MS26/CYP704B is required for anther and pollen wall development in bread wheat (Triticum aestivum L.) and combining mutations in all three homeologs causes male sterility

Manjit Singh *, Manish Kumar, Katherine Thilges, Myeong-Je Cho a, A. Mark Cigan b
DuPont Pioneer, Johnston, Iowa, United States of America

¤ a Current address: University of California, Berkeley, California, United States of America
¤ b Current address: Genus, DeForest, Wisconsin, United States of America
* manjit.singh@pioneer.com

Introduction
Flowering plants have developed specialized structures for the production of male and female gametes. Successful production of male gametes relies on proper formation of male reproductive organs. Pollen grains (microgametophytes) are formed by the anther, the male reproductive organ, and deliver male gametes to organs bearing female gametes. Pollen grains are surrounded by protective pollen walls, intine and exine, to enable survival of pollen in what
supported the decision to publish. The specific roles of these authors are articulated in the ‘author contributions’ section.

**Competing interests:** Manjit Singh, Manish Kumar, Katherine Thilges, Myeong-Je Cho, and A. Mark Cigan are or were employed by DuPont Pioneer. MS and AMC are inventors on pending patent applications on this work and are current or former employees of DuPont Pioneer who owns the pending patent applications PCT/US2015/065768 and provisional patent application number, 62/092,604: Restoration of Male Fertility in Wheat. Materials reported in this paper may be subject to third party ownership and/or to governmental regulations. Availability of materials reported in this paper to academic investigators for non-commercial research purposes under an applicable material transfer agreement will be subject to the requisite permission from any third-party owners of all or parts of the materials and to governmental regulation considerations. Obtaining the applicable permission from such third-party owners will be the responsibility of the requestor. Transgenic materials reported in this paper would only be made available if in full accordance with all applicable governmental regulations. This does not alter our adherence to all the PLOS ONE policies on sharing data, and sharing materials (subject to the above), as detailed online in the guide for authors.

are often adverse environmental conditions. The intine is composed of cellulosic material whereas the major component of exine is sporopollenin (reviewed in Quilichini et al. [1]). Sporopollenin fortifies the exine as the foundation of a skeletal structure as well as through formation of a durable covering. The components of the exine are synthesized by the surrounding tapetum and deposited on the surface of developing microspores within the anther locule [2], whereas the components of the intine are believed to be generated by the microspore vegetative cell [3]. Pollen exine in rice consists of two layers, the tectum (sexine and baculae) and the nexine (foot-layer). Exine development starts with the formation of primexine at tetrad stage (reviewed in Li and Zhang [4]). Primexine, a microfibrillar matrix composed mainly of cellulose, serves as a template for deposition of sporopollenin precursors. Following the release of microspores from tetrad, the tectum is formed on the primexine through deposition of sporopollenin precursors. With the progression in development, the sporopollenin is gradually deposited to thicken and consolidate the exine. Although a detailed biochemical analysis of sporopollenin has proven difficult, it is known to contain phenolics and polyhydroxylated aliphatics, covalently coupled by ether and ester bonds [5–8].

Recently, significant advances have been made in understanding the genes involved in pollen exine formation, including sporopollenin biosynthesis, in Arabidopsis and rice (reviewed in Zhang et al. [9]; Gomez et al. [10]). Several genes involved in synthesis of fatty acid precursors of sporopollenin have been identified [11]. Subfamilies CYP703A and CYP704 of cytochrome P450s have an essential role in hydroxylating the fatty acid constituents of predicted sporopollenin precursors. CYP703As catalyze the in-chain hydroxylation of fatty acids and heterologous CYP703A2 protein from Arabidopsis can catalyze in-chain hydroxylase of fatty acids with chain length from C10 to C16 [12]. Conversely, rice CYP703A3 has been shown to hydroxylase only lauric acid, preferably generating 7-hydroxylated lauric acid [13]. CYP703A3 in rice is required for the development of anther cuticle and pollen exine. The Arabidopsis CYP704B1 catalyzes the in-chain and ω-hydroxylation of fatty acids and is essential for exine biosynthesis [14]. Pollen from cyp704b1 mutant plants lack normal exine, but remain viable and capable of fertilization [14]. The rice OsCYP704B2 gene encodes a long-chain fatty acid hydroxylase capable of metabolizing very similar substrates in vitro as CYP704B1. Pollen grains in cyp704B2 mutants also lack a detectable exine resulting in male sterility. Anthers in these mutants have a defective tapetal layer and undeveloped cuticle [15]. The maize Ms26 gene encodes a cytochrome P450 mono-oxygenase enzyme (CYP704B1) [16, 17] and microspores in ms26 mutants have a defective exine characterized by lack of sporopollenin deposition [18]. Similarly, sorghum mutants lacking functional Ms26/CYP704B are male sterile due to defects in microspore development [19].

The conserved role of MS26/CYP704B in different species provided an opportunity to investigate its role in wheat. Bread wheat (Triticum aestivum L.) is an allohexaploid (2n = 6x = 42) combining ancestral genomes of Triticum urartu, Aegilops speltoides, and Aegilops taushii, contributing the A-, B-, and D-genomes, respectively [20, 21]. Gene expression in bread wheat is often characterized by asymmetrical contribution of the three homoeologous genomes as a result of genetic and epigenetic changes following polyploidization [22–24]. Functional analysis of the homologs of Ms26/CYP704B genes can be important for understanding the role of these genes in wheat reproductive development. Generation of multiple recessive mutant alleles in the wheat homeologs of Ms26/CYP704B gene through a custom-designed homing endonuclease was previously reported [19]. In this study, functional and cytological analyses of these mutants and their combinations were performed. Results indicate the importance of all three homeologs towards male fertility albeit with underlying differences. Apart from elucidating the function of male fertility genes, Ms26/CYP704B mutant alleles provide a novel
source of nuclear male sterility in wheat that can be utilized for the development of hybrid seed production systems and exploitation of heterosis.

Results

Homeologs of TaMs26 are highly similar to grass Ms26/CYP704B genes and are specifically expressed in anthers

To compare the wheat genes homologous to Ms26/CYP704B, genomic sequences corresponding to the three homeologs were obtained through BLAST (Basic Local Alignment Research Tool; [25]) of the TGACv1 wheat genome assembly (T. aestivum cv. Chinese Spring) (http://plants.ensembl.org/Triticum_aestivum/Info/Annotation) with maize Ms26 mRNA sequence (accession NM_001137176). Sequence analysis showed that each of the wheat homeologs have four predicted exons and three introns with a combined exon-intron length of 1926 bp for A-genome and 1920 bp for B- and D-genomes (S1 Fig). These genes represent coding DNA sequences (CDS) of 1665 bp for A-genome homeolog, and 1656 bp for B- and D-genome homeologs, which are capable of encoding for proteins of 554 and 551 amino acids in length, respectively. Comparison of the predicted amino acid sequences showed that the three homeologs are highly similar with 98–99% amino acid identity (Fig 1). The wheat Ms26/CYP704B proteins also show high similarity (96–97% identity) to putative Ms26/CYP704B from barley (BAK08270), while identity with Ms26/CYP704B proteins from rice (XP_015629295.1), maize (NP_011130648.1), sorghum (XP_002465796), and Brachypodium (XP_003558727.1) was 88–89% (Fig 1). Given the high sequence identity of the wheat genes to other grass genes orthologous to the Ms26/CYP704B maize and rice genes, wheat Ms26/CYP704B from here onwards is denoted as TaMs26; and TaMs26 homeologs for A-, B-, and D-genome are denoted as TaMs26-A, TaMs26-B and TaMs26-D, respectively.

To study the RNA expression of the wheat TaMs26 homeologs, RT-PCR was performed with primers located in Exon 4 sequence homologous to all three genomes. qRT-PCR and semi-quantitative RT-PCR revealed that the wheat TaMs26 homeologs are expressed in the

Fig 1. Comparison of MS26 protein sequence across grass species. Sequence differences in rice (XP_015629295.1), wheat (A-, B-, and D-genomes), sorghum (XP_002465796), barley (BAK08270), and Brachypodium (Brachy; XP_003558727.1) as compared to maize MS26 protein (NM_001137176) are indicated as underlined amino acids. A gap in sequence is indicated by a hyphen (‘-’). Within the consensus sequence, sequence corresponding to the 5’ end of exon 4 and the Ems26+ recognition site is highlighted in gray and yellow, respectively. The haem-binding loop is sequence boxed in red.

https://doi.org/10.1371/journal.pone.0177632.g001
anthers from tetrad (late meiosis II) to early uninucleate microspore stages (Fig 2 and S2 Fig) with maximum expression at the uninucleate microspore stage. No expression was detected in the anthers at pre-meiotic and late uninucleate microspore stages or in the ovary, leaf, and root tissues. To estimate the distribution of RNA transcripts from each homeolocus, Illumina deep sequencing was performed on the RT-PCR product from the early uninucleate stage. The number of sequence reads obtained corresponding to $TaMs26-A$, $TaMs26-B$, and $TaMs26-D$ was 256,228 (33%), 282,829 (37%), and 231,026 (30%), respectively, out of 770,083 sequence reads; thus indicating that all three homeologs were expressed in the anthers at comparable levels.

Two wild-type alleles of $TaMs26$ are required for maintenance of male fertility

To analyze the functional contribution of $TaMs26$ to pollen development in wheat, previously generated single recessive mutant alleles in the three homeologs [19] were utilized. For simplicity, homozygous mutations in A-, B- and D-homeologs are denoted as $TaMs26-a$, $TaMs26-b$ and $TaMs26-d$, while heterozygous mutations are denoted as $TaMs26-Aa$, $TaMs26-Bb$ and $TaMs26-Dd$, respectively. Plants containing five non-identical mutations, i) 4 bp $\Delta$ in $TaMs26-A$, ii) 23 bp $\Delta$ in $TaMs26-B$, iii) 81 bp $\Delta$ in $TaMs26-B$, iv) 90 bp $\Delta$ $TaMs26-D$, and v) 96 bp $\Delta$ + 2 bp I in $TaMs26-D$, were used for crossing experiments and further analysis. To track the different alleles, PCR assays with genome-specific primers were designed to genotype mutations in $TaMs26-A$, $TaMs26-B$, and $TaMs26-D$ (see Materials and methods). Previous analysis of single mutants showed that plants homozygous for mutations in any one of the $TaMs26-a$, $TaMs26-b$, or $TaMs26-d$ homeologs were male fertile and capable of generating self-pollinated seed similar to wild-type plants [19]. These results suggested that no single $TaMs26$ homeolog was functionally dominant and that a combination of two or all three homeologs contributed towards male fertility. To examine the impact of homozygous mutations in two genomes on male fertility, $TaMs26-a$, $TaMs26-b$, or $TaMs26-d$ mutations were combined through conventional crossing. As shown in Table 1, double homozygous mutant pairs were generated in combinations that retained a wild-type genome for $TaMs26-A$, $TaMs26-B$ or $TaMs26-D$. These double mutants showed normal vegetative and floral growth and were male fertile, generating viable pollen as indicated by seed set on these plants that was comparable to wild-type plants (Table 1). This data demonstrated that plants wild-type for
TaMs26 in any one genome were competent to maintain male fertility. Moreover, plants that contained a homozygous mutation in one genome and a heterozygous wild-type allele in each of the other two genomes were also male fertile; for example, plants that contained homozygous 4 bp Δ, 81 bp Δ and 90 bp Δ in TaMs26-A, TaMs26-B and TaMs26-D, respectively, were male fertile and set seed similar to wild-type plants. This observation demonstrated that two wild-type Ms26/CYP704B alleles derived either from a single genome or from two different genomes were sufficient for male fertility in wheat.

Triple recessive mutations in TaMs26 result in male sterility

Triple homozygous mutants (Tams26-abd) were generated through conventional crossing to examine male fertility in the absence of a functional copy of TaMs26. Individual plants containing double homozygous TaMs26 mutants were crossed with plants containing a single homozygous TaMs26 mutation. Utilizing the above mentioned five deletions in A-, B- and D-genomes, three sets of triple mutants were generated i) A-4 bp Δ, B-23 bp Δ, D-90 bp Δ; ii) A-4 bp Δ, B-81 bp Δ, D-90 bp Δ; iii) A-4 bp Δ, B-23 bp Δ, D-96 bp Δ + 2 bp Δ. Plants heterozygous for triple mutations (TaMs26-AaBbDd) were allowed to self-pollinate and progeny plants homozygous, heterozygous, and wild-type for each of the three mutations were identified. These plants were evaluated for vegetative and reproductive phenotypes. All triple homozygous mutants (Tams26-abd) were similar to wild-type plants with no obvious differences in vegetative growth characteristics or flowering characteristics (Fig 3A and 3B). Flowers from the Tams26-abd plants contained a high density of vacuolate microspores, while flowers from a wild-type plant contained only a few vestigial microspores. These vestigial microspores were shrunken and appeared to have a deformed pollen wall compared to pollen from wild-type plants when examined by scanning electron microscopy (SEM) (Fig 4).

As has been shown in rice, CYP704B2 also has a role in the formation of anther cuticle. Unlike wild-type anthers, cyp704b2 mutant anthers exhibit abnormal epidermal cuticle formation which is smooth and glossy [15]. To investigate whether TaMs26 is similarly involved in anther cuticle formation, the anther epidermis at vacuolate stage of microspore development in the Tams26

Table 1. Male fertility of double and triple TaMs26 mutants.

| Genotype of TaMs26 homeologs | TaMs26-A | TaMs26-B | TaMs26-D | Male Fertility |
|-----------------------------|----------|----------|----------|---------------|
| TaMs26-Abd                  | WT       | 9 bp Δ   | 96 bp Δ + 2bp l | Fertile       |
| TaMs26-Abd                  | WT       | 81 bp Δ  | 96 bp Δ + 2bp l | Fertile       |
| TaMs26-aBd                  | 4 bp Δ   | WT       | 96 bp Δ + 2bp l | Fertile       |
| TaMs26-abD                  | 4 bp Δ   | 81 bp Δ  | WT        | Fertile       |
| TaMs26-abD                  | 4 bp Δ   | 23 bp Δ  | WT        | Fertile       |
| TaMs26-aBbDd                | 4 bp Δ   | WT: 81 bp Δ | WT: 90 bp Δ | Fertile       |
| TaMs26-AabDd                | WT: 4 bp Δ | 81 bp Δ | WT: 90 bp Δ | Fertile       |
| TaMs26-AaBbd                | WT: 4 bp Δ | WT: 81 bp Δ | 90 bp Δ | Fertile       |

TaMs26 allele represented by uppercase letter (A, B, D) and TaMs26 mutant allele represented by lowercase letter (a, b, d).

Plants with an average seed set >100 were scored as fertile.

https://doi.org/10.1371/journal.pone.0177632.t001
mutants was analyzed by SEM. In contrast to the epidermis of anthers from wild-type plants, which have well-developed cuticular and epicuticular structures (Fig 4A), the epidermal wall of anthers from \( \text{Tams26-abd} \) mutants was smooth and devoid of these cuticular structures (Fig 4C). These observations demonstrated that, like rice, \( \text{TaMs26} \) has a central role both in anther epidermis and pollen wall development in wheat.

One wild-type \( \text{TaMs26} \) allele is insufficient for maintaining male fertility

Interestingly, in addition to the triple homozygous mutants, anther structure and pollen viability was also affected in double homozygous-single heterozygous plants. Despite the presence of a single functional allele, seed number was severely reduced in these plants when compared to wild-type plants (Table 2). Seed set in these mutant plants ranged from 0–32 seeds per plant and was dependent upon which genome contributed the wild-type allele. The lowest seed set was observed in \( \text{Tams26-aBbd} \) mutants and highest in \( \text{Tams26-abDd} \) mutants (Table 2). These observed differences in genome contribution were more readily apparent when microspore development was analyzed in these plants. The most severe pollen phenotype was observed in \( \text{Tams26-aBbd} \) plants, which contained anthers that had severely collapsed and translucent microspores (Fig 3G). The least severe phenotype was observed in anthers from \( \text{Tams26-abDd} \) plants, which contained numerous normal-looking microspores and a nearly equal number of collapsed and shrunken microspores (Fig 3H). The pollen phenotype in \( \text{Tams26-Aabd} \) plants was intermediate to that of \( \text{Tams26-aBbd} \), and \( \text{Tams26-abDd} \) plants (Fig 3F). The cuticle phenotype in the double homozygous-single heterozygous mutant plants also mirrored the observations on microspore phenotype. Anthers in \( \text{Tams26-aBbd} \) plants exhibited the most severe defects and were very similar to those from \( \text{Tams26-abd} \) plants in smoothness of epidermal surface (S3 Fig) while anthers from \( \text{Tams26-abDd} \) plants exhibited the least severe phenotype and closely resembled the wild-type anthers (S3 Fig). The observed differences in anther wall and microspore morphology parallel the seed set across the different single heterozygous \( \text{TaMs26} \) genomes. Together, these results clearly demonstrate that one wild-type \( \text{TaMs26} \) allele
is insufficient to maintain male fertility in wheat and the alleles from three genomes are functionally non-equivalent.

Complementation of triple homozygous mutants requires two Ms26/CYP704B genes

To further ascertain that the male sterility in Tams26-abd plants was directly due to mutations in TaMs26 genes, complementation was tested with the maize Ms26 gene. A copy of the maize Ms26 gene (also referred to as ZmMs26) under control of the native maize Ms26 promoter was introduced into wild-type wheat plants (cv. Fielder) using Agrobacterium-mediated transformation. Two independent single-copy T-DNA insertions were identified by molecular analyses and crossed as females with pollen from wheat plants heterozygous for the TaMs26-A 4 bp Δ, TaMs26-B 81 bp Δ, and TaMs26-D 96 bp Δ + 2bp I mutant alleles. Progeny of these crosses was screened to confirm the presence of ZmMs26 and TaMs26 mutant alleles, and allowed to

Fig 4. Scanning electron microscopy of anther epidermis and microspores at late vacuolate stage of microspore development. Wild type (A and B), Tams26-abd (C and D), and Tams26-abd/ZmMs26-OsMs26 (E and F) plants. Scale bars = 20 μm.

https://doi.org/10.1371/journal.pone.0177632.g004
self-pollinate. F2 seed from this generation were planted and progeny genotyped for the introduced ZmMs26 gene and TaMs26 mutant alleles. Seed from these individual plants was counted as a qualitative measure of male fertility. As shown in Tables 2 and 3, in contrast to the low or no seed set observed with double homozygous-single heterozygous mutants, seed set was restored to wild-type levels when these plants contained a transformed copy of ZmMs26. However, ZmMs26 was unable to restore male fertility to Tams26-abd mutants when present as a single copy, and no seed set was observed from plants containing either of the two independent ZmMs26 insertions (Table 3).

When coupled with the observation of male fertility in the double homozygous mutants and the single homozygous-double heterozygous mutants, observations from single gene complementation strongly suggested that two copies of the Ms26/CYP704B gene would be required to complement triple homozygous mutants. To test this hypothesis, the rice CYP704B2 gene (also referred as OsMs26) was added to a T-DNA expression cassette containing the ZmMs26 gene. Two Ms26 orthologs from maize and rice were selected based on the demonstration of function of these genes in previous studies [15, 19]. This T-DNA expression cassette was transformed into double homozygous mutants (TaMs26-Abd), which were then crossed with A-genome homozygous mutants (TaMs26-aBbDd) to generate a population of triple

### Table 2. Male fertility of triple TaMs26 mutants as determined by seed set.

| Genotype of TaMs26 homeologs | Plants | Total Seed | Seed per Plant |
|-----------------------------|-------|------------|---------------|
|                             | Plants | Total Seed | Seed per Plant |
| Tams26-abd                  | 17     | 4          | 0.25          | 0-3 |
| Tams26-Aabd                 | 37     | 210        | 5.7           | 0-13 |
| Tams26-aBbd                 | 24     | 33         | 1.4           | 0-5 |
| Tams26-abDd                 | 19     | 296        | 15.6          | 7-32 |
| TaMs26-ABD                  | 3      | 444        | 148.0         | 121-186 |

| Plants | Total Seed | Seed per Plant |
|-------|------------|---------------|
| Plants | Total Seed | Seed per Plant |
| Tams26-abd | 17 | 4 | 0.25 | 0-3 |
| Tams26-Aabd | 37 | 210 | 5.7 | 0-13 |
| Tams26-aBbd | 24 | 33 | 1.4 | 0-5 |
| Tams26-abDd | 19 | 296 | 15.6 | 7-32 |
| TaMs26-ABD | 3 | 444 | 148.0 | 121-186 |

| Plants | Total Seed | Seed per Plant |
|-------|------------|---------------|
| Plants | Total Seed | Seed per Plant |
| Tams26-abd | 17 | 4 | 0.25 | 0-3 |
| Tams26-Aabd | 37 | 210 | 5.7 | 0-13 |
| Tams26-aBbd | 24 | 33 | 1.4 | 0-5 |
| Tams26-abDd | 19 | 296 | 15.6 | 7-32 |
| TaMs26-ABD | 3 | 444 | 148.0 | 121-186 |

WT TaMs26 allele represented by uppercase letter (A, B, D) and TaMs26 mutant allele represented by lowercase letter (a, b, d). Results from three combinations of TaMs26 mutations in A-, B-, and D-genomes (i) TaMs26-a 4bp Δ, TaMs26-b 81 bp Δ, and TaMs26-d 96 bp Δ; (ii) TaMs26-a 4bp Δ, TaMs26-b 23 bp Δ, and TaMs26-d 90 bp Δ; and (iii) TaMs26-a 4bp Δ, TaMs26-b 81 bp Δ, and TaMs26-d 96 bp Δ + 2 bp I; Δ, deletion, and I, insertion.

Heads were not bagged. We observed negligible cross pollination in greenhouse conditions; however, residual pollination was possible.

https://doi.org/10.1371/journal.pone.0177632.t002

### Table 3. Complementation of TaMs26 triple recessive mutants.

| Genotype of TaMs26 homeologs | Complementing gene(s) | Plants | Total Seed | Seed per Plant | Male fertile | Male sterile |
|-----------------------------|-----------------------|-------|------------|---------------|--------------|--------------|
| Tams26-Aabd                 | ZmMs26                | 8     | 503        | 62.9          | 8            | 0            |
| Tams26-aBbd                 | ZmMs26                | 5     | 509        | 101.8         | 5            | 0            |
| Tams26-abDd                 | ZmMs26                | 5     | 540        | 108.0         | 5            | 0            |
| Tams26-abd                  | ZmMs26                | 5     | 0          | 0.0           | 0            | 5            |
| Tams26-abd                  | ZmMs26 + OsMs26       | 18    | 1721       | 95.6          | 18           | 0            |
| Tams26-abd                  | N/A                   | 19    | 0          | 0.0           | 0            | 19           |

WT TaMs26 allele represented by uppercase letter (A, B, D) and TaMs26 mutant allele represented by lowercase letter (a, b, d). Results from combination of TaMs26 mutations in A-, B-, and D-genomes: TaMs26-a 4bp Δ, TaMs26-b 81 bp Δ, and TaMs26-d 96 bp Δ + 2 bp I; Δ, deletion.

| Complementing gene(s) | Plants | Total Seed | Seed per Plant | Male fertile | Male sterile |
|-----------------------|-------|------------|---------------|--------------|--------------|
| Complementing gene(s) | Plants | Total Seed | Seed per Plant | Male fertile | Male sterile |
| ZmMs26                | 8     | 503        | 62.9          | 8            | 0            |
| ZmMs26                | 5     | 509        | 101.8         | 5            | 0            |
| ZmMs26                | 5     | 540        | 108.0         | 5            | 0            |
| ZmMs26 + OsMs26       | 18    | 1721       | 95.6          | 18           | 0            |
| N/A                   | 19    | 0          | 0.0           | 0            | 19           |

ZmMs26, maize Ms26; OsMs26, rice Ms26; and N/A, not applicable.

Heads were not bagged. We observed negligible cross pollination in greenhouse conditions; however, residual pollination was possible.

https://doi.org/10.1371/journal.pone.0177632.t003
homzygous mutants with and without the ZmMs26-OsMs26 expression cassette. In total, 37 triple homozygous Tams26-abd mutant plants were analyzed, 18 with one copy of ZmMs26-OsMs26 and 19 lacking the T-DNA expression cassette. All 18 Tams26-abd plants that carried ZmMs26-OsMs26 were male fertile with seed set comparable to wild-type plants and all 19 plants without the T-DNA expression cassette were male sterile (Table 3). It was observed that both the anther epidermis and pollen wall phenotypes were restored to the wild-type phenotype in the plants carrying the ZmMs26-OsMs26 expression cassette (Fig 4E and 4F). Taken together, these results indicated that two functional alleles of Ms26/CYP704B gene are required for anther and pollen development in wheat.

**Discussion**

*Ms26/CYP704B* genes show high conservation of structure and function in wheat and other grasses

Sporopollenin, the main constituent of pollen exine is highly conserved both in structure and composition. Orthologs of the sporopollenin biosynthesis pathway, including Acos5, PksA, PksB, CYP703A and CYP704B, are conserved among flowering plants but are not present in green algae [10]. Several studies for expression and functional analysis of these genes support their conserved role. The *Physcomitrella patens* PpASCL is a functional ortholog of *Arabidopsis* PKSA as it is capable of hydroxyalkylpyrone synthase activity with in vitro preference for hydroxyl fatty acyl-CoA esters [26]. The expression profile of the CYP704B gene family in plant species from bryophytes to angiosperms is conserved among flowering plants but are not present in green algae [10]. Similar to *Arabidopsis*, rice and maize Ms26/CYP704B orthologs that exhibit male reproductive tissue preferential expression [14–16], the expression pattern of *TaMs26* homeologs is anther-specific. Furthermore, all three *TaMs26* homeologs were observed to be expressed at comparable levels implying that each allele contributes to CYP704B function during pollen development in wheat. The role of *MS26/CYP704B* orthologs in pollen exine formation has also been functionally demonstrated across plants. *Arabidopsis*, rice, and maize mutants of *Ms26/CYP704B* orthologs have been shown to have defects in pollen exine structure [14, 15, 18]. Similarly, the triple homozygous wheat (TaMs26-abd) mutants were male sterile due to defects in microspore morphology.

Interestingly, compared to wheat and other grass species, the *Arabidopsis* cyp704B1 mutants exhibit a milder phenotype. Pollen in cyp704B1 mutant plants lack a normal exine but remain viable and can set seed upon self-pollination [14]; whereas *Ms26/CYP704B* mutants in wheat, rice, maize, and sorghum are male sterile. In addition to microspore exine defects, wheat Tams26 and rice cyp704B2 mutants also exhibit defects in anther epidermis [15]. Although it is not known if anther epidermis is similarly affected in *Arabidopsis* cyp704B1 mutants, it is possible that the male sterility in wheat and rice (and possibly maize and sorghum) is due to a combination of anther epidermis and pollen exine defects. If so, then the CYP704B2 genes in wheat and rice (and other grasses) could have assumed a more central role by contributing not only to pollen exine formation but also to anther epidermis formation. In *Arabidopsis*, other members of the CYP86 clan such as those involved in cutin formation [29, 30] may contribute towards anther epidermis development.

**All three TaMs26 homeologs contribute towards male fertility but with underlying differences in contribution**

Bread wheat is an allohexaploid that originated from two hybridization events involving three progenitor species [20, 21]. Such inter-specific hybridization events are generally accompanied...
by genetic or epigenetic resetting to orchestrate gene expression from the complex allopoly- 
ploid genome [31]. Resetting can result in genomic asymmetry or expression of individual 
genes from specific genomes and can lead to partial or complete genome dominance (reviewed 
in Madlung and Wendel [32]). In wheat, such partial or complete genome dominance is evi-
dent for several traits including free threshing phenotype and brittle rachis which are related 
to crop domestication [20]. Analysis of TaMs26 double homozygous and triple homoyzogous 
mutants suggested functional equivalency of all three TaMs26 homeologs. Ms26/CYP704B 
genes have also been analyzed in Brassica napus, a tetraploid species, and it was observed that a 
double knockout in duplicate functionally redundant genes, BnMs1 (BnCYP704B1-1) and 
BnMs2 (BnCYP704B1-1), is required to obtain complete loss of function, which results in male 
sterility. However, in wheat, the analysis of the double homozygous-single heterozygous 
mutants revealed noticeable differences in the contribution of each genome. Pollen and anther 
phenotypes, and consequently seed set, of these mutants were dependent on the genome that 
contributed the heterozygous allele. The fertility of double homozygous-single heterozygous 
mutants was highest when the heterozygous allele was contributed by D-genome (Tams26-
abDd). These observations suggested that the function of Ms26/CYP704B in Tams26-abDd 
plants was likely reduced but still significant. On the other hand, anther and pollen phenotypes 
as well as seed set were lowest when the heterozygous allele was contributed by the B-genome 
(Tams26-aBbd). When analyzed as a single wild-type allele in a background of five mutant 
alleles there are noticeable functional differences between the three TaMs26 homeologs. These 
differences suggest dominance, although subtle, of the D-genome over A- and B-genomes for 
this gene. In contrast, it was observed in Brassica napus that the genotypes BnMs1ms1ms2ms2 
and Bnms1ms1Ms2ms2 exhibit wild-type male fertility phenotype [33, 34]. Transcript counts 
for the three wheat homeologs in anthers showed comparable levels, suggesting that the differ-
ences observed in phenotype were not due to differential transcription of the homeologs. 
These can likely be attributed to post-transcriptional regulation. Differences in protein 
sequences can also lead variable enzymatic activity and although the predicted amino acid 
sequences of the three homeologs show 98–99% amino acid identity, several differences in 
amino acid sequence exist that can have a role in protein function.

Two functional alleles of CYP704B/Ms26 are required for male fertility in wheat

Analyses of double and triple homozygous TaMs26 mutants suggested that two wild-type 
TaMs26 alleles are absolutely required for male fertility. These two wild-type alleles can be 
contributed by a single genome or two different genomes. The complementation experiments 
with one and two heterologous copies of Ms26/CYP704B genes further augmented this conclu-
sion. When one copy of ZmMs26 gene was introduced into a background of triple homozyo-
gous mutants (Tams26-abd), the heterologous gene was unable to restore male fertility. 
However, when one copy of ZmMs26 gene was introduced into a background of double homozyo-
gous-single heterozygous mutants, male fertility was completely restored irrespective of 
which genome contributed the heterozygous alleles. This observation was further borne out by 
the requirement of two exogenously supplied Ms26 genes, ZmMs26 and OsMs26, to restore fer-
tility to Tams26-abd mutant plants. Under the conditions tested in this report, it is evident that 
two functional alleles of an Ms26/CYP704B gene are required for male fertility in wheat 
whether derived from two endogenous wild-type alleles or orthologous genes from different 
species.

Previous studies have shown that a single copy of ZmMs26 gene can complement ms26/
ms26 mutants in maize and sorghum, both diploid species [16, 19]. While a single wild-type
allele of Ms26/CYP704B gene is sufficient for normal function in diploid species, data presented here indicates that hexaploid wheat has evolved to require at least two functional alleles. It appears that due to the presence of six functional alleles, the polyploid wheat has evolved to require a higher dosage of MS26/CYP704B as compared to diploid species. Interestingly, in the case of Ms1 gene, another male fertility gene in wheat, the existing data suggests that only the B-genome homeolog has functional relevance. In certain genetic backgrounds, homozygous recessive mutations in the B-genome homeolog resulted in complete male sterility [35]. While Ms1 functions as a gene in diploid species and heterozygotes (Ms1/ms1) are male fertile, TaMs26 likely represents the other end of the spectrum where all three genomes contribute towards a phenotype, but the minimum requirement for male fertility is two wild-type alleles.

**Conclusion**

Knowledge of genes contributing to reproductive development in wheat has lagged other plant species as polyploidy in wheat makes it less amenable for functional genomics using traditional approaches. However, the development of new biological tools has facilitated the genesis of functional analysis of genes in wheat [36, 37]. In addition to new advances in DNA sequencing, DNA double-strand break (DSB) technologies have provided efficient methods to obtain multiple mutant alleles in the different wheat genomes. Using a custom designed homing endonuclease, mutations were previously generated in each of the three homeologs of the wheat TaMs26 gene [19]. These mutants allowed for the characterization of their gene products to further the understanding of anther and pollen development in wheat. Moreover, the generation of mutant alleles and the ability to restore fertility using orthologous Ms26 genes sets the foundation for the development of a TaMs26-based male fertility control system for hybrid seed production and exploitation of heterosis to increase wheat yields.

**Materials and methods**

**Plant material and growth conditions**

The TaMs26 mutant stocks were derived from the single-genome mutations generated by Cigan et al. [19]. Plants were grown in the greenhouse at DuPont Pioneer research facilities in Johnston, Iowa. The greenhouse conditions were set to 22˚C and 20˚C average day and night temperatures, respectively, with 30% humidity and 16 hr day length period. Standard plant care practices were followed including fertilizer and pesticide applications.

**DNA and protein sequence analysis of TaMs26 homeologs**

To obtain complete genomic sequence for wheat A-, B- and D-homeologs of Ms26/CYP704B, TGACv1 (The Genome Analysis Centre) sequence database was BLAST (Basic Local Alignment Research Tool; Altschul et al. [25]) searched with the maize Ms26 mRNA sequence (accession NM_001137176). TaMs26 sequences were annotated for exons, introns, and CDS and translated into predicted amino acids based on maize sequence. Sequence comparisons and alignments were performed with DNA analysis software: Sequencher (Genecodes) and Vector NTI (Life Technologies Inc.). Sequences of sorghum (XP_002465796), rice (XP_015629295.1), barley (BAK08270), and Brachypodium (XP_003558727.1) orthologs of maize Ms26 were obtained from the NCBI database.

**Genome-specific PCR assay and sequence analysis**

For amplification of TaMs26 genomic fragments, DNA from *T. aestivum* (cv. Fielder) was extracted from four small leaf punches (0.5 cm in diameter) as described in Gao et al. [38].
PCR was performed using Phusion™ High Fidelity PCR Master Mix (New England Biolabs Inc.) according to the manufacturer’s recommendations. PCR amplified fragments were also subcloned for DNA sequence analysis using the pCR2.1-TOPO cloning vector (Life Technologies Inc.). A genomic fragment corresponding to TaMs26 Exon 4 was amplified using primer pair UNIMS26 5'-2 and UNIMS26 3'-1 (S1 Table), which are conserved across the three genomes. Genome-specific fragments were amplified using one genome-specific primer and a second UNIMS primer. A-genome fragment was amplified with primers TAMS26-A and UNIMS26 3'-1; B-genome fragment was amplified with primers TAMS26-B and UNIMS26 5'-2; and D-genome fragment was amplified with primers TAMS26-D and UNIMS26 5'-2 (S1 Table). For genotyping larger deletions (TaMs26-B 81 bp Δ, TaMs26-D 90 bp Δ, and TaMs26-D 96 bp Δ+ 2 bp I), corresponding fragments were amplified with genome-specific primers and size fractionated on 2% agarose gel. Smaller deletions (TaMs26-A 4 bp Δ and TaMs26-B 23 bp Δ) were amplified with corresponding genome-specific primers and part of the amplification product digested with BsiWI, the DNA restriction enzyme that recognizes the sequence 5′-CGTACG-3′, as described in Djukanovic et al. [17]. Products of these reactions were electrophoresed on 1% agarose gels and screened for BsiWI digestion resistant PCR products indicative of mutations at the Ms26 target site.

RT-PCR analysis and transcript counts through deep sequencing

Anthers were collected for stages corresponding to pre-meiotic stage, meiosis II (tetrad) stage, early uninucleate microspores, and late uninucleate microspores. Ovaries were collected at stages corresponding to meiosis and gametogenesis. Leaf and root tissue were collected at seedling stage. RNA was extracted using TRIZOL reagent (Invitrogen Inc.) following manufacturer’s protocol. A total of 1 mg RNA was treated with DNase I enzyme (Invitrogen Inc.) following the manufacturer’s recommendations to remove DNA contamination. 500 ng of DNase-treated RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). The transcribed cDNA was diluted 5X in sterile water and used for PCR. Primer pair TAMS26-F and TAMS26-R was used to amplify TaMs26 transcript and primer pair TAACTIN-F and TAACTIN-R was used to amplify TaActin, and products were analyzed by gel electrophoresis. qRT-PCR was performed with PowerUp SYBR Green Master Mix (ThermoFisher Scientific) with above mentioned primer pairs on a ViiA™7 System (ThermoFisher Scientific). Relative quantification values were determined using the difference in Ct from the TaMs26 and the reference gene (TaActin).

For transcript count by deep sequencing, RT-PCR was performed on the cDNA synthesized above with Phusion™ High Fidelity PCR Master Mix (New England Biolabs) adding on the sequences necessary for amplicon-specific barcodes and Illumina sequencing using “tailed” primers through two rounds of PCR. The primers used in the primary PCR reaction were Ms26F and Ms26R while the primers used in the secondary PCR reaction were F2 and R2. The product amplified by these primers contained two single nucleotide polymorphisms (SNPs) that could discriminate between the three homeologs. The resulting PCR products were concentrated using a Qiagen MinElute™ PCR purification spin column (QIAGEN, Inc.). A portion of the amplification products was visualized on an agarose gel and the remainder was single read 100 nt-length deep sequenced on an Illumina Genome Analyzer IIX (ELIM BioPharmaceuticals, Inc.) with a 30–40% (v/v) spike of PhiX control v3 (Illumina, Inc.) to off-set sequence bias. Two independent reactions were performed. The reads were clustered based on their SNPs and the number of reads was counted using in-house designed sequence analysis software.
Ms26 complementation constructs and transformation

To generate the ms26 fertility complementation T-DNA vector, the Ms45 gene in PHP24490 [39] was replaced by a 3.9 kb DNA fragment [Chromosome 1, nt 4509689–14506399 Maize (B73) RefGen_v2 (MGSC)], which contained the wild-type maize Ms26 gene (also referred to as ZmMs26). The T-DNA expression cassette also carried an α-amylase gene under the control of pollen-specific PG47 promoter along with DsRed gene regulated by a seed-specific promoter as the transformation selectable marker. The α-amylase prevented the pollen-specific transmission of the ZmMs26 gene and resulted in 50:50 segregation of ZmMs26 in transformed plants. The final T-DNA expression cassette, pPG47::Bt1:ZmAA1//Ms26//35 SEN-pEND2::DsRed2, was introduced into Agrobacterium strain LBA4404 as described above. To make the construct with two Ms26 orthologous genes, a 3.2.0 kb genomic fragment of the rice Ms26/CYP704B gene (LOC_Os03g07250.1; chromosome 3 nt 3701612–3704874, Os-Nipponbare ref IRGSP-1.0MSU Rice Genome Annotation Project) including approximately 1.0 kb fragment 5’ of the ATG translation start site and 0.5 kb 3’ of the translation stop site, was amplified from Orzya sativa L. ssp. Japonica, cv. Kitaake, with primers OSMS26-F and OSMS26-R. Amplification and sequencing was performed as described for the wheat TaMs26 genomic fragments. The rice Ms26 ortholog (also referred to as OsMs26) was then cloned into the above mentioned vector, which contained the ZmMs26 gene. The T-DNA expression cassette was transformed into T. aestivum (cv. Fielder) containing homozygous Tams26- a and b mutations as described in Cigan et al. [19]. Transformed plants were crossed with pollen from Tams26aBbd plants and progeny analyzed (see S4 Fig for crossing strategy). Plants transformed with ZmMs26 and ZmMs26-OsMs26 constructs were genotyped with the ZMMS26 TERM primer pair and OSMS26PRO primer pair, respectively (S1 Table).

Microscopy

For scanning electron microscopy (SEM), anthers and pollen were fixed in FAA (formalin, acetic acid, EtOH) and dehydrated in a graded EtOH series (50%, 75%, 95%, 100%). At this point, the samples were critical point dried using liquid carbon dioxide (Denton Vacuum). Anthers and pollen were mounted on aluminum stubs with double-sided sticky tabs, and surrounded with silver paint. The samples were then sputter-coated (Denton Vacuum) with palladium-gold (60:40) and observed using a JEOL 5800 scanning electron microscope (JEOL USA, Inc.) at 10 kV.

For confocal scanning laser microscopy (CSLM), flowers were collected and fixed in 2% paraformaldehyde: 4% glutaaraldehyde in PBS buffer and vacuum infiltrated at 10 psi to increase the penetration of fixative. Samples were then rinsed in PBS and then cleared in graded series of 2,2’-Thiodiethanol (TDE) (10%, 25%, 50%, 75%, and 97%). Samples were mounted on slides in TDE and imaged using the Leica TCS SPE CSLM (Lecia Microsystems) with 405 nm, 488 nm and 532 nm laser lines. 20 anthers from each mutant class and wild type plants were analyzed.

Supporting information

S1 Fig. Comparison of genomic sequence of TaMs26 homeologs of wheat. TaMs26 gene sequences corresponding to A-, B-, and D-genomes are shown with annotations of exons and introns. (PDF)

S2 Fig. Spatial and temporal expression of TaMs26 determined by semi-quantitative RT-PCR. TaActin expression was used as a control. P, pre-meiosis; M, meiosis II; E, early
uninucleate; L, late uninucleate; G, gametogenesis; gDNA, genomic DNA.

S3 Fig. Scanning electron microscopy of anther epidermis from double homozygous-single heterozygous Tams26 mutant wheat plants. Anther epidermis surface at late vacuolate microspore stage from wild type (A), Tams26-Aabd (B), Tams26-abDd (C), and Tams26-aBbd (D) plants. Scale bars = 20 μm.

S4 Fig. Crossing strategy to combine wheat mutations with transformed maize and rice genes for complementation testing.

S1 Table. List of primers used in the study.

Acknowledgments
The authors thank Marc Albertsen and Jim Gaffney for reviewing the manuscript and Iowa State University Microscopy and Nanoimaging Facility for the use of the JEOL 5800 scanning electron microscope. The authors also thank Tracey Fisher and Kara Califf for their assistance and valuable comments in preparation of this manuscript.

Author Contributions
Conceptualization: AMC MS.
Investigation: MS MK KT M-JC.
Writing – original draft: MS AMC.
Writing – review & editing: MS AMC.

References
1. Quilichini TD, Grienenberger E, Douglas CJ. The biosynthesis, composition and assembly of the outer pollen wall: A tough case to crack. Phytochemistry. 2015; 113: 170–182. http://dx.doi.org/10.1016/j.phytochem.2014.05.002. https://doi.org/10.1016/j.phytochem.2014.05.002 PMID: 24906292
2. Ariizumi T, Toriyama K. Genetic regulation of sporopollenin synthesis and pollen exine development. Annu Rev Plant Biol. 2011; 62: 437–460. https://doi.org/10.1146/annurev-arplant-042809-112312 PMID: 21275644
3. Hess MW. Cell-wall development in freeze-fixed pollen: Intine formation of Ledebouria socialis (Hyacinthaceae). Planta. 1993; 189(2): 139–149.
4. Li H, Zhang D. Biosynthesis of anther cuticle and pollen exine in rice. Plant Signaling & Behavior. 2010; 5(9): 1121–1123.
5. Ahlers F, Bubert H, Steuerriegel S, Wiermann R. The nature of oxygen in sporopollenin from the pollen of Typha angustifolia L. Z Naturforsch C. 2000; 55(3–4): 129–36. Epub 2000/05/19. PMID: 10817199
6. Guilford WJ, Schneider DM, Labovitz J, Opella SJ. High resolution solid state 13C NMR spectroscopy of sporopollenins from different plant taxa. Plant Physiol. 1988; 86(1): 134–136. PMID: 16665854
7. Scott RJ. Pollen exine—the sporopollenin enigma and the physics of pattern. In: Scott RJ, Stead AD, editors. Molecular and Cellular Aspects of Plant Reproduction Society for Experimental Biology Seminar Series, Volume 55. 55. Cambridge and New York: Cambridge University Press; 1994. p. 49–80.
8. Wiermann R, Ahlers F, Schmitz-Thom I. Sporopollenin. In: Steinbüchel A, Hofrichter M, editors. Biopolymers. 1. Weinheim, Germany: Wiley-VCH Verlag; 2001. p. 209–227.
9. Zhang D, Shi J, Yang X. Role of Lipid Metabolism in Plant Pollen Exine Development. In: Nakamura Y, Li-Beisson Y, editors. Lipids in Plant and Algae Development. Cham, Switzerland: Springer International Publishing; 2016. p. 315–337.
10. Gómez JF, Taille B, Wilson ZA. Anther and pollen development: A conserved developmental pathway. J Integr Plant Biol. 2015; 57(11): 876–891. https://doi.org/10.1111/jipb.12425 PMID: 26310290

11. Shi J, Cui M, Yang L, Kim Y-J, Zhang D. Genetic and Biochemical Mechanisms of Pollen Wall Development. Trends Plant Sci. 2015; 20(11): 741–753. https://doi.org/10.1016/j.tplants.2015.07.010 PMID: 26442683

12. Morant M, Jorgensen K, Schaller H, Pinot F, Moller BL, Werck-Reichhart D, et al. CYP703 is an ancient cytochrome P450 in land plants catalyzing in-chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. Plant Cell. 2007; 19(5): 1473–87. Epub 2007/05/15. https://doi.org/10.1105/tpc.106.045948 PMID: 17496121

13. Yang X, Wu D, Shi J, Pinot F, Grausen B, et al. Rice CYP703A3, a cytochrome P450 hydroxylase, is essential for development of anther cuticle and pollen exine. J Integr Plant Biol. 2014; 56(10): 979–994. https://doi.org/10.1111/jipb.12212 PMID: 24798002

14. Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, et al. CYP704B1 is a long-chain fatty acid ω-hydroxylase essential for sporopollenin synthesis in pollen of Arabidopsis. Plant Physiol. 2009; 151(2): 574–589. https://doi.org/10.1104/pp.109.144469 PMID: 19700560

15. Li H, Pinot F, Sauveplane V, Werck-Reichhart D, Diehl P, Schreiber L, et al. Cytochrome P450 family member CYP704B2 catalyzes the ω-hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell. 2010; 22(1): 173–190. https://doi.org/10.1105/tpc.109.070326 PMID: 20086189

16. Albertsen MC, Fox T, Huffman G, Trimnell M, Wu Y, Hershey H, inventors; Nucleotide sequences mediating male fertility and method of using same2009 Nov. 3, 2009.

17. Djukanovic V, Smith J, Lowe K, Yang M, Gao H, Jones S, et al. Male-sterile maize plants produced by targeted mutagenesis of the cytochrome P450-like gene (MS26) using a re-designed I-Crel homing endonuclease. Plant J. 2013; 76(5): 888–899. https://doi.org/10.1111/tpj.12335 PMID: 24112765

18. Loukides CA, Broadwater AH, Bedinger PA. Two new male-sterile mutants of Zea mays (Poaceae) with abnormal tapetal cell morphology. Am J Bot. 1995; 82(8): 1017–1023.

19. Cigan AM, Singh M, Benn G, Feigenbutz L, Kumar M, Cho M-J, et al. Targeted mutagenesis of a conserved anther-expressed P450 gene confers male sterility in monocots. Plant Biotech J. 2016; In Press: 1–11.

20. Matsuoka Y. Evolution of polyploid Triticum wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. Plant Cell Physiol. 2011; 52(5): 750–764. https://doi.org/10.1093/pcp/pcr018 PMID: 21317146

21. Petersen G, Seberg O, Yde M, Berthelsen K. Phylogenetic relationships of Triticum and Aegilops and evidence for the origin of the A, B, and D genomes of common wheat (Triticum aestivum). Mol Phylogenet Evol. 2006; 39(1): 70–82. https://doi.org/10.1016/j.ympev.2006.01.023 PMID: 16504543

22. Akhunova AR, Matniyazov RT, Liang H, Akhunov ED. Homeolog-specific transcriptional bias in allopolyploid wheat. BMC Genomics. 2010; 11: 505–520. https://doi.org/10.1186/1471-2164-11-505 PMID: 20849627

23. Brenchley R, Spannagl M, Pfeifer M, Barker GLA, D’Amore R, Allen AM, et al. Analysis of the bread-wheat genome using whole-genome shotgun sequencing. Nature. 2012; 491: 705–710. https://doi.org/10.1038/nature11650 PMID: 23192148

24. Feldman M, Levy AA, Fahima T, Korol A. Genomic asymmetry in allopolyploid plants: wheat as a model. J Exp Bot. 2012; 63(14): 5049–5059.

25. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3): 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 22317122

26. Colpitts CC, Kim SS, Posehn SE, Jepson C, Kim SY, Wiedemann G, et al. PpASC L, a moss ortholog of anther-specific chalcone synthase-like enzymes, is a hydroxalkylpyrone synthase involved in an evolutionarily conserved sporopollenin biosynthesis pathway. New Phytologist. 2011; 192(4): 855–868. https://doi.org/10.1111/j.1469-8137.2011.03858.x PMID: 21883237

27. Nelson DR, Schuler MA, Paquette SM, Werck-Reichhart D, Bak S. Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. Plant Physiol. 2004; 135: 756–772. https://doi.org/10.1104/pp.104.039826 PMID: 15208422

28. Bak S, Beissen F, Bishop G, Hamberger B, Hör f R, Paquette S, et al. Cytochromes P450. The Arabidopsis Book. 2011; 9: e0144. https://doi.org/10.1109/tab.0144 PMID: 22303269

29. Duan H, Schuyler MA. Differential expression and evolution of the Arabidopsis CY86A subfamily. Plant Physiol. 2005; 137(3): 1067–1081. Epub February 11, 2005. https://doi.org/10.1104/pp.104.055715 PMID: 15709153

30. Yeats TH, Rose JKC. The formation and function of plant cuticles. Plant Physiol. 2013; 163(1): 5–20. Epub July 26, 2013. https://doi.org/10.1104/pp.113.222737 PMID: 23893170
31. Marcusse T, Sandve SR, Heier L, Spannagl M, Pfeifer M, The International Wheat Genome Sequencing Consortium, et al. Ancient hybridizations among the ancestral genomes of bread wheat. Science. 2014; 345(6194): 1250092. https://doi.org/10.1126/science.1250092 PMID: 25035499

32. Madlung A, Wendel JF. Genetic and Epigenetic Aspects of Polyploid Evolution in Plants. Cytogenet Genome Res. 2013; 140: 270–285. https://doi.org/10.1159/000351430 PMID: 23751292

33. Lei S, Yao X, Yi B, Chen W, Ma C, Tu J, et al. Towards map-based cloning: fine mapping of a recessive genic male-sterile gene (BnMs2) in Brassica napus L. and syntenic region identification based on the Arabidopsis thaliana genome sequences. Theoretical and Applied Genetics. 2007; 115(5): 643–651. https://doi.org/10.1007/s00122-007-0594-1 PMID: 17605126

34. Yi B, Zeng F, Lei S, Chen Y, Yao X, Zhu Y, et al. Two duplicate CYP704B1-homologous genes BnMs1 and BnMs2 are required for pollen exine formation and tapetal development in Brassica napus. Plant J. 2010; 63(6): 925–938. https://doi.org/10.1111/j.1365-313X.2010.04289.x PMID: 20598092

35. Klindworth DL, Williams ND, Maan SS. Chromosomal location of genetic male sterility genes in four mutants of hexaploid wheat. Crop Sci. 2002; 42: 1447–1450.

36. Borrill P, Adamski N, Uauy C. Genomics as the key to unlocking the polyploid potential of wheat. New Phytol. 2015; 208(4): 1008–22. https://doi.org/10.1111/nph.13533 PMID: 26108556

37. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, et al. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol. 2014; 32(9): 947–951. http://www.nature.com/nbt/journal/v32/n9/abs/nbt.2969.html#supplementary-information.

38. Gao H, Smith J, Yang M, Jones S, Djukanovic V, Nicholson MG, et al. Heritable targeted mutagenesis in maize using a designed endonuclease. Plant J. 2010; 61(1): 176–187. https://doi.org/10.1111/j.1365-313X.2009.04041.x PMID: 19811621

39. Wu Y, Fox TW, Trimnell MR, Wang L, Xu RJ, Cigan AM, et al. Development of a novel recessive genetic male sterility system for hybrid seed production in maize and other cross-pollinating crops. Plant Biotechnol J. 2015; 14: 1046–1054. Epub 2015/10/08. https://doi.org/10.1111/pbi.12477 PMID: 26442654