MutS homologue 4 and MutS homologue 5 Maintain the Obligate Crossover in Wheat Despite Stepwise Gene Loss following Polyploidization.[CC-BY]

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Crossovers (COs) ensure accurate chromosome segregation during meiosis while creating novel allelic combinations. Here, we show that allotetraploid (AABB) durum wheat (Triticum turgidum ssp. durum) utilizes two pathways of meiotic recombination.

The class I pathway requires MSH4 and MSH5 (MutS homologue 4 and MutS homologue 5) that also ensure formation of the obligate CO (Osman et al., 2011; Pyatnitskaya et al., 2019). Zip1 forms the synaptonemal complex (SC) transverse filaments (Sym et al., 1993). Zip2 (orthologous to SHOC1 in plants) and Zip4 mediate a molecular switch of recombination intermediates to MER3-MSH4/MSH5 complexes from association with the chromosome axis to SC central element components (Dubois et al., 2019). Zip3 (orthologous to HEI10 in plants) is an E3 ligase potentially involved in post-translational modification of proteins by SUMOylation or ubiquitylation (Agarwal and Roeder, 2000). Mer3 is a molecular switch of recombination intermediates to MER3-MSH4/MSH5 complexes from association with the chromosome axis to SC central element components (Dubois et al., 2019).

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by Spo11 and MTOPVIB (Keeney et al., 1997; Fu et al., 2016; Robert et al., 2016; Vrielynck et al., 2016) that are repaired as either COs by reciprocal exchange of DNA or noncrossovers when DNA is repaired either using the sister chromatid as a template or via the homologue, which can lead to gene conversion. COs are genetically and cytologically (chiasmata) more likely to be spaced apart than by random chance through a phenomenon known as interference (Jones and Franklin, 2006; Berchowitz and Copenhaver, 2010). The mechanism of interference is poorly understood, but in budding yeast (Saccharomyces cerevisiae) it is imposed by the meiosis-specific ZMM proteins (Zip1, Zip2, Zip3, Zip4, Mer3, MSH4, and MSH5) that also ensure formation of the obligate CO (Osman et al., 2011; Pyatnitskaya et al., 2019). Zip1 forms the synaptonemal complex (SC) transverse filaments (Sym et al., 1993). Zip2 (orthologous to SHOC1 in plants) and Zip4 mediate a molecular switch of recombination intermediates to MER3-MSH4/MSH5 complexes from association with the chromosome axis to SC central element components (Dubois et al., 2019). Zip3 (orthologous to HEI10 in plants) is an E3 ligase potentially involved in post-translational modification of proteins by SUMOylation or ubiquitylation (Agarwal and Roeder, 2000). Mer3 is a molecular switch of recombination intermediates to MER3-MSH4/MSH5 complexes from association with the chromosome axis to SC central element components (Dubois et al., 2019).

Meiosis is a specialized cell division required to accurately segregate chromosomes into haploid gametes. During meiosis I, homologous chromosomes pair, synapse, and recombine, thus ensuring formation of the obligate crossover (CO) necessary to tether chromosome pairs for correct alignment on the metaphase I (MI) plate and subsequent segregation at anaphase I. Homologous recombination is initiated by programmed DNA double-strand breaks (DSBs) catalyzed

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DNA helicase with 3'-to-5' activity that binds to D-loops in vitro and may unwind or migrate recombination intermediates in vivo to promote CO formation (Duroc et al., 2017).

MSH4 and MSH5 are meiosis-specific MutS homologues of the bacterial mismatch repair proteins that bind and stabilize recombination intermediates (Snowden et al., 2004). In budding yeast, MSH4 and MSH5 form heterodimers that promote the formation of class I interference-sensitive COs, thus ensuring formation of the obligate CO (Ross-Macdonald and Roeder, 1994; Hollingsworth et al., 1995; Pyatnitskaya et al., 2019). In Caenorhabditis elegans, recombination is virtually eliminated in the him14 (MSH4 orthologue) mutant, indicating that all COs are mediated by the class I pathway (Zalevsky et al., 1999). Recent evidence suggests that MutSγ (MSH4 and MSH5) protects the nascent recombination intermediates from dissolution by the orthologue of the Bloom’s syndrome RECQ helicase, thus promoting resolution into COs (Woglar and Villeneuve, 2018). In mouse (Mus musculus), no chiasmata were identified at diakinesis in msh5γA mutants, suggesting a similar requirement for MutSγ in promoting all COs, as in C. elegans (Milano et al., 2019). In Arabidopsis (Arabidopsis thaliana), MutSγ ensures the obligate CO and is required for ~85% of chiasmata (Higgins et al., 2004, 2008b). The residual ~15% of chiasmata observed in mutSγ mutants are interference insensitive and form via the class II pathway, which is partially dependent on the MUS81 endonuclease (Berchowitz et al., 2007; Higgins et al., 2008a). Based on a bioinformatics interactome analysis, MutSγ in Arabidopsis is likely to function similarly to that in C. elegans in protecting recombination intermediates from dissolution by RECQ helicases (AbdelGawwad et al., 2019). In rice (Oryza sativa), MutSγ promotes formation of the obligate CO and is required for 78% to 90% of chiasmata (Luo et al., 2013; Zhang et al., 2014; Wang et al., 2016). In addition, a direct physical association between OsMSH4 and OsMSH5 as well as between OsMSH5 and HEI10 was demonstrated using yeast two-hybrid and pull-down assays (Zhang et al., 2014), as were interactions between OsMSH5 and replication protein A family members (OsRPA1a, OsRPA2b, OsRPA1c, and OsRPA2c; Wang et al., 2016) and OsSHOC1 (Zhang et al., 2019). In Arabidopsis and rice mutSγ mutants, immunolocalization of the synaptonemal complex transverse filament proteins appeared normal, indicating a minimal effect on synopsis during prophase I (Higgins et al., 2004; Luo et al., 2013; Zhang et al., 2014). The only MZM gene to be studied in temperate cereals by knockdown is barley (Hordeum vulgare) ZYP1. barley ZYP1RNAi lines exhibited severely impaired synopsis and did not maintain the obligate chiasma (Barakate et al., 2014). Chiasmata were reduced by 73%, but as the ZYP1RNAi lines were knockdowns, it could not be determined if this ZMM protein was required for class I only or class I and class II COs.

Allohexaploid wheat (Triticum aestivum) is a domesticated species, originating from two hybridizations (Matsuoka, 2011). Evidence suggests that Triticum urartu (AA) first hybridized with an unknown Aegilops species (BB) ~500,000 years ago to form tetraploid wheat (Triticum dicoccoides; 2n = 4x = 24; AABBD), followed by domestication into Triticum dicoccum and Triticum turgidum. T. turgidum then hybridized with Aegilops tauschii (DD) ~10,000 years ago to form allohexaploid wheat (2n = 6x = 42; AABBD; Matsuoka, 2011). Pairing homeologous1 (Ph1) has been characterized as the major locus controlling chromosome pairing and homologous COs in polyploid wheat while preventing homeologous COs (Griffiths et al., 2006). It has been fine-mapped to a novel duplicated copy of the ZMM gene ZIP4 and verified using mutants that can be exploited to introgress DNA from distantly related germplasm (Rey et al., 2017). Ph1-ZIP4 on chromosome 5B originated from chromosome 3B, and the ancestral homeologous ZIP4 copies on 3A, 3B, and 3D are still present and expressed (Griffiths et al., 2006; Alabdullah et al., 2019). Therefore, increased ZIP4 gene dosage may bias recombination toward homologous chromosomes over homeologous chromosomes. A bioinformatics analysis revealed that in certain polyploid plant lineages, meiotic recombination genes are the fastest to return to a single copy, and it was proposed that this is a rapid response for adapting meiotic recombination post whole-genome duplication (Blanc and Wolfe, 2004; Lloyd et al., 2014; Sidhu et al., 2017). This is the inverse of ZIP4, which has gained a novel, dominant copy. Recently, it was demonstrated that reducing MSH4 copy number prevents meiotic COs forming between homeologous chromosomes in allohaploid Brassica napus plants generated by microspore culture (Gonzalo et al., 2019). A further bioinformatics analysis revealed that MSH4 systematically returns to a single copy in numerous plant species following independent polyploid events, suggesting an adaptive role during the diploidization of meiosis (Gonzalo et al., 2019). In polyploid species, diploidization refers to the process whereby recombination evolves a bias toward homologues rather than homeologues. A coexpression analysis in wheat revealed mostly balanced homeologous gene expression and a lack of significant meiotic gene loss following polyploidization (Alabdullah et al., 2019). However, as both ZIP4 and MSH4 act in processing recombination intermediates in the class I CO pathway, these opposing data on gene dosage may be complementary rather than antagonistic.

In this study we demonstrate that MutSγ ensures formation of the obligate chiasma in tetraploid wheat even with a minimum gene copy number (msh5a−/−/msh5b−/−). In addition, the functional MSH5 gene from the B subgenome was rapidly mutated posthybridization in the tetraploid lineage and a substantial section of MSH4D was deleted following the formation of hexaploid wheat. In a genetic background of largely unchanged meiotic recombination genes, functional MSH4 and MSH5 copies have decreased in a stepwise manner posthybridization while maintaining the obligate CO between homologues.
This may have occurred by chance due to gene redundancy or may represent an adaptation to allopolyploidy by modulating recombination.

RESULTS

Pseudogenization of MSH5B

To identify MSH5 orthologues in wheat and its wild relatives, BLAST searches were performed using the Arabidopsis MSH5 amino acid sequence. Three MSH5 orthologues were identified in hexaploid wheat T. aestivum: TaMSH5A (TraesCS1A02G315900), TaMSH5B (TraesCS1B02G328200), and TaMSH5D (TraesCS1D02G316200). Two copies were identified in tetraploid wheat T. turgidum: TmMSH5A (TRIDC1AV1G188720) and TmMSH5B (TRITD1Bv1G177460). Two copies were identified in diploid ancestral species, T. urartu (TRUR300283). To confirm that the deletion was not just an artifact from an incomplete database sequence, the region in question was amplified from T. turgidum ‘Kronos’ using flanking primers. The deletion was experimentally confirmed and is predicted to remove exons 2 to 13 (861 bp) from the coding region and to remove 287 amino acid residues from the N terminus of the protein (Supplemental Fig. S1B). This would result in the loss of the entire MutSII domain and a section of the MutSIII domain. MutSII domains bind Holliday junctions and D-loops in vitro (Fukui et al., 2008), so the truncation is likely to lead to a nonfunctional MSH5B protein, indicating that TmMSH5B has degenerated into a pseudogene. The presence of the 5.4-kb deletion in MSH5B appears to have occurred early in the evolution of polyploid wheat, as it was also detected in the ancient, wild tetraploid wheat T. dicoccoides, and has been preserved throughout domestication into modern hexaploid wheat T. aestivum (Fig. 1; Supplemental Fig. S1).

Pseudogenization of MSH4D

To identify MSH4 orthologues in wheat and its wild relatives, BLAST searches were performed using the Arabidopsis MSH4 amino acid sequence. Three copies of MSH4 were identified in hexaploid wheat T. aestivum: TaMSH4AA (TraesCS2A02G171900), TaMSH4AB (TraesCS2B02G198300), and TaMSH4AD (Fig. 1; Supplemental Fig. S1). Approximate dates are given, and the appearance of the novel ZIP4 duplicate in the Ph1 locus on chromosome 5B is also shown. Functional gene copies are highlighted in blue and pseudogenes in red.

![Figure 1. Stepwise gene loss of MSH5B and MSH4D during the evolution of polyploid wheat. Functional MSH5B was naturally mutated following allopolyploidization in early tetraploid wheat T. dicoccoides, and the functional MSH4D was subsequently mutated following the formation of hexaploid wheat T. aestivum. Approximate dates are given, and the appearance of the novel ZIP4 duplicate in the Ph1 locus on chromosome 5B is also shown. Functional gene copies are highlighted in blue and pseudogenes in red.](image-url)
(5.3 kb), due to increased intron sizes. In Ensembl wheat databases, *TaMSH4D* does not possess a gene model (Alabdullah et al., 2019) due to an 8-kb deletion relative to *Ae. tauschii* (the D-genome donor species), presumably disrupting a prediction from the gene annotation software (Supplemental Fig. S2, A and B). The 8-kb deletion is predicted to remove exons 16 to 24 (986 bp) from the coding region and to remove 328 amino acids from the C terminus of the protein (Supplemental Fig. S2C). This would result in the partial loss of the MutSIII domain and the entire MutSV domain, including the helix-turn-helix motif (residues 724–742) required to dimerize with *MSH5* (Obmolova et al., 2000). The disruption of these highly conserved MutS domains suggests that *TaMSH4D* has also degenerated into a nonfunctional pseudogene.

**Wheat MSH4 and MSH5 Possess Disordered Low-Complexity N Termi**

Tetraploid and hexaploid wheat *MSH4A* primary amino acid sequences only differ from the *T. urartu* ancestral diploid A genome sequence by two residues (808/810; Supplemental Fig. S3A) and by five residues in *MSH4B* compared with the diploid *Aegilops* B genome ancestor (802/807; Supplemental Fig. S3B). *MSH5A* and *MSH5D* primary amino acid sequences in tetraploid and hexaploid wheat are identical to their diploid ancestors *T. urartu* and *A. tauschii* (818/818 and 817/817, respectively; Supplemental Fig. S4). Therefore, consensus sequences were created for wheat *MSH4* and *MSH5* for further analysis. Wheat *MSH4* shares 75% amino acid identity with *AtMSH4* but is distinguished by a low-complexity Gly-rich disordered N terminus (MEEGAAGGGGGGGGGVAVA) that is absent in Arabidopsis (Supplemental Fig. 5A). Wheat *MSH4B* shares 27% amino acid identity with *Saccharomyces cerevisiae* *MSH4* but possesses a considerably shorter N terminus (Supplemental Fig. S5B). Wheat *MSH5* shares 67% amino acid identity with *AtMSH5* and contains a low-complexity disordered N terminus (MDEDEEEQLEEEEEVAETGID) that is highly acidic and absent in Arabidopsis (Supplemental Fig. 6A). The *S. cerevisiae* *MSH5* protein shares 27% amino acid identity with wheat *MSH5* and possesses 84 more residues, of which 35 constitute the N terminus (Supplemental Fig. 6B).

**TMSH4A and TMSH4B Are Functionally Redundant**

TMSH4A and TMSH4B are functionally redundant in formation of the obligate chiasma in tetraploid durum wheat (*T. turgidum*). We observed that single mutants for the *A* (*Tmsh4a-1* and *Tmsh4a-2*) and *B* (*Tmsh4b*) subgenomes are fully fertile and indistinguishable from the wild type at meiotic MI (Fig. 4; Supplemental Table S1). There was no significant difference between single *Tmsh4a*b mutants and the wild type for number of bivalents (*P* > 0.05) or the frequency of chiasmatas (*P* > 0.05). However, double mutants (*Tmsh4ab*) were sterile and did not maintain the obligate chiasma, indicated by the presence of univalents (Fig. 4; Supplemental Table S1). Only 3.74 ± 0.18 (n = 72) mean bivalents per cell were observed in *Tmsh4ab-1* compared with 13.9 ± 0.04 (n = 59) in the wild type (*P* < 0.01). Furthermore, *Tmsh4ab-1* MLs predominantly contained rod bivalents (93%) compared with the wild type, which contained mostly rings (82%). The marked increase in univalents and a decrease in ring bivalents coincided with an 85% reduction in the mean chiasmatas per cell, from 26 ± 0.24 (n = 59) in the wild type to 4 ± 0.21 (n = 72) in *Tmsh4ab-1*. No significant differences were observed between *Tmsh4ab-1* and *Tmsh4ab-2* at MI (Fig. 4; Supplemental Table S1).
TtMSH5A Is Required for the Obligate Chiasma

In contrast to MSH4 redundancy, we observed that MSH5A alone is essential for formation of the obligate chiasma in tetraploid wheat and does not act redundantly with MSH5B, further implicating MSH5B as a pseudogene. Single mutants (Ttmsh5a-1 and Ttmsh5a-2) were sterile and exhibited numerous univalents (Fig. 5; Supplemental Table S1). In Ttmsh5a-1, 3.93 ± 0.19 (n = 92) bivalents per cell were observed, compared with 13.9 ± 0.04 (n = 59) in the wild type (P < 0.01). This coincides with an 84% reduction in chiasmata per cell, from 26.22 ± 0.24 (n = 59) in the wild type to 4.29 ± 0.23 (n = 92) in Ttmsh5a-1 (P < 0.01), and no significant differences were observed between Ttmsh5a-1 and Ttmsh5a-2. However, the single B subgenome mutant Ttmsh5b (that retains an intron containing an in-frame stop codon) was fertile and appeared indistinguishable from the wild type at MI for numbers of bivalents (P > 0.05) and the frequency of chiasmata (P > 0.05; Fig. 5; Supplemental Table S1). Moreover, the double mutant (Ttmsh5ab) is not additive compared with the single mutant Ttmsh5a-1, as there was no significant deviation in numbers of bivalents (3.68 ± 0.17, n = 107 versus 3.93 ± 0.19, n = 92, respectively; P > 0.05) or chiasmata (3.97 ± 0.19, n = 107 versus 4.29 ± 0.23, n = 92, respectively; P > 0.05).

The gene dosage of TtMSH5 appears to be in excess of that required for maintaining wild-type levels of chiasmata, even in the absence of a functional TtMSH5B. The heterozygous mutant Ttmsh5a-1/+/ has the minimum dosage of TtMSH5 through a null mutation. Ttmsh5a-1/+/ plants remained fully fertile and meiotic division appeared unperturbed (Fig. 5; Supplemental Table S1). At MI, there was no significant difference in the mean number of bivalents formed per cell between the wild type and Ttmsh5a-1/+/ (13.9 ± 0.04, n = 59 versus 13.96 ± 0.03, n = 52; P > 0.05) or in the mean number of total chiasmata per cell in the wild type and Ttmsh5a-1/+/ (26.22 ± 0.24, n = 59 versus 25.81 ± 0.21, n = 52; P > 0.05). The number of COs between homologous chromosomes, therefore, appears unaffected by the minimum dosage of MSH5.

Early Recombination Events and Synaptonemal Complex Formation Appear Normal in Ttmsh4ab and Ttmsh5a

Even though the obligate chiasma is lost in Ttmsh4ab-1 and Ttmsh5a-1, earlier meiotic stages involving
axis formation and synapsis appeared unperturbed (Supplemental Fig. S7). Immunolocalization of the axis-associated protein ASYNAPSIS1 (ASY1; Armstrong et al., 2002) and synaptonemal complex transverse filament protein ZYP1 (Higgins et al., 2005) appeared indistinguishable from the wild type (Sepsi et al., 2017). In the wild type, Ttmsh4ab-1, and Ttmsh5a-1, ASY1 formed a linear signal along the unsynapsed chromosome axes at leptotene but was depleted during zygotene in the synapsed regions (Supplemental Fig. S7). ZYP1 was initially detected in the wild type, Ttmsh4ab-1, and Ttmsh5a-1 during late leptotene at synapsis initiation sites that extended throughout zygotene until a complete linear signal was observed at pachytene (Supplemental Fig. S7). Furthermore, the number of RAD51 foci that mark early recombination events at leptotene was not significantly different between the wild type (1,408 ± 28, n = 5), Ttmsh4ab-1 (1,346 ± 58, n = 5), and Ttmsh5a-1 (1,395 ± 4, n = 5; P > 0.05; Supplemental Fig. S8), indicating that early recombination events are unaffected in the mutSγ mutants. Vegetative growth and floral development also appeared normal in Ttmsh4ab and Ttmsh5a mutants, indicating that they do not cause observable somatic defects.

Class II COs Are Unaffected by Loss of mutSγ

The 84% to 85% decrease in chiasmata observed at MI in the Ttmsh5a and Ttmsh4ab mutants is consistent with loss of the class I pathway (Higgins et al., 2004, 2008b), but the class II pathway appears unaffected. Class I and class II COs were monitored cytologically by immunofluorescence using antibodies raised against barley HEI10, a class I CO-specific marker (Chelysheva et al., 2012), and TaMUS81, a class II CO-specific marker (Fig. 6; Higgins et al., 2008a). HEI10 localized as a linear signal along the unsynapsed chromosome axes during zygotene in the wild type and the mutSγ mutants and then depleted to form discrete foci during synapsis (Fig. 6A). At pachytene, the mean number of HEI10 foci per meiocyte was 28.8 ± 0.64 (n = 21) in the wild type but only 2.7 ± 0.26 (n = 23) in Ttmsh4ab-1 and 3.1 ± 0.28 (n = 23) in Ttmsh5a-1 (P < 0.01). The residual HEI10 foci in the mutSγ mutants were generally smaller, fainter, and not

Figure 3. TtMSH5 localizes to meiotic chromosomes at prophase I. Representative male meiotic prophase I spreads are shown from tetraploid wheat T. turgidum ‘Kronos’ immunostained for ASY1 (blue), ZYP1 (green), and MSH5 (red). The right column shows magnified views of MutSγ foci directly on the axis/SC. A, The wild type. B, Ttmsh5a. Bars = 10 μm.
associated with the chromosome axes, but a small minority (17.6%, 0.5 ± 0.12 foci per cell) appeared similar to those in the wild type (Fig. 6C). However, the mean number of MUS81 foci per meiocyte at pachytene was not significantly different between Ttmsh4ab (3.82 ± 0.38, n = 27) and Ttmsh5a (4 ± 0.37, n = 21) compared with the wild type (4.03 ± 0.32, n = 40; P > 0.05; Fig. 6, B and D).

In the wild type, chiasma frequency was tightly distributed around the mean and deviated significantly from a Poisson-predicted distribution [$\chi^2(21) = 64.71, n = 59, P < 0.01$]. However, in the Ttmsh4ab (Ttmsh4ab-1 and Ttmsh4ab-2) and Ttmsh5a (Ttmsh5a-1 and Ttmsh5a-2) mutants, the frequency of residual chiasmata was randomly distributed and did not deviate from a Poisson-predicted distribution [$\chi^2(10) = 6.71, n = 127, P > 0.05$; Ttmsh5a, $\chi^2(11) = 1.85, n = 186, P > 0.05$] (Fig. 7, A–C). This indicates that the number of chiasmata per cell is numerically random in Ttmsh4ab and Ttmsh5a, typical of class II COs (Higgins et al., 2004, 2008b).

Although the number of class II COs was numerically random, the physical chromosomal location of chiasmata was significantly more distal in TtmusSγ mutants (80% ± 1.25%, n = 310) than in the wild type (74.6% ± 1.23%, n = 59; P < 0.01; Fig. 7D). It follows that class II COs appear to have a slight but significant predisposition to form at the distal ends of chromosomes than do class I COs in tetraploid wheat. However, whereas class II COs are more likely to form in the distal regions, they are not

Figure 4. TtMSH4A and TtMSH4B are functionally redundant in ensuring the obligate CO. A, TtMSH4 coding region for the A and B subgenomes with TILLING mutations indicated (red/blue) and predicted MutS domains highlighted in yellow. B, Representative 4′, 6-diamino-2-phenylindole-stained male meiotic MIIs from tetraploid wheat T. turgidum 'Kronos'. Bars = 10 μm. C, Mean number of rings, rods, and univalents per male meiocyte. D, Box plot of chiasmata frequency per male meiocyte. n.s., Not significant. Asterisks indicate significant difference by pairwise Wilcoxon rank sum test (**P < 0.01).
confined there, as 20% of interstitial/proximal chiasmata are observed in TmutS γ mutants.

Hexaploid Wheat Maintains the Obligate Chiasma Despite Loss of TaMSH5B and TaMSH4D

Despite pseudogenization of both TaMSH5B and TaMSH4D, meiosis proceeded normally in hexaploid wheat T. aestivum ‘Fielder’ and the obligate chiasma was maintained (Supplemental Fig. S9; Supplemental Table S1). The mean number of bivalents per cell was 20.97 ± 0.03 (n = 36) and the total number of chiasmata per cell was 40 ± 0.28 (n = 36), which are predominantly distally distributed (76.5%). This is consistent with a computational analysis of 13 recombinant inbred mapping populations, which gave values of 40.8 to 51.9 COs per line, typically clustered toward the ends of chromosomes (Gardiner et al., 2019).

DISCUSSION

We have demonstrated that MSH4 and MSH5 (MutSγ) are essential for the class I meiotic recombination pathway in tetraploid wheat, accounting for ~85% of meiotic COs as well as ensuring the obligate chiasma. This complements data from Arabidopsis, rice, tomato (Solanum lycopersicum), and B. napus, where MutSγ is required for ~85% of COs and the obligate chiasma, indicating that this is most likely the major meiotic recombination pathway in the plant kingdom.
Higgins et al., 2004, 2008b; Luo et al., 2013; Anderson et al., 2014; Wang et al., 2016; Gonzalo et al., 2019). The remaining class II COs (~15%) are random in number and are predominantly distally distributed, except for 20% that are observed interstitially. The proportion of class I to class II COs is consistent across Arabidopsis, rice, tomato, and Brassica spp., despite large disparities in chromosome number, genome size, and DSB number. For example, in wild-type tetraploid wheat, ~1,400 RAD51 foci were counted at leptotene as a proxy for DSBs, which mature into 29 HEI10 and four MUS81 foci at pachytene per cell. This closely matches chiasma numbers in the wild type (26) and mut5y (four) mutants. HEI10 and MUS81 foci account for 2% and 0.3% of RAD51 foci, respectively. In Arabidopsis, ~215 DSBs initiate recombination and nine chiasma form, of which 1.1 to 1.5 are dependent on the class II pathway (Higgins et al., 2004, 2008a, 2008b; Choi et al., 2013). Therefore, ~2.3% of DSBs mature into chiasmata in tetraploid wheat and ~4% in Arabidopsis, so that the 85%:15% class I: class II proportion remains constant, even though the class II chiasmata fit a Poisson distribution. In addition, results from tomato demonstrated that the two pathways are not independent because of observed interference between class I and class II COs (Anderson et al., 2014). These data raise the intriguing possibility that the class I and class II CO pathways are intimately associated through an unknown mechanism, possibly by patterning of early recombination intermediates.

In allopolyploid wheat, the obligate chiasma is maintained despite the number of functional copies of MSH5 and MSH4 reducing in a stepwise manner posthybridization. The functional MSH5 from the B subgenome was mutated following the formation of allotetraploid (AABB) wheat, and MSH4 from the D subgenome was mutated following the formation of allohexaploid (AABBDD) wheat. The 5.4-kb deletion in

Figure 6. HEI10 foci are reduced in number in Ttmsh4 and Ttmsh5, whereas MUS81 is unaffected. A, Representative immunostained pachytene from tetraploid wheat T. turgidum ‘Kronos’ for class I marker HEI10 (left) and class II marker MUS81 (right). Bars = 10 μm. B, Quantification of foci number per male meiocyte. Mean values with s.d. are presented, whereas individual counts are represented as dots. n.s., Not significant. Asterisks indicate significant difference by pairwise Wilcoxon rank sum test (***P < 0.01.)
**Figure 7.** Chiasmata are random in number and located predominantly distally in TtmuS5 mutants. A to C, Observed and Poisson-predicted distributions of chiasma frequency per cell. A, The observed wild-type distribution deviates significantly from a Poisson-predicted distribution ($\chi^2 = 64.71$, $P < 0.01$). B, The observed Ttmsh4ab distribution does not deviate from a Poisson-predicted distribution ($\chi^2 = 6.71$, $P > 0.09$). C, The observed Ttmsh5a distribution does not deviate from a Poisson-predicted distribution ($\chi^2 = 1.85$, $P > 0.99$). D, The mean proportion of chiasma (percent) that are distally located. Error bars represent SE. Asterisks indicate significant difference by pairwise Wilcoxon rank sum test (**P < 0.01).

MSH5B is predicted to result in the loss of the entire MutSII domain and a section of the MutSIII domain. MutSII domains bind Holliday junctions and D-loops in vitro (Fukui et al., 2008), so the truncation is likely to lead to a nonfunctional MSH5B protein, providing evidence that TtmS5B is a pseudogene. Cytological evidence supports this prediction, as a reduction of 85% chiasma was observed in msh5a mutants, but there was no additive effect in the msh5ab double mutants. MSH4D contains an 8-kb deletion that is predicted to remove 328 amino acid residues from the C terminus of the protein, resulting in the partial loss of the MutSIII domain and the entire MutSV domain, suggesting that TaMSH4D is also a nonfunctional pseudogene. Even in the absence of MSH5B and MSH4D, the obligate chiasma is still maintained in hexaploid wheat.

Allopolyploid cells possess multiple sets of homologous chromosomes that must pair, recombine, and synapse to ensure accurate chromosome segregation during meiosis to preserve genome stability and reproductive success. In neoallopolyploids, a bias for meiotic recombination to occur between homologues rather than homeologues may not be preadapted, so diploidization will be under strong selection pressure. The mechanisms underlying meiotic stabilization in newly formed allopolyploids are poorly understood, but a recent study in B. napus demonstrated that reducing MSH4 copy number prevented meiotic COs forming between homoeologous chromosomes, whereas homologous COs were unaffected (Gonzalo et al., 2019). Furthermore, MSH4 and MSH5 have been shown to systematically reduce to a single copy in numerous polyploid plant lineages, which is more likely due to convergent selection than by chance (Lloyd et al., 2014; Gonzalo et al., 2019). This is consistent with a potential role for MSH5B/MSH4D gene losses in the diploidization of polyploid wheat.

The functional MSH4 and MSH5 homeologues are highly conserved between wheat species at the primary amino acid level. Wheat MSH4 and MSH5 possess unique low-complexity disordered N termini that are not present in Arabidopsis or S. cerevisiae. The S. cerevisiae MSH4 N terminus destabilizes the protein and is targeted for degradation (He et al., 2020), whereas the disordered wheat MutSy N termini do not contain Ser residues and may stabilize the proteins, although functional studies would be required to test this. A stable MutSy complex would be advantageous in a diploid background but may promote homologous recombination in a neopolyploid. In S. cerevisiae, MutSy stabilizes single-end invasions as well as Holliday junction recombination intermediates (Lahiri et al., 2018). The ability of MutSy to stabilize early recombination intermediates comprising divergent heteroduplex sequences in wheat may have been negated by deletions in MSH5B and MSH4D. The class I CO pathway is regulated in a dosage-dependent manner in mouse and Arabidopsis by RNF212 and HEI10, respectively, as well as by MSH4 in B. napus (Reynolds et al., 2013; Ziolkowski et al., 2017; Gonzalo et al., 2019). Homologous COs appear unaffected by gene duplicate loss in wheat and can maintain the obligate chiasma, despite possessing only one functional MSH5 copy in Ttmsh5a-1/+, the minimum dosage through a null mutation. The dosage of MutSy is therefore in excess of that required for normal levels of homologous COs, but this may not be the case for homoeologous COs, where it is potentially a limiting step. However, this will need to be confirmed experimentally by reconstituting functional MSH5B in tetraploid wheat and MSH5B and MSH4D in hexaploid wheat.

The novel duplicated copy of ZIP4 on chromosome 5B in the Ph1 locus prevents homologous COs forming in hexaploid wheat crossed with distantly related species (Rey et al., 2017). As a constituent of the ZMM complex, ZIP4 may interact with either MSH4 or MSH5 directly or indirectly in wheat. In S. cerevisiae, MSH5 and ZIP4 physically interact in yeast two-hybrid assays,
suggesting that this interaction mediates the association of the MutSy dimer with ZIP2-ZIP4-Spo16 complexes to process recombination intermediates from D-loops into stable single-end invasions and Holliday junctions (De Muyt et al., 2018). It has been suggested that high levels of expression of ZIP4 in hexaploid wheat prevent homoeologous COs (when crossed with a closely related ancestor) in a dosage-dependent manner by overcoming a threshold (Rey et al., 2017). Based on data from Gonzalo et al. (2019), we hypothesize that higher levels of MutSy expression may stabilize recombination intermediates between divergent heteroduplex sequences in wheat (such as homeologues and distantly related chromosomes) but lower levels of MutSy are sufficient to stabilize intermediates between homologues. However, it may be a coincidence that following tetraploidization, wheat evolved an extra copy of ZIP4 while it lost a functional copy of MSH5 followed by mutation of MSH4D in the hexaploid.

CONCLUSION

In summary, MutSy promotes the formation of class I COs in wheat (~85% of all COs) and maintains the obligate chiasma despite stepwise pseudogenization of MSH5B and MSH4D following polyploidization. Loss of MSH5B and MSH4D does not perturb formation of the obligate chiasma and may even play an adaptive role in meiotic recombination in allopolyploid wheat.

MATERIALS AND METHODS

Identification of Wheat MSH4/MSH5

Wheat MSH4 and MSH5 orthologues were identified using the Arabidopsis (Arabidopsis thaliana) amino acid sequences (encoded by AtMSH4 [AT4G17380] and AtMSH5 [AT3G24075]) to BLAST against publicly available databases: Triticum dicoccoides (Avni et al., 2017), WENSeq_v1.0, https://plantsensembl.org/Triticum_dicoccoides; Triticum turgidum (Maccarini et al., 2019), Svevo_v1, https://plants.ensembl.org/Triticum_turgidum; Triticum aestivum (Appels et al., 2018), IWGSC, https://plants.ensembl.org/Triticum_aestivum; Triticum urartu (Ling et al., 2013), ASM4745v1, https://plants.ensembl.org/Triticum_urartu, and (Ling et al., 2018), Tu2.0, https://www.ncbi.nlm.nih.gov/assembly/GCA_003073215.1/Aegilops_speltoides; Aegilops speltoides (Marcussen et al., 2014), TAGC_WCS_speltoides_v1, https://urgi.versailles.inra.fr/blast/; and Aegilops tauschii (Luo et al., 2017), Aet_v4.0, https://plants.ensembl.org/Aegilops_tauschii. Wheat full genomic scaffolds were aligned for the ClustalW algorithm (gap open cost = 15, gap extend cost = 0) and adjusted by eye.

Plant Material

T. turgidum ‘Kronos’ was used as a wild-type control for experiments involving mutant TILLING lines obtained from www.SeedStor.ac.uk: Ttmshka-1 (K2082), Ttmshka-2 (K4365), Ttmshk_b (K4239), Ttmshka-1 (K863), Ttmshka-2 (K4833), and Ttmshk_b (K381); Krasileva et al., 2017). Individual lines were genotyped using single-nucleotide polymorphism-specific primers calibrated by gradient PCR (Supplemental Table S2). Double mutants were generated by crossing single lines for mshk_b (K2082 × K4239 and K4365 × K4239) and mshk_b (K863 × K381). Plants were grown under controlled environmental growth conditions: photoperiod of 16 h, temperature of 21°C (day)/16°C (night), and relative humidity of ~60%.

PCR

Total genomic DNA was isolated from T. turgidum ‘Kronos’ leaf material using the DNeasy Plant Mini Kit (Qiagen). A large, previously unidentified deletion in TMSH5B was verified experimentally by nested PCR with flanking primers designed to amplify over the predicted gap. The first reaction used internal primers TMSH5B_del_F_ext (5’-TGGATGACGGCGAGGAGG-3’) and TMSH5B_del_R_ext (5’-AGATCCTCATTATCTCACCAGGCTG-3’), and the successive reaction used internal primers TMSH5B_del_F_n (5’-AGGGGAGGCTGGCCAGGAGC-3’) and TMSH5B_del_R_n (5’-CTGAAACAGAGAAGCCGCGC-3’). Cycling conditions were annealing temperature = 60°C and extension time = 2 min for both reactions. Amplicons were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek). Sanger sequencing was performed by Eurofins.

Reverse Transcription PCR

Total RNA was extracted from T. turgidum ‘Kronos’ spikes using the ISO-LATE II RNA Mini Kit (Bioline), and cDNA was synthesized by reverse transcription using the Tetro cDNA synthesis kit (Bioline). The coding sequences were amplified using subgenome-specific primers (Supplemental Table S3), ligated into pDrive (Qiagen), and Sanger sequenced (Supplemental Fig. S10). The presence of mutations in the coding sequences was confirmed for the selected TILLING lines. Ttmshka-1 has a CAG-to-TAG mutation in exon 15, which results in a premature stop codon. Ttmshka-2 has a CAA-to-TAA mutation in exon 20, which results in a premature stop codon. Ttmshk_b has a GT-to-GA mutation at the splice donor site between exons 22 and 23, which causes the retention of intron 22 and the formation of a premature stop codon (TAA) 16 codons downstream. Ttmshka-1 has a CAG-to-TAG mutation in exon 8, which results in a premature stop codon. Ttmshka-2 has a CAA-to-TGA mutation in exon 34, which results in a premature stop codon. Ttmshk_b has a GT-to-GA mutation at the splice donor site between exons 27 and 28, which causes the retention of intron 27 and the formation of a premature stop codon (TCA) 14 codons downstream.

Cytological Procedures

Chromosome spreads were performed as described previously (Higgins, 2013; Desjardins et al., 2020). Nikon Ni-E and Eclipse Ci fluorescence microscopes equipped with NIS elements software were used to image chromosomes. The following primary antibodies were used: anti-TaASY1 guinea pig, 1:500 (see below); anti-ArMUS81 rabbit, 1:500 (Osman et al., 2018); anti-ArRAD51 rabbit, 1:200 (Mercier et al., 2003); anti-AMSH4 rat, 1:200 (Higgins et al., 2004); anti-AMSH5 rat, 1:200 (Higgins et al., 2008b); anti-TaMUS81 rabbit, 1:200 (see below); and anti-HvHEI10 guinea pig, 1:250 (see below). Secondary antibodies used at 1:200 were goat anti-guinea pig AMCA (Jackson ImmunoResearch); goat anti-guinea pig Alexa Fluor 488 (Abcam); goat anti-rat AMCA (Jackson ImmunoResearch); goat anti-rat Alexa Fluor 594 (Invitrogen); goat anti-rabbit AMCA (Jackson ImmunoResearch); goat anti-rabbit Alexa Fluor 488 (Invitrogen), and goat anti-rabbit DyLight 594 (Vector Laboratories). Meioocytes were staged with anti-ZIP1 and anti-AASY1 to ensure that foci counts were made at equivalent microsporocytes. The following primary antibodies were used: anti-TaASY1 guinea pig, 1:500 (see below); anti-ArMUS81 rabbit, 1:500 (Osman et al., 2018); anti-ArRAD51 rabbit, 1:200 (Mercier et al., 2003); anti-AMSH4 rat, 1:200 (Higgins et al., 2004); anti-AMSH5 rat, 1:200 (Higgins et al., 2008b); anti-TaMUS81 rabbit, 1:200 (see below); and anti-HvHEI10 guinea pig, 1:250 (see below). Secondary antibodies used at 1:200 were goat anti-guinea pig AMCA (Jackson ImmunoResearch). Immunostained leaf material was observed using the ISO-LATE II RNA Mini Kit (Bioline), and cDNA was synthesized with the Tetro cDNA synthesis kit (Bioline). The coding sequences were amplified using subgenome-specific primers (Supplemental Table S3), ligated into pDrive (Qiagen), and Sanger sequenced (Supplemental Fig. S10). The presence of mutations in the coding sequences was confirmed for the selected TILLING lines. Ttmshka-1 has a CAG-to-TAG mutation in exon 15, which results in a premature stop codon. Ttmshka-2 has a CAA-to-TAA mutation in exon 20, which results in a premature stop codon. Ttmshk_b has a GT-to-GA mutation at the splice donor site between exons 22 and 23, which causes the retention of intron 22 and the formation of a premature stop codon (TAA) 16 codons downstream. Ttmshka-1 has a CAG-to-TAG mutation in exon 8, which results in a premature stop codon. Ttmshka-2 has a CAA-to-TGA mutation in exon 34, which results in a premature stop codon. Ttmshk_b has a GT-to-GA mutation at the splice donor site between exons 27 and 28, which causes the retention of intron 27 and the formation of a premature stop codon (TCA) 14 codons downstream.

Antibody Production

Total RNA was extracted from T. aestivum ‘Cadena’ spikes using the ISO-LATE II RNA Mini Kit (Bioline), and cDNA was synthesized with the Tetro cDNA synthesis kit (Bioline). The wheat AtS1 D subgenome coding region (FrasaCS630DC294100) was used as a template to amplify nucleotides 1 to 696 with Q5 DNA polymerase (New England Biolabs) using primers ASYF (5’-AACATAATTTAGGATGCTGATAAGAG-3’) and ASYR (5’-CTCTCGAGGACAATTCTAATCTCAAGAC-3’). The PCR products were cloned into pDrive (Qiagen) and confirmed by sequencing. The ASY1 fragment was digested by NdeI/XhoI using sites incorporated into the primers (highlighted in boldface),
Supplemental Table S3. Subgenomic-specific primer sequences used to amplify coding TIMS8A and TIMS8B sequences from tetraploid wheat ‘Kronos’.

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Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: TaMSH4A (TraesCS2A02G171900), TaMSH4B (TraesCS2B02G198300), TaMSH5A (TraesCS1A02G315900), TaMSH5B (TraesCS1B02G328200), and TaMSH5D (TraesCS1D02G316200).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Multiple-sequence alignments of MSH5 comparing cultivated polyploidy wheats with their diploid wild relatives.

Supplemental Figure S2. Multiple-sequence alignments of MSH4 comparing cultivated polyploidy wheats with their diploid wild relatives.

Supplemental Figure S3. Functional MSH4 proteins in polyploidy wheats are largely unchanged at the primary protein structure compared with diploid ancestors.

Supplemental Figure S4. Functional MSH5 proteins in polyploidy wheats are unchanged at the primary protein structure compared with diploid ancestors.

Supplemental Figure S5. Wheat MSH4 possesses a disordered low-complexity N terminus.

Supplemental Figure S6. Wheat MSH5 possesses a disordered low-complexity N terminus.

Supplemental Figure S7. Axis formation and synapsis are unaffected in TtMSH4 and TtMSH5 null mutants.

Supplemental Figure S8. Early recombination protein RAD51 loading is unaffected in TtMSH4 and TtMSH5 null mutants.

Supplemental Figure S9. Hexaploid wheat maintains the obligate chiasma despite loss of TaMSH5B and TaMSHHD.

Supplemental Figure S10. TIMS8A and TIMS8B coding sequences from wild-type and mutant lines.

Supplemental Table S1. Reduced severity of chiasma in TtMSH4 and TtMSH5 null mutants.

Supplemental Table S2. Single-nucleotide polymorphism-specific primer sequences and cycling conditions used to genotype ‘Kronos’ wheat TILLING lines for TtMSH4, TtMSH5b, and TtMSH5a.
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