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41

42 **Abstract:**

43 Wheat photo-thermosensitive genic male sterile (PTGMS) line is a vital material in the  
44 two-line hybrid wheat breeding system in which functional pollen production is highly  
45 associated with temperature during early developmental stage. Understanding the potential  
46 mechanism of pollen infertility induced by low temperature in PTGMS wheat is crucial for  
47 the effective utilization of genetic resources to guide wheat breeding. Herein, we combined  
48 full-length single-molecular sequencing and Illumina short reads sequencing data to obtain  
49 the high-resolution spatio-temporal transcriptome map of pollen under low temperature  
50 stress at mother cell, dyad and tetrad stages in PTGMS line BS366. Cytological descriptions  
51 and whole transcriptome analysis revealed a global landscape of low temperature altered  
52 pollen fertility transformation via regulating the transcriptional patterns of cytoskeleton-  
53 related lncRNAs and their target genes, which involved in the calcium signaling and vesicle  
54 trafficking pathways on cytoskeleton homeostasis at different stages of meiosis. Overall,  
55 our results provided the transcriptional and cytological evidences for understanding the low  
56 temperature-induced pollen sterility deficiency in PTGMS wheat line.

57 **Keywords:** Wheat, photo-thermosensitive genic male sterile (PTGMS), cytoskeleton,  
58 alternative splicing, lncRNA

59 **Introduction**

60 Pollen abortion contains multiple physiological, biochemical, and molecular changes such as the  
61 abnormal degradation of the tapetum (Zheng et al., 2019), defective pollen wall (Marianne et al., 2002;  
62 Wu et al., 2015), the level of kinetic of ATPases (Sane et al., 1997), the distribution and concentration of  
63  $Ca^{2+}$  in anther (Tian et al., 1998), the regulation of the cytoskeleton (Tang et al., 2012; Wang et al., 2018),  
64 accumulation ROS in tapetum (Liu et al., 2018a), and the abnormalities of cell signaling transductions (Liu  
65 et al., 2018b). Based on mentions above, it is well known that cytological biological events and related  
66 genes may play important roles in regulation of plants male fertility. In CMS lines wheat, premature or  
67 delayed PCD by tapetal cells disorganized the supply of the nutrients to microspores, thereby resulting  
68 in pollen abortion (Meng et al., 2016). Previous studies suggested this irregular tapetal PCD was tightly  
69 controlled by evolutionarily conserved transcriptional cascades (Liu et al., 2020). In recent years, hybrid  
70 breeding has a remarkable success in several allogamous species such as maize, sunflower, sorghum,  
71 sugar beet, and rye, but not be fully exploited in autogamous crops (Longin et al., 2012). Wheat is an  
72 autogamous crop, however, hybrid seed production requires cross-pollination of the female parent by  
73 pollen from the male parent. Photoperiod and/or thermo-sensitive genic male sterility (P/TGMS) is an  
74 important material in two-line breeding system to explore the potential of heterosis. Previous studies  
75 showed that the male sterility of P/TGMS lines are contributed to abnormal pollen development (Bai et  
76 al., 2017). Further cytological studies showed that P/TGMS wheat, it exhibited disordered distribution

77 of the cytoskeleton, including microfilaments and microtubules when exposed to a sterile environment  
78 during the fertility-sensitive stage (Tang et al., 2011; Wang et al., 2018). Additionally, previous RNA-seq  
79 studies also have mostly focused on the transcripts of male sterility and thousands of differentially  
80 expressed genes have been reported (Liu et al., 2016). As we all know, in male sterile wheat, the process  
81 of pollen abortion reflects extremely complex reprogramming of gene expression involving chromatin  
82 modification, transcription, posttranscriptional processing, posttranslational modification, and protein  
83 turnover. However, the complex posttranscriptional and translational levels molecular mechanisms of the  
84 male sterility, especially the wheat P/TGMS line induced by low temperature, are currently still not clear.

85 As a hexaploid, wheat has a large and complex genome, estimated to reach approximately 17G, which  
86 composes three closely-related and independently maintained genomes that are the result of a series of  
87 naturally occurring hybridisation events. With the continuous advancement of technology, second-  
88 generation sequencing technology (e.g., Illumina sequencing), could feature high-throughput capability  
89 and provide high-quality reads. However, the short-read length potentially introduces errors in  
90 inaccurately identify the transcript results. Third-generation sequencing is a single-molecule real-time  
91 sequencing technology, (e.g., PacBio sequencing), could provide full-length transcript information,  
92 detect single-molecule structure, provide complete mRNA structure and is, therefore, well suited for  
93 transcript recovery and isoform detection in species with well sequenced and/or incomplete genome  
94 sequences (Abdel-Ghany et al., 2016; Wang et al., 2016). In some crop plants, many scientists have been  
95 studied the isoform change when plant response to stress at gene transcriptional levels, such AS level  
96 and long non coding RNA (lncRNA) (Abdel-Ghany et al., 2016; Wang et al., 2016) using combination  
97 of TGS and NGS technology. AS is a widely recognized RNA processing mechanism in eukaryotic  
98 species, playing a major role in the molecular biology of the cell, and within humans it has been  
99 implicated in multiple genetic disorders (Wang et al., 2016). AS is a critical posttranscriptional event  
100 which comes from alternate splice site choices in a single gene locus, including intron retention (IR),  
101 exon skipping (ES), alternative 5' splicing site (Alt5'SS) and alternative 3' splicing site (Alt3'SS) (Wang  
102 et al., 2019). In higher plant, it has been reported that the AS events are involved in a wide range of  
103 developmental and physiological processes including responses to stress. For example, about 60% of  
104 Arabidopsis intron-containing genes are generated by AS (Marquez et al., 2012). It has been  
105 demonstrated that AS is important for cold response when SFs mis-expressed during cold sensitivity or  
106 tolerance treatment (Laloum et al., 2018). In wheat, Liu et al. (2018) performed genome-wide analysis  
107 of alternative splicing (AS) responses to drought stress (DS), heat stress (HS) and their combination (HD)  
108 in wheat seedling to investigate the regulation of AS during these stress processes (Liu et al., 2018c).  
109 More recently, Wang et al. (2019) also investigated the spatio-temporal landscape of heat adaptations in  
110 wheat filling grain and flag leaves at transcriptional and AS levels by hybrid sequencing (second- and  
111 third-generation sequencing). These studies strongly suggest that AS networks are central co-ordinators  
112 of the stress response. Although AS plays an important role in stress response, it is not clear in studies  
113 related to pollen abortion. In addition to AS the lncRNA were also generated by hybrid sequencing (Wang  
114 et al., 2019). Many studies have shown that lncRNA could regulate genes at the transcriptional and post-

115 transcriptional levels by acting as signals, decoys, scaffolds, and guides (Heo and Sung, 2011). However,  
116 virtually nothing is known about the extent and timing of the contribution of AS and lncRNA or how AS  
117 and lncRNA determine the dynamic changes of transcriptome required for regulating male sterility for  
118 P/TGMS line. Here, to illustrate the regulation of these factors and genes on fertility transformation, we  
119 used hybrid sequencing strategy and used a conventional fertile wheat variety as a control and differential  
120 genes background to analyze the role of AS and lncRNA in regulating male sterility during fertility  
121 transformation stages in wheat PTGMS line BS366.

## 122 **Results**

### 123 **Morphological characteristics of mature pollen under different conditions**

124 In order to identify the influence of environment on the fertility of PTGMS line BS366, we observed  
125 the phenotype of BS366 at the trinucleate stage under different fertility conditions and used  
126 conventional wheat Jing411 as control (Fig. 1). Scanning electron microscopy (SEM) examination  
127 at trinuclear stage revealed that epidermis cells of the Jing411 in two conditions and BS366 anthers  
128 in fertile conditions were arranged closely, on the contrary, the epidermis cells of BS366 anthers in  
129 sterile conditions were incomplete and occurred loss. Moreover, the inner epidermal ubisch bodies  
130 of anther for BS366 in sterile conditions were abnormal and accumulated more sparsely distributed.  
131 Based on observations of microspores at the trinucleate stage, the microspores were uniformly  
132 spheroid and had finely reticulate ornamentation on their surface in anther of Jing411 and BS366 in  
133 fertile conditions, while the sterile microspores were extremely atrophied. According to I<sub>2</sub>-KI  
134 staining, BS366 in fertile conditions pollen grains were 60% stained black, whereas pollen grains  
135 were all wrinkled and inadequately stained in sterile condition, and exhibited completely aborted  
136 characteristics, indicating that low temperature could induce the male fertility conversion in PTGMS  
137 line BS366.

### 138 **Overview of sequencing data**

139 Previous study showed that the period from the pollen mother cell stage to the tetrad stage is the  
140 most sensitive to low temperature for the pollen of BS366 (Bai et al., 2017). And previous  
141 cytological studies also showed that there were abnormalities of film-forming body and cell plate  
142 in this process (Tang et al., 2011). To comprehensively investigate the wheat transcriptomes present  
143 during the fertility transition, hybrid sequencing were performed on the anther of BS366 and Jing411  
144 from different fertility condition with three pollen development stages (S1: pollen mother cell stage,  
145 S2: dyad stage, and S3: tetrad stage) (Fig. S1). Totally, 247,486 and 240,993 circular consensus  
146 sequence reads for BS366 and Jing411 were yielded, respectively (Table S2). In total, 209,967 and  
147 190,280 reads of full-length non-chimeric (FLNC) (84.84% and 78.96%) from these circular  
148 consensus sequence reads, were identified based on the inclusion of 5' and 3' primer, and 3' poly(A)  
149 tails, followed by error correction. Then, 209,967 and 190,280 high-quality isoforms were uniquely  
150 mapped to the IWGSC RefSeq v1.0 (Table S3). Finally, 16,000 and 14,864 transcripts were obtained

151 from BS366 and Jing411, followed by error correction using Illumina short reads (Table S4). The  
152 length of transcripts was mainly concentrated in the range of  $10^3$ - $10^4$  for BS366, Jing411 and  
153 IWGSC RefSeq v1.0, suggesting the generated data are accurate and reliable, could be used as a  
154 reference in this study (Fig. 2 and Fig. S2). These transcripts were derived from 13,577 and 13,010  
155 gene loci. Of which, 1,185 and 902 are new gene loci and 6,248 and 4,918 are new transcripts (Table  
156 S4). By comparison with the IWGSC RefSeq v1.0 annotation, PacBio transcripts could be classified  
157 into seven groups including PacBio data, DEG, DAS, lncRNA, fusion gene analysis (Fig. 3).

## 158 **Identification of alternative splicing events during fertility transition**

159 AS is an important biological event of post-transcriptional regulation in organisms (Wang et al.,  
160 2019). A few studies have explored the relationship between AS and fertility transition which  
161 regulated by external environment in plants (Capovilla et al., 2015). Therefore, we analyzed the  
162 patterns of fertility transition-induced AS events from the RNA sequencing data. In this study, four  
163 main AS events including Alternative 3' splice site (Alt3'SS), Exon skipping (ES), Intron retention  
164 (IR) and Mutually exclusive exon (MEX) were identified from three pollen developments stages of  
165 BS366 and Jing411. As shown in Table 1, total 35,248 and 33,099 AS events with 20,312 and 19,490  
166 AS genes were identified from three pollen developments in BS366 and Jing411, respectively. Of  
167 which 11,619, 11,840 and 11,789 AS events in BS366 and 10,980, 11,084 and 11,035 AS events  
168 were determined on subgenomes A, B and D, respectively (Table S5). It was found that ES was the  
169 most abundant (91.08%-91.98%) AS events in both BS366 and Jing411, followed by MEX (4.84%-  
170 5.24%), IR (3.03%-3.67%) in BS366 (Table 1), and some orders in Jing411 and for AS genes (Table  
171 S5).

## 172 **Identification of sterility -related AS events and genes in BS366**

173 The high-resolution temporal transcriptomes allowed us to determine the specific stage at which  
174 differentially spliced genes (DSGs) and differentially expressed genes (DEGs, fold change  $\geq 2.0$ ,  
175 FDR-adjusted  $P$ -value  $< 0.05$ ) showed a significant change, along with the magnitude and trend of  
176 that change. To identify the specific time of significant changes of DSGs and DEGs as well as  
177 analysis the sterility-related DSGs and DEGs in BS366, DSGs and DEGs from three key pollen  
178 development stages of BS366 during fertility transition BS366 were screened. By comparing the  
179 DSGs of three stages, in total, 108, 130 and 141 genes with 118, 141 and 160 AS events were  
180 identified from pollen mother cell stage, dyad stage and tetrad stage respectively after critically  
181 filtering process. For DEGs, in total, 39, 73 and 412 DEGs were identified from three stages (Figure  
182 4A). The change trend of DSGs and DEGs in the three stages was synergistically rising, in which  
183 the DSGs and DEGs contained in tetrad stage have significant changes, which may be the key stage  
184 of fertility transformation.

185 Here four DSGs were found in shared DSGs and DEGs in the three stages (Fig. 4B), in which the  
186 genes encoding zinc finger domain protein 1A (*TraesCS5D02G371100*) and UTP--glucose-1-

187 phosphate uridylyltransferase (*TraesCS5A02G353700*) involved in pollen formation (Chivasa et al.,  
188 2013), and the gene encoding coatamer subunit gamma-2 (*TraesCS1D02G156000*) was associated  
189 with vesicle transport (Hamlin et al., 2014). Moreover, cytoskeleton-related gene kinesin-4  
190 (*TraesCS3B02G196600*), vesicle transport-related gene encoding vacuolar protein sorting-  
191 associated protein (*TraesCS4B02G382900*), and pollen formation-related genes encoding zinc  
192 finger BED domain-containing protein (*TraesCS3B02G126800*) and MYB transcription factors  
193 (*TraesCS3B02G243600*) were found in co-DEGs of the three stages (Figure 4C). Interestingly, the  
194 gene encoding kinesin-4 (*TraesCS3B02G196600*) was differentially expressed in three stages at the  
195 same time and occurred differentially AS in stage 3 (Fig. 4D and E), and the kinesin-4 has been  
196 proved to be involved in vesicle transport and cytoskeleton formation (van Riel et al., 2017), thereby  
197 inferring that AS of this gene could work together with transcriptional regulation to involve in  
198 fertility transformation of PTGMS line BS366. qPCR analysis verified the correctness of RNA-seq  
199 results (Fig. 5F). The primers used in this study were listed in Table S1.

200

## 201 **Comparative analysis of the biological functions regulated at AS and transcription** 202 **levels**

203 GO analysis showed that the most of DSGs were correlated with some cytological and molecular  
204 events in the process of pollen development including cytoskeleton (such as “cytoskeleton” and  
205 “cytoplasmic microtubule organization”), calcium regulation (such as “calcium ion binding” and  
206 “calcium-dependent phospholipid binding”), vesicle transport (such as “vesicle-mediated transport”  
207 and “vacuole”), and pollen formation (such as “cell wall organization” and “glucose-1-phosphate  
208 uridylyltransferase activity”) during the three key stages of fertility conversion (Table S6). It has  
209 been reported these GO terms identified are involved in the male sterility, for example, the DSGs  
210 encoding the protein MOR1 (*TraesCS3B02G371500* in S1, *TraesCS3A02G339900* in S2, and  
211 *TraesCS3B02G371500* in S3) was critical for the orderly assembly of microtubules (Kawamura et  
212 al., 2006). Microtubules and microfilaments control the whole double fertilization process of pollen,  
213 is necessary for the normal development of pollen. Therefore, low temperature induced DSGs was  
214 a crucial further layer of regulation for pollen development, thereby possibly leading to fertility  
215 conversion.

216 To further identify the sterility-related genes, weighted gene co-expression network analysis  
217 (WGCNA) were performed with all DEGs from BS366 based on the seed setting rate (Table S7).  
218 The analysis of module-trait relationships analysis showed that the module ‘Red’ ( $r = -0.98$ ,  $p = 3e$ -  
219  $27$ ) was highly correlated with male sterility in the six samples (Fig. 5B and C). Interestingly, GO  
220 analysis suggested that these DEGs in this module were also mainly concentrated in the GO terms  
221 associated with cytoskeleton, calcium regulation, vesicle transport, and pollen formation, which  
222 were consistent with that of DSGs. Furthermore, cytoskeletal-regulatory complex EF hand

223 (*TraesCS7A02G553100*), actin-related protein 9 (*TraesCS2D02G029100*), and microtubule-  
224 associated protein RP/EB family member 3 (*TraesCS2D02G523000*) were greater connectivity in  
225 these DEGs, suggesting they may be strongly associated with male sterility (Fig. 5D). Thus,  
226 transcriptional regulation may play a major role in cytoskeleton, calcium regulation, vesicle  
227 transport, and pollen formation, which coordinates with AS regulation to induce pollen abortion in  
228 wheat.

## 229 **Differences of diverse transcription factors (TFs) during fertility transition**

230 Transcription factors (TF) that perceive environmental signals and activate the expression of related  
231 genes play master roles in gene regulatory networks in the processes of growth and development  
232 including pollen development in plants (Wang et al., 2018). In this work, total 522 TFs with 633  
233 transcripts from Pacbio data were annotated which belonging to major 48 families (Table S8).  
234 Furthermore, 34 wheat TFs were differentially expressed (DE-TFs), and 6 genes were differentially  
235 spliced (DS-TFs) during fertility transition (S1, S2 and S3) in BS366 after removing background,  
236 respectively (Fig. 6 and Tables S9). In addition, enrichment analysis was performed on these TFs at  
237 each pollen development stage to reveal the fertility transition-related signaling. As shown in Fig. 6,  
238 bZIP family was significantly enriched in DE-TFs at dyad stage, MYB and bHLH families were  
239 enriched in DE-TFs at tetrad stage. More, there were several members of the HMG, ARF, NAC and  
240 bHLH families were differentially expressed at the tetrad stage providing evidence that these  
241 families participated in fertility transition signaling transduction.

## 242 **Function analysis of predicted lncRNAs and their targets**

243 After filtering by CPC, CNCI, CPAT and Pfam, total 53 lncRNAs (>200bp) were obtained from  
244 Pacbio data (Fig. 7A). Generally, lncRNAs regulate gene expression via *cis* (regulation of  
245 neighboring loci) or *trans*-acting mechanisms. It has been proposed that lncRNAs that are  
246 synthesized at a low level are likely to act in *cis*, whereas those accumulate at a higher level are able  
247 to act in *trans* (Kornienko et al., 2013). Identification and analysis of candidate target genes could  
248 provide insight into the functions of lncRNAs in fertility transition of PTGMS line. In this study, 38  
249 ncRNA–mRNA pairs were identified as *cis*-regulation and five were *trans*-regulation lncRNA–  
250 mRNA pairs from 53 lncRNAs after filtering (Table S10). In this five *trans*-regulation lncRNA–  
251 mRNA pairs, only PB.18919.1 have *trans*-regulation targets and other four lncRNAs have both *cis*  
252 and *trans*-regulation targets. There were 14 lncRNAs which no targets were found (Table S10). In  
253 addition, like other non-coding RNA, such as miRNA (Bai et al., 2017), the same lncRNA can  
254 regulate multiple mRNA genes, different lncRNA molecules can also be synergistic regulation of  
255 the same mRNA gene (Table S10), indicating that lncRNAs with their targets may participate in  
256 multiple regulation pathway.

257 To investigate lncRNA functions in regulation of fertility transformation, GO analysis on predicted  
258 targets was performed. As shown in Fig. 7B, the most frequent "molecular function" term was  
259 "peptide alpha-N-acetyltransferase activity", followed by "voltage-gated chloride channel

260 activity", and "microtubule motor activity" for targets. The most frequent "biological process" term  
261 was "microtubule organizing center organization", followed by "microtubule nucleation" and  
262 "spindle assembly". The results indicated that these lncRNA with their targets are involved in  
263 process of cell division and important for male sterility in wheat.

264 qPCR and heatmap analysis were also performed in lncRNAs and their corresponding targets to  
265 verified their expression patterns during different pollen development stages (Fig. 7C and D).  
266 Coordinated expression was found between most lncRNAs and their targets (Figure 7D and Table  
267 S11). PB.51.4, PB.235.1, PB.18919.1 and PB5272.1 with targets (*TraesCS5A02G351600*,  
268 *TraesCS7D02G239700*, *TraesCS5A02G390000* and *TraesCS2D02G553800* down regulated during  
269 pollen development in both fertile condition and sterile condition. However, the other target gene of  
270 PB.18919.1, *TraesCS3D02G288100* (Copper transport protein ATX1) showed up-regulated in  
271 sterile condition and down -regulated in fertile condition (Fig. 7D).

## 272 **RT-PCR validation of the DSGs**

273 In this study, four DSGs were selected randomly from pollen mother cell stage, dyad and tetrad  
274 stage in different condition to validate the accuracy of AS events using reverse transcription  
275 polymerase chain reaction (RT)-PCR (Fig. 8). The isoforms of each DSG were designed primers to  
276 amplify all predicted transcripts and cloned using Sanger sequencing (Table S1). The results as  
277 shown by a gel banding pattern in Figure 8, the size of each amplified fragment was consistent with  
278 that of predicted fragment (Fig. 8). It was also found that expression of transcript isoforms exhibits  
279 a stage-preferential pattern. For example, *TraesCS3B02G326100* (encoding a Lipid-A-disaccharide  
280 synthetase protein), which was produced by an A5'SS event, was preferentially expressed at dyad  
281 stage and tetrad stage in fertile condition and at pollen mother cell stage in sterile condition. It  
282 therefore is example of stage specific RNA isoforms.

## 283 **Effects of altered alternative splicing and gene expression on important cellular** 284 **events**

285 Hybrid sequencing showed that most of DSGs, DESs and lncRNA regulated targets could be related  
286 to chromosomal movement, process of cell division, cytoskeleton activity, cell plate formation. To  
287 further verify the results of the above analysis, the changes of microtubules, microfilaments, cell  
288 plate, chromosomes and calcium throughout critical periods of fertility transformation in pollen  
289 cells of BS366 under fertile and sterile conditions were observed (Fig. 9A). During pollen mother  
290 cell stage, in the fertile condition, the pollen mother cell took on a normal oval shape, the  
291 cytoskeleton was evenly distributed and the polar perinuclear microtubules were initially formed.  
292 However, in the sterile condition, the overall cellular morphology of pollen mother cell was  
293 wrinkled, chromatin was abnormally condensed and arranged scattered in the nuclear region, and  
294 microtubules and microfilaments appear as a radial and disordered filament and the polar  
295 microtubules were not obvious. Up to dyad stage, the fertile pollen mother cell proceeded normal  
296 cytokinesis and formed a distinct cell plate, while the division of sterile cell underwent disruption,  
297 and it's worth noting that the sterile dyad occurred absence of the cell plate. Especially during the

298 tetrad stage, the sterile tetrad happened severe malformation and the cytoskeleton was sparse and  
299 disordered compared with BS366 of fertile condition. Thus, under low temperature stress,  
300 cytoskeleton related genes and lncRNA regulated targets underwent differential alternative splicing  
301 and differential expression, which led to the abnormal meiosis of pollen mother cells, including the  
302 concentration of chromatin, the scattered distribution of cytoskeleton and the absence of cell plates.

303 It was also found that some DEGs such as *TraesCS5B02G336100* (phospholipase D delta),  
304 *TraesCS3B02G456100* (60S ribosomal protein L38) and *TraesCS5B02G160500* (calmodulin-  
305 binding receptor-like cytoplasmic kinase 3) were all up-regulated and associated with  $\text{Ca}^{2+}$   
306 distribution (Fig. 5E). Meanwhile, six DSGs encoding calmodulin related protein also was screened  
307 (Fig. 5A). Many studies have shown that the dynamic distribution of  $\text{Ca}^{2+}$  was related to pollen  
308 sterility in anthers. In order to further verify whether fertility conversion is related to  $\text{Ca}^{2+}$   
309 distribution, we used potassium antimonate to observe the distribution of  $\text{Ca}^{2+}$  in pollen and  
310 tapetum of BS366 under fertile and sterile conditions during pollen development (Fig. 9B). During  
311 the process of pollen mother cell division to tetrad, abundant  $\text{Ca}^{2+}$  precipitates were gradually  
312 accumulated on the cell surface, and the distribution of  $\text{Ca}^{2+}$  in the cytoplasm was very little, which  
313 maintained a low concentration of  $\text{Ca}^{2+}$  distribution environment. However, in the sterile condition,  
314  $\text{Ca}^{2+}$  was less on the surface of sterile pollen cells, and excessive accumulation of  $\text{Ca}^{2+}$  precipitated  
315 in cytoplasm. Therefore, we concluded that the up-regulation of calcium-related DEGs and the  
316 regulation of DSGs may lead to the abnormal function of calcium pump or calcium channel in the  
317 cells and the inability to discharge the excess  $\text{Ca}^{2+}$  out of the cells, thereby leading to the increase  
318 of  $\text{Ca}^{2+}$  concentration in the cytoplasm of sterile pollen, and ultimately leading to pollen abortion.

319 According to the above DE-TFs, DEGs and DSGs analysis, we found many DEGs annotated as  
320 polygalacturonase, glycosyl transferase family 8, ABC transporter were all significantly down-  
321 regulated. In addition, DSGs encoding MYB-related protein, ABC transporter B family member 29,  
322 pectinesterase 31 and UTP--glucose-1-phosphate uridylyltransferase were identified, which may  
323 participate in plant cell wall synthesis (Schubert et al., 2019). In plants, MYB transcription factors  
324 play a very important role in the pollen development process, involving various key steps in the  
325 pollen formation process, including the programmed cell death (PCD) of tapetum, the deposition of  
326 callose, the formation of pollen wall and the accumulation of sporopollenin. Once one of the above  
327 abnormalities occurs, it will lead to pollen abortion (Schubert et al., 2019). Previous studies have  
328 shown that the large superfamily ABC transporter proteins are involved in translocation of a broad  
329 range of substances across membranes using energy from ATP hydrolysis such as transport of  
330 sporopollen (the main component of pollen exine) so they are also required for pollen exine  
331 formation (Chang et al., 2018). The tapetum provides sucrose, proteins, lipids, and sporopollenin to  
332 support the growth and development of the pollen via its degradation and secretion, and the progress  
333 of secreting nutrients to pollen is considered to be vesicular trafficking (Liu et al., 2020). Here,  
334 vesicular trafficking related DEGs encoding Sec23/Sec24 trunk domain protein, coatomer WD

335 associated region were significantly down-regulated, and identified 15 related DSGs (Fig. 5A and  
336 E). To verify the correctness of the above analysis, TEM was used to observe the degradation of  
337 tapetum and the formation of pollen wall of BS366 in fertile and sterile conditions (Fig. 9C). During  
338 pollen mother cell stage and dyad stage, there was no obvious difference between tapetum of BS366  
339 under two conditions. Up to the tetrad stage, the fertile tapetum structure was complete and  
340 connected closely with the middle layer, whereas the tapetum of sterile condition separated from  
341 the middle layer. From the release of microspores from tetrapods to the trinuclear stage, the  
342 degradation of sterile tapetum was significantly faster than that of fertile tapetum. During this  
343 process, the tapetosome of sterile condition were also deformed and missing. Because of the  
344 degradation of tapetum in advance, the callose around the tetrad deposited abnormally, subsequently,  
345 the pollen wall of microspore released from the tetrad also deformed, which was manifested as the  
346 abnormal accumulation and uneven distribution of sporopollenin in the outer wall of pollen. Thus,  
347 we conclude that low temperature stress may induce the change of genes encoding MYB  
348 transcription factor, ABC transporter and glucose metabolism related enzymes, which may lead to  
349 the advanced degradation of tapetum and the deformity of pollen wall.

## 350 **Discussion**

### 351 **Disorder of cytoskeleton is an essential factor for the pollen abortion regulation**

352 In the past decades, many studies showed that cytoskeleton was involved in male sterility in plants.  
353 In the PTGMS rice line Peiai 64S displayed abnormal distribution in microtubules at the meiosis  
354 stage, no polar microtubules in pollen cells at the zygotene stage and rarefied perinuclear  
355 microtubules in diakinesis (Xu et al., 2001). Although Wang et al. (2018) found that disordered and  
356 asymmetrical distribution of microfilaments and microtubules in sterility pollens, nevertheless, the  
357 relationship of cytoskeleton homeostasis with pollen abortion are still unclear in PTGMS wheat line  
358 BS366 (Wang et al., 2018). Some studies showed that microtubules display dynamic instability,  
359 bouts of rapid growth followed by catastrophic shrinking, and the balance between these phases can  
360 be modulated by MAPs which generally increase polymerisation (Kawamura and Wasteneys, 2008).  
361 In our study, a MAPs-related hub gene (*TraesCS2D02G523000*), encoding the microtubule-  
362 associated protein RP/EB family member 3, was significantly up-regulated during the three stages  
363 in the sterile condition in BS366 (Fig. 5E), suggesting that the abnormal expression of MAPs-related  
364 gene may lead to the aggregation disorder of cytoskeleton. For our DSGs analysis, 17 DSGs  
365 including two genes annotated as *MORI* were found to be related to cytoskeleton (Table S6). In  
366 *Arabidopsis thaliana*, *MORI*, the homologue of *Xenopus MAP215*, promoted rapid growth and  
367 shrinkage, and suppressed the pausing of microtubules in vivo (Kawamura and Wasteneys, 2008).  
368 In this study, *MORI* with differential alternative splicing, may hinder the rapid growth of  
369 cytoskeleton abnormally in the critical stage of abortion, thereby affect the development of pollen.  
370 In addition, lncRNAs with their targets were also involved in cytoskeleton. Cell cycle regulation  
371 related gene *NSA2* (Nop seven-associated 2) could blocked the cell cycle in G1/S transition in

372 *Arabidopsis thaliana*. Here, *NSA2* (*TraesCS2B02G131900*) (target of lncRNA PB.3805.1) was up  
373 regulated in SS3 and down regulated in FS3. At the same time, our cytological observation also  
374 verified the above analysis (Fig. 9).

#### 375 **The abnormal calcium messenger system might be related to pollen abortion**

376 At present, it is known that the calcium messenger system is often in the center of signal cascade.  
377 Tian et al. (1998) described the anomalies in the distribution of calcium in anthers of PGMS rice,  
378 which displayed the failure of pollen development and pollen abortion. It was found that calcium  
379 precipitates were abundant in the middle layer and endothecium in sterile anthers, but not in the  
380 tapetum (Tian et al., 1998). In this study, calcium messenger system-related genes were excavated  
381 from DSG and DEG sets, for instance, *TraesCS6D02G176800*, which was annotated as *BON 1*;  
382 *TraesCS4A02G407100*, which was annotated as Calmodulin-binding transcription activator 2  
383 (*CAMTA*); *TraesCS6B02G230900*, which was annotated as Calmodulin binding protein-like  
384 (*CBPL*); *TraesCS5B02G160500*, which was annotated as calmodulin binding receptor like  
385 cytoplasmic kinase 3 (*CRCK3*), and *TraesCS7A02G553100*, which was annotated as cytoskeletal-  
386 regulatory complex EF hand from DEGs, suggesting that calcium messenger system related genes  
387 might play essential roles in pollen fertility transformation after encountering low temperature  
388 during meiosis (Table S6).  $Ca^{2+}$ , Calmodulin (CaM) and CaM binding proteins (CaMBP) are  
389 involved in the process of dynamic distribution of cytoskeleton that regulated by  
390 microtubule/microfilament associated proteins (MAPs). Microfilament assembly will be promoted  
391 when the concentration of  $[Ca^{2+}]_{cyt}$  is decreased, and that will be inhibited when the concentration  
392 of  $[Ca^{2+}]_{cyt}$  is increased (Helper and Callahan, 1987). In resistant cowpea, elevation of  $[Ca^{2+}]_{cyt}$  lead  
393 to deassembly of microtubule during rust fungal infection (Xu and Heath, 1998). To future  
394 investigated the mechanism of calcium in pollen fertility transformation, the potassium antimonate  
395 was used to locate  $Ca^{2+}$  in fertile and sterile anthers of BS366. It was found that pollen cell of BS366  
396 with high concentration of  $[Ca^{2+}]_{cyt}$  and low  $Ca^{2+}$  on the cell surface showed pollen abortion,  
397 suggested that the reason for pollen abortion may be due to the calcium pump or calcium channel  
398 dysfunction, which could not discharge excess  $Ca^{2+}$  outside the cell, so that the middle layer of  
399 anther was abundant in calcium precipitation (Fig. 9). The up-regulation of calcium-related DEGs  
400 and the regulation of DAS may be the reason of calcium message system dysfunction (Fig. 5A and  
401 E).

#### 402 **The abnormal vesicle trafficking is related to pollen abortion**

403 In plants, vesicle trafficking is the main way of material and information exchange between  
404 organelles and is also important for maintaining homeostasis, which is involved in many biological  
405 processes such as cell wall formation, cell secretion and environmental response (Singh et al., 2018).  
406 The membrane vesicle transport machinery includes phospholipids and integral membrane proteins,  
407 such as vesicle-associated membrane proteins (VAMPs), the latter being the major constituent of

408 soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors (SNARE) complexes  
409 (Han et al., 2017). The vesicle-associated proteins (VAPs) are type II integral ER membrane-bound  
410 proteins tethered to the membranes and have been implicated in different processes such as  
411 membrane trafficking, lipid transport and metabolism, and unfolded protein response. SNARE  
412 complexes are responsible for fusion of vesicles with the target membranes, which is involved in  
413 many processes, such as cell plate formation, ion channel regulation, plant growth and development,  
414 plant tropism response (Jena, 2011). Sec23/Sec24, the core component of the coat protein complex  
415 II (COPII), functions to transport newly synthesized proteins and lipids from the endoplasmic  
416 reticulum (ER) to the Golgi apparatus in cells for secretion (Jing et al., 2019). However, information  
417 on the role of vesicle trafficking related proteins in wheat pollen development is scanty. In the  
418 present study, the gene encoding putative vesicle-associated protein 4-2 (VAP4-2) exhibited  
419 significant AS pattern change in PTGMS line BS366. Moreover, the WGCNA analysis results  
420 showed the hub DEGs include a soluble NSF attachment protein (SNAP), a conserved oligomeric  
421 Golgi complex component (COG2), a dynamin-related protein 5A and three Sec23/Sec24 trunk  
422 domain proteins, and it may affect the process of pollen obtaining nutrients through abnormal  
423 vesicle transport. Thus, combined with cytological observation and sequencing analysis, we thought  
424 that the changes of these genes might result in the abnormal development of pollen. It was also  
425 found some DSGs encoding MYB-related protein, UTP--glucose-1-phosphate uridylyltransferase,  
426 and ABC transporter B family member 29, as well as some DEGs encoding cytochrome P450 and  
427 ABC transporter F family member 3, and these abnormal changes may affect sporopollenin  
428 synthesis and formation of the pollen wall (Chang et al., 2016).

#### 429 **Putative cytoskeleton-related and AS mediated pollen sterile network in PTGMS wheat**

430 According to the putative functions and changes in the DSGs, DEGs, DE-TFs, and lncRNA and  
431 their experimental verification in the present study, we propose an intriguing cytoskeleton related  
432 transcriptome and AS response mediated pollen sterile network for PTGMS wheat, as shown in Fig.  
433 10. This network has several functional components comprising the calcium regulation, vesicle  
434 trafficking, distribution of cytoskeleton and pollen development. The abnormal alternative splicing  
435 of these genes encoding kinesin-related protein and myosin as well as the down-regulated genes  
436 encoding dynein may hinder the post-translational modification of microtubules, which may further  
437 lead to the disordered distribution of microtubules in pollen. Therefore, the low temperature  
438 environment, as a signal, may activate or repress the transcription factors, lncRNA or splicing  
439 factors of  $Ca^{2+}$  and vesicle trafficking, and these in turn regulate the transcription or AS of  
440 downstream genes, which in turn disrupted the distribution of the cytoskeleton, thereby hindering  
441 pollen development, and ultimately leading to male sterility in BS366.

#### 442 **Conclusions**

443 In conclusion, through the mechanistic study of the pollen sterility phenotypic change in wheat

444 PTGMS line BS366 by analysis combining second- and third-generation sequencing and  
445 investigations of ultrastructural, we demonstrated that  $Ca^{2+}$  and vesicle trafficking related DEGs,  
446 DSGs and lncRNA affected the assembly and deassembly as well as post-translational modification  
447 of cytoskeleton, thereby causing disorder of cytoskeleton, eventually led to pollen sterility (Fig. 10).  
448 Our study sheds new light on the underlying mechanism of how cytoskeleton contributes to male  
449 sterility in plants and the data could be used as a benchmark for future studies of the molecular  
450 mechanisms of PTGMS in other crops.

## 451 **Materials and methods**

### 452 **Plant materials, growth conditions, and sample collection**

453 In this study, the wheat (*Triticum aestivum* L.) PTGMS line BS366 (Bai et al. 2017) and the  
454 conventional wheat line Jing411 were used as plant materials. All plants were planted in  
455 experimental fields in Beijing (China, N 39°54', E 116°18') in plastic pots in early October and  
456 managed conventionally. The treatments for wheats and anther sample collection were performed  
457 according to Bai et al. (2017). The overall anther development period was divided into six stages:  
458 S1: pollen mother cell stage; S2: dyad stage; S3: tetrad stage; S4: uninucleate stage; S5: binucleate  
459 stage and S6: trinucleate stage as defined in Browne et al. 2018 (Browne et al., 2018). Samples from  
460 fertile and sterile conditions, were collected and named FS1, FS2, FS3, FS4, FS5, FS6 and SS1,  
461 SS2, SS3, SS4, SS5, SS6 for each developmental stage, respectively. Of which S1, S2 and S3 were  
462 for sequencing, S4, S5 and S6 were for phenotypic characterization assistant.

### 463 **Phenotypic characterization at the trinucleate stage**

464 Anthers at the trinucleate stage from fertile and sterile conditions were photographed with a Nikon  
465 E995 digital camera (Nikon, Japan) mounted on a Motic K400 dissecting microscope (Preiser  
466 Scientific, Louisville, KY, USA). To further analyze pollen fertility, the mature pollen grains were  
467 stained using I<sub>2</sub>-KI staining and photographed with a microscope (Zeiss stemi 305). For SEM  
468 analysis, anthers in the trinucleate stage were collected, fixed in 2.5% glutaraldehyde, dehydrated,  
469 air dried in silica, coated with gold-platinum in a sputter coater, and finally examined by SEM  
470 (Hitachi S-3400N) (Yang et al., 2018).

### 471 **RNA Sequencing**

472 After confirming the anther development periods, three critical pollen fertility transformation stages  
473 (S1, S2 and S3) were selected for further sequencing analysis (Figure 1). Illumina RNA seq and Iso-  
474 Seq library were constructed using the method of Wang et al. (2019). The RNAs of 36 samples  
475 (three stages of two cultivars in two different conditions, three biological replicates per stage of two  
476 cultivars) were subjected to 150bp paired-end sequencing using HiSeq X Ten platform (Illumina),  
477 and then the RNAs of 18 samples from each cultivars were mixed in equal concentration and  
478 sequenced on the PacBio RS II platform. Sequencing were performed according to the

479 manufacturer's standard protocol.

#### 480 **Identification of full length transcripts**

481 Raw data obtained from Illumina sequencing were processed and filtered by Illumina pipeline  
482 (<https://www.illumina.com/>) to generated FastQ files. Raw data obtained from PacBio sequencing  
483 were processed using SMRT Pipe analysis workflow of the PacBio SMRT Analysis software suite  
484 (<https://www.pacb.com/products-and-services/analytical-software/smrt-analysis/>). Raw  
485 polymerase reads were filtered and trimmed to generate the ik, subreads and read of inserts (ROIs),  
486 requiring a minimum polymerase read length of 50 bp, a minimal read score of 0.65, a minimum  
487 subread length of 50 bp, Iso-seq pipeline with minFullPass of 0 and a minimum predicted accuracy  
488 of 0.8. Next, full-length, non-chemiric (FLNC) transcripts were determined by searching for the  
489 polyA tail signal and the 5' and 3' cDNA primers in ROIs. ICE (Iterative Clustering for Error  
490 Correction) was used to obtain consensus isoforms and full-length (FL) consensus sequences from  
491 ICE was polished using Quiver. High quality FL transcripts were classified with the criteria post-  
492 correction accuracy above 99%. Then, FL consensus sequences were mapped to reference genome  
493 using Genomic Mapping and Alignment Program (GMAP) using parameters 'cross-species -allow-  
494 close-indels0' and filtered for 99% alignment coverage and 85% alignment identity (Wu and  
495 Watanabe, 2005). Here, 5' difference was not considered when collapsing redundant transcripts.  
496 Integrity assessment for transcripts with no redundant using BUSCO (Simao et al., 2015).

#### 497 **Fusion transcript delectation**

498 The fusion candidates were detected using criteria that: a single transcript must: 1) map to 2 or more  
499 loci, 2) minimum coverage for each loci is 5% and minimum coverage in bp is at least 1 bp, and  
500 total coverage is at least 95% and 3) distance between the loci is at least 10kb.

#### 501 **AS detection and fertility related AS event identification**

502 Transcripts were validated against known reference transcript annotations with the python library  
503 MatchAnnot. In this study, AS events including ES, IR, A5'SS, A3'SS and MEX were detected and  
504 quantified using rMATS and AStalavista tool (version 3.0) (<http://astalavista.sammeth.net/>) (Foissac  
505 and Sammeth, 2007). Candidate splicing event was calculated using reads mapped to splicing  
506 junctions. Differential splicing genes under FS1, FS2, and FS3 compared with SS1, SS2, and SS3,  
507 respectively, were selected with  $FDR \leq 0.05$ .

#### 508 **Identification of lncRNA**

509 The full-length transcripts were aligned to genome of *Triticum aestivum* L. from *Ensembl Plants*  
510 database (<http://plant.ensembl.org/index.html>). Those which could not be aligned were considered  
511 as novel transcripts. The novel transcripts (>200bp) were processed to identify lncRNAs based on  
512 four computational approaches include Coding Potential Calculator (CPC) (Kong et al., 2007),  
513 Coding-Non-Coding Index (CNCI) (Sun et al., 2013), Coding Potential Assessment Tool (CPAT)

514 (Wang et al., 2013) and Pfam. The lncRNA from intersection of these four computational approaches  
515 were used further analysis.

### 516 **Identification of the transcription factors**

517 The TFs were identified based on the domains of known TFs in the plant transcription factor  
518 database PlnTFDB 3.0 (<http://plntfdb.bio.uni-potsdam.de/v3.0/>). The domains of the protein  
519 corresponding to the newly identified transcripts in our analysis and the annotated transcripts in the  
520 IWGSC RefSeq v1.0 (generated from both high-confidence genes and low confidence genes) were  
521 searched against the included domains and excluded domains of each TF in the PlnTFDB database  
522 using the hmm search function of the HMMER software, and only proteins with exactly the same  
523 included domains and not with the excluded domains were regarded as TFs. All TFs were against  
524 DEGs to confirm the fertility-related TFs.

### 525 **Cytological observation**

526 For the transmission electron microscopy (TEM) observation, anthers were fixed, embedded, and  
527 stained as described by Zhang et al. (2014). The ultrathin sections were observed and obtained with  
528 transmission electron microscope (Hitachi, H-7650, Tokyo, Japan) and an 832 charge-coupled  
529 device camera (Gatan, Abingdon, VA, USA). In pollen cells, microfilaments and microtubules were  
530 marked by tetramethylrhodamine isothiocyanate (TRITC)- phalloidin (Sigma, St. Louis, MO, USA)  
531 and anti- $\alpha$ -tubulin (mouse IgG monoclonal anti- $\alpha$ -tubulin, T-9026; Sigma), respectively. The  
532 staining procedures were the same as those described by Wang et al. (2018). For DNA staining, 4',  
533 6-diamidino-2-phenylindole (DAPI) was used for counterstaining. The DAPI staining procedures  
534 were the same as those described by Li et al. (2019). Preparations were observed and images were  
535 captured using a laser scanning confocal microscope (Nikon A1R, Tokyo, Japan).

### 536 **Data access**

537 The data reported in this article have been deposited in the National Genomics Data Center (NGDC)  
538 Genome Sequence Archive (GSA) database under the BioProject accession no PRJCA002516.  
539 (<https://bigd.big.ac.cn/>).Dr

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542

543 **Figure and table Legends**

544 **Figure 1:** Phenotypes of mature anthers and pollen of Jing411 (**A, B**) and BS366 (**C, D**) at the  
545 trinucleate stage under fertile and sterile conditions. Scale bars in anther are equivalent to 1 mm,  
546 in epidermis, ubisch bodies and pollen are equivalent to 50µm. Abbreviations: epidermis (E),  
547 ubisch bodies (Uby).

548 **Figure 2:** Summary of the direct RNA sequencing data of BS366. **A-C:** The bubble scatter plots  
549 show the relationship between the fraction of detected transcripts by the direct RNA sequencing  
550 with the transcript length and the level transcript expression. The violin-boxplots on the right  
551 show the overall distribution of the expression of transcripts. **D:** The histogram plot shows the  
552 distribution of read length of high quality reads obtained from BS366 (red), Jing411 (green) and  
553 IWGSC, respectively.

554 **Figure 3:** CIRCOS visualization of different data at the genome-wide level. The density was  
555 calculated in a 10-Mb sliding window

556 **A:** Karyotype of the wheat genome.

557 **B:** Comparison of transcript density between the IWGSC RefSeq v1.0 annotation and the PacBio  
558 data. From the upper to lower tracks: transcripts in IWGSC RefSeq v1.0, transcripts in BS366  
559 and 411, transcripts in BS366 and 411 in pollen, respectively.

560 **C–E:** Distribution of differentially expressed genes (DEGs) and differentially spliced genes (DSG)  
561 for S3, S2 and S1 of BS366 and Jing411 in between fertile and sterile conditions. From the upper  
562 to lower tracks in each part: DEGs for BS366 in between fertile and sterile conditions, DSGs for  
563 BS366 in between fertile and sterile conditions, DEGs for Jing411 in between fertile and sterile  
564 conditions, DSGs for Jing411 in between fertile and sterile conditions.

565 **F:** Distribution of transcription factors in BS366 (upper track) and Jing411 (lower track).

566 **G-K:** Identified of lncRNAs from pfam (**G**), CPC (**H**), CPAT (**I**), CNCI (**J**) and overlap of them  
567 (**K**).

568 **L:** Linkage of fusion transcripts in BS366(red) and Jing411(blue).

569 **Figure 4:** Identification and comparison analysis of sterility-related AS genes and sterility-related  
570 genes during fertility transition. **A:** The changes of gene number of DSGs and DEGs during anther  
571 development stages.

572 **B-E:** Venn diagram of DSGs (**B**) and DEGs (**C**) in three stages, genes in DSGs and DEGs (**D**), and  
573 genes in common DSGs and common DSGs &DEGs (**E**)

574 **F:** qPCR analysis of seven genes of common DSGs &DEGs

575 **Figure 5:** Analysis male sterility-related DSGs and DEGs. **A:** Heat map for pollen sterility-related  
576 DSGs. **B:** Hierarchical cluster tree showing the modules of co-expressed genes, where the lower  
577 panel shows the Modules in different colors. **C:** Module-trait correlations and corresponding *p*-  
578 values (in parentheses), where the left panel shows the module eigen genes and the right panel  
579 shows a color scale for the module trait correlations ranging from -1 to 1. **D:** Cytoscape  
580 representation of the co-expressed genes in important pathways in the red module. **E:** Heat map  
581 for male sterility-related DEGs.

582 **Figure 6:** Differentially expressed TFs (DE-TFs) and differentially spliced TFs (DS-TFs) in  
583 different anther development stages

584 **Figure 7:** Analysis of identified lncRNAs. **A:** Identified lncRNAs from Pacbio data by using CPC,  
585 CNCI, CPAT and Pfam. **B:** Go enrichment analysis of targets of lncRNAs. **C:** Heat map for male  
586 sterility-related targets of lncRNA. **D:** qPCR analysis the expression of randomly selected  
587 lncRNAs and targets.

588 **Figure 8:** Validation of full-length isoforms using Semi-quantitative RT-PCR. RT-PCR validation  
589 of AS events for three genes. Gel bands in each figure show DNA makers and PCR results in  
590 three stages under two condition. Transcript structure of each isoform is shown in right panel.  
591 Yellow boxes show exons and lines with arrows show introns. PCR primers (F, forward and R,  
592 reverse) are shown on the first isoform of each gene. The length of each full-length isoform is  
593 shown after the transcript structure.

594 **Figure 9:** Cytological observation of BS366 under different conditions. **A:** the distribution of  
595 cytoskeleton of different conditions from pollen mother stage to terad stage. **B:** the distribution of  
596  $Ca^{2+}$  of BS366 under different conditions from pollen mother stage to terad stage. **C:** the  
597 ultrastructural observation of anther, tapletum and pollen cell of BS366 under different conditions  
598 from pollen mother stage to trinucleate stage. FS1: pollen mother cell stage of fertile condition, FS2:  
599 dyad stage of fertile condition, FS3: tetrad stage of fertile condition, FS4: uninucleate stage of fertile  
600 condition, FS5: binucleate stage of fertile condition, FS6: trinucleate stage of fertile condition, SS1:  
601 pollen mother cell stage of sterile condition, SS2: dyad stage of sterile condition, SS3: tetrad stage  
602 of sterile condition, SS4: uninucleate stage of sterile condition, SS5: binucleate stage of sterile  
603 condition, SS6: trinucleate stage of sterile condition. Dd: dyad, E: epidermis, En: endothecium,  
604 PMC: pollen mother cell, T: tapetum, Td: tetrads. Bars are 4  $\mu$ m in A and 1  $\mu$ m in B and C.

605 **Figure 10:** Proposed a cytoskeleton related transcriptome and AS response mediated regulation  
606 networks and the signaling pathway involved in male sterility of PTGMS wheat line BS366.

607 Low temperature activates or repress transcription factors, lncRNA or splicing factors of  $Ca^{2+}$  and  
608 vesicle trafficking and these in turn regulate the transcription or AS of downstream genes, which  
609 in turn disrupted the distribution of the cytoskeleton, thereby hindering pollen development, and  
610 ultimately leading to male sterility in wheat PTGMS line BS366. lncRNA, long non coding

611 RNA; TF, transcription factor; SF, splicing factors; DEG, differentially expressed gene; DSG,  
612 differentially spliced gene.

613 **Figure S1:** Flowchart of sample collection and RNA-sequencing analysis. The anthers with three  
614 stages including pollen mother cell stage, dyad and tetrad stage, were taken from middle of spikes  
615 from two condition in both BS366 and Jing411. In total, 36 samples (three stages for each of the  
616 two varieties in two conditions, three biological replicates per stage) were sequenced using  
617 second-generation sequencing, and two mixed samples (the RNAs of 18 samples from each  
618 variety mixed in equal volume) were sequenced using third-generation sequencing

619 **Figure S2:** Summary of the direct RNA sequencing data of BS366 and Jing411. The bubble scatter  
620 plots show the relationship between the fraction of detected transcripts by the direct RNA  
621 sequencing with the transcript length and the level transcript expression. The violin-boxplots on  
622 the right show the overall distribution of the expression of transcripts.

623 **Table S1:** Primers used in this study

624 **Table S2:** Summary information of circular consensus sequence reads

625 **Table S3:** Summary Statistics of Full-length non-chimeric and isform

626 **Table S4:** Summary Statistics of transcript and gene loci

627 **Table S5:** Statistics of alternative splicing events in different anther development stages during  
628 fertility transition in BS366 and Jing411

629 **Table S6:** GO analysis of pollen sterile related DAS, DSGs and targets of lncRNA

630 **Table S7:** The seed setting rate of BS366 and Jing 411 in different conditions

631 **Table S8:** Identified TFs from Pacbio data

632 **Table S9:** Statistics of differentially expressed TFs (DE-TFs) and differentially spliced TFs (DS-  
633 TFs) in different anther development stages

634 **Table S10:** lncRNA and their corresponding targets

635 **Table S11:** Annotation of targets of lncRNA

636

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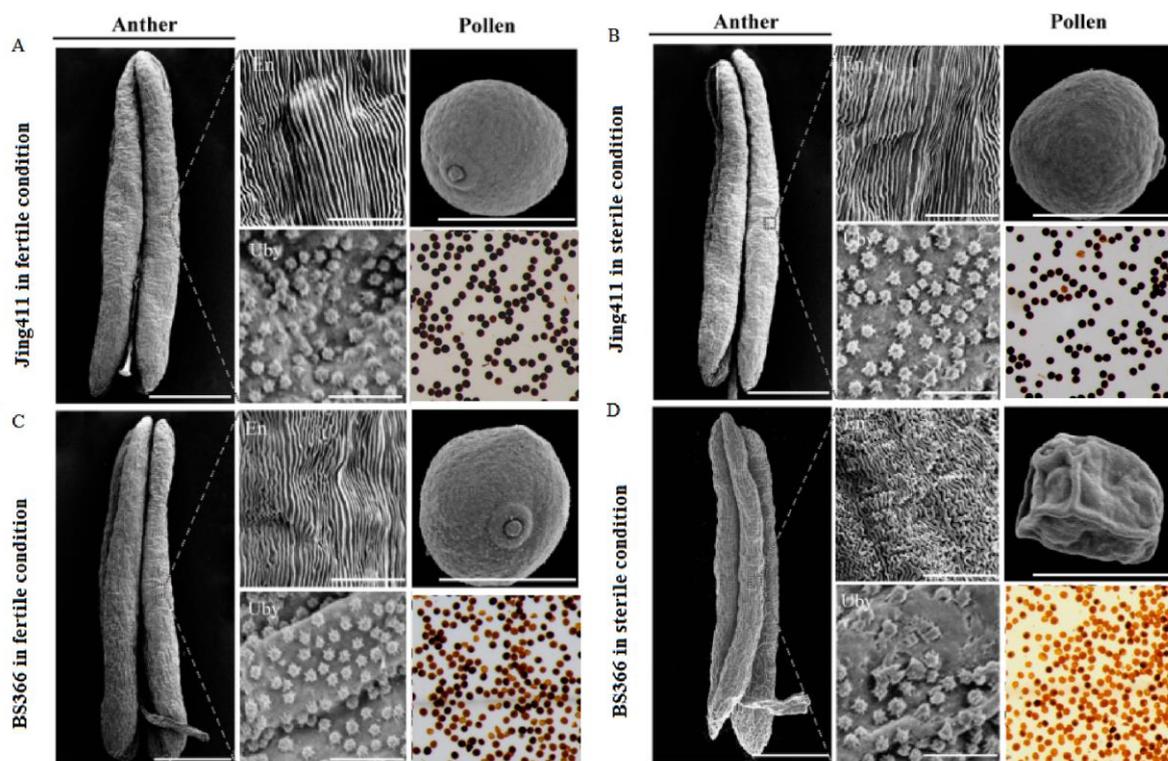
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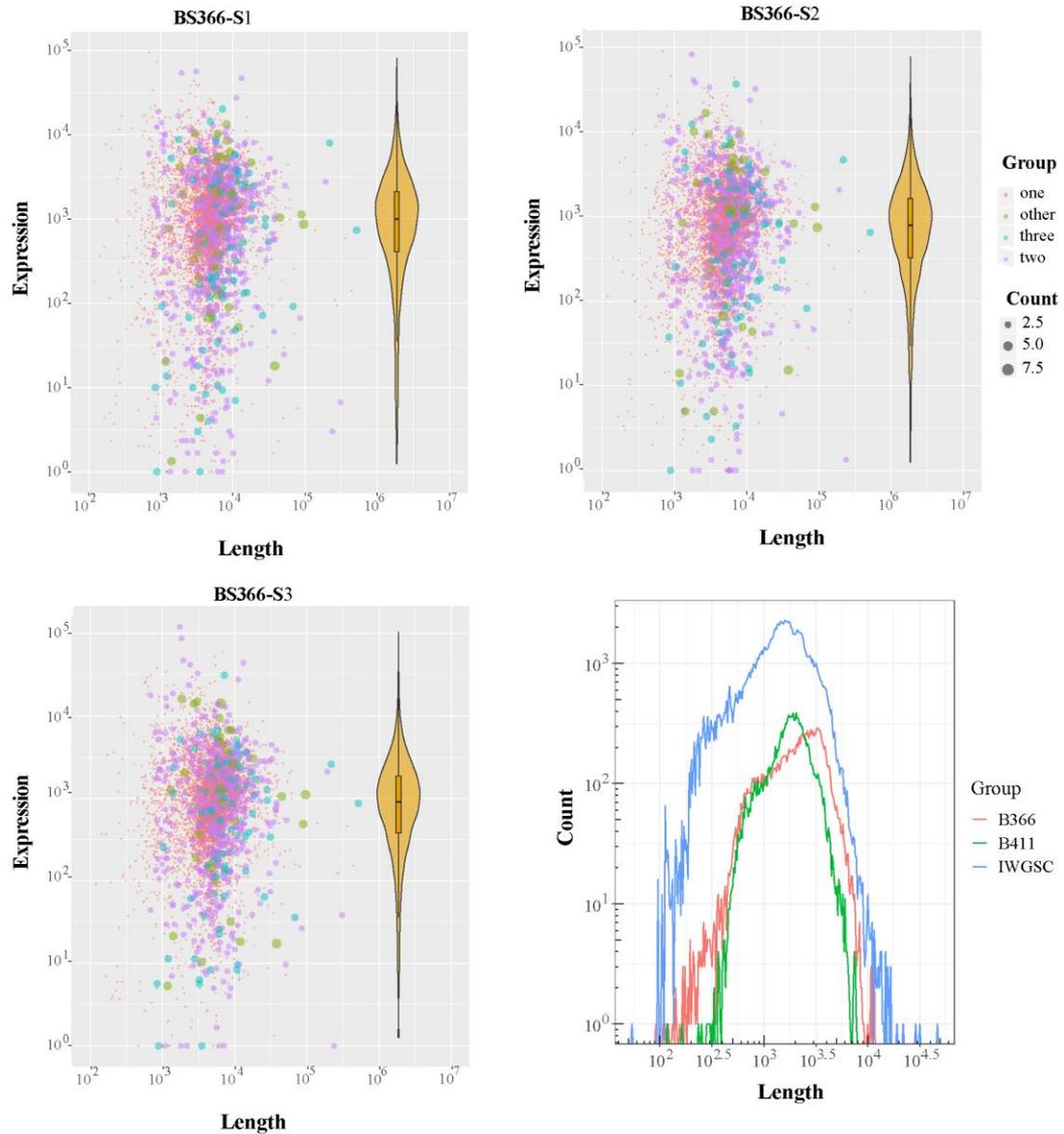
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775 **Figure 1:** Phenotypes of mature anthers and pollen of Jing411 (A, B) and BS366 (C, D) at the  
776 trinucleate stage under fertile and sterile conditions. Scale bars in anther are equivalent to 1 mm,  
777 in epidermis, Ubisch bodies and pollen are equivalent to 50 $\mu$ m. Abbreviations: epidermis (E),  
778 Ubisch bodies (Uby).

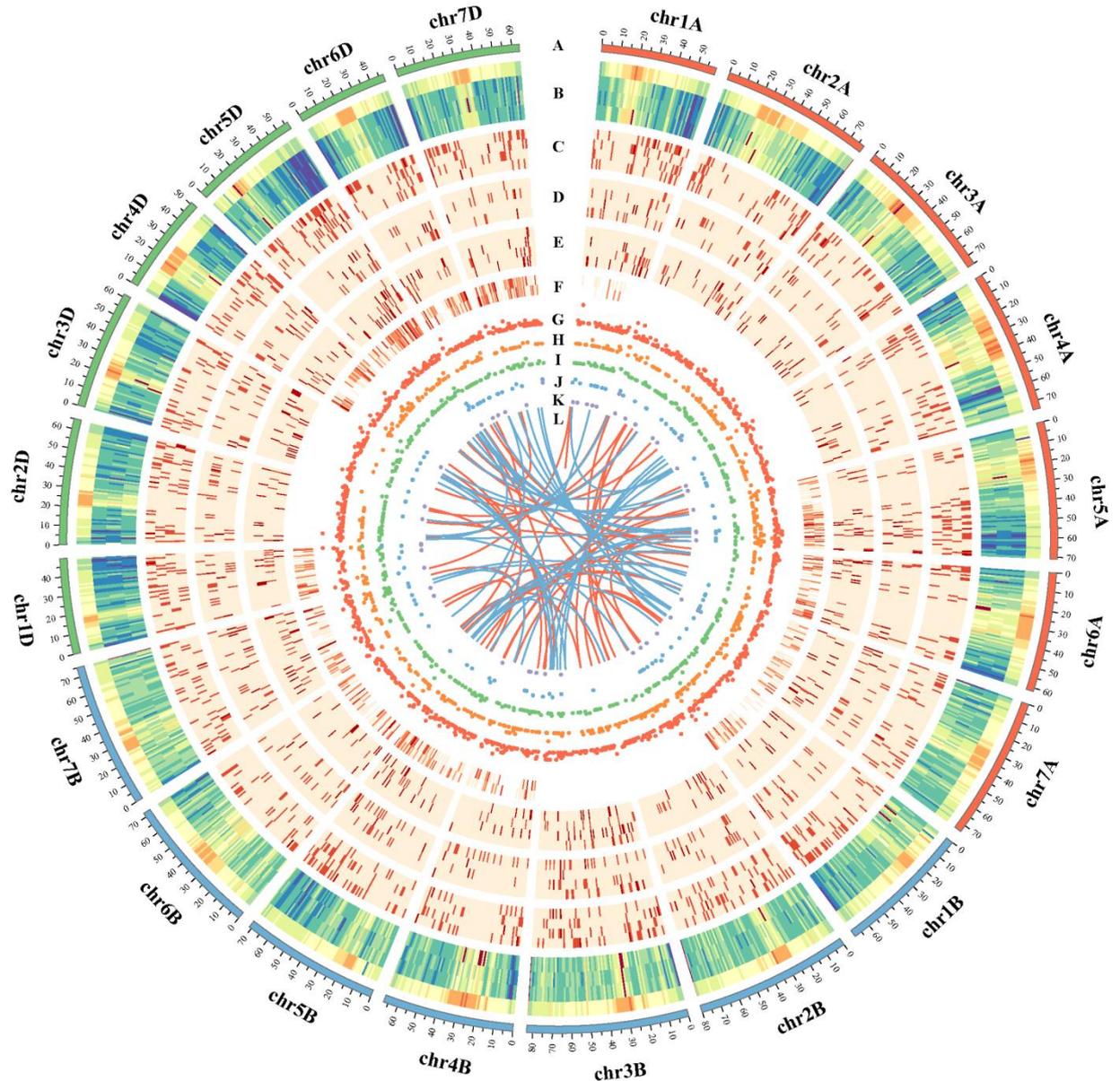
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781 **Figure 2:** Summary of the direct RNA sequencing data of BS366. **A-C:** The bubble scatter plots  
782 show the relationship between the fraction of detected transcripts by the direct RNA sequencing  
783 with the transcript length and the level transcript expression. The violin-boxplots on the right  
784 show the overall distribution of the expression of transcripts. **D:** The histogram plot shows the  
785 distribution of read length of high quality reads obtained from BS366 (red), Jing411 (green) and  
786 IWGSC, respectively.

787



788

789 **Figure 3:** CIRCOS visualization of different data at the genome-wide level. The density was  
790 calculated in a 10-Mb sliding window

791 **A:** Karyotype of the wheat genome.

792 **B:** Comparison of transcript density between the IWGSC RefSeq v1.0 annotation and the PacBio  
793 data. From the upper to lower tracks: transcripts in IWGSC RefSeq v1.0, transcripts in BS366  
794 and 411, transcripts in BS366 and 411 in pollen, respectively.

795 **C–E:** Distribution of differentially expressed genes (DEGs) and differentially spliced genes (DSG)  
796 for S3, S2 and S1 of BS366 and Jing411 in between fertile and sterile conditions. From the upper  
797 to lower tracks in each part: DEGs for BS366 in between fertile and sterile conditions, DSGs for  
798 BS366 in between fertile and sterile conditions, DEGs for Jing411 in between fertile and sterile  
799 conditions, DSGs for Jing411 in between fertile and sterile conditions.

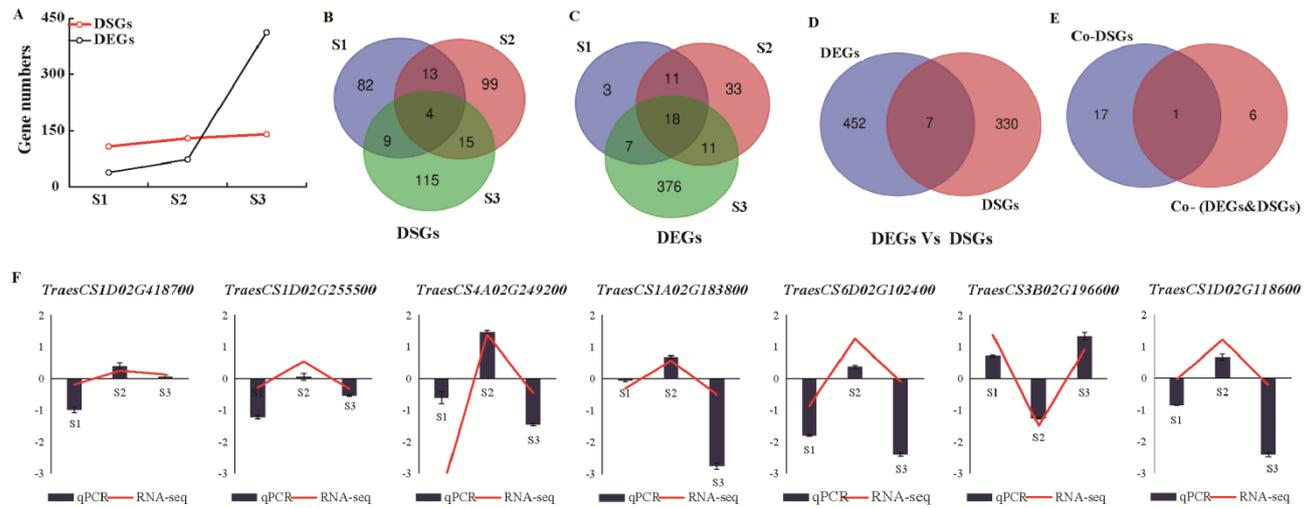
800 **F:** Distribution of transcription factors in BS366 (upper track) and Jing411 (lower track).

801 **G-K:** Identified of lncRNAs from pfam (**G**), CPC (**H**), CPAT (**I**), CNCI (**J**) and overlap of them  
802 (**K**).

803 **L:** Linkage of fusion transcripts in BS366(red) and Jing411(blue).

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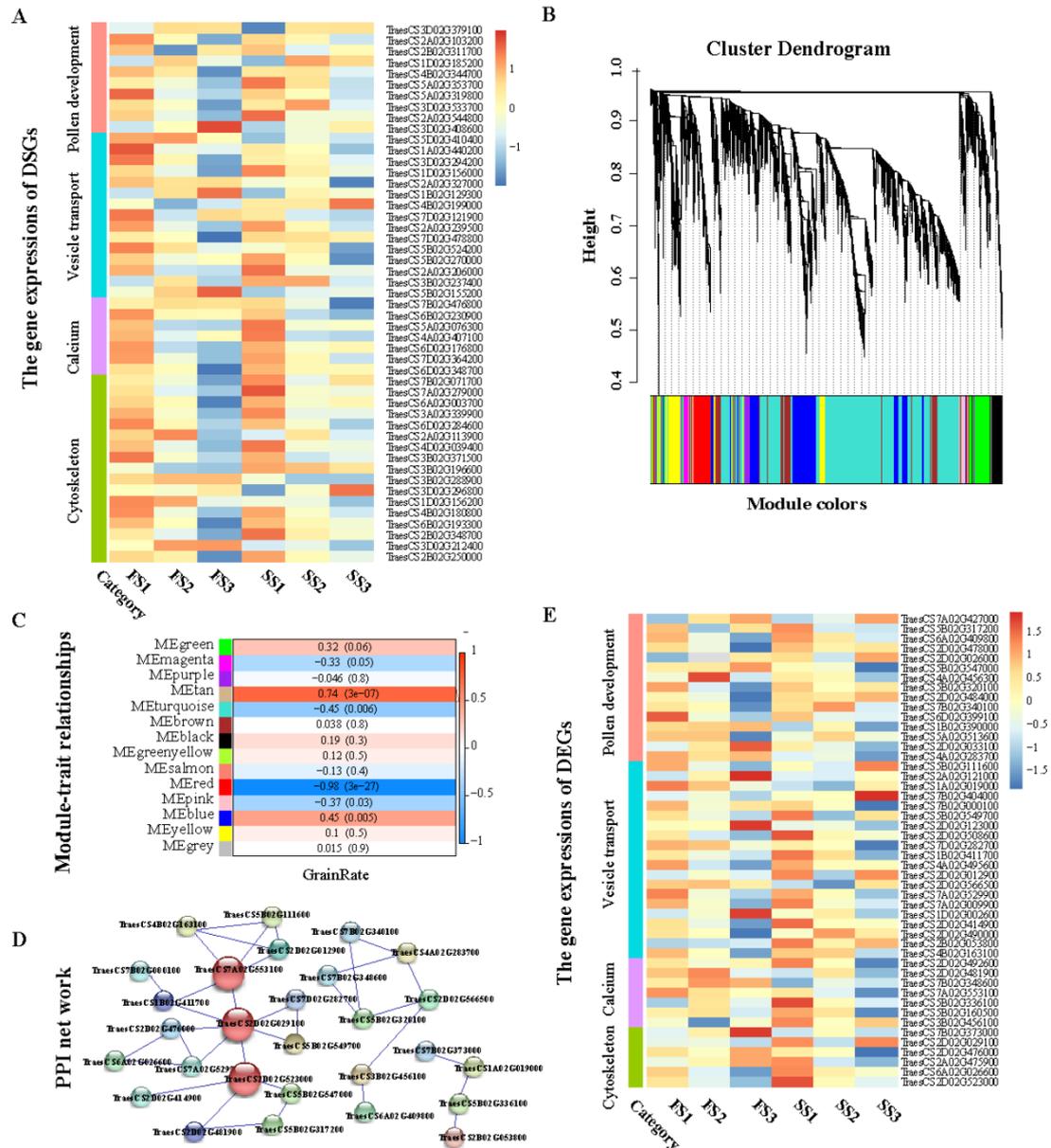
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807 **Figure 4:** Identification and comparison analysis of sterility-related AS genes and sterility-related  
 808 genes during fertility transition. **A:** The changes of gene number of DSGs and DEGs during anther  
 809 development stages.

810 **B-E:** Venn diagram of DSGs (**B**) and DEGs (**C**) in three stages, genes in DSGs and DEGs (**D**), and  
 811 genes in common DSGs and common DSGs & DEGs (**E**)

812 **F:** qPCR analysis of seven genes of common DSGs & DEGs

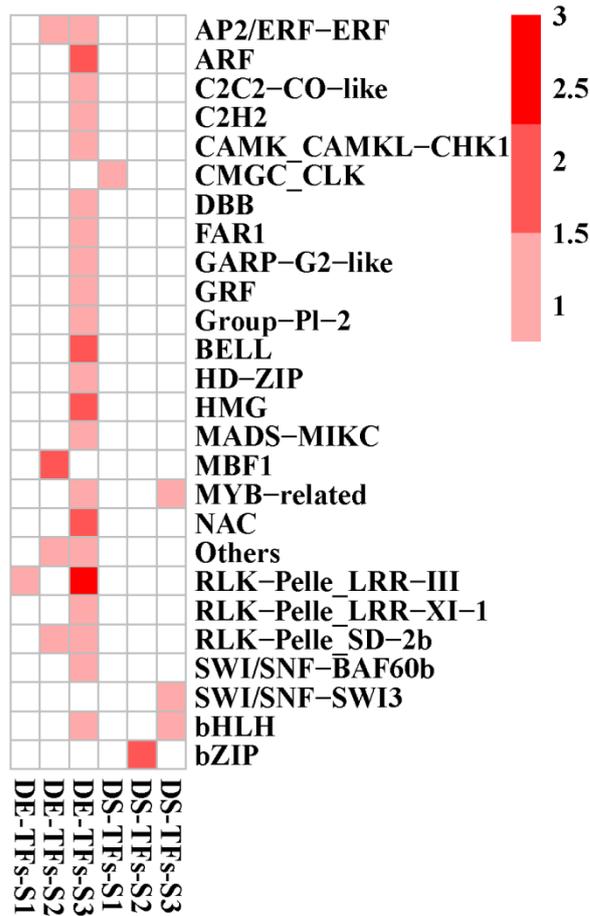
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815 **Figure 5:** Analysis male sterility-related DSGs and DEGs. **A:** Heat map for pollen sterility-related  
 816 DSGs. **B:** Hierarchical cluster tree showing the modules of co-expressed genes, where the lower  
 817 panel shows the Modules in different colors. **C:** Module-trait correlations and corresponding *p*-  
 818 values (inparentheses), where the left panel shows the module eigen genes and the right panel  
 819 shows a color scale for the module trait correlations ranging from -1 to 1. **D:** Cytoscaper  
 820 representation of the co-expressed genes in important pathways in the red module. **E:** Heat map  
 821 for male sterility-related DEGs.

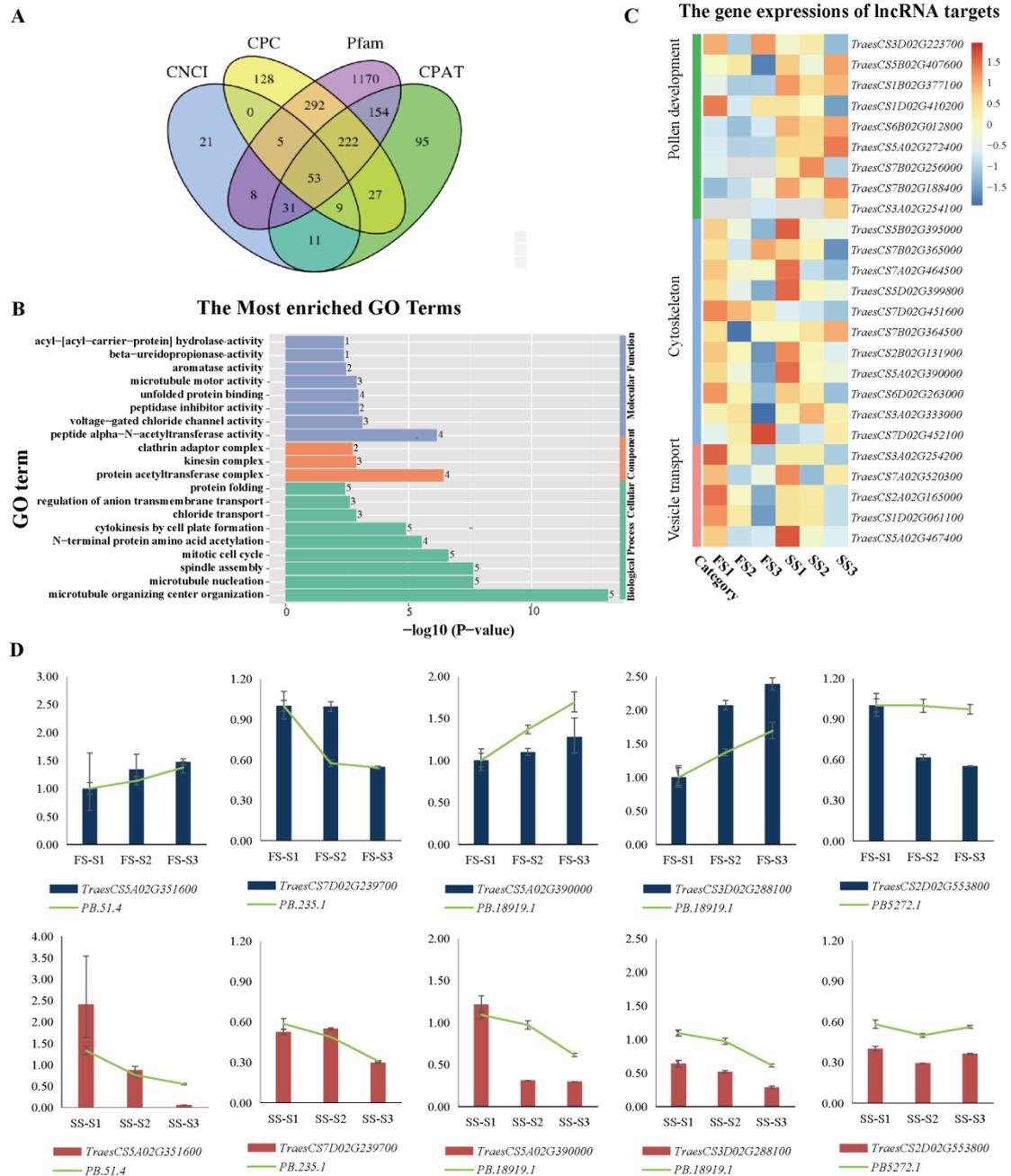
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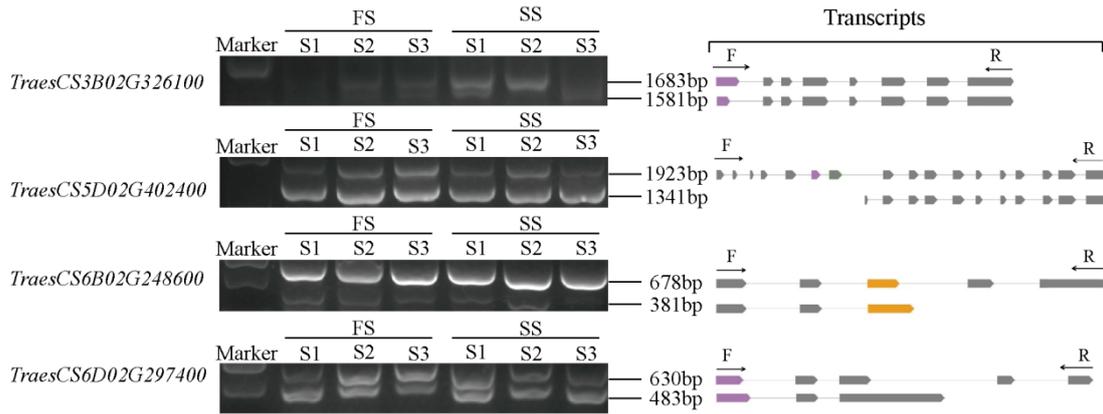
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824 **Figure 6:** Differentially expressed TFs (DE-TFs) and differentially spliced TFs (DS-TFs) in  
 825 different anther development stages

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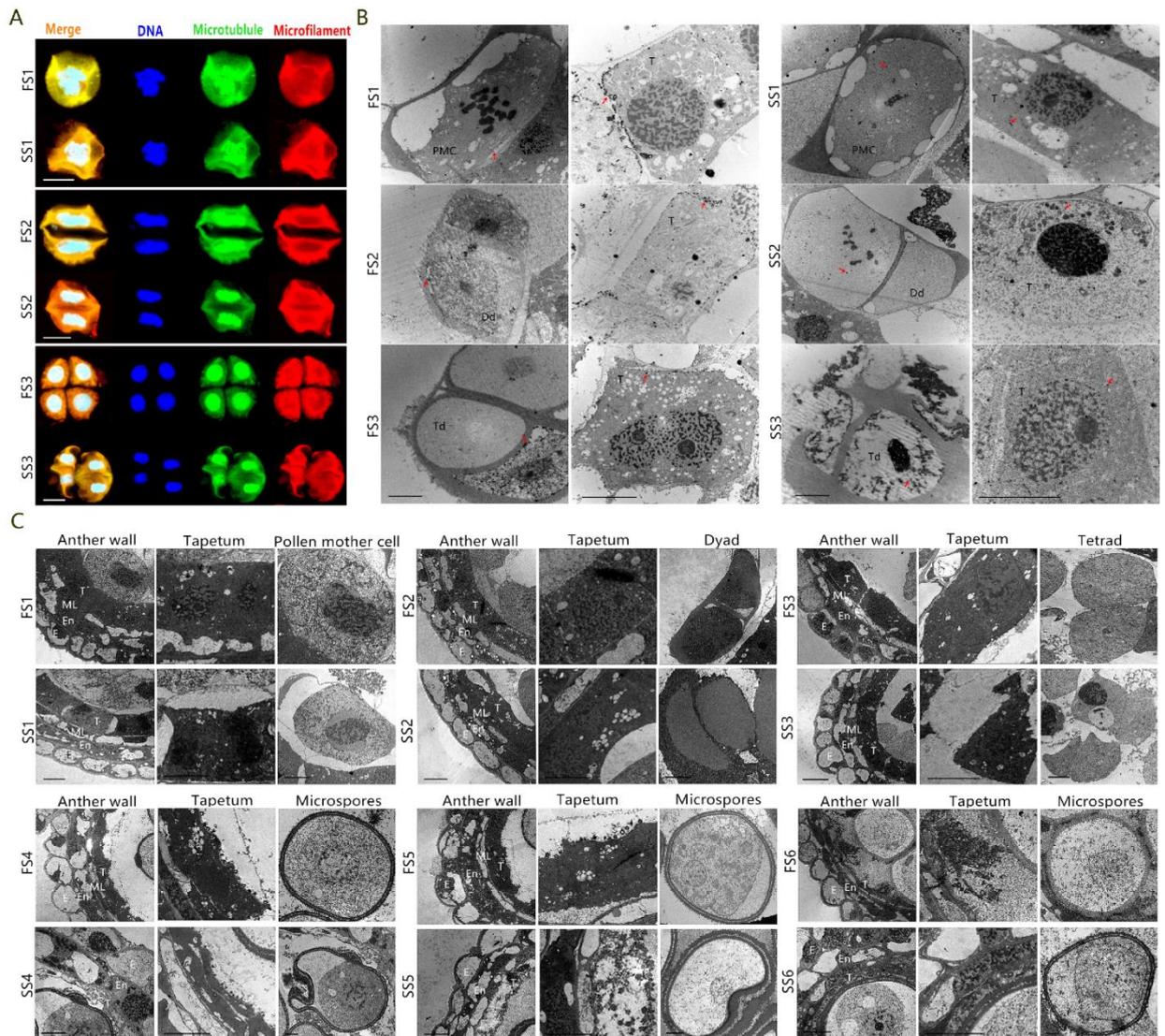
**Figure 7:** Analysis of identified lncRNAs. **A:** Identified lncRNAs from Pacbio data by using CPC, CNCI, CPAT and Pfam. **B:** Go enrichment analysis of targets of lncRNAs. **C:** Heat map for male sterility-related targets of lncRNA. **D:** qPCR analysis the expression of randomly selected lncRNAs and targets.



834

835 **Figure 8:** Validation of full-length isoforms using Semi-quantitative RT-PCR. RT-PCR validation  
836 of AS events for three genes. Gel bands in each figure show DNA markers and PCR results in  
837 three stages under two condition. Transcript structure of each isoform is shown in right panel.  
838 Yellow boxes show exons and lines with arrows show introns. PCR primers (F, forward and R,  
839 reverse) are shown on the first isoform of each gene. The length of each full-length isoform is  
840 shown after the transcript structure.

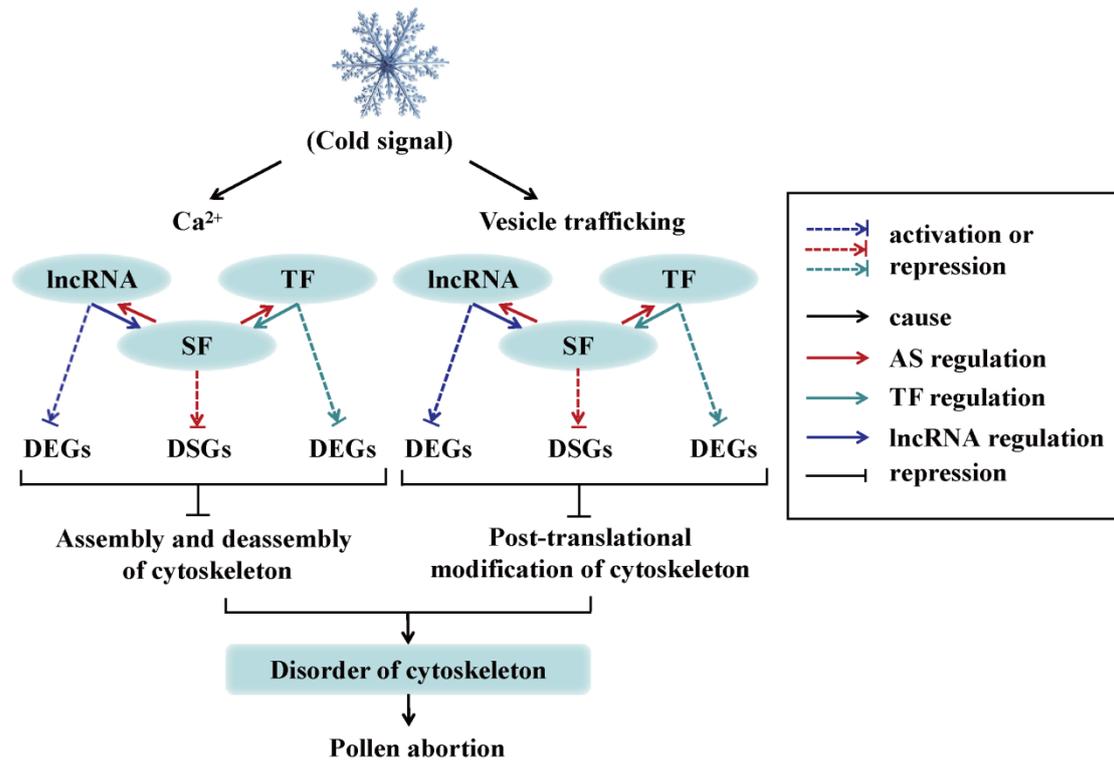
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843 **Figure 9:** Cytological observation of BS366 under different conditions. **A:** the distribution of  
 844 cytoskeleton of different conditions from pollen mother stage to terad stage. **B:** the distribution of  
 845 Ca<sup>2+</sup> of BS366 under different conditions from pollen mother stage to terad stage. **C:** the  
 846 ultrastructural observation of anther, tapletum and pollen cell of BS366 under different conditions  
 847 from pollen mother stage to trinucleate stage. FS1: pollen mother cell stage of fertile condition, FS2:  
 848 dyad stage of fertile condition, FS3: tetrad stage of fertile condition, FS4: uninucleate stage of fertile  
 849 condition, FS5: binucleate stage of fertile condition, FS6: trinucleate stage of fertile condition, SS1:  
 850 pollen mother cell stage of sterile condition, SS2: dyad stage of sterile condition, SS3: tetrad stage  
 851 of sterile condition, SS4: uninucleate stage of sterile condition, SS5: binucleate stage of sterile  
 852 condition, SS6: trinucleate stage of sterile condition. Dd: dyad, E: epidermis, En: endothecium,  
 853 PMC: pollen mother cell, T: tapetum, Td: tetrads. Bars are 4 μm in A and 1 μm in B and C.

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855

856 **Figure 10:** Proposed a cytoskeleton related transcriptome and AS response mediated regulation  
 857 networks and the signaling pathway involved in male sterility of PTGMS wheat line BS366.

858 Low temperature activates or repress transcription factors, lncRNA or splicing factors of  $Ca^{2+}$  and  
 859 vesicle trafficking and these in turn regulate the transcription or AS of downstream genes, which  
 860 in turn disrupted the distribution of the cytoskeleton, thereby hindering pollen development, and  
 861 ultimately leading to male sterility in wheat PTGMS line BS366. lncRNA, long non coding  
 862 RNA; TF, transcription factor; SF, splicing factors; DEG, differentially expressed gene; DSG,  
 863 differentially spliced gene.

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