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ORIGINAL ARTICLE

An RNA-seq transcriptome analysis of floral buds of an interspecific *Brassica* hybrid between *B. carinata* and *B. napus*

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Abstract
Key message **Study on hybrid low fertility.**
Abstract Interspecific hybridizations promote gene transfer between species and play an important role in plant speciation and crop improvement. However, hybrid sterility that commonly found in the first generation of hybrids hinders the utilization of interspecific hybridization. The combination of divergent parental genomes can create extensive transcriptome variations, and to determine these gene expression alterations and their effects on hybrids, an interspecific *Brassica* hybrid of *B. carinata* × *B. napus* was generated. Scanning electron microscopy analysis indicated that some of the hybrid pollen grains were irregular in shape and exhibited abnormal exine patterns compared with those from the parents. Using the Illumina HiSeq 2000 platform, 39,598, 32,403 and 42,208 genes were identified in flower buds of *B. carinata* cv. W29, *B. napus* cv. Zhongshuang 11 and their hybrids, respectively. The differentially expressed genes were significantly enriched in pollen wall assembly, pollen exine formation, pollen development, pollen tube growth, pollination, gene transcription, macromolecule methylation and translation, which might be associated with impaired fertility in the F_1 hybrid. These results will shed light on the mechanisms underlying the low fertility of the interspecific hybrids and expand our knowledge of interspecific hybridization.

Keywords Interspecific hybridization · Transcriptome · RNA-seq · *Brassica* · Hybrid sterility

Introduction

Interspecific hybrids generated from crosses between different species of the same genus have been reported in many crops (Atlagić 2004; Basbag and Gencer 2007; Budashkina et al. 1974; Nuijten et al. 2009). Interspecific hybridization may initiate plant speciation and therefore is of considerable evolutionary significance (Vacher et al. 2011). Moreover, it can promote the transfer of beneficial alleles between species and thus introduce valuable traits of the parental species into a crop (Rieseberg and Willis 2007). Therefore, interspecific hybrids normally exhibit significant heterosis in biomass and stress tolerance (Birchler et al. 2010; Ma et al. 2011). However, different reproductive barriers exist in the generation of interspecific hybrids (Rieseberg and Willis 2007), including the pre-zygotic barriers such as failure of pollen germination and arrest of pollen tube growth, and the post-zygotic barriers that lead to sterility or low fertility in the first generation of hybrids (Bushell et al. 2003; Nasrallah et al. 2007), a phenomenon of hybrid incompatibility that is commonly found in hybrids (Levin 2012; Maheshwari and Barbash 2011), which hinders the use of interspecific hybridization.

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The combination of divergent parental genomes can result in profound gene expression changes (Hegarty et al. 2008; Xu et al. 2012), which may contribute to hybrid incompatibilities in the interspecific F_1 hybrids (Johnson and Porter 2000). Transcriptomic analyses have been reported in allohexaploids derived from trigenomic hybrids between *B. carinata* and *B. rapa* (Zhao et al. 2013), the resynthesized *Brassica napus* (Jiang et al. 2013a), and interspecific sunflower hybrids (Rowe and Rieseberg 2013). However, the gene expression profiles underlying the mechanisms of hybrid incompatibility remain to be determined. RNA sequencing (RNA-seq) using next-generation sequencing (NGS) technologies allows transcriptomic analyses in a very high-throughput and cost-effective manner (Wang et al. 2009). RNA-Seq has clear advantages over traditional approaches, including sequencing and quantifying of transcriptomes at maximal resolution and dynamic range (Marguerat and Bähler 2010), more accurate quantification of differential transcript expression with high levels of sensitivity and reproducibility (Costa et al. 2010), and no requirement of prior knowledge of transcript annotations (Ozsolak and Milos 2011). RNA-seq provides a powerful method for transcriptomic analyses in plants, especially for non-model organisms that lack a reference genome (Birzele et al. 2010).

Interspecific hybridization in *Brassica* has been widely used for genetic improvement. *B. napus* is the most important oilseed crop owing to its superiority over other *Brassica* crops with respect to yield potential and oil quality under favorable environments (Zou et al. 2010). Hybridizations between *B. napus* and its closely related species have been reported (Bajaj et al. 1986; Benabdelmouna et al. 2003; Hauser and Jørgensen 1998; Primard et al. 1988; Quazi 1988). The allotetraploid *B. carinata* possesses valuable agronomic traits for introgression into *B. napus* (Navabi et al. 2011), including heat and drought tolerance (Teklewold and Becker 2006), disease resistance (Fredua-Agyeman et al. 2014), and better performance under late sowing and saline soil conditions (Malik 1990). An interspecific hybrid between *B. napus* and *B. carinata* has been generated to transfer valuable agronomic and disease resistance traits from the B genome of *B. carinata* into *B. napus* (Navabi et al. 2010). A doubled haploid (DH) population was generated in this study, and differences in the lines with and without B-genome chromosomes were significant for both morphological and seed quality traits. The products of male meiosis in microspore-derived progeny from a *B. napus* × *B. carinata* interspecific hybrid were analyzed, and the results suggested that unreduced gametes were preferentially selected in microspore culture (Nelson et al. 2009). Recently, the genotypic effects on homoeologous and homologous recombination frequency were investigated in *B. napus* × *B. carinata* interspecific hybrids. This study demonstrated genetic effects on the frequency of abnormal meiosis between the Brassica A, B and C genomes in the hybrids and found that self-fertility in hybrid progeny decreased as the loss of B-genome loci increased. (Mason et al. 2011).

In this study, an interspecific *Brassica* hybrid was generated from a *B. carinata* × *B. napus* cross. Compared with the parents, some pollen grains of the F_1 hybrid were irregular in shape and exhibited abnormal exine patterns. To determine the gene expression changes in floral buds resulting from interspecific hybridization, RNA-seq-based transcriptome analysis of the F_1 hybrid and its parents was conducted. Comparative analysis of differentially expressed genes (DEGs) identified several candidate transcripts that contributed to the phenotypic differences between the hybrid and its parents. This genome-wide transcriptome comparison could shed light on the mechanisms underlying the low fertility of the interspecific F_1 hybrid and might promote the studies on pollen wall assembly, pollen development and pollen tube growth in plants.

Materials and methods

Plant materials

An interspecific hybrid and its parents *B. napus* (referred to as Bn; AACC, $2n = 38$) and *B. carinata* (referred to as Bc; BBCC, $2n = 34$) were used in this study. The *B. napus* cv. Zhongshuang 11 (ZS11) served as the pollen donor and *B. carinata* W29, provided by Yanyou Wu from Jiangsu University (Chang et al. 2011), was used as the maternal parent. Hand pollination and embryo rescue, as described previously (Xu et al. 2012), generated the F_1 hybrid. Plant materials were grown under glasshouse conditions at the Nanjing Agriculture University agronomy farm, Jiangpu, Nanjing, China. Three biological replicates of the flower buds samples (<2 mm in diameter) of ZS11, W29 and the F_1 hybrid were collected at the initial flowering stage for transcriptome analysis. All tissues were frozen in liquid nitrogen immediately after harvest and kept at −80 °C until use.

Scanning electron microscopy

For scanning electron microscopy (SEM), the opened flowers of the F_1 hybrid and its parents were dissected and pollens from dehiscing anthers were fixed and rehydrated as described previously (Smyth et al. 1990). After critical point drying, the samples were coated with gold and observed using an S-3000N scanning electron microscope (Hitachi, Chiyoda-ku, Tokyo, Japan) at an accelerating voltage of 7 kV in high vacuum mode.

RNA extraction, cDNA library construction and sequencing

For Illumina sequencing, the TRIzol reagent (Invitrogen, Burlington, ON, Canada) was used to extract total RNA, which was digested with RNase-free DNase I (Takara, Dalian, China) according to the manufacturer's protocol. Poly (T) oligo-attached magnetic beads were then used to isolate the mRNA samples from total RNA. cDNA library construction and sequencing were performed as described previously (Zhao et al. 2013). Briefly, cDNA was synthesized using the fragmented mRNA as templates with random hexamer primers (Illumina, San Diego, CA, USA). Short fragments were purified and resolved with elution buffer (EB) and then ligated to sequencing adapters. An Agilent 2100 Bioanaylzer (Agilent Technologies, Santa Clara, CA, USA) and StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) verified the RNA samples and the cDNA libraries. After validation, the cDNA library was sequenced on an Illumina HiSeq 2000 sequencing platform.

RNA-Seq assembly and unigene annotation

The raw reads with adaptors only, those with more than 5 % unknown nucleotides, and reads with more than 20 % low-quality bases (quality value $\leq$10) were filtered out before data analysis. The short reads assembling program "Trinity" (release 2013-02-25) (Grabherr et al. 2011) was then used to assemble the transcriptome de novo using the clean reads. The resulting sequences, defined as unigenes, were analyzed by BLASTX against the NR sequence database, the protein database Swiss-Prot, KEGG and COG with an *E* value cutoff of 10^{-5}. Unigenes that could not be aligned to any database were further scanned using EST-Scan (Iseli et al. 1999) to decide its sequence direction. GO annotations of unigenes according to molecular function (MF), biological process (BP) and cellular component (CC) ontologies were conducted using the Blast2GO program (Conesa et al. 2005). GO functional classification for all unigenes was performed using WEGO (Ye et al. 2006) for a better understanding of the distribution of gene functions at the macro level. Unigenes were aligned to the COG database to predict and classify possible functions of the unigenes. KEGG annotation was conducted to analyze the metabolic pathways and related functions of the unigenes.

Unigene expression analysis and enrichment analysis

For unigene expression analysis, transcripts from flower bud libraries of the three accessions were used. The RPKM method (Mortazavi et al. 2008) was used to calculate the expression level of each gene in each library. RPKM values represent the transcript abundance for each gene and were used for pairwise differential expression analysis using the DESeq method (Dillies et al. 2013). Unigenes with an FDR $<$0.001 and an absolute value of $\log_2$ Ratio $\geq$1 were considered differentially expressed. The differentially expressed transcripts for each comparison of the F_1 hybrid and its parents were clustered, and the software Treeview version 1.6 was used to visualize the results. GO and pathway enrichment analyses of DEGs were performed as previously described (Liu et al. 2013), with a corrected *P* value $<$0.05 as the threshold value.

Quantitative real-time RT-PCR

To validate the results of RNA-seq, quantitative real-time RT-PCR was used to confirm the expression patterns of six randomly selected differentially expressed transcripts. RNA samples for Illumina sequencing were used for the qRT-PCR assay. Primer Premier 5 was used to design gene-specific primers, according to the corresponding unigene sequences. Primers were checked for efficiency using the standard curve method, and their specificities were checked using melting curves after all realtime RT-PCR runs. The sequences of the forward (F) and reverse (R) primers are listed in Supplementary Table 1. SYBR-based qRT-PCR reactions (Toyobo, Osaka, Japan) were performed on an iQ5 Real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the following reaction conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s. The *actin* gene was used as an internal reference gene (Niu et al. 2009), and the relative expression levels of genes were calculated using the delta delta-Ct method (Bio-Rad). All qPCR experiments included three technical replicates and two biological replicates.

Results and discussion

Performances of the interspecific *Brassica* hybrids between *B. carinata* and *B. napus*

Interspecific *Brassica* hybrids of *B. carinata* × *B. napus* were generated by hand pollination and embryo rescue. Reduced fertility and low seed set (average 4–8 per pod) were observed in the hybrid, while the average seed set in *B. carinata* and *B. napus* was 18–22 per pod and 20–26 per pod, respectively. SEM analysis indicated that the majority of pollen grains from F_1 hybrid plants were morphologically normal. However, some of the pollen grains were irregular in shape and exhibited abnormal exine patterns compared with those from the parents (Fig. 1c). Some pollen grains were larger in size, with an abnormal

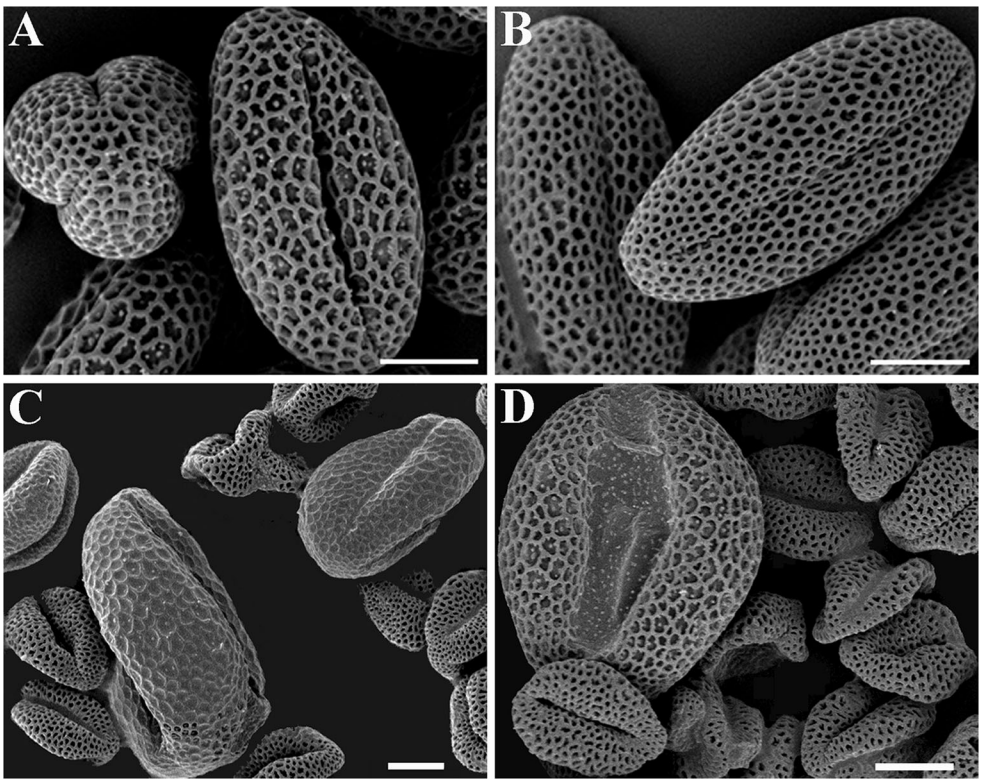

Fig. 1 Scanning electron microscopy (SEM) observations of the pollen phenotype in the F_1 hybrid and its parents. **a** *B. carinata*; **b** *B. napus*; **c**, **d** interspecific F_1 hybrids. *Scale bar* = 10 μm

germination ditch (Fig. 1d). The number of total pollen grains in the F_1 hybrids observed under the SEM was calculated, and the percentages of normal and abnormal pollen grains were 72.4 and 27.6 %, respectively. These defects in pollen structure and development might hinder pollen germination and pollination, and thus reduce the fertility of the F_1 hybrid.

Transcriptome analysis using Illumina sequencing

To determine the gene expression changes underlying the low fertility observed in the interspecific hybrid, transcriptome analysis of the F_1 hybrid and its parents was performed using a high-throughput sequencing approach. A total of 172.8 million reads were obtained using the Illumina HiSeq 2000 platform, and 155,517,734 clean reads were generated after quality checking and raw reads filtering (Table 1). The Illumina reads generated in this study are available at the NCBI GEO database under accession number GSE63088. The assembled unigene lengths ranged from 200 to 12,715 bp; the size distribution of all these unigenes is shown in Supplementary Fig. 1. The distributions of *E* values and similarities were further analyzed. The results are shown in Supplementary Fig. 2. To display the number of transcripts expressed commonly and specifically in the F_1 hybrid and its parents, a Venn diagram was generated (Fig. 2). The results indicated that the F_1 hybrid and its parents shared the transcripts of 24,735 genes. Except for these genes detected across all accessions, the diagram showed that another 11,683 genes were commonly expressed in the F_1 hybrid and *B. carinata*, 5,142 were coexpressed in the F_1 hybrid and *B. napus*, and 819 were coexpressed in *B. carinata* and *B. napus*. Although *B. napus* has more chromosomes than *B. carinata*, there were more genes shared with the F_1 hybrid and its maternal parent, *B. carinata*, than those shared with its paternal parent, *B. napus*. This parent-biased expression pattern in the hybrid has been reported before and might be attributed to cytoplasmic and maternal effects (Zhao et al. 2013). The transcripts of 2,361, 648 and 1,707 genes were specifically detected in *B. carinata*, the F_1 hybrid and *B. napus*, respectively (Fig. 2). Transcriptome analyses of *Brassicas* using different strategies were reported (Trick et al. 2009; Yan et al. 2013; Zhao et al. 2013). In this study, transcripts of 39,598, 32,403 and 42,208 genes were identified in the flower buds of *B. carinata*, *B. napus* and their hybrid, respectively, which will expand our understanding of the overall expression profiles of *Brassica* species.

After assembly, unigene sequences were aligned to the non-redundant NCBI nucleotide database NT (*E* value <0.00001) by BLASTN and aligned to the non-redundant

Table 1 Summary of RNA-seq and de novo assembly of unigenes

Samples	Raw reads	Clean reads	Unigene number	Unigene length (nt)	Mean length (nt)	N50
B. carinata	57,168,688	51,793,494	115,507	61,464,996	532	838
F_1 hybrid	57,711,716	51,250,104	113,520	54,650,269	481	735
B. napus	57,919,374	52,474,136	88,246	55,262,679	626	1,015

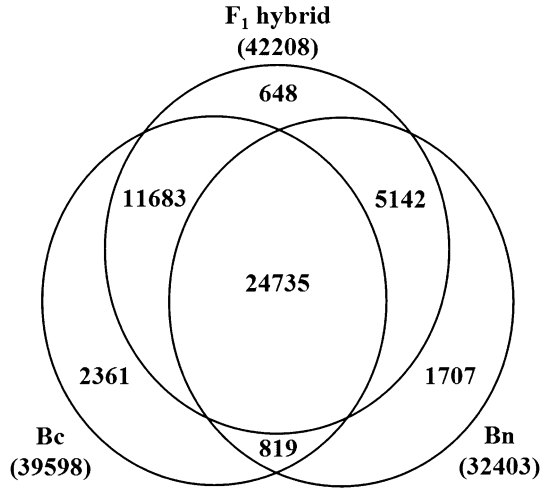

Fig. 2 Distribution of the genes commonly and specifically expressed in the F_1 hybrid and its parents. Bc, *B. carinata*; Bn, *B. napus*

NCBI protein sequence databases (NR), Swiss-Prot, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Group (COG) (*E* value <0.00001) by BLASTX. A total of 147,332 transcripts representing 47,095 genes were annotated in the different databases. Transcripts of 28,226 genes were assigned to 55 functional groups using Blast2GO (version 2.5.0), including 22 functional groups of the BP GO category, 17 functional groups of the CC category and 16 functional groups of the MF category (Fig. 3). The dominant GO terms of BP were cellular process, metabolic process, single-organism process and response to stimulus. Cell part and organelle were the most abundant GO terms for the CC ontology, while the terms binding and catalytic activity were dominant in the MF category. This result is consistent with previous research on anther transcriptomes of chili pepper, which showed that cellular process, cell and binding were the dominant GO terms for the BP, CC and MF ontologies, respectively (Liu et al. 2013). Transcripts of 11,192 genes were classified into 25 COG categories (Fig. 4). "General function prediction only" represented the largest group, followed by "Transcription," "Replication, recombination and repair," "Posttranslational modification, protein turnover, chaperones," "Translation, ribosomal structure and biogenesis" and "Signal transduction mechanisms." Transcripts for 17,902 genes were mapped to 128 KEGG pathways, and the most represented pathways were metabolic pathways, biosynthesis of secondary metabolites, plant hormone signal transduction, plant–pathogen interaction and RNA transport (Table 2). Similar pathway distributions were reported in previous research on synthetic trigenomic allohexaploids of *Brassica* (Zhao et al. 2013).

Gene expression differences between the F_1 hybrid and its parents

Gene expressions of the F_1 hybrid and its parents were analyzed, and gene expression levels were normalized and calculated using the reads per kilo bases per million reads (RPKM) method (Mortazavi et al. 2008). Unigenes with a false discovery rate (FDR) <0.001 and the absolute value of $\log_2$ Ratio ≥1 were defined as DEGs. In the flower buds of the F_1 hybrid, 3,502 genes were upregulated and 4,294 genes were downregulated compared with *B. carinata*, while 6,241 genes were upregulated and 3,312 genes were downregulated compared with *B. napus* (Fig. 5a). These results further confirmed that the gene expression difference was more significant between the F_1 hybrid and its paternal parent.

The transcript patterns of DEGs were further analyzed and distributed into four clusters (I–IV) according to their expression profiles (Fig. 5b). Cluster I contained 7,964 genes that were differentially expressed between the F_1 hybrid and only one of the parents. Cluster II contained 3,384 genes that were upregulated in the F_1 hybrid compared with one parent and downregulated compared with the other. Cluster III had 748 genes with the lowest expression in the F_1 hybrid, while cluster IV comprised 488 genes with the highest expression in the F_1 hybrid. Transcripts that were classified in cluster I accounted for most of the DEGs, followed by transcripts in cluster II. In addition, there were more downregulated transcripts (cluster IV) than upregulated transcripts (cluster III).

The gene expression level in the F_1 hybrid was also compared with the relative mid-parent values (MPV), and genes that showed at least a two-fold change between F_1 hybrid and MPV (FDR <0.001) were considered as non-additively expressed genes. In total, 5,074 non-additive genes were detected in the flower buds (Supplementary Table 2) of the F_1 hybrid. Among these non-additive genes, 3,694 showed differential expression between the hybrid

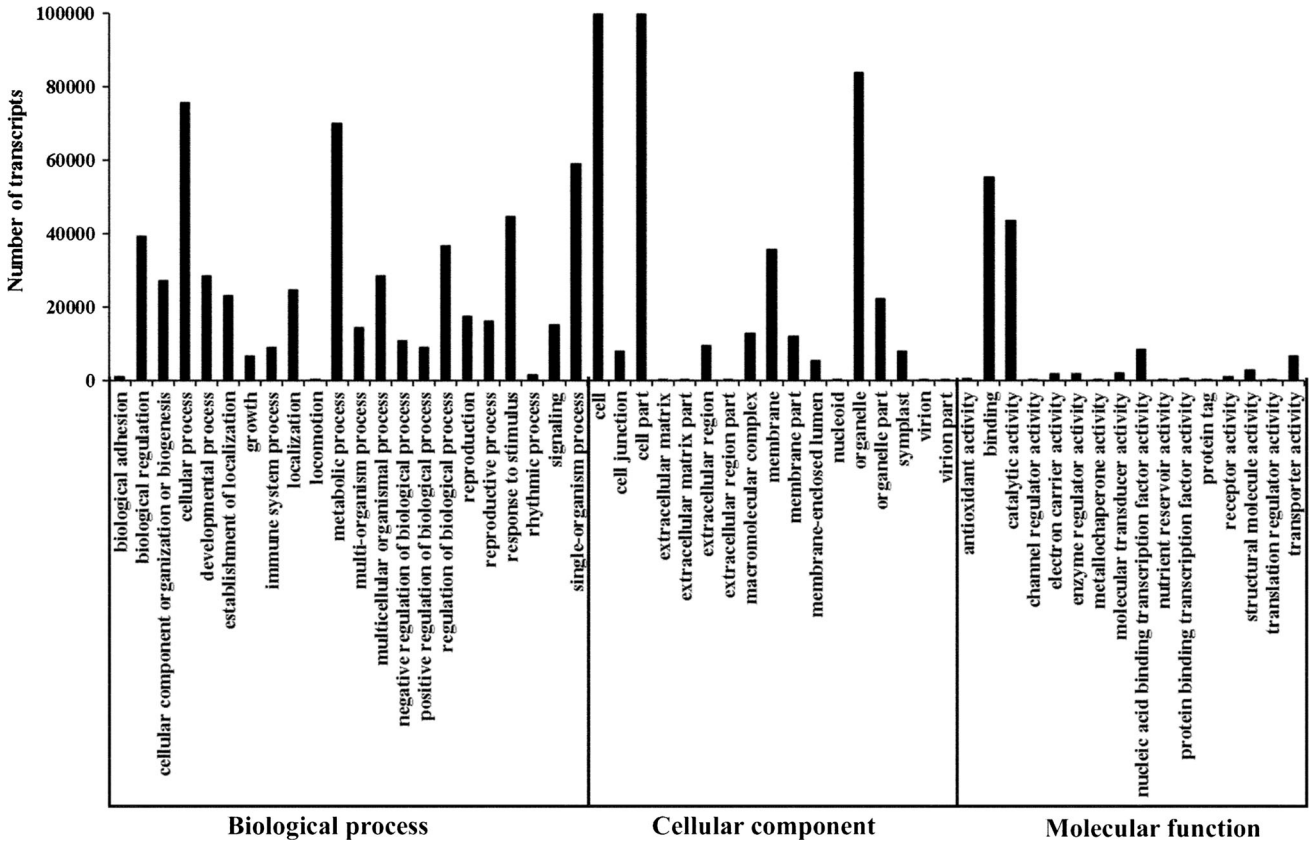

Fig. 3 Gene ontology (GO) classifications of all annotated transcripts

and *B. carinata* and 3,957 showed differential expression between the hybrid and *B. napus*. Non-additive gene expression has been reported in *Arabidopsis* allotetraploids (Kim and Chen 2011; Wang et al. 2006), *Brassica* allohexaploid (Zhao et al. 2013), maize hybrids (Auger et al. 2005; Hoecker et al. 2008), hexaploid wheat (Pumphrey et al. 2009), allopolyploid *Gossypium* (Flagel et al. 2008; Hovav et al. 2008) and citrus allotetraploids (Bassene et al. 2009, 2010), suggesting that this expression pattern is common in hybrids and polyploids. Hierarchical cluster analysis was conducted, and the results also suggested that gene expression in the F_1 hybrid is more similar to that of its maternal parent (Fig. 6).

To confirm the validity of the RNA-seq data, real-time reverse transcription polymerase chain reaction (RT-PCR) analyses for randomly selected transcripts were conducted. The selected genes showed different fold-change values compared with Illumina sequencing; however, the expression trends were generally consistent with the expression profiles estimated from the RNA-seq data (Fig. 7).

Functional analysis of DEGs in flower buds

To clarify the functional distribution of DEGs in the flower buds, GO enrichment analysis was performed (Supplementary Table 3). GO terms with P values ≤ 0.0001 were considered significant. Compared with *B. carinata*, genes related to pollen wall assembly, CC assembly involved in morphogenesis, pollen exine formation, pollen development, gametophyte development and sporopollenin biosynthetic process were significantly downregulated in the F_1 hybrid. GOs associated with ribonucleoprotein complex biogenesis, gene expression, ribosome biogenesis, translation, rRNA metabolic process and RNA methylation were significantly upregulated. Compared with *B. napus*, the downregulated genes in the F_1 hybrid were enriched in 166 GO terms, including plant-type cell wall modification, pollen tube growth, pollination, ion transport, auxin biosynthetic process, pollen wall assembly and pollen exine formation. The upregulated genes in the F_1 hybrid were enriched in 24 GO terms, and the most highly represented GO terms were gene expression, macromolecule methylation, nitrogen compound metabolic process, nucleobase-containing compound metabolic process and translation. These results suggested that the downregulated genes are responsible for the impaired fertility, irregular shape and abnormal exine pattern exhibited by some of the pollens of F_1 hybrid. The upregulated genes were implicated in the regulation of parental bias and non-additive gene expression pattern in F_1 hybrid.

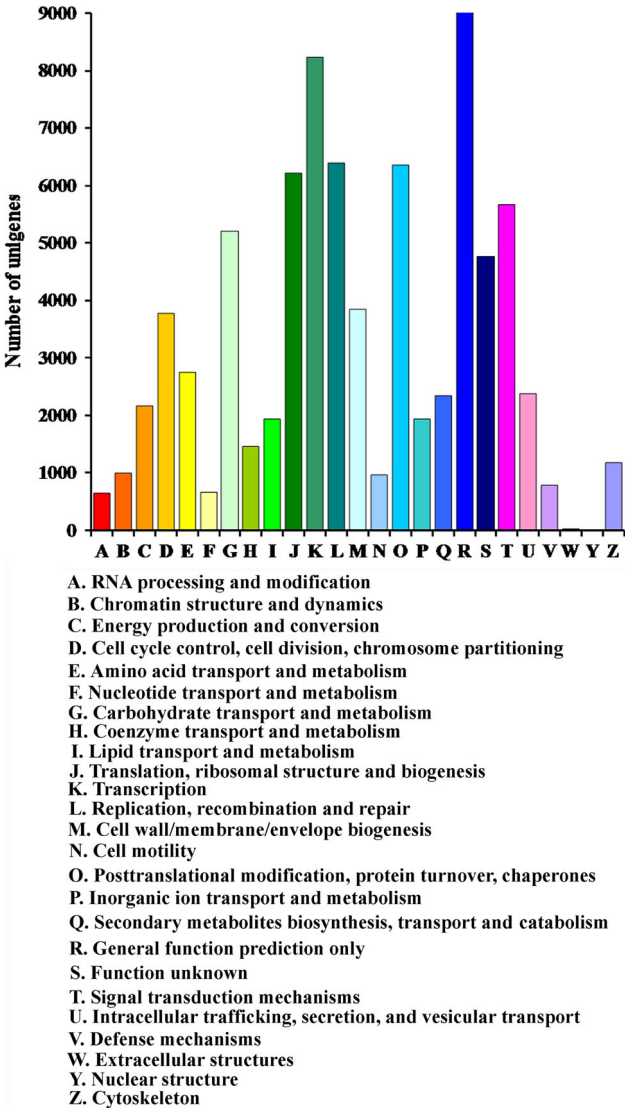

Fig. 4 Clusters of orthologous group (COG) classifications of all annotated transcripts

In addition, KEGG enrichment analysis of DEGs in the flower buds was performed, and pathways with a *Q* value ≤0.05 were considered significantly enriched pathway terms (Supplementary Table 4). The downregulated genes in F_1 hybrid compared with *B. carinata* were mostly enriched in pathways for protein processing in the endoplasmic reticulum, glycerophospholipid metabolism, ether lipid metabolism, flavonoid biosynthesis, endocytosis and glucosinolate biosynthesis. Metabolic pathways, biosynthesis of secondary metabolites, pentose and glucuronate interconversions, starch and sucrose metabolism, plant hormone signal transduction, ether lipid metabolism and glycerophospholipid metabolism were the most enriched pathways involving the downregulated genes and were significantly enriched in the F_1 hybrid compared with *B. napus*. DEGs significantly enriched in ribosome, oxidative

Table 2 The top ten most represented KEGG pathways mapped by all annotated transcripts

Pathway	Number of transcripts	Pathway ID
Metabolic pathways	14,657 (21.85 %)	ko01100
Biosynthesis of secondary metabolites	6,716 (10.01 %)	ko01110
Plant hormone signal transduction	3,743 (5.58 %)	ko04075
Plant–pathogen interaction	3,500 (5.22 %)	ko04626
RNA transport	3,210 (4.79 %)	ko03013
Spliceosome	2,778 (4.14 %)	ko03040
Endocytosis	2,320 (3.46 %)	ko04144
Glycerophospholipid metabolism	2,163 (3.22 %)	ko00564
Protein processing in endoplasmic reticulum	1,842 (2.75 %)	ko04141
Ribosome	1,810 (2.70 %)	ko03010

phosphorylation and spliceosome pathways were upregulated in the F_1 hybrid compared with both parents.

SEM analysis implied that the pollen development of F_1 hybrid plants might be impaired and might thus affect their fertility. This implication was supported by our transcriptome analysis results. The reduced abundance of genes encoding Ca^{2+}-ATPase (ACA7), BTB/POZ and TAZ domain-containing protein 1(BT1), Mitogen-activated protein kinase (MPK4), Polygalacturonase (QRT2) and MYB101 was detected in F_1 hybrid (Table 3). A recent study in *Arabidopsis* suggested that *ACA7* is required for pollen development through regulation of Ca^{2+} homeostasis (Lucca and León 2012). BT1 plays an important role in both male and female gametophyte development in *Arabidopsis* (Robert et al. 2009). AtMPK4 is required for male-specific meiotic cytokinesis (Zeng et al. 2011). QRT2 was implicated in cell-specific pectin degradation (Preuss et al. 1994). Pollen grains are released in tetrads, and the microspores failed to separate during pollen development in the *qrt* mutants of *Arabidopsis* (Preuss et al. 1994; Rhee and Somerville 1998). The pollen-expressed transcription factor MYB101 is important for *Arabidopsis thaliana* pollen tube reception, and *myb97 myb101 myb120* triple mutants failed to discharge their sperm cells into the embryo sac during plant fertilization (Liang et al. 2013).

The pollen wall commonly comprises two layers, and the outer layer, called the exine, protects male sperm from harsh conditions, maintains the integrity of the microspore plasma membrane and facilitates pollination in plant sexual reproduction (Ariizumi and Toriyama 2011; Jiang et al. 2013b). The RNA-seq data indicated that the transcript levels of extracellular lipase 4 (EXL4), tetraketide alpha-pyrone reductase 1(TKPR1) and spermidine hydroxycinnamoyl transferase (SHT) were significantly downregulated in the F_1 hybrid (Table 3). EXL4 is an abundant

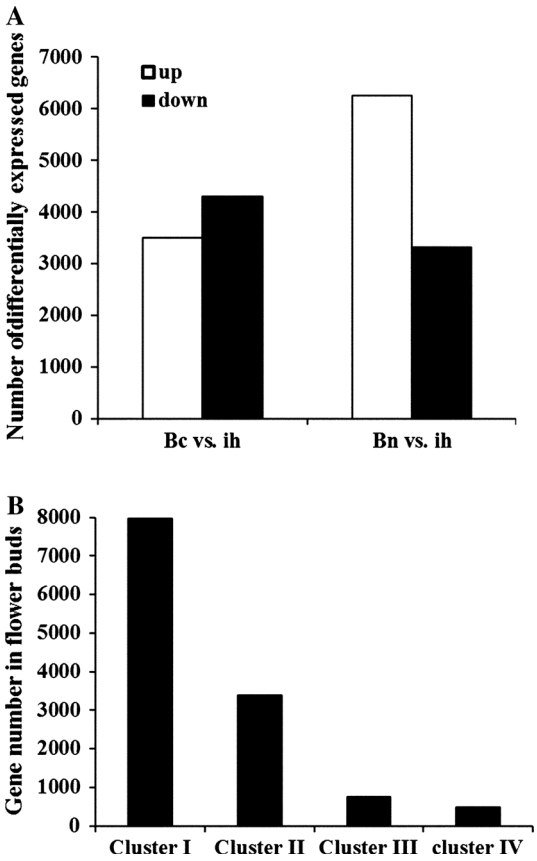

Fig. 5 Number of differentially expressed genes (DEGs) in the F_1 hybrid and its parents. **a** The numbers of upregulated (in *white*) and downregulated genes (in *black*) between the interspecific hybrid (*ih*) and *B. carinata* (*Bc*), interspecific hybrid (*ih*) and *B. napus* (*Bn*) are presented. **b** Cluster distributions of DEGs according to their transcript patterns. *cluster I* consists of DEGs that were differentially expressed between hybrid and only one of the parents; *cluster II* consists of DEGs that were upregulated in hybrid compared to one parent and downregulated compared to another; *cluster III* consists of DEGs with the lowest expression in hybrid; and *cluster IV* consists of DEGs with the highest expression in hybrid

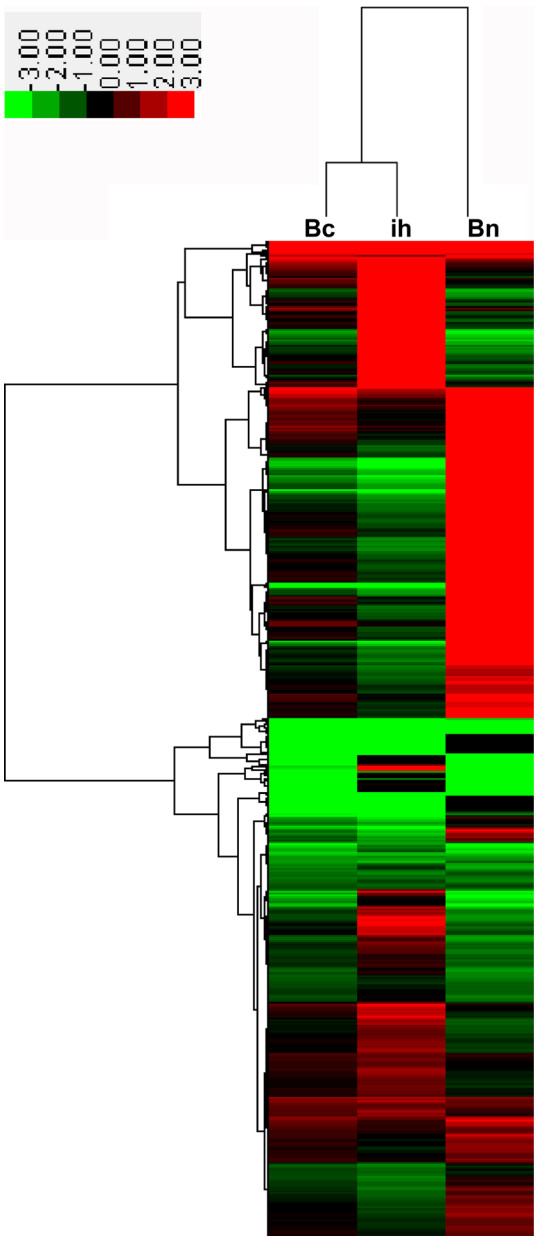

Fig. 6 Hierarchical cluster analyses of differentially expressed transcripts. Bc, *B. carinata*; ih, interspecific hybrid; Bn, *B. napus*. The *column* represents an individual experiment, and the *row* represents the reads per kilo bases per million reads (RPKM)-normalized log2 expression value of each gene. The *color key* represents relative expression levels of the genes. *Red* high expression; *black* moderate expression; *green* low expression

pollen coat protein that plays an important role in promoting pollen hydration on the stigma (Updegraff et al. 2009). Sporopollenin is the major component of the exine, which is derived from the tapetum (Liu and Fan 2013). TKPR1 functions in a conserved biochemical pathway in sporopollenin monomer biosynthesis (Grienenberger et al. 2010). SHT is specifically expressed in anthers and was implicated in the biosynthesis of the flower spermidine derivatives: The pollen wall of the *sht* knock out mutant displayed irregularities and depressions (Grienenberger et al. 2009).

Pollen tube growth plays a critical role in the delivery of the sperm to the egg in sexual reproduction of flowering plants (Krichevsky et al. 2007). Feedback regulation of ROP GTPases, homeostasis of tip calcium gradients, regulation of endocytosis and exocytosis, and production and hydrolysis of phospholipids are key signaling pathways in the regulation of pollen tube growth (Guan et al. 2013). The findings that pollen germination, tube growth, and polarity were significantly impaired in *pip5k4 Arabidopsis* mutant plants revealed the importance of phosphatidylinositol 4-phosphate 5-kinases (PIP5K) in regulating the growth and polarity of pollen tubes (Sousa et al. 2008). Pectin is one of the main components of the pollen tube

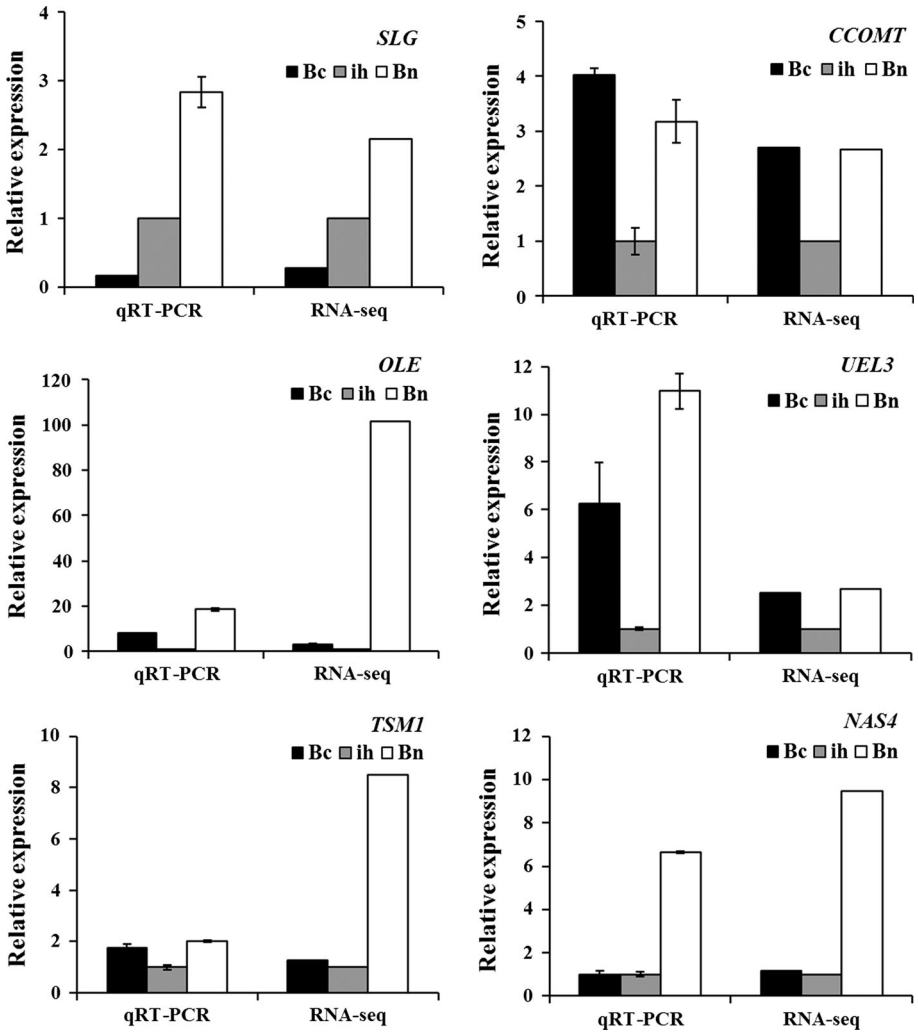

Fig. 7 Real-time PCR validations of six selected differentially expressed transcripts. The transcript levels were normalized to the expression of the *actin* gene, and the level of each transcript in the interspecific hybrid was set at 1.0. *Error bars* represent the SD for three independent experiments. The descriptions of the transcripts and the primers used for each unigene are listed in Supplementary Table 1

cell wall, and the involvement of the multigene family of pectin methylesterases (PMEs) in pollen tubes growth has been reported (Bosch and Hepler 2005; Jiang et al. 2005; Tian et al. 2006). Mutants in exocyst subunits SEC5, SEC6, SEC8 and SEC15a are defective in pollen germination and pollen tube growth, implying the requirement for the putative plant exocyst complex component in the initiation and maintenance of the polarized growth of pollen tubes (Cole et al. 2005; Hála et al. 2008). The reduced abundance of *PIP5K6*, *PME49* and *SEC6* in the F_1 hybrid (Table 3) suggests the role of these genes in inducing impaired pollen growth.

GO enrichment analysis suggested that the upregulated genes in the F_1 hybrid were enriched in regulation of gene expression, including epigenetic regulations, which might account for the parental bias and non-additive gene expression pattern in the hybrid (He et al. 2010; Macdonald 2012). The replication licensing factor CDC10 Target1 (CDT1) plays a role in gametophyte development and maintenance of genome integrity (Castellano Mdel et al. 2004), and overexpression of CDT1a in *Arabidopsis* stimulates DNA replication (Domenichini et al. 2012). The Upregulation of *CDT1a* might be crucial for cell proliferation and genome stability in the F_1 hybrid. The transcript level of COP9 signalosome complex subunit 7 (CSN7) in the F_1 hybrid was threefold higher compared with *B. napus*, while no CSN7 was detected in *B. carinata*. COP9 complex is implicated in transcriptional control, and CSN7 in human may have a direct role in regulating gene expression (Wang et al. 2002). Recent research in Drosophila supports these speculations and demonstrated that CSN7 binds DNA in vitro and functions directly in vivo in transcriptional control of developmentally important pathways (Singer et al. 2014).

Epigenetic regulation is a complex phenomenon that consists of a variety of different processes involved transcriptional gene silencing, DNA methylation, histone modification and chromatin remodelling (Habu et al. 2001). Epigenetic regulation of gene expression in hybrid plants has been well analyzed, and genome-wide, locus-

Table 3 Important DEGs in the flower buds of the F_1 hybrid and its parents

Name	Homologous in At	Accession Nr	Bc RPKM	ih RPKM	Bn RPKM
Pollen development					
ACA7	AT2G22950	gil297821527	0.1073	0	13.4894
BT1	AT5G63160	gil297797307	0.5137	0	1.7949
MPK4	AT4G01370	gil312281951	0.5119	0	1.0204
QRT2	AT3G07970	gil10176984	7.9214	0.9143	24.7941
MYB101	AT2G32460	gil297826689	12.1265	1.4421	3.3650
Pollen exine formation					
EXL4	AT1G75910	gil297839475	155.3997	55.3395	403.2530
TKPR1	AT4G35420	gil15226134	1.3257	0.5509	2.1139
SHT	AT2G19070	gil297832592	0.0737	0	0.4408
Pollen tube growth					
PIP5K6	AT3G07960	gil297829362	1.4086	0.3252	2.6517
PME49	AT5G07420	gil297806773	4.2631	1.3872	2.8199
SEC6	At3G56640	gil297814075	0.4049	0.1577	34.1466
Regulation of gene expression					
CDT1A	AT2G31270	gil38567811	1.0685	3.1914	1.3311
CSN7	AT1G02090	gil42571297	0	5.1203	1.7056
BRM	AT2G46020	gil297824661	0.7661	4.5274	2.1377
FKBP53	AT4G25340	gil297794433	0.6126	1.3788	0.4070

specific variations in different epigenetic components and their association with the changed chromatin states and gene activity of hybrids have been revealed by epigenomic analyses (He et al. 2013). ATP-dependent helicase BRAHMA (BRM) is a subunit of SWI/SNF chromatin remodelling complexes, which plays critical roles in the regulation of transcription and cell proliferation (Archacki et al. 2009). FK506-binding protein53 (FKBP53) was identified as a new histone chaperone in plants, and a recent research showed that AtFKBP53 represses rRNA genes at the chromatin level and thus functions in chromatin remodelling and regulation of transcription in *Arabidopsis* (Li and Luan 2010). The upregulation of *BRM* and *FKBP53* revealed by transcriptome analysis suggested that the epigenetic mechanism may have a potential role in the regulation of gene expression in the interspecific *Brassica* hybrid.

The present study characterized the gene expression of an F_1 hybrid of *B. carinata* and *B. napus* using the RNA-seq approach. The global comparison of the transcriptomes of flower buds from the interspecific *Brassica* hybrid and its parents suggested a role of cytoplasmic or maternal effects in the regulation of gene expression in the interspecific hybrid. Interspecific hybridization created an F_1 hybrid with low fertility, resulting in the downregulation of genes related to pollen structure and development, pollen tube growth and pollination in flower buds of the F_1 hybrid. Real-time PCR confirmed the differential expression patterns of the selected genes. This study shed light on the molecular mechanism underlying hybrid sterility and will promote the use of interspecific hybridization in crop improvement.

Author contribution PC performed the RNA-seq analyses and wrote the manuscript. HL and GY conducted experiments. QY and YW analyzed data. RG conceived and designed research. All authors read and approved the manuscript.

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