Epigenetic regulation of agronomical traits in Brassicaceae

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Abstract

Epigenetic regulation, covalent modification of DNA and changes in histone proteins, are closely linked to plant development and stress response through flexibly altering the chromatin structure to regulate gene expression. In this review, we will illustrate the importance of epigenetic influences by discussing three agriculturally important traits of Brassicaceae. 1) Vernalization, an acceleration of flowering by prolonged cold exposure regulated through epigenetic silencing of a central floral repressor, *FLOWERING LOCUS C*. This is associated with cold-dependent repressive histone mark accumulation, which confers competency of consequence vegetative-to-reproductive phase transition. 2) Hybrid vigor, in which an F_1 hybrid shows superior performance to the parental lines. Combination of distinct epigenomes with different DNA methylation states between parental lines is important for increase in growth rate in a hybrid progeny. This is independent of siRNA-directed DNA methylation but dependent on the chromatin remodeler DDM1. 3) Self-incompatibility, a reproductive mating system to prevent self-fertilization. This is controlled by the *S*-locus consisting of *SP11* and *SRK* which are responsible for self/non-self recognition. Because self-incompatibility in Brassicaceae is sporophytically controlled, there are dominance relationships between S haplotypes in the stigma and pollen. The dominance relationships in the pollen rely on *de novo* DNA methylation at the promoter region of a recessive allele, which is triggered by siRNA production from a flanking region of a dominant allele.

Key words: *Brassicaceae*; epigenetics; vernalization; hybrid vigor; self-incompatibility

1. Introduction

Throughout the lifecycle, angiosperms need to carry out multicellular plant body development and adapt to changing environments. This means that the plant must possess gene sets required for these processes, and each tissue orchestrates the appropriate genes to be transcribed depending on the developmental stage and biotic/abiotic stimuli. Epigenetic regulation, covalent modifications of DNA or histone proteins, controls gene activation or repression by altering the physical properties of the target DNA region beyond the nucleotide sequence, which enables the plant to quickly respond to developmental and environmental cues. These modifications are inherited through multiple cell divisions, but can be removed as necessary to act as reversible switches creating spatially and temporally diverse epigenomes derived from an identical genome sequence. Furthermore, epigenetic silencing of transposable elements (TEs)

must occur in whole plant to protect genome integrity. Thus, by integrating genetic and epigenetic information, plants undergo major developmental transitions and modulate environmental adaptability to successfully complete their lifecycle.

The Brassicaceae are attractive material for basic and applied research, as they include not only the model plant *Arabidopsis thaliana*, but also the *Brassica* genus comprising economically valuable plants, such as oil seed and vegetable crops. Among six species that belong to the *Brassica* genus, three species are diploids (*Brassica rapa*; n=10 (AA genome), *B. nigra*; n=8 (BB), and *B. oleracea*; n=9 (CC)), and the others are allotetraploids, a doubled hybrid of each diploid genome [*B. juncea*; n=18 (AABB), *B. carunata*; n=17 (BBCC), and *B. napus*; n=19 (AACC)]. Additionally, there is a difference in the genome size between *A. thaliana* (130 Mb) and diploid Brassica crops (500-600 Mb) probably due to a whole genome triplication event that likely occurred just after the divergence between the ancestral *Brassica* genus and the Arabidopsis lineages. Recently, it has become clear that epigenetic regulation is closely associated with several agriculturally important traits observed in Brassicaceae. In this review, we will illustrate the importance of epigenetic modifications through three examples of traits in Arabidopsis and Brassica crops, vernalization, hybrid vigor and self-incompatibility.

2. Epigenetic regulation that influences gene expression level

The DNA molecule is compacted into the nuclear space through the formation of higher-order chromatin structure. DNA along with a histone octamer, which consists two copies each of four core proteins, H2A, H2B, H3 and H4, form a basic chromatin unit termed a nucleosome. These histone proteins can be covalently modified at the N-terminal tails by acetylation, methylation, phosphorylation and ubiquitination. Post-translational modifications at a specific residue confer distinct physical properties of chromatin altering an accessibility of general transcription machinery including DNA or RNA polymerases to the DNA strand. For example, acetylation of lysine residues of histone H3 and histone H4 by histone acetyltransferases leads to a transcriptionally active state by loosening association between histone proteins and DNA. Conversely, deacetylation, the removal of an acetyl group by histone deacetylases is associated with transcriptionally inactive chromatin. The case of histone methylation is more complicated, as it is associated with both transcriptional activation and repression depending on the residue and the position where methylation occurs. For example, methylation of histone H3 lysine 4 (H3K4) and histone H3 lysine 36 (H3K36) are associated with transcriptional activation, whereas dimethyl histone H3 lysine 9 (H3K9me2) and trimethyl histone H3 lysine 27 (H3K27me3) are repressive marks. These reactions are mediated by histone methyltransferases containing a conserved SET domain (Pien and Grossniklaus 2007). There are also histone demethylases to remove methyl groups from histones, which establishes chromatin state dynamics in order to respond to developmental programs and environmental signals.

As a component of a nucleosome unit, as well as the canonical histone proteins, multiple histone variants are encoded in the plant genome. Histone variants can be replaced with canonical histone proteins that will influence the physical properties of the nucleosome and nucleosome dynamics. Some variants of H2A and H3 are involved in various processes including transcription, DNA repair and chromatin remodeling, and have distinct distribution in the genome (Talbert et al. 2012). H3.1 is enriched in transcriptionally silent region, whereas H2A.Z and H3.3 are predominantly enriched in actively transcribed genes (Zilberman et al. 2008; Stroud et al. 2012). The deposition of histone variants into chromatin is mediated through various histone chaperones and chromatin remodeling complexes (Schönrock et al. 2006; Choi et al. 2007; Deal et al. 2007; March-Diaz et al. 2008; Nie at al. 2014).

DNA methylation, a covalent addition of the methyl group at the C-5 position of cytosine, is a major epigenetic modification that is widely associated with the

expression levels of transcriptionally activated or silenced regions. Whereas in mammals, DNA methylation is predominantly in the symmetrical CG context, in plants it occurs in three sequence contexts, CG, CHG and CHH (H: A/T/C). The Arabidopsis genome encodes four DNA methyltransferases with distinct biological functions and target specificities. For the maintenance of CG methylation, DNA METHYLTRANSFERASE 1 (MET1) methylates on the newly synthesized strand of the hemi-methylated strand during DNA replication (Finnegan et al. 1996; Ronemus et al. 1996; Kankel et al. 2003). For non-CG contexts, two plant-specific DNA methyltransferases, CHROMOMETHYLASE 2 (CMT2) and CMT3 are responsible for maintaining methylation at CHH and CHG sites, respectively. Both CMT2 and CMT3 are targeted by the H3K9me2 mark that is added by the histone methyltransferase KRYPTONITE (KYP) leading to DNA methylation nearby the methylated histone residues (Bartee et al. 2001; Lindroth et al. 2001; Jackson et al. 2002; Stroud et al. 2014). Methylated cytosine, in turn, can be a substrate for binding by KYP and trigger H3K9me2 creating a self-reinforcing loop between histone and DNA methylation. To establish *de novo* methylation, DOMAINS REARRANGED METHYLASE 1 and DOMAINS REARRANGED METHYLASE 2 (DRM2) function as methyltransferases, and DRM2 plays a central role in the RNA-directed DNA methylation (RdDM) pathway (Cao and Jacobsen 2002; Cao et al. 2003; Matzke and Mosher 2014). Cooperating with two plant-specific RNA polymerases and enzymes involved with 24-nt small interfering RNA (siRNA) production and its guide to nascent sequence, DRMs transfer methyl group onto unmethylated cytosine in all three contexts resulting in transcriptional gene silencing and promotion of heterochromatin formation (Matzke and Mosher 2014). Furthermore, RdDM is also required to maintain CHH methylation without overlapping target sites with CMT2.

In addition to the enzymes that can directly transfer methyl groups, there are proteins whose catalytic activities can indirectly affect DNA methylation levels, such as DECREASED IN DNA METHYLATION1 (DDM1). The *ddm1* mutant shows overall reduction of DNA methylation at both CG and non-CG sites, especially in heterochromatic regions (Vongs et al. 1993; Lippman et al. 2004). *DDM1* encodes a chromatin-remodeling factor, SWI2/SNF2 (Jeddeloh et al. 1999). The first generation of the *ddm1* mutant shows relatively normal growth, but notable developmental defects have been observed after repeated self-pollination for several generations (Kakutani et al. 1996). However, how the DDM1 function leads to changes in the genome wide DNA methylation pattern is still largely unknown.

Genome-wide profiling of epigenetic modifications showed distinct epigenomes depending on the genomic features. DNA methylome analyses revealed that methylated DNA for both CG and non-CG contexts are highly enriched at silenced TEs, which are found primarily in the pericentromeric heterochromatin (Zhang et al. 2006; Zilberman et al. 2007). In the genic regions, CG and non-CG methylation at the promoter is associated with gene silencing, whereas CG methylation is also detected within the transcribed region of moderately expressed genes (Zhang et al. 2006; Zilberman et al. 2007). Several studies on genome-wide landscape of histone modification, most of which focuses on the methylation and acetylation of lysine residue of histone H3, showed that they are enriched in euchromatic regions. These histone modifications are associated with transcriptional states throughout development and stress responses, except H3K9me2 at heterochromatic regions that are required for constitutive TE silencing (He et al. 2011). In many cases, the larger genome size depends on the abundance of retrotransposons. As active TEs may cause genetic instability, epigenetic gene silencing is important to protect the genome integrity in plant species possessing a large number of TEs.

3. Epigenetic regulation of the vernalization response

In flowering plants, the timing of the vegetative-to-reproductive phase transition is one of the most important processes in a lifecycle. It relies on several seasonal cues such as day-length and temperature. For long-day (LD) plants of which Brassicaceae is one, premature flowering during the undesirable cold season severely decreases seed productivity. To avoid this, LD plants establish the requirement for prolonged cold exposure to be competent to initiate inflorescence meristem differentiation, termed a vernalization requirement. This can be explained by up- or downregulation of *FLOWERING LOCUS C* (*FLC*). FLC, a MADS-box protein, acts as a floral repressor in a dose dependent manner by binding to regulatory elements of the floral inducer genes, *FT* and *SOC1*, to block their LD-dependent expression (Michael and Amasino 1999; Sheldon et al. 1999; Helliwell et al. 2006). Within a lifecycle, the *FLC* expression can first be detected during embryo development, and continues throughout the vegetative stage (Sheldon et al. 2008; Choi et al. 2009). On the plant being exposed to cold temperature, *FLC* transcripts gradually decrease proportional to the cold duration, and if the prolonged cold is long enough, the repressed state of *FLC* is maintained even after returning the plants to warm temperature. This sets a floral competency, in terms of derepression of *FT* and *SOC1*, which leads to flowering under the LD conditions (Helliwell et al. 2006). As plants can be vernalized at very young stage, e.g. even at the seed stage in *A. thaliana*, there is temporal separation between cold exposure and actual phase transition. This means that epigenetic modifications must occur to maintain stable silencing of *FLC* through multiple cell divisions, and specific covalent modifications on histone proteins are involved in this process. Vernalization every generation is required to flower except for perennial plants, thus cellular memories of vernalization experience should be reset at the end of the lifecycle. In this chapter, we will provide current understanding of epigenetic regulation in Arabidopsis vernalization focusing on the histone methylation occurring at the *FLC* locus and key players that directly or indirectly cause those modifications, and will discuss similarities and diversities in vernalization-associated epigenetic events between Arabidopsis and some Brassica crops.

3.1 Determinants affecting the basal level of *FLC* before vernalization

Multiple factors can affect the basal *FLC* expression before vernalization. *FRIGIDA* (*FRI*) is a major determinant of natural variation in flowering time by activating the basal expression level of *FLC* before cold exposure (Johanson et al. 2000). FRI interacts with FRIGIDA LIKE 1 (FRL1), SUPRESSOR OF FRIGIDA 4 (SUF4), FLC EXPRESSOR (FLX) and FRIGIDA ESSENTIAL 1 (FES1) to form a complex termed FRI-containing complex (FRI-C) (Choi et al. 2011). As well as increasing in the proportion of 5'-capped *FLC* mRNA, FRI-C enriches the COMPASS-like complex including trxG H3K4 methylase, such as ARABIDOPSIS TRITHORAX 1 (ATX1) and ATX-RELATED 7 (ATXR7) at the *FLC* chromatin that leads to H3K4 trimethylation and *FLC* upregulation (Pien et al. 2008; Geraldo et al. 2009; Jiang et al. 2009; Tamada et al. 2009; Berr et al. 2009). Furthermore, it has been demonstrated that an additional SET domain protein, EARLY FLOWERING IN SHORT DAYS (EFS) with dual substrate-specificity for H3K4 and H3K36, recruits FRI to the *FLC* locus, which leads to further trxG recruitment (Kim et al. 2005; Zhao et al. 2005; Xu et al. 2008; Ko et al. 2010). In contrast to the *FRI*-dependent pathway, an autonomous pathway, composing some independent repressive activities FCA, FPA, FY, FLD, FVE, LUMINIDEPENDENS (LD) and FLOWERING LATE KH DOMAIN (FLK), represses basal *FLC* expression. In this pathway, it is proposed that FCA, an RNA-binding protein physically interacts with FY, a polyadenylation / 3' RNA processing factor, and affects spliced transcript accumulation at *FLC* locus (Macknight et al. 1997; Simpson et al. 2003; Liu et al. 2007).

FRI-C also recruits a SWR1-like chromatin remodeling complex that catalyzes the H2A/H2A.Z replacement to the *FLC* (Deal et al. 2007). Disruption of SWR1-like

complex components resulted in decreased *FLC* expression and early flowering without cold treatment, indicating the requirement of H2A/H2A.Z replacement for basal *FLC* expression (Deal et al. 2007; Choi et al. 2007). On the other hand, removal of H2A.Z is not essential for *FLC* repression by vernalization because this variant still expressed abundant *FLC* even after cold treatment (Finnegan and Dennis 2007).

3.2 Molecular basis of epigenetic silencing of *FLC* during the course of vernalization

Initial events that occur during cold exposure are transient upregulation of COOLAIR, multiple antisense long non-coding RNAs (lncRNAs) and concomitant downregulation of *FLC* (Swiezewski et al. 2009). COOLAIR is transcribed from the region just downstream of the *FLC* polyadenylation site and alternatively spliced. There are two classes of COOLAIR transcript with distinct polyadenylation sites, namely proximally terminated shorter group (Class I) and distally terminated longer group (Class II) (Swiezewski et al. 2009). The quantitative abundance of Class I transcript is important for *FLC* repression, and it is associated with the functions of FCA, FPA and FY as RNA processing factors (Liu et al. 2007, 2010; Marquardt et al. 2014). The choice of this proximally polyadenylated transcript results in FLD-dependent H3K4 demethylation at *FLC* by unknown mechanism (Liu et al. 2007, 2010). While several reports have shown the importance of COOLAIR in the early process of *FLC* repression, its role in the establishment of epigenetic silencing of *FLC* is still controversial (Sheldon et al. 2002; Helliwell et al. 2011).

When the plant is returned to warm conditions, *FLC* repression is stably maintained. Characterization of several flowering mutant lines revealed that Polycomb Repressive Complex 2 (PRC2) including VERNALIZATION 2 (VRN2) contributed to this silencing by increasing H3K27me3 level at the *FLC* locus (Gendall et al. 2001; Bastow 2004; Sung et al. 2006a). Indeed, dynamic changes in the H3K27me3 distribution across the *FLC* gene, namely an initial peak around the nucleation site, a 5'region of intron 1 during the cold, which subsequently spreads across the gene after returning to warm conditions (Sung and Amasino 2004; Angel et al. 2011; Yang et al. 2014). The vernalization-associated PRC2 complex prelocalizes across the *FLC* without cold exposure, but interacts with plant homeodomain (PHD) proteins, VERNALIZATION INSENSITIVE 3 (VIN3), VRN5 and VEL1 to form a PHD-PRC2 complex under cold temperature (Sung and Amasino 2004; Sung et al. 2006b; Wood et al. 2006; De Lucia et al. 2008). This cold-dependent complex is required for further spreading of H3K27me3 after returning to warm temperature.

Following reduction of COOLAIR, another cold-inducible lncRNA, COLDAIR is transiently transcribed from the *FLC* intron 1 in the sense direction (Heo and Sung 2011). Along with *in vivo* interaction of COLDAIR with PRC2 component, similarity of phenotypes between COLDAIR knockdown line and PHD-PRC2 component mutant lines indicate that COLDAIR may take part in epigenetic silencing by recruiting PRC2 to *FLC* (Gendall et al. 2001; Sun and Amasino 2004; Sung et al. 2006b; Mylne et al. 2006; Greb et al. 2007; Heo and Sung 2011). Recently, a third cold-inducible lncRNA has been identified proximal to the *FLC* promoter, and is termed COLDWRAP (Kim and Sung 2017). In contrast to two other lncRNAs, COLDWRAP transcript remains expressed even after returning to warm condition (Kim and Sung 2017). It is proposed that intragenic chromatin loop formation by COLDWRAP is also associated with the maintenance of *FLC* repression through assisting PHD-PRC2 in spreading across the gene.

The epigenetic memories written during vernalization response must be erased at the end of the lifecycle to confer a vernalization requirement to the next generation. Indeed, *FLC* repressed by vernalization is reactivated during the development of reproductive tissue and in early embryogenesis (Sheldon et al. 2008; Choi et al. 2009). EARLY FLOWERING 6 (ELF6), a histone lysine demethylase has been characterized to remove methyl groups from *FLC* chromatin in reproductive tissue

(Crevillén et al. 2014). In a hypomorphic *elf6* mutant line, *FLC* expression could not fully recover and H3K27me3 accumulated at higher level in the next generation as compared with WT, indicating that the presence or absence of H3K27me3 mark is important for resetting of vernalization experience (Crevillén et al. 2014).

3.3 Conservation of basic molecular events underlying vernalization in Brassicaceae

In *B. rapa*, *B. oleracea* and *B. napus*, multiple *FLC* paralogues, with seven exons and a large intron 1 similar to Arabidopsis *FLC*, are encoded, and their functionalities as floral repressors have been shown in part by transgenic experiments (Tadege et al. 2001; Kim et al. 2007; Zou et al. 2012; Shea et al. 2017). In *B. rapa*, four *FLC* orthologues (termed *BrFLC1-3, 5*) are encoded, and QTL analyses and sequence comparative analyses indicated that every *FLC* orthologue could affect flowering time variation among cultivars (Schrantz et al. 2002; Kakizaki et al. 2011; Wu et al. 2012; Kitamoto et al. 2014; Shea et al. 2017). In *B. oleracea*, among four *FLC* orthologues (termed *BoFLC1-3, 5*; *BoFLC2* is also termed *BoFLC4*), *BoFLC2* has been proposed to contribute to determine variation in flowering-time trait thus far (Schrantz et al. 2002; Lin et al. 2005; Okazaki et al. 2007; Ridge et al. 2015; Irwin et al. 2016).

In Chinese cabbage (*B. rapa* var. *pekinensis*), the expression of *BrFLC* paralogues were repressed during cold exposure and stably maintained after returning to warm conditions (Kawanabe et al. 2016a). Active marks, trimethyl histone H3 lysine 4 (H3K4me3) and trimethyl histone H3 lysine 36 (H3K36me3), accumulated at *BrFLCs* under ambient temperature and were reduced by cold treatment, and conversely, H3K27me3 increased after returning to warm conditions, indicating that the roles of epigenetic histone modifications in the vernalization response might be conserved between Arabidopsis and Brassica crops (Kawanabe et al. 2016a). Li et al. (2016) have identified natural antisense transcripts (NATs) derived from the terminator region of *BrFLC2* and grouped them into two classes according to whether they are proximally (Class I) or distally (Class II) terminated. It seemed that upregulation of NATs from Class II are associated with flowering acceleration and *BrFLC2* repression, rather than the proximally terminated transcript (Class I) repressing *FLC* like in Arabidopsis (Swiezewski et al. 2009; Liu et al. 2007, 2010, Li et al. 2016). As is the case in Arabidopsis, in both Chinese cabbage and Cauliflower (*B. oleracea* var. *botrytis*), *VIN3* orthologues were transiently upregulated during cold exposure, indicating that PHD-PRC2-mediated epigenetic silencing might be conserved (Ridge et al. 2015; Kawanabe et al. 2016a). However, the intron 1 of *FLC* and its paralogues appear highly diverse in its size and nucleotide sequence between Arabidopsis and Brassica crops, and COLDAIR-like lncRNA has not been discovered in Brassica crops thus far (Zou et al. 2012; Shea et al. 2017). This suggests that Brassica crops do not have a lncRNA-mediated pathway to stably silence *FLC* paralogues. Interestingly, two tandem *cis*-elements found near the *FLC* nucleation site, RY-1 and RY-2 which are bound by the transcriptional repressors VAL1 and VAL2 are conserved in *B. rapa*, *B. oleracea* and *B. napus* (Qüesta et al. 2016; Yuan et al. 2016). These repressors may be associated with H3K27me3 accumulation at *FLC* and epigenetic silencing by recruiting other negative regulator protein complex (Qüesta et al. 2016; Yuan et al. 2016). The findings above suggested that there are some similarities to *A. thaliana*, but different mechanisms may be controlling the expression profiles of multiple *FLC* paralogues during the vernalization response in Brassica crops. Further exploration of novel or conserved factors, such as more lncRNAs and components of PRC2-PHD-dependent or -independent pathway, will provide new insights into conserved molecular basis and diversities of vernalization response in Brassicaceae.

4. Epigenetic regulation of hybrid vigor

Heterosis or hybrid vigor describes the phenomenon where hybrids exhibit superior performance relative to their parental inbred lines in many traits, such as

biomass, yield, fertility, and abiotic and biotic stress resistance (Lippmann and Zamir 2007). Heterosis has been used in the breeding of crop and vegetable cultivars through F_1 hybrid seed production where F_1 hybrid cultivars have increased production. However, the underlying biological mechanisms are not well understood. Recently developed high-throughput molecular analyses such as transcriptomes, proteomes, metabolomes, epigenomes (including DNA methylome, small RNAomes, and genome wide distribution of histone modifications) allow us to clarify the molecular mechanism of heterosis (Hochholdinger and Hoecker 2007; Birchler et al. 2010; Baranwal et al. 2012; Chen 2013; Groszmann et al. 2013; Schnable and Springer 2013). In this chapter, we introduce recent research in heterosis, focusing on epigenetic regulation in Brassicaceae.

4.1 Historical models of heterosis

Several genetic hypotheses have been presented to explain the development of heterosis. The first hypothesis is the dominance model; superior performance of hybrids results in the suppression/complementation of deleterious recessive alleles from one parent by dominant alleles from the other (Davenport 1908; Bruce 1910; Crow 1998). The second hypothesis is the overdominance model; heterozygosity at individual key loci leads to superior performance compared with either homozygote (East 1936; Crow 1998). A single heterozygous gene, *SINGLE FLOWER TRUSS*, contributes to fruit yield heterosis in tomato, demonstrating the first example of a single overdominant gene in plants (Krieger et al. 2010). The third hypothesis is the epistasis model; interactions between two or more non-allelic genes derived from the parental lines generate superior performance (Richey 1942; Powers 1944; Williams 1959). In 2000's, a genetic approach using QTL (quantitative trait locus) analysis was performed in multiple species and revealed that a large number of genes contribute to heterotic phenotypes by dominance, overdominance, or epistatic effect (Frascaroli et al. 2007; Lippman and Zamir 2007; Radoev et al. 2008; Meyer et al. 2010; Schnable and Springer 2013).

4.2 Heterosis phenotypes in Brassicaceae

In *A. thaliana*, hybrids of combinations of accessions show strong heterosis, especially in vegetative biomass (Barth et al. 2003; Meyer et al. 2004; Groszmann et al. 2014). A heterosis phenotype is seen in early development with hybrids having increased cotyledon size only a few days after sowing (Fujimoto et al. 2012; Meyer et al. 2012, Groszmann et al. 2014). Heterosis in vegetative biomass is largely dependent on a larger leaf size but not on increased leaf number (speed of development) (Meyer et al. 2004, 2012; Fujimoto et al. 2012; Groszmann et al. 2014). The larger leaf area is associated with increased cell size and number of the photosynthetic palisade mesophyll cells. In Chinese cabbage (*B. rapa* var. *pekinensis*), the commercial F_1 hybrid cultivar, 'W39', also showed increased cotyledon area at a few days after sowing compared with parental lines. The F_1 hybrid, 'W39', combines the parental properties, larger cell size of paternal line and increased cell number in maternal line (Saeki et al. 2016). The combination of cell proliferation (increased cell number) and post-mitotic cell expansion (increased cell size) regulates the leaf area (Hisanaga et al. 2015). Difference in cell number or size does not result in a difference in the cotyledon/leaf size between parental lines, but the increased cell number and size in the F_1 hybrid result in an increased cotyledon/leaf size in *A. thaliana* and *B. rapa*, suggesting heterotic hybrids have different mechanism of increasing capacity of increased cell size and numbers.

In *A. thaliana* cell size and chloroplast numbers correlate both in the heterotic hybrid and its parental lines (Fujimoto et al. 2012), suggesting that increased cell numbers and size in hybrids are coordinated with increased chloroplast numbers. Indeed, chlorophyll content per fresh weight and the rate of photosynthesis per unit area are not changed in hybrids (Fujimoto et al. 2012). Heterotic hybrids in rice, wheat, and sorghum also did not show an increased rate of photosynthesis per unit area compared

with parental lines (Yang et al. 2007; Zhang et al. 2007, Tazoe et al. 2016). Transcriptome analysis showed upregulation of chloroplast-targeted genes in F_1 hybrids at a few days in cotyledons, in both *A. thaliana* and *B. rapa*, which might coordinate with increased chloroplast numbers or chlorophyll contents following increased cell size or numbers. A chlorophyll biogenesis inhibitor, norflurazon, treatment on cotyledon stages eliminates the heterosis phenotype (Fujimoto et al. 2012; Saeki et al. 2016), suggesting that photosynthesis and chlorophyll biogenesis are important for increased leaf size in hybrids even at stages after the cotyledon stage in *A. thaliana* and *B. rapa*.

4.3 Changes in expression, siRNAs, and DNA methylation

Interactions between the two different parental genomes lead to the alteration of transcription, small RNA levels, and DNA methylation patterns in F_1, which may be involved in the heterosis phenotype (Birchler et al. 2010; Greaves et al. 2015). Comparison of global transcript profiling between heterotic hybrids and their parents have been performed in many plant species. These studies have revealed additive (gene expression being equal to the average of the parental gene expression level) and non-additive (gene expression being different to the average of the parental gene expression level) gene expression pattern in heterotic hybrids. In many cases, the majority of genes showed additive gene expression and a small proportion of genes showed non-additive gene expression (Swanson-Wagner et al. 2006: Wei et al. 2009; Fujimoto et al. 2012; Meyer et al. 2012; Saeki et al. 2016). In addition, the non-additive gene expression profile is drastically changed through developmental stages even when they differ by only a few days (Fujimoto et al. 2012; Meyer et al. 2012).

Global small RNA expression has been compared between heterotic hybrids and their parents in *A. thaliana*, rice, and maize, and the differences in small RNA levels between them have been observed (He et al. 2010; Groszmann et al. 2011; Barber et al. 2012; Li et al. 2012; Shen et al. 2012). In the heterotic maize hybrid between B73 and Mo17, siRNA clusters were additive in the shoot apex, while siRNA clusters in the ear showed larger deviations, especially falling below midparent levels (Barber et al. 2012). 24-nt siRNAs tended to be downregulated in hybrids compared with their parental lines in rice, maize, and *A. thaliana*, thus global or local reduction in 24-nt siRNAs in hybrids may be a universal phenomenon (He et al. 2010; Groszmann et al. 2011; Barber et al. 2012; Li et al. 2012; Shen et al. 2012). There is a hypothesis that changes in siRNA expression levels in hybrids contribute to non-additive gene expression in hybrids or heterosis. However, maize hybrids having homozygous *mediator of paramutation 1* (*mop1*) mutation, the ortholog of RDR2, or *A. thaliana* hybrids having homozygous mutation in genes involved in 24-nt siRNAs biogenesis do not affect the heterosis phenotype, suggesting that changes in siRNA expression in heterotic hybrids are independent from the heterosis phenotype (Barber et al. 2012; Kawanabe et al. 2016b; Zhang et al. 2016a).

Genetic distance between parental lines might be a good predictor of the level of heterosis, though the relationship between genetic distance and heterosis is controversial (Barth et al. 2003; Geleta et al, 2004; Meyer et al. 2004; Dreisigacker et al. 2005; Yu et al. 2005; Flint-Garcia et al. 2009; Kawamura et al. 2016). In *A. thaliana* and *B. rapa*, there is no correlation between genetic distance and heterosis (Barth et al. 2003; Meyer et al, 2004; Kawamura et al. 2016), suggesting that the different epigenomes of the two parental lines might be involved in heterosis phenotypes as well as the genetic interactions at the specific loci (Greaves et al. 2012a, 2015). DNA methylation has a potential to generate the F_1 specific epigenome because non-additive DNA methylation states caused by trans-chromosomal methylation (TCM) (an increase in methylation at a locus with a previously low methylation allele gaining methylation to resemble the more heavily methylated allele) and trans-chromosomal demethylation (TCdM) (loss of methylation at a genomic segment) in the F_1 have been observed in heterotic *A. thaliana*

(Greaves et al. 2012a, b; Shen et al. 2012). TCM and TCdM events in hybrids are largely dependent on the 24-nt siRNAs, but abolition of the TCM and TCdM by the pol iv or pol v mutations (genes critical for 24-nt siRNA biogenesis) does not affect the heterosis phenotype (Zhang et al. 2016a). More than 10,000 regions of non-additively inherited DNA methylation in epihybrids occur between *met1* and wild type, though these F_1 plants do not show superior performance (Rigal et al. 2016). There is still a possibility that RdDM-independent TCM and TCdM are involved in heterosis and further study will be required to clarify this possibility.

4.4 The chromatin remodeler DDM1 is a key gene for promotion of heterosis

Populations of epigenetic recombinant inbred lines (epi-RILs) between parents, which differed only in epigenetic marks (hybrids between *met1* and WT or between *ddm1* and WT), have been established in *A. thaliana*, and these populations have a variation of phenotypes including biomass (Johannes et al. 2009; Reinders et al. 2009). Several hybrids between WT and specific epiRIL lines derived from the hybrids between *met1* and WT or between *ddm1* and WT showed enhanced vegetative growth, suggesting that epigenetic diversity and epigenetic regulation of transcription plays a role in heterosis (Dapp et al. 2015; Lauss et al. 2016). MutS HOMOLOG1 (MSH1) encodes a protein dually targeted to mitochondria and plastids and is involved in organelle genome stability (Abdelnoor et al. 2003; Xu et al. 2011). Disruption of MSH1 causes change of DNA methylation and enhanced vigor was observed in F_4 generations derived from the hybrids between WT and *msh1*, suggesting that epigenetic reprogramming can result in enhanced growth (Virdi et al. 2015).

Recently two groups showed that DDM1 is a major regulator of heterosis using genetic tests (Zhang et al. 2016b; Kawanabe et al. 2016b). Hybrids between homozygous mutants in some genes involved in epigenetic regulation in the C24 and Col background were developed. The F_1 having homozygous mutations in *rdr2*, *dms3*, *drd1*, *rdm1*, *nrpd1*, *nrpe1*, *ago4*, *ago6*, and *rdm3* showed the same level of heterosis as the wild type F_1, while the F_1 with homozygous mutations in *ddm1* (termed *ddm1* hybrids hereafter) reduced the vegetative heterosis (Zhang et al. 2016b; Kawanabe et al. 2016b). In the hybrid between a heterozygous *ddm1-9* mutation in C24 and a *ddm1-1* homozygous mutant in Col, plants having a homozygous *ddm1* mutation were smaller than those having heterozygous *ddm1* mutation. However, some plants having a *ddm1-1* homozygous mutation showed heterosis as great as the plants having a heterozygous *ddm1* mutation, and some plants having heterozygous *ddm1* mutation reduced heterosis like the *ddm1* mutant hybrid plants (Kawanabe et al. 2016b). Both cases had an identical genetic background except for the *ddm1* mutation. These effects may result from the previous methylation state of the genome in the *ddm1* parent, and the gene or segments important for heterosis coming from the *ddm1* parent might already have an altered level of DNA methylation. By transcriptome analysis, *ddm1* hybrids showed non-additive expression of genes involved in salicylic acid metabolism without any association with DNA methylation (Zhang et al. 2016b). SA concentrations in Col, C24, and wild type hybrids are higher than those in *ddm1* (Col), *ddm1* (C24), and *ddm1* hybrids, respectively. Regardless of whether DDM1 is functional or not, the SA concentrations in C24 is much higher than in Col and the F_1, leading to concentrations in F_1 lower than MPV (mid parent value) (Groszmann et al. 2015; Zhang et al. 2016b). The authors suggested that low endogenous SA concentrations stimulate growth but when the level is beyond a threshold, SA inhibits growth. The endogenous SA concentration in wild type hybrids is best for heterosis, while SA concentrations in *ddm1* hybrids exceed the appropriate range for showing heterosis (Zhang et al. 2016b). However, the difference of SA concentration between F_1 and MPV is largely dependent on the high level of SA concentration in C24, and the difference of SA concentrations between wild type Col and C24 x Col hybrids or between L*er* and C24 x L*er* hybrids are small (Groszmann et al. 2015; Zhang et al.

2016b). Further study will be required to confirm this hypothesis.

There are epigenetic changes in heterotic hybrids. 24-nt siRNAs are changed and affect DNA methylation but these do not appear to be associated with the generation of heterosis. Alterations in DNA methylation in the chromatin remodeler DDM1 affect the level of heterosis, but the mechanism is unclear. Further study will be required to understand how DDM1 regulate heterosis.

5. Epigenetic regulation of self-incompatibility

Self-incompatibility is a classical area in plants involved in the mechanism to prevent self-fertilization (Bateman 1955). This mating system is controlled by the interaction between *S*-locus protein 11/*S* locus cysteine-rich protein (SP11/SCR) on the pollen grain and *S*-receptor kinase (SRK) in the stigma (Schopfer et al. 1999; Takasaki et al. 2000; Takayama et al. 2000). These two proteins are encoded on the single *S*-locus and inherited together from a parent, so they are defined as the '*S*-haplotype'. In Brassicaceae self-recognition is controlled by multiple *S*-haplotypes (*S-1*, *S-2*, …., and *S-n*). There is considerable polymorphism for the two genes in the *S*-haplotypes, and the interaction between SP11 and SRK occur only when they are produced from the same *S*-haplotypes. As a result, this interaction inhibits the germination of pollen carrying the same *S*-haplotype of SP11 (Kachroo et al. 2001; Takayama et al. 2001). Although self/nonself recognition is an interaction between haploid (pollen) and diploid (pistil), pollen changes its behavior by the dominance relationship between the *S*-haplotype of the parent. This phenomenon is due to the fact that *SP11* is expressed in the sporophytic anther tapetum cells, which surround the microspores (Shiba et al. 2002). A dominance relationship between the *S*-haplotype has been reported in some self-incompatible plants such as *Arabidopsis halleri*, *Arabidopsis lyrata*, *B. oleracea*, *B. rapa*, *Ipomoea trifida,* and *Senecio squalidus* (Brennan et al. 2011; Hatakeyama et al. 1998; Kowyama et al. 1994; Kusaba et al. 2001; Llaurens et al. 2008; Thompson and Taylor 1966). Recently it has become clear that epigenetic regulation is involved in this phenomenon. In this chapter, we introduce the latest findings on the epigenetic control of dominance relationship among the *S*-haplotypes in self-incompatibility.

5.1 Dominance relationship in pollen *S* gene

Self-recognition occurs when pollen and pistil have the same *S*-haplotype. The pollen recognition phenotype is determined by one of the two alleles of dominance-recessive interactions between *S* alleles of *SP11*. If the dominance relationship between the two haplotypes is equal (co-dominant), the pollen shows both phenotypes. In Brassicaceae, dozens of *S*-haplotypes have been identified and classified into two classes, class-I and class-II, based on their nucleotide sequence. The class-I *S*-haplotypes are dominant over class-II in the pollen of heterozygotes of class-I and class-II *S*-haplotypes (Nasrallah et al. 1991). Therefore, pollen derived from heterozygous plants of class-I and class-II *S*-haplotype shows phenotype of class-I regardless of the pollen genotype (Figure 1). This phenomenon is due to the reduction of *SP11* mRNA from the class-II *S*-haplotype in the class-I/class-II *S*-heterozygote in the tapetum cells (Kusaba et al. 2002; Shiba et al. 2002).

The dominance-recessive interactions are observed within the same classes of *S*-haplotypes in *B. rapa* (Hatakeyama et al. 1998). Interestingly, class-II *S*-haplotypes exhibit a complicated dominance hierarchy, such as *BrS-44* > *BrS-60* > *BrS-40* > *BrS-29* (Hatakeyama et al. 1998; Kakizaki et al. 2003). Thus, *BrS-44* is most dominant and *BrS-29* is most recessive. Likewise, dominance hierarchy of the five *S*-haplotypes (*AhS-20* > *AhS-12* > *AhS-04* > *AhS-03* > *AhS-01*) is also observed in *A. halleli* (Llaurens et al. 2008). Therefore, the *S*-haplotypes in the middle of the hierarchy (e.g. *BrS-40* and *AhS-12*) act dominantly or recessively dependent on the other partner *S*-haplotype in the heterozygote.

5.2 How do dominance relationships arise?

Because selfed progeny derived from the *S*-heterozygote shows self-incompatibility, repression of *SP11* in the recessive haplotype of *S*-heterozygote is released in the next generation (Shiba et al. 2006). This result suggests that suppression of *SP11* is epigenetically controlled. To elucidate the molecular mechanism of monoallelic expression of *SP11* in a heterozygote, methylation states of genomic DNA for several tissues were examined (Kusaba et al. 2002; Shiba et al. 2006). As a result, monoallelic expression in the anther tapetum was suppressed by DNA methylation of a 300 bp region in the promoter region of the recessive allele but there was no DNA methylation in the promoter region of the dominant allele or in the promoter region of recessive alleles of other tissues (Shiba et al. 2006). In early stage of pollen development, such as the uni-nucleate stage, there is no DNA methylation even in the recessive allele but methylation increases as the anther develop. These observations suggest that *de novo* DNA methylation of a recessive allele occurs in the early stages of anther development just before the initiation of *SP11* transcription. Monoallelic DNA methylation in recessive alleles can explain the dominance relationship of all combinations consistently with phenotypic expression and *SP11* expression, which is considered to be the cause of the dominance-recessive mechanism.

5.3 Dominance modifier

The next question is how *de novo* DNA methylation is controlled. Fujimoto et al. (2006) showed that a class-I haplotype that has a defect in the *SP11* promoter can also suppress the class-II *SP11* expression. This result indicates that the expression of *SP11* is not necessary for the suppression of recessive haplotype and other element(s) are involved in the dominance relationship. Ninety years ago, there was intense discussion among statisticians on the existence of genetic elements controlling the dominance-recessive relation (Billiard and Castric 2011). This element was named 'dominance modifier' but until recently, its entity remained unclear.

It has been known that small RNAs are involved in regulation of gene expression (Carthew and Sontheimer 2009; Voinnet 2009). One of the small RNAs, 24-nt siRNA regulates *de novo* DNA methylation of a homologous region by the RdDM pathway (Daxinger et al. 2009). Tarutani et al. (2010) identified a 24-nt siRNA (named *Smi*) transcribed from the dominant allele (class-I) that directs DNA methylation of the promoter region of recessive *SP11* alleles (class-II) in *B. rapa* (Figure 2a). The precursor of *Smi*, *SP11 Methylation inducer* (*SMI*), flanks the *SP11* and is expressed in the tapetum before *de novo* DNA methylation is initiated (Tarutani et al. 2010). This study provides the first evidence for 24-nt siRNA may act as a 'dominance modifier' thought the allele specific DNA methylation. Interestingly, *SMI* also exist on the class-II alleles, but a single nucleotide substitution in *Smi* causes the recessive *SMI* to be non-functional. Therefore, the more complex dominance hierarchy in the class-II haplotypes in *B. rapa* or *A. halleli* cannot be explained by the function of *Smi* alone. (Kakizaki et al. 2003; Llaurens et al. 2008).

Recently, two independent research teams have proposed different models of complicated dominance hierarchy using different plant species. Durand *et al.* (2014) proposed a 'multiple dominance modifier model'. They identified that the most dominant haplotype has multiple small RNA candidates in *A. halleli* by comprehensive genomic and transcriptome analysis (Durand et al. 2014). The number of small RNA candidates was associated with the dominance hierarchy, and the most recessive haplotype has the least small RNA candidates and many target sites. In this model, an individual small RNA candidate from a dominant haplotype was predicted to target more *SCR* alleles (Figure 2b). Several siRNA candidates were predicted to bind to introns. Although this result suggests the involvement of gene-body-methylation or post-transcriptional gene

silencing, there is no report that these regulations are involved in suppressing the *SCR* expression. Yasuda *et al.* (2016) proposed a different model behind the dominance hierarchy among four class-II *S*-haplotype of *B. rapa* (Figure 2c). They identified a single polymorphic 24-nt small RNA, named *SP11 methylation inducer 2* (*Smi2*), transcribed from downstream of *SRK* in all of class-II *S*-haplotypes (Yasuda et al. 2016). Target sites of *Smi2* were found in all of the promoters of class-II *SP11* but not in class-I *SP11* promoters. This implies that *Smi2* controls dominance hierarchy among class-II haplotypes. They named this model 'Polymorphic dominance modifier model' because the allelic *Smi2* and their targets control dominance hierarchy depending on the similarity of nucleotides. For example, *Smi2-44,* which is derived from the most dominant haplotype, shows high similarity to the promoter of other *SP11* promoter and induces DNA methylation. In contrast, *Smi2-40,* which is derived from the third dominant haplotype, shows similarity to a mots recessive haplotype of *SP11-29* promoter.

The past decade of research in the field of self-incompatibility in Brassicaceae revealed that epigenetic mechanism control monoallelic gene expression. Particularly, more complex dominance relationships, such as dominance hierarchy, were determined by polymorphism between a 24-nt siRNA and its target. Although this epigenetic regulation fits the Brassicaceae, other self-incompatible species which retains dominance hierarchy in the Asteraceae and Convolvulaceae remain unclear. Further analysis will be required to ascertain the commonality of the systems found in Brassicaceae.

6. Conclusion and perspectives

In plants, throughout the life cycle and beyond generation, various epigenetic modifications occur. They bring spatio-temporal dynamics in gene expression associated with important aspects such as plant body development and response to internal or external signals. Interaction between individuals with distinct epiallele or epigenome background can also affect various aspects including vegetative growth rate and reproduction. A series of genetic studies and genome wide profiling of epigenetic states, such DNA methylation, small RNA production and histone modifications have identified a broad range of molecules required for these modifications, and revealed that they play pivotal roles in complex multiple epigenetic layers as introduced in this review. However, there are still a number of questions that await further experimentation as exemplified below.

For vernalization, how different accessions respond differently to varied cold duration is one of the fundamental questions, which is likely associated with sequence variation in the *FLC* intron 1 (Coustham et al. 2012). Moreover, it is proposed that the cold signal is perceived in a digital fashion, namely, every cell is either of one of a bistable state in *FLC* expression, ON or OFF. For hybrid vigor, it is emerging that specific interaction of parents with distinct epigenome background and non-additive gene expression in hybrid determines the level of hybrid vigor through DDM1 function. It is still largely unknown how DDM1 consequently render hybrid vigor. Additionally, whether the function of DDM1 as a chromatin remodeler or genome-wide DNA methylation is important for heterosis phenotype is still to be determined. Understanding how the increase of cell number is accelerated and how its variation depends on sequence diversity is another challenge in hybrid vigor. For self-incompatibility, in general, the canonical RdDM pathway silences TEs in all tissues, whereas *de novo* methylation associated with self/non-self recognition is stage and tissue-dependent. This is the first example of the RdDM pathway not functioning ubiquitously. Identifying the determinant(s) of the first step to confer the stage and tissue-specificity is an interesting theme in the future. Further identification of key factors would extend our understanding and allow us to elucidate fundamental

principles underlying epigenetic regulation important for the plant lifecycle. It is also worthwhile to find potential benefits underlying epigenetic basic research to develop strategies for applying them to agricultural science in the Brassicaceae.

Author contribution statement

EI and TK conceived and designed research. All authors wrote the manuscript.

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Figure regends

Figure 1. Interaction of the *S*-haplotypes in pollen.
Pollen grains from the *S*-heterozygous plant (*S-a* and *S-b*) are compatible for *S-a* homozygous plant (solid line), but incompatible for *S-b* plant (dashed line). In this case, *S-b* haplotype is dominant over the *S-a* haplotype in pollen.

Figure 2. Mode of action of the dominance relationships via trans-acting small RNA in Brassicaceae.
(a) Canonical model for dominance relationship. In S-heterozygote having class-I (dominant) and class-II (recessive) haplotypes, expression of SP11 from recessive allele is repressed by DNA methylation triggered by a 24-nt small RNA "Smi". Smi fails to repress the dominant SP11 expression due to no homologous region in dominant SP11 promoter. Smi derived from recessive haplotype cannot trigger DNA methylation due to the one base mismatch to the recessive SP11 promoter. Black boxes indicates the exons of SP11. Open and solid circles indicates that the unmethylated and methylated status of SP11 promoters, respectively. (b) A mode of "Multiple dominance modifier" model in A. halleli. Dominant S-haplotype have a larger set of small RNA "mirS" precursor genes. (c) The "Polymorphic dominance modifier" model in B. rapa. The single SMI2 gene regulate dominance hierarchy via a homology dependent manner. In all dominant-recessive interactions, Smi2 variants derived from dominant SMI2 region exhibited high similarity to the recessive SP11 promoters.

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Figure legends

Figure 1. Interaction of the *S*-haplotypes in pollen.
Pollen grains from the *S*-heterozygous plant (*S-a* and *S-b*) are compatible for *S-a* homozygous plant (solid line), but incompatible for *S-b* plant (dashed line). In this case, *S-b* haplotype is dominant over the *S-a* haplotype in pollen.

Figure 2. Mode of action of the dominance relationships via trans-acting small RNA in Brassicaceae.
(a) Canonical model for dominance relationship. In S-heterozygote having class-I (dominant) and class-II (recessive) haplotypes, expression of SP11 from recessive allele is repressed by DNA methylation triggered by a 24-nt small RNA "Smi". Smi fails to repress the dominant SP11 expression due to no homologous region in dominant SP11 promoter. Smi derived from recessive haplotype cannot trigger DNA methylation due to the one base mismatch to the recessive SP11 promoter. Black boxes indicates the exons of SP11. Open and solid circles indicates that the unmethylated and methylated status of SP11 promoters, respectively. (b) A mode of "Multiple dominance modifier" model in A. halleli. Dominant S-haplotype have a larger set of small RNA "mirS" precursor genes. (c) The "Polymorphic dominance modifier" model in B. rapa. The single SMI2 gene regulate dominance hierarchy via a homology dependent manner. In all dominant-recessive interactions, Smi2 variants derived from dominant SMI2 region exhibited high similarity to the recessive SP11 promoters.