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Analysis of transcriptional and epigenetic changes in hybrid vigor of allopolyploid *Brassica napus* uncovers key roles for small RNAs

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Running title: Heterosis in the allopolyploid F_1 hybrids of *B. napus*

Key words: *Brassica napus*, heterosis, allopolyploid, transcriptome, miRNA, siRNA, DNA methylation

Summary

Heterosis is a fundamental biological phenomenon characterized by the superior performance of a hybrid compared to its parents. The underlying molecular basis for heterosis, particularly for allopolyploids, remains elusive. In this study, we analyzed the transcriptomes of *Brassica napus* parental lines and their F_1 hybrids at three stages of early flower development. Phenotypically, the F_1 hybrids show remarkable heterosis in silique number and grain yield. Transcriptome analysis revealed that various phytohormones (auxin and salicylic acid) response genes are significantly altered in the F_1 hybrids relative to the parental lines. We also found evidence for decreased expression divergence of the homoeologous gene pairs in the allopolyploid F_1 hybrids and suggest that high-parental expression level dominance plays an important role in heterosis. Small RNA and methylation studies aimed at examining the epigenetic effect of the gene expression level changes in the F_1 hybrids showed that the

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majority of the small interfering RNA (siRNA) clusters had a higher expression level in the F_1 hybrids than parents, and that there was an increase of the genome-wide DNA methylation in the F_1 hybrid. Transposable elements associated with siRNA clusters had a higher level of methylation and a lower expression level in the F_1 hybrid, implying that the non-additively expressed siRNA clusters resulted in lower activity of the transposable elements through DNA methylation in the hybrid. Our data provide insights into the role that gene expression pattern changes and epigenetic mechanisms contribute to heterosis during early flower development in allopolyploid *B. napus*.

Introduction

Heterosis is a complex biological phenomenon in which hybrids exhibit superior phenotypic characteristics, such as enhanced biomass production, developmental rates, grain yields, and stress tolerances, relative to their parents (Birchler et al., 2003; Hochholdinger and Hoecker, 2007; Birchler et al., 2010; Chen, 2010). Although heterosis has been exploited extensively in crop production for many decades, the molecular mechanisms underlying the phenomenon remain largely unknown despite more than a century of study (Birchler et al., 2003; Hochholdinger and Hoecker, 2007; Birchler et al., 2010; Chen, 2010). Dominance, overdominance, and epistasis have each been proposed as genetic explanations of heterosis from a quantitative perspective (Crow, 1948; Yu et al., 1997). However, these hypotheses are largely conceptual and not connected to molecular principles and, therefore, they fall short in

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explaining the molecular basis of heterosis (Birchler et al., 2003; Hochholdinger and Hoecker, 2007; He et al., 2011).

Several studies have shed light on the potential molecular mechanisms of heterosis in plants. A single heterozygous gene, *SINGLE FLOWER TRUSS*, was shown to cause hybrid vigor in tomato (*Solanum lycopersicum*), providing the first molecular evidence of a single overdominant gene driving heterosis (Krieger et al., 2010). Recent work has also shown that hormone-mediated defense and stress response networks contribute to heterosis in *Arabidopsis* F_1 hybrids (Groszmann et al., 2015). There are also data showing that epigenetic variation may contribute to the molecular mechanisms of complex traits, including hybrid vigor (Cubas et al., 1999; Manning et al., 2006; Shindo et al., 2006; Ni et al., 2009; Alonso-Peral et al., 2010). For example, Ni et al. (2009) showed that epigenetic modification of a few regulatory genes (e.g., the circadian oscillators *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*)), were associated with growth vigor in *Arabidopsis thaliana* hybrids.

The genetic composition of hybrid offspring is the result of information inherited from the two parents. Theoretically, no new genes are produced in the F_1 hybrid, so heterosis is likely caused by differences resulting from qualitative or quantitative modifications of gene expression. According to genomic and epigenetic principles, offspring exhibit advantages in growth, stress resistance, and adaptability because of interactions between alleles of parental genomes that change the regulatory network of related genes. Microarray and high-throughput sequencing technologies have enabled detailed investigations of the

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molecular basis of heterosis at the whole genome level (Zhang et al., 2008; Ni et al., 2009; Wei et al., 2009; Lai et al., 2010; Song et al., 2010; Groszmann et al., 2011).

Small RNAs, including microRNA (miRNA) and small interfering RNA (siRNA), affect gene expression by posttranscriptional mechanisms and epigenetic modifications (Vaucheret, 2006; Lu et al., 2012). In intraspecific *Arabidopsis* hybrids, variation in miRNA expression causes non-additive expression of target genes that may affect growth, vigor and adaptation (Ha et al., 2009). Both *cis*- and *trans*-regulation of miRNAs and other genes may underlie natural variation in biochemical and metabolic pathways that affect growth vigor and stress responses (Ng et al., 2011; Ng et al., 2012). Moreover, siRNAs, particularly those associated with transposable elements (TEs), may serve as a buffer against genomic shock and influence gene expression by directing DNA methylation. For example, recent studies suggest that epigenetic diversity between parental lines, such as differences in the levels of the 24-nucleotide (nt) siRNAs in two *Arabidopsis* ecotypes, create allelic variants that influenced the activity of a large number of metabolic and regulatory genes that may contribute to heterotic phenotypes (Groszmann et al., 2011; Shen et al., 2012). These observations indicate that siRNAs may have effect on genome-wide gene expression bias and could be important determinants in plant heterosis.

Brassica napus is a major edible oil-producing crop and its F_1 hybrids have been widely used in commercial production for many years. *B. napus* has a flower development process similar to that of *Arabidopsis*. There have been a few studies on the potential mechanism of heterosis in *B. napus* (Grant and Beversdorf, 1985; Chen et al., 2008). Most of these

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investigations, as well as other studies with *Arabidopsis*, focused on the seedling stage. Investigations of the effects of gene expression on heterosis during flower development are much more limited. Previous studies from our group showed that the early stages of flower development are vital for determine the number of flowers and siliques produced per plant and consequently yield (Zhang et al., 2016b). However, stage-specific transcriptomic changes, which are crucial for understanding the gene regulatory networks underlying flower development and the phenomenon of heterosis in *B. napus*, have not been thoroughly investigated.

The genome sequence of *B. napus* is now available (Chalhoub et al., 2014) and together with the next-generation sequencing technology, provides an unprecedented opportunity to address questions concerning gene expression changes and epigenetic variations between the two parents and their F_1 hybrids that are associated with heterosis in allopolyploid *B. napus*. In this study, we investigated the dynamic transcriptomes, small RNAomes and DNA methylomes of an F_1 hybrid and its two parents at three early flower developmental stages of *B. napus*. Our results revealed that similarities and differences in gene expression and epigenetic changes exist between the F_1 hybrid and its parents at the early flower developmental stage, and these data provide insights into the role of transcriptional and epigenetic changes in heterosis of *B. napus*.

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Results

Heterosis of F_1 hybrids in oilseed rape

The F_1 hybrid (F_1 hereafter) ZYZ108 derived from the cross of MB (female) and ZY50 (male) parents has significantly increased silique numbers and grain yield (Figure 1A-B). Total grain yield of F_1 was 60% higher than the mid-parent value (MPV). Increased yield was mainly the result of significantly increased silique numbers (~50% higher than MPV), because no significant difference was observed for the weight per seed between F_1 and the two parents. The increased silique number of F_1 was not a result of an extended flowering period because the flowering time of F_1 was in-between the two parents and all three genotypes were harvested at the same time. The effect of heterosis on silique number is directly correlated with the increased number of flowers in F_1 (Zhang et al., 2016b). Therefore, we used tissues from three key stages during early flower development to investigate the potential molecular mechanisms underlying heterosis in *B. napus*.

Differentially expressed genes (DEGs) in the two parents are enriched in the F_1 hybrid

Dynamic changes of the transcriptomes in F_1 and its two parents were investigated by analyzing transcripts in shoot apical meristems at the transition meristem (SAM1) and flower meristem initiation (SAM2) stages, and young flower buds (YFB) at the early flower developmental stage (Figure 1C).

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We first analyzed the transcriptomes of each hybrid and compared them to their parents at the same developmental stage (① at Figure 2). Genes in F_1 that had expression levels significantly deviated from the MPV ($p \leq 0.05$; FDR ≤ 0.05) were designated as hybrid-MPV differentially expressed genes (DEGs) (Data S1). Such genes could potentially be responsible for generating the heterosis phenotype. We found that only a small portion of all expressed genes were hybrid-MPV DEGs at the three stages (494-1,592 or 0.81-2.31%) (Figure 3A-C, G). This suggests that the majority of genes in F_1 show expression additivity, whereas only a small part of the expressed genes show non-additivity of expression ($p \leq 0.05$; FDR ≤ 0.05). In F_1, more up-regulated than down-regulated genes were observed in SAM1 and SAM2 (2.9 fold and 3.8 fold, respectively) and these differences were both significant (χ^2 test, $p \leq 0.01$) (Figure 3A-B). In contrast, in YFB, the number of up- and down-regulated genes were similar (Figure 3C).

DEGs between the male (M) and female (F) lines (designated M-F DEGs) were further investigated. In SAM1, SAM2 and YFB, 3,320, 2,347 and 3,838 genes (3.8% - 5.6% of the expressed genes), respectively, were differentially expressed between the two parents ($p \leq 0.05$; FDR ≤ 0.05) (Figure 3D-F). Interestingly, we found a high percentage of the M-F DEGs overlapped with the hybrid-MPV DEGs in all three developmental stages suggesting that genes differing in the expression level between the parents of the hybrid were overrepresented (5-13×) among the genes differentially expressed in the hybrids (Figure 3D-F).

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Gene Ontology (GO) enrichment analyses of the M-F DEGs identified 484, 290 and 556 significantly enriched GO function terms (Fisher's exact test $p \leq 0.05$; FDR ≤ 0.05) in SAM1, SAM2 and YFB, respectively (Figure S1, Data S2). We used REViGO to cluster the functional categories across these lists of significantly enriched GO terms. Based on functional clustered GO terms, the enriched pathways associated with the M-F DEGs were found to be related to several different functional categories, of which "defense and stress response" and "hormone response" were the two prominent GO terms altered between the parental lines.

Hormone response genes are significantly altered in the F_1 hybrid

Gene Ontology (GO) enrichment analyses of the hybrid-MPV DEGs identified 370, 324 and 304 significantly enriched GO function terms (Fisher's exact test $p \leq 0.05$; FDR ≤ 0.05) in SAM1, SAM2 and YFB, respectively (Figure 3H). Using the same approach applied in the analysis of GO terms associated with the M-F DEGs, we found that "defense and stress response", "hormone biosynthetic process" and "hormone response" were the three most prominent functional clusters altered in F_1 (Data S3), which accounted for >50% of the biological terms in SAM1 and SAM2. Interestingly, a large proportion of the GO terms overrepresented between the parental lines and between F_1 and MPV overlapped in each of the three stages (SAM1 56%, SAM2 38%, YFB 36%; Figure S2), consistent with the observation of enrichment of the M-F DEGs in the F_1 hybrid. These results suggest that the variance of the gene expression levels between the two parental lines contributes to hetereosis in *B. napus*.

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Biological processes related to defense responses were among the most prevalent GO terms related to DEGs in the hybrid. A large proportion of terms was involved in defense and stress response in SAM1 (100/253, 27%), SAM2 (128/246, 40%) and YFB (54/304, 17.7%) (Data S4), with all three stages having enrichments in many of the same basal processes. Among the down-regulated defense response genes are several genes well known to be induced by pathogen attack, including *PATHOGENESIS RELATED GENE 1* (*PR1*, *BnaC03g45470D*) and *LIPID TRANSFER PROTEIN 3* (*LTP3*, *BnaA03g13140D*). Many up-regulated genes associated with defense response known to function as repressors of plant defense, including *WRKY33* (*BnaA04g22040D*), *WRKY40* (*BnaA07g35260D*), *PENETRATION 3* (*PEN3*, *BnaCnng64010D*), and *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4* (*ERF4*, *BnaC03g39000D*), were also recognized. The pattern of altered defense-related gene expression observed in F_1 suggests that the hybrid may have decreased basal defense response capabilities. Given the antagonistic relationship between plant immunity and plant growth, the lower level of defense-related cellular activity could be a significant factor in allowing for greater growth of the hybrid.

The altered salicylic acid responses in the F_1 hybrid.

SA is derived mainly from chorismate through two key pathways, the isochorismate (IC) pathway and the phenylalanine (PHE) pathway. The IC pathway is the predominant route for both basal and pathogen induced SA production (Dempsey et al., 2011). Both

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ISOCHORISMATE SYNTHASE 1 (*ICS1*) and *ICS2* were down-regulated in F_1 (Figure 4A, C). In the later steps of the IC pathway, *AVRPPHB SUSCEPTIBLE 3* (*PBS3*) is important for accumulation of SA (Nobuta et al., 2007) and was also down-regulated in F_1 (Figure 4A, C). These results suggested that the IC pathway is down-regulated in F_1. A small proportion of SA is produced through the alternative PHE pathway involving four *PHENYLALANINE AMMONIA-LYASE* (*PAL*) genes (Rohde et al., 2004). In contrast with the down-regulation of the IC pathway, we found that *PAL1* and *PAL2* were significantly up-regulated in F_1 (Figure 4B, C). Interestingly, *C4H*, a downstream gene of the PHE pathway, was also significantly up-regulated in F_1. These results suggested that more cinnamic acid was used for biosynthesis of 4-hydroxycinnamic acid instead of SA (Figure 4B, C). The increasing consumption of chorismate in the PHE pathway leading to 4-hydroxycinnamic acid limited the SA biosynthesis via the major IC pathway. Consistently, we found that the concentration of SA in F_1 was significantly lower than in either parent in SAM1, SAM2 and YFB (Figure 4F).

In addition to the core SA biosynthesis genes, upstream repressors of SA biosynthesis showed up-regulation in F_1 (Figure 4D). To further investigate the role of SA in heterosis, we checked expression changes of the known SA-responsive genes (based on AtGeneExpress) in F_1. A large spectrum of SA-inducible genes ranging from pathogenesis-related genes to *WRKY* transcription factors were differentially expressed and down-regulated in F_1 (Figure 4E). In contrast, the SA-repressed *ETHYLENE RESPONSE FACTORS* (*ERF*) were up-regulated in F_1 (Figure 4E). Increased *ERF4* represses JA signaling and can promote

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vegetative growth (Lyons et al., 2013), whereas *ERF11* down-regulates pathogen defense-related genes (Dombrecht et al., 2007).

The altered auxin and glucosinolate biosynthesis pathways in the F_1 hybrid.

IAA is derived mainly from tryptophan through several key intermediates. While genes involved in the main IAA biosynthetic pathway (through indole-3-pyruvic acid, or IPA) were not altered in the hybrid relative to their MPVs, most genes involved in the indol-3-acetaldoxime (IAOX) pathway, which is specific to the *Brassicaceae* family, were down-regulated in SAM1 and YFB (Figure 5A-C, D). In contrast, in SAM2, genes of the IAOX pathway were significantly up-regulated, but *AOP3* that converts indol-glucosinolates into hydroxyl-glucosinolate (hydroxyl-GS), was also significantly up-regulated in F_1, suggesting that the enhanced IAOX pathway in SAM2 was mainly used in biosynthesis of hydroxyl-GS rather than IAA (Figure 5B, D). In addition, *AMIDASE 1* (*AMI1*), which is involved in the Tryptophan $\rightarrow$ IAOX $\rightarrow$indole-3-acetamide (IAM) $\rightarrow$ IAA route, was down-regulated in all three stages (Figure 5A-C, D). Down-regulation of the auxin-inducible marker genes also reflected the state of the decreased auxin level in F_1 (Figure 5E). These results suggest that the genes involved in the IAA biosynthesis pathway lead to accumulate of IAA were down-regulated in all three stages. This is consistent with the observation that F_1 had a lower level of IAA than either parent in SAM1, SAM2 and YFB (Figure 5F).

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An important role of high-parental expression level dominance in heterosis

Allopolyploids have been found to exhibit parental expression level dominance (ELD), a phenomenon in which some genes that are differentially expressed between the parental lines exhibit expression levels in F_1 that are statistically similar to that of one of the parents (Rapp, 2009; Yoo, 2013). To dissect the expression pattern of the expressed genes (② at Figure 2), we classified genes into 12 male–hybrid-female (M-H-F) expression patterns (Figure 6A) as described in Yoo et al. (2013). We further designated genes with expression levels in F_1 that are statistically similar to those in the male and female parent as ELD-M and ELD-F genes, respectively. A large number of ELD genes were identified in the three different developmental stages. This is in contrast to the limited number of genes found in these samples exhibiting additivity and transgressive regulation. This finding indicates that ELD plays an important role in allopolyploid hybrids. Among the genes exhibiting ELD some were expressed as highly as in the parent showing the highest expression level (high-parental ELDs; types III and V in Figure 6A) while others were expressed as lowly as that of the parent showing the lowest expression level (low-parental ELDs; types IV and VI in Figure 6A). In the three stages, more than 87% (1593/1826), 89% (1068/1191), and 73% (1443/1964) of ELD genes were high-parental ELD ones in SAM1, SAM2 and YFB, respectively (Figure 6B). The number of high-parental ELD genes was significantly higher than that of the low-parental ELD genes in F_1 ($p \leq 0.01$, pairwise *t*-test). Interestingly, a significant number of the hybrid-MPV DEGs overlapped with high-parental ELD genes in each developmental stage (of the total DEGs, 30.5% in SAM1, 21.1% in SAM2 and 9.6% in YFB). We also found

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321, 104 and 153 ELD genes overlapping with the non-additively expressed genes in SAM1, SAM2 and YFB, respectively (Figure S3). These observations suggest that the high-parental ELD genes potentially contributes to heterosis in the hybrid, partly acting together with the non-additive mechanism. To further support this hypothesis, we found that the majority of the IAA biosynthesis pathway genes were non-additively expressed, but *PAL1* (*BnaA04g21240D*) was a high-parental ELD gene (Figure 4B).

We next examined the possible functions of the genes exhibiting high-parental ELD in F_1. A large proportion of the significantly enriched GO terms were found to be overlapping among the three stages [69.5% (221/328) for SAM1, 73.2% (221/302) for SAM2 and 46.6% (221/474) for YFB], indicating that the high-parental ELD genes may be involved in similar biological processes in the three different developmental stages (Figure 6C). We found that the high-parental ELD genes (types Ⅲ+Ⅴ) were significantly enriched for GO terms related to response to defense, stress and hormone stimulus (Figure 6D). These results are consistent with the enrichment results of non-additively expressed genes, implying that ELD genes contributed to the heterosis together with the non-additively expressed genes, and that both non-additivity and parental ELD, especially high-parental ELD, play important roles in heterosis in *B. napus*.

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Significant *trans*-regulation of allelic expression in F_1 hybrid

It is not clear how two alleles of the same gene from the parental lines are regulated in F_1. To distinguish the contribution of each parental allele to the total gene expression level in F_1 (④ at Figure 2), we used parental allelic SNPs to determine the allele origin and its expression level in F_1. To this end, we first sequenced the genomes of the two parents to a 15× coverage and then identified allelic SNPs located in exons of all annotated genes between the two parents by separately mapping their genome sequencing data and RNA-seq data to the *B. napus* reference genome. To ensure the accuracy of parental allele discrimination, only transcripts with allelic SNPs detected in both the transcriptome and re-sequenced genome were included in the read assignment and expression comparison.

To define *cis*- and/or *trans*-regulation of a given allele in F_1 we used the principles documented by Shi et al. (2012) (Figure 7A). We used *p*-value A (p_A, χ^2 test) to distinguish the differences between the two parental alleles expressed in the hybrid and their relative expression patterns in the two parents to determine *trans*-regulation. The alleles are believed to be regulated by *trans*-effect if their relative expression differences between the two parental alleles in F_1 were significantly different from their expression differences in the two parents ($p_A \leq 0.05$). For *cis*-effect, we used *p*-value B (p_B, χ^2 test) to test whether the two parental alleles are expressed equally in the hybrid. If their expression levels were significantly different in the hybrid ($p_B \leq 0.05$), the two parental alleles were suggested to be

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influenced by *cis*-effect. By combining the p_A and p_B test results, four types of regulatory effects could be defined: *trans*-effect-only, *cis*-effect-only, both *cis*- and *trans*-effects, and no *cis*- and *trans*-effects (Figure 7A). After excluding genes with low expression levels (*i.e.* RPKM $\leq$ 2) or with high sequence similarity between the two parental alleles (*i.e.* low SNP coverage), we identified approximately 4,400 to 5,400 genes showing significant allelic expression difference in F_1 in the three developmental stages. In YFB, 147 (3.1%), 319 (6.7%) and 357 (7.5%) genes were found to be regulated by *cis*-effect-only, *trans*-effect-only, and both *cis*- and *trans*-effects (*cis-trans*), respectively (Figure 7B). The remaining 3,959 (82.8%) genes in YFB showed no allelic expression difference between the parents or in F_1 (*i.e*. no *cis-trans*). A similar proportional distribution of the four types of regulatory effects was observed in the two SAM samples.

Among the ELD genes and the hybrid-MPV DEGs, we identified 558 and 299 genes, respectively, showing significant allelic expression difference in F_1 in the three samples. Among the ELD genes, the percentage of *trans*-effect only genes in SAM1 (46.4%), SAM2 (40.0%) and YFB (35.0%) was much higher than that observed among the total genes (6.9% for SAM1, 6.4% for SAM2 and 6.7% for YFB) (Figure 7B). These results underscore the importance of *trans*-regulation for the ELD genes in *B. napus* F_1 hybrid. For the hybrid-MPV DEGs, although *trans*-effects-only played an important role, *cis-trans*-effects seemed to have a similar contribution as *trans*-effects-only to the behavior of these genes (Figure 7B). For both the ELD genes and hybrid-MPV DEGs, *cis*-effects-only was the least detected regulatory effect (Figure 7B). Taken together, these results suggest that *trans*-effects-only

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play a more important role than *cis*-effects-only in shaping the expression profiles of the genes that are differentially expressed in F_1.

Decreased expression divergence of the homoeologous gene pairs in F_1 hybrid

To analyze the expression levels of homoeologous genes in the A_n and C_n subgenomes of F_1 (③ at Figure 2), we first identified homoeologs expressed in the two parents and its F_1 hybrid in the three developmental stages. A total of 17,711, 16,969, and 18,308 such genes were identified in SAM1, SAM2 and YFB, respectively. In general, more C_n homoeologs were expressed at a higher level than their corresponding A_n homoeologs in all samples and genotypes (*i.e.* $C_n > A_n$; Figure 8A). We then calculated the fold changes of the expression level between homoeologous gene pairs in F_1 and the two parents. Compared to its parents, F_1 had fewer differentially expressed gene pairs. For example, the number of homoeologous gene pairs with a 16-fold expression difference [*i.e.* $Log_2(A_n/C_n) \geq 4.0$ or ≤ -4.0] in SAM1 (321), SAM2 (256) and YFB (324) of F_1 was significantly less than that of either parent ($p \leq 0.01$, student's *t*-test) (Figure 8B).

We also considered the magnitude of the expression changes in homoeologous gene pairs between the two parents (MPV) and F_1. Compared to the MPV, F_1 showed less expression difference [*i.e.* $Log_2(F_1/MPV) \leq 0$)] for most homoeologous gene pairs (Figure 8C), suggesting a reduction of homoeolog expression divergence as a result of hybridization. In addition, the homoeologous gene pairs which expressed higher in the C_n subgenome than

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in the A_n subgenome [*i.e.* $Log_2(A_n/C_n \leq 0)$] had larger $Log_2(F_1/MPV)$ values (*i.e.* more deviated from the MPV) than those that expressed higher in the A_n subgenome than in the C_n subgenome [*i.e.* $Log_2(C_n/A_n \leq 0)$] (Figure 8C). This was because the C_n subgenome had more highly expressed homoeologs (Figure 8A). Taken together, these data showed a clear reduction in the expression divergence of homoeologs in F_1 following hybridization.

F_1 hybrid has more non-additively repressed miRNAs

To characterize the potential role of miRNA and siRNA in heterosis of *B. napus*, we investigated the small RNA (sRNA) expression profiles of the two parents and their hybrid at three developmental stages (SAM1, SAM2 and YFB). Consistent with previous reports (Shen et al., 2014; Shen et al., 2015), our data showed that the 24-nt class of sRNAs was the most abundant (>40% of the total reads) (Figure S4). Regarding miRNAs, in addition to the known *B. napus* miRNAs deposited in the miRbase database (release 21) and those reported in recent studies (Shen et al., 2014; Shen et al., 2015), we identified 851 novel miRNAs from 393 families.

A total of 68 conserved miRNAs belonging to 48 families identified in the three stages were found to be non-additively expressed ($p \leq 0.05$, FDR ≤ 0.05). Of the 68 miRNAs, 25, 12 and 12 were non-additively repressed in SAM1, SAM2 and YFB, respectively, while 16, 4 and 20 were non-additively activated in SAM1, SAM2 and YFB, respectively. This set of miRNAs included some of the most functionally well-characterized miRNAs (e.g., miR156,

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miR159, miR165/miR166, miR319, miR393 and miR2111) conserved between monocots and eudicots (Figure 9A). The target genes of these non-additively expressed miRNAs were predicted from the gene models in the *B. napus* genome annotation (Chalhoub et al., 2014) via psRobot analysis (Wu et al., 2012). A total of 117 target transcripts were predicted for 37 of the 68 conserved miRNAs (Data S5). We further investigated the correlations between miRNAs and targets by the Pearson correlation coefficient method (Rodgers and Nicewander, 1988). There were significant negative correlations between the expression levels of the conserved miRNAs and their corresponding target genes (r = -0.83). According to the annotation of the plant transcription factor database PlantTFDB (Jin et al., 2014), 103 targets of the non-additively expressed miRNA encoded 86 TFs from 11 families. Compared with all annotated oilseed rape genes, these target genes were significantly ($p < 0.05$) enriched for 17 biological process and 7 molecular function GO terms, including the IAA-mediated signaling pathway, response to stress and sequence-specific DNA binding TF activity .

In addition to conserved miRNAs, a total of 73 (36 families), 89 (32 families) and 85 (32 families) novel miRNAs were identified in SAM1, SAM2 and YFB, respectively (Data S6). Based on their expression levels in F_1 and parental lines, we found that F_1 had more down-regulated novel miRNAs than up-regulated ones in SAM1 (26/36, 72.22%), SAM2 (28/41, 68.29%) and YFB (23/32, 71.88%). To better understand the possible roles of the repressed miRNAs in heterosis, we performed GO enrichment analysis for their targets. We found that the biological process GO term “protein amino acid phosphorylation” was enriched in both SAM1 ($p = 0.0036$) and SAM2 ($p = 0.0176$) (Figure 9B). We then used our

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previously generated degradome sequencing data (Shen et al., 2015) to validate the interaction between the novel miRNAs and their targets. From this analysis, we were able to confirm miRNA-mediated cleavage for 22 targets, including several genes encoding phosphorylation-related protein kinases, such as *BnaA09g39530D* (Figure 9C). In addition, 128 (12%), 64 (13%) and 107 (7%) non-additively expressed genes, and 189 (12%), 85 (8%) and 100 (7%) ELD genes in SAM1, SAM2 and YFB, respectively, were found to be involved in the phosphorylation pathways (Data S7). These results suggest that the protein amino acid phosphorylation pathways and miRNA-mediated regulation of genes of the pathways might be a potential regulatory process contributing to heterosis.

Non-additive expression of the 24-nt siRNA clusters in F_1 hybrid

To characterize the effects of 24-nt siRNAs on genome stability and gene expression, we surveyed 24-nt siRNA density and expression level in F_1. In SAM1, SAM2 and YFB, about 200,000 ~ 250,000 24-nt siRNA clusters were identified. About 200,000 24-nt siRNA clusters were expressed in both parents and F_1 in each tissue, covering 26.50% ~ 39.86% of the *B. napus* genome. The length of the majority of 24-nt siRNA clusters ranged between 100-nt and 1000-nt with 250-300-nt siRNA clusters dominating all three stages (Figure S5).

Based on comparison of expression levels, we found that 41% - 49% of the 24-nt siRNA clusters were differentially expressed between F_1 and its two parents in three stages. Interestingly, in F_1 the number of 24-nt siRNA clusters with an expression level higher than

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the MPV (64.7% in SAM1, 68.8% in SAM2 and 63.5% in YFB) was significantly higher than the number of 24-nt siRNA clusters with an expression level lower than the MPV (35.3% in SAM1, 31.2% in SAM2 and 36.5% in YFB) in all three developmental stages ($p \leq 0.001$, pairwise student's *t*-test) (Figure 10A). Consistent with this observation, the majority of 24-nt siRNA clusters had a $Log_2(F_1/MPV)$ value of 0.2 (Figure 10B), significantly deviated from the null expectation [*i.e.* $Log_2(F_1/MPV)=0$]. We conducted the same analysis for the 20-nt to 22-nt siRNA clusters and did not observe a similar expression pattern to that found for the 24-nt siRNA clusters. This result indicates that the biased, or non-additive, siRNA expression pattern in F_1 is unique to the 24-nt siRNA clusters.

To further investigate the expression level of the 24-nt siRNA clusters between F_1 and the two parents (MPV), we plotted the number of the 24-nt siRNA clusters identified in F_1 based on their expression level (Figure 10C). Although the peak appeared around the MPV, the distribution of the 24-nt siRNA cluster number was negatively skewed in all three stages (skewness > 0), suggesting more 24-nt siRNA clusters expressed higher than the MPV in F_1.

The 24-nt siRNA clusters were found in a wide range of locations on all chromosomes, and their number in the following three genomic locations were compared: (i) TEs, (ii) gene coding regions, and (iii) ± 1 kb flanking regions of a gene. In both parents and the F_1 hybrid, more than 80% of the 24-nt siRNA clusters co-localized with TEs, although more 24-nt siRNA clusters were found in the ± 1kb flanking regions than in the gene coding regions in all three developmental stages (Figure S6).

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To investigate whether the TE-derived non-additively expressed 24-nt siRNA clusters influence TE transcription, we compared the expression patterns of the TEs, from which 24-nt siRNA clusters were generated, in both parents and F_1. Of the ~ 1,100 - 1,600 TEs identified as being differentially expressed between MPV and F_1 in SAM1, SAM2 and YFB, 60% to 72% of them had a lower expression level in F_1 compared to the MPV, which was significantly higher than the number of the TEs having a higher than the MPV expression level (student's *t*-test $p \leq 0.01$; Figure 10D). Consistent with this observation, the majority of TEs that generate 24-nt siRNAs had a $Log_2(F_1/MPV)$ value of -0.4 to -0.2 in the SAM1 and SAM2, respectively, which deviated from the expected $Log_2(F_1/MPV)$ value of 0 (Figure 10E). Taken together, these results suggest that the activity of the TEs associated with 24-nt siRNA clusters was repressed in F_1.

Distribution analysis of the 24-nt siRNA clusters in the non-additively expressed genes found that most of them were located at the 1kb upstream or downstream regions. In SAM1, SAM2 and YFB, we found 745, 251 and 1,086 non-additively expressed genes, respectively, containing siRNA clusters in their coding regions or 1kb flanking regions. Among them, 438, 195 and 614 genes had siRNA clusters in the 1kb upstream regions, 426, 192 and 615 genes had siRNA clusters in the 1kb downstream regions, and 239, 130 and 412 genes had siRNA clusters in coding regions (Figure S7, Data S8). And 398 (38%), 208 (42%) and 701 (44%) non-additively expressed genes in SAM1, SAM2 and YFB, respectively, showed differential expression of the 24nt-siRNA clusters.

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F_1 hybrid has an increased level of CG methylation

Genome-wide, the average CG (CpG), CHG and CHH methylation levels were 58.2 – 61.1%, 25.7 – 27.4% and 10.4 – 12.6% in the two parents and their F_1 hybrid in YFB. For all three methylation sequence contexts, F_1 always had a slightly higher level than the two parents (Figure 11A). Among the methylated cytosines, the majority were CG sites, followed by CHG and CHH sites in all three genomes. Compared to the two parents, F_1 had a relatively higher proportion of CG sites (77.3%), and a similar proportion of CHG and CHH sites as the female parent (Figure 11B).

According to the frequency distribution of the single base methylation level, in F_1, the number of unmethylated cytosines was decreased and the number of highly methylated cytosines was not increased (Figure 11C), implying that the increased methylation level in F_1 is mainly caused by the reduction of the number of unmethylated cytosines. In F_1, the majority (69%) of the methylated CG sites were inherited from both parents, but a significant number of the methylated CG sites were derived from only one of the parents (6%) or *de novo* methylation (5%) after hybridization (Figure 11D). The latter could explain the increased level of CG methylation in F_1.

Analysis of the location of the CG sites methylated in F_1 but unmethylated in both parents revealed that 78% of these CG sites were associated with genes. Of these sites 23% was found in either the 1kb up- or 1kb down-stream regions of the genes, and 32% was found in the gene coding regions. Only 3% was associated with TEs (Figure 11E). Thus, most of the

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CG sites that were methylated in F_1 but unmethylated in both parents were gene- rather than TE-related. In addition, we found that the 24-nt siRNA clusters had a much higher frequency of methylated cytosines compared with the whole genome. For example, ~98% of cytosines in the 24-nt siRNA clusters identified in the two parents and F_1 were methylated. In addition, we found that the number of methylated CG sites with a higher methylation level in F_1 was ~23% more than the number of methylated CG sites with a lower methylation level in F_1 (insert in Figure 10F), consistent with the observation for the number of the 24-nt siRNA clusters (Figure 11A). This result indicates that the number of the 24-nt siRNA clusters is directly related to the level of CG methylation and the transcriptional activity of TEs (Figure 10D).

Furthermore, among the total 1,592 non-additively expressed genes in F_1, 1,168 (73%), 1,169 (73%), and 1,043 (66%) showed a different level of DNA methylation in 1kb-upstream, 1kb-downstream and coding regions, respectively, implying an impact of the changed DNA methylation level on gene expression in the hybrid. One would assume that down-regulation of the genes should be directly associated with increase of their DNA methylation levels or the expression levels of 24-nt siRNA clusters. However, our results did not support this assumption which is consistent with what have been discovered in the *Arabidopsis* study (Shen et al., 2012).

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Taken together, CG methylation in both TEs and gene coding regions contributed to the increased methylation level in F_1. In other words, genome-wide remodeling of DNA methylation may play a key role in heterosis by influencing both the activity of TEs and changing the expression level of genes.

Discussion

Gene expression level changes related to phytohormone response in the F_1 hybrid

Based on transcriptome comparison between the *B. napus* F_1 hybrid and its two parents in SAM and YFB, we found substantial transcriptional reprogramming following hybridization with 0.8%-2.3% of genes showing altered expression levels in the hybrid. The majority of these genes were up-regulated in F_1. Analysis of the biological process GO terms associated with DEGs in F_1 revealed a preponderance of defense- and hormone-related processes in all three samples. Interestingly, a recent study in *Arabidopsis* (Groszmann et al., 2015) also reported that a large proportion of GO terms of DEGs between F_1 and its parents was involved in defense and stress response. Among the defense- and hormone-related DEGs uncovered in the hybrid were those encoding enzymes involved in biosynthesis of IAA and SA together with downstream genes regulated by the two hormones. These altered gene activities are of potential importance in generating the heterotic phenotype regarding that IAA and SA are key regulators of plant defense responses and are also major controllers of plant structure and growth (Busov et al., 2008; Kazan and Manners, 2009). We found that the

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SA level were significantly down- regulated in F_1. Given the antagonistic relationship between plant immunity and plant growth, the lower level of defense-related cellular activity could be a significant factor in allowing for greater growth of the hybrid. Altered IAA responses have also been observed in rice hybrids (Zhang et al., 2014). Although changes in IAA response are common in hybrids, the molecular triggers appear to be variable amongst different hybrid systems. miRNAs and other small RNAs were linked to changes to the IAA network in hybrid rice (Zhang et al., 2014), whereas in the maize hybrids, allele variants of the IAA-regulated transcription factor *ZmAGROS1* seemed to be the main contributors (Guo et al., 2014). We found that, in the *B. napus* hybrids, the decreased IAA response mainly involves changes of IAA biosynthesis through the IAOX pathway, which is specific to *Brassicaceae*. The decreased IAA concentration in shoot apical meristem and young flower bud potentially contributed to the heterosis through enhancing branching due to a reduced apical dominance (Cline, 1991).

We also compared global patterns of gene expression changes between F_1 and its parents. All possible modes of gene action, including additivity, high- and low-parent dominance, underdominance and overdominance, were observed in the *B. napus* hybrids. The majority of the expressed genes (> 95%) exhibited expression patterns that are not statistically distinguishable from additivity, and 70% - 80% of the non-additive genes exhibited high-parent dominance, which is significantly higher than the proportion (15% - 20%) of the genes exhibited low-parent dominance ($p \leq 0.01$ based on χ^2 test). Interestingly, a number of genes with high-parent dominance were related to defense- and hormone-response in the *B.*

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napus hybrid, coincident with the enriched functional processes found in the DEG analysis. These findings are consistent with the hypothesis that while multiple molecular mechanisms contribute to heterosis, high-parent dominance may be particularly important.

miRNAs are important epigenetic regulators in the F_1 hybrid

In *Arabidopsis* hybrids, variation in miRNA expression causes non-additive expression of target genes that may affect growth vigor (Chen, 2007; Ha and Chen, 2009; Ng et al., 2012; Chen, 2013). A large number of miRNAs, including many functionally well-characterized miRNAs, were non-additively expressed in hybrids of *B. napus*. The observation of a substantial number of miRNAs displaying non-additive expression during early flower developmental stages indicates that those miRNAs may contribute to heterosis of *B. napus*. Up-regulation of miR156 and miR159/miR319 in particular could be beneficial in the plant's adaptation to stress by modulating plant morphological characteristics via transcriptional regulation of the transcription factors *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) and *MYB/TCP* (Palatnik et al., 2007; Wang et al., 2008). *SPL* genes are involved in floral transition and regulation of flowering (Manning et al., 2006). miR156 targets the *SPL* gene family to control the transition from the vegetative phase to the floral phase in *Arabidopsis*, rice and maize (Gang and Poethig, 2006). In rice, *OsSPL14* promotes panicle branching and higher grain productivity (Miura et al., 2010). Notably, three *B. napus* homologs of *OsSPL14* (i.e., *BnaA06g14810D*, *BnaC06g37430D* and *BnaC05g16270D*)

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appeared to be up-regulated in F_1 in both SAM1 and SAM2. Therefore, it is plausible that the non-additively regulated miR156-*SPL* pathway is associated with the heterosis of flower number and grain productivity of the *B. napus* hybrids. In *Arabidopsis*, miR166 is involved in shoot apical meristem and lateral organ formation by regulating its target gene *AtHD-ZIP* (Williams et al., 2005). A *B. napus* homolog of *AtHD-ZIP* (*BnaC08g35630D*) was found to be non-additively expressed in F_1, suggesting that the miR166-*HD-ZIP* pathway might be conserved and contribute to the heterosis phenomenon through its action in the formation of the shoot apical meristem.

TCP transcription factors direct the developmental processes determining leaf size, leaf form, and flower symmetry (Danisman et al., 2012). In a previous study, overexpression of miR319, which specifically down regulates *TCP* mRNAs, was found to result in uneven leaf shape and delayed flowering (Palatnik et al., 2007). Overexpression of miR159 has been reported to reduce *MYB* mRNA accumulation and result in male sterility, whereas plants that overexpress miR159-resistant *MYB33* have upwardly curled leaves, reduced stature, and shortened petioles(Alonso-Peral et al., 2010). In addition, miR164, miR166, miR167 and miR390 have been shown to target *ARF* genes and to be regulated by IAA , suggesting that these miRNAs are also involved in the IAA response pathways (Mallory and Bartel, 2005). These miRNAs are highly conserved in plant species and their targets in *B. napus* have been validated (Shen et al., 2014; Shen et al., 2015). Based on their functionalities in other plant species, the non-additively expressed miRNAs identified in F_1 are also most likely key regulators that are critical for growth vigor and adaptation of the *B. napus* F_1 hybrid.

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Genetic buffering of siRNAs represses TE activity and changes gene expression levels through enhanced methylation level in the F_1 hybrid

siRNA-mediated epigenetic mechanisms have been shown to be important for the maintenance of genome stability of interspecific hybrids and allopolyploids (Ha and Chen, 2009; Lu et al., 2012). In this study, we investigated the relationship between the expression level of 24-nt siRNA clusters and the DNA methylation levels, and their changes in F_1 relative to its parents. Although it is still too early to link the differences found between F_1 and its parents to specific heterotic traits, some interesting alterations in F_1 were strongly correlated with the observed heterosis. We observed up-regulation of the 24-nt siRNA clusters and an increased DNA methylation level in all three sequence contexts, particularly at the CG sites, in the *B. napus* F_1 hybrid (Figure 10A and 10B). We provided evidence to support that the non-additively expressed 24-nt siRNA clusters were linked to the reduced activity of TEs in the hybrid (Figure 10D). The observation of up-regulated 24-nt siRNAs in the *B. napus* hybrid is different from what has been documented in other plant species, including *Arabidopsis*, rice and maize, in which reductions of 24-nt siRNAs following hybridization were observed (Ha and Chen, 2009; He et al., 2010; Groszmann et al., 2011; Barber et al., 2012). More studies are required to determine whether this is unique to the tissues (SAMs of the flower initiation stages and YFB) used in this study or to *B. napus*. Furthermore, it is interesting to note that most of the increased methylation in the *B. napus* hybrid came from cytosines that were not methylated in one or both parents (Figure 11D), indicating the activities of *trans* chromosomal methylation (TCM) (Greaves et al., 2014) and

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de novo DNA methylation. We found that up to three-quarters of the methylated cytosines generated by the TCM and *de novo* DNA methylation mechanisms were associated with protein-coding genes (including 1kb up-/down-stream of genes and gene encoding regions). In view the fact that the expression level of a gene is usually not directly correlated with its methylation level. For example, in *Arabidopsis*, two-thirds of the genes (>20,000) were methylated within the immediate context, including 1 kb upstream and downstream of the annotated gene model (Shen et al., 2012); however, in the *met1* and *ddc* mutants, although nearly all CG and non-CG methylation was eliminated, respectively (Cao and Jacobsen, 2002; Tariq et al., 2003; Zhang et al., 2006; Lister et al., 2008), only hundreds of genes were overexpressed in these two mutants (Zhang et al., 2006). In addition, the RdDM pathway or 24-nt siRNAs seems to be not required for hybrid vigor (Barber et al., 2012; Kawanabe et al., 2016; Zhang et al., 2016a). Therefore, the relationship between gene-associated methylation and the heterosis phenomenon in the *B. napus* F_1 hybrid should be further investigated.

In summary, we examined the differences between an F_1 hybrid and its parents at various developmental stages during floral development using a variety of -omic level technologies, and provided detailed information on the role of critical gene expression alterations and epigenetic mechanisms contributing to heterosis in allopolyploid *B. napus*. Based on the results, we propose that the ELD genes and the non-additively expressed genes together contribute to heterosis mainly through the “defense and stress response” and “hormone response” pathways, which are regulated by both miRNAs and DNA methylation

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caused by changes in 24-nt siRNAs. DNA methylation could also play a role in heterosis by reducing the activity of transportable elements (Figure 12).

Experimental procedures

Plant materials

B. napus F_1 hybrid ZYZ108 (a major commercial variety used in the lower Yangtze river regions of China) derived by crossing the varieties of ZY50 and MB, a male-sterile line used as the male parent (ZY50) and a restorer line used as the female parent (MB), were used in this study. Shoot apical meristems (SAM) at the transition meristem stage (the stage where morphological changes are not visible but induction is taking place; designated sample SAM1) and flower meristem initiation stage (the stage where the first flower primordial are arising on the flanks of the inflorescence meristem; designated sample SAM2), and young flower buds at the early flower developmental stage (designated sample YFB) were collected and used in all experiments, unless otherwise indicated. Each genomic DNA and total RNA sample was extracted from a pool of tissue collected from around 20 to 30 plants.

Library generation for high-throughput sequencing and bioinformatics analyses

Please see Supporting information methods (Methods S1) for details about whole genome DNA re-sequencing, mRNA and sRNA sequencing, MethylC-seq and bioinformatics analyses (Figure S8-9, Data S9-12).

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Accession numbers

Sequence data from this article can be found in the GenBank/EMBL databases with BioProject ID: PRJNA338132.

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Author Contributions

L.F. designed the research and wrote the article. Y.S. performed the experiment, conducted bioinformatics analyses and wrote the article. S.H. performed the field experiment. S.S., E.S., C.Y.Y., D.C. discussed the results. M.P.T. and Q.H.Z. discussed the results and wrote the article.

Supporting Information Legends

Figure S1. Venn diagram showing the number of the significantly enriched GO terms based on the genes differentially expressed between the parental lines.

Figure S2. Overlapping analysis of the GO terms between hybrid-MPV and two parental lines.

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Figure S3. Overlapping analysis between the ELD genes and the non-additively expressed genes in SAM1, SAM2, and YFB.

Figure S4. Length distribution of small RNAs in each sample used in this study.

Figure S5. Length distribution of the 24-nt siRNA clusters in the three samples.

Figure S6. Distribution of the 24-nt siRNA clusters in the 1kb-upstream, 1kb-downstream, transcribed region, and TE in the three tissues.

Figure S7. Venn diagram showing the number of the non-additively expressed genes had siRNA clusters in coding regions or 1kb flanking regions in SAM1, SAM2, and YFB.

Figure S8. Multi-dimensional scaling (MDS) plot showing the clustering of the individual RNA-seq samples.

Figure S9. Correlation analysis of gene expression between the two replicates of SAM1, SAM2 or YFB in each of the three genotypes.

Data S1. Overlapping differentially expressed genes between the SAM1, SAM2 and Young flower bud in hybrid.

Data S2. Gene Ontology enrichment lists for the SAM1, SAM2 and young flower bud (YFB) between the parents.

Data S3. Gene Ontology enrichment lists for the SAM1, SAM2 and Young flower bud (YFB) in hybrid.

Data S4. Hierarchical clustering in the significantly enriched GO terms associated with the differentially expressed genes of the F1 hybrids in SAM1 and SAM2.

Data S5. Conserved miRNAs and the recognized target genes.

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Data S6. Novel miRNAs and miRNA families identified in each sample and each tissue.

Data S7. Lists of the non-additively expressed genes and ELD genes related with phosphorylation pathways.

Data S8. Number of the non-additively expressed genes had siRNA clusters in coding regions or 1kb flanking regions in SAM1, SAM2, and YFB.

Data S9. The experiment design and RNA sequencing information.

Data S10. The experiment design and small RNA sequencing information.

Data S11. The genome sequencing information of both parents.

Data S12. The MethylC sequencing information of the cross combination.

Methods S1. Library generation for high-throughput sequencing and bioinformatics analyses.

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Figure legends

Figure 1. Heterosis of the F_1 hybrid in oilseed rape and in representative plants of the three developmental stages examined in this study.

(A) Comparison of silique numbers among the two parents and their F_1 hybrid.

(B) Comparison of grain yield among the two parents and their F_1 hybrid.

(C) Representative plants of the three developmental stages (SAM1, SAM2 and YFB) from which tissues were analyzed in this study.

Figure 2. Work flow of bioinformatics analysis conducted to investigate the heterosis mechanism in *B. napus*. Two main aspects (gene expression level changes and epigenetic regulation) were targeted in this study and diverse sequence data (in red) were generated by high throughput sequencing.

Figure 3. Overview of the genes differentially expressed in the F_1 hybrid compared with MPV (mean of the two parental values).

(A-C) The number of genes differentially expressed in the F_1 hybrid compared with the expected MPV ($p \leq 0.05$; FDR ≤ 0.05) in SAM1, SAM2 and YFB.

(D-F) Genes that were differentially expressed in the two parents (represented by black bars) were more frequently associated with DEGs in the F_1 hybrid (χ^2 test, $p \leq 0.01$).

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(G) Breakdown of DEGs in the F_1 hybrid showing the number of DEGs that were unique in one sample or overlapping in two or three samples used in this study. The numbers in brackets refer to the number of genes with a UniProtKB number.

(H) The number of significantly enriched GO terms for the DEGs shown in (G).

Figure 4. Altered salicylic acid response in the F_1 hybrid.

Differential expression patterns of genes in (A) isochorismate (IC) and (B) phenylalanine (PHE) SA biosynthesis pathway in SAM1, SAM2 and YFB.

(C) The SA biosynthesis pathway showing routes for production of bioactive SA. Gene names are color-coded to represent their different changes in expression levels in the F_1 hybrid; the same color codes are used for the biosynthetic products to reflect their expected changes.

(D) Differential expression of the repressors of SA biosynthesis.

(E) Differential expression of downstream SA-regulated genes in F_1 hybrid (First three columns); next two columns show change in expression levels of the given gene in response to altered SA concentrations.

(F) Comparison of the SA concentration in SAM1, SAM2 and YFB of the two parents and their F_1 hybrid.

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Figure 5. Altered auxin response in the F_1 hybrid.

(A-C) Auxin biosynthesis pathway showing routes for production of bioactive auxin (IAA). Gene names are color-coded to represent their different changes in expression levels in the F_1 hybrid; the same color codes are used for the biosynthetic products to reflect their expected changes.

(D) Differential expression patterns of genes in auxin biosynthesis.

(E) Differential expression of auxin-regulated genes in F_1 hybrid.

(F) Comparison of the IAA concentration in SAM1, SAM2 and YFB of the two parents and their F_1 hybrid.

Figure 6. Parental expression level dominance (ELD) genes in the hybrid oilseed rape.

Genes with an expression level in the F_1 hybrid similar to the Female parent are designated as ELD-F; genes with an expression level in the F_1 hybrid similar to the Male parent are designated ELD-M.

(A) Expression patterns of twelve types of differentially expressed genes (DEGs). M, male parent; H, hybrid; F, female parent.

(B) Number of genes in each of the twelve types of DEGs in SAM1, SAM2, and YFB.

(C) Venn diagram showing the number of the significantly enriched GO terms based on the genes with a high parental ELD (Ⅲ and Ⅴ in A) in each tissue.

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(D) Prominent GO terms enriched in the genes with a high parental ELD in each tissue.

Figure 7. Distribution of genes with *cis* and/or *trans* effects in SAM1, SAM2 and YFB.

(A) Flow chart showing quantification of *cis* and *trans* effects and classification of the transcripts with *cis* and/or *trans* effects. F: Gene expression level in female parent; M: Gene expression level in male parent; H_F: Female parental allelic expression level in hybrid; H_M: Male parental allelic expression level in hybrid.

(B) Number and percentage of the genes without a *cis* and *trans* effects, with a *cis* or *trans* effects only, or with both *cis* and *trans* effects amongst the ELD genes, DEGs between the F_1 hybrid and MPV, and all expressed genes in each tissue.

Figure 8. The expression patterns of homoeologous genes of the A_n and C_n subgenomes in *B. napus*.

(A) Percentage of the homoeologous genes with a higher or lower expression level in the C_n subgenome than in the A_n subgenome.

(B) Number of homoeologous gene pairs with a 16-fold (i.e. $Log_2(A_n/C_n) \geq 4.0$ or ≤ -4.0) expression difference in the two subgenomes of *B. napus*.

(C) Distribution of the homoeologous gene pairs based on their expression changes between the F_1 hybrid and MPV ($Log_2(F_1/MPV)$ and their expression difference between the A_n and

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C_n homoeologs ($Log_2(A_n/C_n)$) in each tissue.

Figure 9. Changes of miRNAs and their targets in the F_1 hybrid

(A) Expression changes of the differentially expressed conserved miRNAs in the F_1 hybrid compared with MPV in the three tissues.

(B) Significantly enriched GO terms (colored in yellow) of the differentially expressed novel miRNAs targets in the F_1 hybrid.

(C) Examples of candidate targets confirmed to be regulated by novel miRNAs based on degradome analysis.

Figure 10. Expression patterns of the 24-nt siRNA clusters in the F_1 hybrid.

(A) Number of the 24-nt siRNA clusters that have a higher or lower expression level in the F_1 hybrid compared with MPV.

(B) Distribution of the number of the 24-nt siRNA clusters based on their expression level changes between the F_1 hybrid and MPV in each tissue.

(C) Distribution of the number of the 24-nt siRNA clusters based on their expression levels in the F_1 hybrid compared with those in the two parents and MPV.

(D) Number of TEs that were up or down regulated in the F_1 hybrid compared with MPV.

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(Fold change > 1.5)

(E) Distribution of the TE numbers based on their expression level changes in the F_1 hybrid and MPV in each tissue.

(F) Number of cytosine residues that have a higher or lower methylation level in the F_1 hybrid compared with MPV and the distribution of the methylated cytosine numbers based on the methylation level fold change in the F_1 hybrid and MPV in YFB.

Figure 11. Methylation patterns in the two parents and their F_1 hybrid.

(A) The average methylation level of CpG, CHG and CHH context.

(B) Percentage of the methylated cytosines in the CpG, CHG and CHH context.

(C) Histogram showing the distribution frequency of the cytosine methylation level.

(D) Distribution (by percent of total) of the 8 methylation patterns.

(E) Distribution of genomic features with methylated cytosines in the F_1 hybrid but not in either of the two parents.

Figure 12. Hypothetic model of heterosis in *B. napus*.

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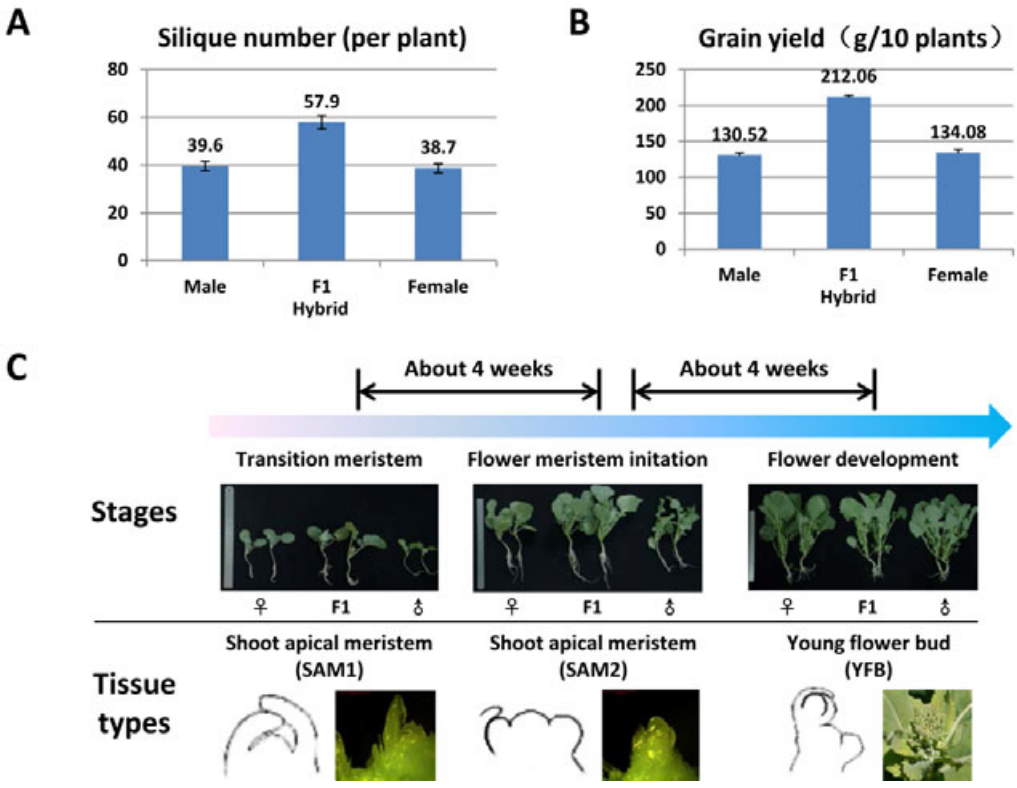

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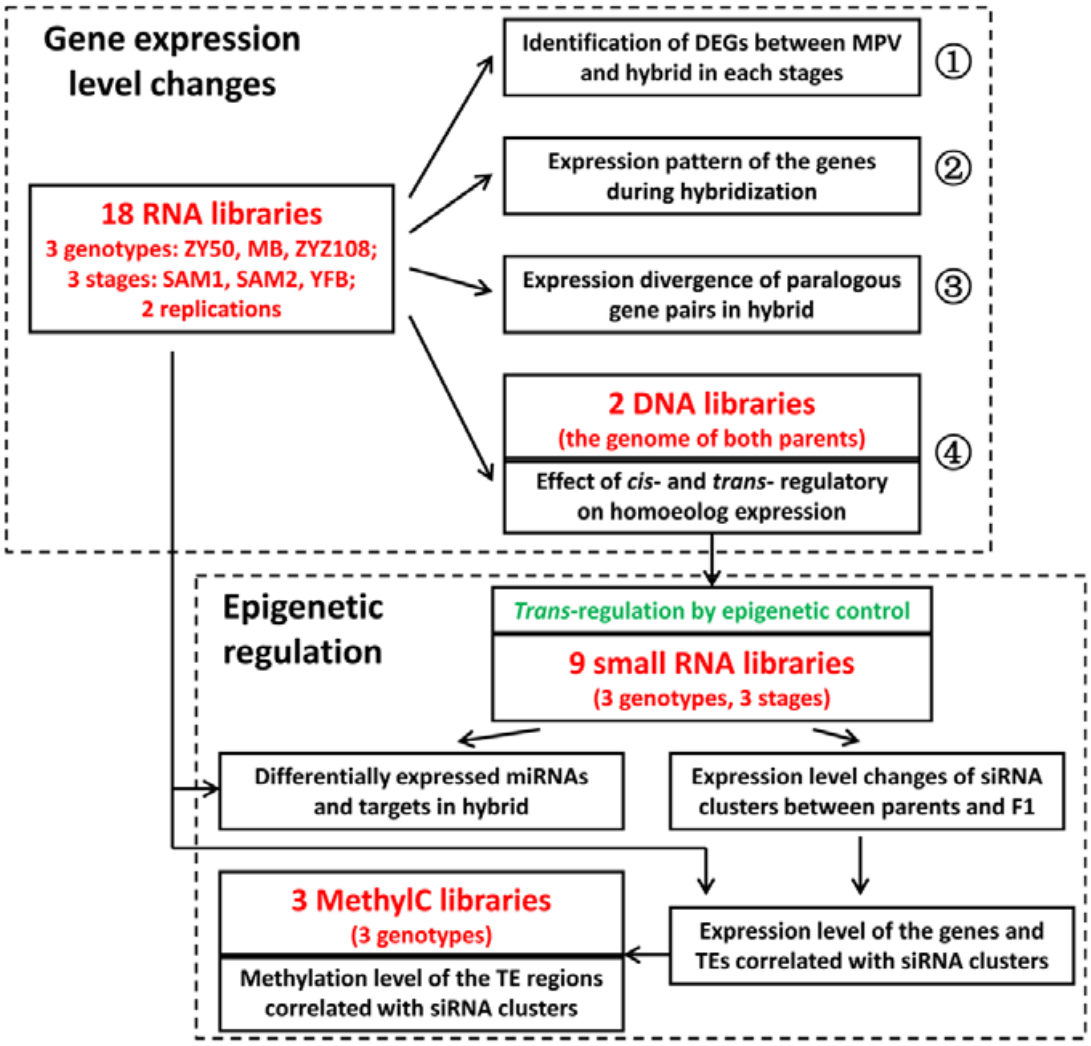

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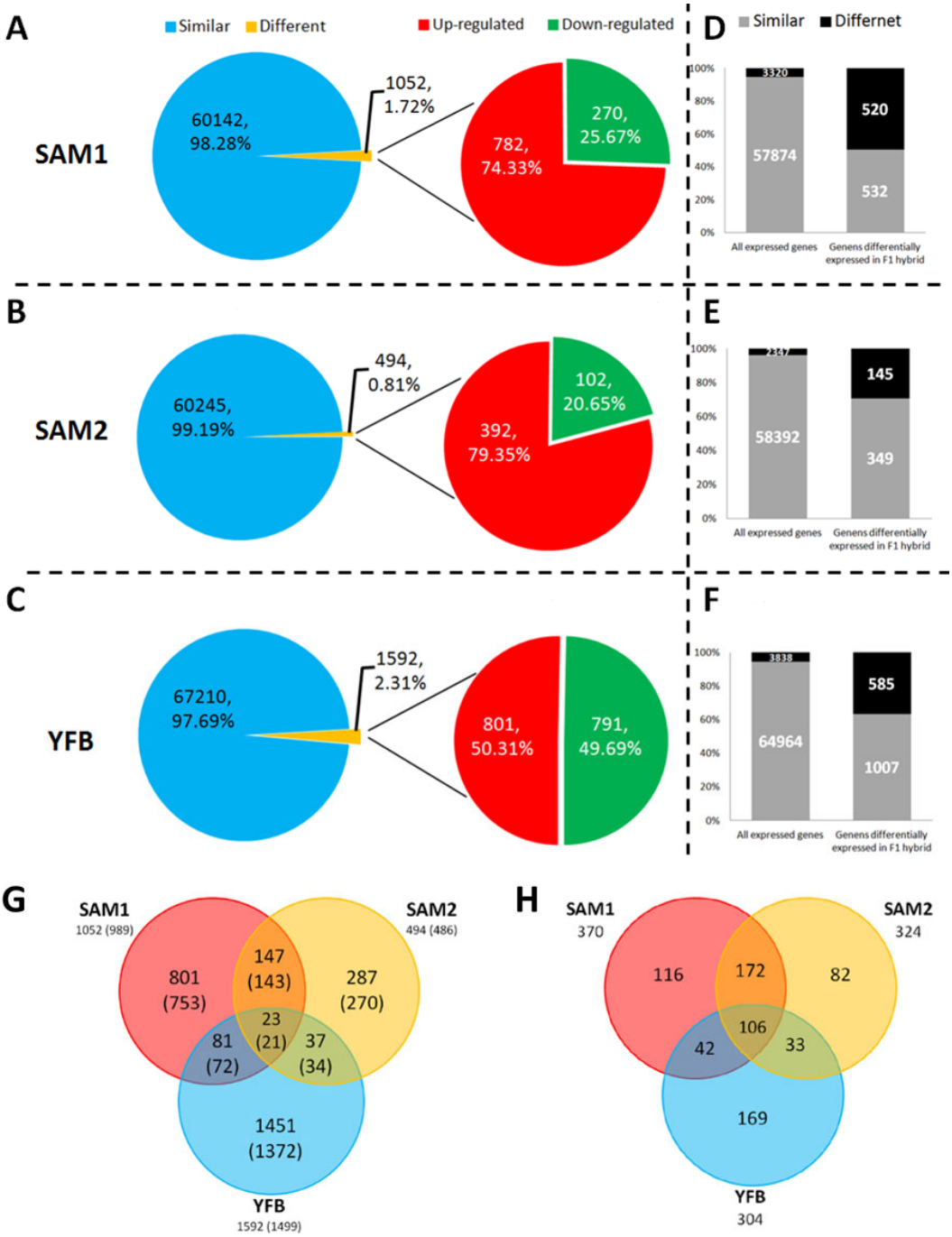

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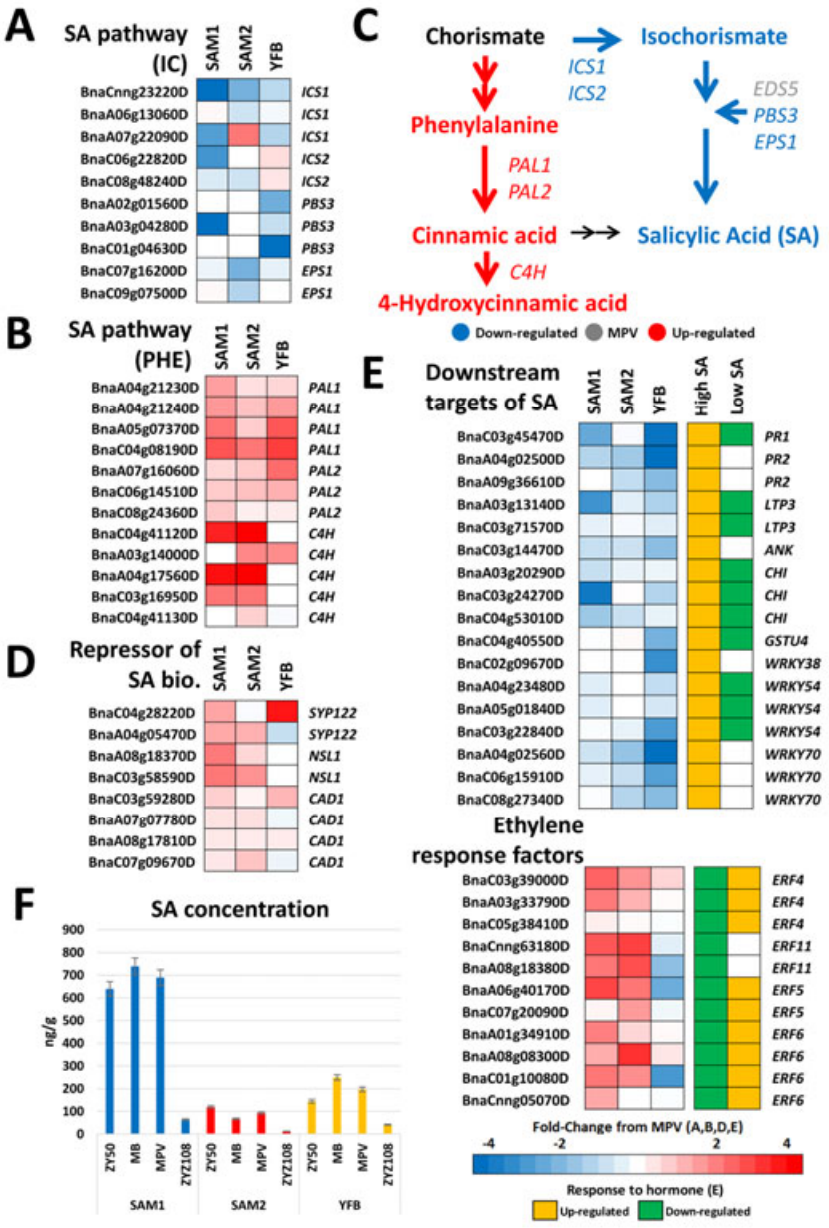

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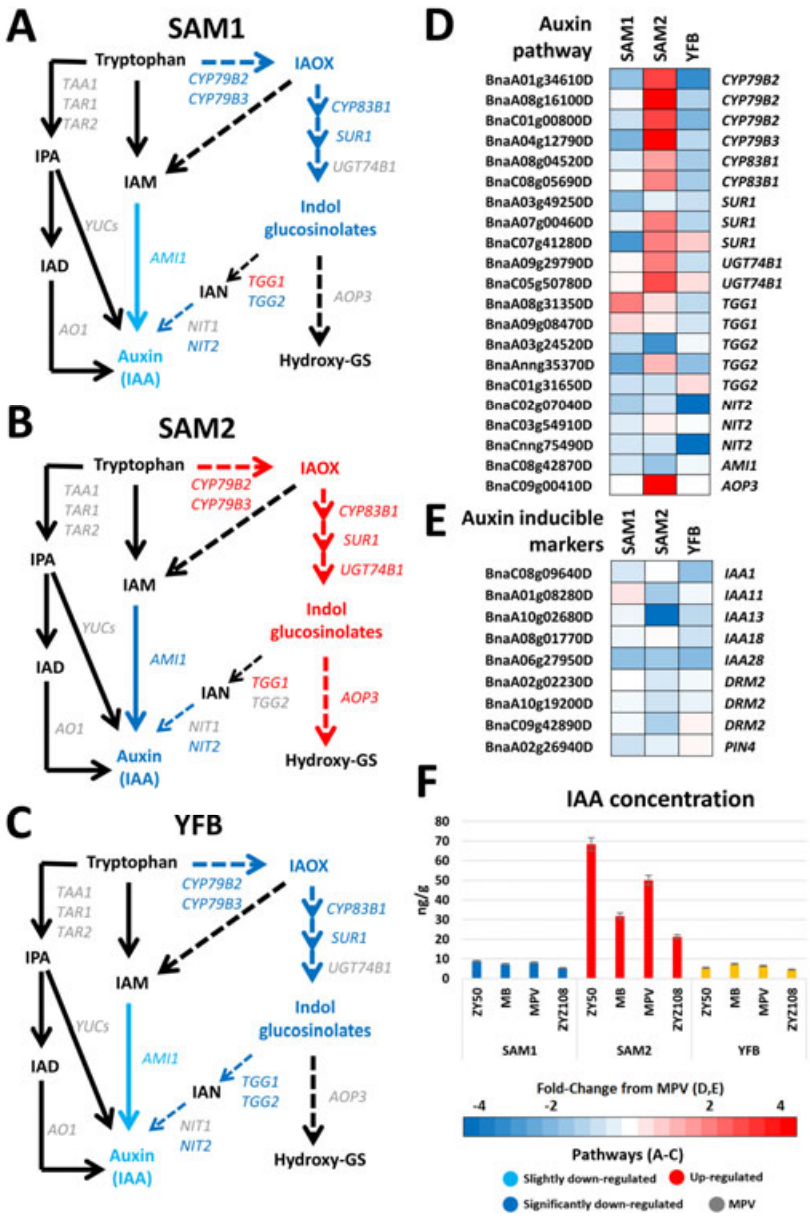

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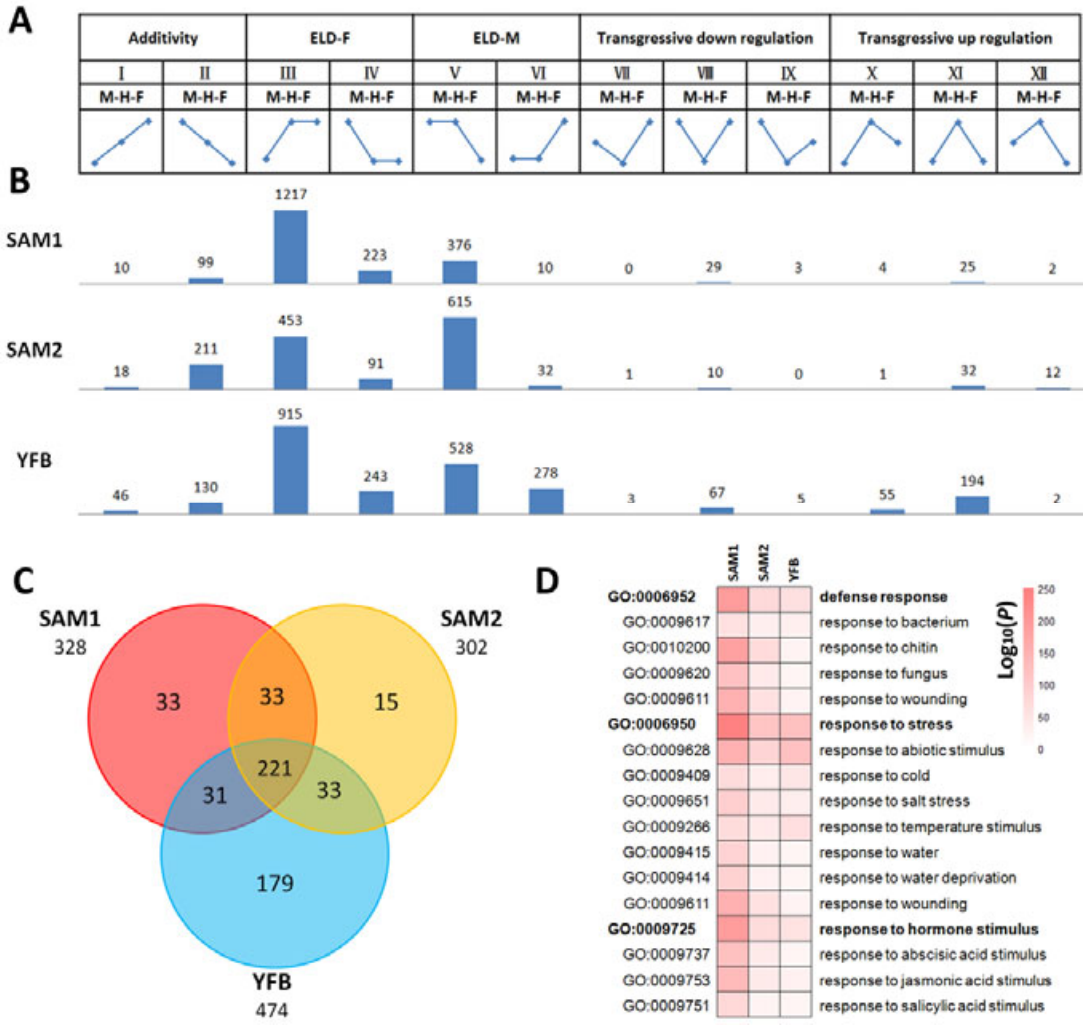

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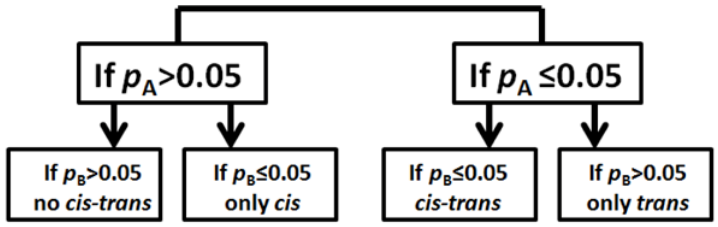
A

p_A

	Female	Male
Expected	$(H_F+H_M)*F/(F+M)$	$(H_F+H_M)*M/(F+M)$
Actual	H_F	H_M

p_B

	Female	Male
Expected	$(H_F+H_M)/2$	$(H_F+H_M)/2$
Actual	H_F	H_M



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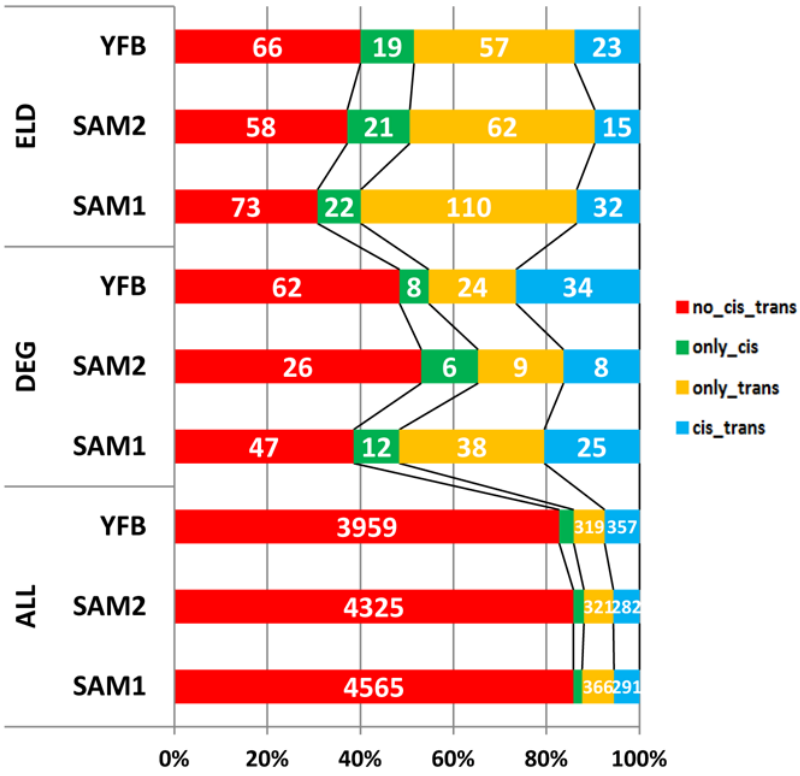

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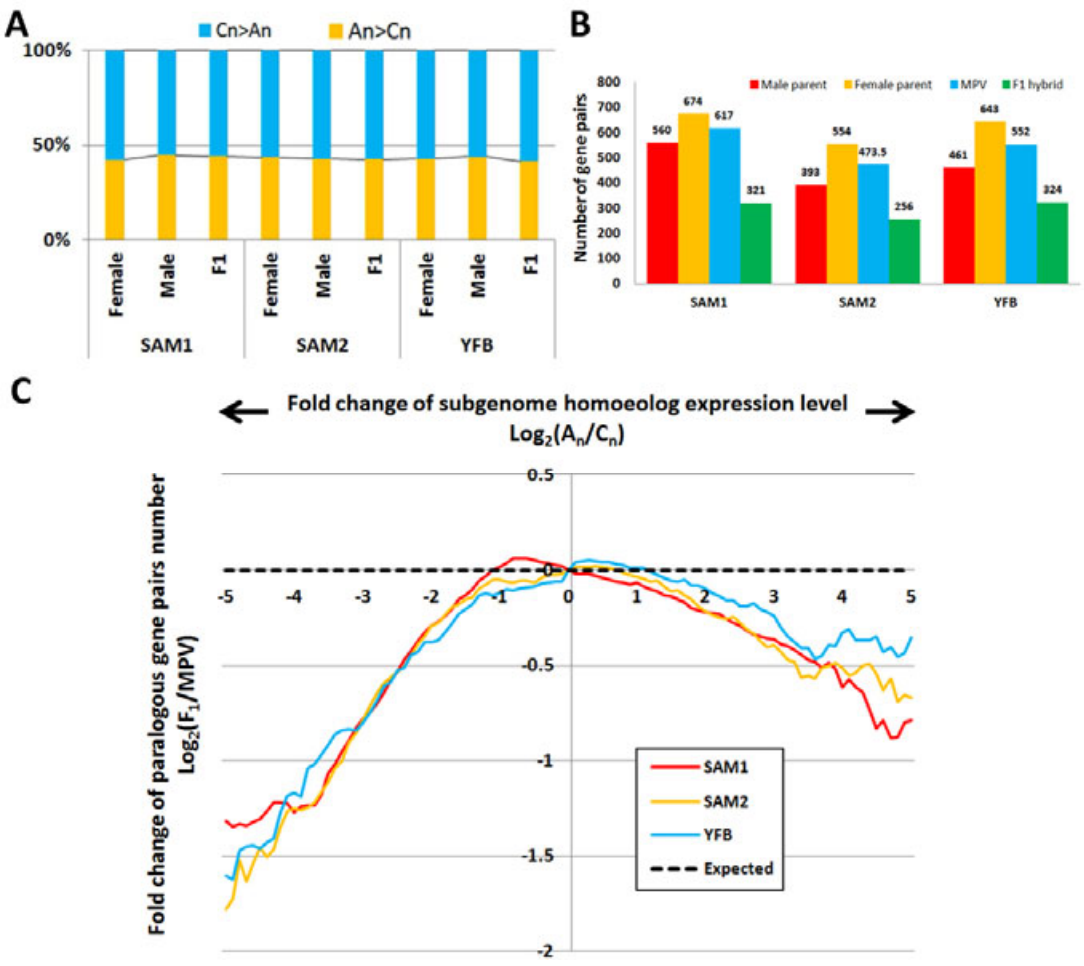

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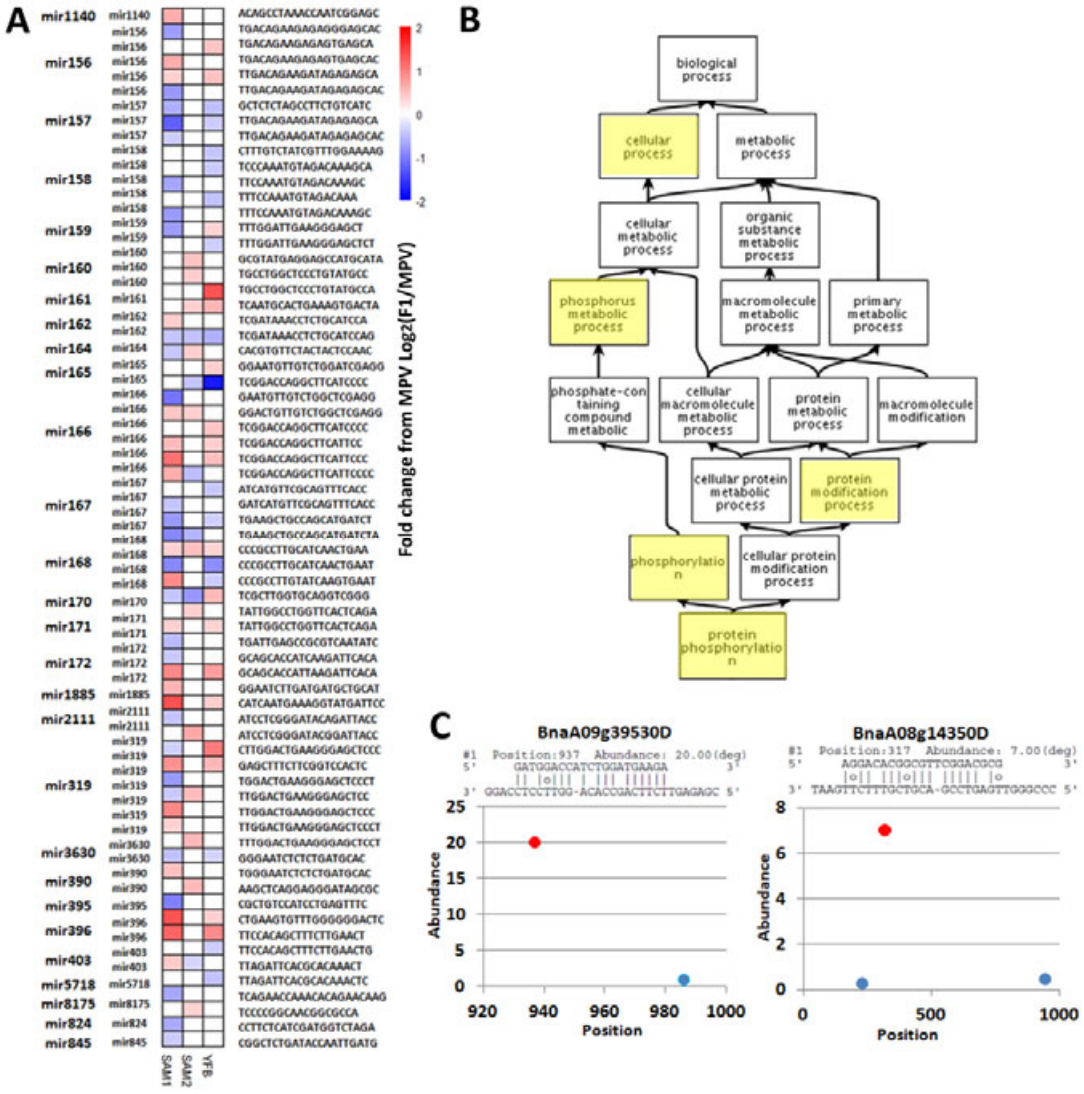

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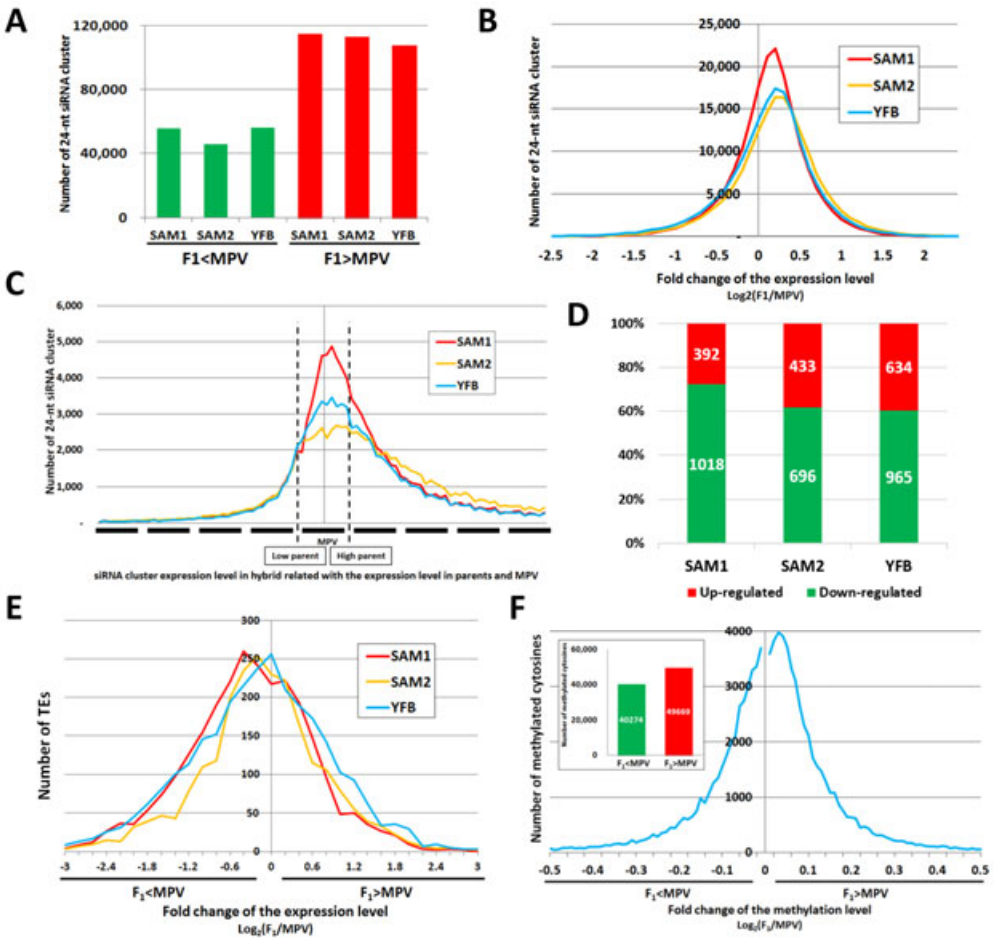

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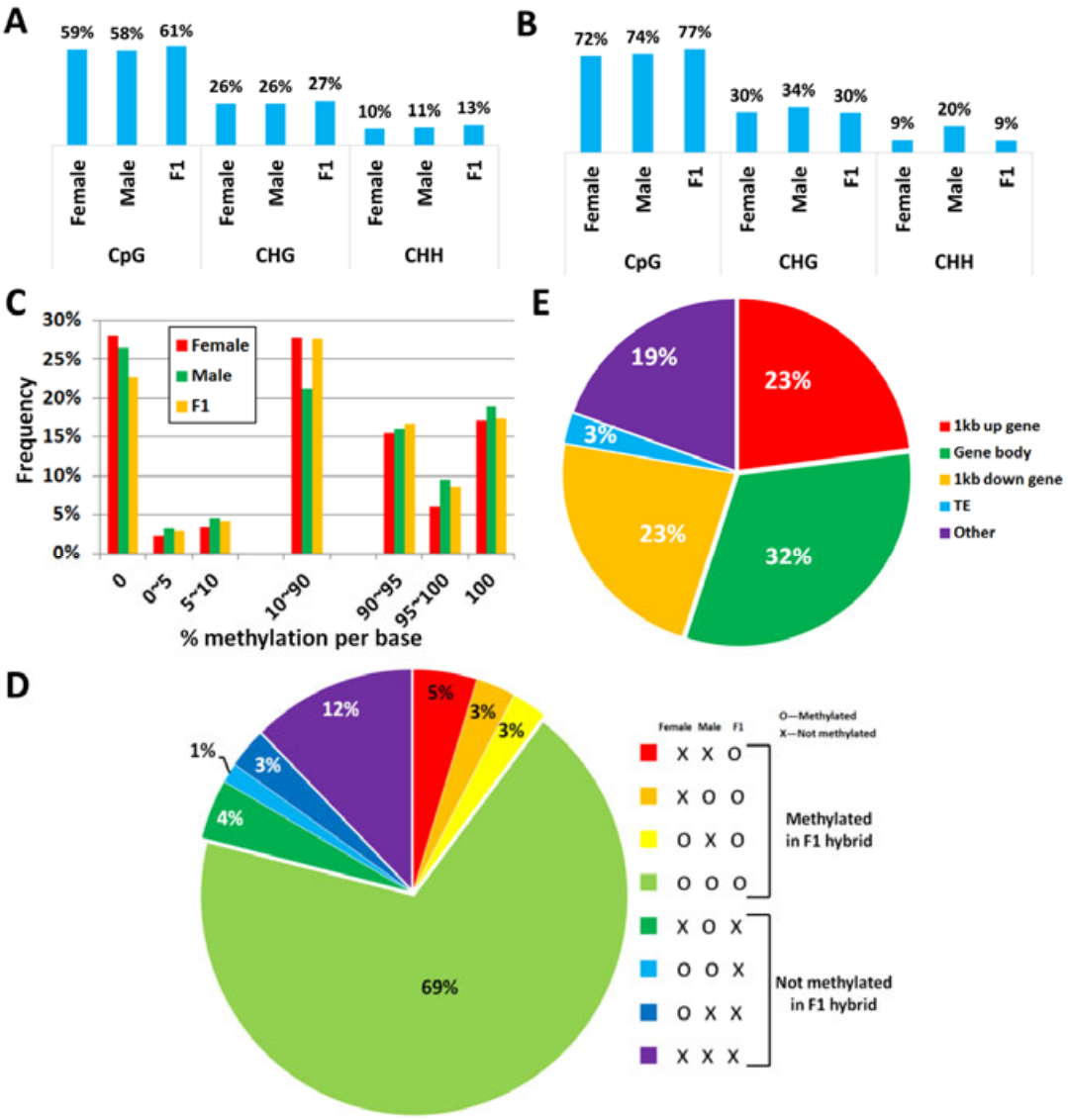

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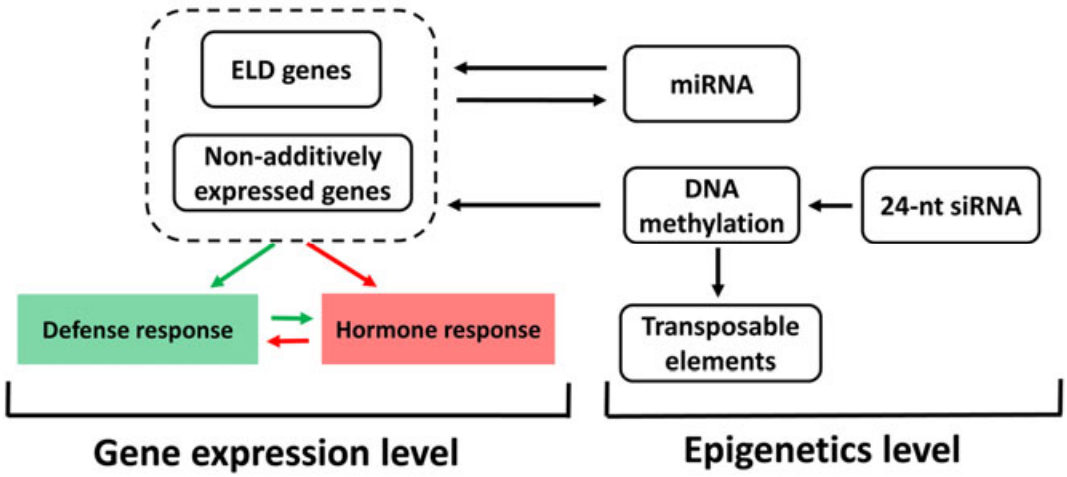

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