



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

Zhixin Jiao¹, Xinxin Zhu¹, Huijuan Li¹, Zhitao Liu^{2,3}, Xinyi Huang², Nan Wu², Junhang An¹, Junchang Li¹, Jing Zhang¹, Yumei Jiang¹, Qiaoyun Li¹, Zengjun Qi² and Jishan Niu¹

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

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

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

ABSTRACT

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 three types in offspring of *dms* plants, with 42, 41 and 40 chromosomes 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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Corresponding author
Jishan Niu, jsniu@henau.edu.cn

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INTRODUCTION

Allohexaploid wheat (*Triticum aestivum* L., $2n = 6x = 42$, genomic constitution AABBDD) has a genome from three diploid species: *Triticum urartu* Thum. (the source of the A genome), *Aegilops speltoides* (Tausch) Gren. or a closely related species (the source of the

B genome), and *Aegilops tauschii* Coss. (the source of the D genome) (Huang et al., 2002). Because allohexaploid wheat has high level of functional redundancy, it can host a range of diverse whole-chromosome aneuploids (Zhang et al., 2013; Zhang et al., 2017). There are various types aneuploid variations available in wheat, such as nulli-tetrasomic lines, nullisomic lines, monosomic lines, ditelosomic lines, as well as chromosome fragmental deletion lines (Endo & Gill, 1996; Qi et al., 2003). The aneuploid stocks are useful in wheat gene mapping, and especially, genes can be located to a small segment using a series of chromosome fragmental deletion lines (Endo & Gill, 1996; Qi et al., 2003). A set of homozygous chromosome deletion lines were obtained in cv. Chinese Spring (Endo & Gill, 1996). Most of the homozygous chromosome 2A short arm deletion lines are sterile; genes involved in male development are located on the chromosome 2A short arm (Endo & Gill, 1996).

Presently, at least five stable genic male sterility (GMS) genes are known in bread wheat. They are *ms1* on 4BS (Wang et al., 2017), *Ms2* on 4DS (Xia et al., 2017), *Ms3* on 5AS (Maan et al., 1987), *Ms4* on 4DS (Maan & Kianian, 2001) and *ms5* on 3AL (Pallotta et al., 2019), but the molecular regulation mechanisms of the male sterility lines are largely unknown.

Anther and pollen developments are complex biological processes, determining wheat male fertility. Pollen development starts from a single cell layer, which undergoes a series of cell divisions and differentiation to form the innermost meiocytes encased within four somatic anther cell layers; from inner to outer these are the tapetum, middle layer, endothecium and epidermis (Zhang & Yang, 2014). The tapetum serves as a nutritive tissue, providing metabolites, nutrients, and cell wall precursors for the development of pollen grains (Goldberg, Beals & Sanders, 1993). The regulatory genes involved in pollen exine patterning are known in Arabidopsis and rice (Pearce et al., 2015; Lin et al., 2017). For example, *AtMS1* (Male Sterility 1) (Wilson et al., 2001), *AtDRL1* (Dihydroflavonol 4-Reductase-Like 1) (Tang et al., 2009), *AtLAP3* (Less Adherent Pollen 3) (Dobritsa et al., 2009), *AtLAP5* (Less Adherent Pollen 5) (Dobritsa et al., 2010) in Arabidopsis, and *OsGAMYB* (GA, gibberellin; MYB, *v-myb* avian myeloblastosis viral oncogene homolog) (Aya et al., 2009), *OsNP1* (No Pollen 1) (Liu et al., 2017), *OsDPW2* (Defective Pollen Wall 2) (Xu et al., 2017) in rice are involved in the biosynthesis of sporopollenin, which is a major constituent of exine in the outer pollen wall. However, pollen developmental studies in wheat have lagged behind these plants.

Previously, we reported a mutant of dwarf, multi-pistil and male sterile *dms* in wheat (Duan et al., 2015; Zhu et al., 2016). Pollen vigor and hybridization tests demonstrated that *dms* mutant was male sterile. Male sterility and male fertility followed a segregation ratio of 1:3 [D:(T +M) = 1:3] (Duan et al., 2015). However, the genetic basis of this mutant is unknown. The present study is to discover the genetic basis at the cytological and molecular levels. We also identified a set of genes playing potential key roles accounting for male sterility on chromosome 2A.

MATERIALS & METHODS

Plant materials

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

Morphological and histochemical observations

Phenotype of plants, young spikes and stamens of *dms* were observed at different developmental stages (Zadoks, Chang & Konzak, 1974). Developmental stages of the young spike primordia were referenced as previously described (Vahamidis *et al.*, 2014). The florets with anthers and pistils at different developmental stages were dissected from spikes using an anatomical needle. Anthers were observed using scanning electron microscope (SEM) (SU8010, Hitachi, Tokyo, Japan) as previously described (Jiao *et al.*, 2019). Structural and histochemical observation on the anthers of T and D plants were carried out using a method previously described by Geng *et al.* (2018).

RNA extraction and mRNA sequencing

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

mRNA data analysis

The mRNA reads of the fifteen samples were aligned to the draft assembly (IWGSC v1.0) of the wheat genome survey sequence (<http://www.wheatgenome.org/>) using Tophat2 tool software (Kim *et al.*, 2013). Reads distribution on 21 wheat chromosomes of T, M and D plants were analyzed and drawn using Circos tool software (<http://mkweb.bcgsc.ca/tableviewer/>). Gene functional annotation was carried out as previously described by us (He *et al.*, 2018). Gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped (FPKM) (Florea, Song & Salzberg, 2013). Differentially expressed genes (DEGs) between two sample groups were analyzed using DESeq R package (Wang *et al.*, 2010). The FDR < 0.01 (false discovery rate) and FC ≥ 2 were set as the thresholds for significantly DEGs.

Principal components were calculated using the FPKM values (Schulze *et al.*, 2012). For the single gene, heat maps were drawn using software Hem I v 1.0.3.7 according to the FPKM values. Tissue specific expression patterns of wheat genes were analyzed using *k*-means cluster on the BMKCloud platform (<https://www.biocloud.net/>). Clustering was performed in R using the *k*-means function, where *k* = 8 within the cluster package by Euclidean distance. Wheat tissue type, expression profile data sources and sample

description in origin article (*Garcia-Seco et al., 2017*; *Feng et al., 2017*; *Li et al., 2019*) were listed in [Table S1](#).

Single nucleotide polymorphism (SNP) association analysis

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

Simple sequence repeat (SSR) analysis

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

Cytological analysis

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

qRT-PCR for mRNAs

Anthers of T and D plants at 3-nucleate pollen stage were prepared for real-time PCR. The experimental samples were consistent with the samples of RNA-seq. The qRT-PCR was performed as previously described by us (*He et al., 2018*). Reverse transcription was performed with 1 µg RNA using Hifair[®] II 1st Strand cDNA Synthesis SuperMix (11123ES60, Yeasen, Shanghai, China). Real-time PCRs of mRNAs were performed using Hieff[®] qPCR SYBR Green Master Mix (1201ES08, Yeasen, Shanghai, China) and a CFX

Connect™ Real-Time System (Bio-Rad, Hercules, CA, USA) following the production instructions. The wheat actin gene was used as an internal control. All primer sequences are listed in [Table S4](#). The gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method ([Livak & Schmittgen, 2001](#)). The SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis for the qRT-PCR.

RESULTS

Male abortion in D plants of *dms*

There were three typical phenotypes in the progeny of *dms*, tall (T), semi-dwarf (M) and dwarf (D) plants ([Fig. S1](#)). D plants were male sterile according to the result of I₂-KI staining ([Duan et al., 2015](#)). To discover the morphological causes of the male abortion in D, scanning electron microscope was employed to observe the anther ultrastructure ([Fig. 1](#)). Unlike the fertile T plants, the anthers of D plants were not dehiscent at the trinucleate stage, and no mature pollen grains were released ([Figs. 1A and 1B](#)). The outer anther epidermal cells of T plants were arranged neatly, whereas these of D plants were irregular ([Figs. 1C and 1D](#)). Moreover, compared with the anthers of the T plants, the inner epidermal of D plants showed aberrant sized Ubisch bodies, which suggested a structural abnormality ([Figs. 1E–1H](#)). The pollen grains of T plants were plump and rounded; by contrast, the pollen grains of D plants were extremely shriveled and atrophied ([Figs. 1I and 1L](#)). Furthermore, we performed a histological analysis of the anthers in the *dms* mutant and the wild type. In the initial stages of pollen development ([Figs. S2A and S2B](#)), there were no differences between the wild type and *dms* mutant. The young microspores were normal ([Fig. S2C and S2D](#)). However, compared with the wild type, degenerated pollen grains were observed in the *dms* mutant at 3-nucleate pollen stage ([Figs. S2E and S2F](#)).

The female organs in flowers of D plants developed unwell, most of the pistils were normal, a few were multi-pistils ([Fig. S3](#)). D plants were male sterile but female fertile, it can be used as a female parent to cross with other wheat genotypes. We got F₁ seeds from dwarf plants (as female parent) crossed with Guomai 301 (as pollen parent) ([Fig. S4](#)).

The most SNPs were on chromosome 2A

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

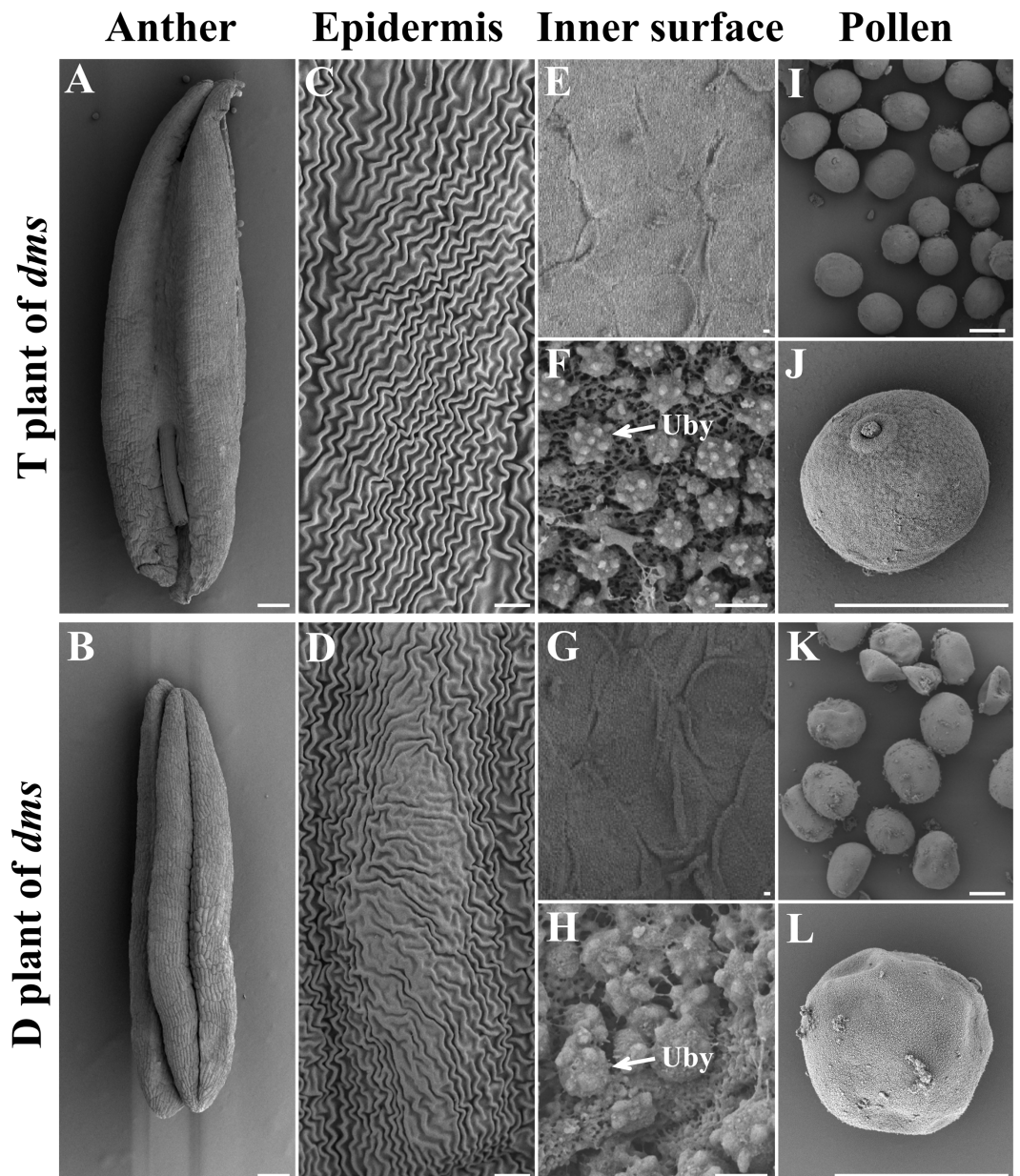


Figure 1 The scanning electron micrographs of the anthers, anther epidermis, anther inner surface, and pollen grains in the T and D plants of *dms* at the trinucleate stage. Uby: Ubisch bodies. Scale bars represent 1 mm in anthers, 500 μm in pollen, 50 μm on the epidermis surface and 10 μm on the inner surface.

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

In order to further verify that the genetic variation of *dms* occurred on chromosome 2A, eleven plants including three typical phenotypes of tall, semi-dwarf and dwarf were randomly selected for SSR genotyping. SSR markers evenly distributed across wheat A, B

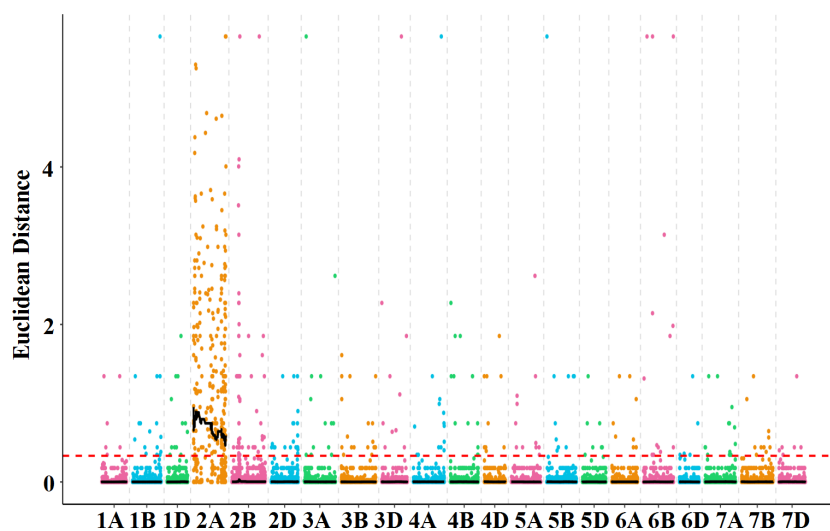


Figure 2 Chromosome distribution of the SNPs between stamens of T and D plants. ED is Euclidean distance. Each dot represents a SNP identified between T and D. The serial numbers of wheat chromosomes are indicated on the X-axis. The red dotted line indicates significance threshold ($ED = 0.33$). The wavy line above the significance threshold indicates the variant section.

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and D genomes were used to detect the polymorphism between tall and dwarf plants of *dms*. The result showed that all the primers specifically amplifying fragments on chromosomes 2A (*Xgwm312*, *Xgpw2229*, *Xgwm95*, *Xgwm445*, *Xwmc794*, *Xgwm328*, *Xgwm425*, *Xbarc212* and *Xbarc122*) couldn't amplify the expected products in the dwarf plants of *dms* (Fig. 4; lane 4, 7, 9, 11), which implied that wheat chromosome 2A was missing in the dwarf plants.

The SSR markers were used to quantitatively amplify the DNA templates of tall, semi-dwarf and dwarf plants. The amounts of the amplified products of the tall plants (Fig. 4; lane 1, 3, 5, 8) were about double that of the semi-dwarf plants (Fig. 4; lane 2, 6, 10), and dwarf plants without PCR products (Fig. 4). In another word, the SSR primers specifically amplifying fragments on 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

To explore the chromosome number of *dms*, 42 plants derived from M plants were investigated. Among them, 17 plants had 42 chromosomes, 21 had 41 chromosomes and 4 had 40 chromosomes (Table 1). After chromosome analysis, the seedlings were planted in field. The 17 plants with 42 chromosomes showed regular plant height, pistil and fertility, the 21 plants with 41 chromosomes showed medium plant height and regular pistil and fertility, while all the 4 plants with 40 chromosomes showed dwarf status, multi-pistil and complete male sterility (Fig. S7).

To validate and identify the chromosome constitution of *dms*, high-resolution chromosome painting was applied using eight single strand oligonucleotide probes. The result showed that all 21 wheat homoeologous chromosome pairs can be reliably discriminated. Total 45 plants derived from M plants were clearly characterized, among

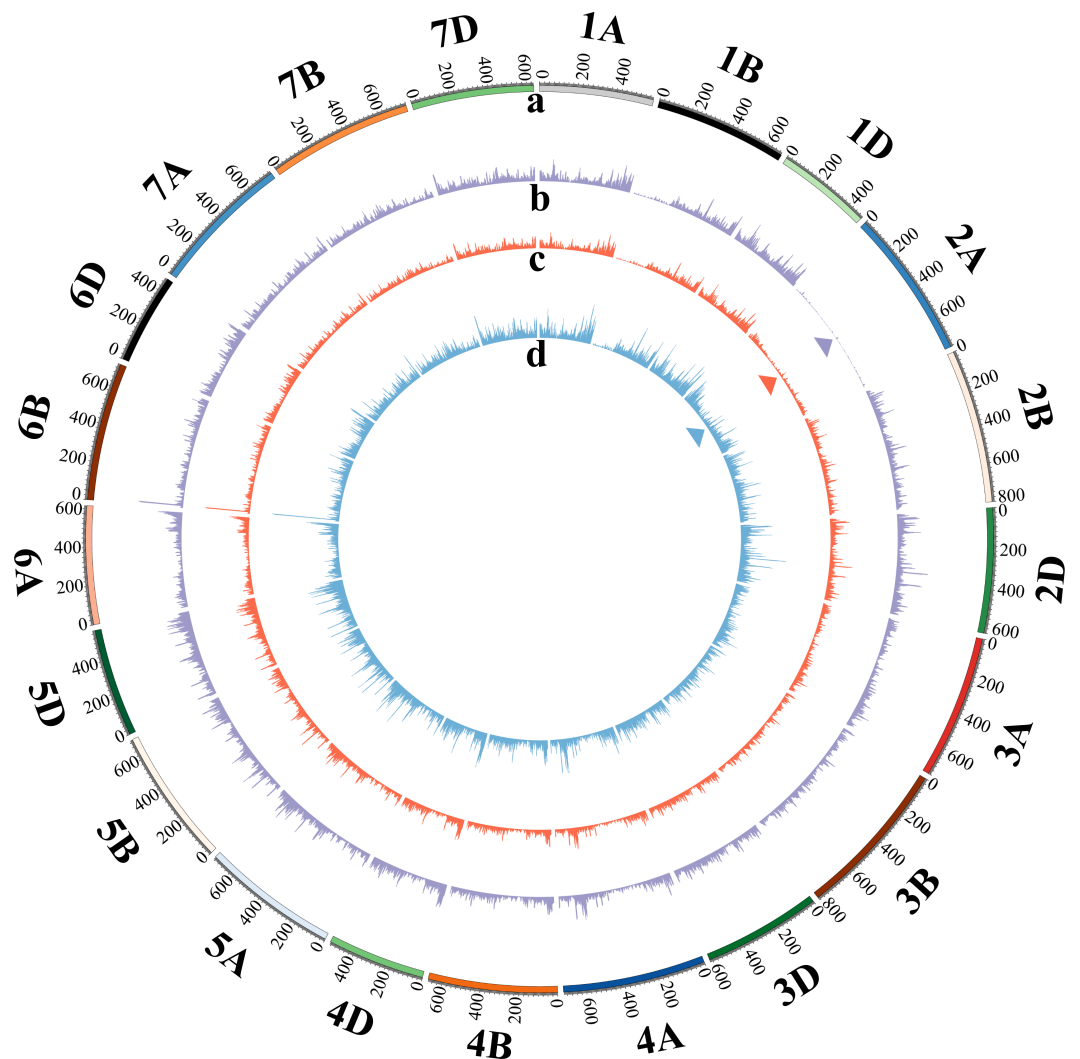


Figure 3 Circular diagram of the reads distributed on the 21 wheat chromosomes of T, M and D plants. The tracks toward the center of the circle display (A) chromosome name and size (Mb); (B) read segments density of D plant; (C) read segments density of M plant; (D) read segments density of T plant. The triangles highlight reads distributions on chromosome 2A.

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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)

them, 18 plants had 42 chromosomes and regular karyotypes (Figs. 5A and 5B), 20 plants had 41 chromosomes and lacked one chromosome 2A (Figs. 5C and 5D), while the remaining 7 plants had 40 chromosomes and lacked a pair of chromosomes 2A (Figs. 5E and 5F).

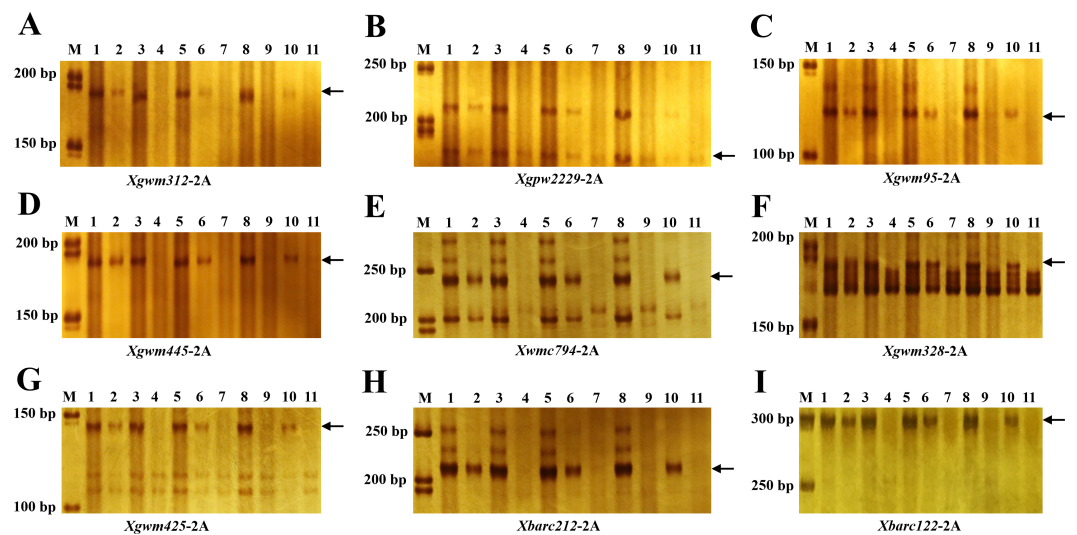


Figure 4 SSR markers on chromosome 2A were polymorphic. M, DNA molecule weight markers; lanes 1–11, the offspring of *dms* mutant. lanes 1, 3, 5 and 8 were tall plants; lanes 2, 6 and 10 were semi-dwarf plants; lanes 4, 7, 9 and 11 were dwarf plants. The arrows indicate the SSR products.

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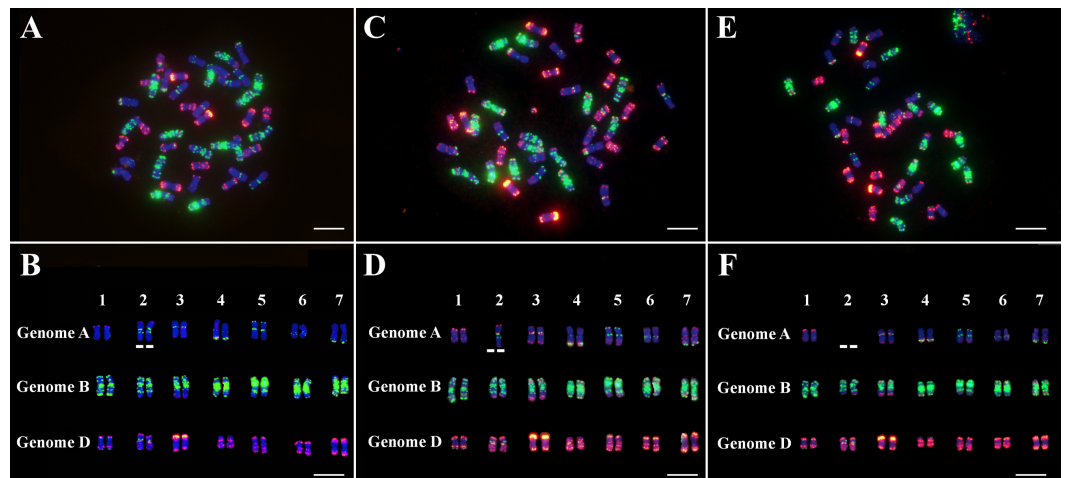


Figure 5 High-resolution chromosome painting analysis of the plants derived from M plants at metaphase of mitosis. (A) and (B), one karyotype of T plant, which had 42 normal chromosomes. (C) and (D), one karyotype of M plant, which had 41 chromosomes, lacked one chromosome 2A. (E) and (F), one karyotype of D plant, which had 40 chromosomes, lacked a pair of chromosomes 2A. 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 μ m.

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Chromosome 2A carries key genes determining fertility

We assessed the effects of several different chromosome 2As by making six crosses (Table 2). At F₁ generation, all six crosses segregated into two phenotypes, tall plants (T) and Semi-dwarf plants (M). Combined with the data above, we knew that the genotypes of the cross

Table 2 Phenotypes of the six crosses at F₁ and F₂ generations.

Cross.		F ₁ ^a		F ₂ ^b	
Female	Male (M plants of <i>dms</i>)	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)

Notes.

^aAt F₁ generation, all six cross combinations segregated into two phenotypes, the total number of T plant was 37, the total number of M plant was 37.

^bAll seeds harvested from M plants at F₂ generation will segregate into T, M and D plants at F₃ generation as *dms*.

Zhoumai 18 × M at F₁ generation were 2A_{Zhoumai18}2A_{*dms*} and 2A_{Zhoumai18}, the corresponding phenotypes were T and M plants. This demonstrated that the transmitting frequency of null-2A pollens was about half of the 2A pollens (Table 2). All the T plants of the six crosses at F₁ generation didn't segregate at F₂ generation, but all M plants at F₁ generation segregated into T, M and D plants at F₂ generation. All M plants at F₂ also segregated into T, M and D plants at F₃ (Table 2). The 2As played key roles during whole plant development in all the monosomic lines of 2As from Taishan 4429, Jing 08-426, Yangmai 11, Yuyou 1 and Guomai 301. These data demonstrated that the six different origin chromosomes 2As had the similar function as the chromosome 2A in *dms*. In addition, the heterozygous genetic backgrounds of the six crosses, including chromosomes 2Ds and 2Bs couldn't complement the effects of chromosome 2A. D plants were used as a female parent to cross with other wheat genotypes, so as to construct inter-cultivar chromosome 2A substitution lines (Fig. S8). At F₁ generation, all the lines had one chromosome 2A, they were male fertile, which indicated chromosome 2A carries key genes determining male fertility.

Stamen-specifically expressed genes on chromosome 2A

To identify the key genes determining fertility in wheat, 5939 genes located on chromosome 2A (TraesCS2A01G000100 - TraesCS2A01G593900) were analyzed. Total 4613 genes on chromosome 2A were identified and found to be homeologous to genes on other chromosomes (Table S7). Deletion of these genes on chromosome 2A might not affect the phenotype of D plants because of gene redundancy. Total 1326 2A-specific genes were identified (Table S8). Tissue specific expression analysis showed that 188 genes on chromosome 2A were stamen specifically expressed genes (Fig. 6A; Table S9). Venn

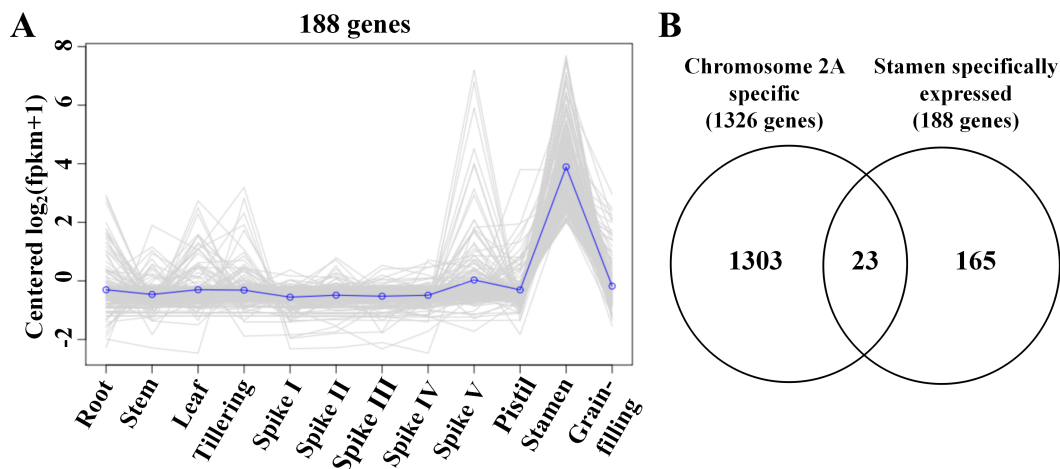


Figure 6 Chromosome 2A carries key genes involved in pollen development. (A) The expression profiles of the stamen specifically expressed genes on 2A. (B) Venn diagram showed the chromosome 2A and stamen specifically expressed genes.

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diagram analysis showed that 23 genes were chromosome 2A specific and stamen specifically expressed genes (Fig. 6B; Table S9). Among them 6 genes were reported to be involved in pollen development related biological processes (Table 3), they may be the key genes determining male fertility.

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

A total of 5199 genes were significantly differentially expressed ($FC \geq 4$) between stamens of T and D plants. Among them 4761 DEGs expressed less, only 438 DEGs expressed highly in D-ST (Table S10). Obviously, the expressions of most DEGs in D plants of *dms* were lower because lacking of the chromosome 2A. We identified 229 putative TF DEGs between T-ST and D-ST. They belonged to 47 TF families. The top three with the most number of DEGs were MYB, C2H2 zinc finger protein (C2H2) and APETALA 2/ethylene-responsive element binding factor (AP2/ERF) transcription factor families and most of them expressed less in D-ST (Table S11).

Totally 45 DEGs were associated with auxin homeostasis, such as biosynthesis, response, signaling and metabolism (Fig. S9). Among auxin signal transduction-related genes, homologs of auxin response factor (ARF) genes and several auxin biosynthesis-related genes expressed less in D-ST. Reduced expression of auxin biosynthesis and signal transduction related genes were closely related to male sterility (Su et al., 2019). Abnormality of auxin homeostasis might be a major factor leading to the phenotype of *dms*.

DEGs involved in pollen development related metabolism in *dms*

Enrichment analysis of the DEGs between T-ST and D-ST revealed that most of them were involved in pollen development related metabolism such as the GO terms of 'lipid metabolism', 'cell wall biogenesis' and 'pollen development' (Fig. 7). Among lipid metabolism related genes, fatty acid biosynthetic related genes expressed less and lipid

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	<i>AT5G40260/AtSWEET</i>	SWEET sugar transporter	A role in glucose efflux for pollen nutrition / Male sterility	<i>Chen et al. (2010)</i>
TraesCS2A01G051800	<i>AT5G40260/AtSWEET</i>	SWEET sugar transporter	A role in glucose efflux for pollen nutrition / Male sterility	<i>Chen et al. (2010)</i>
TraesCS2A01G261500	<i>At4g05330/AtAGD13</i>	C2H2 transcription factor	Involved in pollen development / Disrupted pollen cell wall	<i>Reňák, Dupřáková & Honys (2012)</i>
TraesCS2A01G442500	<i>Os01g0293100/OsTIP2</i>	bHLH transcription factor	Control of anther cell differentiation / Male sterility	<i>Fu et al. (2014)</i>
TraesCS2A01G521900	<i>At5g49180/AtPME58</i>	A putative pectin methylesterase	Involved in pollen germination and tube growth	<i>Leroux et al. (2015)</i>
TraesCS2A01G527700	<i>At5g13930/AtCHS</i>	Chalcone synthase	Involved in flavonoid biosynthesis	<i>Dobritsa et al. (2010)</i>

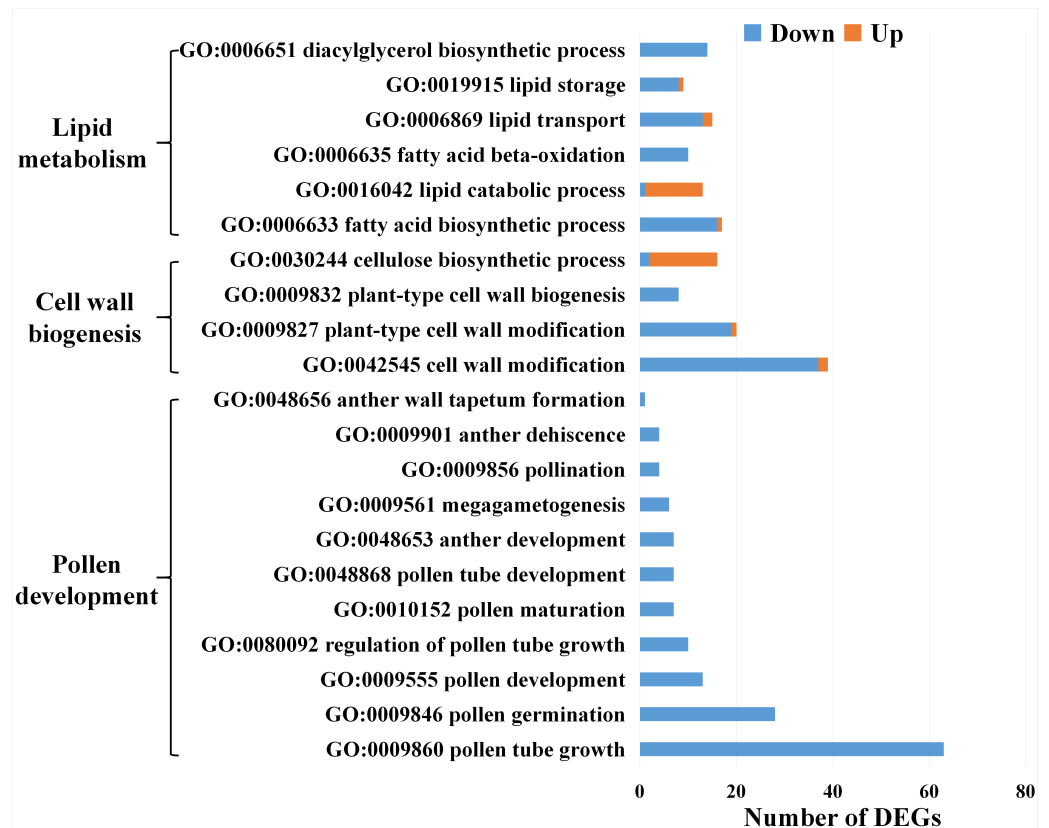


Figure 7 The DEGs of GO category in lipid metabolism, cell wall biogenesis and pollen development between stamens of T plants and D plants. Down, the genes were expressed less in stamens of D plants; Up, the opposite.

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catabolic process related genes expressed highly in D-ST. Among cell wall biogenesis related genes, cell wall modification related genes expressed less and cellulose biosynthetic process related genes expressed highly in D-ST (Fig. 7). This indicated that fatty acid metabolic and cell wall assembly disorder might be the critical factors causing male sterility in *dms*.

DEGs involved in abnormal pollen development of *dms*

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

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			
*TraesCS1B01G024700	2	0	<i>At5g66020/SAC1B</i>	Involved in pollen exine formation	<i>Despres et al. (2003)</i>
*TraesCS1D01G020200	31	9			
TraesCS1A01G187500	7	49			
TraesCS1B01G195300	6	48	<i>Os10g0524500/NP1</i>	Required for male fertility	<i>Liu et al. (2017)</i>
TraesCS1D01G189200	8	53			
*TraesCS2A01G288400	300	0			
*TraesCS2B01G305200	297	57	<i>Os04g0398700/PS1</i>	Pollen specific protein	–
*TraesCS2D01G286700	323	62			
*TraesCS4A01G000100	151	27			
*TraesCS4D01G000100	130	24	<i>TaPhl p 5</i>	Causes grass pollen allergy	–
TraesCS5A01G233600	10	19			
TraesCS5B01G232100	7	18	<i>At5g22260/MS1</i>	Required for male sterility	<i>Wilson et al. (2001)</i>
TraesCS5D01G240500	10	31			
TraesCS7A01G269700	1341	837			
TraesCS7B01G167900	395	105	<i>Os08g0546300/C4</i>	Required for pollen wall development	<i>Li & Zhang (2010)</i>
TraesCS7D01G270200	353	88			
TraesCS7A01G458700	41	5			
TraesCS7B01G357900	48	6	<i>Os01g0812000/GAMYB</i>	Required for tapetum function and pollen wall formation in rice	<i>Aya et al. (2009)</i>
TraesCS7D01G446700	53	7			
*TraesCS1A01G133200	15	1			
*TraesCS1B01G152700	11	1	<i>AT3G12690/AGC1.5</i>	Involved in polarized growth of pollen tubes	<i>Li et al. (2018a)</i>
*TraesCS1D01G124200	5	3			
*TraesCS1A01G134100	26	4			
*TraesCS1D01G135900	35	11	<i>Os03g0170900/SUT1</i>	Essential for normal pollen germination	<i>Hirose et al. (2010)</i>
*TraesCS3A01G157100	13	1			
*TraesCS3B01G183500	8	3	<i>AT1G79250/AGC1.7</i>	Involved in polarized growth of pollen tubes	<i>Li et al. (2018a)</i>
*TraesCS3D01G164700	13	1			
*TraesCS3A01G270400	70	2			
*TraesCS3B01G304200	40	0	<i>AT3G24620/RopGEF8</i>	Required for pollen tube growth	<i>Gu et al. (2006)</i>
*TraesCS3D01G270200	98	4			
TraesCS4A01G001100	9	0			
TraesCS4B01G002300	13	0	<i>AT3G04690/ANXUR1</i>	Involved in premature pollen tube rupture	<i>Boisson-Dernier et al. (2009)</i>
TraesCS4D01G001000	3	0			
TraesCS4A01G185600	12	2			
TraesCS4B01G133100	13	2	<i>AT3G07960/PIP5K6</i>	Regulates clathrin-dependent endocytosis in pollen tubes	<i>Zhao et al. (2010)</i>
TraesCS4D01G128000	13	2			
*TraesCS6A01G200700	13	2			
*TraesCS6B01G226800	14	1	<i>AT5G35700/FIM5</i>	Required for pollen germination and pollen tube growth	<i>Su et al. (2017)</i>
*TraesCS6D01G189200	19	2			

(continued on next page)

Table 4 (continued)

ID	T-ST_FPKM	D-ST_FPKM	Homologous gene/ Gene name	Reported function	Reference
TraesCS7A01G281300	232	53		Involved in pollen tube growth	<i>Verdino et al. (2002)</i>
TraesCS7B01G179800	277	66	<i>Os08g0560700/Phl p 7</i>		
TraesCS7D01G280000	471	109			

Notes.

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

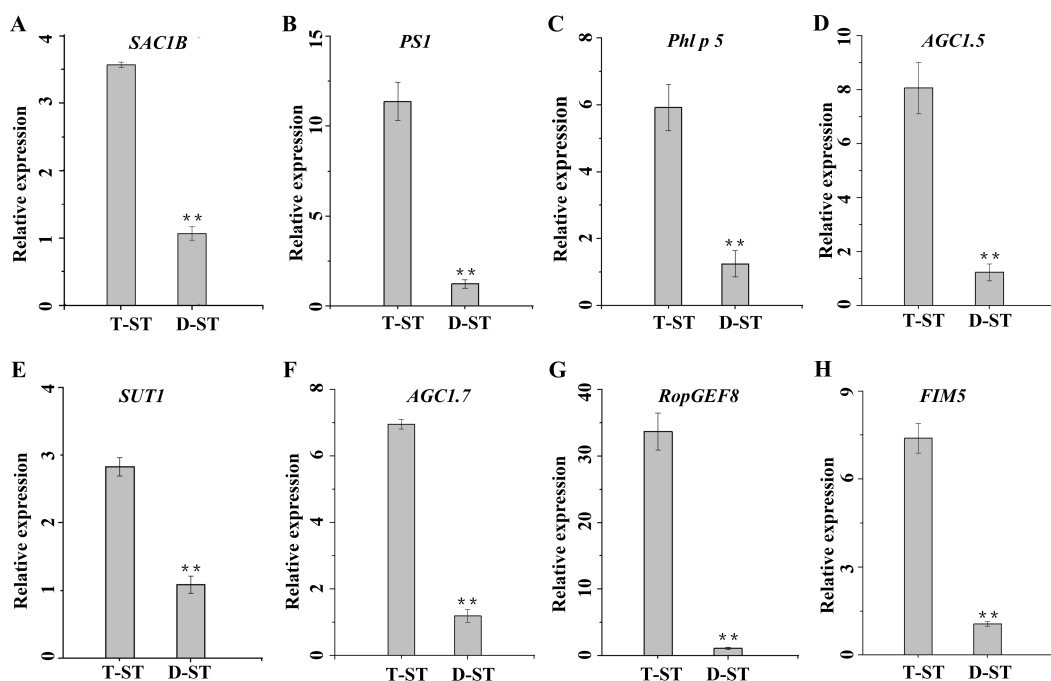


Figure 8 Spatiotemporal expression profiles of the key genes involved in abnormal pollen development of *dms*. (A) SAC1B, (B) PSI, (C) Phl p 5, (D) AGCL5, (E) SUT1, (F) AGCL7, (G) RopGEF8, (H) FIM5. The asterisks indicate the significant difference between different samples at $**P = 0.01$. All qRT-PCR reactions were replicated thrice.

Full-size DOI: 10.7717/peerj.10275/fig-8

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

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

DISCUSSION

Aneuploids are large scale mutations greatly affect cellular physiology and have significant phenotypic consequences (Zhang et al., 2017). The typical phenotype of *dms* was significantly different from its parent Zhoumai 18 at three traits, plant height, pistil number and male fertility. Preliminary, this mutant was considered as a SNP mutation.

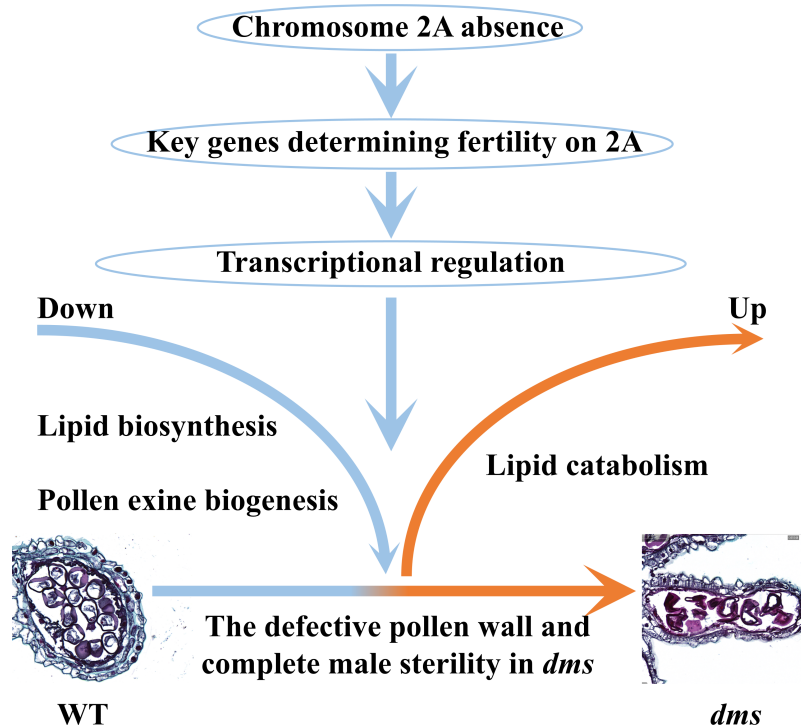


Figure 9 A hypothesis on the molecular regulatory network in *dms*. The modules coloured in light red are highly expressed in *dms* and modules coloured in light blue are expressed less in *dms*.

Full-size DOI: [10.7717/peerj.10275/fig-9](https://doi.org/10.7717/peerj.10275/fig-9)

Till the SNP association analysis showed that a large amount of SNPs between T and D plants, we thought their chromosomes should be clarified. High resolution chromosome painting is a new and efficient method for distinguishing chromosomes, which has many advantages including high sensitivity and resolution (Du *et al.*, 2017). Using this method, we successfully distinguished the karyotypes of D, M and T plants derived from M plants of mutant *dms*, and their phenotypes were corresponded with their chromosome constitutions. Now it was clear that the mutant *dms* was resulted from the absence of chromosome 2A. Our data showed that there were 23 2A-specific genes were highly expressed in stamen. However, it can't exclude other genes on 2A involved in pollen development also. In the case of wheat *Ms1* gene on 4BS, it has homoeologs on 4A and 4D. Mutant *ms1* is responsible for male sterile phenotype due to that homoeologous *Ms1* on 4A and 4D were not expressed (due to methylation) (Wang *et al.*, 2017). Some genes in the list of 23 2A-specific genes could be candidates of key genes responsible for the male sterility, but not exclude other possibilities. The molecular regulatory network can be elucidated well till the key gene/genes have been investigated.

Endo and Gill reported a set of nullisomic, monosomic, trisomic and tetrasomic lines from Chinese Spring (CS) (Endo & Gill, 1996). Among them, the 2A nullisomic line could not be maintained for they were sterile in both sexes. However, a stable self-fertile 2A nullisomic line was obtained from common wheat 'Abbondanza'. Although the pistils and stamens of the 2A nullisomic line were fertile, its female flower organ developed unwell

(Xue *et al.*, 1991). Different genetic backgrounds lead to nullisomic lines of ‘Abbondanza’ are greater vigor and fertility than those of Chinese spring (Ji, Xue & Wang, 1992). Our 2A nullisomic line *dms* is multi-pistil and male sterile, that is different from the other 2A nullisomic lines from ‘Abbondanza’ and CS (Endo & Gill, 1996). A set of chromosome deletion stocks from CS are reported, five out of the nine chromosome 2AS deletion lines have irregular meioses with many univalents at metaphase I, and they are highly sterile in both sexes, the seed settings of the four chromosome 2AL deletion lines are reduced after selfing. In our study, the meiosis of the pollen development is normal in *dms* mutant, which is different from the 2A nullisomic lines from CS (Endo & Gill, 1996).

MYB TFs play pivotal roles in plant development and stress response (Verma, 2019; Zheng *et al.*, 2018). Many MYB TFs have been functionally characterized in pollen development of *Arabidopsis* and rice, such as *AtMYB32* (Preston *et al.*, 2004), *OsGAMYB* (Aya *et al.*, 2009) and *OsTDF1* (Cai *et al.*, 2015). In *dms*, three homologs of *OsGAMYB*, *TraesCS7A01G458700*, *TraesCS7B01G357900* and *TraesCS7D01G446700*, expressed less. In rice, *GAMYB* is essential for pollen development, and it directly binds to promoter of β -KETOACYL REDUCTASE (*KAR*), a key enzyme essential for fatty acid synthesis. *GAMYB* activates the expression of *KAR* and other genes involved in the synthesis of sporopollenin, and are involved in the formation of exine and Ubisch bodies (Aya *et al.*, 2009). Reduced expression of *GAMYB* might lead to the shriveled and atrophied pollen grains of D plants. Change in the levels of *AtMYB32* expression influence pollen development by affecting the composition of the pollen wall in *Arabidopsis* (Preston *et al.*, 2004). Knocking out *OsTDF1* impaired tapetum development, leading to male sterility in rice (Cai *et al.*, 2015). Similarly, some MYB TF genes expressed less in *dms*, their functions involved in exine formation in wheat needs further research.

Pollen germination is critical for double fertilization in angiosperms (Zhang, He & McCormick, 2009). The polarity of tip-growing pollen tubes is maintained through dynamic association of active Rho GTPases in plants (ROP-GTP) (Li *et al.*, 2018a). Guanine nucleotide exchange factors for ROPs (RopGEFs) catalyze the activation of ROPs and thereby affect spatiotemporal ROP signaling (Gu *et al.*, 2006). Deletion *RopGEF* mutant has the defects in pollen tube polar growth (Gu *et al.*, 2006). AGC1.5 and AGC1.7 kinases phosphorylate RopGEFs to control pollen tube growth. Loss functions of AGC1.5 and AGC1.7 in pollen tubes results in meandering and depolarized growth morphology (Zhang, He & McCormick, 2009). In summary, the AGC1.5/1.7-RopGEFs-ROPs signaling pathway is involved in pollen germination and tip growth in *Arabidopsis* (Li *et al.*, 2018a; Huang *et al.*, 2019). In our research, the homologs of *AtAGC1.5*, *AtAGC1.7* and *AtRopGEF8* involved in ROP signaling expressed less in *dms*. Similarly, all the DEGs associated with various aspects of pollen germination related biological processes, such as pollen tube growth, regulation of pollen tube growth, pollen tube development and pollination, expressed less in *dms*. These data demonstrated that pollen germination and pollen tube growth might be suppressed in *dms*. Further experiments should be carried out to test the hypothesis.

In our study, all the six chromosome 2As from different cultivars and the genetic backgrounds of the six heterozygotes of the crosses had similar functions. These indicated

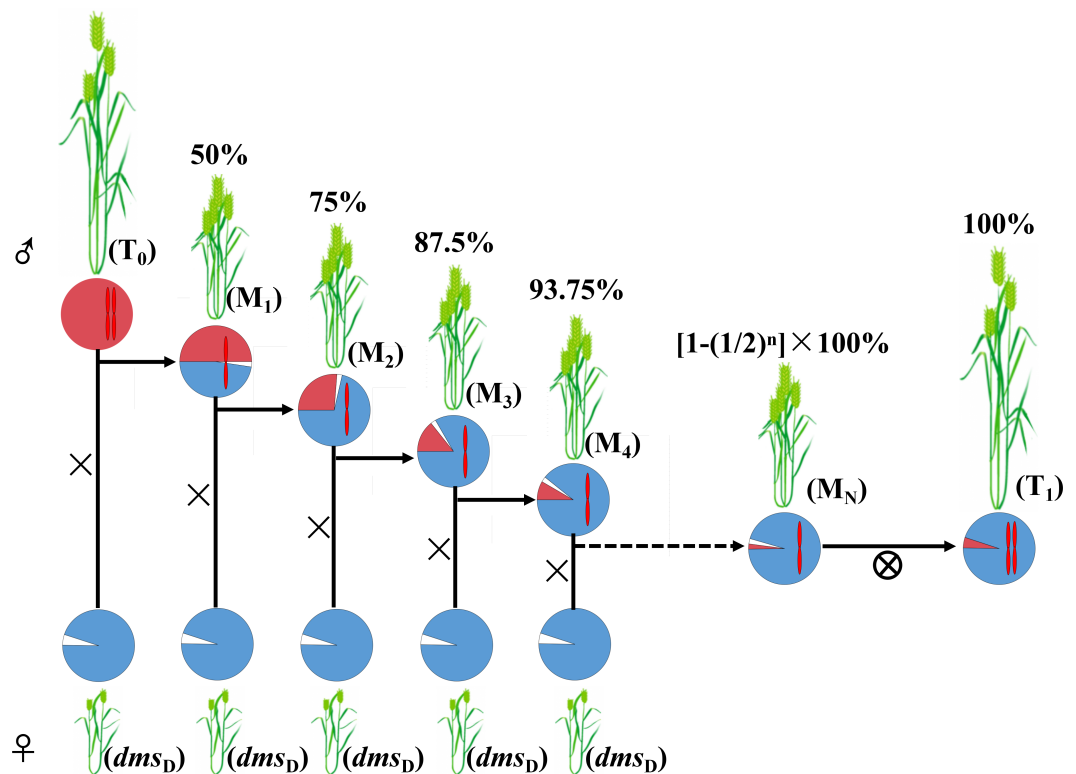


Figure 10 A program for wheat 2A translocation line construction with the D plants of *dms*. The modules coloured in red are the genetic backgrounds of chromosome 2A donor, modules coloured in blue are the genetic backgrounds of D plants, and modules coloured in white are the chromosome 2A lost in D and M plants. T₀: chromosome 2A donor plants; M₁–M_N: the plants harbored one donor chromosome 2A; *dms*_D: the D plants of *dms*; T₁: 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.

Full-size [DOI: 10.7717/peerj.10275/fig-10](https://doi.org/10.7717/peerj.10275/fig-10)

that the interactions among 2A, 2B and 2D were similar to that in *dms*. Wheat chromosome 2A has many important agronomy trait genes such as powdery mildew resistance gene *PmLK906* (Niu *et al.*, 2008), photoperiod response locus *Ppd-A1* (Beales *et al.*, 2007), reduced height (*Rht*) genes *Rht7* (Worland, Law & Shakoor, 1980). Some quantitative trait loci (QTL) for thousand grain weight (Quan, Sean & Sparkes, 2015), floret primordia development (Guo *et al.*, 2017) and grain protein-content (Groos *et al.*, 2003) are also mapped on chromosome 2A. Because the 2A nullisomic line *dms* derived from Zhoumai 18 is male sterile but female fertile, it can be used as a female parent to cross with other wheat genotypes, so as to construct inter-cultivar chromosome 2A substitution lines. Backcross to *dms* can construct series lines with highly similar genetic backgrounds but different 2As (Fig. 10), which can be used to evaluate the functions of different 2As and wheat design breeding targeting 2As.

CONCLUSIONS

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

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Zhixin Jiao conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Xinxin Zhu and Huijuan Li performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Zhitao Liu analyzed the data, prepared figures and/or tables, and approved the final draft.
- Xinyi Huang, Nan Wu and Junhang An analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Junchang Li performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Jing Zhang, Yumei Jiang and Qiaoyun Li performed the experiments, prepared figures and/or tables, and approved the final draft.

- Zengjun Qi and Jishan Niu conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Permission prior to accessing the field site was provided by Haolong Yang, administrator at the Henan Seed Company.

Data Availability

The following information was supplied regarding data availability:

The sequence data is available at NCBI BioProject: [PRJNA612424](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA612424).

Raw data are available in the [Supplemental Files](#).

Supplemental Information

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