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1 **SPO11.2 is essential for programmed double strand**
2 **break formation during meiosis in bread wheat**
3 **(*Triticum aestivum* L.)**

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20

21 Summary

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

40

41 Significance statement

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

48

50 Most eukaryote species multiply through sexual reproduction. This involves the fusion of
51 specialized haploid cells, the gametes, leading to restoration of the initial ploidy level of the
52 parents. These haploid cells are produced through meiosis, a cell-cycle process that consists
53 of two successive cell divisions following one round of DNA replication (Mercier *et al.*, 2015).
54 The first division separates the pairs of homologous chromosomes coming from both parents
55 and the second separates sister chromatids, leading to four balanced haploid cells carrying
56 one set of chromosomes. An accurate and balanced segregation of the homologous
57 chromosomes during the first meiotic division relies on appropriate pairing of chromosomes
58 that form specific assemblies called bivalents (Hunter 2015; Mercier *et al.*, 2015; Zickler and
59 Kleckner 2015). Each chromosome is physically linked to its homologue through one or several
60 chiasmata, which are the visible cytological manifestation of a reciprocal exchange between
61 two homologous chromatids (also called crossover, CO; Grelon 2016). At least, one CO per
62 bivalent is required to ensure faithful segregation of homologues and to produce viable
63 gametes (Zamarolia *et al.*, 2014; Mercier *et al.*, 2015). COs occur during the early step of
64 meiosis (prophase I). They are initiated by DNA double-strand breaks (DSBs) catalysed by a
65 complex of several proteins (De Massy, 2013; Lam and Keeney 2015). Over the last 20 years,
66 genetic approaches on different model species have identified about 20 essential proteins
67 involved in meiotic DSB production (Cole *et al.*, 2010; Lam and Keeney, 2015; Jing *et al.*, 2019).
68 In budding yeast, the complex is composed of ten proteins (Lam and Keeney 2015), among
69 which, SPO11 acts via a topoisomerase-like reaction (De Massy *et al.* 1995; Keeney and
70 Kleckner 1995; Liu *et al.*, 1995; Bergerat *et al.*, 1997; Keeney *et al.*, 1997; Keeney 2008). SPO11
71 is a conserved protein, homologous to the A-subunit of topoisomerase VI (TOPOVIA) from the
72 Archae, *Sulfolobus shibatae* (Bergerat *et al.*, 1994, 1997; Nichols *et al.*, 1999). Despite the key
73 importance of the activity of SPO11, its action mechanism remains largely unknown. TOPOVI
74 proteins operate through the assembly of two subunits, A and B, and the question of the
75 existence of a B subunit in eukaryotes, necessary for the SPO11 activity, has only been
76 resolved by the recent identification of the B subunit (TOPOVIB) in vertebrates (Robert *et al.*,
77 2016a) and plants (Vrielynck *et al.*, 2016) (reviewed in Robert *et al.*, 2016b).

78 Homologs of the SPO11 protein have been identified in many species. Their analysis reveals
79 strong evolutionary conservation with archaeal proteins (Malik *et al.*, 2007), especially in the
80 DNA binding and the divalent-cation interaction domains (respectively 5Y-CAP (catabolite
81 gene activator protein) and TOPRIM (topoisomerase-primase), common to topoisomerases
82 and primases), the two main domains of the TOPOVIA protein (De Massy *et al.*, 1995; Keeney
83 and Kleckner 1995; Liu *et al.*, 1995; Bergerat *et al.*, 1997; Keeney *et al.*, 1997; Nichols *et al.*,
84 1999; Diaz *et al.*, 2002; Schoeffler and Berger, 2005). The absence of SPO11 protein results in
85 a lack of meiotic DSBs and thus defective CO formation (Klapholz *et al.*, 1985, Baudat and De
86 Massy, 2004). This leads to chromosome segregation errors in meiosis I and eventually to
87 sterility (Bergerat *et al.*, 1997; Keeney *et al.* 1997; Dernburg *et al.*, 1998; Cervantes *et al.*, 2000;
88 Hartung *et al.*, 2007).

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

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

108 hybridizations involving three related diploid species (for details, see International Wheat
109 Genome Sequence Consortium, IWGSC 2014; 2018). The first occurred about 0.8 million years
110 ago (MYA) and took place between *T. urartu* (AA genome) and a yet-unknown species related
111 to the *Sitopsis* section (SS genome related to wheat BB genome). This natural cross gave rise
112 to tetraploid species (*T. diccoides*), that further evolved to give *T. turgidum*, the ancestor of
113 current *durum wheat*. The second cross arose ~0.4 MYA and involved this newly created
114 tetraploid species and *Aegilops tauschii* (DD genome) leading to hexaploid bread wheat. Thus,
115 in bread wheat, 85.6% of the genes are present in two or three homoeologous copies and 6%
116 have more than three copies (IWGSC 2018; Ramirez-Gonzalez *et al.*, 2018). Moreover, 70% of
117 the genes that exhibit three homoeologous copies, are expressed in a balanced manner
118 between the three copies (IWGSC 2018; Ramirez-Gonzalez *et al.*, 2018). For the remaining 30%,
119 one or two copies are more expressed but without detectable differences between the
120 homoeologous genomes, suggesting the absence of any sub-genome dominance (Alabdullah
121 *et al.*, 2019). Similar results were also observed for wheat meiosis-specific genes (Lloyd *et al.*,
122 2014).

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

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

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

141 Results

142 Three homoeologous copies of *SPO11-2* are present in the wheat genome but 143 slightly differ between each other

144 We used *Arabidopsis SPO11-2* protein sequence (AT1G63990.1) as a query to perform a
145 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
146 three high-confidence proteins located on the homoeologous group 7 chromosomes and
147 annotated as *TraesCS7A02G300300.1*, *TraesCS7B02G201200.1* and *TraesCS7D02G296000.2*.
148 For convenience, they will further be named as *TaSPO11-2-7A*, *TaSPO11-2-7B* and *TaSPO11-2-7D*
149 respectively. The amino acid (aa) identity percentages shared with *Arabidopsis* protein
150 ranged from 64.9 to 66.7% (Table 1) suggesting a strong conservation of *SPO11-2* protein
151 between these two species. The genomic wheat-gene lengths were 3085 bp, 3299 bp and
152 3026 bp for the A, B and D copies respectively. This was 50% more than the gene from
153 *Arabidopsis* (2063 bp), confirming longer introns in wheat as described earlier (IWGSC 2014;
154 2018). Differences in size between the copies were mainly due to small insertions/deletions
155 (indels) in introns 8 and 10 (Figure S1).

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

168 The *TaSPO11-2-7A* genomic sequence is highly similar to the B and D homoeologous copies
169 (95.3% and 96% identity respectively, Figure 1B). Two amino acids are inserted just after the
170 predicted translation starting site (ATG; Figure 1C). In addition, this copy exhibits a deletion of
171 two nucleotides (CC) in exon 2 (Figure 1C). This deletion results in a frameshift inducing a
172 premature stop codon (PSC) at the end of the exon 3 suggesting that the A copy is not
173 functional. *Ab initio* annotation proposed an alternative model where exon 2 is skipped
174 leading to a shorter protein (359 aa; Figure 1A), albeit more similar to the Arabidopsis protein
175 (66.7% identity). These results prompted us to investigate whether the mutation on the A copy
176 is also present in the diploid and tetraploid ancestors of wheat and in other varieties.

177 **The mutation of the A copy is conserved between cultivars and with tetraploid** 178 **species but is absent from the diploid ancestors**

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

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

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

197 Since the *SPO11-2* sequence is extremely well conserved between wheat and Arabidopsis, we
198 tested whether the function of *SPO11-2* is also conserved during evolution. We expressed the
199 *TaSPO11-2-7D* cDNA in the Arabidopsis *spo11-2* mutant and evaluated its ability to restore
200 fertility. Strikingly, all 10 *spo11-2* homozygous mutant plants expressing the *TaSPO11-2-7D*
201 transgene that we obtained, exhibited a significant increase in fertility compared to *Atspo11-*
202 *2* mutant (Figure 3; Table S2). The residual fertility in the different transformants ranged from
203 50% to 100% of that of the wild-type, with most lines exhibiting fertility around 80-90%. This
204 is in strong contrast with the 5% residual fertility of the *Atspo11-2* mutants (Hartung *et al.*,
205 2007). Cytogenetic analysis of meiotic progression in pollen mother cells of *Atspo11-2*
206 transformants expressing the *TaSPO11-2-7D* transgene revealed normal meiotic stages
207 (Figure 3C). In particular, Metaphase I with five bivalents could be observed in *Atspo11-*
208 *2/TaSPO11-2-7D* transgenic plants (mean of 4.4 bivalent/cell, n = 35) contrasting with the
209 presence of univalents in *Atspo11-2* (Hartung *et al.*, 2007). These results demonstrate that
210 wheat *TaSPO11-2-7D* gene can substitute for Arabidopsis *SPO11-2* and thus exhibits
211 evolutionary conserved function in meiosis.

212 **Only the B and D copies of *TaSPO11.2*, but not the A-copy, ensure wheat** 213 **fertility**

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

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

228 and double *ab*^{-/-} and *ad*^{-/-} mutants exhibited between 90.5% and 96% of unaltered pollen. On
229 the contrary, only 12% and 30% of the pollen grains were viable in the triple *abd*^{-/-} and the
230 double *bd*^{-/-} mutants respectively (Figure S3B). Total RNAs of cvs Renan and CS anthers were
231 collected at the leptotene stage and semi-quantitative RT-PCR performed to analyse
232 expression of the different *TaSPO11-2* copies. All three copies were expressed (Figure S4A)
233 and they were more highly expressed in Renan compared to Chinese Spring. In both varieties,
234 the D-copy was more expressed than the B-copy that was more expressed than the A-copy
235 (Figure S4B). For the A copy, using available RNASeq data (Lloyd *et al.*, 2014; Martin *et al.*,
236 2018; Ramirez-Gonzalez *et al.*, 2018), we showed that both isoforms (with alternative ATG or
237 with exon-2 skipping) were expressed (Figure S4C) and that the main expressed form was the
238 one with exon-2 skipping. All together, these results showed that both B and D copies of the
239 *SPO11-2* gene are functional, while the A copy is altered and no longer plays its full role.
240 Despite its alteration, the A copy remains expressed, but significantly less so than the other
241 two copies. A single functional copy of either the B or D genome is sufficient to ensure normal
242 fertility.

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

244 Mutation of *SPO11-2* affects meiosis in Arabidopsis (Hartung and Puchta 2000, 2001). We thus
245 established an atlas of meiotic stages for the wheat mutants. In control plants (*ABD*^{+/+}) meiosis
246 is normal, with 21 bivalents at Metaphase I, well-balanced anaphases and final meiotic
247 products composed of four balanced nuclei (tetrads; Figure 5A). Normal meiotic stages were
248 also observed for all single sub-genome mutants (*a*^{-/-}, *b*^{-/-}, *d*^{-/-}), and for the *ab*^{-/-} and *ad*^{-/-} double
249 mutants (Figure S5). In contrast, extensive meiotic defects were observed in the *abd*^{-/-} triple
250 mutant, with only univalents observed at Metaphase I and random segregation of these at
251 Anaphase I. This leads to unbalanced polyads at the end of Meiosis II in these plants (Figure
252 5A, Figure S6). The *bd*^{-/-} double-mutant showed the same phenotypes as *abd*^{-/-} triple mutant,
253 confirming that the A copy is non-functional. Quantification of numbers of chiasmata per cell
254 in control plants (*ABD*^{+/+}, *ABD*^{+/-}), the single (*a*^{-/-}, *b*^{-/-}, *d*^{-/-}), the double (*ab*^{-/-}, *ad*^{-/-}) mutants,
255 showed 21 bivalents and a mean of 40.4 ± 2.0 , 38.7 ± 3.5 and 38.1 ± 3.4 chiasmata per cell,
256 respectively (Figure 5B). In sharp contrast, the *bd*^{-/-} double mutant exhibited 4.6 ± 2.4
257 chiasmata per cell and no chiasmata were observed in the *abd*^{-/-} triple mutant. Thus, as in
258 Arabidopsis, *TaSPO11-2* is required for CO formation in wheat. However, only the B and D

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

261 ***TaSPO11.2* is necessary for chromosome synapsis and meiotic DSB formation** 262 **in hexaploid wheat**

263 We established the kinetics of the synaptonemal complex in our control (Figure S7). At the
264 beginning of meiotic prophase, ASY1 (Asynaptic 1) associates with chromosome axes, forming
265 visible filaments. At the leptotene stage, the ZYP1 (Zipper 1) protein is present on
266 chromosome axes as foci and these extend as synaptonemal complex (SC) formation proceeds
267 through zygotene and pachytene stages. These filaments disassemble at the diplotene stage
268 to fix only the chiasmata. In wheat, the SC is thus set up in the same way as in other species
269 (Colas *et al.*, 2017). We focussed on the pachytene stage to see if synapsis was affected by the
270 mutation of SPO11-2 protein in wheat. In the *abd* mutant, ASY1 is normally loaded onto the
271 chromosomes, forming long filaments along chromosome axes and eventually starts
272 disassembling at pachytene. In contrast, ZYP1 signal appears mostly in dots and do not form
273 long filaments typical of synapsis, although a few partial stretches could be observed (Figure
274 6A). Normal pachytene stages were not observed. As expected, sub-genome single-mutants
275 showed normal synapsis while triple-mutant was unable to form SC. Similar defects were
276 observed for the *bd*^{-/-} double mutant, confirming the inactivity of A copy (Figure 6A). The other
277 double-mutants (*ab*^{-/-}, *ad*^{-/-}) had normal complete synapsis. Meiotic progression is thus
278 dramatically affected in absence of *TaSPO11-2* and the SPO11-2 protein is necessary for the
279 establishment of the SC in bread wheat.

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

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

298

299 Discussion

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

301 Using SPO11-2 Arabidopsis protein as query, we found three copies of the orthologous gene
302 in the wheat genome, mapping on each of the three homoeologous genomes. This was not
303 unexpected since 47% of the wheat genes are present in triads with a single gene copy per
304 sub-genome (an A:B:D configuration of 1:1:1; IWGSC 2018). The three copies of hexaploid
305 wheat and those from diploid (*T. urartu* and *Ae. tauschii*) and tetraploid (*T. diccoides*) wheat
306 ancestors are highly conserved. The D copy was the best conserved between wheat and *Ae.*
307 *tauschii*, which is consistent with its more recent hybridization with the AABB tetraploid
308 genome progenitor and with a limited number of D-genome progenitors (Giles and Brown
309 2006; Marcussen *et al.*, 2014). This high level of similarity was also observed between wheat
310 D-copy and Arabidopsis *SPO11-2* gene, confirmed by the successful complementation of
311 Arabidopsis *spo11-2* mutant with the wheat SPO11-2-D protein. This latter result is
312 reminiscent of the data obtained with *TaSPO11-1* (Da Ines, Michard *et al.*, in press) and
313 demonstrate that both *TaSPO11-1* and *TaSPO11-2* can functionally replace Arabidopsis
314 *AtSPO11-1* and *AtSPO11-2*. Collectively, our data reveal important functional conservation of
315 the *SPO11* complex features.

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

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

339 Alternatively, exon skipping (or alternative splicing) removing exon 2 gives a protein of 359 aa,
340 with all catalytic sites and with the first amino acids involved in the interaction with MTOPVIB.
341 Alternative splicing is observed for *SPO11* in mammals (Romanienko and Camerini-Otero
342 1999) and for *SPO11-1* in Arabidopsis (Hartung and Puchta 2000; Reddy *et al.* 2013), with up
343 to seven isoforms observed for *SPO11-2* in Arabidopsis because of either intron retention or
344 exon skipping (Sprink and Hartung 2014). Several isoforms have also been observed in *Brassica*
345 *rapa* (6 isoforms), *Carica papaya* (6), *Oryza sativa* (2) and *Physcomitrella patens* (2). Isoform
346 β^* from *P. patens* corresponds to the skipping of exon 2 as we suggest for the A copy of wheat
347 and this form was found in generative tissues only (Sprink and Hartung 2014).

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

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

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

356 **TaSPO11-2 is necessary for meiotic double strand break formation**

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

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

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

371 In Arabidopsis, ~150-250 DSBs are observed at Leptotene (Vignard et al., 2007; Serrentino and
372 Borde 2012) while in maize, two-fold more (~500) DSBs are observed (Anderson et al., 2003;
373 Pawlowski et al., 2003) for a genome that is ~20 times larger. The wheat genome is more than
374 130 times larger than that of Arabidopsis and ~6 times larger than the maize genome (~14.5
375 Gb, IWGSC 2018). According to this non-linear relationship between genome-size and number
376 of DSBs, we expected ~800-3200 DSBs in wheat. Using γ -H2AX staining, Gardiner et al.,
377 estimated the number of DSBs to more than 2100 (Gardiner et al., 2019). Using DMC1
378 antibody, we estimated for the first time in wheat, the number of DMC1 foci as being between
379 365-1563 (mean 833), which corresponds to the lower boundary of our estimates. The slight
380 discrepancy between γ -H2AX and DMC1 counts may be explained by several parameters such
381 as lab growing conditions, cell staging and/or choice of antibody. DMC1 marks active DSB sites
382 engaged in homologous recombination and thus, although very close to reality, DMC1 foci

383 may slightly underestimate the real number of initial DSBs. On the contrary, γ -H2AX may not
384 always indicate the presence of DSBs (de Feraudy *et al.*, 2010; Cleaver *et al.* 2011; Revet *et al.*,
385 2011) and thus use of γ -H2AX could slightly overestimate DSBs. Nevertheless, both
386 approaches gave a similar order of magnitude and number of DSBs obtained is in the range of
387 what was expected. Thus, compare to Arabidopsis, the bread wheat genome exhibit around
388 ten times more DSBs while its genome is 130 times larger. This means that the DSBs density is
389 much lower in the bread wheat genome. Interestingly, recombination mainly occurs in the
390 distal regions of the chromosomes in wheat, while pericentromeric regions are almost devoid
391 of COs (Saintenac *et al.*, 2009; Choulet *et al.*, 2014). It is tempting to speculate that this CO
392 distribution is explained (at least partly) by a low amount of DSBs compared to the size of the
393 genome and by a preferential location of these DSBs in the telomeric regions to the expense
394 of pericentromeric regions. However, this remains to be demonstrated.

395

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

402

403 Experimental procedures

404 Plant material and growth conditions

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

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

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

421 **Identification of wheat *SPO11-2* copies**

422 DNA sequences of *SPO11-2* were identified in the hexaploid wheat ([https://wheat-
423 urgi.versailles.inra.fr/Data](https://wheat-urgi.versailles.inra.fr/Data)), tetraploid wheat *T. durum* (Avni *et al.*, 2017
424 <https://wewseq.wixsite.com/consortium>), and diploid wheat-relatives *T. urartu* (Ling *et al.*,
425 2018 <http://www.mbkbase.org/Tu/>) and *Ae. tauschii* (Luo *et al.*, 2017
426 <https://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA341983&cmd=DetailsSearch>) genome
427 through a BLAST-P research using the Arabidopsis *SPO11-2* protein sequence (AT1G63990.1)
428 as query. The three copies of wheat *SPO11-2* (TraesCS7A02G300300.1,
429 TraesCS7B02G201200.1, TraesCS7D02G296000.2) further named TaSPO11-2-7A, TaSPO11-2-
430 7B, TaSPO11-2-7D respectively) as well as *T. turgidum ssp dicoccoides* (TRIDC7AG041910.1
431 and TRIDC7BG033100.4), *T. urartu* (TuG1812G0700003251.01) and *Ae. tauschii*
432 (AET7Gv20751300.7) copies were then aligned with MAFFT7 (Multiple Alignment using Fast
433 Fourier Transform; <https://mafft.cbrc.jp/alignment/server/>; Kato *et al.*, 2002).

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

436 Intron/exon boundaries were identified using the genomic and CDS sequences from
437 Arabidopsis as model. We aligned *SPO11-2* genomic and CDS sequences exon 2 with MAFFT7.
438 A deletion of two nucleotides (CC) was found in the A-copy.

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

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

445 **Gene expression**

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

453 **Complementation in Arabidopsis**

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

463 **Meiocyte isolation**

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

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

477 **Design of DMC1 antibody**

478 The DMC1 protein shares very strong homology with the RAD51 protein (50% of amino-acid
479 positions) and to avoid cross-reactivity we aligned TaRAD51 and TaDMC1 proteins and those
480 of *Arabidopsis thaliana* (AT5G20850.1 and AT3G22880.1). We selected sequences used by
481 Sanchez-Moran *et al.*, (2007) considered as the most immunogenic in our case (amino-acids
482 27 to 42). We validated the uniqueness of this sequence *in silico* by doing a BLAST-P against
483 the IGWSC database using the short function for the small sequences. BLAST-analysis results
484 confirmed the presence of hits exclusively in TaDMC1-5-CS. Two peptides for TaDMC1-5-CS
485 were designed after the bioanalysis. We used competition with the two peptides synthesized
486 for the immunization of 2 chickens by Eurogentec to validate experimentally the specificity of
487 the TaDMC1-5-CS antibody. Antibody is available on request.

488 **Immunostaining**

489 Samples were prepared according to Colas *et al.*, (2016, 2017) and performed with slight
490 modifications for wheat mutants. Anthers at prophase I stage were collected on ice in a watch
491 glass containing 1 mL phosphate buffer (PBS) 1X with 1 μ L of protease inhibitor cocktail
492 (Sigma) then fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature.
493 Anthers were washed twice with 1 mL PBS 1X for 5 min at 4°C, transferred at room
494 temperature in 1 mL permeabilization solution (PBS 1X with 0.05% Triton X100, 1 mM EDTA)
495 for 15 min then in 1 mL blocking solution consisting in 3% bovine serum albumin (BSA) in PBS
496 1X, 0.1% Tween-20, 1mM EDTA for 15 min. Meiocytes of three anthers per slide were isolated
497 manually under binocular in 20 μ L of PBS 1X on Poly-L-Lysine coated slide. The meiocyte
498 suspension was left at room temperature to dry slightly. Fifty microliters per slide of primary
499 antibody solutions (dilutions 1:400 anti-TaASY1 (rabbit, Agrisera,) Boden *et al.*, 2007, 2009,

500 1:200 anti-TaZYP1 (guinea pig) supplied by A.C. Martin, John Innes Centre, UK and a mixture of
501 two peptides by 1:20 anti-TaDMC1 (chicken, Eurogentec)) were deposited on the slide then
502 covered with a piece of parafilm. The slides were placed in humid chamber at 4°C for 36 h to
503 48 h. Slides were washed three times for 5 min with PBS 1X. Fifty microliters of secondary
504 antibody solutions (dilution in blocking solution 1:400 anti-rabbit Alexa Fluor 568, 1:300 anti-
505 guinea pig Alexa Fluor 647, 1:300 anti-chicken Alexa Fluor 488, Fisher Scientific) were
506 deposited on each slide, covered with parafilm and incubated in a humid chamber at room
507 temperature for 1 h. Slides were washed tree times with PBS 1X and mounted with
508 Vectashield-DAPI.

509 **Confocal microscopy and image analysis**

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

517 **Viability of pollen and fertility**

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

525 **Statistics**

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

528 testing with default values (and other tests: from Anderson and Darling, from Lillifors and the
529 Jarque Bera test). If the values follow a normal distribution, a student test was applied with
530 the Two test and test module, keeping the defaults (alpha=0.05). If the values do not follow a
531 normal distribution we applied a non-parametric Kruskal-Wallis test with an alpha error to
532 0.01.

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544 Supporting Information

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

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

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

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

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

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

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

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

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

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

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

558

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827

828

829 Tables

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

838

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_PGSA_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

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

840

841 Figure legends

842 **Figure 1.** Characterization of SPO11-2 homoeologous genes of bread wheat. **A.** Structure of
 843 the genes (light-grey; exons are coloured) and of the proteins (dark-grey; the seven domains
 844 (see Table S1-B; Bergerat *et al.*, 1997; Malik *et al.*, 2007; Hartung *et al.*, 2010; Vrielynck *et al.*,
 845 2016) are coloured); **B.** Ternary graphic of the percentages of identity between homoeologous
 846 proteins (left) and genomic sequences –right); **C.** *TaSPO11-2* MAFFT alignment of exon-1 and
 847 exon-2 of the three homoeologous wheat copies; translation initiation sites are squared in
 848 green and intron/exon boundaries in red; deletion of two nucleotides in *TaSPO11-2-7A-CS* is
 849 highlighted with a grey circle.

850

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

857

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

866

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

873 **Figure 5.** Progression of meiosis from metaphase I to telophase II (tetrads) in WT and mutants.

874 **A.** Mutants *bd^{-/-}* and *abd^{-/-}* show abnormalities during meiosis such as presence of univalents
875 in metaphase I (orange circle) and polyad formation at anaphase II (red circle). Scale bar: 5
876 μ m. **B.** Chiasma number per cell. Since values do not follow normality law, Kruskal-Wallis test
877 was applied with alpha = 0.01 and Monte-Carlo methods with 10,000 simulations. Values were
878 significantly different for the double (*bd^{-/-}*) and triple (*abd^{-/-}*) mutants (red) only (*: p-value <
879 0.0001; NS: not significant).

880 **Figure 6.** Synaptonemal complex (SC) and DMC1-foci analyses of the mutants during prophase
881 I. **A.** Immuno-localization of synaptonemal proteins ASY1 (green) and ZYP1 (red). Control
882 $ABD^{+/+}$: the synapse is set up correctly until complete co-localization of the two proteins at
883 pachytene. $abd^{-/-}$ and $bd^{-/-}$ mutants: alteration of the SC with ZYP1 protein aggregates leading
884 to synapse failure. Scale bar: 5 μ m. **B.** Triple immuno-localization of ASY1 (red), ZYP1
885 (magenta) and DMC1 (green) meiotic proteins at leptotene stage. Scale bar : 5 μ m. **C.** number
886 of DMC1 foci per cell at mid-zygotene for the genotypes Renan (RE), Chinese spring (CS),
887 control $ABD^{+/+}$, and mutants $bd^{-/-}$ and $abd^{-/-}$. Since values follow normality law, a T-Test was
888 applied between the control ($ABD^{+/+}$; blue) and the mutants (red). *: p-value <0.0001; NS: not
889 significant.