Transcript Profiling Analysis and ncRNAs’ Identification of Male-Sterile Systems of *Brassica campestris* Reveal New Insights Into the Mechanism Underlying Anther and Pollen Development

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Male-sterile mutants are useful materials to study the anther and pollen development. Here, whole transcriptome sequencing was performed for inflorescences in three sterile lines of Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino, syn. *B. rapa* ssp. *chinensis*), the genic male-sterile line (A line), the Polima cytoplasmic male-sterile (CMS) line (P line), and the Ogura CMS line (O line) along with their maintainer line (B line). In total, 7,136 differentially expressed genes (DEGs), 361 differentially expressed long non-coding RNAs (lncRNAs) (DELs), 56 differentially expressed microRNAs (miRNAs) (DEMs) were selected out. Specific regulatory networks related to anther cell differentiation, meiosis cytokinesis, pollen wall formation, and tapetum development were constructed based on the abortion characteristics of male-sterile lines. Candidate genes and lncRNAs related to cell differentiation were identified in sporocyteless P line, sixteen of which were common to the DEGs in *Arabidopsis spl/nzz* mutant. Genes and lncRNAs concerning cell plate formation were selected in A line that is defected in meiosis cytokinesis. Also, the orthologs of pollen wall formation and tapetum development genes in *Arabidopsis* showed distinct expression patterns in the three different sterile lines. Among 361 DELs, 35 were predicted to interact with miRNAs, including 28 targets, 47 endogenous target mimics, and five precursors for miRNAs. Two lncRNAs were further proved to be functional precursors for bra-miR156 and bra-miR5718, respectively. Overexpression of bra-miR5718HG in *B. campestris* slowed down the growth of pollen tubes, caused shorter pollen tubes, and ultimately affected the seed set. Our study provides new insights into molecular regulation especially the ncRNA interaction during pollen development in *Brassica* crops.

**Keywords:** *Brassica campestris*, pollen development, long non-coding RNA, miRNA, endogenous target mimic, miRNA precursor
INTRODUCTION

Anther development starts from an archesporial cell that produces sporogenous cells that further differentiate into the endothecium, middle layer, tapetum, and pollen mother cells (PMCs) (Murmur et al., 2010). In Arabidopsis, Sporocyteless/Nazelle (SPL/NZZ) is the key regulator of anther cell differentiation, whose mutation blocks the formation of sporocytes (Yang et al., 1999). The basic leucine zipper transcription factors TGAGCG motif-binding protein 9 (TGAB9) and TGA10 work downstream of SPL/NZZ and interact with floral CC-type glutaredoxins ROXY1 and ROXY2 (Murmur et al., 2010). Ath-miR156 is downregulated in spl/nzz and its overexpression in spl8 mutant leads to similar phenotypes of spl/nzz mutant (Xing et al., 2010). Although several key regulators have been found, molecular networks controlling anther cell specification remain largely unknown.

Pollen development can be divided into two major phases: the developmental phase and the functional phase (Hafidh and Honys, 2021). The latter refers to the interaction between pollen and stigma in which pollen grains rehydrate and germinate with pollen tubes to accomplish double fertilization (Johnson et al., 2019). The developmental stage of pollen is germinate with pollen tubes to accomplish double fertilization and Honys, 2021). The latter refers to the interaction between the developmental phase and the functional phase (Hafidh anther cell specification remain largely unknown.

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together with nuclear genes. Polima (Pol) and Ogura (Ogu) CMS are widely used in the breeding of Brassica crops. In Pol CMS lines, sporogenous cells fail to differentiate into the endothecium, middle layer, and tapetum, and ultimately no pollen sac is formed (An et al., 2014). In Ogu type CMS lines, the obvious malformation is discovered during the late tetrad and uninucleate stage where tapetal cells are radially expanded and vacuolated, and the deposition of sporopollenin is delayed (Kang et al., 2014; Xing et al., 2018). As a typical GMS line, Chinese cabbage (B. campestris ssp. chinensis cv. Aijiaohuang) ‘Bcajh-97-01A’ has been demonstrated to undergo aberrant cytokinesis during male meiosis with defective exine formation, and premature tapetal PCD (Huang et al., 2009; Shen et al., 2019). To systematically explore the molecular mechanisms underlying anther/pollen development in Brassica crops, we created the GMS line ‘Bcajh-97-01A’, a Pol CMS line ‘Bcpol-97-05A’ and an Ogu CMS line ‘Bcogu-97-06A’ of Chinese cabbage (hereafter called A line, P line, and O line, respectively) that shared the same maintainer line ‘Bcajh-97-01B’ (B line) by successive selection and back-crossing (Huang et al., 2008; Liang et al., 2019). In this study, whole transcriptome sequencing was performed to disclose the regulatory networks between mRNAs and ncRNAs, and interactions of different types of ncRNAs during anther and/or pollen development in B. campestris. Differentially expressed genes (DEGs) and ncRNAs associated with pollen development were identified in different sterile lines. We also constructed the interplay between IncRNAs and miRNAs, which will broaden our knowledge on the molecular mechanisms underlying male sterility and proceed with its utilization in breeding.

**MATERIALS AND METHODS**

**Plant Materials**

The Ogu CMS line (B. napus) was transferred into ‘Aijiaohuang’ (B. campestris) through successive backcrossing four times. And then, the fertile plant (B line) in the ‘Aijiaohuang’ two-type line ‘Bcajh-97-01A/B’ were backcrossed to Ogu CMS in B. campestris for four generations. The fertility and morphology characteristics like height and width of backcross progeny were observed over successive years. Finally, we established an O line that shared the common maintainer line (B line) with the A line.

The three sterile lines (A line, P line, and O line) and their common maintainer line (B line) in Chinese cabbage were cultivated in the experimental farm of Zhejiang University, Hangzhou, China at the same time. Inflorescences were harvested from plants at the full flowering stage with the removal of open flowers. Each sample of inflorescences was collected from more than 10 individual plants, frozen in liquid nitrogen immediately, and stored at −75°C for further use. Three replicates were performed.

**Whole Transcriptome Sequencing**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer’s instructions. Libraries for mRNA-Seq and lncRNA-Seq were generated using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, NEB, Ipswich, MA, United States) as the instruction manual described. Libraries for miRNA-Seq were generated using NEBNext Ultra™ small RNA Sample Library Prep Kit for Illumina® (NEB) following the manufacturer’s recommendations and index codes were added to attribute sequences to each sample. RNA-Seq was performed by the Biomarker Technologies Co., Ltd., Beijing, China on an Illumina Hi-Seq platform.

**Identification of ncRNAs and Differential Expression Analysis**

The transcriptome was assembled using the StringTie package based on the reads mapped to the reference genome downloaded from the Brassica database, Institute of Vegetables and Flowers, Beijing, China (version 1.5, BRAD) and annotated using the gffcompare program. Coding-Non-Coding Index (CNCI), coding potential assessment tool (CPAT), coding potential calculator (CPC), and Pfam were used to predict IncRNAs. The secondary structure of IncRNAs was generated by the Mfold web server. Known miRNAs were identified by aligning the mature miRNAs’ sequences in miRBase, University of Manchester, Manchester, United Kingdom (version 21). Reads were identified as known miRNAs when the alignment was identical. Novel miRNAs were predicted by a modified miRDeep2, Max Delbrück Center for Molecular Medicine, Berlin, Germany with plant-specific parameters (version 2.0.5). DESeq R package, Dana-Farbr Cancer Institute, MA, United States (version 1.10.1) was used for screening DEGs, differentially expressed IncRNAs (DELs), DECs, and differentially expressed miRNAs (DEMs). The criteria were a [fold change (FC)] ≥ 2 and a false discovery rate (FDR) ≤ 0.05.

**Construction of IncRNA-mRNA, IncRNA-miRNA, and Transcription Factors Networks**

Adjacent genes within 100 kb of IncRNAs were considered to be their cis-targets and trans-targets, which were predicted by LncTar software (Casero et al., 2015). Pearson bivariate correlation was adopted to estimate the expression relationships between ncRNAs and their targets. The correlation is significant at a confidence level (bilateral) ≤ 0.05 (P < 0.05) and extremely significant when P-value ≤ 0.01. Targets of DEMs were predicted by TargetFinder, Beijing Institute of Radiation Medicine, Beijing, China (version 1.6). LncRNAs as potential miRNA targets were selected by the psRNATarget website with the default parameter. LncRNAs as miRNA precursors were predicted by comparing IncRNAs with miRNA precursor sequences. LncRNAs as miRNA eTFMs were predicted by a local Perl script (Ye et al., 2014). The transcription factor network was constructed by iGRN, a newly developed tool for transcriptional networks, and visualized using Cytoscope, Institute for Systems Biology, Washington, WA, United States (version 3.8) (Meng et al., 2021).

1 http://www.biomarker.com.cn
2 http://brassicadb.cn/#/
3 http://unafold.org/RNA_form.php
4 http://www.mirbase.org/
5 http://plantgrn.noble.org/psRNATarget/
**Nicotiana benthamiana** Transient Expression Assay

Fragments of *bra-miR156HG* and *bra-miR5718HG* were amplified and inserted into the pBI121 vector under two CaMV 35S promoters, respectively. Primers used for vector construction are listed in **Supplementary Table 1**. Plasmids were transformed into Agrobacterium tumefaciens strain GV3101 and then infiltrated into tobacco leaves when OD_{600} reached 1.0–1.2. The leaves were collected, frozen in liquid nitrogen immediately, and stored at −75°C for RNA extraction after 48 h of infiltration.

**Semi-Quantitative and Real-Time Quantitative RT-PCR**

For transient expression assay, semi-quantitative reverse transcription (RT)-PCR of lncRNAs was performed with 2 × TSINGKE master mix (Tsingke Biotechnology Co., Ltd., Beijing, China) and *nbe-18S rRNA* was used as a reference. Expression of miRNAs was detected by real-time quantitative PCR (RT-qPCR) on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, United States) using TB Green Premix Ex Taq II (TaKaRa, Dalian, China) and *nbe-5.8S rRNA* was used as a reference. For validation of RNA-Seq, RT-qPCR was performed using the same samples for RNA-Seq. The expressions of DEGs and DELs were normalized against *BraUBC10* and those of DEMs were normalized against *bra-5.8S rRNA*. The relative expression levels were calculated using the 2−ΔΔCT method. The cDNAs used for the RT-qPCR analysis of miRNA were synthesized by a Mir-X™ miRNA First-Strand Synthesis Kit (TaKaRa). All primers are shown in **Supplementary Table 1**.

**Data Availability**

Transcriptome data supporting the findings of this study have been deposited in National Center for Biotechnology Information Sequence Read Archive (SRA) under the accession number PRJNA753197. All other relevant data are available from the corresponding author on request.

**RESULTS**

**Transcripts Were Identified Genome-Wide in Brassica campestris**

As the general morphology of inflorescences and flowers of the three sterile lines did not differ from those of the common fertile maintainer line except for the anthers, inflorescences without opened flowers of A line, P line, O line, and B line were taken for transcriptome sequencing to analyze the transcriptome during pollen and anther development (**Figure 1A**). For lncRNAs and mRNAs, a total of 141.89 GB of clean data were obtained for 12 samples, with an average of 10.20 GB of clean data per sample. For microRNA, a total of 253.31 M clean data were obtained, and clean reads of each sample no less than 14.40 M. A total of 41,133 mRNAs, 13,879 putative lncRNAs, and 342 miRNAs were identified (**Supplementary Figures 1A–C**). The identified lncRNAs were classified into lncRNAs, antisense lncRNAs, sense lncRNAs, and intronic lncRNAs, which were evenly distributed on different chromosomes (**Supplementary Figures 1D,E**). The expression difference of transcripts between each male-sterile line and the maintainer line was then compared respectively. Totally, 7,136 DEGs, 361 DELs, 56 DEMs were selected out (**Figures 1B–D**). A total of 2,277 DEGs, 77 DELs, and 11 DEMs were common to all sterile lines. Further, 608, 1,139, and 715 DEGs were specific to A line, P line, and O line, respectively (**Figures 1B–D**). As for DELs, 22, 52, and 78 DELs were specifically expressed in the A line, P line, and O line, respectively. No line-specific DEMs were found in the A line, 20 DEMs were specifically expressed in the P line, and only six DEMs were specifically expressed in the O line. Compared with the B line, 3,376, 4,779, and 4,480 DEGs were identified in A line, P line, and O line, respectively, 79% of which on average were downregulated (**Figures 1B,E**). Among the three sterile lines, the largest number of DELs was discovered in O line (242), followed by P line (179), and the least was in A line (142) (**Figures 1C,F**). The number of miRNAs expressed in four lines was comparatively lower than other transcripts. There were 18, 47, and 32 differentially expressed microRNAs (DEMs) identified in A line, P line, and O line, respectively (**Figures 1D,G**). All the expression data of DEGs, DELs, and DEMs were concluded in **Supplementary Data 1**. Interestingly, like miRNAs, the proportion of downregulated non-coding transcripts in sterile lines including lncRNAs and miRNAs was significantly higher than that of upregulated transcripts (**Figures 1E–G**). Further, we carried out the RT-qPCR experiments of seven DEGs, six DELs, and three DEMs to validate the results of RNA-Seq. The results showed that the expression pattern of these candidate genes was consistent with the results of RNA-Seq, which suggest the transcriptome data were reliable (**Supplementary Figure 2**).

**Using the P Line We Found Candidate Transcripts Involved in Anther Cell Differentiation**

To excavate genes related to anther cell differentiation, firstly, the expression of genes that were experimentally confirmed to function in anther differentiation (reviewed by Walbot and Egger, 2016) was analyzed in the B line and the sterile lines. Our results showed that five out of them were DEGs in sterile lines compared with the fertile line (**Figure 2A**). Homolog genes of *Arabidopsis* SPL/NZZ, *Bra026359* (NZZ-1), and its putative downstream genes, *Bra018634* (TGA9-1), *Bra031622* (TGA9-2), *Bra009233* (TGA10-1), *Bra005914* (TGA10-2), and *Bra009231* (*STUBBELIG*-receptor family 2, SRF2) were downregulated in P line, and so did miR156. While the expression of another homolog to *Arabidopsis* SPL/NZZ, *Bra019056* (NZZ-2) and four downstream genes except for *Bra009231* (SRF2) was also decreased in the O line and only miR156 was downregulated in the A line. Additionally, through target prediction and expression correlation analysis, five lncRNAs were found to target SPL/NZZ and its downstream genes (**Supplementary Data 2**). Two lncRNAs target *Bra009233* and one targets *Bra026359*, *Bra019056*, *Bra009233*, and *Bra005914*. MSTRG:2229.1 was predicted to positively regulate *Bra026359* (NZZ-1) while *Bra019056* (NZZ-2) was negatively related with
IncRNA MSTRG.17700.1, MSTRG54890.1 was predicted to target Bra009233 (TGA10-1).

Considering that molecular networks regulating anther cell differentiation are not well studied, it is reasonable that only several function-known DEGs related to it were identified in the three sterile lines. To further elucidate molecular networks in anther cell differentiation, all line-specific DEGs were filtered by gene ontology (GO) term “cell differentiation.” The number of DEGs annotated in P line (72) was approximately twice that of A line (31) and O line (38) (Figure 2B). For further analysis, only DEGs specifically expressed in the P line were included because a previous study described that sporogenous cells in Pol CMS anthers failed to differentiate, which was largely different from the phenotype of A line and O line (An et al., 2014). As B. campestris and Arabidopsis have a common ancestor, functional analysis of homologous genes in...
FIGURE 2 | Analysis of genes in anther cell differentiation in three sterile lines of *Brassica campestris*. (A) Genic network of anther cell differentiation. (B) Venn diagram of differentially expressed genes (DEGs) number involved in cell differentiation in fertile line and sterile lines. (C) Regulatory network of transcription factors annotated to participate in cell differentiation predicted by integrated gene regulatory network (iGRN). Genes in red mean upregulated DEGs, while genes in green represent down-regulated DEGs. B line, the fertile line ‘Bcajh97-01B’; A line, the sterile line ‘Bcajh97-01A’; P line, Polima (Pol) cytoplasmic male-sterile (CMS) line ‘Bcpol97-05A’; O line, Ogura (Ogu) CMS line ‘Bcogu97-06A’; BAM1/2, β-amylase 1/2; SDG2, SET domain protein 2; EMS1, excess microsporocytes 1; AG, agamous; SPL/NZZ, sporocyteless/nozzle; SPL8, SQUAMOSA promoter binding protein-like 8; MAPK3/6, mitogen-activated protein kinase 3/6; ROXY1/2, CC-type glutaredoxin 1/2; TGA9/10, TGACG (TGA) motif-binding protein 9/10; BIM1, brassinosteroid insensitive 1 (BRI1)-EMS-suppressor 1 (BES1)-interacting MYC-like protein 2; SRF2, STRUBBELIG-receptor family 2; CUL2, culin 2; GUN4, genomes uncoupled 4; NUC, nutcracker; GSTF2, Glutathione s-transferase phi 2; DUR, defective uge in root; bHLH071, basic helix-loop-helix protein 71; GS02, gassoho 2; MEE25, maternal effect embryo arrest 25; MYB7/56/105, MYB domain protein 7/56/105; FRP1, GBF’s pro-rich region-interacting factor 1; CDC5/48B, cell division cycle 5/48B; BR6OX1, brassinosteroid-6-oxidase 1; HSI2, high-level expression of sugar-inducible gene 2; IDD16, indeterminate(id)-domain 16; TLP-3, thaumatin-like protein 3; RLP35, receptor like protein 35; PSK5, phytosulfokine 5 precursor; GPAT4, glycerol-3-phosphate sn-2-acyltransferase 4; CXE12, carboxylesterase 12; PI, pistillata; ROPGAP3, ROP guanosine triphosphatase (GTPASE)-activating protein 3.
Down Bra002042 8.613 3.164 Down Bra009055 2.000 0.198 Down Down Bra008175 134.316 6.473 Down Bra006422 1.419 0.580 Down Bra029913 56.944 6.537

Consequently, 16 common DEGs between involved in anther cell differentiation (Wijeratne et al., 2007).

As the phenotype of the P line was similar to the spl/nzz mutant, all P line-specific DEGs and the microarray data from anthers of spl/nzz mutant, all P line-specific DEGs and the microarray data

sugar metabolism (Bowman et al., 1989; Jeong et al., 2015).

Network prediction in a newly developed tool for transcription factors regulatory in B. campestris can provide a reference to the function of genes in Arabidopsis.

Table 1: Common DEGs between Polima cytoplasmic male-sterile of Brassica campestris and the Arabidopsis spl/nzz mutant.

| ID in BRAD | Expression in B line | Expression in P line | Regulated | Homologous genes in Arabidopsis | Gene annotation |
|---|---|---|---|---|---|
| Bra021188 | 2.043 | 0.345 | Down | AT3G16650 Pleotropic regulatory locus 2 (PRL2) | RNA processing and modification |
| Bra029913 | 56.944 | 6.537 | Down | AT3G48690 Carboxylesterase 12 (CXE12) | Serine hydrolases hydrolyzing 2,4-D-methyl |
| Bra002042 | 8.613 | 3.164 | Down | AT2G16720 MYB domain protein 7 (MYB7) | Cell differentiation, regulation of flavanol biosynthetic process |
| Bra009231 | 2.731 | 0.958 | Down | AT5G06820 Strubbelig-receptor family 2 (SRF2) | Protein phosphorylation |
| Bra006422 | 1.419 | 0.580 | Down | AT5G17800 MYB56 | Negative regulation of cell division |
| Bra008175 | 134.316 | 6.473 | Down | AT1G75030 Thaumatin-like protein 3 (TLP-3) | Defense response |
| Bra002513 | 1.757 | 0.045 | Down | AT5G60080 - | Protein phosphorylation |
| Bra009055 | 2.000 | 0.198 | Down | AT5G10140 Flowering locus C (FLC) | Floral transition repression and temperature compensation of the circadian clock |
| Bra003995 | 1.451 | 0.393 | Down | AT1G69560 MYB105 | Boundary specification, meristem initiation and maintenance, and organ patterning |
| Bra006978 | 1.560 | 0.197 | Down | AT3G63230 Cell division cycle 48B (CDC48B) | Mitotic spindle disassembly |
| Bra006953 | 0.449 | 1.612 | Up | AT5G47700 Gaspho2 (GSO2) | Regulation of cell division and cell fate specification |
| Bra003889 | 1.895 | 4.182 | Up | AT1G72180 C-terminally encoded peptide (CEP) receptor 2 (CEPR2) | Receptor for CEP1 peptide |
| Bra028006 | 8.508 | 19.272 | Up | AT1G33590 | Response to Karrakin, signal transduction |
| Bra017533 | 0.699 | 2.011 | Up | AT5G46690 Basic helix-loop-helix protein 71 (bHLH071) | Interacting with FAMA to regulate stomatal differentiation |
| Bra026499 | 0.661 | 1.526 | Up | AT5G23400 | Signal transduction |
| Bra011403 | 2.550 | 5.431 | Up | AT4G32980 Arabidopsis thaliana homeobox 1 (ATH1) | Regulation of gibberellin biosynthetic genes |

The expression of genes was presented as fragments per kilobase of exon model per million mapped fragments. B line and P line refer to the fertile line ‘Bcajh97-01B’ and the Polima cytoplasmic male-sterile of B. ampestris, respectively. Bold characters in the column “regulated” represent genes that showed the same expression change and non-bold ones represent genes that showed the opposite change in the Arabidopsis spl/nzz mutant and P line of B. ampestris. DEG, differentially expressed gene; TAIR, the Arabidopsis information resource; BRAD, Brassica database.

Arabidopsis can provide a reference to the function of genes in B. campestris. Integrated gene regulatory network (iGRN), a newly developed tool for transcription factors regulatory network prediction in Arabidopsis was used to investigate the relationship between homologs of the 72 P line-specific DEGs. Finally, 15 DEGs were screened out to form a regulatory network (Figure 2C). Twenty genes were found to interact with Pistillata (PI, AT5G20240) that controls the differentiation of petals and stamens, and 13 genes were found to interact with Nutcracker (NUC, AT5G44160) which affects flowering time via sugar metabolism (Bowman et al., 1989; Jeong et al., 2015). As the phenotype of the P line was similar to the Arabidopsis spl/nzz mutant, all P line-specific DEGs and the microarray data from anthers of spl/nzz were compared to discover more genes involved in anther cell differentiation (Wijeratne et al., 2007). Consequently, 16 common DEGs between spl/nzz and P line were identified and 13 of them showed the same expression changes, among which six transcription factors (FLC, bHLH071, ATH1, MYB56, MYB105, and MYB7) were also identified with iGRN (Table 1). Five lncRNAs and three miRNAs were predicted to interact with seven DEGs (Supplementary Data 2). Notably, Bra002042 (MYB7) were predicted to be the target of one lncRNA, MSTRG.33978.1, and two miRNAs, bra-miR159a and bra-miR319-3p (Supplementary Data 2).

Utilizing A Line We Selected Potential Transcripts Related to Cell Plate Formation During Meiotic Cytokinesis

Previous cytological observations showed that failure of tetrads formation in the A line was caused by the aberrant cytokinesis (Huang et al., 2009; Shen et al., 2019). To explore the network that participated in male meiotic cytokinesis, DEGs concerning it were selected via GO analysis, Swiss-Prot, and NR annotation. Finally, 13 DEGs, 11 of which were A line-specific and two were expressed in both A line and P line were identified (Table 2). Generally, dicotyledonous male meiocytes undergo simultaneous-type cytokinesis where cell plates determined by
TABLE 2 | Specifically expressed genes involved in cytoplasmic cell plate formation in ‘Bcajh97-01A’ of Brassica campestris.

| ID in BRAD | Expression in B line | Expression in A line | Regulated | Homologous genes in Arabidopsis | Swiss-Prot annotation | NR annotation |
|------------|----------------------|----------------------|-----------|-------------------------------|----------------------|--------------|
| Bra014865  | 50.911               | 25.592               | Down      | AT3G53750 Actin 3 (ACT3)       | ACT3                 | ACT3         |
| Bra033236  | 12.236               | 3.862                | Down      | AT1G01750 Actin-depolymerizing factor 10 (ADF10) | ADF10 | A hypothetical ADF (CAR18B_v10011760mg) |
| Bra020294  | 1.205                | 3.251                | Up        | AT5G59730 Exo70 family protein H7 (EXO70H7) | –                   | EXO70H7      |
| Bra031138  | 84.051               | 40.448               | Down      | AT2G20760 Clathrin light chain 1 (CLC1) | CLC1                | CLC protein |
|           |                      |                      |           | Bra033472 2.534 6.372 Up AT3G51890 CLC3 |                  |              |
| Bra002635  | 0.141                | 0.672                | Up        | Bra013771 0.638 0.255 Down AT2G20760 Clathrin light chain 1 (CLC1) |                  |              |
| Bra037232  | 2.729                | 1.305                | Down      | AT2G18170 Mitogen-activated protein kinase 7 (MAPK7) | MAPK7 | P-loop containing nucleoside triphosphate hydrolases superfamly protein |
| Bra016220  | 2.731                | 1.439                | Down      | AT1G70430 – | MAPKK1 | Kinase family protein |
| Bra021794  | 0.544                | 3.531                | Up        | AT2G32510 MAPK kinase 17 (MAPK17) | MAPKK3 | MAPKK17 |
| Bra030001  | 0.187                | 0.927                | Up        | AT3G50310 MAPKK20 | MAPKK20 | MAPKK20 |
| Bra034123  | 12.917               | 6.961                | Down      | AT3G10525 Siamese related 1 (SMR1) | SMR1 | Cyclin-dependent protein kinase inhibitor SMR1 |
| Bra001367  | 12.750               | 6.011                | Down      | AT3G10525 SMR1 | Cyclin-dependent protein kinase inhibitor SMR1 | Hypothetical protein (ARALDRAFT_478388) |

The expression of genes was presented as fragments per kilobase of exon model per million mapped fragments. B line, the fertile line ‘Bcajh97-01B’; A line, the genic male-sterile line ‘Bcajh97-01A’; TAIR, the Arabidopsis information resource; BRAD, Brassica database.

radial microtubule arrays form between all four haploid nuclei at the same time (Figure 3A). As one of the main components of radial microtubule arrays, actin plays an important role in cytokinesis (De Storme and Geelen, 2013). Bra014865 (ACT3) and Bra033236 (ADF10) annotated to encode an actin protein and an actin-depolymerizing factor 10, respectively, were downregulated in A line (Table 2). Clathrin light protein was reported to be the coating materials of vesicles secreted from the Golgi/Trans-Golgi network in somatic cytokinesis (Buschmann and Muller, 2019). In A line, two genes, Bra031138 (Clathrin light chain 1, CLC1) and Bra033472 (CLC3) were annotated to encode clathrin light protein (Table 2). During cytokinesis, a fusion of vesicles carrying substances required for cell plate synthesis is a crucial process, which is regulated by the Exocyst complex (De Storme and Geelen, 2013). Mutation of Exocyst complex subunit EXO70A1 is compromised the initial cell plate assembly (Fendrych et al., 2010). In A line, Bra020294 was annotated as the Exocyst subunit exo70 and downregulated in A line (Table 2). Moreover, genes responsible for callose synthesis were downregulated in the A line as well (see below Figure 4). Three lncRNAs interacted with Bra033472 (CLC3) and Bra033236 (ADF10), two of which were targeted Bra033472 (CLC3) (Supplementary Data 3).

In mitotic cytokinesis, the phosphorylation of MAP65 protein affects the expansion of phragmoplast, which is regulated by a classical MAPK cascade signaling pathway (De Storme and Geelen, 2013). To explore the MAPK pathway involved in male meiosis of B. campestris, DEGs specifically expressed in A line were screened out and a putative cascade signaling pathway was proposed according to the annotation of DEGs (Figure 3B). In this cascade signaling pathway, three MAPKKks (NPK1, MAPKK17, and MAPKK20) worked downstream of NPK1-activating kinase 1 (NACK1; then, three MAPKKks targeted MAPK1 that was encoded by Bra016220, and subsequently, MAPK7 was activated to phosphate the protein MAP65. Expression analysis showed that the expression of Bra037232 encoding the protein MAPK7 was decreased (Supplementary Data 1). Eleven lncRNAs were presumed to target three MAPKKks (Supplementary Data 3). Seven of them targeted Bra021794 (MAPKK17) while two targeted Bra002635 (NPK1) and two targeted Bra030001 (MAPKK20), respectively (Supplementary Data 3).

Transcripts Related to Pollen Wall Formation Showed Distinct Expression Patterns in Different Sterile Lines

Previous morphological observation showed that a rough and irregular surface rather than a reticulate exine structure existed on pollen grains in A line (Shen et al., 2019). Although the differentiation of sporogenous cells was arrested, a few anther sacs with withered pollen still reside in Pol CMS (An et al., 2014).
Defective in exine formation of the syngenic gene of B. campestris, but not in O line. The expression of Bra029746 by two lncRNAs, and two targeted by three lncRNAs. In contrast, Fifteen DEGs were targeted by only one lncRNAs, three targeted by two lncRNAs, and two targeted by three lncRNAs. In contrast to the similar expression pattern between MSTRG.16387.1 and Bra013041 (AMS), MSTRG.16374.1 showed the reverse expression trend with Bra013041 (AMS).

**Tapetum Degradation-Related Genes Were Downregulated in Three Sterile Lines**

It has been reported that tapetum cells in A line exhibited premature PCD after meiosis (Shen et al., 2019), however, in Ogu CMS, tapetum cells became vacuolized and elongated radially at the uninucleate stage with delayed degradation (Kang et al., 2014; Xing et al., 2018). To unearth the underlying regulatory network of tapetum development, the expression of genes related to it in the three sterile lines was analyzed. Thirty protein-coding genes were included based on (Parish and Li, 2010). Among these genes, 12 of them were DEGs (Figure 5). Further analysis of gene expression changes showed that 11 of them were downregulated in the P line, half in the O line, and only four in the A line. Compared with the B line, all homogenous genes to the DYT1-TDF1-AMS-MYB80-MS1 pathway except Bra013519 (DYTI) were downregulated in the P line. Expression of genes correlated with tapetum formation and differentiation was not discrepant in the O line and A line, but the expression of genes interrelated with tapetum degradation declined in the two sterile lines. All copies of three homogenous genes of Arabidopsis VGD1, GLOX1, and RD19C relevant to tapetum degradation were downregulated, but Bra000615, a homolog to CEPI, was upregulated in the O line (Figure 5).

ncRNAs that interact with protein-coding genes in the network were selected out via target prediction and expression correlation analysis. Finally, 21 IncRNAs and one miRNA targeted 14 DEGs were obtained (Supplementary Data 5). The expression of Bra025337 (TDF1) was negatively related to a newly defined miRNA, unconservative_A03_9803 (P = 0.039). Three IncRNAs, MSTRG.33891.1, MSTRG.33874.2, and
FIGURE 4 | Metabolism pathways of pollen wall components in the three sterile lines of Brassica campestris. Differentially expressed genes associated with the metabolism of sporopollenin, cellulose, pectin, and callose were indicated in the figure. The solid arrows represent a direct relationship, and the dotted arrows indicate an indirect relationship. B line, the fertile line ‘Bcajh97-01B’; A line, the genic male-sterile line ‘Bcajh97-01A’; P line, the Polima cytoplasmic male-sterile line ‘Bcpol97-05A’; O line, the Ogura cytoplasmic male-sterile line ‘Bcogu97-06A’; MYB80, MYB domain protein 80; MS2, male sterility 2; ACOS5, acyl-CoA synthetase 5; COMT, caffeate α-methyltransferase; 4CL, 4-coumarate:CoA ligase; PKSB, polyketide synthase B; CYP703A2, cytochrome P450 family 703 subfamilies A polypeptide 2; CYP704B1, cytochrome P450 family 704 subfamily B polypeptide 1; AMS, aborted microspores; VLCFAs, very-long-chain fatty acids; TKPR1/2, tetraketide α-pyrone reductase 1/2; ABCG, Adenosine triphosphate (ATP)-binding cassette, subfamily G; UGP, uridine diphosphate (UDP)-glucose pyrophosphorylase; Cals, callose synthase; DEK1, defective in exine formation 1; RPG1/2, ruptured pollen grain 1/2; CDM1, callose defective microspore 1; cwINV, cell wall invertase; FRK, fructokinase; GAUT, galacturonosyl transferase.
MSTRG.33884.1 were found to target the meristem control gene Bra039894 (WUS).

Extensive Interactions Between DELs and miRNAs Were Found During Anther and Pollen Development

LncRNAs and miRNAs have been shown to play critical roles in regulating gene expression via complex interaction networks (Han et al., 2021). To further investigate their functions in anther/pollen development, the interaction between DELs and miRNAs in the three sterile lines was explored. As a result, 35 DELs were predicted to interact with miRNAs, with most of which were identified in P line (23), followed by O line (21), and the least in A line (11) (Table 3). Twenty-eight DELs were putative targets of 43 miRNAs. Notably, six common DELs targeted by miRNAs were downregulated in the three sterile lines. Although 47 lncRNAs were predicted to work as eTMs for miRNA, only MSTRG.30876.5 was DEL in the P line and O line, and no DEL functioned as eTM. In the inflorescences of *B. campestris*, the expression pattern of these two lncRNAs was positively correlated with that of bra-miR156 and bra-miR5718 (Figure 6A), respectively. To confirm whether bra-miR156HG and bra-miR5718HG could generate corresponding miRNAs or not, the fragments of the two lncRNAs were inserted into the pBI121 vector (Figure 6B), respectively, and a transient transformation assay in tobacco was performed. Semi-quantitative RT-PCR showed that both lncRNAs were successfully expressed in tobacco leaves (Figure 6C). The expression levels of bra-miR156 and bra-miR5718 were significantly increased in leaves injected with relevant specific fragments of host genes (Figure 6D). These results proved that bra-miR156HG and bra-miR5718HG can generate mature miRNAs in vivo.

Overexpression of bra-miR5718HG in *Brassica campestris* Upregulated bra-miR5718 and Affected Pollen Tube Growth and Seed Set

To answer whether the host gene of miRNA would function in pollen development or not, we created the bra-miR5718HG-overexpressed transgenic plants in *B. campestris*. Two transgenic lines, namely OE-83 and OE-91, with successful overexpression were obtained. The expression of bra-miR5718 was correspondingly increased while its target gene braPAP10...
### TABLE 3 | Summary of differentially expressed lncRNAs acting as miRNAs’ targets, precursors, and eTMs in *Brassica campestris*.

| miRNA | Different roles of DELs | | |
|-------|-------------------------|---------------------|---------------------|
|       | eTM                      | Target              | Host gene           |
|       | A line | P line | O line | A line | P line | O line | A line | P line | O line |
|       |         |         |         |         |         |         |         |         |         |
| Bra-miR156c | – | – | – | – | – | – | MSTRG.50087.1 | – |
| Bra-miR156f | – | – | – | – | – | – | MSTRG.50087.1 | – |
| Bra-miR158-5p | – | – | – | MSTRG.20734.1 | MSTRG.20734.1; MSTRG.20734.2 | – | MSTRG.50087.1 | – |
| Bra-miR162-5p | – | – | – | – | – | – | – | – |
| Bra-miR403-5p | – | MSTRG.30876.5 | – | – | – | – | – |
| Bra-miR5719 | – | MSTRG.6099.1 | MSTRG.6099.1; MSTRG.6099.1 | – | – | – |
| Bra-miR9408-3p | – | MSTRG.12676.1 | – | – | – | – | – |
| Unconservative_A01_124 | – | – | – | – | – | – | MSTRG.10680.1; MSTRG.25550.1; MSTRG.25551.3 |
| Unconservative_A02_5263 | – | – | – | MSTRG.26116.1 | – | – | MSTRG.21862.1 |
| Unconservative_A02_5624 | – | – | – | MSTRG.3172.12 | – | – | – |
| Unconservative_A02_9048 | – | – | – | MSTRG.47571.3 | MSTRG.22869.1 | – | – |
| Unconservative_A03_10008 | – | – | – | – | MSTRG.20239.1 | – | – |
| Unconservative_A03_12816 | – | – | – | MSTRG.6099.1 | MSTRG.6099.1 | – | – |
| Unconservative_A03_13897 | – | – | – | – | – | – | MSTRG.50087.1 |
| Unconservative_A04_16774 | – | – | – | MSTRG.22869.1 | – | – | – |
| Unconservative_A04_16775 | – | – | – | MSTRG.22869.1 | – | – | – |
| Unconservative_A04_16989 | – | – | – | – | – | – | – |
| Unconservative_A05_19537 | – | – | – | MSTRG.13936.3 | – | – | – |
| Unconservative_A06_25702 | – | – | – | – | – | – | MSTRG.22869.1 |
| Unconservative_A07_27582 | – | – | – | MSTRG.41319.2 | MSTRG.41319.2 | – | – |
| Unconservative_A07_29750 | – | – | – | MSTRG.8026.1 | – | – | – |
| Unconservative_A08_33893 | – | – | – | – | – | – | – |
| Unconservative_A10_40369 | – | – | – | MSTRG.51803.1 | MSTRG.51803.1 | – | – |
| Unconservative_A10_41812 | – | – | – | MSTRG.22869.1 | – | – | – |
| Unconservative_A10_41813 | – | – | – | MSTRG.22869.1 | – | – | – |
| Unconservative_A10_42634 | – | – | – | MSTRG.22869.1 | – | – | – |
| Unconservative_A10_42699 | – | – | – | MSTRG.56864.1 | MSTRG.56864.1 | – | – |
| Unconservative_scaffold 036722_49811 | – | – | – | – | – | – | – |

Long non-coding RNAs (lncRNAs) in bold font were upregulated and lncRNAs non-bolded were downregulated in sterile lines. eTMs, endogenous target mimics; A line, the genic male-sterile line ‘Bcajh97-01A’; P line, the Polima cytoplasmic male-sterile line ‘Bcpol97-05A’; O line, the Ogura cytoplasmic male-sterile line ‘Bcogu97-06A’.
was downregulated in these lines (Figure 7A). Compared with wild type (WT), no obvious abnormality was observed during the vegetative phase, but the flowering time of transgenic plants was slightly delayed for 3 days (Figure 7B). Alexander staining of pollen grains showed that pollen viability was not affected in the two transgenic lines (Figure 7C). However, in vitro germination assay showed that the length of pollen tubes in transgenic plants was significantly shorter than that in WT plants, thus, a time-series in vitro germination assay was performed to observe the growth rate of pollen tubes in the transgenic plants. At 20 min after germination, the average pollen tube length of WT was 17.34 µm, yet it was 14.27 µm of OE-83 and OE-91 (Figures 7D,E). With the increase of germination time, the difference in pollen tube length was more obvious. The average pollen tube length of WT reached 74.95 µm at 60 min after germination, while it was only 64.27 µm of OE-83 and OE-91, which was significantly shortened ($P < 0.01$) (Figures 7D,E). Further, to see if the shorter pollen tubes affect seed set, we crossed OE-83 (♀) with WT (♂) through pollinating the stigma of emasculated OE-83 (♀) flowers with WT (♂) pollen grains. The resultant pollinated OE-83 (♀) flowers were 100% fertile and produced normal silique in which vigor seeds developed. By contrast, the reciprocal WT (♂) × OE-83 (♀) cross caused shorter silique and fewer seeds inside. Such infertility was also observed in OE-83 self-crossed plant but not in WT self-crossed plant. This result suggested that over-expression of bra-miR5718HG influenced the seed set in B. campestris (Figures 7F,G).

**DISCUSSION**

**Whole Transcriptome Analysis Helps Us to Deeply Understand the Gene Regulation During Anther and Pollen Development in Brassica Crops**

Sporocyte formation and meiosis are the early stages of pollen development. SPL/NZZ is required for the initiation of sporogenesis in Arabidopsis (Wijeratne et al., 2007). In the present study, Bra026359 (SPL/NZZ) were degraded in P line. Analysis of P line-specific DEGs identified candidate genes for anther cell differentiation (Figure 2A and Table 1). Most of P line-specific DEGs were transcription factors and their homologous genes in Arabidopsis formed a network, in which PI seemed to target most genes. PI could form a higher-order heterotetrametric complex with Agamous-like 13 (AGL13)-AG-Apetala 3 (AP3)
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FIGURE 7 | Bra-miR5718HG slow down pollen tube growth via working as host genes for miR5718 in B. campestris. (A) Relative expression of bra-miR5718HG, bra-miR5718, and braPAP10 in transgenic and wild-type (WT) plants. (B) Photograph of bra-miR5718HG overexpression transgenic lines and WT. (C) Alexander staining of pollen grains from bra-miR5718HG overexpressing plants in B. campestris. (D) Representative photograph of pollen germination in vitro of overexpression lines and WT. (E) Pollen tubes length of overexpression lines and WT at different times after germination. (F) Representative photograph of siliques from reciprocal cross and self-cross of transgenic and WT plants. (G) Statistics of seed number of siliques from reciprocal cross and self-cross of transgenic and WT plants. Data were presented as mean ± SD and the t-test was used for significance analysis. **P < 0.01. Triplicate experiments were conducted.

to regulate the expression of SPL/NZZ (Hsu et al., 2014). Thirteen DEGs displayed the same expression changes between P line and spl/nzz mutant and half of them belong to the MYB or Receptor-like protein kinases (RLKs) families. MYB7, MYB56, and MYB105 were MYB family members who worked in different parts of the plant reproductive process. AtMYB7 and its homologs AtMYB4 affected pollen exine formation, AtMYB56 negatively regulated the flowering time, and the double mutants of AtMYB105 and AtMYB117 presented disordered floral organ boundary specification, and meristem initiation and maintenance.
SPL transcription factors and differentiation (Racolta et al., 2014). Interactions between these were explored, although its roles in anther cell differentiation remain to be explored, GSO2 positively regulates root cell proliferation and differentiation (Racolta et al., 2014). Interactions between these transcription factors and SPL/NZZ will create new windows of pollen development.

Whole transcriptome analysis showed that expression changes of well-known networks in anther/pollen development varied in different male sterile lines with different abortion phenotypes (Figures 4, 5). Pollen wall and tapetum are essential structures for functional pollen grains and the synthesis or regulatory pathways for them have been well studied (Parish and Li, 2010; Shi et al., 2019), the participation of ncRNAs, especially newly defined lncRNAs, and the results of transient transformation assay, it can be assumed that bra-miR156HG and bra-miR5718HG contributed to pollen development considering its relatively high expression level in Ogu CMS Chinese cabbages (Yu et al., 2012; Wei et al., 2015). The extremely significant positive correlation between the expression of two host genes and corresponding miRNAs, and the results of transient transformation assay, it can be assumed that bra-miR156HG and bra-miR5718HG generated the corresponding miRNAs in vivo (Figure 6).

**Bra-miR5718HG Negatively Regulated Pollen Tube Growth in Brassica campestris**

In the top30 GO enrichment items, “nucleus” and “plasma membrane” were included in most of the DEGs. Furtherer, we found that 461 DEGs were enriched to the term “pollen tube growth” (Supplementary Figure 4). Our transgenic assay revealed that overexpression of bra-miR5718HG led to the upregulation of bra-miR5718 and reduced expression of braPAP10 (Figure 7A). Delayed pollen tube growth *in vitro* of the transgenic plants overexpressing bra-miR5718HG was observed, which resulted in shorter pollen tubes and finally caused less seed set in *B. campestris* (Figures 7D–G). *AtPAP10* (AT2G16430), the *Arabidopsis* homolog of *braPAP10*, is a member of the purple acid phosphatase (PAP) family (Xie and Shang, 2018). PAPs are usually involved in the acquisition and distribution of phosphorus in plants, yet more functions of them have been revealed besides Pi deficiency adaption (Bhadouria and Giri, 2021). Overexpression of *AtPAP2* improved sucrose phosphate synthase (SPS) activity and the levels of sugars and tricarboxylic acid (TCA) metabolites, resulting in earlier bolting and higher seed yield (Sun et al., 2012). Interestingly, many PAPs showed relatively higher expression in reproductive tissues, and 21 PAP genes in *Arabidopsis* were detected expression in dry pollen and germinated pollen tubes in microarray analysis (Kuang et al., 2009; Qin et al., 2009; Xie and Shang, 2018). *AtPAP10*, *AtPAP18*, and *AtPAP27* were upregulated two or threefolds in the transcriptome of pollen tubes compared to the mature pollen, which meant that they might have a positive role in pollen tube growth (Yu et al., 2012). *AtPAP15* (At3g07130),

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(see the original paper for references and further details)
a PAP with phytase activity, showed an obvious GUS signal in mature pollen grains and germinated pollen tubes (Kuang et al., 2009). Consistent with its expression, mutants of *AtPAP15* presented lower pollen germination (30–35%) compared with WT (78%), which can be explained by its phytase activity since the major storage form of phosphorus in pollen grains is phytate (Kuang et al., 2009). Biochemical properties analysis of *AtPAP10* revealed that it has hydrolysis activity on ATP, ADP, dATP, pyrophosphate, polyphosphate, and phytate (Wang et al., 2011). In this study, shorter pollen tubes were observed in *bra-miR5718HG* overexpressing plants and the expression of *braPAP10* was downregulated. These results indicated that *braPAP10* may play a positive role in pollen tube growth of *B. campestris* and its expression was regulated by *bra-miR5718HG*.

Above all, it is apparent that interactions between mRNAs and ncRNAs, as well as different ncRNAs, are universal and widespread during pollen and anther development, and the discovery of these interactions can help to better understand the molecular mechanism underlying this process. Moreover, our study provides many candidate genes and ncRNAs, and further confirmation for the function and the interaction of them will unveil the complex network for pollen development.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA753197.

**AUTHOR CONTRIBUTIONS**

LH designed the research. DZ, ZJ, JWC, and DL analyzed the transcriptome data. DZ, CC, and TL generated the transgenic plants and performed the experiments. JSC constructed the male sterile lines. DZ, XX, SL, and LH wrote the manuscript. All authors have read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.806865/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.