

PDF issue: 2025-05-02

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(Citation) Breeding Science,68(2):145-158

(Issue Date) 2018-03

(Resource Type) journal article

<mark>(Version)</mark> Version of Record

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(URL) https://hdl.handle.net/20.500.14094/90007944



Invited Review

Recent research on the mechanism of heterosis is important for crop and vegetable breeding systems

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Heterosis or hybrid vigor is a phenomenon where hybrid progeny have superior performance compared to their parental inbred lines. This is important in the use of F_1 hybrid cultivars in many crops and vegetables. However, the molecular mechanism of heterosis is not clearly understood. Gene interactions between the two genomes such as dominance, overdominance, and epistasis have been suggested to explain the increased biomass and yield. Genetic analyses of F_1 hybrids in maize, rice, and canola have defined a large number of quantitative trait loci, which may contribute to heterosis. Recent molecular analyses of transcriptomes together with reference to the epigenome of the parents and hybrids have begun to uncover new facts about the generation of heterosis. These include the identification of gene expression changes in hybrids, which may be important for heterosis, the role of epigenetic processes in heterosis, and the development of stable high yielding lines.

Key Words: heterosis, hybrid vigor, epigenetics, non-additive gene expression.

Introduction

In plants, F_1 hybrids between specific combinations of parental lines show superior performance relative to the parental lines; the F_1 hybrids have hybrid vigor or heterosis. The heterosis phenotype was first described in 'The effects of cross and self-fertilization in the vegetable kingdom' by Charles Darwin. George Harrison Shull introduced the term "heterosis" in 1914 to replace the more cumbersome word "heterozygosis", which did not express the superior performance of the hybrids (Shull 1948). Heterosis is agronomically important because the superior performance can appear as biomass, yield, and abiotic and biotic stress tolerance. Breeding of F_1 hybrid cultivars based on heterosis is used in many crops and vegetables.

In 1909, Shull found increased grain yields in hybrid corn and he proposed this as a method of corn breeding. His idea was accepted and led to the transition from openpollinated to four-way cross hybrids (double cross hybrid), and then to two-way cross hybrids (single cross hybrid)

Communicated by Takeshi Nishio

Received December 25, 2017. Accepted January 29, 2018.

(Crow 1998). In the Midwestern Corn Belt state of Iowa, hybrid corn was less than 10% of the crop in 1935, but it was over 90% four years later. Hybrid corn was the main crop in the 1950s in the United States, and yield advanced from one ton per hectare in 1930 to four tons per hectare in 1960 and approximately twelve tons per hectare in 2017. The reason for the rapid spreading of hybrid corn was due not only to increased yield, but also to uniformity in growth and flowering, which allowed machine harvesting (Crow 1998, Duvick 2001). Now, single cross hybrid corn has developed into a major industry worldwide. In the 1970s Yuan Longping developed hybrid rice with a significant yield advantage (10-20% greater) over inbred parental varieties. Hybrid rice was accepted in China, and about half of the total area of rice in China was planted to hybrids by the early 2000s (Cheng et al. 2007). Hybrids are also used in crops such as sorghum, sunflower, canola, and in a number of vegetables. Attempts have been made to produce hybrid seed in wheat (Kempe et al. 2014).

Although heterosis contributes to increased yield in many crops and vegetables, the underlying biological mechanisms of heterosis are not well understood. For a long time, research has aimed to clarify this mystery. This review describes recent research on heterosis.

First Published Online in J-STAGE on April 12, 2018.

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Fig. 1. Three hypotheses to explain the genetic mechanism of heterosis. Phenotype is the sum of gene effects (A, B, A + B). (a) The dominance model; dominant alleles (A and B) suppress or complement the recessive alleles (a and b). (b) The overdominance model; heterozygosity (B_1/B_2) at the key locus contributes to heterosis leading to superior performance. (c) The epistasis model; non-allelic genes (A₂ and B₁) inherited from the parental lines interact and contribute to heterosis.

Traditional models of heterosis

Attempts to understand the genetic basis of heterosis began in the 1990s and several hypotheses were put forward to explain the mechanism of heterosis. All addressed levels of gene activity. The dominance hypothesis was the first to be proposed (Bruce 1910, Crow 1998, Davenport 1908, Jones 1917) and heterosis results in the suppression/complementation of deleterious recessive alleles from one parent by dominant alleles from the other parent (Fig. 1). This model predicts it would be possible to create an inbred line of equal performance to the F₁ hybrid cultivar by eliminating all deleterious alleles and/or accumulating favorable alleles. The second proposal is the overdominance hypothesis, which attributes heterozygosity at individual loci as leading to heterosis (Fig. 1) (Crow 1998, East 1936, Shull 1908). The third suggestion is the epistasis hypothesis in which interactions between two or more non-allelic genes derived from the parental lines generate heterosis (Fig. 1) (Powers 1944, Richey 1942, Williams 1959). These hypotheses have been fundamental to heterosis research, but it is not clear that any one model explains the molecular mechanism of heterosis. These hypotheses are not mutually exclusive, and it is possible that heterosis is an accumulation or interaction of alleles with contributions from each mechanism.

Genetic analysis of heterosis

Any positive correlation between genetic distance and heterosis has been debated (Birchler *et al.* 2010). Initially, in maize, it was accepted that crosses of more genetic divergent parental lines lead to greater heterosis. There was a positive correlation between genetic distance and heterosis within a range of diverged parental lines, although there was a negative correlation between genetic distance and heterosis when the genetic distance was extremely high (Moll *et* *al.* 1965). An efficient method for predicting reliable hybrid performance from parental lines is desired in F_1 hybrid breeding because hybrid production is expensive, time consuming, and is labor intensive to test large number of hybrids in field trials. Recently development of DNA marker and sequence technology allows calculation of the genetic distance with high accuracy. However, positive correlation is not always observed between genetic distance and heterosis (Barth *et al.* 2003, Girke *et al.* 2012, Kawamura *et al.* 2016, Yang *et al.* 2017). Prediction of hybrid performance by genomic selection using general or specific combining ability has also been challenged (Zhao *et al.* 2015)

Quantitative trait loci (QTL) analysis has been performed in maize, rice, sorghum, tomato, rapeseed, and cotton in attempts to understand the genetic basis of heterosis (Lippman and Zamir 2007). Most heterosis QTL studies focus on yield-related traits in biparental populations (Lippman and Zamir 2007). Other researchers tried to identify a QTL for general or specific combining ability in hybrids using multiparental populations (Giraud et al. 2017, Zhen et al. 2017). In maize, pioneering QTL research concluded that overdominance was the main cause of heterosis (Stuber et al. 1992), and this was supported by a later study (Li et al. 2017). Other research programs led to dominance or epistasis to be considered as the main cause of heterosis in maize (Frascaroli et al. 2007, Lu et al. 2003). In rice, there is support for both the dominance hypothesis (Xiao et al. 1995) and the overdominance and epistasis hypotheses (Li et al. 2001, Luo et al. 2001). In tomato, a single heterozygous gene, SINGLE FLOWER TRUSS, contributes to fruit yield heterosis, the first example of a single overdominant gene in plants (Krieger et al. 2010). It appears that heterosis cannot be explained by any single hypothesis, and the causes of heterosis depend on the species, traits, and parental combination (Li et al. 2008).

Single nucleotide polymorphism (SNP) data of large populations have enabled a comparison of genetic architecture in a number of lines. In addition, genome-wide association studies (GWAS) using large SNP data have been incorporated into a genetic approach. In rice, genomes of 10,074 F_2 lines derived from 17 hybrids were sequenced for genotyping, and QTLs for yield traits identified. Positive dominance effects tended to be overrepresented in the grainyield-related traits, with the contribution of male alleles being larger than female alleles in most of the grain-yield QTLs (Huang et al. 2016). In winter wheat, grain yield was assessed in 1,604 hybrids and their 15 male and 120 female parental elite lines (approximately four million plants) in 11 environments, and a 90,000-SNP-array used for genotyping. Relative contributions of the genetic components of mid-parent heterosis for grain yield were estimated, and dominance, additive-by-additive, additive-by-dominance, and dominance-by-dominance variance were 16%, 50%, 21%, and 13%, respectively, suggesting that epistatic effects are greater than dominance effects in grain-yield heterosis of wheat (Jiang et al. 2017).

How additively or non-additively expressed genes can contribute to heterosis

Transcriptome analyses initially using microarray technology and later, RNA-sequencing (RNA-seq) have been used to compare parental lines with their F_1 hybrids to identify genes potentially involved in heterosis.

The mode of gene action in F_1 hybrids is classified as having additive and non-additive expression. Additive gene expression is where the gene expression level in F_1 hybrids is equal to the average level of parental gene expression (termed mid-parent value; MPV) (Fig. 2). Non-additive gene expression occurs when the gene expression level in F_1 hybrids is different from the MPV. Gene expression is further classified into high-parent dominance, low-parent dominance, over-dominance, and under-dominance (Fig. 2). High- or low-parent dominance is when expression levels in hybrids are equal to one parent but is significantly different from the second parent (Fig. 2). Over- and underdominance is when gene expression levels in hybrids are outside the parental range (Fig. 2). In seedlings of maize, transcriptome analysis using microarrays on the parental inbred lines, B73 and Mo17, and their heterotic F1 hybrids showed all possible modes of gene action in F₁ hybrids, with most genes having additive gene expression (Swanson-Wagner et al. 2006).

Transcriptome analyses have been performed for seedlings, roots, embryos, and immature ears in maize (Hu *et al.* 2016, Jahnke *et al.* 2010, Paschold *et al.* 2012, Stupar *et al.* 2008, Swanson-Wagner *et al.* 2006). In any tissue, there were more additively expressed genes than non-additively expressed genes, and non-additively expressed genes were tissue/stage specific. Seedlings of six F_1 hybrids with a diversity of heterosis levels showed similar proportions of additively and non-additively expressed genes, suggesting that heterosis is not a consequence of the proportion of higher levels of additive or non-additive expression in F_1 hybrids (Stupar *et al.* 2008).

In rice, transcriptome analyses have been performed for



Fig. 2. Classification of the mode of gene action in F_1 hybrid (F_1) compared with parental gene expression level (P_1 or P_2). LP; low parent, HP; high parent, MPV; mid-parent value.

seedlings, leaves, roots, and inflorescence buds (Li et al. 2016, Song et al. 2010, Wei et al. 2009, Zhai et al. 2013). Using super-hybrid rice Lian-You-Pei-Jiu 9 (LYP9) and its parental lines, transcriptome analyses by microarray in seven tissues (leaves at seedling and tillering stages, flag leaves at booting, heading, flowering, and filling stages, and panicles at filling stages) were performed. Cluster analysis revealed transcriptome profiles of LYP9 were similar to the maternal parent in the early developmental stages but closer to the paternal parent at later stages. Non-additively expressed genes range from 0.5 to 1.4% in seven tissues, and differentially expressed genes showed tissue specificity (Wei et al. 2009). RNA-seq using young inflorescence buds at the four early developmental stages in LYP9 and its parental lines showed from 30 to 50% of genes had nonadditive expression. Enriched pathways were translation, transcription, circadian rhythm, carbon fixation, and starch and sucrose metabolism (Li et al. 2016). Several genes differentially expressed between F₁ hybrids and its parents were located at yield-related QTL regions, suggesting they are candidates for contributing to heterosis (Li et al. 2016). Using another super-hybrid rice *Liangyou-2186* (LY2186) and its parents, transcriptome analysis by serial analysis of gene expression (SAGE) was performed in leaves at the grain-filling stage. Photosynthesis-related categories were over-represented in the differentially expressed genes, and genes involved in carbon-fixation pathway were up-regulated in F₁ hybrids. The activity of enzymes involved in the carbon fixation pathways and the net photosynthetic rate were increased in the F₁ hybrids, suggesting that carbon fixation is enhanced, which could be associated with heterosis (Song et al. 2010).

In *B. napus* shoot apical meristems and young flower buds (three stages of early flower developments), most genes showed additive expression in the F_1 hybrids. Non-additively expressed genes were differentially expressed between parental lines and had high-parent dominance. These genes had over-representation of the categories related to defense and hormone responses (Shen *et al.* 2017).

The underlying hypothesis for a transcriptome approach is that genes whose expression changes in F_1 hybrids (non-additively expressed genes) may be involved in heterosis and include genes categorized into specific pathways that may have altered regulation. The hypothesis that the mid-parent expression pattern in F_1 hybrids might play a role in heterosis is considered because in many cases, the number of differentially expressed genes between parental lines was more than those between parental lines and F_1 hybrids. The majority of genes showed additive gene expression, with only a small proportion of genes showing nonadditive gene expression (Springer and Stupar 2007). There is no clear conclusion as to which is more important, additive or non-additive expression for generating heterosis, but both classes probably contribute.

Transcriptional changes in heterotic hybrids at the early developmental stages in *A. thaliana*

In F_1 hybrids in A. thaliana such as C24 × Columbia-0 (Col) and C24 × Landsberg erecta (Ler), a heterosis phenotype is seen at early developmental stages with hybrids having increased cotyledon size only a few days after sowing (DAS) (Fujimoto et al. 2012a, Groszmann et al. 2014, Meyer et al. 2012). In C24 \times Col hybrids as early as 3 DAS, 724 genes showed increased expression and 329 genes showed decreased transcription compared with MPV. There was a particularly high over-representation of genes with increased transcription whose products are active in the chloroplast (187 genes out of the 724) and these were mainly genes involved in chlorophyll biosynthesis and photosynthesis. The increased transcription of the chloroplast-targeted genes seen at 3 DAS was transient and was not present in cotyledons at 6 or 7 DAS. Up-regulation of the chloroplast-targeted genes was observed in the first and second leaves at 6 or 7 DAS. This up-regulation was also transient and was not present in these leaves at 10 DAS. At 10 DAS in the aerial tissue, 118 genes showed differential expression between F₁ hybrids and the MPV of which 95 were up-regulated and 23 down-regulated genes. Genes categorized into response to biotic stimulus tended to be up-regulated and genes categorized into response to stress tended to be either up- or downregulated. A small number of genes overlapped between the 3 and 10 DAS sets of differentially expressed genes. At 3 DAS, up-regulated genes showed an over-dominance mode, while non-additively expressed genes showed high-parent or low-parent dominance gene action at 10 DAS (Fujimoto et al. 2012a). Transcriptomes of Col, C24, and the reciprocal hybrids at 4, 6, and 10 DAS were also compared (Meyer et al. 2012). Seventy-five percent of differentially expressed genes showed differences in the transcript levels at only one time point, and 10% of genes were significantly different at all time points. Genes involved in defense response tended to be differentially expressed at 6 DAS, while there was no over-representation in any categories at other time points. The gene expression levels in the F₁ hybrids were intermediate between the levels of parental lines or close to the level of one of the two parents, suggesting that mid-parent expression in F_1 hybrids can lead to heterosis (Meyer *et al.*) 2012). These results indicate that the non-additive gene expression profile is drastically changed in developmental stages even when they differ by only a few days (Fujimoto et al. 2012a, Meyer et al. 2012).

In C24 × Ler hybrids, transcriptome analyses have been performed on developing embryos and seedlings, and 15-day-old seedlings (Alonso-Peral *et al.* 2017, Groszmann *et al.* 2015, Shen *et al.* 2012, Zhu *et al.* 2016). In the early embryo at four days after pollination (globular stage) and six days after pollination (heart stage), the transcriptome profile of F_1 hybrids was similar to the maternal line (Alonso-Peral *et al.* 2017). Transcriptome analyses in the early seedlings at 0, 3, 5, and 7 DAS showed the number of non-additively expressed genes at 3 DAS was more than those at the other time points, and the proportion of the over- and under-dominance gene mode at 3 DAS was higher than at the other time points. However, non-additively expressed genes at 3 DAS did not show non-additive gene expression at 5 or 7 DAS, suggesting that non-additive expression during early stages of seedling developments is mainly a consequence of differences in the timing of the gene expression pattern between F1 hybrids and parents (Zhu et al. 2016). At 0 DAS, a small number of non-additively expressed genes overlapped between reciprocal hybrids, while more non-additively expressed genes overlapped between reciprocal hybrids at 3 DAS, suggesting a weakening of the maternal effect, which is established in developing embryos from 0 to 3 DAS (Alonso-Peral et al. 2017, Zhu et al. 2016). At 3 DAS, many up-regulated genes in F₁ hybrids are photosynthesis-related genes (Zhu et al. 2016), similar to the results in C24 \times Col hybrids (Fujimoto *et al.* 2012a). In 15-day-old seedlings, differentially expressed genes tended to be down-regulated in C24 × Ler hybrids, and genes involved in flavonoid biosynthesis were down-regulated in reciprocal hybrids (Groszmann et al. 2015, Shen et al. 2012). Flavonoids inhibit auxin transport, and F_1 hybrids showed faster indole-3-acetic acid (IAA) transport than parental lines, suggesting that heterosis is at least partially due to increased auxin signaling (Shen et al. 2012). Genes involved in salicylic acid (SA) biosynthesis were downregulated in C24 × Ler and C24 × Col hybrids in 15-dayold seedlings and a decrease in SA concentration in C24 \times Ler compared with MPV was observed, suggesting that a reduction in SA concentration and associated changes in gene expression levels in the C24 \times Ler hybrids contribute to heterosis (Groszmann et al. 2015).

Photosynthetic capacity is important for heterosis

In A. thaliana, F₁ hybrids between C24 and Col and between C24 and Ler showed earlier up-regulation of chloroplast-targeted genes in 3-day-old cotyledons (Fujimoto et al. 2012a, Zhu et al. 2016). Genes involved in photosynthesis-related categories were also up-regulated in leaves of super-hybrid rice LY2186 at the grain-filling stage (Song et al. 2010). A commercial F₁ hybrid cultivar of Chinese cabbage (Brassica rapa var. pekinensis), W39, showed vegetative heterosis at early developmental stages and yield heterosis (25% greater than the better parent) (Saeki et al. 2016). Increased cotyledon area in W39 was obvious from 2 to 4 DAS, and genes involved in chloroplasttargeted genes were up-regulated at 2 DAS in W39. As in A. thaliana, this up-regulation was transient (Saeki et al. 2016). In the C24 \times Col hybrids, transient up-regulation of chloroplast-targeted genes did not lead to increased photosynthesis activity per unit area or chlorophyll content per unit fresh weight (Fujimoto et al. 2012a), while superhybrid rice LY2186 showed increased activity of enzymes involved in carbon fixation pathways and net photosynthetic

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rate (Song *et al.* 2010). In the heterotic hybrid, Tentaka, in *Sorghum bicolor*, the CO_2 assimilation rate per leaf area did not differ from its parental lines (Tazoe *et al.* 2016). In all cases, the amount of photosynthesis per individual plant in F_1 hybrids is larger than that of parental lines because of increased leaf area in heterotic hybrids.

The relationship between transient up-regulation of chloroplast-targeted genes and increased leaf area is not clear. In A. thaliana, cell size and chloroplast numbers per cell were correlated (Fujimoto et al. 2012a, Pyke and Leech 1991), and photosystem II chlorophyll a/b-binding polypeptide gene (Lhcb) transcription is coordinated with cell size (Meehan *et al.* 1996). When the leaf area is increased in F_1 hybrids, cell number and/or cell size increased with increased chloroplast number or chlorophyll content, suggesting that up-regulation of chloroplast-targeted genes might be involved in this step. To confirm the importance of a relationship between chlorophyll biosynthesis/photosynthesis and heterotic biomass, norflurazon, an inhibitor of phytoene desaturase and hence chlorophyll synthesis and photosynthesis, was applied. Treatment of seedlings at 3 DAS with norflurazon resulted in the heterotic response of the hybrid being absent at 21 DAS in A. thaliana (Fujimoto et al. 2012a). In Chinese cabbage, seeds were grown on Murashige and Skoog (MS) medium for one week, and transferred to MS medium with 1 µM norflurazon and grown a further two weeks. In this case, heterosis was obvious in the first and second leaves of the W39 hybrid in spite of leaves being without chlorophyll. In contrast, seeds grown on MS medium with 1 µM norflurazon for one week and transferred to MS medium without norflurazon for three



Fig. 3. Shutdown of chlorophyll biosynthesis in the cotyledon by norflurazon preventing increase of leaf area in the F₁ hybrid. Left panel; plants were grown on Murashige and Skoog (MS) medium for two weeks. F₁ hybrids show heterosis in the first and second leaves. Middle panel; seeds were sown on MS medium and grown for one week. The seedlings were transferred to MS medium with 1 μ M norflurazon and grown for another two weeks. F₁ hybrids show heterosis in the first and second leaves. Right panel; seeds were sown on MS medium with 1 μ M norflurazon and grown for one week, then transferred to MS medium for three weeks. Plants did recover chlorophyll biosynthesis after removal of norflurazon. However, F₁ hybrids do not show heterosis in the third and fourth leaves.

weeks did not show any heterosis in the third and fourth leaves, which were green (**Fig. 3**) (Saeki *et al.* 2016). These results suggest that chlorophyll biosynthesis or photosynthesis in the first week of cotyledon growth is important for increased leaf size in F_1 hybrids and even after the cotyledon stage. The up-regulation of chloroplast-targeted genes in the cotyledon and photosynthesis at the cotyledon stage are important for increased leaf area in F_1 hybrids, and this increased leaf area could lead to an increased amount of photosynthesis per individual plant, leading to yield heterosis (Saeki *et al.* 2016).

Is circadian-mediated gene expression involved in heterosis?

The possibility that heterosis is caused by a change of gene expression of master genes affecting downstream gene expression has been discussed. Altered expression of the central clock genes in the F_1 increases expression levels of downstream genes involved in energy and metabolism including photosynthesis and starch pathways, and could lead to biomass heterosis (Ni *et al.* 2009).

Inter-specific hybrids show non-additive gene expression and a hybrid vigor-like phenotype (Fujimoto et al. 2011, Osabe et al. 2012a, Tonosaki et al. 2016). The progeny of allotetraploids synthesized by inter-specific hybridization between A. thaliana and A. arenosa showed growth vigor and non-additive expression of over 1,400 genes. Of these non-additively expressed genes, many genes involved in energy and metabolism pathways are up-regulated. Some genes contain at least one CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) binding site or evening element. CCA1 and LATE ELONGATED HYPOCOTYL (LHY) expression levels in allotetraploids were lower than the MPV at Zeitgeber time (ZT) 6-12 and higher than the MPV at ZT15 (ZT reflects the hours since dawn). TIMING OF CAB EXPRESSION 1 and GIGANTEA expression were inversely correlated with CCA1 and LHY expression. These results indicate that amplitude but not the phase of the circadian clock is altered in allotetraploids, suggesting that this change leads to up-regulation of genes in chlorophyll and starch metabolic pathways and an increased amount of chlorophyll and starch. Similar results were observed in an intra-specific hybrid between C24 and Col accessions of A. thaliana, suggesting that altered expression of circadian oscillator genes is associated with heterosis in $C24 \times Col$ hybrids as well as in allotetraploids. Ni et al. (2009) showed 12% more total chlorophyll and 10% more starch in $C24 \times Col$ hybrids than the higher parent. By contrast, chlorophyll content per unit fresh weight and photosynthetic activity per unit area in C24 × Col hybrids was not increased (Fujimoto *et al.* 2012a). The increased expression of genes reported by Ni et al. (2009) was not apparent in the transcriptome analyses by two other groups using the same $C24 \times Col$ hybrids (Fujimoto *et al.* 2012a, Meyer *et al.* 2012). As the non-additively expressed genes are stagespecific (Fujimoto *et al.* 2012a, Meyer *et al.* 2012), there is a possibility that a difference in developmental timing is responsible for the differences reported in the transcriptomes.

In the hybrid between C24 and Ler, expression levels of CCA1 were 20% to 30% lower than the MPV at ZT6 and *CCA1* expression peaks in $C24 \times Ler$ were lower than Ler \times C24 hybrids at ZT6 (e.g. ZT6 is six hours from dawn). This difference of CCA1 expression between reciprocal hybrids was correlated with difference of starch accumulation; lower CCA1 expression at ZT6 correlates with increased biomass. The DNA methylation level at CHH sites (H is A, T, or C) in the promoter region of CCA1 in Ler \times C24 hybrids was higher than that in C24 \times Ler hybrids, suggesting there was negative correlation between DNA methylation level and *CCA1* expression. In addition, Ler \times argonaute 4 (ago4) (Ler) hybrids showed lower CCA1 expression peaks, higher CHH methylation levels in the promoter region of CCA1, and greater dry weight than those in ago4 (Ler) × Ler hybrids. These results suggest that parent-of-origin effect on CHH methylation via RNA-directed DNA methylation (RdDM) affects CCA1 expression amplitude and leads to growth difference between reciprocal hybrids (Ng et al. 2014). It is not clear how this model is related to the heterosis shown in both reciprocal hybrids (C24 \times Ler or Ler \times C24 hybrids).

Unlike *A. thaliana*, in maize, there is a temporal shift of ZmCCA1-binding to targets to the early morning; the proportion of ZmCCA1-binding peaks at ZT3 was higher than at ZT9 or ZT15 in hybrids but there was no difference between ZT3 and ZT9 in parental lines. The target genes of this temporal shift to the early morning showed upregulation in the morning in hybrids compared with parental lines. From these results, a phase-shift model for heterosis was predicted; the altered temporal binding of ZmCCA1 to photosynthetic and metabolic genes causes non-additive gene expression levels and increased carbon fixation capacity, leading to heterosis (Ko *et al.* 2016).

It is certain that circadian rhythm is important for the growth of plants (Ni *et al.* 2009), but further research will be necessary to determine whether an alteration of the amplitude or phase-shift of the circadian rhythm directly up-regulates the expression of genes involved in chlorophyll and starch metabolic pathways and increases the amount of chlorophyll or starch in heterotic hybrids.

Inter- and Intra-specific hybridization cause nonadditive small RNA expression

Small RNAs are involved in a broad range of function; micro RNAs (miRNAs) are predominantly 21 nucleotides (nt) in length and involved in plant development (Cui *et al.* 2017, Guo *et al.* 2016). Small interfering RNA (siRNAs) are predominantly 24 nt in length and involved in heterochromatin maintenance and silencing of transposable elements (TEs) (Wendte and Pikaard 2017). 24-nt siRNAs are involved in the process of *de novo* DNA methylation, termed



Fig. 4. Schematic diagram illustrating the maintenance and *de novo* DNA methylation via RNA-dependent DNA methylation (RdDM) pathway. AGO4, ARGONAUTE 4; CMT2, CHROMOMETHYLASE 2; CMT3, CHROMOMETHYLASE 3; DCL3, DICER-LIKE 3; DDM1, DECREASE IN DNA METHYLATION 1; DRM2, DOMAINS REARRANGED METHYLTRANSFERASE 2; KYP, KRYPTONITE; MET1, METHYLTRANSFERASE 1; Pol IV, RNA Polymerase IV; Pol V, RNA Polymerase V RDR2, RNA-DEPENDENT RNA POLYMERASE 2; 24-nt siRNA, 24 nucleotide small interfering RNA. mCG, mCHG, and mCHH represent DNA methylation at CG, CHG, and CHH sites (H is A, T, or C), respectively.

RdDM. Two plant specific RNA polymerases, RNA Polymerase IV (Pol IV) and Pol V, along with RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), DICER-LIKE 3 (DCL3), and AGO4 all function in this RdDM pathway in *A. thaliana* (**Fig. 4**) (Wendte and Pikaard 2017). Pol IV and RDR2 are involved in the biogenesis of 24-nt siRNAs and are physically associated in both *A. thaliana* and maize (Haag *et al.* 2012, 2014, Law *et al.* 2011).

Several reports have examined global small RNA profiles using heterotic F_1 hybrids and their parental lines in maize, rice, and A. thaliana (Barber et al. 2012, Groszmann et al. 2011a, Ha et al. 2009, He et al. 2010, Kenan-Eichler et al. 2011, Li et al. 2012, Shen et al. 2012). The first report comparing small RNA profiles between the hybrids and parents used inter-specific hybrids between A. thaliana and A. arenosa, which display growth vigor. siRNAs were reduced in the F_1 , but their levels recovered in the F_7 (Ha et al. 2009). In another case of inter-specific hybrids between tetraploid Triticum turgidum ssp. durum (AABB genome) and the diploid Aegilops tauschii (DD genome), siRNAs were not changed in hybrids (ABD genome), but were decreased in allopolyploids (AABBDD genome) (Kenan-Eichler *et al.* 2011). The inter-specific hybrid of a different parental combination, T. turgidum ssp. dicoccon (AABB) and Ae. tauschii ssp. strangulata (DD), was also used for small RNA profiles in three types of tissues, 7-day-old seedling, heading-stage young spikes, and immature seed at 11 days after flowering. In all three tissues, the levels of 24-nt siRNAs in Ae. tauschii were smaller than those in T. turgidum, and in allohexaploid progeny levels appeared to be similar to those in T. turgidum (Li et al. 2014). These results indicate changes of expression of 24-nt siRNA occur in some tissues of the allopolyploids compared with the progenitor species.

In the case of intra-specific hybrids, 24-nt siRNAs tended to be down-regulated in heterotic hybrids compared with their parental lines. In rice heterotic hybrid between 'Nipponbare' and 93-11, siRNAs were down-regulated in the hybrid and most showed a low parent pattern (He et al. 2010), although this conclusion has been changed following re-analysis of the data by the same group; only a small fraction of siRNAs (<0.8%) changed between hybrid and parental lines (Shen et al. 2012). In B. napus, the number of 24-nt siRNA clusters in F₁ hybrids was higher than the MPV in the shoot apical meristems or young flower buds (Shen et al. 2017). The heterotic hybrid of A. thaliana, $C24 \times Ler$, showed a reduction of 24-nt siRNAs relative to the parental lines at loci where the parents had different 24-nt siRNAs levels (Groszmann et al. 2011a). Another research group showed that most siRNA clusters (>93%) were not differentially expressed between parents and hybrids, though differentially expressed siRNA clusters tended to be at low parent value (Shen et al. 2012). The global small RNA profile has been examined using another combination of A. thaliana accessions, Ler and Col, which showed biomass heterosis in short day conditions. Sixty percent of siRNA clusters showed non-additive expression and 80% were at low parent values (Li 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 below mid-parent levels (Barber et al. 2012).

Global or local reductions in 24-nt siRNAs in hybrids may be a universal phenomenon but whether the reduction of 24-nt siRNAs is responsible for heterosis is not clear. 24nt siRNAs are involved in RdDM and it is generally considered that RdDM can lead to transcriptional repression not only in TEs but also in protein-coding genes through DNA methylation; reduction of 24-nt siRNAs may result in changes of DNA methylation levels leading to change of expression level in TEs and some protein-coding genes. As siRNAs have the ability to modify epigenetic marks in trans, siRNA derived from one parental allele can modify the epigenetic state in the other parental allele in hybrids (Greaves et al. 2015, Groszmann et al. 2013). The number of re-activated transposons in an RdDM defective mutant was much smaller than in the hypomethylated decrease in DNA methylation 1 (ddm1) mutant (Zemach et al. 2013), suggesting that the effect of silencing of transposons by RdDM is not very large; siRNA-targeted TEs are associated with reduced gene expression in both A. thaliana and A. lyrata (Hollister et al. 2011). In C24 × Ler hybrids, decreased 24-nt siRNAs are largely associated with flanking regions of protein-coding genes and there was a correlation between change of siRNA levels with DNA methylation and expression levels at several loci (Groszmann *et al.* 2011a).

In the *rdr2* mutant, which has defects in 24-nt siRNA biogenesis, only 70 genes had differential expression compared with wild type (wt) plants, and 26 of these 70 genes had siRNAs in their flanking regions (Kurihara *et al.* 2008),

indicating RdDM has only a small impact on expression of protein-coding genes. To clarify the relationship between siRNAs and heterosis, mutants in biogenesis of 24-nt siRNAs were analyzed in maize and A. thaliana (Barber et al. 2012, Kawanabe et al. 2016, Zhang et al. 2016a). Heterosis was fully maintained in maize hybrids, homozygous for mediator of paramutation 1 (mop1), the ortholog of RDR2 (Barber et al. 2012). The heterosis phenotype is not affected in hybrids between Col and C24 accessions in A. thaliana with homozygous mutations in either rdr2 or nuclear RNA polymerase IV, subunit 1 (nrpd1; largest subunits of Pol IV), which are key genes involved in biogenesis of 24-nt siRNAs (Kawanabe et al. 2016, Zhang et al. 2016a, 2016b). Hybrids having mutations in ago4, ago6, or nuclear *RNA polymerase V, subunit 1 (nrpe1; largest subunit of Pol* V), which are also components of RdDM, maintained heterosis (Zhang et al. 2016b). Hybrids having mutations in defective in meristem silencing 3 (dms3), defective in RNA-directed DNA methylation 1 (drd1), or RNA directed DNA methylation 1 (rdm1), which are components of the DRD1-DMS3-RDM1 (DDR) complex involved in Pol V function, also maintained heterosis (Zhang et al. 2016b). These results indicate that the RdDM pathway is not required for the establishment of heterosis in maize or A. thaliana.

Is DNA methylation a key factor in heterosis?

A comparison of DNA methylation states between parental lines and their progenies generated from single seed descent over 30 generations has shown that the rate of spontaneous changes of DNA methylation is higher than the rate of spontaneous genetic mutations (Becker et al. 2011, Ossowski et al. 2010, Schmitz et al. 2011), suggesting that sequenceindependent epialleles play important roles in phenotypic diversity (Becker et al. 2011, Fujimoto et al. 2012b, Schmitz et al. 2011). DNA methylation, which is one epigenetic modification, is classified into two types, de novo (see above) and maintenance DNA methylation. In maintenance DNA methylation, CG context methylation is maintained by METHYLTRANSFERASE 1 (MET1), and non-CG contexts are maintained by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), CHROMOMETH-YLASE 2 (CMT2), and CMT3 (Osabe et al. 2012b, Zemach et al. 2013). In addition to DNA methyltransferases, a chromatin remodeling factor, DDM1, is involved in the maintenance of DNA methylation (Fig. 4) (Fujimoto et al. 2008, Sasaki et al. 2011, Vongs et al. 1993, Zemach et al. 2013).

To identify loci with epigenetic regulation causing phenotypic variation, populations of epigenetic recombinant inbred lines (epi-RILs) between parents, which differed only in epigenetic marks, such as hybrids between *met1* and wt Col, or between *ddm1* and wt Col have been generated in *A. thaliana* (Johannes *et al.* 2009, Reinders *et al.* 2009, Teixeira *et al.* 2009). Stable inheritance of complex traits such as flowering time, primary root length, plant height,

and biomass has been observed in these epi-RIL populations, providing evidence that epigenetic variation can contribute to complex traits (Cortijo et al. 2014, Johannes et al. 2009, Reinders et al. 2009). Enhanced growth similar to heterosis was observed in several of the hybrids between wt and specific epiRIL lines. In the case of epiRIL lines derived from hybrids between met1 and wt, 34 selected epiRILs were crossed with wt Col, and rosette sizes in F₁ hybrids were examined. Twenty-eight of 34 hybrids did not exceed the better parent. Two F₁ hybrids had enlarged rosettes at a young stage but not after 14 days. The remaining four F_1 hybrids showed increased rosette size until bolting, and the F₁ hybrid between one epiRIL, epi31, and wt showed consistent vigorous growth. The $epi31 \times wt$ hybrids showed this vigorous growth, but wt \times epi31 did not, suggesting that parent-of-origin effects influence hybrid growth (Dapp et al. 2015). This research supports the involvement of epigenetic regulation and interactions in heterosis (Dapp et al. 2015). In the case of other epiRIL lines derived from the hybrids between ddm1 and wt, the F₁ hybrids between Col and 19 epiRILs were generated, and three F_1 hybrids showed greater than 15% increased leaf area compared with the better parent, suggesting that heterosis in F_1 hybrids generated from epigenetically divergent lines may be a general phenomenon (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 plant phenotypes such as leaf variegation, dwarfism, altered leaf structure, and late-flowering in A. thaliana. An altered phenotype caused by disruption of MSH1 was also observed in other species such as sorghum, tomato, and soybean (Xu et al. 2012). Disruption of MSH1 causes changes of DNA methylation in A. thaliana (Virdi et al. 2015). Enhanced vigor was observed in F₄ generations derived from the hybrids between wt and msh1 in A. thaliana (Virdi et al. 2015). MSH1-RNA interference (RNAi) suppression in sorghum or tomato showed altered phenotypes, which were inherited even in transgenenull offspring. Crossing between wt and transgene-null plants with developmental alterations results in enhanced heritable growth vigor in the progeny lines in sorghum and tomato (de la Rosa Santamaria et al. 2014, Yang et al. 2015b). These results suggest that epigenetic reprogramming can result in enhanced growth patterns; this may lead to its use in epigenetic breeding in crop plants (Virdi et al. 2015, Yang et al. 2015b).

Recent progress has pointed to the possibility that epigenetic regulation might contribute to heterosis (Greaves *et al.* 2015, Groszmann *et al.* 2011b, 2013, Springer 2013). DNA methylation states have been compared between heterotic F_1 hybrids and parental lines in rice, *B. napus*, and *A. thaliana* (Chodavarapu *et al.* 2012, Greaves *et al.* 2012, He *et al.* 2010, Shen *et al.* 2012, 2017, Zhang *et al.* 2016a). In *B. napus*, F_1 hybrids had a slightly higher level of DNA methylation at all three sequence contexts, CG, CHG, and CHH sites (H is A, T, or C). The number of unmethylated cytosines in F_1 hybrids was decreased, while the number of highly methylated cytosines was not increased. As increased 24-nt siRNA clusters were observed in F_1 hybrids, the authors suggested that increased 24-nt siRNAs are directly correlated with increased DNA methylation levels (Shen *et al.* 2017). In rice using fully expanded leaves from sixweek-old plants in 'Nipponbare' and *indica* 93-11, and their reciprocal hybrids, DNA methylation levels in the F_1 hybrids were intermediate to parental methylation levels. Differential methylation was strongly associated with regions where siRNA production was unequal in the two parents (Chodavarapu *et al.* 2012).

In the case of A. thaliana using 15-day-old seedlings of C24, Ler, and its reciprocal hybrids, increased DNA methylation levels at all three contexts in both reciprocal hybrids relative to parental lines were observed. F1 hybrids had increased DNA methylation relative to parental lines in both protein-coding genes and TEs, and the increase in TEs was greater than in protein-coding genes. In addition, increased DNA methylation in F₁ hybrids occurred predominantly in regions covering siRNA clusters, especially the regions that were differentially methylated between the parents (Shen et al. 2012). In the case of this parental combination using the immature floral buds, F1 hybrids showed differences of DNA methylation compared with parental lines, especially the regions differentially methylated between the parental lines. Increases in DNA methylation were more frequent than decreases in F_1 hybrids (Greaves *et al.* 2012). Change of DNA methylation states in the F₁ hybrids results from 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) or trans-chromosomal demethylation (TCdM) (loss of methylation at a genomic segment) (Fig. 5), and TCM and TCdM events in hybrids are largely dependent on 24-nt siRNAs (Greaves et al. 2012). In the case of A. thaliana using 14-day-old seedlings of C24, Col



Fig. 5. Change of DNA methylation state in the F_1 hybrid. Transchromosomal methylation (TCM); gaining DNA methylation of an allele with low methylation. siRNA derived from parent with high methylation level can cause TCM. Trans-chromosomal demethylation (TCdM); loss of DNA methylation by loss of siRNA at a genomic segment.

and their reciprocal hybrids, increased DNA methylation levels in hybrids were observed in all three contexts. Regions differentially methylated by TCM and TCdM were observed in F_1 hybrids and 24-nt siRNAs were associated with them.

In the hybrids having mutations in both *nrpd1* (*pol iv* mutant) and *nrpe1* (*pol v* mutant), TCM and TCdM were abolished, but hybrids maintained the heterosis phenotype, suggesting that these DNA methylation interactions are not critical for heterosis (Zhang *et al.* 2016a). More than 10,000 regions of non-additively inherited DNA methylation in epi-hybrids occur between *met1* and wt, though these F_1 plants do not show superior performance, suggesting that creation of new non-parental epialleles in F_1 hybrids is independent of heterosis (Rigal *et al.* 2016).

Recently two groups showed that DDM1 is a major regulator of heterosis (Kawanabe et al. 2016, Zhang et al. 2016b). The F_1 hybrids having homozygous mutations in ddm1 had reduced vegetative heterosis in C24 × Col hybrids (Kawanabe et al. 2016, Zhang et al. 2016b). The reduction of vegetative heterosis by defects in DDM1 function was also observed in the C24 \times Cvi and the Col \times Cvi hybrid, the level of reduction of heterosis being dependent on the combination of parental lines (Kawanabe et al. 2016). In the hybrid between a heterozygous *ddm1-9* mutant in C24 and a homozygous *ddm1-1* mutant in Col, plants homozygous for the *ddm1* mutation were smaller than heterozygotes. However, some plants homozygous for *ddm1* showed heterosis as great as the plants having a heterozygous *ddm1* mutation, and some plants with heterozygous *ddm1* reduced heterosis to the level in the homozygous ddm1 hybrid plants (Kawanabe et al. 2016). In both cases there was 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 genes or chromosomal segments important for heterosis coming from the *ddm1* parent having an altered level of DNA methylation.

To examine the involvement of MET1 in heterosis, two approaches were tested; using met1-RNAi knockdown lines in a C24 background or BC_2F_1 lines obtained by backcrossing of C24 to a hybrid with a met1 mutant of Col. Hybrids between *met1*-RNAi (C24) and *met1-1* (Col) or BC_2F_1 $(C24) \times metl-l$ (Col) hybrid with homozygous metl did not show reduced level of heterosis, indicating that MET1dependent maintenance of CG methylation is not involved in heterosis in C24 \times Col hybrids (Kawanabe *et al.* 2016). An F₁ hybrid between hypomethylated plants was developed by crossing *ddm1-9* in C24 and *met1-1* in Col. The $ddm1-9 \times met1-1$ hybrid (DDM1/ddm1, MET1/met1) showed heterosis levels like the wild type $C24 \times Col$ hybrids, indicating that a hypomethylated state of parental lines does not cause a decreased heterosis phenotype (Kawanabe et al. 2016).

The results from vigorous growth in F_1 hybrids between epiRILs and wt or reduction of heterosis levels by disrup-

tion of DDM1 function, which is involved in maintenance of DNA methylation in all three sequence contexts, suggests that alterations in DNA methylation affect the level of heterosis. However, the mechanism is unclear and further study will be required to understand how DDM1 regulates heterosis.

Heterosis and stress tolerance

Most heterosis research has been focused on growth vigor or increased yield, and there is little research on heterosis and stress tolerance. However, if stress tolerance and yield heterosis could be combined, these hybrids could be excellent cultivars.

The relationship between biomass heterosis and change of gene expression in stress response has been investigated. Transcriptomes of three-week-old seedlings at ZT0, ZT6, and ZT15 were compared between a C24 \times Col hybrid and its parental lines. Genes categorized into photosynthetic and stress-responsive pathway tended to show differential expression between F₁ and MPV. At ZT6, genes involved in the photosynthetic pathway were mostly up-regulated in F_1 hybrids, while genes involved in abiotic or biotic stress tended to be down-regulated. Using the ACCELERATED CELL DEATH 6 (ACD6) or COLD REGULATED 78 (COR78) gene as a biotic or abiotic stress marker gene, respectively, gene expression levels of these two genes at ZT0, ZT9, and ZT18 were examined in ten accessions of A. thaliana. Ten accessions were divided into two groups, those where ACD6 genes showed a higher expression level at ZT18 and those where COR78 genes showed a higher expression level at ZT6. The biomass heterosis was correlated with difference of expression levels of ACD6 at ZT18 or COR78 at ZT9 between parental lines, suggesting that biomass heterosis is dependent on larger expression differences in stress-responsive genes between parental lines (Miller et al. 2015). A model of balancing stress response to promote biomass heterosis has been proposed (Miller et al. 2015). Hybrid necrosis, which causes slow growth, wilting, discoloration, and lethality in intra- and inter-specific hybrids, would result from an autoimmune-like response through epistatic interaction between resistance genes (Tonosaki et al. 2016). In F_1 hybrids, heterosis is opposite to necrosis in plant growth, suggesting that the repression of genes related to biotic stress may result in heterosis because necrosis is caused by induction of biotic stress genes. In some plant species, genes involved in biotic stress in F_1 hybrids with growth vigor tended to be down-regulated compared with those in their parental lines, and there could be a negative trade-off between expression of defense response genes and growth heterosis (Fig. 6) (Miller et al. 2015).

In the F_1 hybrids between C24 and L*er*, reduction of the expression levels in defense response genes was confirmed, but hybrids did not show greater susceptibility to *P. syringae* in F_1 hybrids than in its parental lines (Groszmann *et al.* 2015). In other combinations of parental lines in *A. thaliana*,



Fig. 6. A trade-off between expression of defense response genes and growth heterosis. Repressors affect defense response genes (R1 or R2), and repression of these genes result in enhanced growth (G1 or G2). Parents (P_1 or P_2) have a balance between expression of defense response genes and growth, while F_1 emphasizes growth at the expense of the expression of defense response genes.

there was no relationship between change of gene expression in biotic stress pathways and biomass heterosis (Yang *et al.* 2015a). In the case of Fusarium head blight (FHB) resistance in wheat, 1,604 hybrids and 120 female and 15 male parental lines were evaluated for FHB resistance and heterosis levels. The mean better parent heterosis of FHB resistance was 2% and there was no correlation between FHB resistance and plant height, indicating growth heterosis is independent of FHB resistance (Miedaner *et al.* 2017). In the case of Fusarium yellows resistance gene in Chinese cabbage, resistance was largely dependent on the resistance gene itself as generally thought (Pu *et al.* 2012, Shimizu *et al.* 2014, 2015), and the growth vigor does not contribute to Fusarium yellows resistance (Kawamura *et al.* 2015, 2016, Saeki *et al.* 2016).

Though there is little evidence of a positive relationship between biomass heterosis and stress tolerance, there is some evidence of heterosis in stress tolerance. In A. thaliana, heterosis in disease resistance against P. syringae pv. tomato (*Pst*) DC3000 was examined using F_1 hybrids between Col and 20 accessions. Two F_1 hybrids (Col × Ler, Col × Sei-0) showed heterosis in Pst DC3000 resistance, while the other 18 F₁ hybrids did not have any heterosis. The Col \times Sei-0 hybrids showed up-regulation of genes involved in the SA biosynthesis pathway compared with parental lines, contrasting to Col × Aa-0 hybrids, which did not show heterosis in disease resistance. In addition, Col × Sei-0 hybrids accumulated more SA than parental lines at one day postinoculation, but Col × Aa-0 hybrids did not. PHYTOALEXIN DEFICIENT 4 (PAD4) functions upstream of the SA biogenesis pathway, and the $Col \times Ler$ hybrids with homozygous pad4 mutation did not show heterosis in disease resistance, indicating that increased levels of SA resulting from enhanced activation of the SA biosynthesis pathway in hybrids are important for heterosis in disease resistance (Yang et al. 2015a).

Heterosis in freezing tolerance in reciprocal F₁ hybrids between C24 and Col accessions was observed in both acclimated and non-acclimated plants. The content of soluble sugars such as fructose, glucose, raffinose, and sucrose in leaves was increased more in the F₁ hybrids than the parental lines in the acclimated state. Expression levels of some cold-regulated genes were examined after three hours or 14 days of cold treatment, but all genes in F_1 hybrids showed expression levels within the parental range (Rohde et al. 2004). Further combinations of A. thaliana accessions were tested and heterosis in freezing tolerance was observed in some F₁ hybrids. Heterosis was observed more frequently in crosses involving C24 than in crosses involving Col. There was a moderate correlation between heterosis effects in freezing tolerance and sugar contents or flavonol content (Korn et al. 2008). In addition, metabolic profiles in five parental accessions and eight F₁ hybrids in A. thaliana revealed that the pathway leading to raffinose or tricarboxylic acid cycle intermediates can be used to predict heterosis in freezing tolerance (Korn et al. 2010).

The data are not extensive but overall there was no direct relationship between biomass heterosis and heterosis in freezing tolerance or disease resistance. However, it may be possible to produce better cultivars if the biomass heterosis and the stress tolerance heterosis are combined.

Perspective

Plant breeders have been very successful in increasing crop yields and the phenomenon of heterosis has contributed to these advances for about 100 years. However, global food shortage due to population increase and global climate change is imminent, so further increases of crop production are critical. While heterosis has already contributed to increased yield, we do not understand the molecular mechanisms of heterosis. Recently, the advent of high-throughput



Fig. 7. Possible pathway of heterosis in *A. thaliana*. Heterosis is dependent on the increased photosynthesis by increased leaf area, and DECREASE IN DNA METHYLATION 1 (DDM1) plays a role in increasing leaf area of F_1 hybrids. Left bottom picture represents seed-lings at 28 days after sowing, and the F_1 having homozygous *ddm1* mutations reduces vegetative heterosis.

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sequencing has enabled the comparison of levels of gene expression between hybrids and their parents and identification of metabolic pathways altered in hybrids. Photosynthesis and chloroplast-targeted genes are up-regulated in hybrids relative to parents suggesting earlier development in heterotic hybrids (Fig. 7) (Fujimoto et al. 2012a, Saeki et al. 2016, Zhu et al. 2016). High throughput sequencing has also enabled dissection of the parental allelic contributions to gene expression and epigenetic states (Chodavarapu et al. 2012, Saeki et al. 2016). The chromatin remodeler DDM1 appears essential for the development of full heterosis (Fig. 7) (Kawanabe et al. 2016, Zhang et al. 2016b). Fixing heterosis so that it continues beyond the F_1 generation is a challenging strategy for increasing yield, and "Hybrid Mimics", which are pure breeding lines having the characteristics of hybrids and fix heterosis in subsequent generations, would be an option (Wang et al. 2015, 2017). Combining heterosis in different characters such as yield heterosis and stress tolerance heterosis could enhance yield. Research on the big issue of heterosis should continue as a part of enhancing food production.

Acknowledgments

This work was supported in part by an Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects (14544567), Scientific Research on Innovative Areas (24113509), Grant-in-Aid Young Scientists (A) (15H05614) (JSPS), and PREST (JPMJPR12B8) (JST) to R. Fujimoto.

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