Magnesium Increases Homoeologous Crossover Frequency During Meiosis in ZIP4 (Ph1 Gene) Mutant Wheat-Wild Relative Hybrids

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Wild relatives provide an important source of useful traits in wheat breeding. Wheat and wild relative hybrids have been widely used in breeding programs to introduce such traits into wheat. However, successful introgression is limited by the low frequency of homoeologous crossover (CO) between wheat and wild relative chromosomes. Hybrids between wheat carrying a 70 Mb deletion on chromosome 5B (ph1b) and wild relatives, have been exploited to increase the level of homoeologous CO, allowing chromosome exchange between their chromosomes. In ph1b-rye hybrids, CO number increases from a mean of 1 CO to 7 COs per cell. CO number can be further increased up to a mean of 12 COs per cell in these ph1b hybrids by treating the plants with Hoagland solution. More recently, it was shown that the major meiotic crossover gene ZIP4 on chromosome 5B (TaZIP4-B2) within the 70 Mb deletion, was responsible for the restriction of homoeologous COs in wheat-wild relative hybrids, confirming the ph1b phenotype as a complete TaZIP4-B2 deletion mutant (TaZIP4-B2 ph1b). In this study, we have identified the particular Hoagland solution constituent responsible for the increased chiasma frequency in TaZIP4-B2 ph1b mutant-rye hybrids and extended the analysis to TaZIP4-B2 TILLING and CRISPR mutant-Ae variabilis hybrids. Chiasma frequency at meiotic metaphase I, in the absence of each Hoagland solution macronutrient (NH4H2PO4, KNO3, Ca(NO3)2·4H2O or MgSO4·7H2O) was analyzed. A significant decrease in homoeologous CO frequency was observed when the Mg2+ ion was absent. A significant increase of homoeologous CO frequency was observed in all analyzed hybrids, when plants were irrigated with a 1 mM Mg2+ solution. These observations suggest a role for magnesium supplementation in improving the success of genetic material introgression from wild relatives into wheat.

Keywords: wheat, wild relatives, magnesium, Ph1 locus, TaZIP4-B2, homoeologous crossover, CRISPR/Cas9 system
INTRODUCTION

Despite possessing related ancestral genomes (genome AABBD), bread wheat behaves as a diploid during meiosis. Deletion of chromosome 5B in tetraploid and hexaploid wheat results in a level of incorrect chromosome pairing and exchange, visualized as a low level of multivalents at metaphase I, and homoeologous crossovers (COs) between related chromosomes in wheat-wild relative hybrids (Riley and Chapman, 1958; Sears and Okamoto, 1958). From these observations, it was proposed that chromosome 5B carries a locus termed Pairing homoeologous 1 (Ph1), which evolved on wheat's polyploidalization and restricted chromosome pairing and COs to true homologs (Riley and Chapman, 1958). A hexaploid wheat cv. Chinese Spring (CS) line carrying a 70 Mb deletion on the long arm of chromosome 5B (ph1b) has been exploited over the last 40 years to allow exchange between wild relative and wheat chromosomes. Recently, it was shown that on wheat's polyploidalisation, a segment of 3B carrying the major crossover gene ZIP4 and a block of heterochromatin, duplicated and inserted between two CDK2-like genes within a cluster of CDK2-like and methyl-transferase genes (Griffiths et al., 2006; Al-Kaff et al., 2008; Martin et al., 2014, 2017). Using exploitation of TILLING mutants, it was shown that the duplicated ZIP4 gene (TaZIP4-B2) within this cluster, both promotes homologous CO and restricts homoeologous CO (Rey et al., 2017). Therefore, TaZIP4-B2 within the 70 Mb ph1b deletion region is responsible for the effect on homoeologous CO in wheat-wild relative hybrids, and as such the ph1b line can be described as a complete-deletion (or complete loss-of-function) mutant of TaZIP4-B2 (TaZIP4-B2 ph1b mutant). In terms of the effect on chromosome synopsis/pairing, cell biological studies reveal that the ph1b deletion in wheat has little effect, with most synopsis occurring during clustering of the telomeres as a bouquet. Furthermore, in wheat-wild relative hybrids, which only possess homoeologues, the ph1b deletion also has little effect on the level of synopsis, except that most pairing occurs after dispersal of the telomere bouquet. In wheat itself, a few chromosomes also undergo delayed pairing until after dispersal of the bouquet, with the subsequent incorrect pairing leading to the low level of multivalents observed at metaphase I (Martin et al., 2014, 2017).

For the last 40 years, the wheat CS ph1b deletion line has been exploited in crosses with wild relatives to allow exchange between chromosomes at meiosis. As indicated previously, in these hybrids, the extent of chromosome synopsis is similar whether the line carries the ph1b deletion or not. Moreover, on the synapsed chromosomes, similar numbers of MLH1 sites (normally a marker for CO), are observed (Martin et al., 2014). However significant site CO frequency is only observed in those hybrids carrying the ph1b deletion. However even in this case, the frequency of resulting COs still does not reflect the number of available MLH1 sites (Martin et al., 2014). This implies that there is potential for increased processing of MLH1 sites into COs. Fortuitously, it has been observed that a nutrient solution (Hoagland's solution) added to the soil when TaZIP4-B2 ph1b mutant-rye hybrids are growing resulted in increased CO frequency, although it was not known which nutrient component was responsible for the effect (Martin et al., 2017).

Mineral elements are essential nutrients for plants to complete their life cycle. They are classified into macro and micronutrients, which are required in relatively large and small amounts, respectively (Hoagland and Arnon, 1950). The importance of each of these macronutrients has been reported in numerous physiological processes, such as plant growth, cell division, and metabolism (Huber, 1980; Maathuis, 2009). However, limited studies have been performed as to their effect on meiosis. Early studies have previously reported that alterations of external factors, such as temperature, or nutrient composition, can produce profound effects on chiasma frequency (Grant, 1952; Wilson, 1959; Law, 1963; Bennett and Rees, 1970; Fedak, 1973).

The main objective of the present study was to determine whether a specific macronutrient present in the Hoagland solution was responsible for the observed increased homoeologous CO frequency in Tazip4-B2 ph1b mutant-rye hybrids described in Martin et al. (2017). We also analyzed whether this macronutrient increased homoeologous CO frequency in each of the Tazip4-B2 ph1b (complete deletion), TILLING (point mutation) and CRISPR (partial deletion) mutant-Ae. variabilis hybrids.

MATERIALS AND METHODS

Plant Material

Plant material used in this study included: Triticum aestivum (2n = 6x = 42; AABBD) cv. Chinese Spring Tazip4-B2-ph1b mutant line (Sears, 1977); Triticum aestivum cv. Chinese Spring-rye hybrids—crosses between the Tazip4-B2-ph1b mutant line hexaploid wheat and rye [Secale cereal cv. Petkus (2n = 2x = 14; RR)]; Triticum aestivum cv. Chinese Spring-Aegilops variabilis hybrids—crosses between Tazip4-B2-ph1b mutant line hexaploid wheat and rye [Ae. variabilis Egl. (2n = 4x = 28; UUS’S’)]; Triticum aestivum cv. Cadenza-Ae. variabilis hybrids—crosses between Cad1691 and Cad0348, Tazip4-B2 TILLING mutants and Ae. variabilis (Krasileva et al., 2017; Rey et al., 2017); and Triticum aestivum cv. Fielder-Ae. variabilis hybrids—crosses between Tazip4-B2 CRISPR mutant and Ae. variabilis (see Production of TaZIP4-B2 knock-out using RNA-guided Cas9, section Materials and Methods).

Nutrient Solution Treatments

The total number of plants used in this work is described in Table S1. All seedlings were vernalized for 3 weeks at 7°C under a photoperiod of 16 h light/8 h dark, and then transferred to a controlled environmental room until meiosis (approximately 2 months later for all genotypes used in this study). The growth conditions were 16 h/8 h, light/dark photoperiod at 20°C day and 15°C night, with 70% humidity. At least 2 weeks before meiosis, irrigation of plants with a Hoagland solution (100 mL per plant) was commenced following the method previously described in Martin et al. (2017). Briefly, plants were irrigated once a week with a Hoagland solution (100 mL) from the stem elongation stage of the vegetative stage (stage 7–8, Feeke’s scale).
The composition of the Hoagland solution was: (macronutrients) KNO₃ (12 mM), Ca (NO₃)₂-2H₂O (4 mM), NH₄H₂PO₄ (2 mM), MgSO₄.7H₂O (1 mM); and (micronutrients) NaFe-EDTA (60 mM), KCl (50 µM), H₃BO₃ (25 µM), MnSO₄·H₂O (2 µM), ZnSO₄ (4 µM), CuSO₄·5H₂O (0.5 µM), H₂MoO₄·2H₂O (0.5 µM). Four treatments were carried out to analyze the effect of the absence of NH₄H₂PO₄, KNO₃, Ca (NO₃)₂-2H₂O or MgSO₄·7H₂O from the Hoagland solution on homoeologous CO frequency in *Tazip4-B2-ph1b* mutant-rye hybrids. For each treatment, a different Hoagland solution was prepared in the absence of each macronutrient (NH₄H₂PO₄, KNO₃, Ca (NO₃)₂-2H₂O or MgSO₄·7H₂O). Moreover, the effect of the presence of only MgSO₄·7H₂O (MgSO₄·7H₂O is designed as Mg²⁺ in the manuscript) in only water rather than in Hoagland solution on CO frequency was also analyzed in *Tazip4-B2-ph1b* mutant-rye hybrids in comparison to the effects of Hoagland solution. Also, two different concentrations of Mg²⁺ (1 and 2 mM of Mg²⁺ in water) were used to assess the homoeologous CO frequency in *Tazip4-B2-ph1b* mutant-rye hybrids. The treatment with either Mg²⁺ in water alone or Hoagland solution in *Tazip4-B2-ph1b* mutant-*Ae. variabilis*, and *Tazip4-B2 TILLING and CRISPR mutant-*Ae. variabilis* hybrids was also assessed.

Assessment of the addition of Mg²⁺ in water alone on homoeologous CO frequency was also made on non-irrigated plants, by injecting into *Tazip4-B2 ph1b* mutant-rye hybrids tillers a solution containing 1 mM Mg²⁺ in water (0.5 mL per spike) just above every spike at the stage 9 on the Feekes’s scale (three spikes with water alone and three spikes with Mg²⁺ in water). All spikes were analyzed 24–48 h after the injection.

**Feulgen-Stained Analysis**

After either irrigating with Hoagland or Mg²⁺ solution, or injecting the Mg²⁺ solution, tillers were harvested when the flag leaf was completely emerged, and only anthers at meiotic metaphase I were collected and fixed in 100% ethanol/acetic acid 3:1 (v/v) for at least 24 h. Cytological analysis of meiocytes was performed using Feulgen reagent as previously described in [Sharma (2014)](https://www.frontiersin.org/articles/10.3389/fpls.2014.00956/full). Metaphase I meiocytes were observed under a phase contrast microscope equipped with a Leica DFC450 camera and controlled by LAS v4.4 system software (Leica Biosystems, Wetzlar, Germany). The digital images were used to determine the meiotic configurations of the meiocytes by counting the number of univalents, rod (1 chiasma) and ring (2 chiasmata) bivalents and multivalents (trivalents (1–2 chiasmata), tetravalents (3 chiasmata) and pentavalents (4 chiasmata)). Two different methods depending on the number of chiasma (single or double chiasmata) were used to calculate chiasma frequency per meiocyte (see Figure S1 for examples of the scored structures). Images were processed using Adobe Photoshop CS5 (Adobe Systems Incorporated, US) extended version 12.0 × 64.

**Production of *TaZIP4-B2* CRISPR Mutants Using RNA-Guided Cas9**

Three single guide RNAs (sgRNA) were designed manually to specifically target *TaZIP4-B2*. These guides were in the limited regions where there was sufficient variation between ZIP4 on 5BL and homoeologous group 3 chromosomes (Figure 3). The specific guides were: Guide 4: 5′GATGAG CGACGATCTCTGTG3′, Guide 11: 5′GATGCGTGCTGCT ATCCCTCGG3′ and Guide 12: 5′GAAGAAAGATGCGGCC TTGAG3′ (Figure 3). Two constructs were assembled using standard Golden Gate assembly ([Werner et al., 2012](https://www.frontiersin.org/articles/10.3389/fpls.2012.00379/full)) with each construct containing the Hygromycin resistance gene under the control of a rice Actin1 promoter, Cas9 under the control of the rice ubiquitin promoter and two of the sgRNAs each under the control of a wheat U6 promoter (Figure 3). Construct 1 contains guides 4 and 12 and construct 2 contains guides 11 and 12. To produce each gRNA, a PCR reaction was performed using Phusion High-Fidelity Polymerase (Thermo Scientific M0530S) with a forward primer containing the gRNA sequence, and a standard reverse primer 5′TGTTGGTC TCCAAGCGTAATGCCAATTTGTAC3′ using the plasmid pICSL7001::U6p::gRNA (Addgene plasmid 46966) as template. Each gRNA was cloned individually into the level 1 vectors pICH47751 (gRNA4 & 11) and pICH47761 (gRNA12). Level 1 construct pICH47802-RActpro::Hpt::NosT (selection maker), pICH47742-ZmUbipro::Cas9::NosT and the gRNAs were then assembled in the binary Level 2 vector pGoldenGreenGate (pGGG) a Golden Gate compatible vector based on pGreen ([Hellens et al., 2000](https://www.frontiersin.org/articles/10.3389/fpls.2000.00001/full)) (Figure 3).

The two constructs were introduced to *T. aestivum* cv. Fielder by Agrobacterium-mediated inoculation of immature embryos. 450 immature embryos were inoculated with Agrobacterium strain AGL1 containing each construct. Briefly, after 3 days co-cultivation with Agrobacterium, immature embryos were selected on 15 mg/l hygromycin during callus induction for 2 weeks and 30 mg/l hygromycin for 3 weeks in the dark at 24°C on Murashige and Skoog medium (MS; Murashige and Skoog, 1962) 30 g/l Maltose, 1.0 g/l Casein hydrolysate, 350 mg/l Myo-inositol, 690 mg/l Proline, 1.0 mg/l Thiamine HCl ([Harwood et al., 2009](https://www.frontiersin.org/articles/10.3389/fpls.2009.00751/full)) supplemented with 2 mg/l Picoloram, 0.5 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D). Regeneration was under low light (140 µmol.m-2.s-1) conditions on MS medium with 0.5 mg/l Zeatin and 2.5 mg/l CuSO₄·5H₂O.

Primary transgenic plants (T0) were analyzed by PCR across the region of interest. The sequences for the forward and reverse primers used for the screening in T0 were 5′GCCGCCCAT GACGATCTCTGGAG3′ and 5′GGACGGCGAGGGAGCGAGAG3′, respectively ([Rey et al., 2017](https://www.frontiersin.org/articles/10.3389/fpls.2017.00809/full)) followed by direct sequencing. The PCR was performed using RedTaq ReadyMix PCR Reaction
Magnesium Increases Homoeologous COs

RESULTS

Magnesium Increases Homoeologous COs in Tazip4-B2 ph1b Mutant-Rye Hybrids

The Tazip4-B2 ph1b mutant-rye hybrids were obtained by crosses between the hexaploid wheat cv. Chinese Spring Tazip4-B2 ph1b mutant and rye. These hybrids were used to analyse which macronutrient (NH4·H2PO4, KNO3, Ca(NO3)2·4H2O or MgSO4·7H2O) present within in the Hoagland solution detailed in Martin et al. (2017) could be responsible for the increased CO number observed in the Tazip4-B2 ph1b mutant-rye hybrids. To assess the effect of the absence of each macronutrient in homoeologous CO frequency in meiotic metaphase I, we irrigated several Tazip4-B2 ph1b mutant-rye hybrids with: (1) Hoagland solution; (2) water alone; (3) Hoagland solution minus KNO3; (4) Hoagland solution minus Ca(NO3)2·4H2O; (5) Hoagland solution minus NH4·H2PO4; (6) Hoagland solution minus MgSO4·7H2O (MgSO4·7H2O is designed as Mg2+ in the manuscript) (Table 1). The absence of each Hoagland solution macronutrient caused a slight increase in homoeologous CO frequency, except for the treatment lacking Mg2+, where a significant decrease in homoeologous CO frequency per meiocyte was observed at meiotic metaphase I in these hybrids (Table 1).

No significant differences in CO frequency at metaphase I were observed between hybrids treated with water alone and those

Statistical Analyses

Statistical analyses were performed using STATISTIX 10.0 software (Analytical Software, Tallahassee, FL, USA). Analysis of variance (ANOVA) in Tazip4-B2 ph1b mutant-rye hybrids, Tazip4-B2 TILLING mutant-Ae. variabilis hybrids and Tazip4-B2 CRISPR mutant-Ae. variabilis hybrids was based on a completely randomized design. Several transformations were carried out: tangent (ring bivalents), arcsine (trivalents), and logarithm (double CO) transformations in the analysis of the effect of absence of each macronutrients in homoeologous CO frequency in Tazip4-B2 ph1b mutant-rye hybrids; exponential (ring bivalents) transformation in Tazip4-B2 ph1b mutant-Ae. variabilis hybrids; exponential (rod bivalents, rings bivalents and trivalents) transformation in Tazip4-B2 TILLING mutant (Cad1691)-Ae. variabilis hybrids; and square root (ring bivalents) transformation in Tazip4-B2 ph1b mutant-rye hybrids. These hybrids were used to analyse which macronutrient (NH4·H2PO4, KNO3, Ca(NO3)2·4H2O or MgSO4·7H2O) present within in the Hoagland solution detailed in Martin et al. (2017) could be responsible for the increased CO number observed in the Tazip4-B2 ph1b mutant-rye hybrids. To assess the effect of the absence of each macronutrient in homoeologous CO frequency in meiotic metaphase I, we irrigated several Tazip4-B2 ph1b mutant-rye hybrids with: (1) Hoagland solution; (2) water alone; (3) Hoagland solution minus KNO3; (4) Hoagland solution minus Ca(NO3)2·4H2O; (5) Hoagland solution minus NH4·H2PO4; (6) Hoagland solution minus MgSO4·7H2O (MgSO4·7H2O is designed as Mg2+ in the manuscript) (Table 1). The absence of each Hoagland solution macronutrient caused a slight increase in homoeologous CO frequency, except for the treatment lacking Mg2+, where a significant decrease in homoeologous CO frequency per meiocyte was observed at meiotic metaphase I in these hybrids (Table 1).
treated with the Hoagland solution minus Mg\(^{2+}\) [a mean of 7.91 chiasmata for hybrids treated with water alone and 8.09 chiasmata for hybrids treated with Hoagland solution minus Mg\(^{2+}\) (Table 1)].

Additionally, we scored all meiocytes for the occurrence of double chiasmata in the metaphase 1 chromosomal configurations (examples highlighted by arrows in Figure S1). When double chiasmata were considered in the chiasma frequency, a mean of 8.15 chiasmata and 8.62 chiasmata was observed respectively in Tazip4-B2 ph1b mutant-rye hybrids treated with water alone, and those treated with the Hoagland solution minus Mg\(^{2+}\) (Table 1). As expected, no significant differences were observed between the two treatments when double chiasmata were considered in these Tazip4-B2 ph1b hybrids.

Once the absence of Mg\(^{2+}\) was demonstrated to decrease homoeologous CO frequency in Tazip4-B2 ph1b mutant-rye hybrids, the effect of irrigating with only Mg\(^{2+}\) present at a final concentration of 1 mM in water rather than in the Hoagland solution, was also analyzed on homoeologous COs in Tazip4-B2 ph1b mutant-rye hybrids (Figure 1). Treatment with a solution containing only Mg\(^{2+}\) also increased homoeologous COs at metaphase I per meiocyte in these hybrids, showing no significant difference in comparison to the Hoagland solution treatment. A mean of 11.09 chiasmata was observed after treatment with 1 mM Mg\(^{2+}\) in water following injection into the tillers of Tazip4-B2 ph1b mutant-rye hybrids (Figure 1). When double chiasmata were considered in the chiasma configurations (examples highlighted by arrows in Figure S1), a mean of 11.09 chiasmata and 11.30 chiasmata considering double chiasmata in the hybrids treated with 1 mM Mg\(^{2+}\) was observed in the hybrids considering a single chiasma (Table 2) and a mean of 9.67 chiasmata and 11.30 chiasmata considering double chiasmata (Table 2).

### Table 2

| A | No. of cell examined | Univalents | Red bivalents | Ring bivalents | Trivalents | Chiasma frequency |
|---|----------------------|------------|--------------|---------------|------------|-------------------|
|   |                      | Mean ± SE  | Mean ± SE    | Mean ± SE     | Mean ± SE  | Mean ± SE         |
|   |                      | (Range)    | (Range)      | (Range)       | (Range)    | Single Chiasma    |
|   | With Hoagland solution | 109        | 12.36 ± 0.21\(^{a}\) | 4.39 ± 0.14\(^{a}\) | 2.57 ± 0.10\(^{a}\) | 0.58 ± 0.07\(^{a}\) | 10.74 ± 0.16\(^{a}\) |
|   | With 1mM Mg\(^{2+}\) | 117        | 12.26 ± 0.25\(^{a}\) | 3.38 ± 0.13\(^{a}\) | 2.81 ± 0.10\(^{a}\) | 0.79 ± 0.07\(^{a}\) | 11.09 ± 0.19\(^{a}\) |
|   | With 2mM Mg\(^{2+}\) | 107        | 14.62 ± 0.24\(^{a}\) | 4.09 ± 0.14\(^{a}\) | 1.89 ± 0.12\(^{a}\) | 0.46 ± 0.06\(^{a}\) | 8.84 ± 0.18\(^{a}\) |
| P-value | 0.0000 | 0.0116 | 0.0000 | 0.0022 | 0.0000 | 0.0000 |

**FIGURE 1** | Effect of either 1 mM or 2 mM Mg\(^{2+}\) on homoeologous CO frequency of T. aestivum cv. Chinese Spring Tazip4-B2 ph1b mutant-rye hybrids. (A) Frequencies of univalents, bivalents, trivalents, and chiasma frequency (single and double chiasmata) were scored at meiotic metaphase 1 in Tazip4-B2 ph1b mutant-rye hybrids treated with either Hoagland solution, 1 mM Mg\(^{2+}\) or 2 mM Mg\(^{2+}\) solution. Values in parenthesis indicate range of variation between cells. \(P < 0.05\) indicates significant differences according to LSD test. Different letters indicate significant differences between treatments. (B) Representative meiotic configurations of Tazip4-B2 ph1b mutant-rye hybrids. From left to right: treatment with Hoagland solution, 1 mM Mg\(^{2+}\) or 2 mM Mg\(^{2+}\) solution. Bar: 20µm.
Magnesium Increases Homoeologous COs in Wheat Tazip4-B2 TILLING Mutant-Ae. variabilis Mutant Hybrids

Recently we reported that Tazip4-B2 TILLING mutants crossed with Ae. variabilis exhibited homoeologous COs at meiotic metaphase I (Rey et al., 2017). We therefore decided to analyse whether the level of homoeologous COs induced by Tazip4-B2 TILLING mutants was also affected by treatment with 1 mM Mg^{2+} solution. To assess the effect of 1 mM Mg^{2+} on homoeologous CO frequency at metaphase I, we added 100 mL per plant of a solution of either 1 mM Mg^{2+} solution in comparison to when Mg^{2+} was absent (a mean of 11.14 and 1.99, respectively, after treatment with 1 mM Mg^{2+} and a mean of 12.49 and 1.60, respectively, after treatment with water alone were observed in Figure 2). With regard to the Hoagland solution treatment, significant differences were observed between hybrids treated with water alone and hybrids treated with Hoagland solution (Figure 2). Hoagland solution treatment showed the highest chiasma frequency, followed by 1 mM Mg^{2+} and water alone [means of 16.53, 15.31, and 14.15 chiasmata were observed, respectively, when a single chiasma was considered, and means of 18.05, 16.54, and 15.10 chiasmata were observed, respectively, when double chiasmata were considered (Figure 2)].

**TABLE 2** | Effect of injecting 1 mM Mg\(^{2+}\) solution into the tillers of Tazip4-B2 ph1b mutant-rye hybrids.

| No. of cell examined | Univalents | Trivalents | Rod bivalents | Ring bivalents | Double chiasmata |
|----------------------|------------|------------|---------------|----------------|------------------|
|                      | Mean ± SE  | Mean ± SE  | Mean ± SE     | Mean ± SE      | Mean ± SE        |
|                      | (Range)    | (Range)    | (Range)       | (Range)        | (Range)          |
| Water alone          | 87         | 15.02 ± 0.26\(^a\) | (3–20)        | 12.32 ± 0.26\(^b\) | (6–18)           |
| With 1 mM Mg\(^{2+}\)+ | 79         | 3.43 ± 0.16\(^b\) | (1–6)         | 4.32 ± 0.17a | (1–8) |
| P-value              | 0.0001     | 0.1079     | 0.0001        | 0.0001         | 0.0001           |

Frequencies of univalents, bivalents, trivalents, and chiasma frequency (single and double chiasmata) were scored at meiotic metaphase I in Tazip4-B2 ph1b mutant-rye hybrids treated with water alone and 1 mM Mg\(^{2+}\) solution. Values in parenthesis indicate range of variation between cells. P < 0.05 indicates significant differences according to LSD test. Different letters indicate significant differences between treatments.
respectively, after treatment with 1 mM Mg$^{2+}$ and means of 12.21 and 12.23 single chiasma frequency, respectively, in water alone (Table 3; Figure S2). Significant differences were also observed when double chiasmata were scored in both mutant lines (Table 3; Figure S2). Numbers of univalents and trivalents were also affected by treatment with 1 mM Mg$^{2+}$ in both mutant lines as in the Tazip4-B2 ph1b mutant-Ae. variabilis hybrids described in the previous section. Numbers of univalents were significantly decreased both in Tazip4-B2 TILLING mutant (Cad1691)-Ae. variabilis and Tazip4-B2 TILLING mutant (Cad0348)-Ae. variabilis hybrids treated with 1 mM Mg$^{2+}$ [means of 12.74 and 12.11 univalents respectively with Mg$^{2+}$ and means of 14.74 and 14.63 univalents respectively with water alone (Table 3)]. Numbers of trivalents were significantly increased both in wheat (Cad1691)-Ae. variabilis and wheat (Cad0348)-Ae. variabilis hybrids, after treatment with 1 mM Mg$^{2+}$ [means of 1.63 and 1.93 trivalents respectively with Mg$^{2+}$, and means of 1.05 and 1.27 trivalents respectively with water alone (Table 3)].

Finally, we assessed the effect of treating with Hoagland solution and with water alone, finding significant differences in homoeologous COs between the two treatments, both in Tazip4-B2 TILLING mutant (Cad1691)-Ae. variabilis and Tazip4-B2 TILLING mutant (Cad0348)-Ae. variabilis hybrids. Numbers of univalents and trivalents were also affected to the same extent (Table 3). In the Tazip4-B2 TILLING mutant (Cad1691)-Ae. variabilis hybrid, means of 14.74 univalents and 1.05 trivalents were observed in hybrids treated with water alone, and means of 11.94 univalents and 1.50 trivalents observed in hybrids treated with Hoagland solution (Table 3). In the Tazip4-B2 TILLING mutant (Cad0348)-Ae. variabilis hybrid, means of 14.63 univalents and 1.27 trivalents were observed in hybrids with water alone and means of 12.48 univalents and 1.76 trivalents in hybrids treated with Hoagland solution (Table 3).

### Table 3

| TREATMENT                        | No. of cells examined | Univalents | Rod bivalents | Ring bivalents | Trivalents | Tetravalents | Pentavalents | Chiasma frequency |
|----------------------------------|-----------------------|------------|---------------|---------------|------------|--------------|--------------|-------------------|
|                                  |                       | Mean ± SE  | Mean ± SE     | Mean ± SE     | Mean ± SE  | Mean ± SE    | Mean ± SE    | Mean ± SE         |
|                                  |                       | (Range)    | (Range)       | (Range)       | (Range)    | (Range)     | (Range)     | (Range)           |
| Without 1mM Mg$^{2+}$            | 136                   | 12.49 ± 0.59$^a$ | 6.54 ± 0.33$^a$ | 1.84 ± 0.20$^a$ | 1.60 ± 0.21$^b$ | 0.18 ± 0.08$^b$ | 0.05 ± 0.04$^b$ | 14.15 ± 0.42$^c$ |
|                                  |                       | (4-22)     | (2-10)        | (0-5)         | (0-4)      | (0-2)       | (0-1)       | (5-19)            |
| With 1mM Mg$^{2+}$               | 175                   | 11.14 ± 0.22$^b$ | 6.32 ± 0.13$^b$ | 2.11 ± 0.10$^b$ | 1.99 ± 0.08$^c$ | 0.15 ± 0.03$^c$ | 0.08 ± 0.02$^c$ | 15.31 ± 0.16$^d$ |
|                                  |                       | (1-17)     | (2-11)        | (0-6)         | (0-4)      | (0-2)       | (0-1)       | (11-23)           |
| With Hoagland solution           | 104                   | 10.44 ± 0.27$^c$ | 5.74 ± 0.16$^c$ | 2.86 ± 0.13$^c$ | 2.01 ± 0.09$^d$ | 0.20 ± 0.04$^d$ | 0.11 ± 0.03$^d$ | 16.53 ± 0.21$^e$ |
|                                  |                       | (1-17)     | (2-11)        | (0-5)         | (0-4)      | (0-2)       | (0-1)       | (12-23)           |

P-values: 0.0000, 0.0012, 0.0000, 0.0008, 0.6642, 0.2913, 0.0000, 0.0000.

### Figure 2

Effect of either 1 mM Mg$^{2+}$ or Hoagland solution on homoeologous CO frequency of T. aestivum cv. Chinese Spring Tazip4-B2 ph1b mutant-Ae. variabilis hybrids. (A) Frequencies of univalents, bivalents, trivalents, tetravalents, pentavalents and chiasma frequency (single and double chiasmata) were scored at meiotic metaphase I in Tazip4-B2 ph1b mutant-Ae. variabilis hybrids treated with either 1 mM Mg$^{2+}$ or Hoagland solution. Values in parenthesis indicate range of variation between cells. P < 0.05 indicates significant differences according to LSD test. Different letters indicate significant differences between treatments. (B) Representative meiotic configurations of Tazip4-B2 ph1b mutant-Ae. variabilis hybrids. From left to right: water alone, 1 mM Mg$^{2+}$ treatment and Hoagland solution treatment. Bar: 20 µm.

### Phenotypic Analysis of Tazip4-B2 Mutants Generated by CRISPR/Cas9 System

Firstly, eighty-one primary transgenic plants (T0) were analyzed by PCR followed by direct sequencing. Four plants were identified with edits in the target region. One plant had a perfect 115 bp deletion between guides G11 and G12. Twenty-four T1 plants from this line were screened and 5 homozygous edited plants with the 115 bp deletion were recovered. These plants were used to score the number of univalents, bivalents, and multivalents, and total chiasma frequency in the Tazip4-B2 mutant CRISPR lines (Figure 3). Wild-type Fielder lines were used as control plants (Figure 3). The Tazip4-B2 CRISPR mutant lines exhibited a significant reduction in ring bivalents, from a mean of 18.33 to 14.84 in the wild-type Fielder and CRISPR mutant lines respectively (Figure 3). A significant increase in the number of univalents and rod bivalents was also observed, from means of 0.51 univalents and 2.38 rod bivalents in the wild-type Fielder line, to means of 1.16 univalents and 4.93 rod bivalents in the CRISPR mutant lines (Figure 3). This indicates a significant reduction in homologous COs in these Tazip4-B2 mutant lines (Figure 3). Chiasma frequency decreased from a mean of 39.07 single chiasma and 40.50 double chiasma in the wild-type Fielder line, to a mean of 35.55 single chiasma and 37.11 double chiasma in the Tazip4-B2 CRISPR mutant (Figure 3).
Magnesium Also Increases Homoeologous COs in Wheat Tazip4-B2 CRISPR Mutant-Ae. variabilis Mutant Hybrids

For this study, a wild-type Fielder and a Tazip4-B2 CRISPR Fielder mutant line were crossed with Ae. variabilis to assess the level of homoeologous COs in the resulting hybrids (Table S2). Frequency of univalents, bivalents and multivalents, and total chiasma frequency were scored at meiotic metaphase I (Table S2). Tazip4-B2 CRISPR mutant hybrids exhibited a significant increase in single chiasma frequency, from a mean of 3.15 in the wild-type Fielder-Ae. variabilis hybrid to 16.66 in the Tazip4-B2 CRISPR-Ae. variabilis hybrid (Table S2). Double chiasma frequency was also increased in the Tazip4-B2 CRISPR mutant hybrids (Table S2). There was also a similar increase in the chiasma frequency to that reported previously in Tazip4-B2 TILLING-Ae. variabilis hybrids (Rey et al., 2017).

Having observed the effect of treatment with Mg\(^{2+}\) on homoeologous CO frequency in the Tazip4-B2 TILLING mutant hybrids, we also analyzed the effect of this ion on Tazip4-B2 CRISPR mutants-Ae. variabilis hybrids. We added 100 mL of a solution of 1 mM Mg\(^{2+}\) in water or Hoagland solution once a week to the soil in which the hybrids were growing. As expected, the addition of nutrients to these mutant hybrids caused a significant increase in chiasma frequency (Table 4; Figure S2). Tazip4-B2 CRISPR-Ae. variabilis hybrids treated with water alone exhibited means of 16.66 single chiasma frequency and 18.10 double chiasma frequency. Addition of 1 mM Mg\(^{2+}\) caused a significant increase in chiasma frequency of these mutant hybrids (means of 17.67 and 18.75 single and double chiasma frequency respectively) (Table 4). Also, the addition of Hoagland solution increased the homoeologous COs in these Tazip4-B2 CRISPR hybrids. Means of 18.34 and 19.82 single and double chiasma frequency respectively, were observed in those plants treated with Hoagland solution (Table 4).

DISCUSSION

Introgression of genetic material from relative species into bread wheat has been used in plant breeding for over 50 years, although classical plant breeding methods to introgress wild relative segments into wheat are both inefficient and time consuming (Ko et al., 2002). Recent availability of SNP based arrays, combined with classical cytogenetic approaches, significantly enhanced our ability to exploit wild relatives (King et al., 2017a,b), using lines carrying a deletion of either the whole of chromosome 5B, or a smaller 70 Mb segment (ph1b) (Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977), to increase the level of homoeologous crossovers between wild relatives and wheat chromosomes. Recombination between wild relative chromosomes and wheat chromosomes is, however, still limited. Thus, there is a need to find abiotic or biotic treatments such as temperature, nutritional availability, DNA-damaging agents, among others (Lambing et al., 2017) to enhance recombination. Martin et al. (2017) recently reported an alternative tool to increase CO number in Tazip4-B2 ph1b mutant-rye hybrids, using the addition of a Hoagland solution.
to the soil in which the plants are grown. Martín et al. (2017) also showed that the presence of the Hoagland solution did not affect the homoeologous CO number in wild-type wheat-rye hybrids.

Here, we report the successful identification of the particular Hoagland solution constituent responsible for the observed increase in homoeologous CO frequency. After analyzing Tazip4-B2 ph1b mutant-rye hybrids in the absence of each separate Hoagland solution macronutrient, we observed a significant reduction in homoeologous CO frequency when the Mg$^{2+}$ ion was absent. This suggests that the Mg$^{2+}$ ion is mainly responsible for the effect of Hoagland solution on homoeologous COs described previously by Martín et al. (2017). These observations were obtained after cytogenetic analysis of meiotic configurations at meiotic metaphase I. The analysis involved scoring single and double chiasmata in the chromosomal structures (Figure S1).

Single chiasma counting has commonly been used in many
studies to measure chiasma frequency in wheat (Dhaliwal et al., 1977; Sears, 1977; Roberts et al., 1999). However, other studies have suggested that double chiasmata may occur in these chromosomal configurations (Gennaro et al., 2012; Dreissig et al., 2017). Double chiasmata were considered in the present study, as a high number of MLH1 sites were previously reported in Tazip4-B2 ph1b mutant-rye hybrids in Martin et al. (2014). In our studies, up to 19 chiasmata were scored in Tazip4-B2 ph1b mutant-rye hybrids, which is similar to the number of MLH1 sites observed previously (Martin et al., 2014).

The effect of treatment with a solution of 1mM Mg$_{2+}$ in water, was analyzed to confirm whether that the Mg$_{2+}$ ion was responsible for the increase in homoeologous COs observed in these hybrids. The effect of treatment with this solution was assessed either by irrigation of, or injection into Tazip4-B2 ph1b mutant-rye hybrids. Surprisingly, both methods of application increased homoeologous CO frequency in the Tazip4-B2 ph1b mutant-rye hybrids. Thus, the results from the injection method of application suggested that the 1 mM Mg$_{2+}$ concentration was directly responsible for the increased homoeologous CO effect seen in the Tazip4-B2 ph1b mutant-rye hybrids, rather than through indirect effects on the plant growth or development. However, homoeologous CO frequency was decreased when the Mg$_{2+}$ concentration was increased further (Figure 1). This reduction in COs was associated with a significant increase in the number of univalents, and decrease in the number of ring bivalents and trivalents.

A recent study revealed that TaZIP4-B2 within the 5B region defined by the 70 Mb ph1b deletion, was responsible for the suppression of homoeologous COs in hybrids (Rey et al., 2017). Tazip4-B2 TILLING mutants (one with a missense mutation and another with a nonsense mutation), when crossed with Ae. variabilis, exhibit similar levels of homoeologous CO that observed in ph1b-Ae. variabilis hybrids (Rey et al., 2017). It was therefore important to assess the effect of 1mM Mg$_{2+}$ solution on these Tazip4-B2 TILLING mutant-Ae. variabilis hybrids to confirm that the effect was associated with Tazip4-B2, and that the Mg$_{2+}$ effect could also be observed in a different hybrid. Moreover, we also applied the CRISPR/Cas9 genome editing system in hexaploid wheat cv. Fielder to the mutant TaZIP4-B2 to compare its mutant phenotype with those observed in TILLING mutant lines, and their Ae. variabilis hybrids. Tazip4-B2 CRISPR mutants showed a significant decrease in homologous COs compared to control plants (TaZIP4-B2 wild type wheat), similar to that already reported for Tazip4-B2 TILLING mutants (Rey et al., 2017). Also, as expected, a significant increase was observed in Tazip4-B2 CRISPR mutant-Ae. variabilis hybrids, similar to that observed in both ph1b-Ae. variabilis and Tazip4-B2 TILLING mutant-Ae. variabilis hybrids. Furthermore, the addition of 1 mM Mg$_{2+}$ to all these hybrids increased the frequency of homoeologous CO. This confirms that the Mg$_{2+}$ effect is associated with Tazip4-B2, and occurs in different hybrids. The only difference observed with the Tazip4-B2 CRISPR and TILLING mutants was the occurrence of multivalents in the CRISPR mutants compared to the TILLING mutants (Figure 3 and Rey et al., 2017). This suggests that
TaZIP4-B2 not only promotes homologous COs and restricts homoeologous COs, but also contributes to the efficiency of homologous pairing. We hypothesize that the CRISPR deletion disrupts more of the TaZIP4-B2 function than the TILLING mutants. Interestingly, in rice, ZIP4 mutants have previously been reported to show a delay in completing homologous synopsis (Shen et al., 2012), however, in that diploid species, this does not lead to homoeologous COs because only homologs are present. However, in the ph1b mutant, delayed pairing of some homologs is observed until after the telomere bouquet, allowing some subsequent homologous pairing to take place. This delayed pairing of homologs in the ph1b mutant is consistent with a ZIP4 mutant phenotype.

Magnesium is one of the most important nutrients, mainly involved in the general promotion of plant growth and development. In terms of CO function, Mg^{2+} may affect multiple proteins in the class I interference crossover either in a positive or negative manner. For example, recent studies have suggested that Mg^{2+} is required for the endonuclease activity of the MLH1-MLH3 heterodimer (Rogacheva et al., 2014). The MLH1-MLH3 heterodimer shows a strong preference for HJs in the absence of Mg^{2+} (Ranjha et al., 2014). Whatever the target, our present study reveals that homoeologous COs can be increased by the 1 mM Mg^{2+} treatment of Tazip4-B2 (ph1b, TILLING or CRISPR derived) mutant-wild relative hybrids. Thus, this treatment can be used as a tool to enhance the introgression of wild relative traits into wheat.

**AUTHOR CONTRIBUTIONS**

M-DR, AM, PS, and GM: conceived and designed the study; MS, SH, and WH: participated in the development of the Tazip4-B2 mutant in bread wheat cv. Fielder by CRISPR/Cas9 system; M-DR: analyzed the research results and wrote the first draft; PS and GM: modified the paper. All authors have read and approved the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

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M-DR: analyzed the research results and wrote the first draft; PS and GM: modified the paper. All authors have read and approved the final version of the manuscript.

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