Cytological and molecular characterizations of a novel 2A nullisomic line derived from a widely-grown wheat cultivar Zhoumai 18 conferring male sterility

Zhixin Jiao [1], **Xinxin Zhu** [1], **Huijuan Li** [1], **Zhitao Liu** [2,3], **Xinyi Huang** [2], **Nan Wu** [2], **Junhang An** [1], **Junchang Li** [1], **Jing Zhang** [1], **Yumei Jiang** [1], **Qiaoyun Li** [1], **Zengjun Qi** [2], **Jishan Niu** [Corresp. 1]

[1] Henan Agricultural University, National Centre of Engineering and Technological Research for Wheat / National Key Laboratory of Wheat and Maize Crop Science, Zhengzhou, Henan, China

[2] Nanjing Agricultural University, State key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing, Jiangsu, China

[3] Sichuan Academy of Agricultural Sciences, Crop Research Institue, Chengdu, Sichuan, China

Corresponding Author: Jishan Niu
Email address: jsniu@henau.edu.cn

A dwarf, multi-pistil and male sterile *dms* mutant was previously reported by us. However, the genetic changes in this *dms* are unclear. To examine the genetic changes, single nucleotide polymorphism (SNP) association, chromosome counting, and high-resolution chromosome fluorescence *in situ* hybridization (FISH) techniques were employed. By comparing tall plants (T) with dwarf plants (D) in the offspring of *dms* mutant plants, SNP association analysis indicated that most SNPs were on chromosome 2A. There were 3 types in offspring of *dms* plants, with 42, 41 and 40 respectively. High-resolution chromosome painting analysis demonstrated that T plants had all 42 wheat chromosomes; the medium plants (M) had 41 chromosomes, lacking one chromosome 2A; while D plants had 40 wheat chromosomes, and lacked both 2A chromosomes. These data demonstrated that *dms* resulted from a loss of chromosome 2A. We identified 23 genes on chromosome 2A which might be involved in the development of stamens or pollen grains. These results lay a solid foundation for further analysis of the molecular mechanisms of wheat male sterility. Because D plants can be used as a female parent to cross with other wheat genotypes, *dms* is a unique germplasm for any functional study of chromosome 2A and wheat breeding specifically targeting genes on 2A.

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[2] State key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China

[3] Crop Research Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China

*Corresponding Author:
Jishan Niu[1]
Email address: jsniu@henau.edu.cn

Abstract

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40 genetic changes in this *dms* are unclear. To examine the genetic changes, single nucleotide
41 polymorphism (SNP) association, chromosome counting, and high-resolution chromosome
42 fluorescence *in situ* hybridization (FISH) techniques were employed. By comparing tall plants
43 (T) with dwarf plants (D) in the offspring of *dms* mutant plants, SNP association analysis
44 indicated that most SNPs were on chromosome 2A. There were 3 types in offspring of *dms*
45 plants, with 42, 41 and 40 respectively. High-resolution chromosome painting analysis
46 demonstrated that T plants had all 42 wheat chromosomes; the medium plants (M) had 41
47 chromosomes, lacking one chromosome 2A; while D plants had 40 wheat chromosomes, and
48 lacked both 2A chromosomes. These data demonstrated that *dms* resulted from a loss of
49 chromosome 2A. We identified 23 genes on chromosome 2A which might be involved in the
50 development of stamens or pollen grains. These results lay a solid foundation for further analysis
51 of the molecular mechanisms of wheat male sterility. Because D plants can be used as a female
52 parent to cross with other wheat genotypes, *dms* is a unique germplasm for any functional study
53 of chromosome 2A and wheat breeding specifically targeting genes on 2A.
54
55 **Keywords** Wheat (*Triticum aestivum* L.), *dms*, Chromosome, Nullisomic line, Pollen
56 development
57

Introduction

59 Allohexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42, genomic constitution AABBDD) has a
60 genome from three diploid species: *Triticum urartu* Thum. (the source of the A genome),
61 *Aegilops speltoides* (Tausch) Gren. or a closely related species (the source of the B genome), and
62 *Aegilops tauschii* Coss. (the source of the D genome) (**Huang et al., 2002**). Because
63 allohexaploid wheat has high level of functional redundancy, it can host a range of diverse
64 whole-chromosome aneuploids (**Zhang et al., 2013; Zhang et al., 2017**). There are various
65 types aneuploid variations available in wheat, such as nulli-tetrasomic lines, nullisomic lines,
66 monosomic lines, ditelosomic lines, as well as chromosome fragmental deletion lines (**Endo &
67 Gill, 1996; Qi et al., 2003**). The aneuploid stocks are useful in wheat gene mapping, and
68 especially, genes can be located to a small segment using a series of chromosome fragmental
69 deletion lines (**Endo & Gill, 1996; Qi et al., 2003**). A set of homozygous chromosome deletion
70 lines were obtained in cv. Chinese Spring (**Endo & Gill, 1996**). Most of the homozygous
71 chromosome 2A short arm deletion lines are sterile; genes involved in male development are
72 located on the chromosome 2A short arm (**Endo & Gill, 1996**).
73   Presently, at least five stable genic male sterility (GMS) genes are known in bread wheat.
74 They are *ms1* on 4BS (**Wang et al., 2017**), *Ms2* on 4DS (**Xia et al., 2017**), *Ms3* on 5AS (**Maan

75 **et al., 1987**), *Ms4* on 4DS (**Maan & Kianian, 2001**) and *ms5* on 3AL (**Pallotta et al., 2019**), but
76 the molecular regulation mechanisms of the male sterility lines are largely unknown.
77   Anther and pollen developments are complex biological processes, determining wheat male
78 fertility. Pollen development starts from a single cell layer, which undergoes a series of cell
79 divisions and differentiation to form the innermost meiocytes encased within four somatic anther
80 cell layers; from inner to outer these are the tapetum, middle layer, endothecium and epidermis
81 (**Zhang & Yang, 2014**). The tapetum serves as a nutritive tissue, providing metabolites,
82 nutrients, and cell wall precursors for the development of pollen grains (**Goldberg, Beals &**
83 **Sanders, 1993**). The regulatory genes involved in pollen exine patterning are known in
84 Arabidopsis and rice (**Pearce et al., 2015; Lin et al., 2017**). For example, *AtMS1* (*Male Sterility*
85 *1*) (**Wilson et al., 2001**), *AtDRL1* (*Dihydroflavonol 4-Reductase-Like 1*) (**Tang et al., 2009**),
86 *AtLAP3* (*Less Adherent Pollen 3*) (**Dobritsa et al., 2009**), *AtLAP5* (*Less Adherent Pollen 5*)
87 (**Dobritsa et al., 2010**) in Arabidopsis, and *OsGAMYB* (*GA*, gibberellin; *MYB*, *v-myb* avian
88 myeloblastosis viral oncogene homolog) (**Aya et al., 2009**), *OsNP1* (*No Pollen 1*) (**Liu et al.,**
89 **2017**), *OsDPW2* (*Defective Pollen Wall 2*) (**Xu et al., 2017**) in rice are involved in the
90 biosynthesis of sporopollenin, which is a major constituent of exine in the outer pollen wall.
91 However, pollen developmental studies in wheat have lagged behind these plants.
92   Previously, we reported a mutant of dwarf, multi-pistil and male sterile *dms* in wheat (**Duan et**
93 **al., 2015; Zhu et al., 2016**). Pollen vigor and hybridization tests demonstrated that *dms* mutant
94 was male sterile. Male sterility and male fertility followed a segregation ratio of 1:3
95 [D:(T+M)=1:3] (**Duan et al., 2015**). However, the genetic basis of this mutant is unknown. The
96 present study is to discover the genetic basis at the cytological and molecular levels. We also
97 identified a set of genes playing potential key roles accounting for male sterility on chromosome
98 2A.

Materials & Methods

Plant materials

101   Mutant *dms* used in this study was obtained as previously described by us (**Duan et al., 2015**).
102 All plant materials were planted in our experimental field at Houwang Village, Xingyang City,
103 Henan, P. R. China (34°51′N, 113°35′E, 49 m above sea level). We have got permission prior to
104 accessing the field site by Henan Seed Company, and the administrator's name is Haolong Yang.

Morphological and histochemical observations

106   Phenotype of plants, young spikes and stamens of *dms* were observed at different
107 developmental stages (**Zadoks et al., 1974**). Developmental stages of the young spike primordia
108 were referenced as previously described (**Vahamidis et al., 2014**). The florets with anthers and
109 pistils at different developmental stages were dissected from spikes using an anatomical needle.
110 Anthers were observed using scanning electron microscope (SEM) (SU8010, Hitachi, Tokyo,
111 Japan) as previously described (**Jiao et al., 2019**). Structural and histochemical observation on

112   the anthers of T and D plants were carried out using a method previously described by **Geng et**
113   **al. (2018)**.

RNA Extraction and mRNA Sequencing

115   Young spikes of T, M and D plants at floret primordium visible stage (stage 7) (**Vahamidis et**
116   **al., 2014**) and anthers of T and D plants at 3-nucleate pollen stage (stage 12) (**Browne et al.,**
117   **2018**) were sampled for RNA extraction. All the five samples for mRNA sequencing had 3
118   biological replicates. Total RNAs of fifteen samples were extracted respectively with TRIzol®
119   reagent (TransGen Biotech, Beijing, China). RNA sequencing and basic analysis were carried
120   out in BioMarker Company (Beijing, China).

mRNA Data Analysis

122   The mRNA reads of the fifteen samples were aligned to the draft assembly (IWGSC v1.0) of
123   the wheat genome survey sequence (http://www.wheatgenome.org/) using Tophat2 tool software
124   (**Kim et al., 2013**). Reads distribution on 21 wheat chromosomes of T, M and D plants were
125   analyzed and drawn using Circos tool software (http://mkweb.bcgsc.ca/tableviewer/). Gene
126   functional annotation was carried out as previously described by us (**He et al., 2018**). Gene
127   expression levels were estimated by fragments per kilobase of transcript per million fragments
128   mapped (FPKM) (**Florea, Song & Salzberg, 2013**). Differentially expressed genes (DEGs)
129   between two sample groups were analyzed using DESeq R package (**Wang et al., 2010**). The
130   FDR < 0.01 (false discovery rate) and FC ≥ 2 were set as the thresholds for significantly DEGs.
131   Principal components were calculated using the FPKM values (**Schulze et al., 2012**). For the
132   single gene, heat maps were drawn using software Hem I v 1.0.3.7 according to the FPKM
133   values. Tissue specific expression patterns of wheat genes were analyzed using k-means cluster
134   on the BMKCloud platform (https://www.biocloud.net/). Clustering was performed in R using
135   the k-means function, where $k = 8$ within the cluster package by Euclidean distance. Wheat tissue
136   type, expression profile data sources and sample description in origin article (**Garcia-Seco et al.,**
137   **2017; Feng et al., 2017; Li et al., 2019**) were listed in **Table S1**.

Single nucleotide polymorphism (SNP) association analysis

139   Clean reads data of the two bulks (young spikes of T and D plants) were selected for SNPs
140   identification. After the reads were aligned to the Chinese Spring genome, the SNPs were called
141   using module 'HaplotypeCaller' software GATK v3.6 (**McKenna et al., 2010**). Euclidean
142   distance (ED) algorithm was used to calculate the grade of different SNPs between T and D
143   plants (**Hill et al., 2013**).

Simple sequence repeat (SSR) analysis

145   Genomic DNAs of T, M and D plants were extracted from young leaves using cetyltrimethyl
146   ammonium bromid (CTAB)-method (**Clarke, 2009**). DNA concentration was measured with a
147   DU800 Nucleic Acid Protein Analyzer (Beckman coulter, Fullerton, California, USA). SSR
148   markers distributed on wheat chromosome 2A were used to analyze the genetic polymorphisms
149   among T, M and D plants. SSR primers employed in this study included those of GWM (**Röder**
150   **et al., 1998**), WMC (**Somers, Isaac & Edwards, 2004**), BARC (**Song et al., 2005**) and GPW
151   (**Gupta et al., 2002**). PCR amplification reactions were performed using a method previously
152   described by **Zhu et al. (2019)**. PCR products were separated on an 8% PAGE gel with a
153   standard DNA molecular weight marker in the first lane of the gel. The products were run at 60
154   W for about 1.0 h. Then the gel was removed from the apparatus and stained using the silver
155   staining method (**Li et al., 2018b**). Primers used for SSR analysis are list in **Table S2**.

Cytological analysis

157   Chromosome configurations and high-resolution chromosome painting analysis of plants
158   derived from M plants at metaphase of mitosis. Chromosome samples were prepared as
159   previously described (**Du et al., 2017**). For chromosome painting, eight single strand
160   oligonucleotides were used to form a modified multiplex probes for karyotype analysis in wheat,
161   which included TAMRA (6-carboxytetramethylrhodamine)-modified oligonucleotides pAs1-1,
162   pAs1-3, pAs1-4, pAs1-6, AFA-3 and AFA-4, and two FAM (6-carboxyfluorescein)-modified
163   oligonucleotides pSc119.2-1 and $(GAA)_{10}$. Oligonucleotide probes used for FISH are list in
164   **Table S3**. The FISH procedure was tested as previously described (**Du et al., 2017**).
165   Chromosomes were visualized with microscope Olympus BX51 and pictures were captured with
166   SPOT CCD (SPOT Cooled Color Digital Camera). Image analysis was conducted using
167   Photoshop v6.0.

qRT-PCR for mRNAs

169   Anthers of T and D plants at 3-nucleate pollen stage were prepared for real-time PCR. The
170   experimental samples were consistent with the samples of RNA-seq. The qRT-PCR was
171   performed as previously described by us (**He et al., 2018**). Reverse transcription was performed
172   with 1 μg RNA using Hifair® Ⅱ 1st Strand cDNA Synthesis SuperMix (11123ES60, Yeasen,
173   Shanghai, China). Real-time PCRs of mRNAs were performed using Hieff® qPCR SYBR Green
174   Master Mix (1201ES08, Yeasen, Shanghai, China) and a CFX ConnectTM Real-Time System
175   (Bio-Rad, Hercules, CA, USA) following the production instructions. The wheat actin gene was
176   used as an internal control. All primer sequences are listed in **Table S4**. The gene expression
177   levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (**Livak & Schmittgen, 2001**). The SPSS
178   version 17.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis for the
179   qRT-PCR.
180

Results

181

Male abortion in D plants of *dms*

182

183   There were three typical phenotypes in the progeny of *dms*, tall (T), semi-dwarf (M) and dwarf
184 (D) plants (**Fig. S1**). D plants were male sterile according to the result of I_2–KI staining (**Duan et
185 al., 2015**). To discover the morphological causes of the male abortion in D, scanning electron
186 microscope was employed to observe the anther ultrastructure (**Fig. 1**). Unlike the fertile T plants,
187 the anthers of D plants were not dehiscent at the trinucleate stage, and no mature pollen grains
188 were released (**Fig. 1A and 1B**). The outer anther epidermal cells of T plants were arranged
189 neatly, whereas these of D plants were irregular (**Fig. 1C and 1D**). Moreover, compared with the
190 anthers of the T plants, the inner epidermal of D plants showed aberrant sized Ubisch bodies,
191 which suggested a structural abnormality (**Fig. 1E-1H**). The pollen grains of T plants were
192 plump and rounded; by contrast, the pollen grains of D plants were extremely shriveled and
193 atrophied (**Fig. 1I-1L**). Furthermore, we performed a histological analysis of the anthers in the
194 *dms* mutant and the wild type. In the initial stages of pollen development (**Figure S2A and S2B**),
195 there were no differences between the wild type and *dms* mutant. The young microspores were
196 normal (**Figure S2C and S2D**). However, compared with the wild type, degenerated pollen
197 grains were observed in the *dms* mutant at 3-nucleate pollen stage (**Figure S2E and S2F**).
198   The female organs in flowers of D plants developed unwell, most of the pistils were normal, a
199 few were multi-pistils (**Fig. S3**). D plants were male sterile but female fertile, it can be used as a
200 female parent to cross with other wheat genotypes. We got F_1 seeds from dwarf plants (as female
201 parent) crossed with Guomai 301 (as pollen parent) (**Fig. S4**).

The most SNPs were on chromosome 2A

202

203   In order to clarify the genetic changes in *dms*, SNP association analysis was carried out. A
204 total of 176.73 Gb data were obtained from the five super bulked samples of *dms* by mRNA
205 Sequencing: young spikes of T plants (T-YS; T1a, T1b, T1c) (**Fig. S5A**), young spikes of M
206 plants (M-YS; T2a, T2b, T2c) (**Fig. S5B**), young spikes of D plants (D-YS; T3a, T3b, T3c) (**Fig.
207 S5C**), stamens of T plants (T-ST; T4a, T4b, T4c) (**Fig. S5D**), stamens of D plants (D-ST; T5a,
208 T5b, T5c) (**Fig. S5E**). SNP association analysis indicated that the most SNPs between T and D
209 plants were on chromosome 2A (**Fig. 2**). Total 523 SNPs were significantly different between T-
210 ST and D-ST, ED>0.33, in them, 230 were on chromosome 2A, which occupied 44% of the total
211 SNPs (**Fig. S6; Table S5**). The result implied that the mutation occurred on chromosome 2A.
212 The results were consistent with the sequencing reads distribution on chromosome 2A of young
213 spikes in T, M and D plants (**Fig. 3**). About 5.24% of the total reads was identified on
214 chromosome 2A in T plants, which was about twice that of M plants (2.86%) (**Table S6**).

SSR markers on chromosome 2A were polymorphic among tall, semi-

215

dwarf and dwarf plants

216

217   In order to further verify that the genetic variation of *dms* occurred on chromosome 2A, eleven
218   plants including three typical phenotypes of tall, semi-dwarf and dwarf were randomly selected
219   for SSR genotyping. SSR markers evenly distributed across wheat A, B and D genomes were
220   used to detect the polymorphism between tall and dwarf plants of *dms*. The result showed that all
221   the primers specifically amplifying fragments on chromosomes 2A (*Xgwm312*, *Xgpw2229*,
222   *Xgwm95*, *Xgwm445*, *Xwmc794*, *Xgwm328*, *Xgwm425*, *Xbarc212* and *Xbarc122*) couldn't
223   amplify the expected products in the dwarf plants of *dms* (**Fig. 4; lane 4, 7, 9, 11**), which implied
224   that wheat chromosome 2A was missing in the dwarf plants.
225   The SSR markers were used to quantitatively amplify the DNA templates of tall, semi-dwarf
226   and dwarf plants. The amounts of the amplified products of the tall plants (**Fig. 4; lane 1, 3, 5, 8**)
227   were about double that of the semi-dwarf plants (**Fig. 4; lane 2, 6, 10**), and dwarf plants without
228   PCR products (**Fig. 4**). In another word, the SSR primers specifically amplifying fragments on
229   chromosomes 2A could be used to distinguish the T, M and D plants in the progeny of *dms*.

Chromosome 2A was absent in D plants

231   To explore the chromosome number of *dms*, 42 plants derived from M plants were
232   investigated. Among them, 17 plants had 42 chromosomes, 21 had 41 chromosomes and 4 had
233   40 chromosomes (**Table 1**). After chromosome analysis, the seedlings were planted in field. The
234   17 plants with 42 chromosomes showed regular plant height, pistil and fertility, the 21 plants
235   with 41 chromosomes showed medium plant height and regular pistil and fertility, while all the 4
236   plants with 40 chromosomes showed dwarf status, multi-pistil and complete male sterility (**Fig.
237   S7**).
238   To validate and identify the chromosome constitution of *dms*, high-resolution chromosome
239   painting was applied using eight single strand oligonucelotide probes. The result showed that all
240   21 wheat homoeologous chromosome pairs can be reliably discriminated. Total 45 plants derived
241   from M plants were clearly characterized, among them, 18 plants had 42 chromosomes and
242   regular karyotypes (**Fig. 5A and 5B**), 20 plants had 41 chromosomes and lacked one
243   chromosome 2A (**Fig. 5C and 5D**), while the remaining 7 plants had 40 chromosomes and
244   lacked a pair of chromosomes 2A (**Fig. 5E and 5F**).

Chromosome 2A carries key genes determining fertility

246   We assessed the effects of several different chromosome 2As by making six crosses (**Table 2**).
247   At F_1 generation, all six crosses segregated into two phenotypes, tall plants (T) and Semi-dwarf
248   plants (M). Combined with the data above, we knew that the genotypes of the cross Zhoumai 18
249   × M at F_1 generation were $2A_{zhoumai18}2A_{dms}$ and $2A_{zhoumai18}$, the corresponding phenotypes were T
250   and M plants. This demonstrated that the transmitting frequency of null-2A pollens was about
251   half of the 2A pollens (**Table 2**). All the T plants of the six crosses at F_1 generation didn't
252   segregate at F_2 generation, but all M plants at F_1 generation segregated into T, M and D plants at
253   F_2 generation. All M plants at F_2 also segregated into T, M and D plants at F_3 (**Table 2**). The 2As

254 played key roles during whole plant development in all the monosomic lines of 2As from
255 Taishan 4429, Jing 08-426, Yangmai 11, Yuyou 1 and Guomai 301. These data demonstrated
256 that the six different origin chromosomes 2As had the similar function as the chromosome 2A in
257 *dms*. In addition, the heterozygous genetic backgrounds of the six crosses, including
258 chromosomes 2Ds and 2Bs couldn't complement the effects of chromosome 2A. D plants were
259 used as a female parent to cross with other wheat genotypes, so as to construct inter-cultivar
260 chromosome 2A substitution lines (**Fig. S8**). At F_1 generation, all the lines had one chromosome
261 2A, they were male fertile, which indicated chromosome 2A carries key genes determining male
262 fertility.

Stamen-specifically expressed genes on chromosome 2A

264 To identify the key genes determining fertility in wheat, 5939 genes located on chromosome
265 2A (TraesCS2A01G000100 - TraesCS2A01G593900) were analyzed. Total 4613 genes on
266 chromosome 2A were identified and found to be homeologous to genes on other chromosomes
267 (**Table S7**). Deletion of these genes on chromosome 2A might not affect the phenotype of D
268 plants because of gene redundancy. Total 1326 2A-specific genes were identified (**Table S8**).
269 Tissue specific expression analysis showed that 188 genes on chromosome 2A were stamen
270 specifically expressed genes (**Fig. 6A; Table S9**). Venn diagram analysis showed that 23 genes
271 were chromosome 2A specific and stamen specifically expressed genes (**Fig. 6B; Table S9**).
272 Among them 6 genes were reported to be involved in pollen development related biological
273 processes (**Table 3**), they may be the key genes determining male fertility.

DEGs involved in pollen development related signal transduction in *dms*

276 A total of 5199 genes were significantly differentially expressed (FC ≥ 4) between stamens of
277 T and D plants. Among them 4761 DEGs expressed less, only 438 DEGs expressed highly in D-
278 ST (**Table S10**). Obviously, the expressions of most DEGs in D plants of *dms* were lower
279 because lacking of the chromosome 2A. We identified 229 putative TF DEGs between T-ST and
280 D-ST. They belonged to 47 TF families. The top three with the most number of DEGs were
281 MYB, C2H2 zinc finger protein (C2H2) and APETALA 2/ethylene-responsive element binding
282 factor (AP2/ERF) transcription factor families and most of them expressed less in D-ST (**Table
283 S11**).
284 Totally 45 DEGs were associated with auxin homeostasis, such as biosynthesis, response,
285 signaling and metabolism (**Fig. S9**). Among auxin signal transduction-related genes, homologs
286 of auxin response factor (ARF) genes and several auxin biosynthesis-related genes expressed less
287 in D-ST. Reduced expression of auxin biosynthesis and signal transduction related genes were
288 closely related to male sterility (**Su et al., 2019**). Abnormality of auxin homeostasis might be a
289 major factor leading to the phenotype of *dms*.

DEGs involved in pollen development related metabolism in *dms*

290

291   Enrichment analysis of the DEGs between T-ST and D-ST revealed that most of them were
292   involved in pollen development related metabolism such as the GO terms of 'lipid metabolism',
293   'cell wall biogenesis' and 'pollen development' (**Fig. 7**). Among lipid metabolism related genes,
294   fatty acid biosynthetic related genes expressed less and lipid catabolic process related genes
295   expressed highly in D-ST. Among cell wall biogenesis related genes, cell wall modification
296   related genes expressed less and cellulose biosynthetic process related genes expressed highly in
297   D-ST (**Fig. 7**). This indicated that fatty acid metabolic and cell wall assembly disorder might be
298   the critical factors causing male sterility in *dms*.

DEGs involved in abnormal pollen development of *dms*

299

300   The DEGs associated with various aspects of pollen development, such as pollen tube growth,
301   pollen germination, megagametogenesis, anther dehiscence and anther wall tapetum formation,
302   expressed less in D-ST (**Fig. 7**). A series of pollen development related genes were identified
303   (**Table 4**). The homologs of *AtSAC1B* and *OsGAMYB* involved in exine formation and
304   *AtAGC1.5, OsSUT1, AtRopGEF8, AtAGC1.7, AtFIM5* and etc. involved in pollen germination
305   and pollen tube growth expressed less in D-ST (**Table 4**). The qRT-PCR results showed that the
306   expression patterns of the representative genes were well consistent with that of the sequencing
307   results (**Fig. 8**).

A hypothesis of the molecular regulatory network in *dms* wheat lines

308

309   In summary, we put forward a hypothesis on the molecular regulatory network in *dms* (**Fig. 9**).
310   Several evidences support this hypothesis: (1) Chromosome 2A is absent in D plants (**Fig. 4**); (2)
311   key genes involved in pollen development are identified on chromosome 2A in *dms* (**Table 3**);
312   and (3) fatty acid biosynthetic related genes expressed less and catabolic related genes expressed
313   highly in *dms* (**Fig. 7**).

Discussion

314

315   Aneuploids are large scale mutations greatly affect cellular physiology and have significant
316   phenotypic consequences (**Zhang et al., 2017**). The typical phenotype of *dms* was significantly
317   different from its parent Zhoumai 18 at three traits, plant height, pistil number and male fertility.
318   Preliminary, this mutant was considered as a SNP mutation. Till the SNP association analysis
319   showed that a large amount of SNPs between T and D plants, we thought their chromosomes
320   should be clarified. High resolution chromosome painting is a new and efficient method for
321   distinguishing chromosomes, which has many advantages including high sensitivity and
322   resolution (**Du et al., 2017**). Using this method, we successfully distinguished the karyotypes of
323   D, M and T plants derived from M plants of mutant *dms*, and their phenotypes were
324   corresponded with their chromosome constitutions. Now it was clear that the mutant *dms* was

325 resulted from the absence of chromosome 2A. Our data showed that there were 23 2A-specific
326 genes were highly expressed in stamen. However, it can't exclude other genes on 2A involved in
327 pollen development also. In the case of wheat *Ms1* gene on 4BS, it has homoeologs on 4A and
328 4D. Mutant *ms1* is responsible for male sterile phenotype due to that homoeologous *Ms1* on 4A
329 and 4D were not expressed (due to methylation) (**Wang et al., 2017**). Some genes in the list of
330 23 2A-specific genes could be candidates of key genes responsible for the male sterility, but not
331 exclude other possibilities. The molecular regulatory network can be elucidated well till the key
332 gene/genes have been investigated.
333   Endo and Gill reported a set of nullisomic, monosomic, trisomic and tetrasomic lines from
334 Chinese Spring (CS) (**Endo & Gill, 1996**). Among them, the 2A nullisomic line could not be
335 maintained for they were sterile in both sexes. However, a stable self-fertile 2A nullisomic line
336 was obtained from common wheat 'Abbondanza'. Although the pistils and stamens of the 2A
337 nullisomic line were fertile, its female flower organ developed unwell (**Xue et al., 1991**).
338 Different genetic backgrounds lead to nullisomic lines of 'Abbondanza' are greater vigor and
339 fertility than those of Chinese spring (**Ji, Xue & Wang, 1992**). Our 2A nullisomic line *dms* is
340 multi-pistil and male sterile, that is different from the other 2A nullisomic lines from
341 'Abbondanza' and CS (**Endo & Gill, 1996**). A set of chromosome deletion stocks from CS are
342 reported, five out of the nine chromosome 2AS deletion lines have irregular meioses with many
343 univalents at metaphase I, and they are highly sterile in both sexes, the seed settings of the four
344 chromosome 2AL deletion lines are reduced after selfing. In our study, the meiosis of the pollen
345 development is normal in *dms* mutant, which is different from the 2A nullisomic lines from CS
346 (**Endo & Gill, 1996**).
347   MYB TFs play pivotal roles in plant development and stress response (**Verma, 2019; Zheng
348 et al., 2018**). Many MYB TFs have been functionally characterized in pollen development of
349 *Arabidopsis* and rice, such as *AtMYB32* (**Preston et al., 2004**), *OsGAMYB* (**Aya et al., 2009**) and
350 *OsTDF1* (**Cai et al., 2015**). In *dms*, three homologs of *OsGAMYB*, *TraesCS7A01G458700*,
351 *TraesCS7B01G357900* and *TraesCS7D01G446700*, expressed less. In rice, GAMYB is essential
352 for pollen development, and it directly binds to promoter of *β-KETOACYL REDUCTASE* (*KAR*),
353 a key enzyme essential for fatty acid synthesis. GAMYB activates the expression of *KAR* and
354 other genes involved in the synthesis of sporopollenin, and are involved in the formation of exine
355 and Ubisch bodies (**Aya et al., 2009**). Reduced expression of *GAMYB* might lead to the shriveled
356 and atrophied pollen grains of D plants. Change in the levels of *AtMYB32* expression influence
357 pollen development by affecting the composition of the pollen wall in *Arabidopsis* (**Preston et
358 al., 2004**). Knocking out *OsTDF1* impaired tapetum development, leading to male sterility in
359 rice (**Cai et al., 2015**). Similarly, some MYB TF genes expressed less in *dms*, their functions
360 involved in exine formation in wheat needs further research.
361   Pollen germination is critical for double fertilization in angiosperms (**Zhang, He &
362 McCormick, 2009**). The polarity of tip-growing pollen tubes is maintained through dynamic
363 association of active Rho GTPases in plants (ROP-GTP) (**Li et al., 2018a**). Guanine nucleotide
364 exchange factors for ROPs (RopGEFs) catalyze the activation of ROPs and thereby affect

365   spatiotemporal ROP signaling (**Gu et al., 2006**). Deletion *RopGEF* mutant has the defects in
366   pollen tube polar growth (**Gu et al., 2006**). AGC1.5 and AGC1.7 kinases phosphorylate
367   RopGEFs to control pollen tube growth. Loss functions of AGC1.5 and AGC1.7 in pollen tubes
368   results in meandering and depolarized growth morphology (**Zhang et al., 2009**). In summary, the
369   AGC1.5/1.7-RopGEFs-ROPs signaling pathway is involved in pollen germination and tip growth
370   in Arabodopsis (**Li et al., 2018a; Huang et al., 2019**). In our research, the homologs of
371   *AtAGC1.5*, *AtAGC1.7* and *AtRopGEF8* involved in ROP signaling expressed less in *dms*.
372   Similarly, all the DEGs associated with various aspects of pollen germination related biological
373   processes, such as pollen tube growth, regulation of pollen tube growth, pollen tube development
374   and pollination, expressed less in *dms*. These data demonstrated that pollen germination and
375   pollen tube growth might be suppressed in *dms*. Further experiments should be carried out to test
376   the hypothesis.
377   In our study, all the six chromosome 2As from different cultivars and the genetic backgrounds
378   of the six heterozygotes of the crosses had similar functions. These indicated that the interactions
379   among 2A, 2B and 2D were similar to that in *dms*. Wheat chromosome 2A has many important
380   agronomy trait genes such as powdery mildew resistance gene *PmLK906* (**Niu et al., 2008**),
381   photoperiod response locus *Ppd-A1* (**Beales et al., 2007**), reduced height (*Rht*) genes *Rht7*
382   (**Worland, Law & Shakoor, 1980**). Some quantitative trait loci (QTL) for thousand grain
383   weight (**Quan, Sean & Sparkes, 2015**), floret primordia development (**Guo et al., 2017**) and
384   grain protein-content (**Groos et al., 2003**) are also mapped on chromosome 2A. Because the 2A
385   nullisomic line *dms* derived from Zhoumai 18 is male sterile but female fertile, it can be used as
386   a female parent to cross with other wheat genotypes, so as to construct inter-cultivar
387   chromosome 2A substitution lines. Backcross to *dms* can construct series lines with highly
388   similar genetic backgrounds but different 2As (**Fig. 10**), which can be used to evaluate the
389   functions of different 2As and wheat design breeding targeting 2As.

390   Conclusions

391   We characterized a dwarf, multi-pistil and male sterile mutant *dms* derived from a widely-grown
392   wheat cultivar Zhoumai 18. Cytological and molecular analyses demonstrated that mutant *dms*
393   was a novel wheat 2A nullisomic line. Twenty-three stamen and pollen development related
394   genes are identified specifically on chromosome 2A. We put forward a hypothesis on the
395   molecular regulatory network of the sterility trait in *dms*. *dms* is a unique germplasm for gene
396   functional study about chromosome 2A and wheat design breeding targeting 2A.
397

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406

Competing Interests

408 The authors declare there are no competing interests.

409

Author Contributions

411 Zhixin Jiao performed the experiments, analyzed the data, contributed
412 reagents/materials/analysis tools, prepared figures and/or tables, drafted the manuscript.
413 Xinxin Zhu, Huijuan Li, Junhang An, Junchang Li and Jing Zhang helped with sowing, sample
414 preparation and data processing.
415 Zhitao Liu, Xinyi Huang, Nan Wu and Zengjun Qi contributed to the high-resolution
416 chromosome fluorescence in situ hybridization experiments.
417 Yumei Jiang and Qiaoyun Li contributed to the field experiments and maintenance of the wheat
418 accessions.
419 Jishan Niu found the mutant, conceived and designed the experiments, authored or reviewed
420 drafts of the paper, approved the final draft.

421

Data Availability

423 The sequences have been deposited at the Sequence Read Archive of the National Center for
424 Biotechnology under BioProject numbers PRJNA612424.

425

Supplemental Information

427 Supplemental information for this article can be found online at http://xxx.

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669

Figure 1

The scanning electron micrographs of the anthers, anther epidermis, anther inner surface, and pollen grains in the T and D plants of *dms* at the trinucleate stage.

Uby: Ubisch bodies. Scale bars represent 1 mm in anthers, 500 μm in pollen, 50 μm on the epidermis surface and 10 μm on the inner surface.

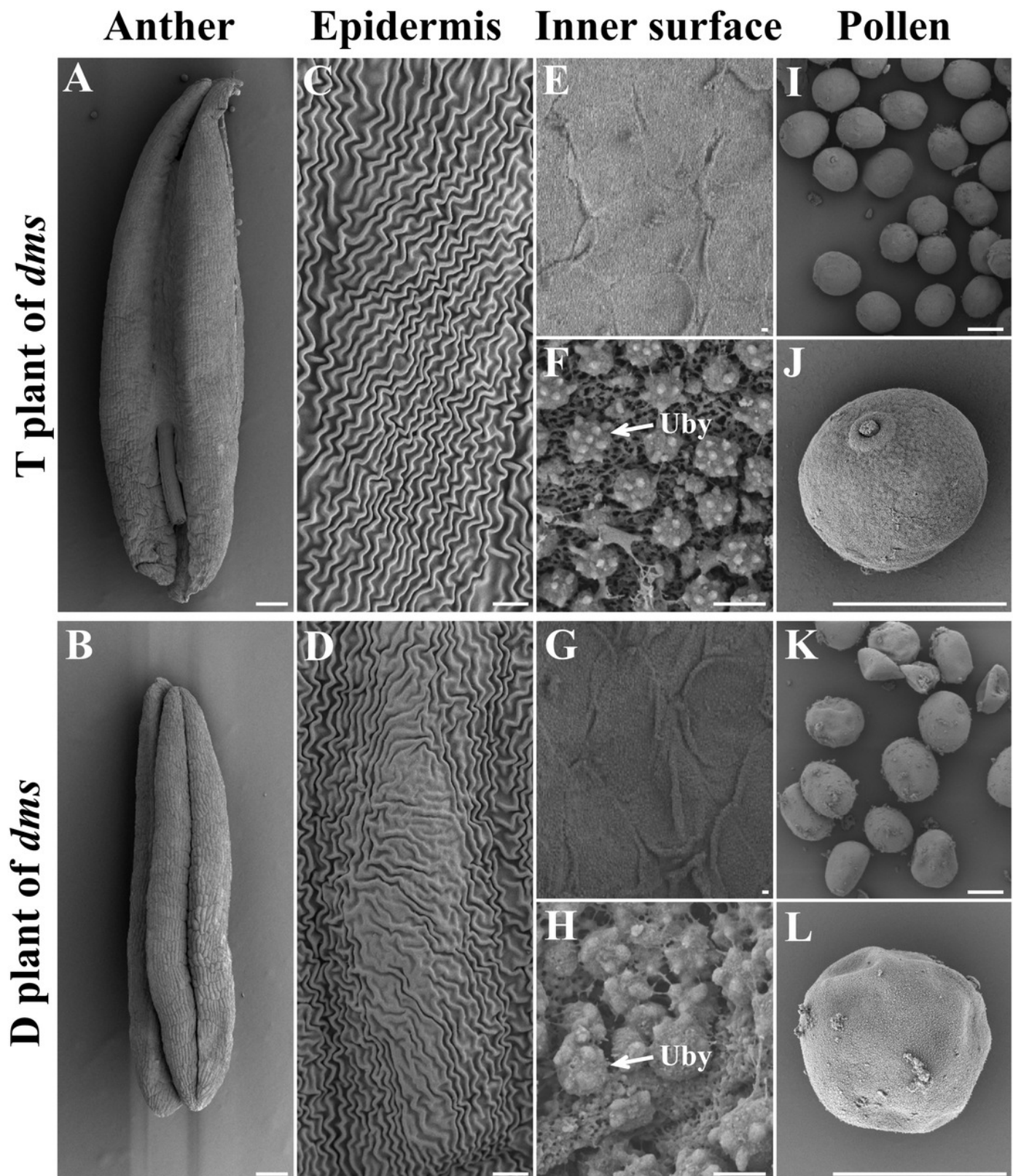



Figure 2

Chromosome distribution of the SNPs between stamens of T and D plants.

ED is Euclidean distance. Each dot represents a SNP identified between T and D. The serial numbers of wheat chromosomes are indicated on the X-axis. The red dotted line indicated significance threshold (ED = 0.33). The wavy line above the significance threshold indicated variant section.

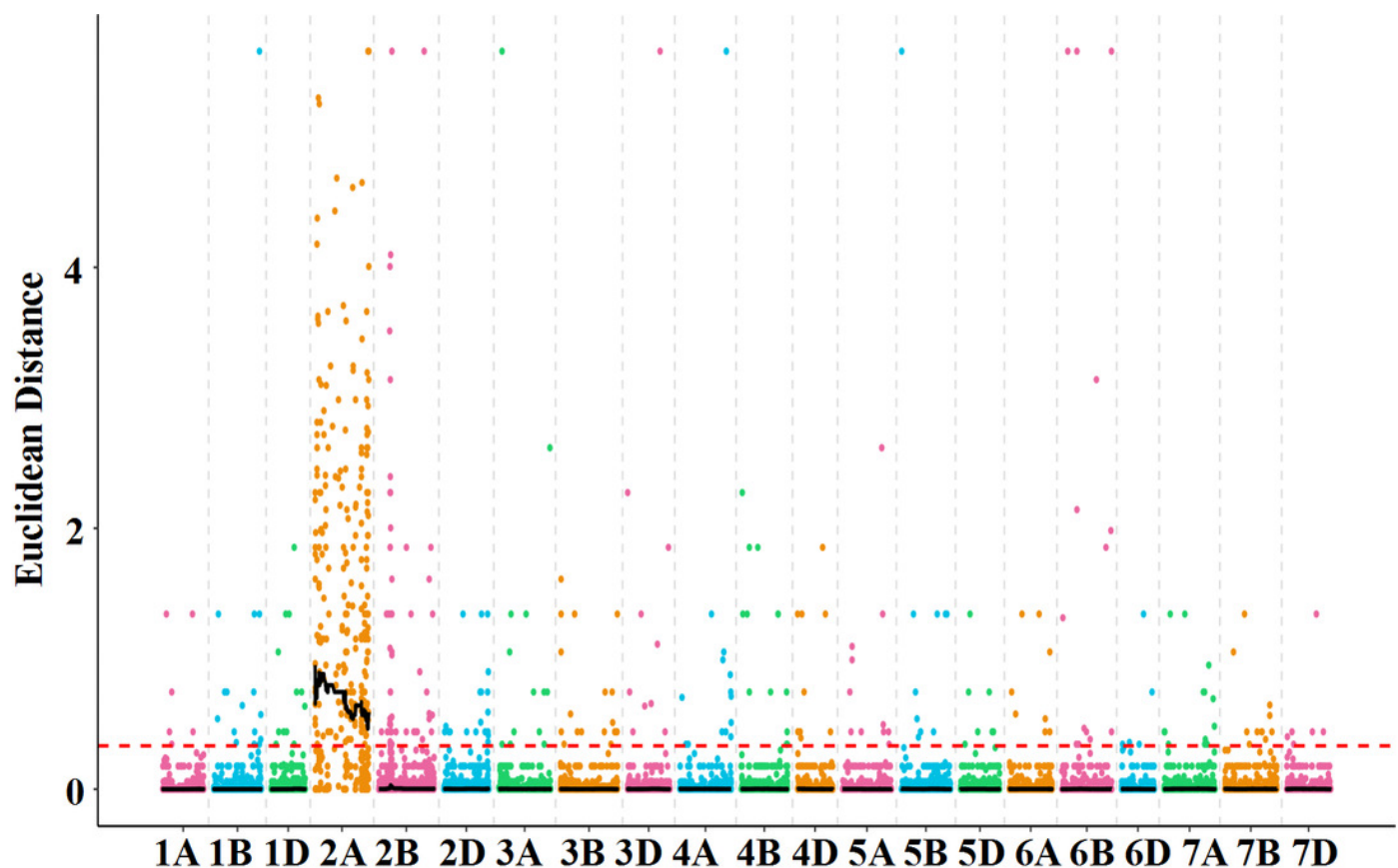



Figure 3

Circular diagram of the reads distributed on the 21 wheat chromosomes of T, M and D plants.

The tracks toward the center of the circle display **(a)** chromosome name and size (Mb); **(b)** read segments density of D plant; **(c)** read segments density of M plant; **(d)** read segments density of T plant. The triangles highlight reads distributions on chromosome 2A.

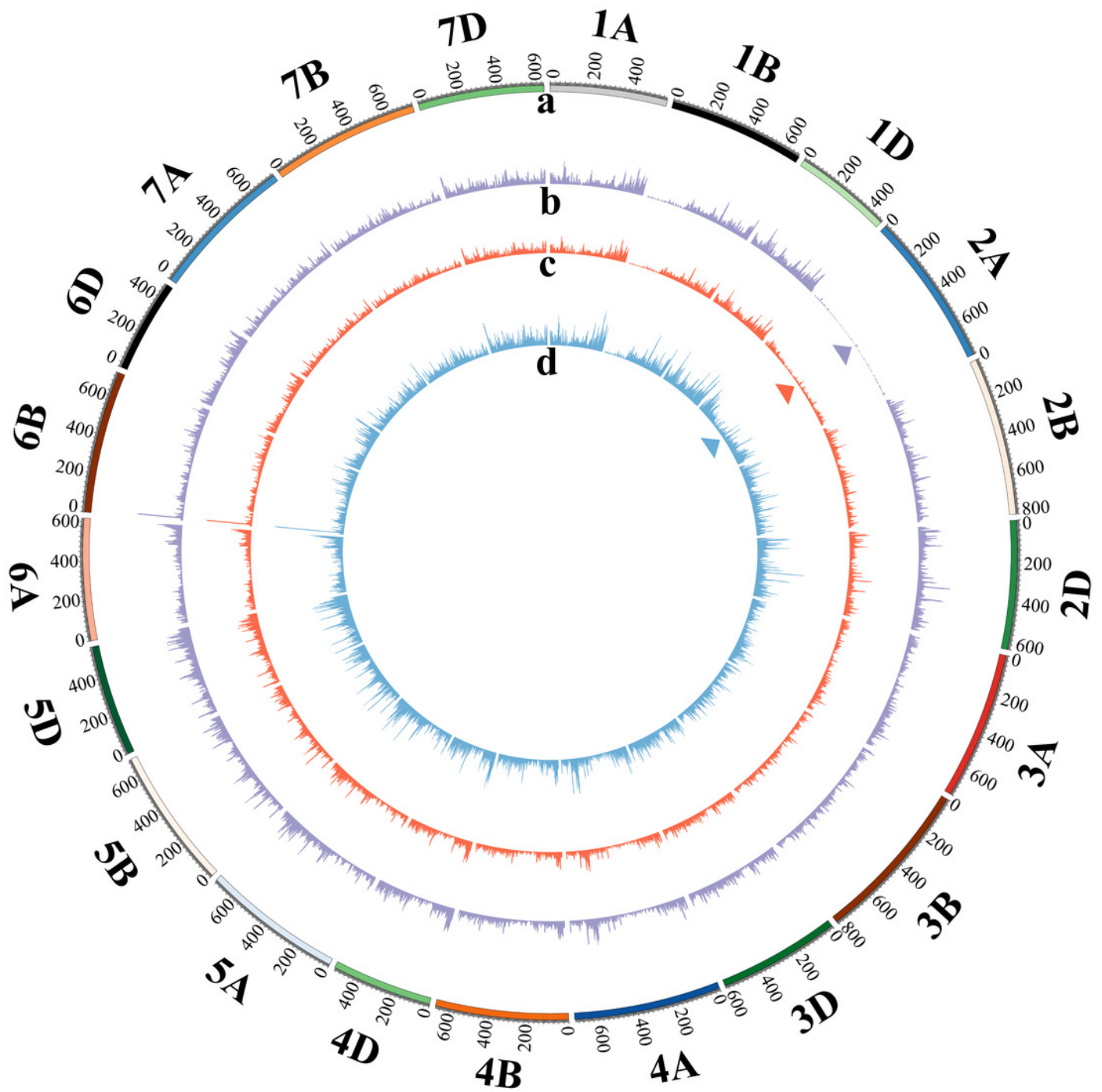



Figure 4

SSR markers on chromosome 2A were polymorphic.

M, DNA molecule weight markers; lanes 1-11, the offspring of *dms* mutant. lane 1, 3, 5 and 8 were tall plants; lane 2, 6 and 10 were semi-dwarf plants; lane 4, 7, 9 and 11 were dwarf plants. The arrows indicate the SSR products.

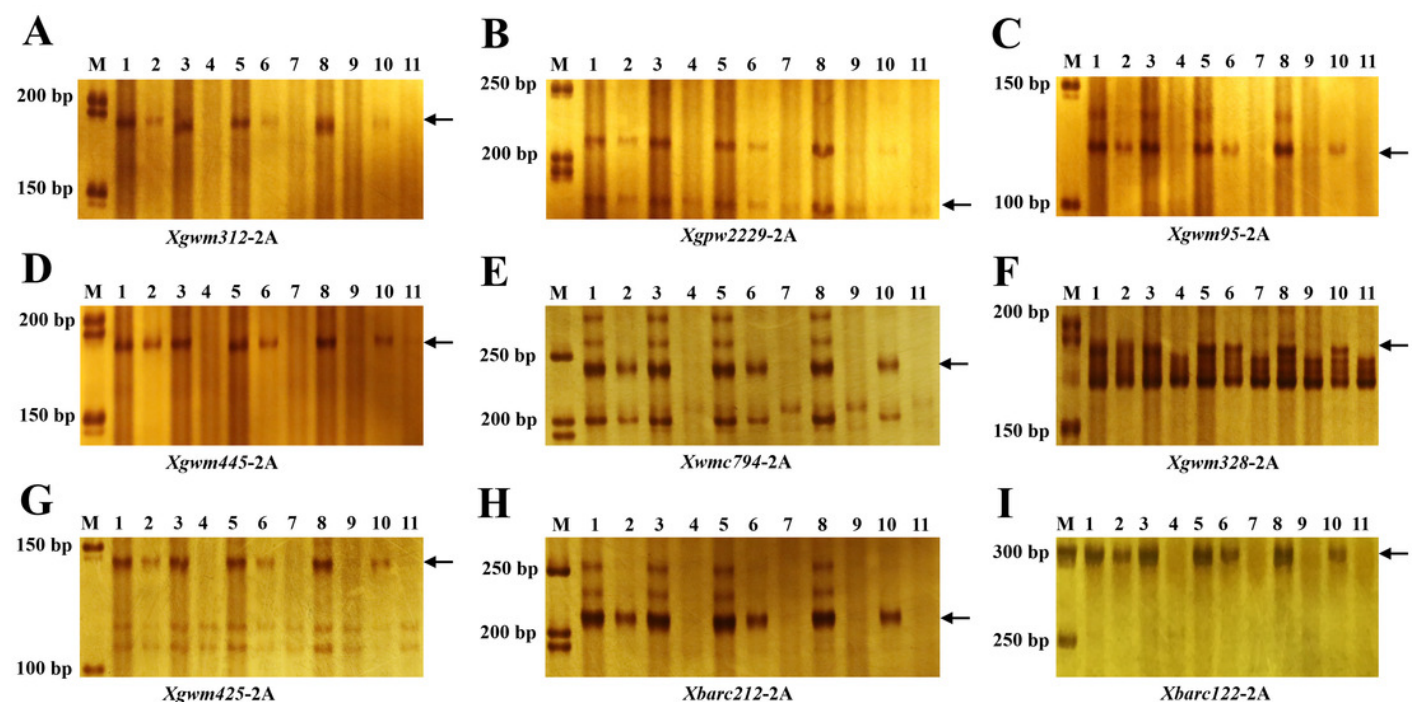



Figure 5

High-resolution chromosome painting analysis of the plants derived from M plants at metaphase of mitosis.

A and B, one karyotype of T plant, which had 42 normal chromosomes. **C and D,** one karyotype of M plant, which had 41 chromosomes, lacked one chromosome 2A. **E and F,** one karyotype of D plant, which had 40 chromosomes, lacked a pair of chromosomes 2A. ChrA, ChrB and ChrD in Figure **B**, **D** and **F** represent subgenome A, B and D. Blue color, chromosomes counterstained with DAPI; green, signals of oligos pSc119.2-1, $(GAA)_{10}$; red, signals of oligos AFA-3, AFA-4, pAs1-1, pAs1-3, pAs1-4 and pAs1-6. Scale bar = 10 μm.

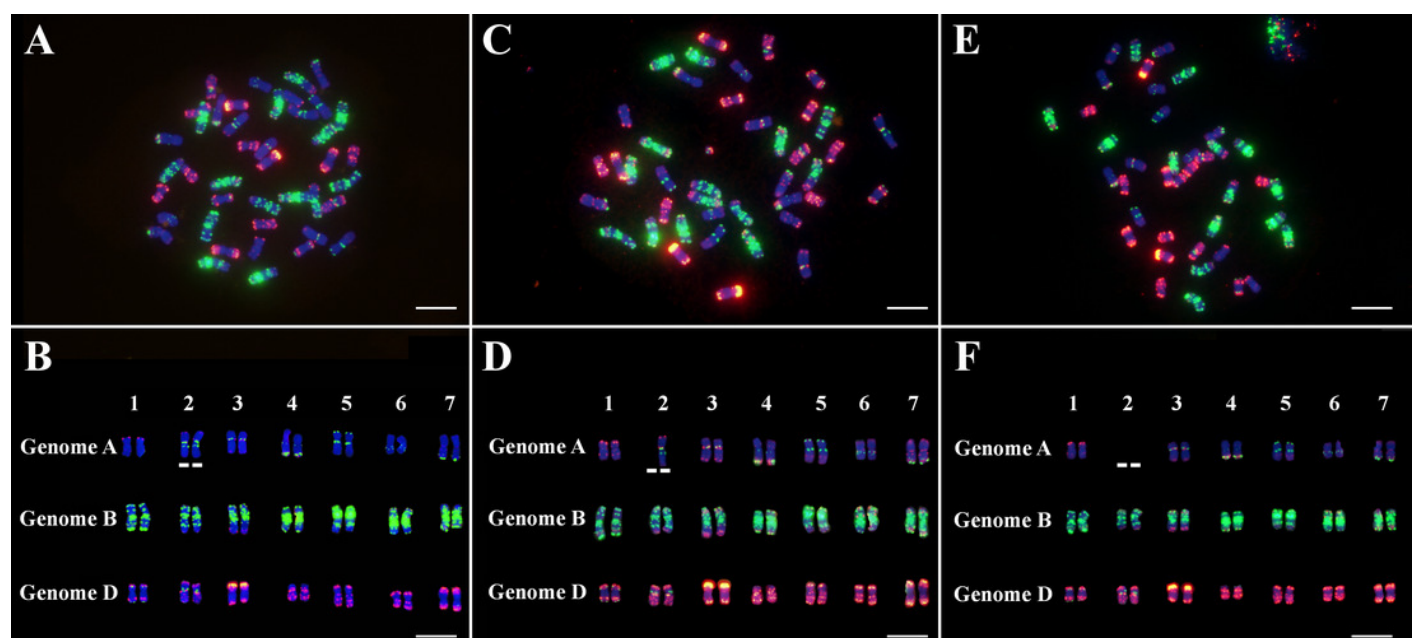



Figure 6

Chromosome 2A carries key genes involved in pollen development.

(A) The expression profiles of the stamen specifically expressed genes on 2A. **(B)** Venn diagram showed the chromosome 2A and stamen specifically expressed genes.

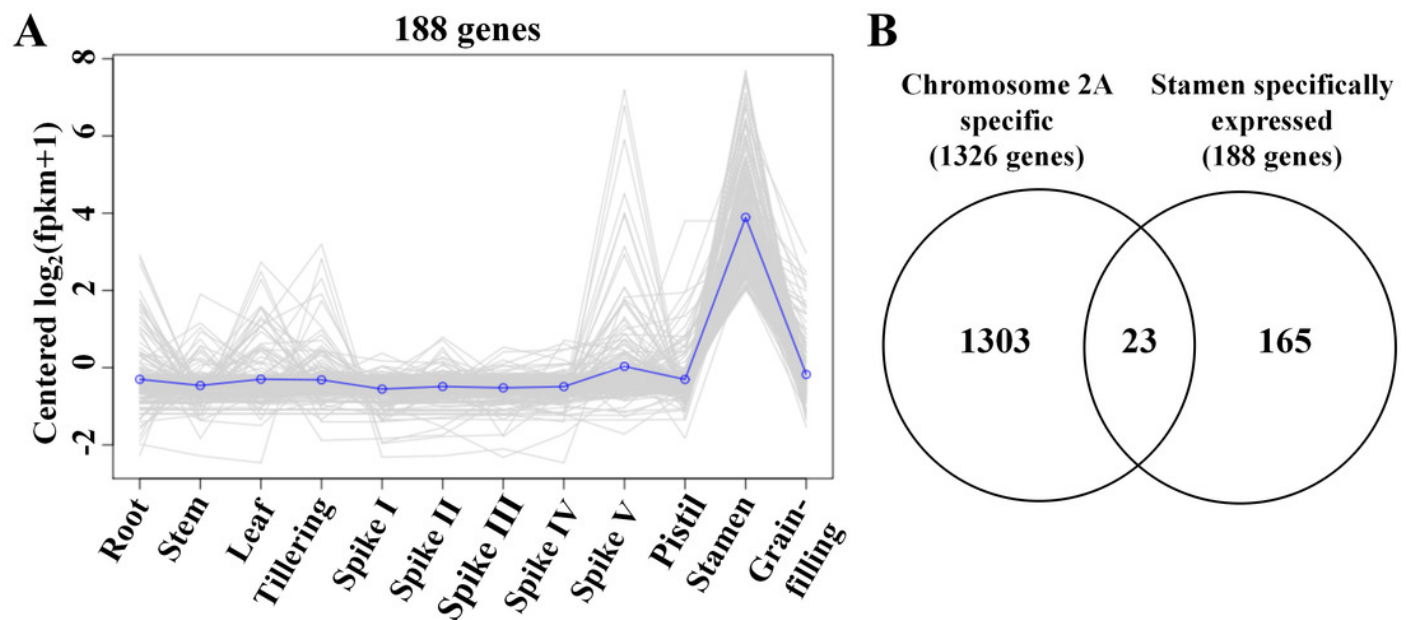



Figure 7

The DEGs of GO category in lipid metabolism, cell wall biogenesis and pollen development between stamens of T plants and D plants.

Down: the genes were expressed less in stamens of D plants; Up: the opposite.

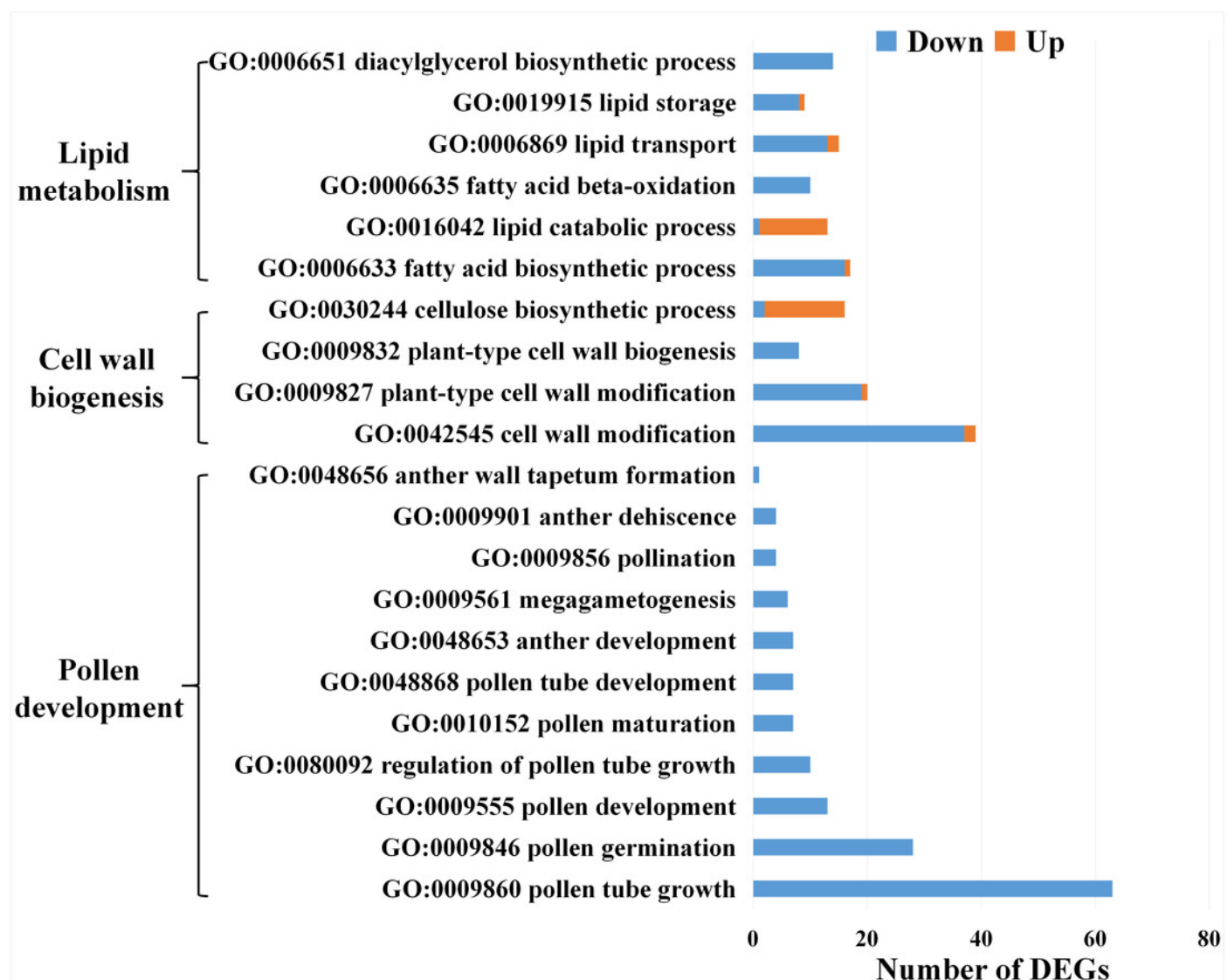



Figure 8

Spatiotemporal expression profiles of the key genes involved in abnormal pollen development of *dms*.

The asterisks indicate the significant difference between different samples at **P = 0.01. All qRT-PCR reactions were replicated thrice.

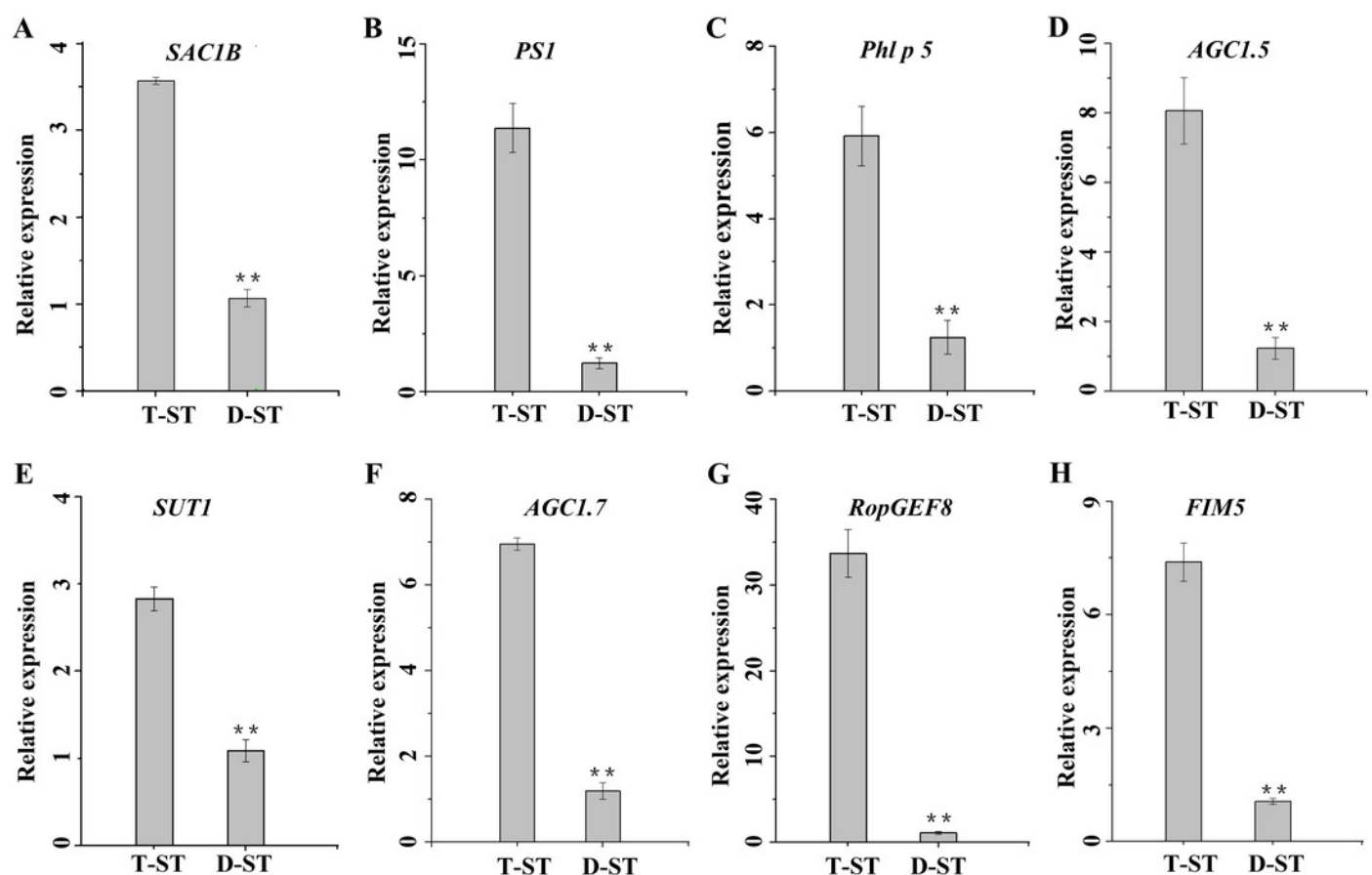



Figure 9

A hypothesis on the molecular regulatory network in *dms*.

The modules coloured in light red are highly expressed in *dms* and modules coloured in light blue are expressed less in *dms*.

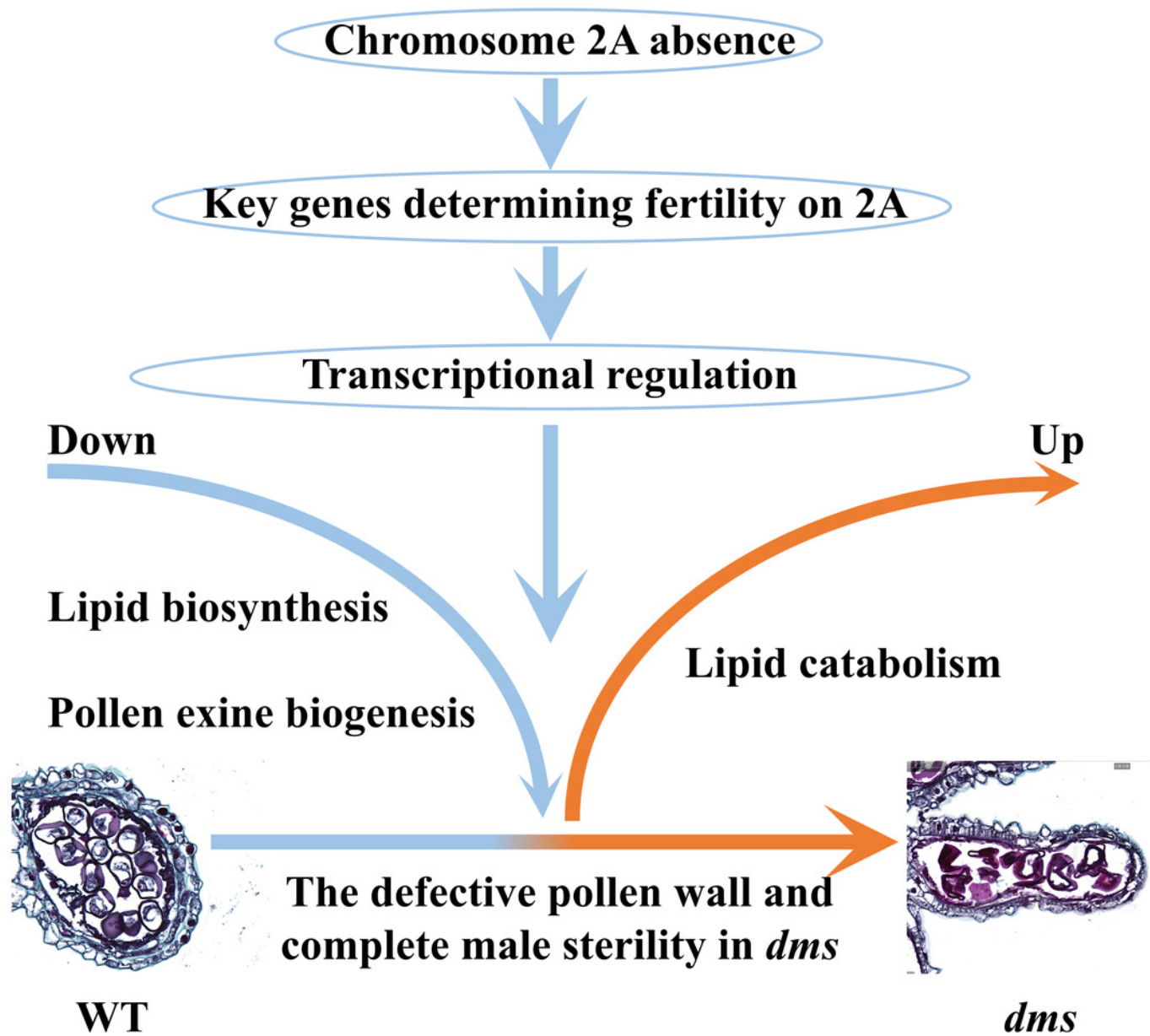
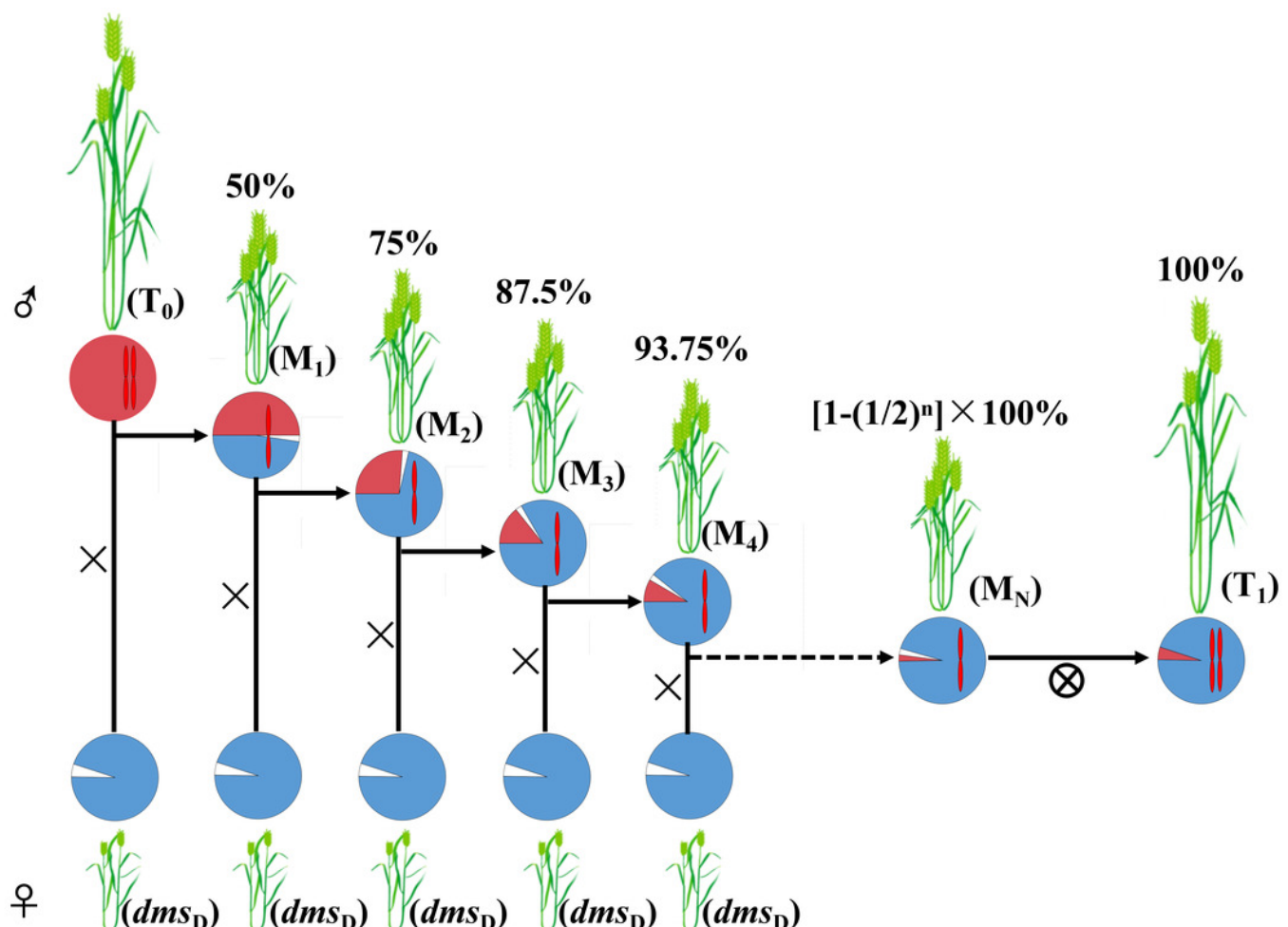



Figure 10

A program for wheat 2A translocation line construction with the D plants of *dms*.

The modules coloured in red are the genetic backgrounds of chromosome 2A donor, modules coloured in blue are the genetic backgrounds of D plants, and modules coloured in white are the chromosome 2A lost in D and M plants. T_0: chromosome 2A donor plants; M_1-M_N: the plants harbored one donor chromosome 2A; dms_D: the D plants of *dms*; T_1: New normal wheat lines with two donor chromosome 2As and genetic background of *dms*. The chromosomes in the modules are chromosome 2As. The number above the plants are the percentages of genetic backgrounds from D plants except for chromosome 2As.

Chromosome numbers and phenotypes of the progeny derived from M plants.

1   **Table 1** Chromosome numbers and phenotypes of the progeny derived from M plants.

Seedling number detected	Chromosome number	Phenotype
17	42	Normal tall plant (T)
21	41	Semi-dwarf plant (M)
4	40	Dwarf, multi-pistil and male sterility (D)

Phenotypes of the 6 crosses at F_1 and F_2 generations.

1   **Table 2** Phenotypes of the 6 crosses at F_1 and F_2 generations.

Cross.		F_1*	F_2**		
Female	Male (M plants of *dms*)	Phenotype and observed lines (number)	Phenotype and observed lines (number)		
Zhoumai18	M	T (22)	T		
		M (10)	T(12)	M(23)	D(10)
Taishan 4429	M	T (3)	T		
		M (6)	T(10)	M(21)	D(9)
Jing 08-426	M	T (3)	T		
		M (3)	T(8)	M(15)	D(6)
Yangmai 11	M	T (3)	T		
		M (6)	T(13)	M(26)	D(12)
Yuyou 1	M	T (3)	T		
		M (6)	T(15)	M(30)	D(14)
Guomai 301	M	T (3)	T		
		M (6)	T(12)	M(24)	D(9)

2   *At F_1 generation, all six cross combinations segregated into two phenotypes, the total

3   number of T plant was 37, the total number of M plant was 37. ** All seeds harvested

4   from M plants at F_2 generation will segregate into T, M and D plants at F_3 generation as

5   *dms*.

The key genes determining male fertility in wheat on chromosome 2A.

1 **Table 3** The key genes determining male fertility in wheat on chromosome 2A.

ID	Homologous gene/ Gene name	Protein product	Reported function / Mutant phenotype	Reference
TraesCS2A01G043400	*AT5G40260/ AtSWEET*	SWEET sugar transporter	A role in glucose efflux for pollen nutrition / Male sterility	Chen et al. 2010
TraesCS2A01G051800	*AT5G40260/ AtSWEET*	SWEET sugar transporter	A role in glucose efflux for pollen nutrition / Male sterility	Chen et al. 2010
TraesCS2A01G261500	*At4g05330/ AtAGD13*	C2H2 transcription factor	Involved in pollen development / Disrupted pollen cell wall	Reňák, Dupl'áková & Honys, 2012
TraesCS2A01G442500	*Os01g0293100/ OsTIP2*	bHLH transcription factor	Control of anther cell differentiation / Male sterility	Fu et al. 2014
TraesCS2A01G521900	*At5g49180/ AtPME58*	A putative pectin methylesterase	Involved in pollen germination and tube growth	Leroux et al. 2015
TraesCS2A01G527700	*At5g13930/ AtCHS*	Chalcone synthase	Involved in flavonoid biosynthesis	Dobritsa et al. 2010

Key genes involved in abnormal pollen development of *dms*.

1   **Table 4** Key genes involved in abnormal pollen development of *dms*.

ID	T-ST_FPKM	D-ST_FPKM	Homologous gene / Gene name	Reported function	Reference
*TraesCS1A01G020700	32	9	*At5g66020/*		
*TraesCS1B01G024700	2	0		Involved in pollen exine formation	Despres et al. 2003
*TraesCS1D01G020200	31	9	*SAC1B*		
TraesCS1A01G187500	7	49	*Os10g0524500/*		
TraesCS1B01G195300	6	48		Required for male fertility	Liu et al., 2017
TraesCS1D01G189200	8	53	*NP1*		
*TraesCS2A01G288400	300	0			
*TraesCS2B01G305200	297	57	*Os04g0398700/*	Pollen specific protein	-
*TraesCS2D01G286700	323	62	*PS1*		
*TraesCS4A01G000100	151	27			
*TraesCS4D01G000100	130	24	*TaPhl p 5*	Causes grass pollen allergy	-
TraesCS5A01G233600	10	19	*At5g22260/*		
TraesCS5B01G232100	7	18		Required for male sterility	Wilson et al., 2001
TraesCS5D01G240500	10	31	*MS1*		
TraesCS7A01G269700	1341	837	*Os08g0546300/*		
TraesCS7B01G167900	395	105		Required for pollen wall development	Li & Zhang, 2010
TraesCS7D01G270200	353	88	*C4*		

TraesCS7A01G458700	41	5	Os01g0812000/ GAMYB	Required for tapetum function and pollen wall formation in rice	Aya et al., 2009
TraesCS7B01G357900	48	6			
TraesCS7D01G446700	53	7			
*TraesCS1A01G133200	15	1	AT3G12690/ AGC1.5	Involved in polarized growth of pollen tubes	Li et al., 2018a
*TraesCS1B01G152700	11	1			
*TraesCS1D01G124200	5	3			
*TraesCS1A01G134100	26	4	Os03g0170900/ SUT1	Essential for normal pollen germination	Hirose et al., 2010
*TraesCS1D01G135900	35	11			
*TraesCS3A01G157100	13	1	AT1G79250/ AGC1.7	Involved in polarized growth of pollen tubes	Li et al., 2018a
*TraesCS3B01G183500	8	3			
*TraesCS3D01G164700	13	1			
*TraesCS3A01G270400	70	2	AT3G24620/ RopGEF8	Required for pollen tube growth	Gu et al., 2006
*TraesCS3B01G304200	40	0			
*TraesCS3D01G270200	98	4			
TraesCS4A01G001100	9	0	AT3G04690/ ANXUR1	Involved in premature pollen tube rupture	Boisson-Dernier et al., 2009
TraesCS4B01G002300	13	0			
TraesCS4D01G001000	3	0			
TraesCS4A01G185600	12	2	AT3G07960/ PIP5K6	Regulates clathrin-dependent endocytosis in pollen tubes	Zhao et al., 2010
TraesCS4B01G133100	13	2			
TraesCS4D01G128000	13	2			
*TraesCS6A01G200700	13	2	AT5G35700/	Required for pollen	Su et al., 2017

*TraesCS6B01G226800	14	1	*FIM5*	germination and pollen tube growth	
*TraesCS6D01G189200	19	2			
TraesCS7A01G281300	232	53	*Os08g0560700/*	Involved in pollen tube growth	Verdino et al., 2002
TraesCS7B01G179800	277	66	*Phl p 7*		
TraesCS7D01G280000	471	109			

2   *The asterisks indicate the genes verified by real-time qRT-PCR.