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Review

The importance of reproductive barriers and the effect of allopolyploidization on crop breeding

Kaoru Tonosaki*[1)], Kenji Osabe[2)], Takahiro Kawanabe[3)] and Ryo Fujimoto[3)]

[1)] *Kihara Institute for Biological Research, Yokohama City University*, 641-12 Maioka, Totsuka, Yokohama, Kanagawa 244-0813, Japan
[2)] *Okinawa Institute of Science and Technology*, 1919-1 Tancha, Onna-son, Kunigami, Okinawa 904-0495, Japan
[3)] *Graduate School of Agricultural Science, Kobe University*, Rokkodai, Nada-ku, Kobe 657-8501, Japan

Inter-specific hybrids are a useful source for increasing genetic diversity. Some reproductive barriers before and/or after fertilization prevent production of hybrid plants by inter-specific crossing. Therefore, techniques to overcome the reproductive barrier have been developed, and have contributed to hybridization breeding. In recent studies, identification of molecules involved in plant reproduction has been studied to understand the mechanisms of reproductive barriers. Revealing the molecular mechanisms of reproductive barriers may allow us to overcome reproductive barriers in inter-specific crossing, and to efficiently produce inter-specific hybrids in cross-combinations that cannot be produced through artificial techniques. Inter-specific hybrid plants can potentially serve as an elite material for plant breeding, produced through the merging of genomes of parental species by allopolyploidization. Allopolyploidization provides some benefits, such as heterosis, increased genetic diversity and phenotypic variability, which are caused by dynamic changes of the genome and epigenome. Understanding of allopolyploidization mechanisms is important for practical utilization of inter-specific hybrids as a breeding material. This review discusses the importance of reproductive barriers and the effect of allopolyploidization in crop breeding programs.

Key Words: inter-specific hybrid, pre-zygotic barrier, endosperm barrier, hybrid necrosis, heterosis, genomic rearrangements, non-additive expression.

Introduction

Crop species have been improved for consumption by selecting for useful traits, and accumulating desirable genes from genetic resources. "Hybridization breeding" introduces agriculturally valuable traits into existing cultivars, such as biotic or abiotic stress tolerance, from breeding stocks or wild progenitors by intra-specific or inter-specific crossing. As genetic resources are limited in intra-species, inter-specific crossing is expected to contribute to further development of plant breeding programs.

In the process of hybridization breeding by inter-specific crossing, reproductive barriers, whose mechanisms maintain the genetic integrity of species or prevent gene flow with other species, challenge hybrid production. Reproductive barriers are roughly classified into two types, pre- (before fertilization) and post-zygotic (after fertilization) barriers. Pollination with other species is restricted by their natural habitat (geographic isolation), flower structure (i.e., shape or color), flowering time or pollen mediator, which are involved in pre-zygotic barriers (Lowry *et al.* 2008). Inter-specific incompatibility is the inhibition of pollen tube germination or growth. Inter-specific incompatibility is involved in post-zygotic barriers and occurs in the pistils when the pollen from other species pollinate the stigma (Dresselhaus *et al.* 2011). Even if pollen germination and fertilization are successful, embryo abortion or abnormal growth of endosperm (over- and under-proliferation of endosperm) causing disturbance of normal seed development are observed as post-zygotic barriers (Haig and Westoby 1991). Embryo abortion is thought to be caused by endosperm abnormality. Endosperm abnormality depends on the cross combination of species, direction of hybridization, and ploidy levels of parental species (Kinoshita 2007).

After hybrid seed formation, there are still reproductive barriers. Inter-specific hybrids sometimes show developmental defects compared to the parental lines, which is termed hybrid necrosis (or hybrid weakness). Furthermore, hybrid sterility caused by epistatic interactions between nuclear genes or between the nuclear genome and the maternally derived mitochondrial genome, termed cytoplasmic

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*Corresponding author (e-mail: tonosaki@yokohama-cu.ac.jp)

male sterility (CMS), remains as a post-zygotic barrier. However, male sterility is sometimes useful for breeding, and CMS is successfully applied to harvest the F_1 hybrid seeds (Yamagishi and Bhat 2014). Several genes involved in hybrid sterility or CMS in rice have been isolated by a genetic approach (Fujii and Toriyama 2008). Overcoming reproductive barriers expands the possibility of hybridization breeding, and understanding the molecular mechanism of reproductive barriers is becoming more important.

There are polyploid species not only in wild species, but also in cultivated crops. Polyploids include important crops, such as maize, cotton, wheat, canola, tobacco, potato, and strawberry, indicating that polyploidization can potentially generate useful traits for cultivation. In plant speciation, whole-genome duplication that can lead to genetic redundancy and functional divergence of paralogous genes is considered as an important event related to the increase of genetic diversity (Innan and Kondrashov 2010). There are two types of polyploid species, autopolyploid and allopolyploid. Autopolyploid species are formed by multiplication of the genome from a single species. Genome size is increased by genome multiplication and the multiplied genes can provide useful traits for agriculture, such as enhanced photosynthesis or enlargement of fruit size by expansion of cell size (Lavania *et al.* 2012). Allopolyploid species are formed by merging genomes from two or more distantly related species. The merging genomes from divergent species not only leads to functional divergence of paralogous genes, but also heterozygosity (Chen 2010, Comai 2005, Osborn *et al.* 2003). Heterozygosity has advantages such as heterosis, an increase in genetic diversity, and phenotypic variability. Heterosis can give rise to growth vigour and an increase in crop yield. Genetic diversity and phenotypic variability are accompanied by various changes including genomic rearrangements by multivalent chromosome formation, homoeologous recombination, chromosome elimination, and transcriptome alteration (Adams *et al.* 2003, Gaeta and Chris Pires 2010, Otto 2007), some of which may play a role in generating desirable traits for cultivation.

This review focuses on the reproductive barriers in inter-specific hybridization and the dynamic genetic or epigenetic changes in inter-specific hybrids. We introduce the technical or genetic methods to overcome these reproductive barriers, molecular mechanism of reproductive barriers (especially pollen rejection, endosperm barriers and hybrid necrosis), and the dynamic changes caused by allopolyploidization.

1. Overcoming reproductive barriers

Producing inter-specific hybrids is an effective way to increase genetic diversity for breeding programs. However, it is necessary to overcome reproductive barriers. Artificial techniques to overcome the reproductive barriers have been developed for producing inter-specific hybrids. The use of genetic information to determine the crossability is also effective for hybridization breeding. In many plant species, different accessions of parental species have different crossability in inter-specific crossing, indicating that some genetic factors are important for the efficient production of inter-specific hybrids. Identification of key genes for inter-specific hybridization will allow rapid production of hybrids and contribute to accelerating the breeding program.

1-1. Artificial techniques for producing inter-specific hybrids

Techniques for producing inter-specific hybrids have been developed in many crops. There are several methods for overcoming inter-specific incompatibility as a pre-zygotic barrier. It has been suggested that these barriers are weaker at the early floral development stage; therefore, the bud-pollination method (pollinating stigmas of buds two to three days before anthesis) has been used in Brassicaceae (Hiscock and Dickinson 1993, Udagawa *et al.* 2010) and Solanaceae species (Gradziel and Robinson 1991, Kuboyama *et al.* 1994). Inter-specific incompatibility occurs at the stigma or style, and pollinating to a cut section of a stigma or style (cut-style pollination method) has been used in Liliaceae (Van Tuyl *et al.* 1988). Heat treatment of pistils or pollen has also been successful to overcome pre-zygotic barriers in Liliaceae (Matsubara 1981).

As a post-zygotic barrier, embryo abortion occurs during inter-specific crossing, especially between distantly related species. To overcome this barrier, embryo rescue techniques, including ovary, ovule, and embryo culture, have been developed (Van Tuyl *et al.* 1991). The timing of embryo abortion is different for each parental combination, and suitable method for embryo rescue in each inter-specific cross is required. If the embryo abortion occurs at the early stage, immediately after fertilization, ovary culture can be applied where young ovaries collected after pollination can be grown on tissue culture medium. Mature seeds can be obtained through ovary culture in some inter-specific crosses (Takahata 1990, Takahata and Takeda 1990). When mature seeds cannot be obtained by ovary culture or hybrid embryo because of abortion caused by the mismatch between embryo and endosperm development, ovule and embryo culture methods are suitable (Bajaj *et al.* 1986, Iwai *et al.* 1986). The embryo culture method rescues immature embryos by growing the embryo on the medium, and it is the most effective method applied in many inter-specific crosses (Sharma *et al.* 1996). The ovule culture method removes the immature ovules from the ovaries, and the ovules are germinated on the medium. This method is useful for plant species with small ovules when the dissection of immature embryos from the ovule is technically difficult.

Inter-specific hybrids between distantly related species are generally sterile or partially sterile. One of the reasons for hybrid sterility is reduced chromosome paring during meiosis. Hybrid sterility can often be restored by zygotic or somatic chromosome doubling through cell cycle disruption by antimitotic agents such as colchicine, oryzalin, or trifluralin

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(Dhooghe *et al.* 2011). Nitrous oxide (N_2O) is also applied as a polyploidizing agent in lilies (Nukui *et al.* 2011).

1-2. Key genes for inter-specific hybridization

Key genes for crossability have been identified by genetic analysis of intra-specific genetic variation. In wheat (*Triticum aestivum*), two loci carrying dominant genes, *Kr1* and *Kr2*, located on the long arm of chromosome 5B and 5A, respectively, affect the crossability in inter-specific crossing between wheat and rye (*Secale cereale*) (Lein 1943). Dominant alleles of *Kr1* and *Kr2* show poor crossability caused by inhibition of pollen germination and pollen tube growth before fertilization (Jalani and Moss 1980, Lange and Wojciechowska 1976, Riley and Chapman 1967). Most wheat cultivars with dominant alleles (*Kr1* and *Kr2*) show low crossability in inter-specific crosses with rye; however, 'Chinese spring' and 'Hope', which have recessive alleles (*kr1* and *kr2*), show high crossability (Riley and Chapman 1967). Other crossability loci, *Kr3* located on chromosome 5D (Krolow 1970), *Kr4* on chromosome 1A, and *SKr* on the short arm of chromosome 5B (Tixier *et al.* 1998) have been identified. These *Kr* genes have different effects on the crossability between wheat and rye; *Kr1* has a stronger effect than other *Kr* genes (Krolow 1970, Riley and Chapman 1967). Moreover, wheat *Kr* genes affect the crossability with cultivated barley *Hordeum vulgare* (Koba and Shimada 1992), wild barley *Hordeum bulbosum* (Snape *et al.* 1979), sorghum *Sorghum bicolor* (Inagaki and Mujeeb-Kazi 1995) and *Aegilops squarrosa* (syn. *tauschii*) (Koba and Shimada 1993). Although fine mapping of *Kr1* (Bertin *et al.* 2009) and the *SKr* locus have been carried out (Alfares *et al.* 2009), *Kr* genes have not been isolated and their molecular functions are not clear.

In Brassicaceae, the two lines of *Brassica rapa*, 'Shogoin-kabu' and 'Chiifu' show different crossability to *Raphanus sativus*; 'Shogoin-kabu' can produce several seeds when crossed with *R. sativus*, whereas 'Chiifu' does not produce any seeds. Three QTLs for crossability, *qBrHFA-1*, *qBrHFA-2*, and *qBrHFA-3*, which control embryo abortion, have been identified using a segregating population between 'Shogoin-kabu' and 'Chiifu' (Tonosaki *et al.* 2013). Moreover, the combination of *qBrHFA-1* and *qBrHFA-3* is important for increasing crossability with *R. sativus*.

Although most cultivars of wheat and *B. rapa* do not have high crossability for inter-specific crossing, a few cultivars such as "Chinese spring" of wheat and "Shogoin-kabu" of *B. rapa* show high crossability. This suggests that these cultivars gained high crossability by the mutation of the key genes during breeding steps. Thereby, varieties of other crops may also have a high crossability caused by mutation of these key genes. The generation of DNA markers based on this genetic information may be useful for effective production of inter-specific hybrids, and hybridization breeding.

2. Molecular evidence of reproductive barriers

2-1. Pre-zygotic barriers

Pre-zygotic barriers in inter-specific crossing may be composed of multi-steps. Pollen tube guidance in angiosperms is a multi-stage mechanism (Okuda and Higashiyama 2010), which leads the pollen tube to the ovule from the stigma via the pistil by recognition of its own pollen (**Fig. 1A–1F**). It is thought that defective pollen tube guidance interferes with the plant reproductive mechanisms; therefore, all steps of pollen tube guidance potentially lead to a pre-zygotic barrier. Some studies on pollen tube guidance provide insights into pre-zygotic barriers by inter-specific pollen recognition (**Fig. 1G–1J**).

Pre-zygotic barriers and self-incompatibility

The first pre-zygotic barrier is pollen rejection; the inhibition of pollen hydration, germination, or pollen tube growth (**Fig. 1G**, **1H**). These behaviors of pollen rejection are similar to that of self-incompatibility, which is regulated by a single multi-allelic locus, the *S* locus, in dicots (Dresselhaus *et al.* 2011, Murfett *et al.* 1996). In sporophytic self-incompatibility of Brassicaceae plants, the inhibition of pollen hydration or germination starts from an *S* haplotype specific interaction between the female *S*-determinant (*S*-locus receptor kinase (SRK)) and the male *S*-determinant (*S*-locus protein 11/*S*-locus-cysteine-rich protein (SP11/SCR)) in the stigma (Watanabe *et al.* 2003). In addition to genes, M-locus protein kinase (MLPK) was identified as a factor of self-incompatibility (Murase *et al.* 2004). In Solanaceae, Rosaceae, and Plantaginaceae plants with gametophytic self-incompatibility, the female *S*-determinant (S-RNase), which has ribonuclease activity, and the male *S*-determinant (*S*-locus F-box (SLF/SFB) protein), which is a component of the E3 ubiquitin ligase complexes, have been identified (Entani *et al.* 2003, Lai *et al.* 2002, Sijacic *et al.* 2004). Additionally, modifier genes, asparagine-rich HT proteins (HT-B) in tobacco and two HT proteins (HT-A and HT-B) in tomato, which play a role in pollen rejection and do not contribute to *S* specificity, were identified (Goldraij *et al.* 2006, O'Brien *et al.* 2002).

In inter-specific crosses, unilateral incompatibility, which occurs when pollination between species is successful in only one direction, is often observed. It often occurs in crosses between self-compatible and self-incompatible species in Solanaceae species. Pollen from self-compatible species is rejected by pistils of self-incompatible species, while pollen from self-incompatible species is not rejected by pistils of self-compatible species. This relationship is called the "SI × SC rule". The molecular mechanism controlling self-incompatibility and inter-specific unilateral incompatibility may be linked in both Brassicaceae species with sporophytic self-incompatibility (Hiscock and Dickinson 1993) and Solanaceae species with gametophytic self-incompatibility (Lewis and Crowe 1958, Murfett *et al.* 1996).

Two lines of *B. rapa* stigmas pollinated with *Brassica oleracea* pollen grains show different pollen tube growth.

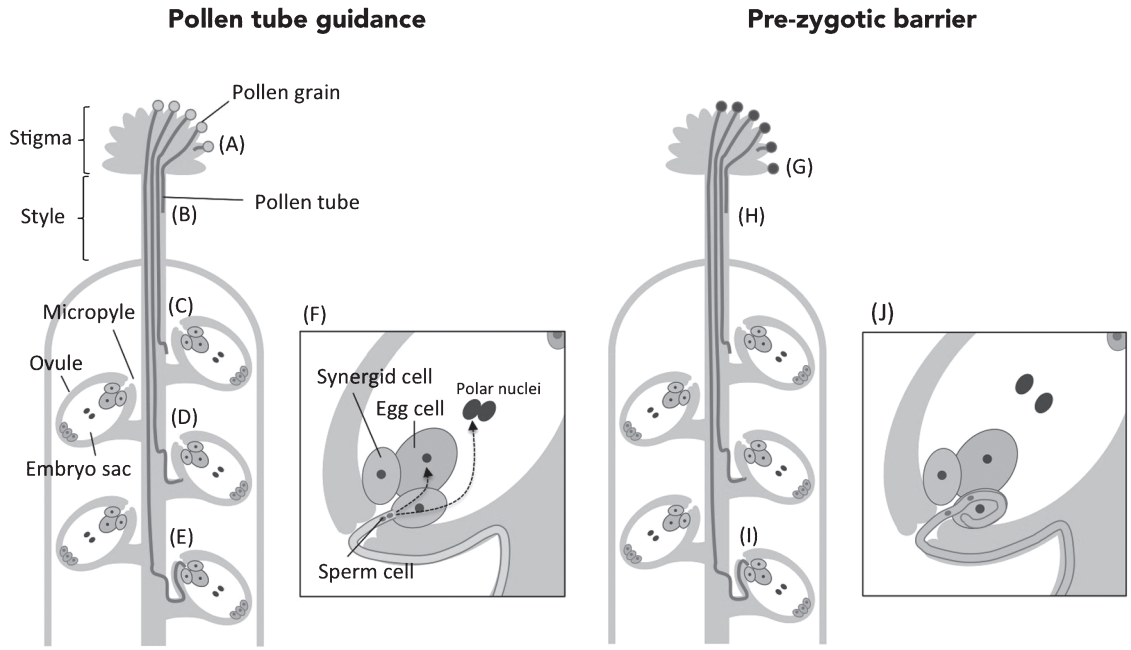

Fig. 1. Schematic representations of multi-stage controlled pollen tube guidance and pre-zygotic barriers. Pollen tube guidance involves multi-stage controls (A–F). In pollen tube guidance, the pollen tube enters the stigma after pollen germination (A), and elongates the pollen tube through the stigma and style (B). The pollen tube penetrates the transmitting tract (C), grows on the inner surface of the ovule (D), and enters the micropyle toward the synergid cell (E). Finally, the pollen tube releases the sperm cells (F). Pre-zygotic barriers can also be a multi-stage mechanism (G–J). In the case of pollination with other species, pollen hydration and germination is inhibited on the stigma (G) and pollen tube elongation through stigma and style is prevented (H). In some inter-specific hybrids, these steps (G–H) may involve *S*-locus genes, which regulate self-incompatibility. The pollen tube is attracted by attractants, LURE peptide, in a species-preference manner (I). Pollen tubes entering the micropyle cannot burst to release the sperm cells, and overgrow inside the female gametophyte (J).

Pollen from *B. oleracea* is rejected on pistils of self-incompatible lines of *B. rapa*, while pollen from *B. oleracea* is not rejected on pistils of self-compatible lines of *B. rapa*, which lack functional SP11, SRK, and MLPK (Fujimoto *et al.* 2006, Murase *et al.* 2004). However, this difference of inter-specific incompatibility in stigma may not involve the female determinants of self-incompatibility, SRK, and MLPK (Udagawa *et al.* 2010). Molecular genetic evidence that links the mechanisms of self-incompatibility and inter-specific unilateral incompatibility has not been clarified in Brassicaceae species.

Cultivated tomato (*Solanum lycopersicum*) is self-compatible and has lost the function of *S*-RNase, HT-A, and HT-B (Kondo *et al.* 2002). The introduction of functional *S*-RNase and HT genes from self-incompatible wild tomato (*Solanum peruvianum*) into self-compatible cultivated tomato causes inter-specific unilateral incompatibility, while a functional *S*-RNase alone did not (Tovar-Mendez *et al.* 2014). Similarly, in another Solanaceous genus, *Nicotiana*, transgenic plants with *S*-RNase and *HT* expression in *Nicotiana tabacum* show inter-specific unilateral incompatibility (Hancock *et al.* 2005, Murfett *et al.* 1996). These results suggested that the mechanism of pollen rejection of unilateral incompatibility overlaps with that of *S*-RNase dependent self-incompatibility. However, there are cases of an *S*-RNase-independent pollen rejection system.

In the genetic analysis of inter-specific unilateral incompatibility of the pollen side, two loci, *ui1.1* and *ui6.1*, have been identified as factors of male-determinant for inter-specific unilateral incompatibility (Chetelat and Deverna 1991, Li *et al.* 2010). *ui1.1* and *ui6.1* encode SLF-23 and Cullin1 proteins, which are components of the SCF-type (Skp1, Cullin1, F-box) and ubiquitin ligase complexes (Li and Chetelat 2010, 2015), respectively. Cultivated tomato species and two wild relatives of cultivated tomato, which are self-compatible species, lack *SLF-23* and have a loss-of-function mutation in *Cullin1*. Two transgenes, *SLF-23* and *Cullin1* from self-incompatible species, introduced into cultivated tomato, are sufficient to convert pollen rejection phenotype from unilateral incompatibility to compatible in inter-specific crossing (Li *et al.* 2010, Li and Chetelat 2015). Moreover, knockdown of *Cullin1* loses self-incompatibility in *Solanum arcanum* (Li and Chetelat 2014), indicating that the SCF-type ubiquitin ligase complex may be involved not only in inter-specific unilateral incompatibility but also in self-incompatibility.

Species-preference of pollen tube attraction

Synergid cells play an important role in pollen tube guidance (**Fig. 1E**) (Higashiyama *et al.* 2006). In *Torenia fournieri*, LUREs, which are secreted cysteine-rich polypeptides derived from synergid cells, have been identified as attractants (Okuda *et al.* 2009). LUREs of *T. fournieri* do

not attract the pollen tube of the closely related species, *Lindernia micrantha*, indicating pollen tube guidance by LUREs acts in a species-preferential manner (**Fig. 1I**) (Okuda *et al.* 2009). LUREs are also attractants in *A. thaliana* and *A. lyrata*. AtLURE1 peptide showed species-preference, while AlLURE1 peptide did not. In transgenic plants of *T. fournieri* expressing AtLURE1 in synergid cells, the pollen tube of *A. thaliana* reached the embryo sac of *T. fournieri*, suggesting that expression of *AtLURE1* is sufficient to overcome inter-specific barriers in micropylar pollen tube guidance and penetration of the embryo sac (Takeuchi and Higashiyama 2012). Inter-specific hybrids between *A. thaliana* and *A. lyrata* can be obtained by pollinating *A. thaliana* stigma with pollen from *A. lyrata* by the cut-style pollination method; however, *A. thaliana* pollen cannot germinate on the *A. lyrata* stigma (Fujimoto *et al.* 2008). However, AlLURE1 has the ability to attract *A. thaliana* pollen, suggesting that the avoidance of pollen rejection on stigma by the cut-style pollination method may be applied to obtain reciprocal hybrids.

Species specificity of the pollen tube burst

The pollen tube bursts after entering into the micropyle in the last steps of pollen tube growth, and discharges two sperm cells into the female gametophyte for double fertilization (**Fig. 1F**) (Hamamura *et al.* 2011). In the *A. thaliana feronia* mutant, the pollen tube overgrows in the female gametophyte, and is unable to burst after entering the micropyle (**Fig. 1J**) (Huck *et al.* 2003, Rotman *et al.* 2003). The *FERONIA* gene encodes a receptor-like kinase, and is localized to the filiform apparatus of synergid cells. It has been suggested that FERONIA receives a male ligand, and triggers a signal cascade of feedback from the synergid cell to the pollen tube, causing an arrest of pollen tube growth, and release of sperm cells (Escobar-Restrepo *et al.* 2007). In inter-specific crossing of *A. thaliana* with pollen from a related Brassicacese species, the pollen tube showed overgrowth in the female gametophyte, similar to the phenotype observed in the *feronia* mutant (**Fig. 1J**). In the cross with *A. lyrata*, around half of the ovules received pollen tubes and were fertilized, but the others had pollen tubes that overgrew inside the female gametophyte. In the cross with a more distant relative, *Cardamine flexuosa*, around 70% of pollen tubes entered *A. thaliana* ovules, and displayed a *feronia*-like phenotype. This phenotype in inter-specific crossing correlated with high sequence divergence in the extracellular domain of FERONIA (Escobar-Restrepo *et al.* 2007), allowing us to speculate that the FERONIA pathway is involved in the pre-zygotic barrier just before fertilization in inter-specific crossing.

2-2. Reproductive barriers in endosperm

Embryo abortion in inter-specific crossing is thought to be caused by endosperm abnormality. In flowering plant, double fertilization gives rise to the embryo and endosperm (Berger *et al.* 2008). The endosperm is a triploid tissue with an unequal parental genomic contribution (the sperm nucleus (n) fuses with the two polar nuclei (2n) in the central cell), and it is a storage tissue for the control of nutrient transfer to the embryo during seed development and for seedling development after germination. In many cases of inter-specific crossing, embryo abortion phenotypes are observed together with endosperm abnormality, and can be overcome by embryo rescue techniques compensating with nutrients from the medium. This suggests that the major factor that is responsible for embryo abortion is the scarcity of nutrient supply during embryo development due to the failure of endosperm development.

The mechanism of endosperm abnormality

The abnormality of hybrid endosperm shows contrary phenotypes depending on the direction of the cross, especially when the parental lines have different ploidy levels. For example, when the ploidy level of the female parent is higher (maternal excess), under-proliferation of endosperm and the promotion of seed storage synthesis are observed. By contrast, when the ploidy level of the male parent is higher (paternal excess), over-proliferation of the endosperm and alteration of seed storage synthesis are observed (Scott *et al.* 1998, Sekine *et al.* 2013). Both under- and over-proliferation of hybrid endosperm are observed in many plant species, and it may be due to a general mechanism for preventing hybrid seed development. In classical genetic studies, models of 'endosperm balance number' in potatoes (Johnston *et al.* 1980) or 'polar nuclei activation' in oats (Nishiyama and Yabuno 1978) have suggested that the genome balance between male and female parents is important for hybrid endosperm development in inter-specific crossing. In inter-specific crossing, the balancing of parental genome dosages by manipulation of ploidy levels is required for successful hybrid seed development (Johnston and Hanneman 1982, Josefsson *et al.* 2006). These results support the importance of parental genome balance in hybrid endosperm.

Determinants of the parental genome balance

Recent studies suggest that disturbance of the genome balance is caused by the epigenetic misregulation of imprinted genes (Kinoshita 2007). Genome imprinting is when the two alleles of a gene are expressed at different levels depending on their parent of origin, and is known to be regulated by epigenetic mechanisms. In flowering plant, genomic imprinting mainly occurs in the endosperm, and many imprinted genes have been identified by high-throughput sequencing techniques (Hsieh *et al.* 2011, Luo *et al.* 2011, Waters *et al.* 2011, Wolff *et al.* 2011). In *A. thaliana*, many mutants showing endosperm abnormality have been identified, some of which are imprinted genes. For example, the mutants of *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT SEED2* (*FIS2*), which encode components of the Polycomb repressive complex 2 (PRC2), show extension of the syncytial phase, delay of cellularization of the endosperm (over-proliferation), and arrest of embryo development (Chaudhury *et al.* 1997, Grossniklaus *et al.* 1998, Kiyosue *et al.* 1999). In particular, the *fis2* mutant not only

delays the timing of endosperm cellularization, but also decreases the translocation rate of hexoses to the embryo (Hehenberger *et al.* 2012). *MEA* and *FIS2* are imprinted genes in the endosperm, expressed only from the maternal allele (Jullien *et al.* 2006, Kinoshita *et al.* 1999). The PRC2, including MEA and FIS2, has an important role in the development of endosperm.

The PRC2 complex is conserved in mammals, Drosophila, and plants, and has methyltransferase activity, trimethylation of lysine 27 on histone H3 (H3K27me3) at target genes. Type-I MADS transcription factors, *AGAMOUS-LIKE GENE 62* (*AGL62*) and *PHERES1* (*PHE1*), are downstream genes regulated by PRC2 (Kang *et al.* 2008, Köhler *et al.* 2005). The delay of cellularization of the endosperm and decrease of the translocation rate of hexoses to embryo observed in *fis2* mutant are suppressed in double mutants of *fis2* and *agl62*, suggesting that AGL62 regulates endosperm development, especially during cellularization (Hehenberger *et al.* 2012). *PHE1* is an imprinted gene expressed only from the paternal allele and plays a role in endosperm development, because suppression of *PHE1* in the *mea* mutant background prevents over-proliferation of the endosperm (Köhler *et al.* 2005).

Endosperm abnormality and PRC2 regulation

Endosperm abnormality is not only observed in inter-specific crosses, but also in crosses between parents of different ploidy (inter-ploidy cross). Increased paternal contribution by paternal excess during inter-ploidy crossing shows over-proliferation of the endosperm (Scott *et al.* 1998, Sekine *et al.* 2013). In the abnormal endosperm of paternal excess, expression of *MEA* is decreased, and expression of PRC2 target genes is increased; moreover, over-expression of *MEA* can restore normal endosperm development (Erilova *et al.* 2006). This indicates that reduced PRC2 function is likely to be responsible for endosperm abnormality in inter-ploidy crossing. On the other hand, in inter-specific crossing between *A. thaliana* and its close relative, *A. arenosa*, the hybrid endosperm shows over-proliferation and increased expression of PRC2 target genes such as *PHE1* and *AGL62* (Josefsson *et al.* 2006, Walia *et al.* 2009). Similarly, endosperm abnormality and the alteration of expression of *OsMADS87*, a *PHE1* orthologue, are observed in inter-specific hybrids between rice and related wild species (Ishikawa *et al.* 2011). The maternal *phe1* mutation improves the production of inter-specific hybrid seeds (Josefsson *et al.* 2006), suggesting that endosperm abnormality is attributed to misexpression of PRC2 target genes, which is caused by alteration of PRC2 regulation, in the hybrid endosperm. Some PRC2 components were identified as being involved in the post-zygotic barrier in the seeds of inter-specific hybrids in the inter-specific crossing of *Arabidopsis* (Burkart-Waco *et al.* 2013) or *Brassica* crops (Tonosaki *et al.* 2013). These results support PRC2 being related to endosperm abnormality in inter-specific crossing.

In inter-specific crossing, manipulation of ploidy levels was able to overcome endosperm abnormality (Johnston and Hanneman 1982), and moderate the misexpression of PRC2 target genes (Bushell *et al.* 2003, Johnston and Hanneman 1982). Disturbance of the genome balance may alter PRC2 regulation by a change in PRC2 dosage between parental species, and give rise to misexpression of PRC2 target genes (**Fig. 2A**). It has been suggested as a "quantitative difference" in inter-specific hybrids (Schatlowski and Köhler 2012). On the other hand, the endosperm abnormality and alteration of gene expression are observed in inter-specific crossing between cultivated rice and its close relatives, which have the same genome size and chromosome number (Ishikawa *et al.* 2011), indicating that endosperm abnormality occurred in inter-specific crossing without the quantitative difference between parental species. Due to the diversity of the amino acid sequences of the PRC2 component from different species in hybrid endosperm, the complex can be adversely affected. The formation of the functional PRC2 complex of maternally derived polycomb genes is limited in the hybrid endosperm (Ishikawa and Kinoshita 2009). Therefore, there is not enough functional PRC2 to regulate target genes of each allele from the two parental species leading to a "qualitative difference" in interspecies (**Fig. 2B**).

Other factors in post-zygotic barriers

From the comparison of transcriptomes between hybrid seeds three days after pollination (DAP) in *A. thaliana* Columbia × *A. arenosa* (incompatible hybrid) and *A. thaliana* C24 × *A. arenosa* (compatible hybrid), misexpression of endosperm and seed regulator genes, and activation of ribosomal, photosynthesis, stress-related, and immune response genes have been identified (Burkart-Waco *et al.* 2013). The loss-of-function mutants of *TRANSPARENT TESTA GLABRA2* (*ttg2*) and *HAIKU1* (*iku1*) genes, which are endosperm growth factors, used as the maternal parent, showed partial improvement in seed survival when crossed to *A. arenosa*, suggesting that misregulation of genes involved in endosperm development causes hybrid incompatibility (Burkart-Waco *et al.* 2013). The loss-of-function of *NON-EXPRESSOR OF PATHOGENESIS RELATED 1* (*NPR1*) and *SALICYLIC ACID INDUCTION-DEFICIENT 2* (*SID2*), whose functions are in the defense response, also showed increased crossability, suggesting that a defense response dependent on the salicylic acid pathway is involved in the reproductive barrier.

2-3. Hybrid necrosis

Hybrid necrosis is one of the negative results of both intra- and inter-specific hybrids. Necrosis can be temperature dependent (Bomblies and Weigel 2007), and cause slow growth, wilting, discoloration, and lethality. Recent studies revealed that hybrid necrosis is caused by an autoimmune-like response through epistatic interaction between resistance (*R*) genes (Bomblies *et al.* 2007, Jeuken *et al.* 2009), and that the Dobzhansky-Muller model explains hybrid necrosis caused by the deleterious interaction between lineage-specific alleles at two or more loci

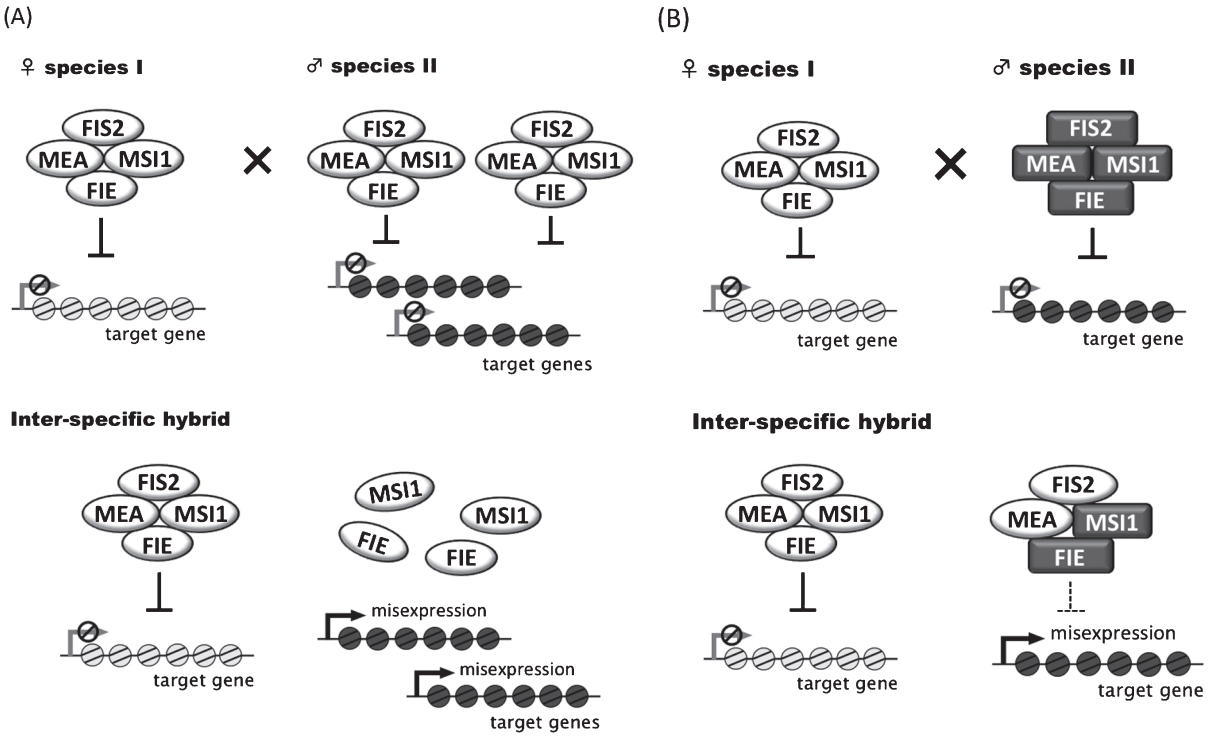

Fig. 2. Regulation of target genes by PRC2 in inter-specific hybrid endosperm. (A) Alteration of PRC2 regulation by quantitative difference. The PRC2 proteins MEA, FIS2, FIE and MSI1 form a complex and repress target genes. The balance of PRC2 and target genes is different between parental species. In hybrid endosperm between maternal species I and paternal species II, misexpression of target genes is observed because maternally imprinted genes, *MEA* and *FIS2*, are not expressed from paternal species II. (B) Alteration of PRC2 regulation by qualitative difference. Two types of PRC2 complex, functional PRC2 and chimera complex, formed in hybrid endosperm. Chimera complex is assembled from MEA and FIS2, which are expressed only when they are derived from maternal species I allele, and FIE and MSI1 from species II allele. Chimera complex may be adversely affected by the diversity of the amino acid sequence of the PRC2 component.

(Dobzhansky 1937, Muller 1942).

In inter-specific hybrids in lettuce (*Lactuca sativa* and *Lactuca saligna*), hybrid necrosis is caused by the interaction between the *L. sativa* allele at the C6 locus and the *L. saligna* allele at the C9 locus. Segregation analysis found *Rin4* at the C9 locus, which has the highest sequence similarity to *RIN4* (RPM1 INTERACTING PROTEIN 4) of *A. thaliana*. *Rin4* of *L. saligna* can cause severe necrosis in *L. sativa* when the C6 locus of *L. sativa* is present, but *Rin4* of *L. sativa* and *L. virosa* does not lead to necrosis. There are six amino acid differences between Rin4 of *L. sativa* and *L. saligna*, and three of these changes in *L. sativa* Rin4 are required for hybrid necrosis (Jeuken *et al.* 2009).

Triploid hybrids (ABD) between tetraploid wheat (AABB) and diploid *Aegilops tauschii* (DD) showed abnormal growth phenotypes, some that are caused by two types of hybrid necrosis (type II and type III); type II necrosis occurs under low temperature conditions (4°C), while type III occurs gradually without temperature dependence (Mizuno *et al.* 2010). Segregation tests revealed that type II and type III necrosis are associated with a single genetic locus on the short arm of the D genome, chromosome 7D and 2D, respectively (Mizuno *et al.* 2010, 2011). Temperature-dependent hybrid weakness is also observed in inter-specific hybrids in rice (Chen *et al.* 2014, Fu *et al.* 2013). One high-temperature dependent hybrid weakness is caused by the interaction between *Hwi1* in wild rice, *Oryza furipogon*, and *Hwi2* in indica rice, *Oryza sativa* (Chen *et al.* 2014). *Hwi1* and *Hwi2* encode two LRR-RLK genes and putative subtilisin-like protease, respectively, and it has been considered that the activation of the autoimmune response by the interaction between these two genes causes hybrid weakness (Chen *et al.* 2014).

3. Effects of allopolyploidization

3-1. Heterosis

In the process of generating inter-specific hybrids, heterosis phenotype caused by heterozygosity of different species can be fixed through chromosome doubling, and may contribute to adaptation of allopolyploids and selection for breeding. Artificially synthesized *Brassica juncea* (AABB genome) sometimes show biomass heterosis relative to the parental species of *B. rapa* (AA genome) and *B. nigra* (BB genome), but are dependent on the hybrid combinations of parental lines (Bansal *et al.* 2012). Biomass heterosis is also observed in inter-specific hybrids between allopolyploid species *B. napus* (AACC genome) and one of its progenitor

species, *B. rapa* (Liu *et al.* 2002, Qian *et al.* 2003). Inter-subgenomic heterosis has been observed in *B. napus* introgressed with partial *B. rapa* genome, and introgression of the *B. rapa* genetic component into *B. napus* has been used for generating a new type of *B. napus* (Qian *et al.* 2005). In a study of *B. rapa* and *B. napus*, novel genomic alterations post-hybridization were associated with the greatest number (compared to allelic contribution by *B. rapa*) of QTLs for agronomic traits (Zou *et al.* 2011).

Hybrids between *A. thaliana* and related species of *A. arenosa*, *A. lyrata* or *A. halleri* show a hybrid-vigour-like phenotype (Fujimoto *et al.* 2011, Ni *et al.* 2009). In the inter-specific hybrid between *A. thaliana* and *A. arenosa*, key genes of circadian rhythm, such as *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), *LHY1* (*LATE ELONGATED HYPOCOTYL 1*), *TOC1* (*TIMING OF CAB EXPRESSION 1*), and *GI* (*GIGANTEA*), showed a differential expression pattern between the hybrid and its parental lines (Ni *et al.* 2009). Circadian rhythm plays a role in plant growth and development, and the transgenic plants down-regulated in *CCA1* expression during the day increased its biomass. Thus, the authors considered that alteration of circadian rhythm genes in inter-specific hybrids results in the vigour phenotype (Ni *et al.* 2009).

3-2. Chromosome dynamics

Hybridization can lead to dynamic changes of the chromosomes in response to the "genomic shock" of the merging genomes. During this process, major (elimination, disjunction and doubling of chromosome) and minor (deletion or inclusion of DNA sequences, gene conversion and rDNA loci changes) chromosome changes are known to occur. Many of these chromosome changes occur after allopolyploidization events (hybridization of different species leading to doubling of the genome), and the effect of hybridization is thought to have greater influences on gene expression than the doubling of homologous chromosomes (Adams 2007). These chromosomal changes induced by hybridization can cause transcriptional changes that can ultimately affect the phenotype of the plants.

Homoeologous chromosome pairing

During meiosis, homologous/homoeologous chromosomes are paired to form so-called bivalents (association of three or more chromosomes is called multivalent). The formation of bivalents involves homologous recombination, which exchanges genetic information between paired homologous/homoeologous chromosomes, and results in segregation of a novel set of chromosomes into gametophytic cells. Understanding of the molecular mechanism of homologous/homoeologous pairing may be useful to control desired traits for breeding programs. For example, homoeologous recombination is well documented in *B. napus* (Cifuentes *et al.* 2010, Gaeta and Chris Pires 2010, Nicolas *et al.* 2008, 2009, Szadkowski *et al.* 2010), and homoeologous chromosome exchange in *B. napus* has been reported to affect seed yield (Osborn 2004) and Sclerotinia resistance (Zhao *et al.* 2006).

Chromosomally distant species hybrids generally show sterility due to chromosomal rearrangement on meiotic pairing. Generally, homologous chromosomes need to be associated for the allopolyploid hybrid plant to reduce chromosome number and recombine during meiosis. Pairing of chromosomes tend to favor homologs relative to homoeologs (Soltis and Soltis 2000), probably due to their similarity. The majority of chromosomes in wheat × barley hybrids was univalent, and an average of 0.7 bivalents per pollen mother cells was reported (Islam and Shepherd 1980). Similarly, bivalent formation was seen in allotriploid hybrids of *Musa acuminata* × *Musa balbisiana*, allotetraploid *Festuca pratensis* × *Lolium perenne*, and allohexaploid *Lycopersicon esculentum* × *Lycopersicon peruvianum* (Jeridi *et al.* 2011, Parokonny *et al.* 1997, Zwierzykowski *et al.* 2008). The occurrence of bivalent and multivalent pairings is naturally low, but is important for novel genetic exchanges between species.

In wheat, suppression of homoeologous chromosome pairing is known to be controlled by two key genes (*Ph1* and *Ph2*) and a number of minor genes (Mello-sampayo 1971). Homoeologous pairing in allohexaploid wheat and *Aegilops speltoides* (wild relative of wheat) was shown to be controlled by the *Ph1* locus, where deletion of *Ph1* allowed homoeologous chromosomes to pair (Riley and Chapman 1958, Sears 1976). Increased (average of 5.03–6.63) bivalent formation per pollen mother cell, and a small number of trivalents and quadrivalents were seen in wheat × barley hybrids using the *Ph* mutant of wheat (Sethi *et al.* 1986). Homoeologous pairing between wheat and *Haynaldia villosa* chromosomes was also demonstrated by inhibiting *Ph* function (Bhullar *et al.* 2014, Chen *et al.* 1994), supporting *Ph* as a key player in homoeologous pairing. Another gene, *PrBn*, in *B. napus* has also been identified as an important gene controlling homoeologous pairing (Jenczewski *et al.* 2003).

Uniparental chromosomal elimination

Uniparental chromosomal elimination is another genomic change seen in hybrids, which biases the inheritance of chromosomes from one parent, and eliminates the chromosomes from the other parent. Complete chromosome elimination prevents an opportunity for genetic recombination between parental genomes, suggesting that this phenomenon can be regarded not only as allopolyploidization events, but also as a factor of post-zygotic barriers. Chromosome elimination can be temperature dependent (Pickering and Morgan 1985, Sanei *et al.* 2011), and genomic reorganization may be triggered when the merging parental genomes of the hybrid are genetically distant (Lim *et al.* 2004). However, complete chromosomal elimination has been reported in inter-specific hybrids between closely related species *Hordeum vulgare* or *Hordeum parodii* × *H. bulbosum*, and *Hordeum marinum* × *H. vulgare*; hybrids of Brassica species (Finch 1983, Kasha and Kao 1970, Li *et al.* 2004, Subrahmanyam 1977), between remotely related parental

species (*Aegilops spp*, ryegrass (*Lolium multiflorum*), barley, oat, rye (*S. cereal*), and in crosses between wheat and *Pennisetum glaucum*, *S. bicolor*, *Tripsacum dactyloides*, *H. bulbosum*, *Coix lacryma-jobi*, *Imperata cylindrical*, pearl millet, or *Zea mays* (Barclay 1975, Chen *et al.* 1991, Gernand *et al.* 2005, Inagaki and Mujeeb-kazi 1995, Komeda *et al.* 2007, Laurie and Bennett 1988, 1989, Mochida and Tsujimoto 2001, Rines and Dahleen 1990, Zenkteler and Nitzsche 1984). Partial somatic elimination of chromosomes from one of the parent species was reported in *Hordeum lechleri* × *H. vulgare* (Linde-Laursen and von Bothmer 1999), *Avena sativa* × *Z. mays* (Riera-Lizarazu *et al.* 1996), or *Triticum aestivum* × *H. vulgare* (Barclay 1975). However, allopolyploids of *Gossypium* and *Spartina* did not show genomic rearrangements after hybridization (Ainouche *et al.* 2004, Liu *et al.* 2001). Chromosome elimination is seen not only in genetically distant hybrids, but also in closely related hybrids, suggesting that genetic distance is not the only factor determining elimination.

Observations in maize crosses and wheat × pearl millet crosses demonstrated that chromosome elimination occurs after pollination, during embryo development (Gernand *et al.* 2005, Zhao *et al.* 2013). Several hypotheses have been presented to explain uniparental chromosome elimination during hybrid embryo development in plants. Some of these are: differences in timing of essential mitotic processes due to asynchronous cell cycles (Gupta 1969), asynchrony in nucleoprotein synthesis leading to a loss of the most retarded chromosomes (Bennett *et al.* 1976, Laurie and Bennett 1989), degradation of alien chromosomes by host-specific nuclease activity (Davies 1974), spatial separation of genomes during interphase (Linde-Laursen and von Bothmer 1999) and metaphase (Schwarzacher-Robinson *et al.* 1987), formation of multipolar spindles (Subrahmanyam and Kasha 1973), and parent-specific inactivation of centromeres (Finch 1983, Jin *et al.* 2004, Kim *et al.* 2002, Mochida *et al.* 2004).

CENH3 was identified as a key player in uniparental chromosome elimination between *H. vulgar* × *H. bulbosum* inter-specific hybrids (Sanei *et al.* 2011). CENH3 encodes the Centromere-specific Histone H3 variant that functions in centromere formation and chromosome segregation, and is contained in all eukaryotic genomes. From this study, inactivity of the centromere caused the failure to assemble kinetochores that attach the microtubules for chromosome separation, leading to the loss of parental chromosome (**Fig. 3**). Modification of CENH3 has been demonstrated to efficiently produce homozygous diploid lines that may benefit breeding of hybrid lines by allowing rapid fixing of the homozygous locus linked to desired traits (Copenhaver and Preuss 2010, Ravi and Chan 2010).

Genomic rearrangement

In addition to major chromosomal changes, finer rearrangement of the genome can occur immediately after hybridization, and ultimately affect transcription. Mutation in simple sequence repeats (SSR), chromosomal rearrangements (translocation, duplications, inversions and deletions), and transposon activation were observed immediately after hybridization ($A^rA^rC^nC^n$) between *B. napus* ($A^nA^nC^nC^n$) and *B. rapa* (A^rA^r) (Zou *et al.* 2011). Wheat allopolyploid showed DNA sequence elimination as an immediate response to allopolyploid formation (Feldman *et al.* 1997, Kashkush *et al.* 2002, Ozkan *et al.* 2001, Shaked *et al.* 2001). Loss, gain, and rearrangement of the nucleolus organizer region (NOR) and 5S rRNA gene were also seen immediately after hybridization in synthetic Arabidopsis allotetraploid (Pontes *et al.* 2004). Long-terminal-repeat-retrotransoposon (LTR-RTN) can be reactivated in allopolyploidization and inter-specific hybridization (Labrador *et al.* 1999, Liu and Wendel 2000), and it has been shown that transposable element (TE) activation can affect gene expression (Bennetzen 2005, Kashkush *et al.* 2003). An introgression of <0.1% of wild rice genomes into cultivated rice resulted in extensive genomic variation, and affected up to 30% of genomic loci including TE and protein-coding genes (Wang *et al.* 2005). In a study of *B. rapa* and *B. napus* hybrids, genomic changes that occurred post-hybridization were associated with a large number of QTLs (compared to the allelic contribution by *B. rapa*) for agronomic traits (Zou *et al.* 2011). This demonstrates that even minor

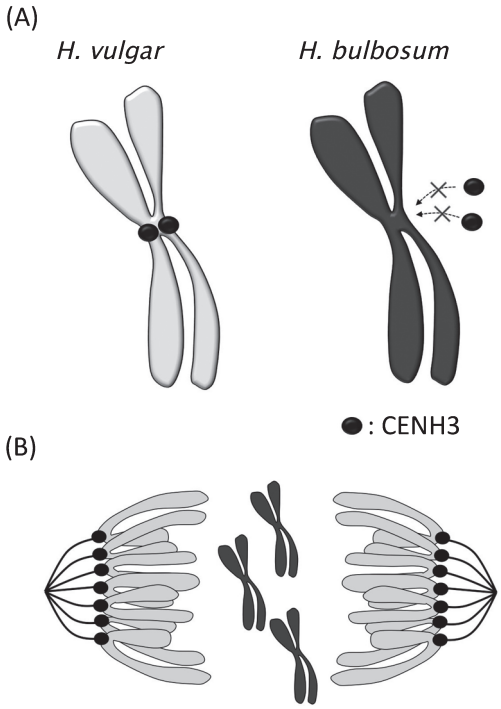

Fig. 3. Role of CENH3 in uniparental chromosome elimination in inter-specific hybrid embryos between *H. vulgare* × *H. bulbosum*. CENH3 (Filled circle) is loading into the centromere of *H. vulgare* but not of *H. bulbosum*, and incorporates only in the centromere of *H. vulgare* during the G2 phase (A). Sister chromatids of *H. vulgare* are separated and moved to opposite ends by attached microtubules in anaphase. However, chromosomes of *H. bulbosum* are abandoned, and are not inherited to daughter cells (B). As a result, chromosomes of *H. bulbosum* are eliminated in the inter-specific hybrid embryo.

rearrangements caused by hybridization can have a significant effect on gene expression and phenotype.

Most molecular and cytological studies of allopolyploids have been on synthesized polyploids, but chromosomal changes have been observed in a recently formed natural allopolyploid, *Tragopogon* (Chester *et al.* 2012). Chromosome rearrangement occurs in response to hybridization of different genomes, to adjust genome expression that may also lead to phenotypic novelty. Further understanding of the mechanisms may allow finer control and rapid introgression of desired traits after hybridization.

3-3. Non-additive expression in protein coding genes and micro RNAs

Allopolyploidization causes changes of gene expression, and these changes may ultimately affect the plant phenotype leading to heterosis (see 3-1 section) or stress tolerance, and ultimately to plant adaptation or domestication (Comai 2005). Following this concept, many studies have compared the transcriptome between artificially synthesized allopolyploids and their parental lines, or between natural allopolyploid species and their putative parental species (Adams and Wendel 2005, Chen 2007). These studies revealed that the majority of genes showed additive expression patterns (gene expression being equal to the average of the parental gene expression level) and some part of the genes showed non-additive gene expression (gene expression being different from the average of the parental gene expression level) in allopolyploids (Adams and Wendel 2005, Chen 2007).

Genome dominance (genes in one parental genome tend to show higher expression levels than the other parent in allopolyploids) is reported in some allopolyploids (Cheng *et al.* 2012, Grover *et al.* 2012, Rapp *et al.* 2009, Wang *et al.* 2006). Nucleolar dominance (rRNA genes derived from one of the parental species inactivated in allopolyploids) is well known as an example of genome dominance, and silencing of one of the parental rRNA genes is epigenetically regulated (Earley *et al.* 2006, 2010, Lawrence *et al.* 2004, Preuss and Pikaard 2007). In inter-specific hybrids between *A. arenosa* and *A. thaliana*, between *A. lyrata* and *A. thaliana*, and between *A. halleri* and *A. thaliana*, *A. thaliana*-derived rRNA is silenced. Similarly, *B. oleracea*-derived rRNA is silenced in inter-specific hybrids between *B. rapa* and *B. oleracea* (Chang and Pikaard 2005, Fujimoto *et al.* 2011, Lewis *et al.* 2007). In *A. arenosa* × *A. thaliana* and *B. rapa* × *B. oleracea* hybrids, genes derived from *A. thaliana* and *B. oleracea* genomes tend to be silenced, respectively, suggesting that genome dominance in protein-coding genes is consistent with nucleolar dominance (Cheng *et al.* 2012, Wang *et al.* 2006). By contrast, genome dominance is not observed in protein coding genes in both *A. thaliana* × *A. lyrata* and *A. thaliana* × *A. halleri* hybrids (Fujimoto *et al.* 2011), suggesting that non-additive gene expression is independent from nucleolar dominance. In *Aegilops longissima* (SS) × *Triticum uratu* (AA) hybrids and *T. uratu* (AA) × *Ae. tauschii* (DD) hybrids, A-genome-derived rRNA is silenced, and A- and D-genome-derived rRNA are silenced in synthetic hexaploid wheat (BBAADD), indicating that the dominance order of rRNA is B > D > A genome. Silencing of rRNA is consistent with increased DNA methylation

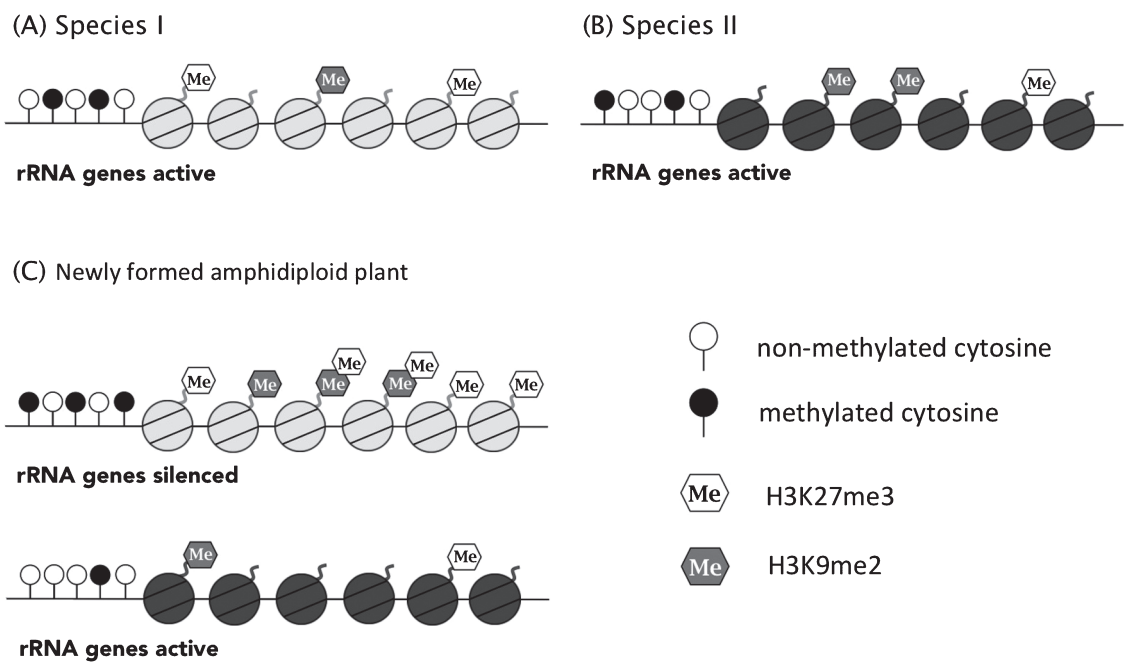

Fig. 4. Silencing of rRNA by epigenetic modification in newly formed amphidiploid plants. rRNA genes active in genomes of species I (A) and II (B). After formed amphidiploid plants, rRNA derived from species I is silenced by increased DNA methylation and enrichment of histone modification (H3K27me3 and H3K9me2) (C).

levels (especially CHH (where H = A, T, or C) and CHG methylation), and enrichment of H3K27me3 and H3K9me2 modifications, indicating that silencing of rRNA is epigenetically controlled (**Fig. 4**) (Guo and Han 2014).

In allopolyploids, parental-biased gene expression change refers to biased expression of homoeologous genes (up- or down-regulated) depending on the parent-of-origin, which has been reported in wheat and cotton (Grover *et al.* 2012, Li *et al.* 2014, Li and Chetelat 2014, Shen *et al.* 2014, Yoo *et al.* 2013). In wheat, genes that have a differential expression between *Ae. tauschii* (DD) and *Triticum turgidum* (AABB), and an equal expression level between *Ae. tauschii* (DD) and *T. aestivum* (AABBDD), tend to include transcription factors such as key floral developmental genes. While genes that have differential expression between *Ae. tauschii* (DD) and *T. turgidum* (AABB), and equal expression levels between *T. turgidum* (AABB) and *T. aestivum* (AABBDD) tended to categorize into stress response. Thus, the authors suggested that merging of characteristic expression patterns for the two parental lines is important for adaptation in wheat (Li *et al.* 2014). Recent technical advances in next-generation sequencing have allowed not only large-scale analysis of transcriptome profiling (RNA-Sequencing), but also to distinguish the parental alleles of allopolyploids using nucleotide sequence differences between parental species (Akama *et al.* 2014).

Changes in transcription are not only observed in protein coding genes, but are also observed in small RNAs (Ha *et al.* 2009, Kenan-Eichler *et al.* 2011, Li *et al.* 2014). Micro RNAs (miRNAs) are a 21-nucleotide class of small RNAs, and they repress the target genes by mRNA cleavage or translational repression. Up to 25% of analyzed miRNAs displayed non-additive gene expression in synthetic hexaploid wheat, and negative correlations of expression levels between miRNA and their target genes among hybrids and their parental lines, suggesting that non-additive gene expression in some protein coding genes are due to non-additively expressed miRNAs (Li *et al.* 2014). Negative correlations between miRNA and target mRNA levels are also found in *A. thaliana* × *A. arenosa* hybrids, and the authors suggested that non-additive accumulation of miRNAs explains approximately 58% expression changes in their target mRNA in the *A. thaliana* × *A. arenosa* hybrids (Ha *et al.* 2009, Ng *et al.* 2012). Among *A. thaliana*, *A. arenosa*, and its synthetic F_8 allotetraploids, miR163 expression in leaves and flowers is higher in *A. thaliana* than in *A. arenosa* and F_8 allotetraploids. One of the target genes of miR163 encodes farnesoic acid methyltransferase, which converts farnesoic acid to methyl farcesoste, and changes in miR163 expression lead to changes in secondary metabolites, suggesting that miRNA regulation is also involved in adaptive evolution of allopolyploids (Ng *et al.* 2012).

Conclusions and perspectives

Reproductive barriers are challenges that must be overcome for developing efficient breeding programs by inter-specific crossing. Traditional techniques (medium culture methods) are effective methods, and have greatly contributed to producing inter-specific hybrids in various crops. However, inter-specific crossing requires different techniques depending on the cross-combinations. In many plant species, different accessions of parental species have different crossability in inter-specific crossing; therefore, cultivars that can be used by traditional techniques are limited.

Recent findings indicate that some reproductive barriers are controlled by the interaction between molecules from each species, suggesting that reproductive barriers may be overcome by modification of molecules related to the reproductive barrier. The pollen rejection behavior in inter-specific crossing is thought to be controlled by common factors related to the process during pollen recognition during pollen tube guidance. The loss of function mutation of either male or female *S*-determinants leads to the loss of the ability for self-incompatibility. It is possible that pre-zygotic barriers can also be overcome by the loss of function in male or female molecules related to inter-specific pollen recognition. One of the post-zygotic barriers, hybrid necrosis, is caused by an autoimmune-like response through epistatic interaction between two genes, indicating that the specific combination of causal genes is important. Hybrid necrosis can be efficiently avoided by crossing between species with a compatible combination of epistatic interaction. Endosperm abnormality may be caused by alteration of PRC2 regulation. In inter-specific hybrid endosperm, PRC2 regulation is affected by "quantitative difference" or "qualitative difference" in interspecies. In either case, it may be possible to overcome endosperm abnormality by modification of PRC2 dosage such as ploidy manipulation. The PRC2 genes, *MEA* and *FIS2*, are regulated epigenetically; therefore, if the expression levels of PRC2 genes are elevated by the modification of epigenetic gene regulation, PRC2 regulation in hybrid endosperm can be balanced with their target genes. As a result, endosperm abnormality of inter-specific crosses may be overcome without ploidy manipulation.

Allopolyploidization, caused by merging genomes, not only contributes to adaptation of allopolyploid species, but also for plant breeding programs. Heterozygosity, changes in chromosome and non-additive gene expression by allopolyploidization, will provide various advantages, and there is a potential to create novel agronomic traits. Indeed, some favorable traits, such as biomass heterosis (Bansal *et al.* 2012, Liu *et al.* 2002, Qian *et al.* 2003), seed yield (Osborn 2004), Sclerotinia resistance (Zhao *et al.* 2006) and loss of self-incompatibility (Okamoto *et al.* 2007), have been gained through allopolyploidization. Although the molecular mechanisms of allopolyploidization are not completely known, advances in new technologies enable new approaches, and will help elucidate the molecular mechanisms. Therefore, understanding the mechanisms of allopolyploidization may enable us to fine-tune the phenotype of inter-specific hybrids and to rapidly introduce desirable

traits to cultivars from other species, and will provide further development for plant breeding of inter-specific hybrids.

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