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Summary

Meiotic recombination is initiated by formation of DNA double strand breaks (DSBs). This involves a protein complex that includes in plants the two similar proteins, SPO11-1 and SPO11-2. We analysed the sequences of SPO11-2 in hexaploid bread wheat (*Triticum aestivum*) as well as in its diploid and tetraploid progenitors. We investigated its role during meiosis using single, double and triple mutants. The three homoeologous SPO11-2 copies of hexaploid wheat exhibit high nucleotide and amino acid similarities with those of the diploids, tetraploids and Arabidopsis. Interestingly however, two nucleotides deleted in exon-2 of the A copy lead to a premature stop codon and suggest that it encodes a non-functional protein. Remarkably, the mutation was absent from the diploid A-relative *T. urartu* but present in the tetraploid *T. dicoccoides* and in different wheat cultivars indicating that the mutation occurred after the first polyploidy event and has since been conserved. We further show that triple mutants with all three copies (A, B, D) inactivated are sterile. Cytological analyses of these mutants show synapsis defects, accompanied by severe reductions in bivalent formation and numbers of DMC1 foci, thus confirming the essential role of *TaSPO11-2* in meiotic recombination in wheat. In accordance with its 2-nucleotide deletion in exon-2, double mutants for which only the A copy remained are also sterile. Notwithstanding, some DMC1 foci remain visible in this mutant, suggesting a residual activity of the A copy, albeit not sufficient to restore fertility.

Significance statement

Analysis of the three homoeologous copies of *TaSPO11-2* of bread wheat and of meiosis in the corresponding mutants, reveals that *TaSPO11-2-7A* is non-functional due to a two-nucleotide deletion in exon-2 which occurred after the first polyploidisation event. Consequently, numbers of DMC1-foci (and presumably of double-strand breaks) are strongly reduced in triple and double *Taspo11-2* mutants, accompanied by strong meiotic defects and confirming the essential role of *TaSPO11-2* in meiosis.

Introduction

Most eukaryote species multiply through sexual reproduction. This involves the fusion of specialized haploid cells, the gametes, leading to restoration of the initial ploidy level of the parents. These haploid cells are produced through meiosis, a cell-cycle process that consists of two successive cell divisions following one round of DNA replication (Mercier *et al.*, 2015). The first division separates the pairs of homologous chromosomes coming from both parents and the second separates sister chromatids, leading to four balanced haploid cells carrying one set of chromosomes. An accurate and balanced segregation of the homologous chromosomes during the first meiotic division relies on appropriate pairing of chromosomes that form specific assemblies called bivalents (Hunter 2015; Mercier *et al.*, 2015; Zickler and Kleckner 2015). Each chromosome is physically linked to its homologue through one or several chiasmata, which are the visible cytological manifestation of a reciprocal exchange between two homologous chromatids (also called crossover, CO; Grelon 2016). At least, one CO per bivalent is required to ensure faithful segregation of homologues and to produce viable gametes (Zamarolia *et al.*, 2014; Mercier *et al.*, 2015). COs occur during the early step of meiosis (prophase I). They are initiated by DNA double-strand breaks (DSBs) catalysed by a complex of several proteins (De Massy, 2013; Lam and Keeney 2015). Over the last 20 years, genetic approaches on different model species have identified about 20 essential proteins involved in meiotic DSB production (Cole *et al.*, 2010; Lam and Keeney, 2015; Jing *et al.*, 2019). In budding yeast, the complex is composed of ten proteins (Lam and Keeney 2015), among which, SPO11 acts via a topoisomerase-like reaction (De Massy *et al.* 1995; Keeney and Kleckner 1995; Liu *et al.*, 1995; Bergerat *et al.*, 1997; Keeney *et al.*, 1997; Keeney 2008). SPO11 is a conserved protein, homologous to the A-subunit of topoisomerase VI (TOPOVIA) from the Archae, *Sulfolobus shibatae* (Bergerat *et al.*, 1994, 1997; Nichols *et al.*, 1999). Despite the key importance of the activity of SPO11, its action mechanism remains largely unknown. TOPOVI proteins operate through the assembly of two subunits, A and B, and the question of the existence of a B subunit in eukaryotes, necessary for the SPO11 activity, has only been resolved by the recent identification of the B subunit (TOPOVIB) in vertebrates (Robert *et al.*, 2016a) and plants (Vrielynck *et al.*, 2016) (reviewed in Robert *et al.*, 2016b).

Homologs of the SPO11 protein have been identified in many species. Their analysis reveals strong evolutionary conservation with archaeal proteins (Malik *et al.*, 2007), especially in the DNA binding and the divalent-cation interaction domains (respectively 5Y-CAP (catabolite gene activator protein) and TOPRIM (topoisomerase-primase), common to topoisomerases and primases), the two main domains of the TOPOVIA protein (De Massy *et al.*, 1995; Keeney and Kleckner 1995; Liu *et al.*, 1995; Bergerat *et al.*, 1997; Keeney *et al.*, 1997; Nichols *et al.*, 1999; Diaz *et al.*, 2002; Schoeffler and Berger, 2005). The absence of SPO11 protein results in a lack of meiotic DSBs and thus defective CO formation (Klapholz *et al.*, 1985, Baudat and De Massy, 2004). This leads to chromosome segregation errors in meiosis I and eventually to sterility (Bergerat *et al.*, 1997; Keeney *et al.* 1997; Dernburg *et al.*, 1998; Cervantes *et al.*, 2000; Hartung *et al.*, 2007).

While a single gene encodes *SPO11* in most organisms, plants have several *SPO11* genes. Three copies were isolated in Arabidopsis (*SPO11-1*, *-2*, *-3*) but only *SPO11-1* and *SPO11-2* are required for accurate meiosis, *SPO11-3* apparently only being involved in somatic endo-reduplication (Hartung and Puchta 2000; Grelon *et al.*, 2001; Hartung and Puchta 2001; Hartung *et al.,* 2002; Sugimoto-Shirasu *et al.,* 2002; Yin *et al.* 2002; Stacey *et al.,* 2006; Hartung *et al.* 2007; Sprink and Hartung 2014). Five *SPO11* orthologues have been identified in rice (*OsSPO11-1, -2, -3, -4, -5*; An *et al.*, 2011). Mutation of *OsSPO11-1* and *OsSPO11-2* affects meiotic progression and results in sterility, confirming crucial roles in meiosis (Yu *et al.*, 2010; Fayos *et al.,* 2019). The OsSPO11-4 protein was initially described as having DNA-cleavage activity (An *et al.*, 2011; Shingu *et al.*, 2012), with RNAi experiments showing aberrant meiosis and reduced fertility, suggesting an important role in meiosis (An *et al.*, 2011). However, contrasting results were observed regarding the interaction between OsSPO11-4 and OsMTOPVIB proteins (Fu *et al.*, 2016; Xue *et al.*, 2016) and recent data suggest that the additional *OsSPO11-4* gene plays no major role in rice meiosis (Fayos *et al.,* 2020).

Polyploidy adds another layer of intricacy in this already complex process. All angiosperm species have undergone at least one round of whole genome duplication during their evolution (Soltis and Soltis 2009, Van de Peer *et al.,* 2009) and about 30% of them are current polyploids (Wood *et al.,* 2009). Among these, bread wheat (*Triticum aestivum* L.) is an allo-hexaploid species (AABBDD; 2n = 6x = 42) derived from two successive interspecific

hybridizations involving three related diploid species (for details, see International Wheat Genome Sequence Consortium, IWGSC 2014; 2018). The first occurred about 0.8 million years ago (MYA) and took place between *T. urartu* (AA genome) and a yet-unknown species related to the *Sitopsis* section (SS genome related to wheat BB genome). This natural cross gave rise to tetraploid species (*T. diccocoides*), that further evolved to give *T. turgidum*, the ancestor of current *durum wheat*. The second cross arose ~0.4 MYA and involved this newly created tetraploid species and *Aegilops tauschii* (DD genome) leading to hexaploid bread wheat. Thus, in bread wheat, 85.6% of the genes are present in two or three homoeologous copies and 6% have more than three copies (IWGSC 2018; Ramirez-Gonzalez *et al.,* 2018). Moreover, 70% of the genes that exhibit three homoeologous copies, are expressed in a balanced manner between the three copies (IWGSC 2018; Ramirez-Gonzalez al., 2018). For the remaining 30%, one or two copies are more expressed but without detectable differences between the homoeologous genomes, suggesting the absence of any sub-genome dominance (Alabdullah *et al.*, 2019). Similar results were also observed for wheat meiosis-specific genes (Lloyd *et al.,* 2014).

Until now, only a few meiotic genes have been deeply studied in wheat and data are often limited to a description of the differences between homoeologous copies and expression analyses (Boden *et al.,* 2007; Devisetty *et al.,* 2010; Khoo *et al.,* 2012). In-depth functional analyses are mainly restricted to *Ph1* (Pairing homoeologous locus 1), a gene involved in homoeologous recombination (Griffith *et al.,* 2006, Rey *et al.,* 2017, Martin *et al.,* 2017).

Here, we present a thorough analysis of *TaSPO11-2* and its role in bread wheat meiosis. We first identified and characterized the sequences of the three homoeologous copies of this gene. The A-copy has a two-nucleotide deletion in exon 2 leading to a premature stop-codon and presumably to a non-functional protein. This mutation was conserved in the wheat tetraploid ancestor but not in the diploid species indicating that mutation occurred after the first polyploidy event of wheat. We then isolated single mutants for each copy and developed a full set of single, double and triple mutant lines derived from heterozygous hybrids. Analysis of meiosis in these mutants confirmed that only the B and D homoeologous copies are functional. In particular, *TaSpo11-2* triple mutants showed significantly reduced numbers of DSBs, impaired synapsis and defective CO formation. Our data thus demonstrate the essential role of *TaSPO11-2* in meiotic recombination initiation in bread wheat. Finally, we show that

expression of the *TaSPO11-2-7D* copy restores fertility of Arabidopsis *spo11-2* mutant demonstrating the strong functional conservation of the plant *SPO11-2* gene.

Results

Three homoeologous copies of SPO11-2 are present in the wheat genome but slightly differ between each other

We used Arabidopsis SPO11-2 protein sequence (AT1G63990.1) as a query to perform a BLAST-P analysis (Altschul *et al.,* 1997) against the IWGSC database (https://wheat-urgi.versailles.inra.fr/Data). Considering the hexaploid status of bread wheat, we identified three high-confidence proteins located on the homoeologous group 7 chromosomes and annotated as *TraesCS7A02G300300.1*, *TraesCS7B02G201200.1* and *TraesCS7D02G296000.2*. For convenience, they will further be named as *TaSPO11-2-7A*, *TaSPO11-2-7B* and *TaSPO11-2-7D* respectively. The amino acid (aa) identity percentages shared with Arabidopsis protein ranged from 64.9 to 66.7% (Table 1) suggesting a strong conservation of SPO11-2 protein between these two species. The genomic wheat-gene lengths were 3085 bp, 3299 bp and 3026 bp for the A, B and D copies respectively. This was 50% more than the gene from Arabidopsis (2063 bp), confirming longer introns in wheat as described earlier (IWGSC 2014; 2018). Differences in size between the copies were mainly due to small insertions/deletions (indels) in introns 8 and 10 (Figure S1).

We made *ab-initio* annotations of the three homoeologous copies and compared them to the annotation from Arabidopsis to study their structure thanks to TAIR prediction for exon positions and putative motifs for the Arabidopsis protein structure (Figure 1A). The coding sequence (CDS) from Arabidopsis covers 1152 bp corresponding to 383 aa and consists of 10 introns and 11 exons. *Ab initio* annotation of wheat *SPO11-2* genes predicted 10 exons for *TaSPO11-2-7A* and 11 exons for the other two B and D copies. This corresponds to 359, 386 and 386 aa for the A, B and D copies respectively. The seven motifs (Bergerat *et al.,* 1997; Malik *et al.,* 2007) known for this gene and the essential motifs for the catalytic activity (Y124) (Hartung *et al.,* 2007) and DNA binding capacity (G215, R222, R226) (Shingu *et al.,* 2012) are conserved in all copies (Figure 1A; Table S1). The B and D proteins are identical in size (386 aa, Figure 1B) and almost identical (97.7% identity).

The *TaSPO11-2-7A* genomic sequence is highly similar to the B and D homoeologous copies (95.3% and 96% identity respectively, Figure 1B). Two amino acids are inserted just after the predicted translation starting site (ATG; Figure 1C). In addition, this copy exhibits a deletion of two nucleotides (CC) in exon 2 (Figure 1C). This deletion results in a frameshift inducing a premature stop codon (PSC) at the end of the exon 3 suggesting that the A copy is not functional. *Ab initio* annotation proposed an alternative model where exon 2 is skipped leading to a shorter protein (359 aa; Figure 1A), albeit more similar to the Arabidopsis protein (66.7% identity). These results prompted us to investigate whether the mutation on the A copy is also present in the diploid and tetraploid ancestors of wheat and in other varieties.

The mutation of the A copy is conserved between cultivars and with tetraploid species but is absent from the diploid ancestors

We aligned sequences of *TaSPO11-2-7A* exon 2 from cultivars Arche (AR), Apache (AP), Renan (RE) and W7984 (W7; synthetic wheat; Nelson *et al.,* 1995) with the *TaSPO11-2-7A-CS* reference sequence from Chinese spring (CS; Figure 2A). The five sequences were identical showing that the two nucleotides are also deleted in these cultivars. In addition, we isolated the homoeologous copies from the two diploid ancestors *T. urartu* (AA genome) and *Ae. tauschii* (DD genome) and from a tetraploid wild accession from *T. turgidum ssp dicoccoides* (AABB, cv. Zavitan). As expected, we found only one copy of *SPO11-2* in the diploid species and two copies in the tetraploid. Genomic sequences ranged from 2976 to 3293 bp (Table 1) which was similar to what was observed for Chinese Spring.

We then aligned all these CDS sequences (Figure 2B). Interestingly, the deletion of two nucleotides in the second exon was also present in *TtSPO11-2-7A-Za* but absent in *T. urartu* (Figure 2B). With these two nucleotides, the copy from *T. urartu* is thus almost identical to the B and D copies (95,9% and 94,3% identity respectively) and should correspond to the functional copy. This suggests that the deletion occurred after the first polyploidization event between *T. urartu* and the donor of the B genome. This mutation has been maintained until the second polyploidization event between *T. turgidum ssp dicoccoides* and *Ae. tauschii*, and beyond during tetraploid and hexaploid wheat evolution and domestication.

Wheat *TaSPO11-2-7D* can complement Arabidopsis *Atspo11-2* mutant

Since the *SPO11-2* sequence is extremely well conserved between wheat and Arabidopsis, we tested whether the function of *SPO11-2* is also conserved during evolution. We expressed the *TaSPO11-2-7D* cDNA in the Arabidopsis *spo11-2* mutant and evaluated its ability to restore fertility. Strikingly, all 10 *spo11-2* homozygous mutant plants expressing the *TaSPO11-2-7D* transgene that we obtained, exhibited a significant increase in fertility compared to *Atspo11-2* mutant (Figure 3; Table S2). The residual fertility in the different transformants ranged from 50% to 100% of that of the wild-type, with most lines exhibiting fertility around 80-90%. This is in strong contrast with the 5% residual fertility of the *Atspo11-2* mutants (Hartung *et al.,* 2007). Cytogenetic analysis of meiotic progression in pollen mother cells of *Atspo11-2* transformants expressing the *TaSPO11-2-7D* transgene revealed normal meiotic stages (Figure 3C). In particular, Metaphase I with five bivalents could be observed in *Atspo11-2*/*TaSPO11-2-7D* transgenic plants (mean of 4.4 bivalent/cell, n = 35) contrasting with the presence of univalents in *Atspo11-2* (Hartung *et al.*, 2007). These results demonstrate that wheat *TaSPO11-2-7D* gene can substitute for Arabidopsis *SPO11-2* and thus exhibits evolutionary conserved function in meiosis.

Only the B and D copies of *TaSPO11.2*, but not the A-copy, ensure wheat fertility

TaSPO11-2 is essential for meiosis but the question of the functionality of the three wheat homoeologous copies remains to be ascertained. We isolated a mutant plant heterozygous for the three homoeologous copies of *TaSPO11-2-7-RE* gene ($ABD^{+/-}$) in our irradiated population derived from Renan (Figure S2). We analysed a series of 73 sister plants for all combinations of single and multiple *Taspo11*-2 mutants. All mutants show normal vegetative development. The spikes are physiologically normal (Figure 4A) and all lines exhibit a non-significant decrease in seed numbers compared to the control except the triple mutant ($abd^{-/-}$) and the double $bd^{-/-}A^{+/-}$ or $bd^{-/-}A^{+/+}$ mutants, for which no grains were obtained (Figure 4B). Interestingly, the double $ab^{-/-}D^{+/-}$ and $ad^{-/-}B^{+/-}$ mutants that remained heterozygous for the D or B copies, respectively, showed normal fertility, indicating that only one functional copy of *TaSPO11-2* is sufficient to ensure a fully fertile plant. This suggests that there is no dosage effect for *SPO11-2*.

We next investigated pollen viability in the mutants using Alexander staining according to Jahier (1992) (Figure S3A). In control plants, pollen remained intact. Similarly, the three single

and double *ab*$^{-/-}$ and *ad*$^{-/-}$ mutants exhibited between 90.5% and 96% of unaltered pollen. On the contrary, only 12% and 30% of the pollen grains were viable in the triple *abd*$^{-/-}$ and the double *bd*$^{-/-}$ mutants respectively (Figure S3B). Total RNAs of cvs Renan and CS anthers were collected at the leptotene stage and semi-quantitative RT-PCR performed to analyse expression of the different *TaSPO11-2* copies. All three copies were expressed (Figure S4A) and they were more highly expressed in Renan compared to Chinese Spring. In both varieties, the D-copy was more expressed than the B-copy that was more expressed than the A-copy (Figure S4B). For the A copy, using available RNASeq data (Lloyd *et al.*, 2014; Martin *et al.*, 2018; Ramirez-Gonzalez *et al.,* 2018), we showed that both isoforms (with alternative ATG or with exon-2 skipping) were expressed (Figure S4C) and that the main expressed form was the one with exon-2 skipping. All together, these results showed that both B and D copies of the *SPO11-2* gene are functional, while the A copy is altered and no longer plays its full role. Despite its alteration, the A copy remains expressed, but significantly less so than the other two copies. A single functional copy of either the B or D genome is sufficient to ensure normal fertility.

TaSPO11-2 is necessary for normal meiotic progression in bread wheat

Mutation of *SPO11-2* affects meiosis in Arabidopsis (Hartung and Puchta 2000, 2001). We thus established an atlas of meiotic stages for the wheat mutants. In control plants (ABD$^{+/+}$) meiosis is normal, with 21 bivalents at Metaphase I, well-balanced anaphases and final meiotic products composed of four balanced nuclei (tetrads; Figure 5A). Normal meiotic stages were also observed for all single sub-genome mutants (*a*$^{-/-}$, *b*$^{-/-}$, *d*$^{-/-}$), and for the *ab*$^{-/-}$ and *ad*$^{-/-}$ double mutants (Figure S5). In contrast, extensive meiotic defects were observed in the *abd*$^{-/-}$ triple mutant, with only univalents observed at Metaphase I and random segregation of these at Anaphase I. This leads to unbalanced polyads at the end of Meiosis II in these plants (Figure 5A, Figure S6). The *bd*$^{-/-}$ double-mutant showed the same phenotypes as *abd*$^{-/-}$ triple mutant, confirming that the A copy is non-functional. Quantification of numbers of chiasmata per cell in control plants (ABD$^{+/+}$, ABD$^{+/-}$), the single (*a*$^{-/-}$, *b*$^{-/-}$, *d*$^{-/-}$), the double (*ab*$^{-/-}$, *ad*$^{-/-}$) mutants, showed 21 bivalents and a mean of 40.4 ± 2.0, 38.7 ± 3.5 and 38.1 ± 3.4 chiasmata per cell, respectively (Figure 5B). In sharp contrast, the *bd*$^{-/-}$ double mutant exhibited 4.6 ± 2.4 chiasmata per cell and no chiasmata were observed in the *abd*$^{-/-}$ triple mutant. Thus, as in Arabidopsis, *TaSPO11-2* is required for CO formation in wheat. However, only the B and D

copies are functional and the mutation of the A copy strongly affects (although not completely) its activity.

TaSPO11.2 is necessary for chromosome synapsis and meiotic DSB formation in hexaploid wheat

We established the kinetics of the synaptonemal complex in our control (Figure S7). At the beginning of meiotic prophase, ASY1 (Asynaptic 1) associates with chromosome axes, forming visible filaments. At the leptotene stage, the ZYP1 (Zipper 1) protein is present on chromosome axes as foci and these extend as synaptonemal complex (SC) formation proceeds through zygotene and pachytene stages. These filaments disassemble at the diplotene stage to fix only the chiasmata. In wheat, the SC is thus set up in the same way as in other species (Colas *et al.,* 2017). We focussed on the pachytene stage to see if synapsis was affected by the mutation of SPO11-2 protein in wheat. In the *abd* mutant, ASY1 is normally loaded onto the chromosomes, forming long filaments along chromosome axes and eventually starts disassembling at pachytene. In contrast, ZYP1 signal appears mostly in dots and do not form long filaments typical of synapsis, although a few partial stretches could be observed (Figure 6A). Normal pachytene stages were not observed. As expected, sub-genome single-mutants showed normal synapsis while triple-mutant was unable to form SC. Similar defects were observed for the $bd^{-/-}$ double mutant, confirming the inactivity of A copy (Figure 6A). The other double-mutants ($ab^{-/-}$, $ad^{-/-}$) had normal complete synapsis. Meiotic progression is thus dramatically affected in absence of *TaSPO11-2* and the SPO11-2 protein is necessary for the establishment of the SC in bread wheat.

Meiosis is initiated by the formation of DSBs by the SPO11 complex and SPO11-2 is an essential member of this complex in Arabidopsis (Hartung and Puchta 2000). We analysed the formation of DSBs in a control and in *Taspo11-2* mutants by performing immuno-localization of the DMC1 (Disrupted Meiotic cDNA 1) recombinase protein as a marker of DSBs. At leptotene, TaDMC1 protein is essentially located in the nucleolus and starts to spread to the rest of the nucleus (Figure S8). DMC1 foci peak at zygotene and the signal decreases at pachytene (Figure 6B). In control plants ($ABD^{+/+}$), a mean of 727 foci (± 193) were counted at zygotene; this number is ~20% lower compared to Renan or Chinese spring (Figure 6C) probably because this plant also derives from irradiated mutants. We found a drastic ten-fold decrease in the mean number of DMC1 foci 71 ± 29 at zygotene in the triple mutant.

Interestingly, we also found a strong, but lower reduction (4-fold reduction, with 174 ± 53 DMC1 foci) in the *bd*$^{-/-}$ double mutant (p-value < 0.0001, Figure 6B-C and Table S3). Thus, DMC1 loading is dramatically affected in both mutants. Yet, there are significantly more DMC1 foci in the *bd*$^{-/-}$ double mutant than in the triple mutant and this concords with chiasma number and pollen viability (see above) and confirms the residual activity of the A copy of TaSPO11-2. Eventually, DMC1 foci were still present at zygotene in other mutants (Figure 6B). Overall, these data demonstrate that TaSPO11-2 is necessary for DSB formation and initiation of meiosis in bread wheat.

Discussion

The A copy of *SPO11-2* is following a pseudogenisation process

Using SPO11-2 Arabidopsis protein as query, we found three copies of the orthologous gene in the wheat genome, mapping on each of the three homoeologous genomes. This was not unexpected since 47% of the wheat genes are present in triads with a single gene copy per sub-genome (an A:B:D configuration of 1:1:1; IWGSC 2018). The three copies of hexaploid wheat and those from diploid (*T. urartu* and *Ae. tauschii*) and tetraploid (*T. diccocoides*) wheat ancestors are highly conserved. The D copy was the best conserved between wheat and *Ae. tauschii*, which is consistent with its more recent hybridization with the AABB tetraploid genome progenitor and with a limited number of D-genome progenitors (Giles and Brown 2006; Marcussen *et al.,* 2014). This high level of similarity was also observed between wheat D-copy and Arabidopsis *SPO11-2* gene, confirmed by the successful complementation of Arabidopsis *spo11-2* mutant with the wheat SPO11-2-D protein. This latter result is reminiscent of the data obtained with *TaSPO11-1* (Da Ines, Michard *et al.,* in press) and demonstrate that both *TaSPO11-1* and *TaSPO11-2* can functionally replace Arabidopsis *AtSPO11-1* and *AtSPO11-2*. Collectively, our data reveal important functional conservation of the *SPO11* complex features.

On the contrary, the A copy diverged slightly from that of the other homoeologous genomes, with a deletion of two nucleotides in the second exon. This deletion leads to a frameshift and to the occurrence of a premature stop codon giving a truncated non-functional protein. This mutation was present in the tetraploid species but absent from the diploid progenitor. This

suggests that mutation occurred after the first polyploidization event (~0.8 MYA), in the tetraploid ancestor that further hybridized with *Ae. tauschii* to give current bread wheat, since the same mutation is conserved in different hexaploid varieties as well as in tetraploid species. Non-functionality of the A-copy was confirmed in the $bd^{-/-}$ double mutants, which were sterile. However, we observed a few viable pollen grains as well as a few DMC1 foci in these mutants, suggesting some residual activity of the SPO11 complex. The A copy is expressed, albeit significantly less than the other two copies. These results were in accordance with RNA-Seq data in the literature derived from Chinese Spring developing anthers (Lloyd *et al.,* 2014) or from *ph1* mutants (Martin *et al.*, 2018). Thus, the A copy is normally transcribed and translated, but deletion of two nucleotides (CG in *T. urartu* A progenitor or CC in B and D genomes; Figure 2B) in *SPO11-2* exon-2 sequence creates a premature stop codon. Interestingly, *ab initio* prediction gave us two possibilities for the expression of the A-copy: an alternative translation-starting site on exon 3 or alternative splicing with exon-2 skipping. The first hypothesis gives a protein of 290 aa missing the first 96 aa, but keeping the essential motifs for the catalytic activity Y124 (Hartung *et al.,* 2007) and DNA binding capacity G215, R222, R226 (Shingu *et al.,* 2012). However, given that the N-Terminal part of SPO11-2 protein is involved in the interaction with MTOPVIB in Arabidopsis (Vrielynck *et al.,* 2016), if a complex is present, it would only be partially active. Proteomic analyses will be needed to evaluate this hypothesis.

Alternatively, exon skipping (or alternative splicing) removing exon 2 gives a protein of 359 aa, with all catalytic sites and with the first amino acids involved in the interaction with MTOPVIB. Alternative splicing is observed for *SPO11* in mammals (Romanienko and Camerini-Otero 1999) and for *SPO11-1* in Arabidopsis (Hartung and Puchta 2000; Reddy et al. 2013), with up to seven isoforms observed for *SPO11-2* in Arabidopsis because of either intron retention or exon skipping (Sprink and Hartung 2014). Several isoforms have also been observed in *Brassica rapa* (6 isoforms), *Carica papaya* (6), *Oryza sativa* (2) and *Physcomitrella patens* (2). Isoform β* from *P. patens* corresponds to the skipping of exon 2 as we suggest for the A copy of wheat and this form was found in generative tissues only (Sprink and Hartung 2014).

The very low level of pollen viability we observed, suggests either of the two forms (or even the two) remains partly active. However, because the form with exon-2 skipping is normally expressed (Figure S4C), we thus favour the hypothesis that skipping of exon 2 produces a less-efficient TaSPO11-2-7A protein. This would explain the presence of a few bivalents and some

viable pollen grains in the *bd*$^{-/-}$ double mutants, even if this is not sufficient to fully restore fertility.

All these results suggest that the A copy from SPO11-2 of bread wheat is losing its functionality and is on the way of pseudogenisation.

TaSPO11-2 is necessary for meiotic double strand break formation

Plants missing *SPO11-2* (*bd*$^{-/-}$ or *abd*$^{-/-}$ mutants) are sterile, as this is the case for Arabidopsis *spo11-2* mutants (Hartung and Puchta 2000).

We developed specific wheat antibodies to describe for the first time the progression of wheat prophase I in both wild type and *spo11-2* mutant backgrounds. In wheat, the SC is set up in the same way as in most species (Khoo et al., 2012, Boden et al., 2007, Barakate et al., 2014). The SPO11-2 protein is necessary for the establishment of the SC, since mutants are unable to synapse and to reach the pachytene stage, leaving cells blocked at a zygotene-like stage. *SPO11-2* is involved in the formation of bivalents, with the triple mutants giving only univalents, while the double *bd-/-* mutant gave 4.8 ring bivalents instead of the 21 seen in the wild type or in other single (*a*$^{-/-}$, *b*$^{-/-}$, *d*$^{-/-}$) or double (*ab*$^{-/-}$, *ad*$^{-/-}$) mutants.

Although Drosophila and *C. elegans* SC is established independently of recombination (Dernburg *et al.,* 1998, McKim *et al.*, 1998), in general recombination is required for meiotic chromosomal synapsis (*e.g.* yeast (Alani *et al.*, 1990; Bishop *et al.*, 1992), mouse (Yoshida *et al.,* 1998; Romanienko *et al.,* 2000), Arabidopsis (Grelon *et al.,* 2001; Stacey *et al.,* 2006)).

In Arabidopsis, ~150-250 DSBs are observed at Leptotene (Vignard *et al.*, 2007; Serrentino and Borde 2012) while in maize, two-fold more (~500) DSBs are observed (Anderson *et al.,* 2003; Pawlowski *et al.,* 2003) for a genome that is ~20 times larger. The wheat genome is more than 130 times larger than that of Arabidopsis and ~6 times larger than the maize genome (~14.5 Gb, IWGSC 2018). According to this non-linear relationship between genome-size and number of DSBs, we expected ~800-3200 DSBs in wheat. Using γ-H2AX staining, Gardiner *et al.,* estimated the number of DSBs to more than 2100 (Gardiner *et al.,* 2019). Using DMC1 antibody, we estimated for the first time in wheat, the number of DMC1 foci as being between 365-1563 (mean 833), which corresponds to the lower boundary of our estimates. The slight discrepancy between γ-H2AX and DMC1 counts may be explained by several parameters such as lab growing conditions, cell staging and/or choice of antibody. DMC1 marks active DSB sites engaged in homologous recombination and thus, although very close to reality, DMC1 foci

may slightly underestimate the real number of initial DSBs. On the contrary, γ-H2AX may not always indicate the presence of DSBs (de Feraudy *et al.,* 2010; Cleaver et al. 2011; Revet *et al.,* 2011) and thus use of γ-H2AX could slightly overestimate DSBs. Nevertheless, both approaches gave a similar order of magnitude and number of DSBs obtained is in the range of what was expected. Thus, compare to Arabidopsis, the bread wheat genome exhibit around ten times more DSBs while its genome is 130 times larger. This means that the DSBs density is much lower in the bread wheat genome. Interestingly, recombination mainly occurs in the distal regions of the chromosomes in wheat, while pericentromeric regions are almost devoid of COs (Saintenac *et al.,* 2009; Choulet *et al.,* 2014). It is tempting to speculate that this CO distribution is explained (at least partly) by a low amount of DSBs compared to the size of the genome and by a preferential location of these DSBs in the telomeric regions to the expense of pericentromeric regions. However, this remains to be demonstrated.

In this study, we have identified and characterised *SPO11-2*, a gene involved in the formation of double strand breaks at the onset of meiosis. Its functionality during the formation of the synaptonemal complex is described for the first time in wheat. We show that only two of the three homoeologous copies present in the wheat genome are functional. This is an important step in precisely deciphering the way recombination can be improved in a polyploid species such as bread wheat.

Experimental procedures

Plant material and growth conditions

To study the impact of the mutation of *SPO11-2*, we used the reference line Chinese Spring as a control. The variety Renan was used to develop the set of 4,500 irradiation lines (gamma rays, 150 Gy) from which, the various *Spo11-2* mutants were derived. Single-copy mutants were identified and crossed manually between each other to generate a heterozygous line for the three copies of *SPO11-2* (Figure S2). This line was self-pollinated and the progeny was screened by Q-PCR to isolate 73 plants with zero, one, two or three mutated copies. Two different individuals (replicates) per combination were randomly selected for further analyses. Since *SPO11-2* is located on homoeologous group 7, we used the nulli-tetrasomic (NT) stock

of group 7 chromosomes (lines missing one pair of homologous chromosomes that is replaced by a pair of homoeologous chromosomes: N7AT7B, N7AT7D, N7BT7A, N7BT7D, N7DT7A, N7DT7B; Sears, 1954; Sears, 1966; Sears and Sears, 1978) for expression analysis of the copies of *SPO11-2*.

Seeds were germinated in growth chamber at 18°C ± 1°C. Cold treatment (8°C) was applied at a three-leaf stage for two months with an 8h-light photoperiod. Plantlets were potted (4 L) in the greenhouse at 23°C day and 18°C night with a 16-h day photoperiod. Commercial progressive release fertilizer Nutricote (Fertil) was used to fertilize the plants during watering.

Identification of wheat *SPO11-2* copies

DNA sequences of *SPO11-2* were identified in the hexaploid wheat (https://wheat-urgi.versailles.inra.fr/Data), tetraploid wheat *T. durum* (Avni *et al.,* 2017 https://wewseq.wixsite.com/consortium), and diploid wheat-relatives *T. urartu* (Ling *et al.,* 2018 http://www.mbkbase.org/Tu/) and *Ae. tauschii* (Luo *et al.,* 2017 https://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA341983&cmd=DetailsSearch) genome through a BLAST-P research using the Arabidopsis SPO11-2 protein sequence (AT1G63990.1) as query. The three copies of wheat SPO11-2 (TraesCS7A02G300300.1, TraesCS7B02G201200.1, TraesCS7D02G296000.2) further named TaSPO11-2-7A, TaSPO11-2-7B, TaSPO11-2-7D respectively) as well as *T. turgidum ssp dicoccoides* (TRIDC7AG041910.1 and TRIDC7BG033100.4), *T. urartu* (TuG1812G0700003251.01) and *Ae. tauschii* (AET7Gv20751300.7) copies were then aligned with MAFFT7 (Multiple Alignment using Fast Fourier Transform; https://mafft.cbrc.jp/alignment/server/; Kato *et al.,* 2002).

We used ACT (Artemis Comparison Tool; Carver *et al.*, 2005) to display pairwise comparisons between the three homoeologous sequences.

Intron/exon boundaries were identified using the genomic and CDS sequences from Arabidopsis as model. We aligned *SPO11-2* genomic and CDS sequences exon 2 with MAFTT7. A deletion of two nucleotides (CC) was found in the A-copy.

To isolate *SPO11-2* sequences in cultivars Renan, Apache, Arche and W7984 (synthetic wheat; Nelson *et al.,* 1995), we exploited the SNP BreedWheat TaBW280K array (Rimbert *et al.,* 2018), which used an exome capture to target approximately 57 MB of coding sequences in 43 bread wheat accessions. We aligned all reads matching with *SPO11-2* genomic sequence on the

corresponding reference Chinese Spring sequences and we visualized the correspondences with IGV software (Genomic Visualisation and interpretation).

Gene expression

To study the gene expression, we extracted mRNAs in pre-meiotic and leptotene anthers of plant controls (three biological replicates) using Renan (Re), Chinese spring (CS) and CS Nulli-tetrasomic lines. We then generated genome-specific primers for *SPO11-2* in the CDS sequence. We quantified the signal intensity of each *SPO11-2* cDNA and normalized it against housekeeping gene (Ta54227: ATPase Paolacci *et al.*, 2009) using Quantity One software (Bio-Rad, Hercules, CA, USA). RNA-Seq data were those described in Lloyd *et al.,* (2014), Martin *et al.,* (2018) and Ramirez-Gonzalez *et al.*, (2018).

Complementation in Arabidopsis

For expression of *TaSPO11-2* in Arabidopsis, the *TaSPO11-2-7D* coding sequence was synthesized and cloned into a GATEWAY destination vector pMDC32 in which the 35S promoter was replaced by the Arabidopsis *RAD51* promoter. This plasmid was then inserted in an *Agrobacterium tumefaciens* C58C1 strain, which was subsequently used to transform Arabidopsis *SPO11-2-3/spo11-2-3* (Vrielynck *et al.,* 2016) heterozygous plants by the floral dip method (Clough and Bent 1998). Seeds from the *Agrobacterium*-treated plants were sown on soil and transformants were selected for Hygromycin resistance. Cytological analysis of meiotic progression in pollen mother cells was performed as previously described (Ross and Murphy 1996).

Meiocyte isolation

Immature wheat inflorescences were harvested and placed in ice. Anthers were extracted using two roll pins. The identification of the stage was determined from one of the three anthers of the same spikelet after Acetocarmine (Carmin 10 g/L with acetic acid 45%) staining under light microscope. For meiotic atlas, two synchronized anthers were fixed in Carnoy solution (EtOH 100-acetic acid v/v: 3:1) during 48H then placed in EtOH 70% and stored at 4°C. Anthers were placed on Poly-L-Lysine coated slides in 15 µL of fresh 45% acetic acid and then opened under binocular with two roll pins to liberate meiocytes. The slides were frozen in

liquid nitrogen. The slides were mounted with Vectashield DAPI (Eurobio Ingen) and observed on ZEISS Axio Observer Z1 microscope (Carl ZEISS Microscopy). For meiotic behaviour studies, fixed chromosomes of anthers at metaphase I stage were spread according to Jahier (1992). The slides were photographed under brightfield light on ZEISS Axio Observer Z1 microscope. The number of chiasmata and pairing types were counted on at least 50 cells per genotype to calculate the means.

Design of DMC1 antibody

The DMC1 protein shares very strong homology with the RAD51 protein (50% of amino-acid positions) and to avoid cross-reactivity we aligned TaRAD51 and TaDMC1 proteins and those of *Arabidopsis thaliana* (AT5G20850.1 and AT3G22880.1). We selected sequences used by Sanchez-Moran *et al.,* (2007) considered as the most immunogenic in our case (amino-acids 27 to 42). We validated the uniqueness of this sequence *in silico* by doing a BLAST-P against the IGWSC database using the short function for the small sequences. BLAST-analysis results confirmed the presence of hits exclusively in TaDMC1-5-CS. Two peptides for TaDMC1-5-CS were designed after the bioanalysis. We used competition with the two peptides synthesized for the immunization of 2 chickens by Eurogentec to validate experimentally the specificity of the TaDMC1-5-CS antibody. Antibody is available on request.

Immunostaining

Samples were prepared according to Colas *et al.,* (2016, 2017) and performed with slight modifications for wheat mutants. Anthers at prophase I stage were collected on ice in a watch glass containing 1 mL phosphate buffer (PBS) 1X with 1 µL of protease inhibitor cocktail (Sigma) then fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature. Anthers were washed twice with 1 mL PBS 1X for 5 min at 4°C, transferred at room temperature in 1 mL permeabilization solution (PBS 1X with 0.05% Triton X100, 1 mM EDTA) for 15 min then in 1 mL blocking solution consisting in 3% bovine serum albumin (BSA) in PBS 1X, 0.1% Tween-20, 1mM EDTA for 15 min. Meiocytes of three anthers per slide were isolated manually under binocular in 20 µL of PBS 1X on Poly-L-Lysine coated slide. The meiocyte suspension was left at room temperature to dry slightly. Fifty microliters per slide of primary antibody solutions (dilutions 1:400 anti-TaASY1 (rabbit, Agrisera,) Boden *et al.*, 2007, 2009,

1:200 anti-TaZYP1 (guinea pig) supplied by A.C. Martin, John Ines Centre, UK and a mixture of two peptides by 1:20 anti-TaDMC1 (chicken, Eurogentec)) were deposited on the slide then covered with a piece of parafilm. The slides were placed in humid chamber at 4°C for 36 h to 48 h. Slides were washed three times for 5 min with PBS 1X. Fifty microliters of secondary antibody solutions (dilution in blocking solution 1:400 anti-rabbit Alexa Fluor 568, 1:300 anti-guinea pig Alexa Fluor 647, 1:300 anti-chicken Alexa Fluor 488, Fisher Scientific) were deposited on each slide, covered with parafilm and incubated in a humid chamber at room temperature for 1 h. Slides were washed tree times with PBS 1X and mounted with Vectashield-DAPI.

Confocal microscopy and image analysis

The fluorescence optical images of meiocytes were obtained with confocal LSM 800 microscope (Carl ZEISS) for three channels (488, 568, 647 nm) and Zeiss ZEN2 image analysis software. Image acquisition was made with high resolution AIRYSCAN module. Detection parameters were 850V for power laser and an intensity adjustment for each channel. 3D confocal stack images were acquired and deconvoluted with ZEN2 software image analysis (Carl ZEISS). Image analysis was made with Imaris software 7.6 (https://imaris.oxinst.com/) with Spot detection method (defaults parameters) for counting DMC1 foci.

Viability of pollen and fertility

Pollen of one anther was deposited on a slide and stained with Alexander reagent according to Jahier (1992) for 3 min. Two repetitions were done by genotype. Slides were observed in brightfield on Axio Observer Z1 (Carl ZEISS) microscope. Images full field were acquired with tiles module of ZEN2 image analysis software. Around 1500 pollen grains per repetition were counted with Image J software. We conducted a fertility study by working on three to five plants per genotype and we isolated four master spikes per plant and counted the number of grains produced by self-pollination.

Statistics

Statistical inference tests were done with the XLSTAT software (https://www.xlstat.com/fr/). Normality of values was verified with the normality test module including Shapiro and Wilk

testing with default values (and other tests: from Anderson and Darling, from Lillifors and the Jarque Bera test). If the values follow a normal distribution, a student test was applied with the Two test and test module, keeping the defaults (alpha=0.05). If the values do not follow a normal distribution we applied a non-parametric Kruskal-Wallis test with an alpha error to 0.01.

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Supporting Information

Figure S1. Synteny analysis between the three homoeologous copies of *TaSPO11-2-CS*.

Figure S2. Experimental plan of the production of mutants. Nomenclature of mutants.

Figure S3. Analysis of pollen viability of mutants using Alexander-dye staining.

Figure S4. Expression analysis of homoeologous copies of *TaSPO11-2*.

Figure S5. Meiotic behaviour of *TaSPO11-2* mutants.

Figure S6. Analysis of terminal meiotic products in WT and mutants using aceto-carmine staining.

Figure S7. ASY1, ZYP1, meiotic protein kinetic in the wild-type ($ABD^{+/+}$).

Figure S8. DMC1 kinetic during prophase of the wild-type ($ABD^{+/+}$).

Table S1. Complete annotation of *TaSPO11-2*-CS homoeologous copies.

Table S2. Seed number per silique in *AtSpo11-2* primary transformants after complementation with *TaSPO11-2-7D-CS*.

Table S3. Descriptive and inference statistics (Quantitative data) of DMC1 foci per cell.

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Tables

Table 1: Results of BLAST-P analysis using AtSPO11-2 protein as query. Arabidopsis protein was BLASTed against corresponding wheat Databases: IWGSC= *T. aestivum* cv. Chinese Spring (hexaploid AABBDD); WheatTu= *T. monococcum ssp urartu* (diploid AA); AET= *Ae. tauschii* (diploid DD); TRIDC= *T. diccocoides* cv. Zavitan (tetraploid AABB). Sequences producing significant alignments were sorted out using parseblast.pl (Choulet *et al.* 2014) with default parameters; %Id: percentage of identity with Arabidopsis protein; Prot.: length of the proteins (aa); Gene: length of the genomic sequences (exons+introns in bp); CDS: length of the coding sequences (bp).

Query = AtSPO11-2_AT1G63990.1; Length=383 aa						
Databases	Sequences producing significant alignments	Protein	%Id	Prot. (aa)	Gene (bp)	CDS (bp)
IWGSC_v1.1_HC_20170706_pep.fasta	TraesCS7A02G300300.1*	TaSPO11-2-7A-CS	66.7	359	3085	1080
	TraesCS7A02G300300.2**	TaSPO11-2-7A-CS-ISO2	69	290	3085	873
	TraesCS7D02G296000.2	TaSPO11-2-7D-CS	65.1	386	3026	1161
	TraesCS7B02G201200.1	TaSPO11-2-7B-CS	64.9	386	3299	1161
WheatTu.pros	TuG1812G0700003251.01	TuSPO11-2	65.7	386	3151	1161
AET_HighLow_confidence_gene_protein	AET7Gv20751300.7	AetSPO11-2	65.1	393	2979	1182
TRIDC_WEWseq_PGSB_20160501_Proteins	TRIDC7AG041910.1	TtSPO11-2-7A-Za	66.7	384	2976	1155
	TRIDC7BG033100.4	TtSPO11-2-7B-Za	65.7	385	3293	1158
Arabidopsis_thaliana.TAIR10.pep	AT1G63990.1	AtSPO11-2	100	383	2544	1152

*: isoform with exon-2 skipping; **: isoform with alternative starting site.

Figure legends

Figure 1. Characterization of SPO11-2 homoeologous genes of bread wheat. **A.** Structure of the genes (light-grey; exons are coloured) and of the proteins (dark-grey; the seven domains (see Table S1-B; Bergerat *et al*., 1997; Malik *et al.*, 2007; Hartung *et al.*, 2010; Vrielynck *et al.*, 2016) are coloured); **B.** Ternary graphic of the percentages of identity between homoeologous proteins (left) and genomic sequences –right); **C.** *TaSPO11-2* MAFTT alignment of exon-1 and exon-2 of the three homoeologous wheat copies; translation initiation sites are squared in green and intron/exon boundaries in red; deletion of two nucleotides in *TaSPO11-2-7A-CS* is highlighted with a grey circle.

Figure 2. Comparative analysis of exon-2 from A-copy of different varieties and species. **A.** Alignment of *TaSPO11-2* exon-2 from cultivars Chinese Spring (CS), Renan (RE), Apache (AP), Arche (AR) and W7984 (W7, synthetic wheat; Nelson *et al.* 1995). **B.** Alignment of *TaSPO11-2* exon-2 from *T. monococcum ssp urartu* (*TuSPO11-2-G1812*; diploid AA), *T. turgidum ssp diccocoides* (*TtSPO11-2-7-Za*; Tetraploid AABB), *Ae. tauschii* (*AetSPO11-2-St*; diploid DD) and *T. aestivum* cv. Chinese Spring (*TaSPO11-2-7-CS;* hexaploid AABBDD).

Figure 3. Wheat *TaSPO11-2-7D* complements fertility of the Arabidopsis *spo11-2* mutant. **A.** Photography of siliques in wild-type, *Atspo11-2* mutants and *Atspo11-2 TaSPO11-2* complemented plants. Both wild-type and *Atspo11-2 TaSPO11-2* plants have long siliques full of seeds, while *Atspo11-2* mutants are sterile and exhibit short siliques. **B.** Number of seeds per silique in Wild-type, *Atspo11-2*, and 10 *Atspo11-2 TaSPO11-2* independent primary transformants. Each dot represents the number of seeds in one silique. **C to N.** Meiotic progression in wild-type **(C-F)**, *Atspo11-2* **(G-J)** and *Atspo11-2 TaSPO11-2*-complemented plants **(K-N).** (Scale Bar: 10 µm).

Figure 4. Wheat mutant fertility analysis. **A.** Photography of spikes and 10 seeds (when produced) for all genotypes used in this study. **B.** Number of seeds per genotype and spike (3 plants minimum per genotype and 4 spikes per plants). Since values do not follow normality law, Kruskal-Wallis test was applied with alpha = 0.01 and Monte-Carlo methods with 10,000 simulations. Values were significantly different for the double ($bd^{-/-}A^{+/-}$ or $bd^{-/-}$) and triple ($abd^{-/-}$) mutants (red) only (*: p-value< 0.0001; NS: not significant).

Figure 5. Progression of meiosis from metaphase I to telophase II (tetrads) in WT and mutants. **A.** Mutants $bd^{-/-}$ and $abd^{-/-}$ show abnormalities during meiosis such as presence of univalents in metaphase I (orange circle) and polyad formation at anaphase II (red circle). Scale bar: 5 µm. **B.** Chiasma number per cell. Since values do not follow normality law, Kruskal-Wallis test was applied with alpha = 0.01 and Monte-Carlo methods with 10,000 simulations. Values were significantly different for the double ($bd^{-/-}$) and triple ($abd^{-/-}$) mutants (red) only (*: p-value< 0.0001; NS: not significant).

Figure 6. Synaptonemal complex (SC) and DMC1-foci analyses of the mutants during prophase I. **A.** Immuno-localization of synaptonemal proteins ASY1 (green) and ZYP1 (red). Control $ABD^{+/+}$: the synapse is set up correctly until complete co-localization of the two proteins at pachytene. $abd^{-/-}$ and $bd^{-/-}$ mutants: alteration of the SC with ZYP1 protein aggregates leading to synapse failure. Scale bar: 5 µm. **B.** Triple immuno-localization of ASY1 (red), ZYP1 (magenta) and DMC1 (green) meiotic proteins at leptotene stage. Scale bar : 5 µm. **C.** number of DMC1 foci per cell at mid-zygotene for the genotypes Renan (RE), Chinese spring (CS), control $ABD^{+/+}$, and mutants $bd^{-/-}$ and $abd^{-/-}$. Since values follow normality law, a T-Test was applied between the control ($ABD^{+/+}$; blue) and the mutants (red). *: p-value <0.0001; NS: not significant.