2009). This family includes a number of economically important plants such as Chinese cabbage, turnip (*Brassica rapa*), rapeseed (*Brassica napus*), broccoli, cauliflower, cabbage (*Brassica oleracea*), radish (*Raphanus sativus*), horseradish (*Armracia rusticana*), thale-cress (*Arabidopsis thaliana*) and many others (Gómez-Campo, 1980).

Brassicaceae family is reported to have appeared in past 100 million years from Middle East. Several features in *Brassicaceae*, there are supported that *Brassicaceae* is monophyletic group (Price et al., 1994; Galloway et al., 1998). However among several morphologies of the *Brassicaceae* family, leaf and fruit shape is various a lots supporting an idea that some phenotypes are widely differentiated from the other features in the early phylogeny, called homoplasy (Galloway et al., 1998; Franzke et al., 2011).

Flower shapes of *Brassicaceae* family plants are highly invariable in that typical *Brassicaceae* flowers have a cruciform corolla with four saccate sepals, four symmetric petals and tetradynamous (four long and two short) stamens. The pistil bears two carpels and style (Hall et al., 2002), and after pollination, goes through significant longitudinal growth eventually developing into the fruit with two capsulated structure inside which fertilized seeds reside. The fruit of *Brassicaceae* plants is commonly called a silique. There are two types of siliques in *Brassicaceae*. A certain type of silique is long and thin containing, two modified carpels. After the silique is matured, it spontaneously opens by two valves through the replum (dehiscence type). The other is short and thick, and does not open after maturity (indehiscence type). It is formed as single valve and cannot find the

replum (Hall et al., 2006; Dardick et al., 2014). The former includes *Arabidopsis* and Chinese cabbage, and the letter includes radish and some rapeseed. Additionally, some *Brassicaceae* plants have mixed fruit shape of those two types with the joint at middle. It is called heteroarthrocarpy which is characterized by bisected heteromorphic parts of the fruit and by partially of perfectly indehiscence (Hall et al., 2006; 2011).

From 1826 by Sageret (Oost, 1984), the intergeneric hybrids in *Brassicaceae* plants have been continuously reported (Karpechenko, 1924; 1927; Mcnaught, 1973; Dolstra, 1982; Lee et el., 2011). There are many attempts to generate intergeneric hybrid between *Brassica* and *Raphanus*, only a few successful examples were reported. The recently developed cultivar, x*Brassicoraphanus* 'Baemoochae', was derived from a cross *Brassica rapa* and *Raphanus sativus*. It was generated by embryo rescue after crossing and microspore culture following induced mutation. It has been self-fertilized over 10 generations with stable fertility (2.3 seeds per silique) (Lee et el., 2011). One of the common phenotypes appeared in hybrid of *Brassica* and *Raphanus* is a heteroarthrocarpy which was also described by Karpechenko as "their lower part is distinctly 2-celled like a cabbage pod, but the upper part is more like a radish pod" (Karpechenko, 1924; 1928).

x*Brassicoraphanus*, newly synthesized allopolyploid, was stabilized through genome hybridization and following polyploidization and provides a model to address questions beyond the processes. The intergeneric hybrid between *B. rapa* (AA; n=10) and *R. sativus* (RR; n=9), x*Brassicoraphanus* (AARR; n=19) has been expected to contain transcriptomic changes considering its generally intermediate phenotypes compared to its parents (Lee et el., 2011).

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of x*Brassicoraphanus*, *Brassica rapa* cv. Chiifu-401-42, and *Raphanus sativus* cv. WK10039 were surface sterilized in 30% hypochlorite solution with 0.5% sodium dodecyl sulfate for 5minutes, followed by five washes with sterile distilled water. The seeds were plated on one-half-strength Murashige & Skoog medium (Duchefa, Haarlem, The Netherlands) supplemented with gamborg B5 vitamins with 2% (w/v) sucrose and 0.8% (w/v) plant agar. The plates with seeds were placed at 24 ^{o}C incubator with 16 hours of light and 8hours of dark. Then, plants were vernalized at 4 ^{o}C with 16 h of light and 8 h of dark for 4 weeks. Plants were moved to bed soil in pots and placed in the growth chamber with the same light condition at 24 ^{o}C until bolting. After flowering, they were transferred and grown in a green house. All flower bud were sampled at the greenhouse. Some flower buds were hand-pollinated to get mature fruit.

Light microscopy

For histological studies, inflorescences including flowers and mature silique after pollination by hand were harvested and fixed in FAA solution (formaldehyde, acetic acid and alcohol). Samples were dehydrated through an ethyl alcohol series, infiltrated with tert-butyl alcohol (TBA), and embedded in paraplast (Sigma Aldrich, MO, USA) according to standard procedures (Berlyn and Miksche, 1976). Samples were sectioned to 10um on a microtome (MICROM Lab., HM 340 E, Germany). The sections were affixed to glass slides, deparaffinized and stained with Toluidine blue O (Sigma Aldrich, MO, USA). For lignin staining, sections

were stained lignin-specific staining solution (2% phloroglucinol solution in 95% ethyl alcohol) for 2 minutes then photographed in 50% hydrochloric acid. Light microscopy images were obtained using photomicroscope (Carl Zeiss, Axiophot, Germany) and images were captured with a digital camera (Carl Zeiss, AxioCam MRc, Germany) at the National Instrument Center for Environmental Management (NICEM, http://nicem.snu.ac.kr). Sections were recorded digitally using a microscope and measurements were performed on captured images using ZEN 2012 blue edition software (Carl Zeiss, Germany).

Scanning Electron Microscopy

For SEM studies, pistils were harvested and fixed in FAA solution. Samples were dehydrated through an ethyl alcohol series and critical point dried with CO_2 in a critical point dryer. Samples mounted on aluminum stubs with conductive tape, coated with platinum using critical point dryer (Leica, CPD300, Germany). Samples were coated using sputter coater (Leica, EM ACE200, Austria), and put in the field-emission scanning electron microscope (Carl Zeiss, SUPRA 55VP, Germany), and observed in detail.

RNA Extraction for RNA-seq

Total RNA was extracted from inflorescences including flowers of *B. rapa*, x*Brasicoraphanus* and *R. sativus* with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. For transcriptome analysis, mRNA libraries were constructed in strand-specific manner using TruSeq adapter (Illumina, CA, USA). Sequencing was performed on an Illumina HiSeq 2000

system by paired end 101bp sequencing method at the National Instrument Center for Environmental Management (NICEM, http://nicem.snu.ac.kr). The RNA-seq data was analyzed by Hosub Shin.

RESULTS

Heteroarthrocarpic mature fruits of x*Brassicoraphanus*.

The mature fruits of x*Brassicoraphanus* and its parents, *B rapa*, and *R. sativus* were observed (Figure 1). A *B. rapa* fruit has a capsular shape with two valves. After maturation, it completely dehisces by opening of the two valves through the replum and the seeds are dispersed. In addition, the beak, a most distal part of the silique, is devoid of seeds and fully dehiscent in *B. rapa* (Figure 1A).

In contrast, the *R. sativus* fruits have no valve and the replum also does not exist. The silique is completely indehiscent even after maturation and seeds cannot be dispersed. In a mature silique, the gynophore, a stalk that supports the gynoecium, is the only abscission part. The pointed tip of *R. sativus* silique is much longer than that of *B. rapa* (Figure 1C).

Fruit of x*Brassicoraphanus* have distinct characteristics compared to its parents. The silique has two completely separate parts displaying mixed features of both *B. rapa* and *R. sativus*. The upper half of the x*Brassicoraphanus* fruit (referred to as “distal segment”) resembles that of *R. sativus*, while the lower half (referred to as “proximal segment”) is reminiscent of *B. rapa* with a split running longitudinally towards the calyx. The distal segment is indehiscent after maturation like *R. sativus*. It is replumless, but there are 2-3 seeds set inside the distal segment. The shape of proximal segment of x*Brassicoraphanus* fruit coincides with that of *B. rapa* silique. At maturity when the silique is completely dried, the valves in the

proximal segment separate into two parts through the replum, which allows the seeds to disperse. Interestingly, an unusual transverse cleft is present at the junction of distal and proximal segments, forming a joint region structure. The *Raphanus*-like proximal and the *Brassica*-like distal segments are precisely divided by the joint region (Figure 1B). This is a typical characteristic of heteroarthrocarpic fruits (Al-Shehbaz, 1985). Heteroarthrocarpic fruits are bisected into heteromorphic segments which are partially or completely indehiscent at the joint region (Hall et al., 2006). Many of the *Brassicaceae* family members (~40%) are known to have heteroarthrocarpic fruits, and x*Brassicoraphanus* apparently has typical heteroarthrocarpic siliques.

Heteroarthrocarpic structure of x*Brassicoraphanus* fruit is formed before fertilization

To observe an early development event of heteroarthrocarpic structure in x*Brassicoraphanus* silique, the pistil before fertilization was examined by stereomicroscopy. The flower buds of *B. rapa*, x*Brassicoraphanus* and *R. sativus* were collected before opening of the flower. The *B. rapa* pistil is the shortest among them which is approximately a half of *R. sativus* pistil in length (Figure 2). However, the *B. rapa* pistil has a larger diameter than the others (Figure 2). Two valves were clearly observed, divided by the replum that extended longitudinally from a base to the style. There are a number of ovules formed inside the ovary. Also, *B. rapa* has a short beak at the tip of silique (Figure 2).

The pistil of *R. sativus* is the longest among the three. It has small structure considered as valves at the basal part of the pistil (Figure 2). The distal part is composed of both ovary and style elements. Ovules reside inside the lower part of the distal segment, but the uppermost part is sterile.

As in the mature fruit, the x*Brassicoraphanus* pistil displays compound characteristics derived from *B. rapa* and *R. sativus* such as a pistil size and the valve length. The pistil size and the valve length of x*Brassicoraphanus* are intermediate between *B. rapa* and *R. sativus*. The proximal segment resembled the valves of *B. rapa*, whereas the distal segment is similar to the *R. sativus* pistil. After fertilization, the pistil develops into the fruit. In x*Brassicoraphanus*, the proximal segment is dehiscent, while the distal segment is always indehiscent.

The heteromorphic segments, a combination of proximal and distal parts, of silique are separated by the joint region. The joint region exists in all the fruits of *B. rapa*, *R. sativus* and x*Brassicoraphanus* even though it may or may not abscise (Figure 2).

Epidermal cells of *B. rapa,* x*Brassicoraphanus* and *R. sativus* pistils.

Epidermal cell shape is one of the indicators important for pistil structure. To compare epidermal cell shapes for pistils of *B. rapa*, *R. sativus*, *xBrassicoraphanus*, the scanning electron microscopy was employed. Pistils immediately after fertilization were used for examination. In *B. rapa*, the boundary of distal and proximal segments is clear (Figure 3A-D), in which the proximal

segment is composed of two valves, whereas the distal segment is a sterile style (Figure 3A). The replum of the proximal segment is clearly demarcated by the furrow between the two valves. Valves are attached to each other by the small cells around the replum. Stomata in the valves are undifferentiated, but fully differentiated and opened in the distal segment (Figue3B-D).

In x*Brassicoraphanus*, the distal and proximal segments are separated by the joint (Figure 3E-H). Both segments have the replum, which extends longitudinally along the pistil (Figure 3E). In the proximal segment, the valves are composed of large, interlocking cells and the replum forms a deep furrow. In contrast, the distal segment has relatively small cells and a prominent replum structure. The joint is formed at the boundary between the distal and proximal segments. The epidermal cells of the joint region have longer and more rectangular shapes than those of the distal segment and the valves at the proximal segment. Therefore, it was possible to successfully distinguish between the distal, proximal segments and the joint region by cell type (Figure 3F-H).

The distal segment of *R. sativus* pistil is longer than the proximal segment (Figure 3I). The proximal segment is short and composed of two valves with the replum in the middle. The replum of proximal segment has a groove. The joint separated by the two segments are composed of small cells. In the distal segment, the replum is externally as long as the length of the distal segment. It is not protruded or furrowed. Epidermal cells of the replum are apparently brick-like. It is distinguished from irregularly shaped cells near the replum (Figure 3J-L).

Different carpel and replum structures of *B. rapa*, *R. sativus* and x*Brassicoraphanus*.

In the longitudinal section of the unopened flower of *B. rapa*, the septum is in the middle of the carpel and the ovules are located at both sides of the septum inside the valves (Figure 4A). The two replums are connected by the septum. Dehiscence occurred at the replum, which is bound by a valve margin (Figure 4B). Valve margin and the repum are surrounded by small and tightly packed cells. Inside the replum, there are endocarps *a*, and *b*, a lignified layer, and a separation layer. A lignified layer and a separation layer together are called the valve margin (Figure 4C). As fruits mature after fertilization, the cells become increased and lignified. Transverse sections of the replum and the valve margin of a mature fruit treated with the lignin specific stain reveal the endocarp *b* of the valve and a lignified layer of the valve margin. Dehiscence occurred at the lignified layer near the separation layer of valve margin. Endocarp *b* and *a* lignified layer are connected, and lignin is preferentially accumulated in the endocarp *b*. The internal structure and a lignified layer explains why dehiscence easily takes place in the *B. rapa* fruit (Figure 4D).

In *R. sativus*, the septum exists throughout the entire length of the ovary, and the ovules are present in a zigzag conformation at the side of the septum (Figure 4L). Growth of this single ovule causes the septum to be internally appressed to one side leading to a zigzag shape. Parts considered as the replum are present in several places. The replum of *R. sativus* is significantly different from that of *B. rapa* in shape (Figure 4M). The valve margin is not found. In addition,

there are no lignified layer and separation layer formed inside the *R. sativus* pistil. A boundary between the endocarps *a* and *b* is not clear either (Figure 4N). Even though the endocarp of *R. sativus* becomes lignified, its morphology is clearly distinct from that of *B. rapa* causing indehiscence at maturation (Figure 4O).

In the transverse and longitudinal sections of the unopened flower of x*Brassicoraphanus*, the positions of the septum and ovules are similar to *B. rapa* (Figure 4H). The proximal segment (basal part of carpel) has the replum and a valve margin. There are endocarps *a* and *b* inside the valves. In a magnified view, the boundary between the endocarps *a* and *b* was clearly observed (figure 4E, F). Lignified layers observed in endocarp *b* of the valve and the valve margin are similar to the sections of *B. rapa* (Figure 4B, E). After fruits matured, the silique dehisces at the two valves through the separation layer of the valve margin (Figure 4G).

The replum of the distal segment of x*Brassicoraphanus* is reminiscent that of *R. sativus*. Only the replum, but not a valve margin including lignified and separation layers is observed in a transverse section of the distal part of the x*Brassicoraphanus* pistil (Figure 4I). Inside the valve, the endocarps *a* and *b* are adjoined to each other (Figure 4J). The lignin accumulation pattern at the endocarp is similar to *R. sativus* suggesting that indehiscence of the distal segment after maturation is similar to *R. sativus* (Figure 4I-K, M-O).

Joint region of x*Brassicoraphanus* carpel with higher cell density.

In the longitudinal section of the x*Brassicoraphanus* pistil, the joint region is present between the distal and proximal segments, defined by a specific cell shape (Figure 5A). The valve margin at the joint region forms a niche in a magnified view (Figure 5B). The joint region is composed of small and tightly packed cells compared with nearby cells (figure 5C). These compact cells in the joint region cause the fruit to separate into two pieces after maturation, which is observed only in x*Brassicoraphanus* but not in *B. rapa* or *R. sativus*. Lignification of the joint region may be crucial for abscission of the two segments.

Alteration of gene expressions which control gynoecium and fruit development

Genes involved in gynoecium and fruit development have been categorized into three groups in *Arabidopsis*; apical to basal axis of gynoecium development, mediolateral axis of gynoecium development, and post-fertilization fruit development (Dinney and Yanofsky, 2004). Most of the genes were found as multiple copies reflecting the polyploidy origin of *Brassica* and *Raphanus*. Interestingly, some of the fruit development gene orthologues such as *SHP1*, *RPL2*, and *RPL3* are not found in *R. sativus*. Deletion of these genes might be responsible for the absence of valve margin development in *R. sativus*. Notably, *R. rapa*-originated *ETT2*, *CRC*, and *ANT3* genes in x*Brassicoraphanus* showed elevated expression compared to *R. sativus*. *ETT* gene is known to promote the ovary formation by inhibiting *SPT* expression (Dinney and Yanofsky, 2004). *CRC* and *ANT* genes are involved in mediolateral gynosecium development. The complex fruit structure of x*Brassicoraphanus* is hard to explain with several gene

expression patterns. However, such alterations in gene expression patterns may be closely related to a unique developmental mechanism of x*Brassicoraphanus* pistil forming an intermediate fruit shape between *B. rapa* and *R. sativus*.

Table 1. List of genes controlling gynoecium and fruit development in *B. rapa*, x*Brassicoraphanus*, and *R. sativus*

Gene name	*A. thaliana*	*B. rapa*	x*Brassicoraphanus*		*R. sativus*
ETT1	AT2G33860.1	Bra005465	xB78573b	xB07382r	Rsa27612
ETT2		Bra021885	xB54518b	xB32861r	Rsa15284
SPT1	AT4G36930.1	Bra011740	xB32503b	xB59199r	Rsa07757
SPT2		Bra010591	xB17370b	xB06352r	Rsa33078
STY1.1	AT3G51060.1	Bra038880	xB74411b	xB12192r	Rsa06104
STY1.2		Bra036838	xB55197b	xB21725r	Rsa26310
STY2.1	AT4G36260.1	Bra017754	xB70788b	xB04583r	Rsa11281
STY2.2		Bra011675	xB32436b	xB59135r	Rsa07679
KAN1.1	AT5G16560.1	Bra008613	xB10060b	xB19289r	Rsa29458
KAN1.2		Bra023570	xB58725b	xB12149r	Rsa06033
KAN1.3		Bra006360	xB44546b	xB00776r	Rsa10511
KAN2.1	AT1G32240.1	Bra023254	xB65494b	xB68680r	Rsa01990
KAN2.2		Bra033844	xB79153b	xB55772r	Rsa19169
CRC	AT1G69180.1	Bra004364	xB03337b	xB60144r	Rsa35362
ANT1	AT4G37750.1	Bra017852	xB70883b	xB04668r	Rsa11211
ANT2		Bra010610	xB17389b	xB06331r	Rsa33057
ANT3		Bra011782	xB32550b	xB59240r	Rsa07803
LUG1	AT4G32551.2	Bra037051	xB63858b	xB04405r	Rsa11450
LUG2		Bra011355	xB32128b	xB49375r	Rsa07425
LUG3		Bra040085	xB16984b	ND	ND
FUL1	AT5G60910.1	Bra029347	xB38797b	xB01810r	Rsa24037
FUL2		Bra004007	xB02989b	xB20752r	Rsa30306
FUL3		Bra035952	xB76601b	xB54152r	Rsa38261
FUL4		Bra012997	xB83380b	ND	ND
SHP1.1	AT3G58780.3	Bra003356	xB02319b	ND	ND
SHP1.2		Bra007419	xB35525b	ND	ND
SHP1.3		Bra014552	xB67575b	ND	ND
SHP2.1	AT2G42830.2	Bra004716	xB46321b	xB45266r	Rsa28150
SHP2.2		ND	ND	xB64093r	Rsa15765
IND	AT4G00120.1	ND	xB15809b	xB57987r	Rsa51868
RPL1	AT5G02030.1	Bra028883	xB55895b	xB11557r	Rsa05495
RPL2		Bra009618	xB43837b	ND	ND
RPL3		Bra005703	xB75930b	ND	ND

***** ND : Not determined

Figure 1. Mature heteroarthrocarpic fruits in *Brassicaceae*.

Matured fruits of B. rapa, xBrassicoraphanus and R. sativus (A, B and C) in lateral view before dehiscence (on the left) and medial view (on the right) after dehiscence. Arrows indicate joint regions. **(A)** Dehiscence of valves through the replum. **(B)** Transverse cleft appears at the joint region (arrow). Only the distal part is dehiscent. **(C)** Indehiscence of matured fruit and abscission at the gynophore (arrow).

Scale bars = 1 cm

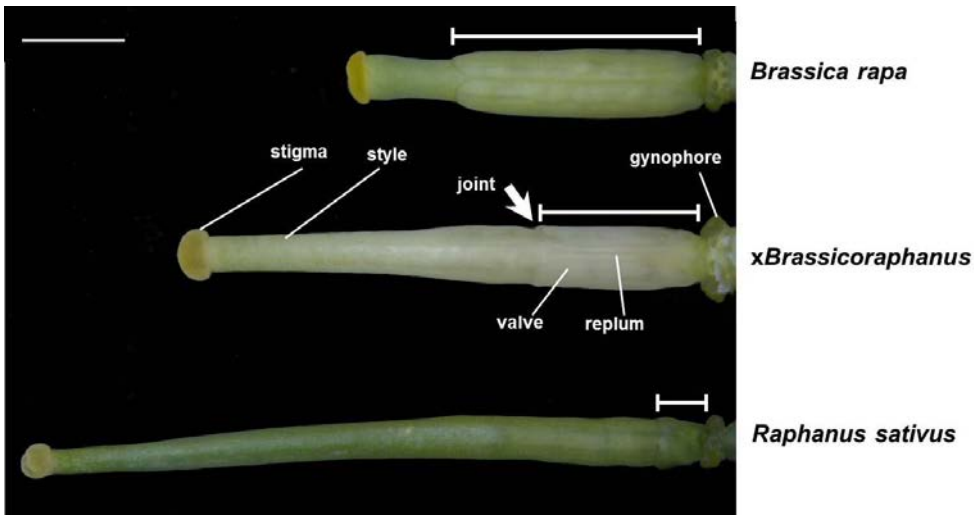

Figure 2. Comparison of pistil shape in *B. rapa, R. sativus* and x*Brassicoraphanus*.

(Top to bottom) Medial view of pistil showing proximal and distal segments separated by the joint region. White bars are valve length of proximal segment.

Scale bars = 2 mm

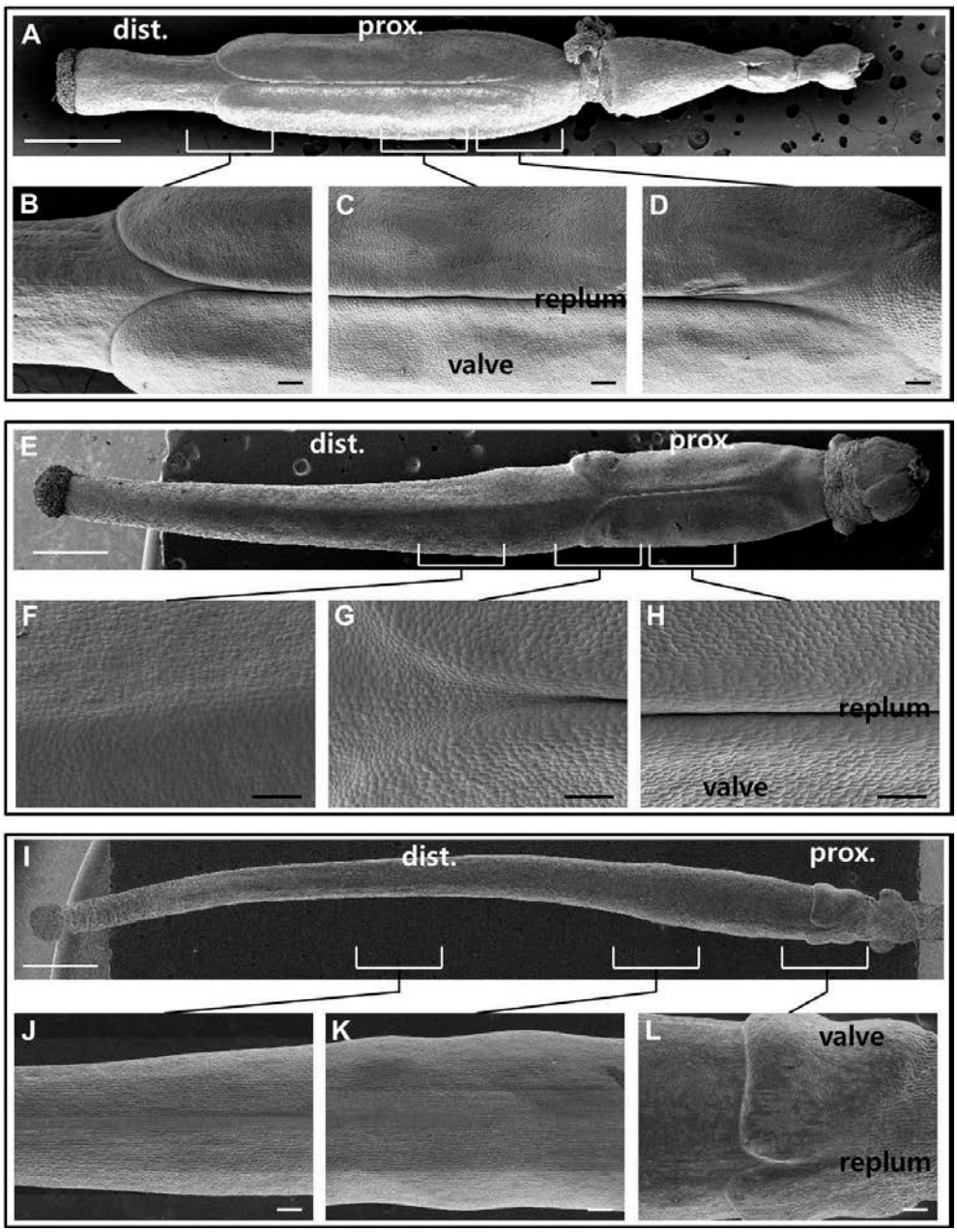

Figure 3. Close views in the different parts of pistils in *B. rapa*, x*Brassicoraphanus* and *R. sativus*.

Scanning electron micrographs of *B. rapa* (A to D), x*Brassicoraphanus* (E to H) and *R. sativus* (I to L) pistil epidermal cell.

(A) Medial view of *B. rapa* pistil.

(B) Magnified view of boundary between the distal and proximal segment.

(C) Magnified view of valves in proximal segment.

(D) Magnified view of basal part of valves.

(E) Medial view of x*Brassicoraphanus* pistil showing distal and proximal segment separated by the joint region.

(F) Magnified view of distal segment showing the prominent replum.

(G) Magnified view of joint region between the distal and proximal segment.

(H) Magnified view of the replum and valves in proximal segment.

(I) Medial view of *R. sativus* pistil.

(J) Magnified view of distal segment upper part showing the replum in the middle.

(K) Magnified view of distal segment.

(L) Magnified view of boundary between the distal and proximal segment.

Scale bars = 1 mm (A, E, I), 100 ㎛ (B-D, F-H, J-L)

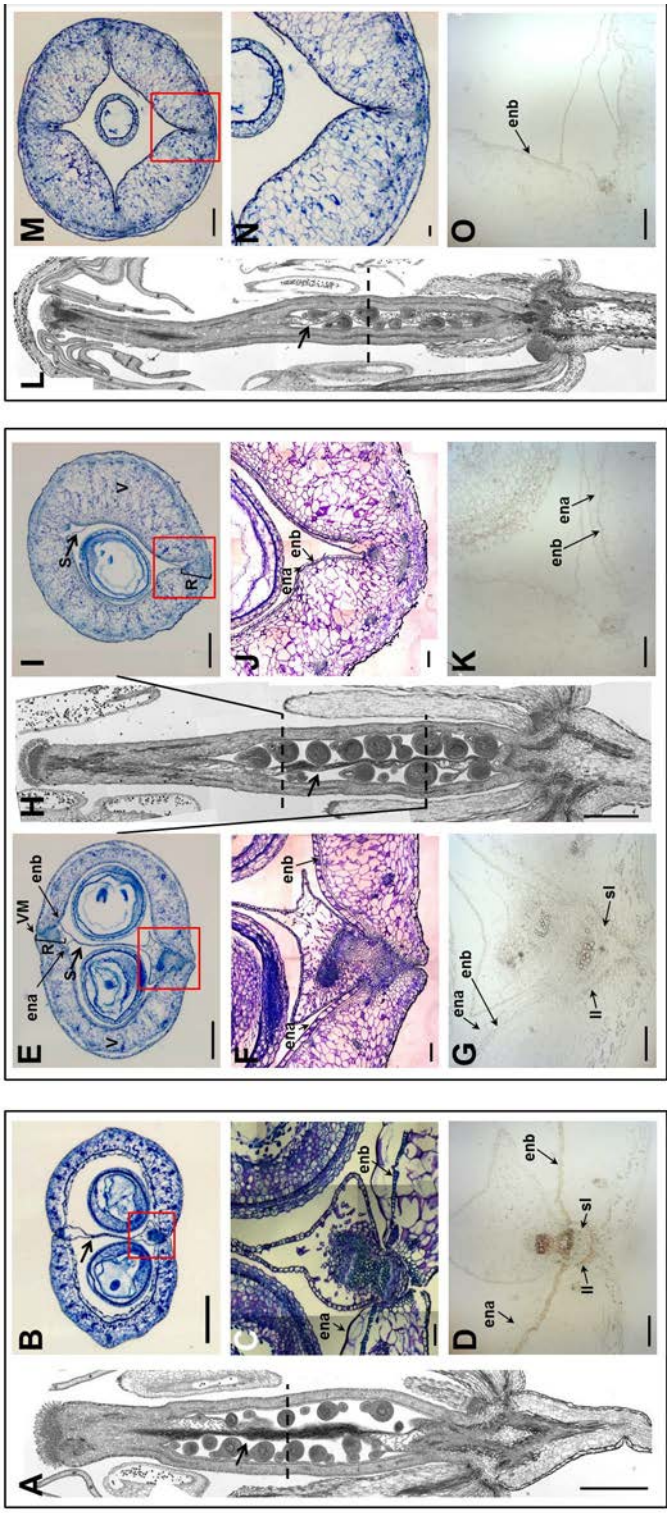
A
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Figure 4. Carpel and replum structures in *B. rapa*, x*Brassicoraphanus* and *R. sativus*.

(A-D) *Brassica rapa.* **(E-K)** x*Brassicoraphanus.* **(L-O)** *Raphanus sativus*.

(A, H and L) Scanning election micrographs with longitudinal section of flower bud for *B. rapa*, x*Brassicoraphanus* and *R. sativus*. Black arrows indicate septum.

(B, E, I and M) Transverse section of fruit stained with toluidine blue O.

(C, F, J and N) Magnified medial view of the replum and valve margin.

(D, G, K and O) Transverse **s**ections stained with phloroglucinol, which colors lignified cells pink.

Scale bars = 1 mm (A, B, E, H, I, L, M), 200 μm (C, D, F, G, J, K, N, O). V = Valve; S = Septum; R = Replum; VM = Valve Margin; ena = endocarp *a*; enb = endocarp *b*; ll = lignified layer; sl = separation layer.

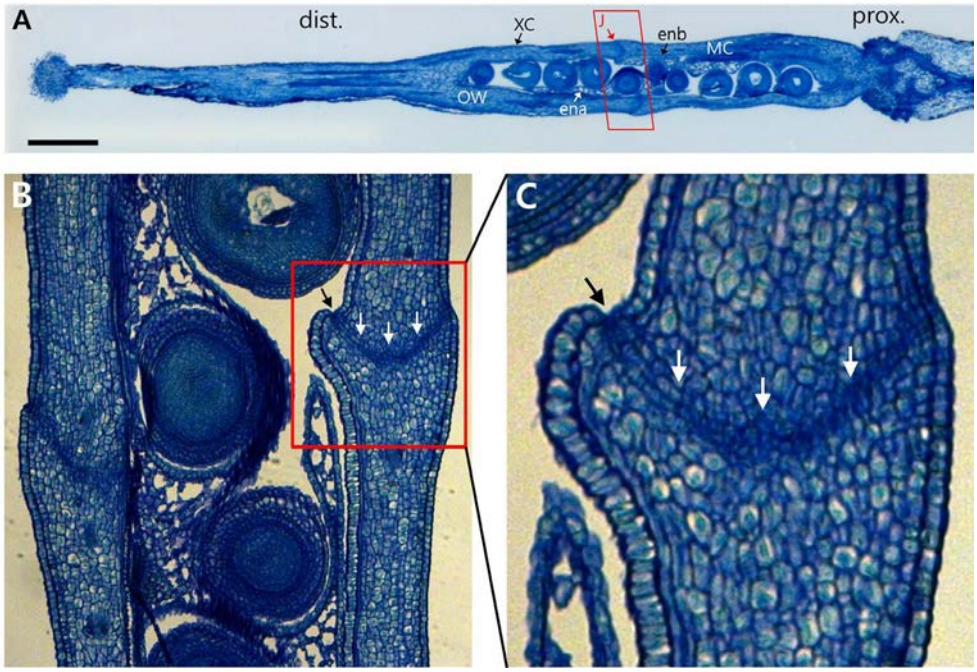

Figure 5. Joint region of x*Brassicoraphanus* carpel with higher cell density.

(A) Longitudinal section of the x*Brassicoraphanus* carpel. Joint region is indicated by red box. **(B)** Magnified image of the joint region from (A). **(C)** The joint region forms a furrow (black arrow) with a number of compact cells (white arrows). Sections were stained with Toluidine Blue O. Scale bars = 1 mm (A). dist. = distal segment; prox. = proximal segment; J = joint region; XC = exocarp; MC = mesocarp; OW = ovary wall; ena = endocarp *a*; enb = endocarp *b*.

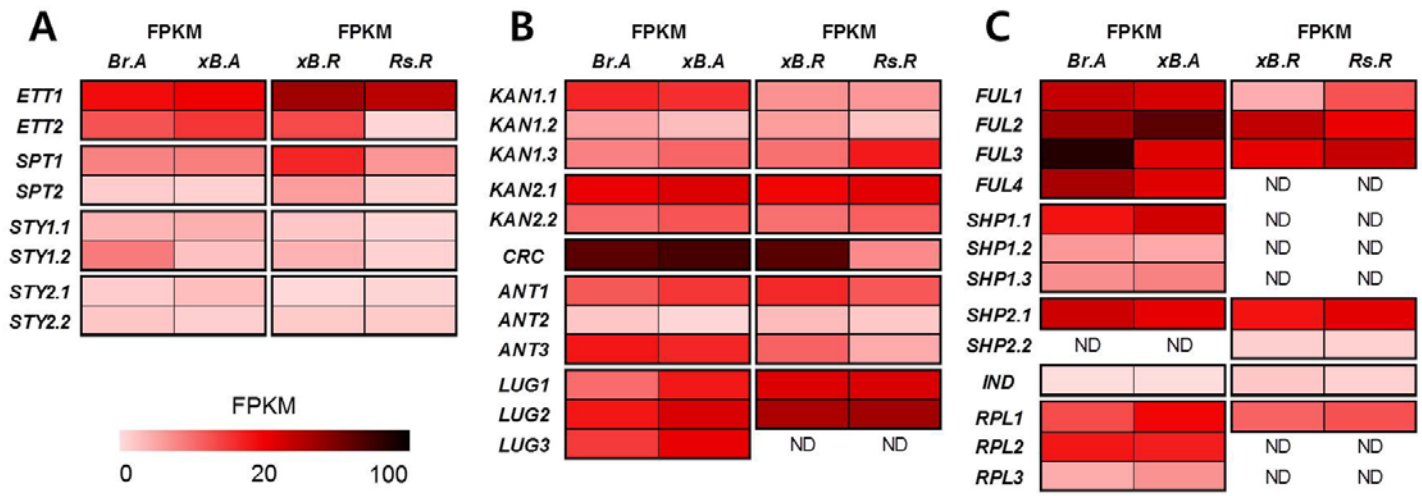

Figure 6. Expression levels of genes controlling gynoecium and fruit development from RNA-seq

FPKM values from RNA-seq of inflorescence **(A)** Genes involved in gynoecium development (apical to basal axis). **(B)** Genes related to mediolateral axis development. **(C)** Genes of post-fertilization fruit development. * ND : Not determined

Br. A = A genome of *Brassica rapa*; *Rs*. R = R genome of *Raphanus sativus*; x*B*. A, R = A, R genome of x*Brassicoraphanus*;

ETT = ETTIN; *SPT = SPATULA*; *STY1, 2 = STYLISH1, 2*; *KAN1, 2 = KANADI1, 2*; *CRC = CRABSCLAW*; *ANT = AINTEGUMENTA*;

LUG = LEUNIG; *FUL = FRUITFULL*; *SHP1, 2 = SHATTERPROOF1, 2*; *IND = INDEHISCENT*; *RPL = REPLUMLESS*

DISCUSSION

Heteroarthrocarpy of the hybrid of *Brassica* and *Raphanus* has long been described and its intermediate fruit shape has been used as an indicator of the hybridity. However neither the microscopic morphology nor the expression of fruit development genes has intensely been observed. In this study, I examined the fruit morphology and fruit development gene expression in a genetically stabilized intergeneric hybrid, x*Brassicoraphanus* obtained from a cross between *B. rapa* and *R. sativus*.

In microscopy analysis, the intermediate structures of x*Brassicoraphanus* pistil and silique were examined not only for external shapes but also for internal structures. The mature *B. rapa* fruit is composed of the two valves with the replum, displaying dehiscence (Figure 1A). In contrast, the fruit of *R. sativus* forms a single valve without the replum. It is always indehiscent after maturation (Figure 1C). The x*Brassicoraphanus* fruit have blended features of *B. rapa* and *R. sativus*. The distal segment resembles the fruit of *R. sativus*, which is an indehiscent part, while the proximal segment is dehiscent and similar to the *B. rapa*. The valves of proximal segment separate into two parts and then seeds are dispersed (Figure 1B). This is heteroarthrocarpy which has a bisected fruit shape consisting of two heteromorphic segments. This heteroarthrocarpic shape is observed in the pistil of x*Brassicoraphanus* as well. This feature of x*Brassicoraphanus* fruit is likely to be formed before fertilization (Figure 2).

I also observed the epidermal cells of *B. rapa*, x*Brassocpraphanus*, and *R. sativus* pistils. In x*Brassicoraphanus*, the boundary between the distal and

proximal segments is clear. The cell shapes of the replum and the valves of the proximal segment of x*Brassicoraphanus* pistils are similar to those of *B. rapa*, whereas the replum epiderma cells resemble those of *R. sativus*. The proximal and distal segments are completely separated by the joint (Figure 3).

One of the specific features of x*Brassicoraphanus* fruit is the formation of the joint region. The joint region exists in all fruits of three crops, but it is not clearly formed in *B. rapa* and *R. sativus*. This region is involuted with specific epidermal cell shapes (Figure 5). However, this feature is not unique to x*Brassicoraphanus*. The joint region is found not only in x*Brassicoraphanus* but also in its parents, *B. rapa* and *R. sativus*, and already formed during pistil development. The joint regions in *B. rapa* and *R. sativus* siliques are not fully developed and not detached. More observations are required for the details of the joint regions in *B. rapa* and *R.sativus* for lignification pattern and ovary wall structures.

After maturation, the x*Brassicoraphanus* fruit displays a dehiscent proximal part and an indehiscent distal part due probably to differential lignin accumulation in the replum and valves. In *B. rapa* and x*Brassicoraphanus* proximal segments, dehiscence occurs at the replum, which is bound to the valve margin. During maturation of fruit, a number of cells around the replum are lignified. Especially, lignin is accumulated in the endocarp *b* layer in valves and in the lignified layer at the valve margin. Dehiscence occurs in the separation layer of valve margin near the lignified layer. Both *B. rapa* and x*Brassicoraphanus* have similar valve margin structures,and therefore pod shattering and seed dispersal easily take place (Figure 4B-G). The replum of *R. sativus* and the distal segment of

x*Brassicoraphanus* are significantly different from the replum of *B. rapa*. Existence of the valve margin is unclear, and lignified and separation layers are hardly found. For this reason, the *R. sativus* fruit and the distal segment of x*Brassicoraphanus* remain indehiscent after maturation (Figure 4I-K, M-O).

These intermediate phenotypes of x*Brassicoraphanus* may result from altered gene expression of duplicated genes. Many genes that control fruit development have been studied in a model plant *Arabidopsis*. As a member of the same *Brassicaceae* family, most of the orthologous genes have been identified and many of them exist as multiple-copies (Figure 6). It has been proposed that altered gene expression after allopolyploidization is responsible for neomorphic and intermediate phenotypes. Heteroarthrocarpy of x*Brassicorphanus* would be a good example to test this hypothesis.

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초 록

지구상에 많은 현화식물의 진화 과정에서 교잡과 배수화는 중요한 역할을 했다. 특히 이종간의 교잡은 새로운 종의 출현을 가져온다. 일반적으로 종,속간교잡을 통해 새로운 식물체를 얻는 것은 매우 힘들고 그 식물체는 정상적인 임성을 갖지 못한다고 알려져 있다. 그러나 **'배무채'** (x*Brassicoraphanus*)는 배추 (*Brassica rapa* L.)와 무 (*Raphanus sativus* L.) 사이에서 성공적으로 교잡과 배수화를 거친 속간이질사배체 식물이다. 배무채는 여러 가지 특징을 가지고 있는데 눈에 띄는 특징 중에 하나는 암술과 꼬투리 즉, 과실의 복합적인 형태이다. 배무채의 암술과 과실은 배추와 무의 암술과 과실의 특징을 함께 가지고 있다. 본 연구에서는 암술과 과실의 형성과 과실의 열개에 초점을 맞추어 배무채의 생식기관의 발달을 연구하였다. 배무채의 화기구조는 부모인 배추와 무의 모양과 매우 비슷하지만 과실 모양은 배추와 무의 중간 형태를 띠고 있다. 예를 들어, 배무채 과실의 윗부분 (distal segment)은 무의 과실과 닮았다. 이 부분은 원통형의 형태로 되어있고 과실이 성숙한 후에 열개가 일어나지 않는다. 그러나 배무채 과실의 아랫부분 (proximal segment)은 배추의 과실과 일치하는 것을 확인하였다. 이 부분은 격막(replum)을 중심으로 두개의 밸브(valve)로 형성되어 있는데 과실이 성숙한 후에 이 구역에서 밸브가 분리되고 열개가 일어난다. 또한 흥미로운 것은 배무채 과실의 윗부분과 아랫부분

사이 연결지점에 가로로 놓인 특이한 틈이 선명하게 존재한다. 이를 접합지역 (joint region)이라 하는데 이 부분에 의하여 배추와 무를 닮은 부분이 명확하게 구분된다. 이렇게 배무채에서 과실의 형태와 발달에 관여하는 중복 유전자의 변화를 알아보기 위하여 모델식물인 애기장대의 과실 발달의 유전적 경로에 있는 주요한 유전자들의 상동 유전자 (orthologous gene)를 찾았다. 본 연구에서는 배무채와 그 부모인 배추와 무에서 유전자 발현 정도를 비교함으로써 과실의 형태와 발달을 설명하였다. 이는 유전체 교잡 후에 나타나는 새로운 형질에 관여하는 중복 유전자들의 발현이 어떻게 변화하는지 보여주는 좋은 예가 될 것이다.

주요어: 속간교잡, 배수화, 배무채, 이질사배체, 과실, 열개, 중복유전자.

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