Analysis of small RNA changes in different *Brassica napus* synthetic allopolyploids

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Allopolyploidy is an evolutionary and mechanisticaly intriguing process involving the reconciliation of two or more sets of diverged genomes and regulatory interactions, resulting in new phenotypes. In this study, we explored the small RNA changes of eight F2 synthetic *B. napus* using small RNA sequencing. We found that a part of miRNAs and siRNAs were non-additively expressed in the synthesized *B. napus* allotetraploid. Differentially expressed miRNAs and siRNAs differed among eight F2 individuals, and the differential expression of miR159 and miR172 was consistent with that of flowering time trait. The GO enrichment analysis of differential expression miRNA target genes found that most of them were concentrated in ATP-related pathways, which might be a potential regulatory process contributing to heterosis. In addition, the number of siRNAs present in the offspring was significantly higher than that of the parent, and the number of high parents was significantly higher than the number of low parents. The results have shown that the differential expression of miRNA lays the foundation for explaining the trait separation phenomenon, and the significant increase of siRNA alleviates the shock of the newly synthesized allopolyploidy. It provides a new perspective between small RNA changes and trait separation in the early stages of allopolyploid polyploid formation.

1 **Analysis of small RNA changes in different *Brassica napus* synthetic**

2 **allopolyploids**

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9 **Abstract**

10 Allopolyploidy is an evolutionary and mechanisticaly intriguing process involving the reconciliation of two or

11 more sets of diverged genomes and regulatory interactions, resulting in new phenotypes. In this study, we

12 explored the small RNA changes of eight F2 synthetic *B. napus* using small RNA sequencing. We found that a

13 part of miRNAs and siRNAs were non-additively expressed in the synthesized *B. napus* allotetraploid.

14 Differentially expressed miRNAs and siRNAs differed among eight F2 individuals, and the differential

15 expression of miR159 and miR172 was consistent with that of flowering time trait. The GO enrichment analysis

16 of differential expression miRNA target genes found that most of them were concentrated in ATP-related

17 pathways, which might be a potential regulatory process contributing to heterosis. In addition, the number of

18 siRNAs present in the offspring was significantly higher than that of the parent, and the number of high parents

19 was significantly higher than the number of low parents. The results have shown that the differential expression

20 of miRNA lays the foundation for explaining the trait separation phenomenon, and the significant increase of

21 siRNA alleviates the shock of the newly synthesized allopolyploidy. It provides a new perspective between small

22 RNA changes and trait separation in the early stages of allopolyploid polyploid formation.

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24 **Key words:** small RNA; synthetic *Brassica napus*; trait separation

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Introduction

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Polyploidy, or whole-genome duplication (WGD), is prevalent in nature and is particularly common in

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angiosperms, increasing biodiversity and providing new genetic material for evolution (Wendel 2000). Synthetic

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polyploidy is often associated with novel and presumably advantageous ecological attributes such as range

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expansion (Hijmans et al. 2007), novel secondary chemistry and morphology (Leitch and Leitch 2008), and

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increased pathogen resistance (Nuismer and Thompson 2001). Previous studies have investigated synthetic

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allopolyploids and show that various genetic (Song et al. 1995; Xiong et al. 2011) and epigenetic (Adams et al.

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2003; Cui et al. 2013; Ge et al. 2013) changes, as well as alterations in gene expression levels (Wang et al. 2006;

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Chelaifa et al. 2010; Yoo et al. 2013) occur at the initial stage of allopolyploidization. At the genetic level, loss

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of parental and/or appearance of novel sequences at the initial stage of allopolyploidization are common events.

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Non-homologous chromosome exchanges occur in synthetic *B. napus*, resulting in the addition and/or deletion

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of sequences (Gaeta et al. 2007). At the epigenetic level, changes in small RNA and DNA methylation patterns

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occur at the initial stage of allopolyploidization. Shen et al. reported higher siRNA and DNA methylation levels

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in F1 hybrids (Shen et al. 2017). The role of heredity and epigenetics leads to changes in gene expression, which

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in turn leads to novel phenotypes (Chen 2007).

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Non-coding small RNAs are widely found in eukaryotes, which are endogenous with a length of about 20-24 nt.

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Many studies have shown that small RNAs play an important role in gene expression regulation through

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transcriptional level gene silencing, or post-transcriptional level gene silencing (Baumberger and Baulcombe

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2005). Their first report was the phenomenon of RNA interference in nematodes (Lee et al. 1993), and later the

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phenomenon of gene silencing or inhibition was discovered (Napoli et al. 1990; Carvalho et al. 1992; Hannon

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2002). Shortly after these studies, the researchers confirmed that post-transcriptional gene silencing in plants is

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associated with small RNA activity (Hamilton and Baulcombe 1999). These small RNAs regulate various

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biological processes by interfering with the translation of mRNA. In plants, small RNAs can be divided into two

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major categories depending on their synthesis and function: miRNA and siRNA. miRNAs and siRNAs are

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considered to be highly conserved and are important gene expression regulators in plants (Jones-Rhoades et al.

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2006; Axtell and Bowman 2008). Small RNA is a molecule and the approach is a molecular biological tool to

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control gene expression at the transcriptional and post-transcriptional levels.

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Oilseed rape (*B. napus*, AACC, 2n = 38), which is generally thought to be naturally crossed and doubled between

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B. rapa (AA, 2n = 20) and *B. oleracea* (CC, 2n = 18), was formed 7500 years ago and is a good model for

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exploring allopolyploids (U 1935; Chalhoub et al. 2014). In addition, oilseed rape is now one of the most

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important oilseed crops in the world and is inseparable from people's lives. However, due to the short history of

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domestication between 300 and 400 years ago, the genetic basis of oilseed rape was narrower than that of the

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parental species (Go´mez-Campo C 1999), which further led to the restriction of oilseed rape breeding and

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utilization of heterosis. Therefore, it is necessary to explore the molecular mechanism of the distant hybridization

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between *B. rapa* and *B. oleracea* in order to obtain synthesis *B. napus* and to expand the germplasm resources

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of *B. napus*.

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Previous studies have explored sRNA changes and regulatory patterns in different generations of resynthesized

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B. napus (Fu et al. 2016b). However, these patterns in allopolyploids with different traits have not been fully

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examined to answer the relationship between trait differences and small RNA changes. The present study

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analyzed small RNA changes of eight F2 synthetic *B. napus*. We found that a part of miRNAs and siRNAs were

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81 non-additively expressed in the synthesized *B. napus* allotetraploid and the number of siRNAs present in the
82 offspring was significantly higher than that of the parent, which can alleviate the effects of "genome shock".
83 Differentially expressed miRNAs and siRNAs differed among eight F2 individuals. The differential expression
84 of miR159 and miR172 was consistent with that of flowering time trait.Differential expression of small RNAs
85 further affects the expression of the traits by affecting the expression of the target genes, thereby explaining the
86 phenomenon of partial trait separation. . It provides a new perspective of small RNA changes and trait separation
87 in the early stages of allopolyploid formation.
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89 **Materials and methods**
90 **Plant materials**
91 For this study, we used 10 accessions, including the female parent Cai-Xin, male parent Chinese kale, and eight
92 F2 synthetic allopolyploids (Fig. 1). First, by embryo rescuing, F1 haploid (AC) hybridization between Cai-Xin
93 (P1) and Chinese kale (P2) was performed. Then, F1 allopolyploids (AACC) were obtained by colchicine
94 doubling (Wei et al. 2017). Seeds were collected by F1 (AACC) budding self-pollination. The eight F2 plants
95 and the parents were planted in the greenhouse of the Chinese Academy of Agricultural Sciences Institute of
96 Vegetables and Flowers (Beijing, China). We investigated the field traits during the flowering period: flower
97 time and flower size.
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99 **sRNA library construction and sequencing**
100 Young leaves next to bud (5 cm in length) were collected, frozen in liquid nitrogen, and stored at -80°C until
101 extraction. RNA was extracted from three biological replicates using TRIzol reagent (Invitrogen, Life
102 Technologies) following standard protocols. The quality and quantity of the extracted RNA were assessed using
103 the agarose gel electrophoresis, NanoPhotometer® spectrophotometer, Qubit and Agilent 2100 (Beijing China).
104 Then a linker was added to both ends of the small RNA and reverse transcription to synthesize cDNA.
105 Subsequently, after PCR amplification, the target DNA fragment was separated by PAGE gel electrophoresis,
106 and the cDNA library was recovered by gelatinization. The final PCR products were sequenced using Hiseq
107 2500 at Nuohe company (Beijing, China).
108
109 **Identification of miRNAs and siRNA clusters**
110 After trimming adaptor sequence at the 5' and 3' ends of the sequenced reads, the cellular structural RNAs (e.g.,
111 rRNAs, snoRNAs, snRNAs) were removed using in-house Perl scripts. Clean reads of 18 to 30 nt were aligned
112 to the *B. rapa* (Wang et al. 2011) *and B. olearea* (Liu et al. 2014) genome by the bowtie2 software (Langmead
113 and Salzberg 2012) with parameter setting for a perfect match. The sequences of P1 were aligned to the *B. rapa*
114 (Wang et al. 2011), the sequenses of P2 were aligned to *B. oleracea* (Liu et al. 2014), and the sequences of F2
115 were aligned to the merge genome of *B. rapa* and *B. olearea* (Wang et al. 2011; Liu et al. 2014). A miRNA was
116 considered as conserved if its mature sequence had two or fewer nucleotide mismatches compared with the
117 miRNAs in miRBase (http://www.mirbase.org, release 21) (Meyers et al. 2008).
118 After removing sRNAs aligned tomiRNA, the remaining sRNA reads were then used to identify siRNA clusters.
119 Only reads mapped to unique loci were counted for subsequent analyses. A siRNA cluster or locus was defined
120 as a genomic region matched by at least three sRNA reads. If one cluster resided within 200 nt of another, they
121 were merged and regarded as a single cluster.

122

Differential expression analysis of miRNA and siRNA

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124   sRNAs were counted as miRNA reads when they were fully or partly ($\geq$ 1 nt) overlapping with the mature

125   miRNA sequence. The expression levels of miRNAs were normalized to reads per million (RPM) that was

126   calculated using the formula RPM = number of miRNA reads /total number of clean reads $\times 10^6$.

127   The expression level of a siRNA cluster was estimated by uniquely mapped reads. Only reads with a full-length

128   perfect match were accepted as hits. And the expression levels were normalized to reads per million (RPM) for

129   further analysis. The expression differences of miRNAs and siRNA clusters were determined by DEGseq (Wang

130   et al. 2010).

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132   **miRNA target prediction and GO enrichment analysis of target genes**

133   The targets of miRNAs in *B. rapa* and *B. olearea* were predicted using the psRNATarget (Dai et al. 2018).

134   Default parameters were used to filter candidates.

135   GO   enrichment   analysis   was   implemented   by   the   GOseq   R   package

136   (http://www.bioconductor.org/packages/release/bioc/html/goseq.html) in which gene length bias was corrected.

137   AgriGO (a Web-based tool and database for gene ontology analysis; http://bioinfo.cau.edu.cn/ agriGO/) (Du et

138   al. 2010), was also used in this study. GO terms with a corrected FDR $\leq$ 0.05 were considered to be significantly

139   enriched.

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141   **Data Accessibility Statement**

142   The small RNA data we sequenced would be uploaded to genebank database after the article is published.

143

144   **Results**

145   **sRNAs of resynthesized *B. napus* and its parents**

146   The reads obtained by parental sequencing were 16.1 million and 14.0 million, respectively. The reads of eight

147   F2 plants were 15.3 million, 15.2 million, 15.8 million, 13.2 million, 16.0 million, 18.3 million, 14.5 million and

148   11.0 million respectively (Fig. 2a). After removing low quality reads, 10.6 − 15.7 million clean reads were

149   obtained (96.79% - 98.97% of total reads). Among them, 21-24nt reads account for the largest proportion, which

150   was 15% - 16% in the ten samples (Fig. 2b). The reads size distribution is not the same with that of previous

151   reports (Fu et al. 2016b), which may be due to different materials used in the experiment.

152   Average 93.69% of the total reads in the eight F2 samples, 96% and 70% of two parents samples, could be

153   perfectly mapped to the *B. rapa* genome and *B. olearea* genome with no mismatch (Fig. 2c). The alignment ratio

154   of the eight F2 samples was relatively high, which was inconsistent with previous research results (Shen et al.

155   2017). May be because the method of comparison was different. Shen et al (2017)'s experimental method was

156   to compare the sequencing data with the genome of *B. napus*. This experiment was to compare the sequencing

157   data with the genome of *B. rapa* and *B. oleracea*. The high ratio indicates that our method was reasonable and

158   the utilization of reads was relatively high. In addition, the perfectly mapped reads consisted of various types of

159   sRNAs, including miRNA, rRNAs, tRNAs, snoRNAs, snRNAs, and unannotated sRNAs (Fig. 2d). A large

160   fraction of perfectly mapped unique reads (approximately 88.58 %) was not annotated and probably includes

161   new siRNA candidates (Fig. 2d).

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163   **Identification and comparison of miRNAs in eight synthetic *B. napus* and its parents**

164   We identified known miRNAs by aligning clean reads with miRBase databases 22.1. 72050 – 173239 known

165   miRNAs of eight F2 samples, 22208 and 1410 known miRNAs of two parent samples were detected. The number

166   of miRNAs found in F2 was much higher than that of the parent, indicating that hybridization and

167   polyploidization caused changes in the number of miRNAs (Table S1). Due to the impact of the "genome shock",

168   the miRNAs of the hybrid progeny vary greatly, and the miRNA relate to gene regulation during the

169   physiological process plays a role in relieving the impact of the "genome shock" (Guan et al. 2014, Ghani et al.

170   2014, Shen et al. 2015).

171   To further explore the changes in miRNAs caused by hybridization and doubling, we compared the expression

172   quantity between eight F2 plants and mid-parent value (MPV). Under the assumption of additive expression,

173   average expression in each of the eight F2 samples was quite different with the MPV.

174   A total of 33 conserved bra-miRNAs in the eight F2 samples were found to be non-additively expressed (P≤0.05,

175   FDR≤0.05). Of the 33 miRNAs, 11, 1, 9, 8, 5, 4, 11 and 6 were non-additively activated in eight F2

176   allotetraploids, respectively, while 7, 8, 5, 7, 7, 8, 6 and 5 were non-additively repressed in eight F2

177   allotetraploids, respectively (Fig. 3a). A total of 13 conserved bol-miRNAs in the eight F2 samples were found

178   to be non-additvely expressed (P≤0.05, FDR≤0.05). Of the 13 miRNAs, 5, 5, 2, 3, 3, 4, 4 and 4 were non-

179   additively activated in eight F2 allotetraploids, respectively, while 5, 4, 7, 5, 5, 6, 8 and 5 were non-additively

180   repressed in eight F2 allotetraploids, respectively (Fig. 3b). The target genes of these non-additively expressed

181   miRNAs were predicted from the gene models in the *B. rapa* and *B. olearea* genome annotation via

182   psRNATarget analysis (Wu et al. 2012). A total of 300 target transcripts were predicted for 33 conserved bra-

183   miRNAs. A total of 269 target transcripts were predicted for 13 conserved bol-miRNAs.

184   Compared with all annotated *B. rapa* genes, these target genes were significantly (P < 0.01) enriched for 16

185   biological process, 4   cellular component and 19 molecular function GO terms, including the ATP biosynthetic

186   process, phospholipid transport and auxin efflux transmembrane transporter activity (Fig. 3c). Compared with

187   all annotated *B. olearea* genes, these target genes were significantly (P < 0.01) enriched for 6 biological process,

188   1 cell cellular component and 8 molecular function GO terms, including phospholipid transport, ATP

189   biosynthetic process and ATPase activity, coupled to transmembrane movement of ions, phosphorylative

190   mechanism (Fig. 3d).

191   These results suggest that the protein amino acid phosphorylation pathways and miRNA-mediated regulation of

192   genes of the pathways might be a potential regulatory process contributing to heterosis.

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194   **Identification and comparison of siRNAs in eight synthetic *B. napus* and its parents**

195   To characterize the effects of siRNAs on genome stability and gene expression, we surveyed siRNA density and

196   expression level in different allotetraploids. In eight F2 samples, about 18744–38962 siRNA clusters were

197   identified. 19 and 101 siRNA clusters were identified in two parents. To further investigate the expression level

198   of the siRNA clusters between F2 and the two parents, we found that high_parent in the eight F2 samples were

199   38963, 26014, 27654, 20226, 22592, 37995, 25629, 18746, much higher than low_parent 81, 81, 80, 80, 80, 80,

200   79, 81 (Fig. 4c). Most of the siRNAs in the progeny showed high_parent expression. The results indicate that

201   hybridization and polyploidization caused a surge in siRNA. siRNA has the effect of mitigating polyploidy

202   shock, which was consistent with the previous conclusions (Fu et al. 2016b).

203   In addition, the number of siRNAs in eight F2 plants was 249275 – 516295, which was much higher than the

204 number of parents (1260) (Fig. 4a). AACC1 had the highest number of siRNAs, followed by AACC6. The results
205 showed that there was a difference in number of siRNA clusters in eight F2 plants.
206 Based on comparison of expression levels, we found that 387 - 679 of the siRNA clusters were differentially
207 expressed between F2 and its two parents (P≤0.05, pairwise Student's t-test). Interestingly, to AA genome, the
208 number of siRNA clusters in F2 plants with an expression level higher than the MPV (220, 194, 172, 221, 225,
209 307, 149, 174 in eight F2 samples respectively) was significantly higher than the number of siRNA clusters with
210 an expression level lower than the MPV (8, 7, 7, 3, 3, 7, 7, 7 in eight F2 samples respectively). To CC genome,
211 the number of siRNA clusters in F2 plants with an expression level higher than the MPV (208, 130, 151, 177,
212 162, 242, 126, 113 in eight F2 samples respectively) was significantly higher than the number of siRNA clusters
213 with an expression level lower than the MPV (76, 75, 77, 51, 50, 77, 77, 76 in eight F2 samples respectively)
214 (Fig. 4b). In both *B. rapa* and *B. olearea* genomes, the number of up-regulated siRNAs expression in F2 plants
215 is much higher than the number of down-regulated expression. The results showed that there are more up-
216 regulated expression siRNAs to AA genome, while there are more down-regulated expression siRNAs to CC
217 genome. Therefore, two genomes (AA\CC genomes) showed significant differences in response to WGD. It is
218 indicated that hybridization and polyploidization produce many new siRNAs, which can alleviate the effects of
219 "genome shock".
220 Consistent with this observation, the majority of siRNA clusters had a log2(F1/MPV) value of 7 (Fig. 4d),
221 significantly deviating from the null expectation [log2(F1/MPV) = 0]. This result indicates that the distribution
222 trend of eight F2 plants was consistent, AACC1 changed the most, and AACC8 changed the least. In short, there
223 was a difference in siRNA differential expression of eight F2 plants. Because the newly formed allotetraploid is
224 unstable, there are differences in the small RNA variation of different F2 plants, which lays a foundation for the
225 performance of different plant traits.
226
227 **Flowering trait analysis**
228 Among the differentially expressed miRNAs, we found that bra-miR159 was down-regulated in AACC2 and
229 up-regulated in other individuals. We used psRNATarget to predict the 14 target genes of bra-miR159 online,
230 which were MYB101 and MYB65 transcription factors. These two transcription factors promote the expression
231 of the LHY gene, which in turn promotes flowering (Liu and Chen 2009; Wu et al. 2009; Anwesha and Thomas
232 2010) (Fig. 5a). In addition, bol-miR172 was up-regulated in AACC1 and down-regulated in other individuals.
233 It was predicted that bol-miR172a has 6 target genes, which were AP2-like and AP2 transcription factors. These
234 two transcription factors inhibit the expression of FLOWERING LOCUS T (FT) (Wu et al. 2009; Wollmann et
235 al. 2010; Zhu and Helliwell 2011), which in turn delays flowering (Fig. 5b). The results of field traits showed
236 that AACC1 flowered at the earliest and AACC2 flowered earlier. The results show that small RNA could affect
237 the trait by regulating the expression of the target gene.
238
239 **Discussion**
240 **Changes in miRNA in the eight synthetic *B. napus* allopolyploids**
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242 Small RNA plays an important role in polyploid inheritance and gene expression by altering chromatin structure
243 and regulating gene expression. Therefore, studying the expression level of small RNA is helpful to study the
244 regulation mechanism of polyploid gene expression (Yao et al. 2007; Ng et al. 2012; Xie and Zhang 2015).

245   Studies have analyzed the expression and distribution of miRNAs and siRNAs in allotetraploids of *A. thaliana*
246   and found that small RNAs act as a buffer to buffer the genomic shocks of Arabidopsis polyploids (Ha et al.
247   2009). Xie et al. studied *G. hirsutum* (genomic AADD) and found that cotton has an increased miRNA relative
248   to its two diploid ancestors (Xie and Zhang 2015). In addition, in the Brassica, Fu et al. found that the number
249   and expression levels of miRNAs in the newly synthesized *B. napus* from different generations increased
250   compared with the parents (Fu et al. 2016a). These conclusions are consistent with our findings, indicating that
251   the process of hybridization and doubling of the multiploidization results in a general increase in miRNAs. In
252   addition, we found that the number of miRNAs in the newly synthesized *B. napus* was much higher than that of
253   the parents. Loss of miRNAs and new phenomena also exist (Table S1). Our results are inconsistent with the
254   results of Fu et al. (2016a)'s research, possibly due to material differences: the parent we use was the follower
255   Chinese cabbage, and the parent used by Fu et al. (2016a) was *B. campestris*. It is speculated that due to genetic
256   differences among different subspecies, the number of differentially expressed miRNAs in newly synthesized *B.*
257   *napus* is different.
258   Besides, there are few reports on the changes in sRNAs between different traits in allopolyploid. It was found
259   that the number of miRNAs differed between different individuals. Some miRNAs are expressed much higher
260   in one F2 plant than orther F2 plants. Some miRNAs are expressed in one F2 plant and are not expressed in
261   another F2 plant. Zhang et al. (2016) analyzed the transcriptomes of different individual plants of synthesis *B.*
262   *napus*, and found that there were differences in gene differential expression. This experiment shows that
263   miRNAs also differ between different plants. This result indicates that the initial genome of allopolyploid
264   formation was unstable, and there are differences in miRNA expression in self-crossing progeny.
265   All of these results suggest that miRNAs play important roles in the regulation of interspecific hybridisation and
266   polyploidization processes, and hence in subsequent evolution of polyploidy crops (Fu et al. 2016, Shen et al.
267   2015, Shen et al. 2017).

268

269   **Insights into non-additively miRNA regulation in the synthetic *B. napus* allopolyploids**
270   Non-additively gene expression occurs during polyploidization, which results in the complexity of gene
271   expression and phenotye. The miRNA regulate the expression of target molecules (mRNA). Therefore, the
272   miRNA gene is non-additively expressed or is itself mutagenized to form a base mutation, which may lead to
273   the generation of a new target site or the loss of the old target site, resulting in a new phenotype. The comparative
274   analysis of miRNAs and siRNAs between *A. arenosa* and their parents showed that the expression patterns were
275   highly variable between tetraploids and diploids, and most miRNAs were non-additively (Ha et al. 2009). Li et
276   al. (2014) simulated the early generations of common wheat by using new synthetic wheat derived from crossing
277   between *Triticum turgidum* (AABB) and *Agilops tauschii* (DD) and chromosome doubling. It was found that a
278   high proportion of miRNAs were non-additively expression in synthetic wheat, which resulted in differential
279   expression of important target genes. Dynamic regulation of some homologous genes mediated by small RNA
280   may be responsible for the heterosis of new hexaploid wheat (Li et al. 2014). In our study, high-throughput
281   sequencing was used to compare miRNA expression between eight F2 plants and their parents. Our results show
282   that 48.9% of miRNAs are non-additively expressed in the synthetic *B. napus*. Fu et al. (2016) found that
283   approximately 86.6% of miRNAs were non-additively expressed in different generations of synthetic *B. napus*
284   (Fu et al. 2016a).The reason for this difference may be the different materials used in the experiment. Fu's
285   experimental materials were different plants of different generations, and our materials were different plants of

286   the same generation. The experimental results show that the differences between generations may be greater
287   than the differences between the same generation.
288   In addition, small RNA affects the character performance by regulating the expression of target genes. Guan et
289   al. found miR828 and miR858 regulate homoeologous MYB2 gene in polyploidy cotton, which in turn affects
290   the fabric trait (Guan et al. 2014). In allopolyploid Arabidopsis, target genes of miR163 encode a family of small
291   molecule methyltransferases involved in secondary metabolite biosynthetic pathways (Ng et al. 2011). In our
292   experiment, non-additively expression differs among eight plants. The differential expression of bra-miR159
293   and bol-miR172 in eight F2 plants was consistent with the results of flowering time in the field, which further
294   explained the differences in flowering traits among eight individuals. The transcription factors of bra-miR159
295   are MYB101 and MYB65, which in turn promote the expression of the LHY gene. The transcription factors of
296   bol-miR172 are AP2-like and AP2, which in turn inhibit the expression of FLOWERING LOCUS T (FT).
297   Eventually, changes in small RNA lead to changes in flowering time. The results further indicate that epigenetic
298   variation is widespread in the early stage of allopolyploid formation. Differential expression of small RNAs
299   further affects the expression of the traits by affecting the expression of the target genes, thereby explaining the
300   phenomenon of partial trait separation.
301   The non-additively expression of miRNAs in synthetic heteropolyploids may increase polyploid fitness. In
302   Arabidopsis allotetraploids, non-additively expressed genes are involved in multiple biological processes, which
303   may provide an evolutionary mechanism for heterologous polyploid selection and adaptability (Wang et al.
304   2006). In our experiments, the relevant target genes of non-additively expressed miRNAs were significantly
305   enriched in ATP-related pathways. These findings suggest that non-additively mRNA/miRNA may play an
306   important role in the growth and non-additively phenotypes of polyploids.

307   **Changes in siRNA in the eight synthetic *B. napus* allopolyploids**

308   siRNA-mediated epigenetic mechanisms have been shown to be important for maintaining the genomic stability
309   of allopolyploids (Misook Haa 2009; Lu et al. 2012), and the increasing of siRNAs has the effect of slowing
310   geome shock. siRNA is generally produced by endogenous transposons and repetitive sequences in plants. In
311   addition, siRNA is non-additively expressed in newly synthesized allopolyploids, which may promote the
312   variation and adaptability of polyploids (Shen et al. 2017). In this study, the relationship of the siRNA clusters
313   expression levels between eight F2 plants and parents were studied. The number of up-regulated expression of
314   siRNA cluster between eight F2 plants and the parents was found to be greater than the number of down-
315   regulated expression. Ha et al. found that the newly synthesized Arabidopsis allopolyploid siRNA clusters were
316   reduced (Ha et al. 2009). This indicates that the expression patterns of siRNA among different species are not
317   the same, and the expression of siRNA of other allopolyploids needs further investigation. Another possibility
318   is speculated to be related to different experimental analytical methods. Previous research generally use
319   allopolyploid genomes (rapeseed, Arabidopsis, and cotton) for data aligning. The experimental method is to
320   align with the parents's (*B. rapa* and *B. olearea*) genomes respectively. In the early stage of allopolyploid
321   formation, genomic information of synthetic allopolyploid was closer to the parents' genome than to the
322   allopolyploid genome after evolution, so the method could identify more differences. In addition, the number of
323   non-additively expressed siRNAs in eight F2 plants was different, AACC1 had the largest variation and AACC8
324   had the smallest variation. The experimental results further indicated that the siRNA increased significantly in
325   the early stage of allopolyploid formation to alleviate the genome shock, and there was a difference between the
326   different allopolyploids, which may be related to the trait separation.

327

Conclusions

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329 The study explored the small RNA changes of eight F2 synthetic B. napus using small RNA sequencing. The
330 results have shown that the differential expression of miRNA lays the foundation for explaining the trait
331 separation phenomenon, and the significant increase of siRNA alleviates the shock of the newly synthesized
332 allopolyploidy. It provides a new perspective between small RNA changes and trait separation in the early stages
333 of allopolyploid polyploid formation.

334

335 **Auther Contribution statement**
336 Rifei Sun and Yunxiao Wei designed the experimental design and wrote the article. Fei Li, Shujiang Zhang,
337 Shifan Zhang and Hui Zhang contributed to the interpretation of the results and coordinated the study.
338 Yunxiao Wei performed the experiment. All the authors read and approved the final manuscript.

339

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346 **Compliance with ethical standards**

347

348 **Competing interests**
349 The authors declare that they have no conflict of interest.

350

351 **Data accessibility statement**
352 The small RNA data we sequenced was uploaded to figshare web. The link address was
353 https://figshare.com/s/838240fd8fbc7349b7d0.

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487

Figure 1

Fig. 1. Plant materials

$$\boxed{\text{AA}} \times \boxed{\text{CC}}$$

$$\downarrow$$

$$\boxed{\text{F1}:\text{AC}\rightarrow\text{AACC } (2n=38)}$$

$$\downarrow$$

$$\boxed{\text{F2:AACC1,AACC2、 AACC3...AACC8}}$$
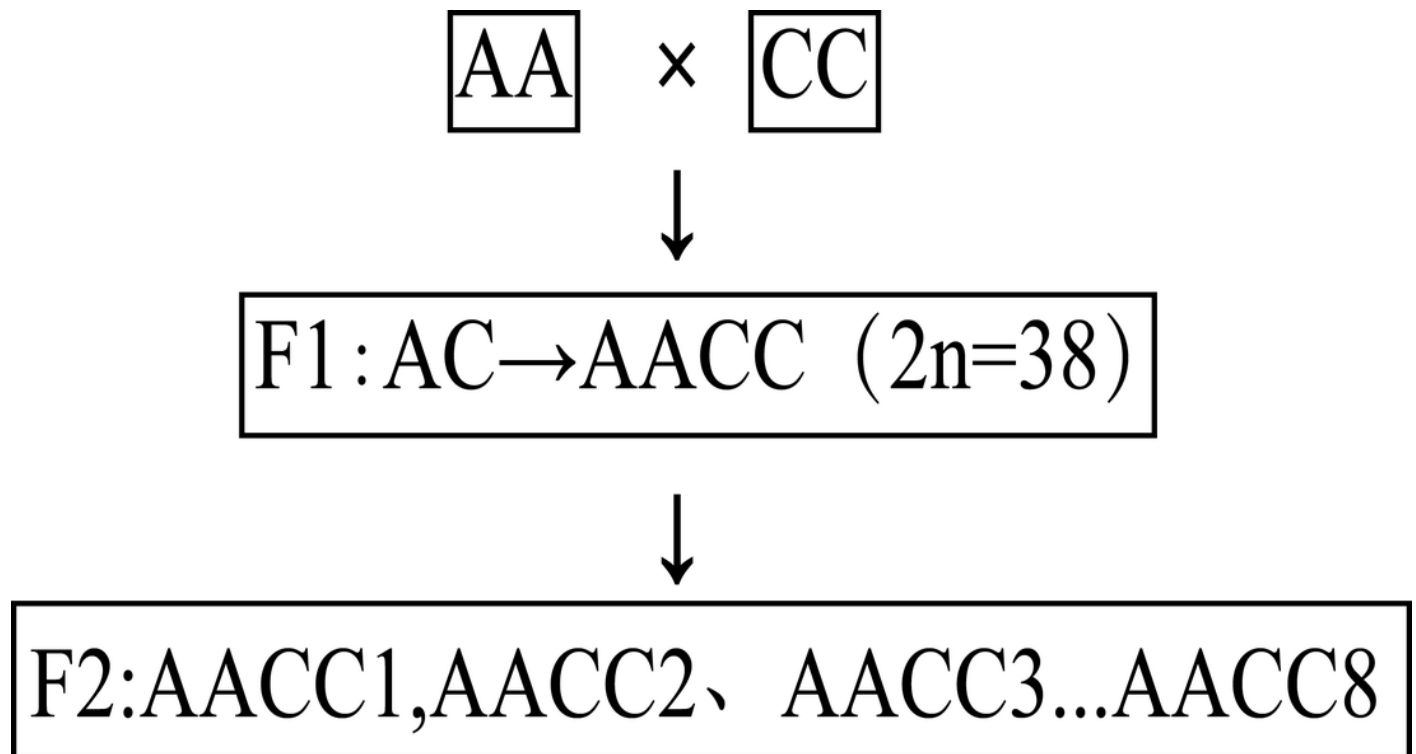
Figure 2

Fig. 2. Small RNA sequencing-quality analysis and length distribution

a: Number of clean reads for small RNA sequencing in each sample; b: Distribution of small RNA in each sample; c: Ratio of all sRNAs in each sample to the genome (*B. rapa* and *B. olearea*); d: Annotated information of small RNA in each sample and its proportion to total small RNA.
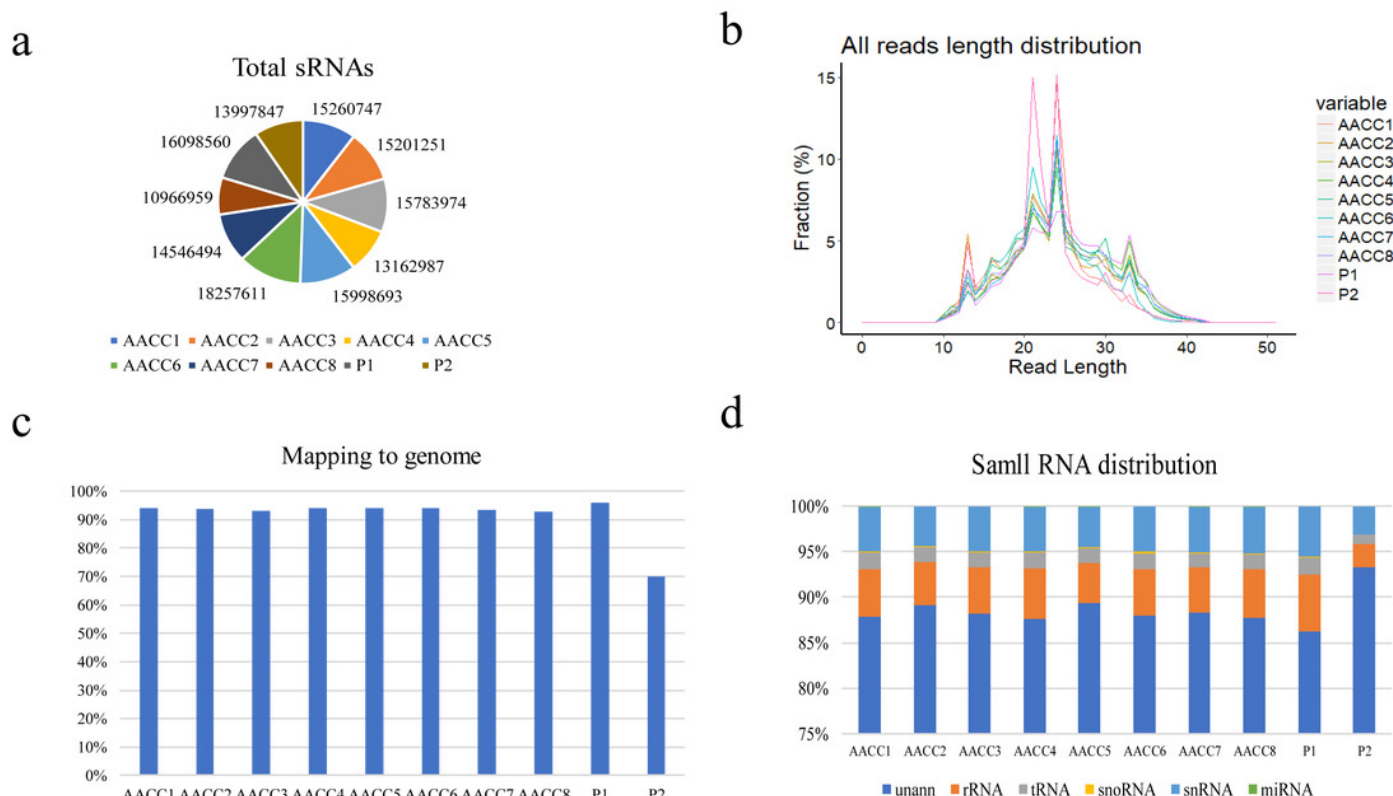
Figure 3

Fig. 3. miRNAs and their targets in the F2 hybrids

a: Expression changes of the differentially expressed conserved miRNAs in the F2 hybrids compared with the MPV when aligning *B. rapa* genome; b: Expression changes of the differentially expressed conserved miRNAs in the F2 hybrid compared with the MPV when aligning *B. olearea* genome; c: Gene Ontology terms of the differentially expressed miRNAs targets in F2 hybrids when aligning *B. rapa* genome; d: Gene Ontology terms of the differentially expressed miRNAs targets in F2 hybrids when aligning *B. rapa* genome.
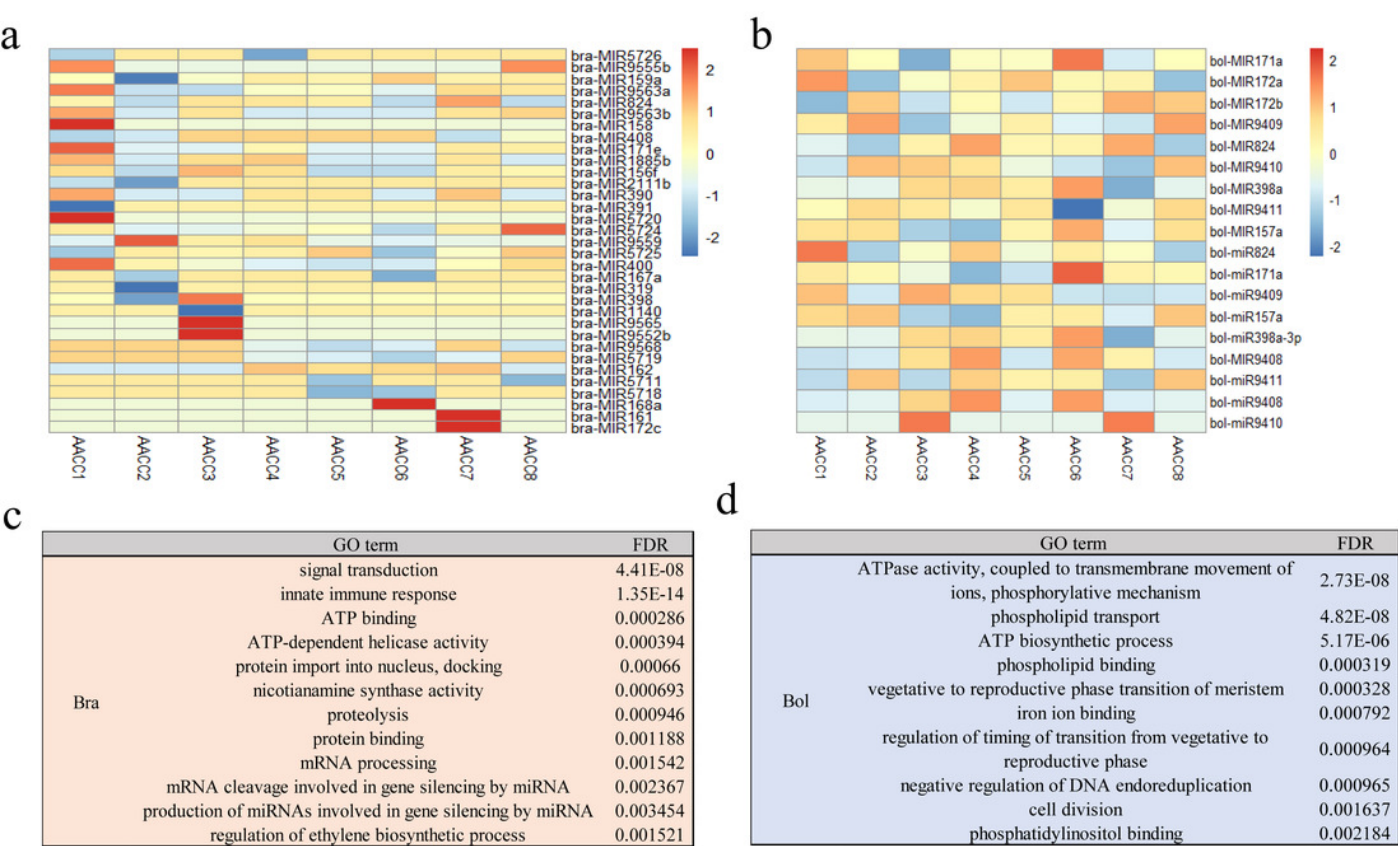
Figure 4

Fig. 4. Expression patterns of the siRNA clusters in F2 hybrids

a: Number of the siRNA clusters in F2 hybrids and MPV; b: Number of siRNA clusters that were up- or down-regulated in F2 hybrids compared with MPV; c: Number of siRNA clusters that were high- or low-parent in F2 hybrids; d: Distribution of the number of the siRNA clusters based on their expression level changes between F2 hybrids and MPV.
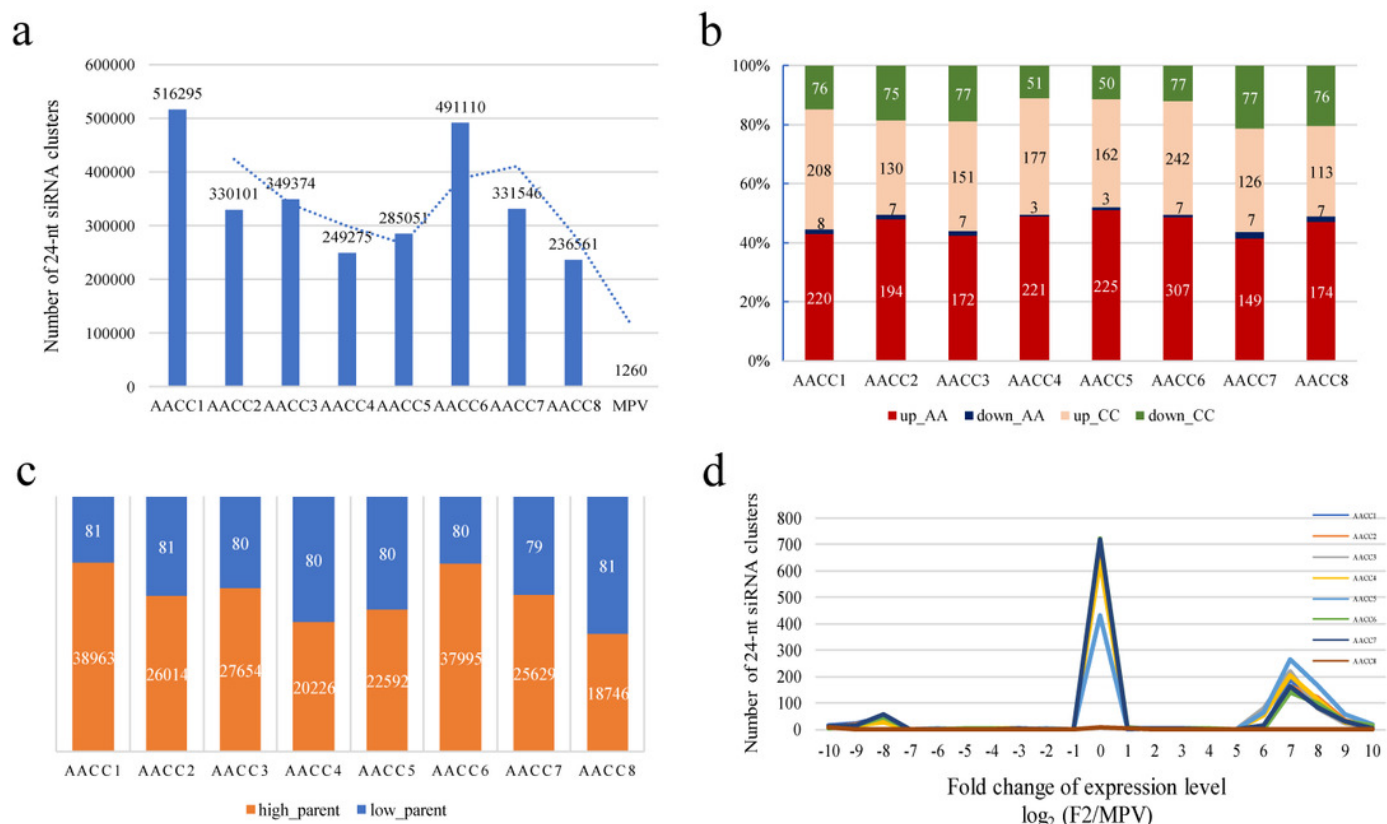
Figure 5

Fig. 5. Analysis of miR159 and miR172 in F2 hybrids

a: miR159 difference analysis in the F2 hybrids; b: miR172 difference analysis in the F2 hybrids.