


Article

Wild and Cultivated Homoeologous Barley Chromosomes Can Associate and Recombine in Wheat in the Absence of the *Ph1* Locus

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Abstract: Bread wheat is an allohexaploid that behaves as a diploid during meiosis, the cell division process to produce the gametes occurring in organisms with sexual reproduction. Knowledge of the mechanisms implicated in meiosis can contribute to facilitating the transfer of desirable traits from related species into a crop like wheat in the framework of breeding. It is particularly interesting to shed light on the mechanisms controlling correct pairing between homologous (equivalent) chromosomes and recombination, even more in polyploid species. The *Ph1* (*Pairing homoeologous 1*) locus is implicated in recombination. In this work, we aimed to study whether homoeologous (equivalent chromosomes from different genomes) *Hordeum chilense* (wild barley) and *H. vulgare* (cultivated barley) chromosomes can associate and recombine during meiosis in the wheat background in the absence of the *Ph1* locus. For this, we have developed *H. chilense* and *H. vulgare* double monosomic addition lines for the same and for different homoeology group in wheat in the *ph1b* mutant background. Using genomic in situ hybridization, we visualized the two (wild and cultivated) barley chromosomes during meiosis and we studied the processes of recognition, association, and recombination between homoeologous chromosomes in the absence of the *Ph1* locus. Our results showed that the *Ph1* locus does not prevent homoeologous chromosome pairing but it can regulate recombination.

Keywords: *Triticum*; *Hordeum*; homoeologous pairing; chromosome introgressions; meiosis; chromosome recognition; recombination; *Ph1* locus



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1. Introduction

Wheat is one of the oldest crops in the world. Its cultivation dates back 10,000 years ago during the Neolithic Revolution and it is considered one of the main reasons for which humans transformed from hunter-gatherer nomad to settled farmer [1]. Presently, wheat is the third most produced cereal in the world (734 million tons), only behind maize (1.1 million tons) and rice (782 million tons) (data taken from 2018; <http://faostat3.fao.org/>). Nevertheless, this production needs to be increased on account of the growing human population, which means that wheat crops need to be more productive and better adapted to specific agro-climatic conditions [2]. In this framework, plant breeders play an essential role to increase wheat quality and productivity by trying to understand gene functions and interactions, as well as exploiting the existing genetic variability in the development of new varieties better adapted to the environment. In fact, wide crossing is an important tool in plant breeding and sometimes the results are the starting point for new crops. Examples of new plant species developed in the Triticeae tribe are \times *Triticosecale*, obtained after crossing wheat and rye, or \times *Tritordeum*, an amphyploid between the wild barley *H. chilense* Roem. et Schult. and wheat [3,4]. Hence, in these cases, related species have been used by breeders as genetic donors for widening the genetic basis of wheat to get, for example,

wheat cultivars better adapted to specific agro-climatic conditions, higher grain quality, or carrying resistance to diseases [5–8]. The use of relative species to transfer valuable genes into crops to improve grain nutritional quality and increase the total yield potential is based on interspecific hybridization of many cultivars with their wild relatives [9]. In the case of wheat, hybridization between wheat chromosomes and those from the wild related species produces only a low level of chromosome pairing and recombination, increasing the difficulty of introgressing desirable traits from the related species into it. Thus, going deeper into the knowledge of wheat genetics and its genome organization seems to be essential in the framework of plant breeding.

Bread wheat is an allopolyploid with three subgenomes (A, B, and D) which contain seven pairs of homologous (equivalent) chromosomes each one. Equivalent chromosomes from different subgenomes are named homoeologous chromosomes, which complicates the process of recognition and pairing during meiosis. Despite this genome complexity, hexaploid wheat behaves as a diploid during meiosis. This means that chromosomes associate regularly in pairs of homologues (equivalent chromosomes from the same subgenome), being homoeologous pairing prevented through the action of *Ph* (*Pairing homoeologous*) genes [10]. Among *Ph* genes, the *Ph1* locus, located on the short arm of chromosome 5B, plays a major effect on chromosome associations during meiosis [11]. Other loci such as the *Ph2* and the *Ph3*, located on the short arm of chromosomes 3D and 3A, respectively, have a weaker effect on meiosis than the *Ph1* locus [12–14]. Due to its implication in meiosis, the *Ph1* locus has been studied for decades and was initially described in charge of homologous chromosome pairing in bread wheat [11,15–17], although the molecular mechanisms behind these observations are still unclear. More recently, other functions of the *Ph1* locus during meiosis have appeared. The *Ph1* locus also suppresses recombination between homoeologous chromosomes [18–20] although it does not prevent chromosome associations between homoeologues [21]. It is clear that the precise control of chromosome pairing is crucial for conferring meiotic regularity, and hence reproductive stability in allopolyploids [22].

Several studies have explored chromatin arrangements in interphase nuclei, chromosome interactions, and movements during meiotic prophase I, and mechanisms that ensure correct segregation of chromosomes during anaphase. These studies have shed some light on chromosome dynamics in *Arabidopsis thaliana* as well as in plants with large and complex genomes such as wheat and maize [23]. In wheat, at the onset of meiosis, homologous chromosomes are sorted in pairs and are intimately associated along their lengths while a proteinaceous structure, the synaptonemal complex (SC), is assembled between them. Despite all the existing information regarding these processes, how homologues find each other to associate correctly in pairs is one of the least understood meiotic events [24–26]. In wheat, when a chromosome recognizes its homologue and not another chromosome, a localized conformational change in adjacent chromatin is triggered in both partners, facilitating recognition and pairing of homologous versus homoeologous chromosomes, this process also being under the effect of the *Ph1* locus [14,27]. Thus, the *Ph1* locus has a big influence on fertility but in contrast, has a negative effect for plant breeding purposes since it avoids pairing and recombination between wheat chromosomes and those from related species. Therefore, the study of chromosome associations during meiosis in wheat is necessary and can contribute to developing valuable tools in the framework of breeding.

The development of alien chromosome additions has an important utility not only for breeding but also for plant genetic studies. The availability of chromosome introgressions into a crop like wheat make these lines powerful tools for fundamental research, contributing, for example, to elucidating the processes of homoeologous recombination, distribution of chromosome-specific markers, and repetitive DNA sequences, and regulation of heterologous gene expression [28]. Sets of both cultivated (*H. vulgare*) and wild (*H. chilense*) barley addition lines in a hexaploid wheat background were developed several decades ago [29–31] and still currently have an enormous potential in plant meiosis studies. These lines allow the targeting of one specific pair of chromosomes or just a chromosome section

within the wheat background using genomic in situ hybridization (GISH), and as a result, rearrangements and associations in a pair of homologues can be studied individually [32].

In this study, we developed double monosomic addition lines of cultivated and wild barley, *H. vulgare* and *H. chilense*, respectively, in the wheat *ph1b* mutant background for the same and for different homoeology group with the aim of going deeper into the knowledge of chromosome associations during meiosis. These double monosomic addition lines allow the visualization of chromosomes from two different barley species in wheat in the *ph1b* mutant background and analyze whether the *Ph1* locus might play a role in chromosome recognition/pairing at early meiosis in the absence of homologues. Results showed that the *Ph1* locus does not impede homoeologous recognition and pairing between *H. chilense* and *H. vulgare* chromosomes during meiosis but recombination, which only occurs in the absence of the *Ph1* locus.

2. Materials and Methods

2.1. Plant Material

In this work we used *H. vulgare* and *H. chilense* monosomic addition lines in bread wheat (*Triticum aestivum* cv. Chinese Spring) in the *ph1b* mutant background carrying one *H. vulgare* or one *H. chilense* chromosome (AABBDD *ph1bph1b* + H^v and AABBDD *ph1bph1b* + H^{ch}, respectively), which were developed previously in our lab [33]. These lines were allowed to self-pollinate to obtain *H. chilense* and *H. vulgare* disomic addition lines in the wheat *ph1b* mutant background to be used as parental lines with the aim of developing *H. chilense* and *H. vulgare* double monosomic addition lines in the wheat *ph1b* mutant background. *Hordeum chilense* and *H. vulgare* double monosomic addition lines in the presence of the *Ph1* locus were also used [21].

Seeds were germinated on wet filter paper in the dark for 5 days at 4 °C, followed by a period of 24 h at 25 °C. emerging seedling roots were cut, incubated during 4 h in 0.05% *w/v* colchicine at 25 °C, fixed in Carnoy's solution (three parts 100% ethanol plus, one part glacial acetic acid) and stored at 4 °C until their use. Plants were then grown in the greenhouse at 26 °C during the day and 18 °C during the night (16 h photoperiod). Immature spikes were collected and were also preserved in Carnoy's solution and used to characterise chromosome pairing at meiosis I.

2.2. DNA Characterization

Genomic DNA was extracted from frozen seedling leaves following the protocol described by [34] with some modifications [35]. The absence of the *Ph1* locus in the lines used in this work was verified using the ABC920 SCAR marker [36]. The presence of each *Hordeum sp.* chromosome was confirmed by both PCR [37,38] and in situ hybridization.

2.3. Fluorescence In Situ Hybridization

Associations between *H. vulgare* and *H. chilense* chromosomes in wheat in the *ph1b* mutant background were studied using fluorescence genomic in situ hybridization (GISH) [39]. Root tips and spikes in meiosis were collected and pre-treated as described previously [21]. Chromosome spreads were obtained from both root tips and anthers in meiosis as described [21]. Genomic DNA extracted from *H. vulgare* and *H. chilense* seedling leaves was used as probes for GISH experiments. *Hordeum vulgare* and *H. chilense* genomic DNA were labeled by nick translation with biotin-11 (Boehringer Mannheim Biochemicals, Germany) and digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN, USA), respectively. The in situ hybridization protocol was performed according to [21].

2.4. Fluorescence Microscopy and Image Processing

Hybridization signals were visualized using a Nikon Eclipse 80i epifluorescence microscope. Images were captured with a Nikon CCD camera using the Nikon 3.0 software (Nikon Instruments Europe BV, Amstelveen, The Netherlands) and processed with Photoshop 11.0.2 software (Adobe Systems Inc., San Jose, CA, USA).

3. Results

3.1. Development of Double Monosomic *H. vulgare*-*H. chilense* Addition Lines in Wheat in the *ph1b* Mutant Background

With the aim of obtaining double monosomic *H. chilense*-*H. vulgare* addition lines in wheat in the *ph1b* mutant background, we cultivated 50 monosomic *H. chilense* and *H. vulgare* addition lines for chromosomes 5H^{ch}, 7H^{ch}, and 7H^v, which were previously developed in our lab in the absence of the *Ph1* locus [35]. These monosomic addition lines were self-pollinated in order to identify disomic addition lines for each *Hordeum* chromosome in the segregation population in the wheat *ph1b* mutant background. F2 seeds were germinated and plants were screened by PCR using molecular markers to confirm the presence of the *H. chilense* or *H. vulgare* chromosomes and the *ph1b* deletion. Those plants carrying either the *H. chilense* or *H. vulgare* chromosomes in the absence of the *Ph1* locus were selected and analyzed by GISH to identify disomic addition lines for each 5H^{ch}, 7H^{ch}, and 7H^v chromosomes. GISH analysis was required to distinguish among the positive plants for a specific chromosome, those carrying two copies of the desirable *H. chilense* or *H. vulgare* chromosome. Only 8, 5, and 5 wheat plants were identified carrying two copies of each 5H^{ch}, 7H^{ch}, and 7H^v, respectively, in the *ph1b* mutant background (Table 1). After in situ hybridization experiments, these 5H^{ch}, 7H^{ch}, and 7H^v disomic addition lines in the *ph1* mutant background were cultivated until grain was obtained. Then, seeds were germinated, and plants were cultivated and used as parental lines for genetic crosses to generate double monosomic *H. chilense*-*H. vulgare* addition lines in wheat in the *ph1b* mutant background. A summary of the process and the number of plants obtained is shown in detail in Figure 1 and Table 1.

Table 1. (a) Plants used for genetic crosses to develop wheat lines carrying a *Hordeum* disomic addition in the *ph1b* mutant background. (b) Plants obtained after genetic crosses between disomic addition lines.

| (a) | | | | | |
|---|---|---|---|---|---|
| parental lines <i>ph1bph1b</i> | | | self-pollinated progeny <i>ph1bph1b</i> | | |
| 7H ^v monosomic addition line | 5H ^{ch} monosomic addition line | 7H ^{ch} monosomic addition line | 7H ^v 7H ^v disomic addition line | 5H ^{ch} 5H ^{ch} disomic addition line | 7H ^{ch} 7H ^{ch} disomic addition line |
| 50 | 50 | 50 | 5 | 8 | 5 |
| (b) | | | | | |
| F1 progeny <i>ph1bph1b</i> | | | F2 progeny <i>ph1bph1b</i> | | |
| 7H ^v L5H ^{ch} double monosomic addition line | 7H ^v L7H ^{ch} double monosomic addition line | 7H ^v L5H ^{ch} double monosomic addition line | 7H ^v L7H ^{ch} double monosomic addition line | 7H ^v L monosomic addition line | 7H ^v L5H ^{ch} aneusomaty |
| 26 | 20 | 5 | 3 | 1 | 1 |

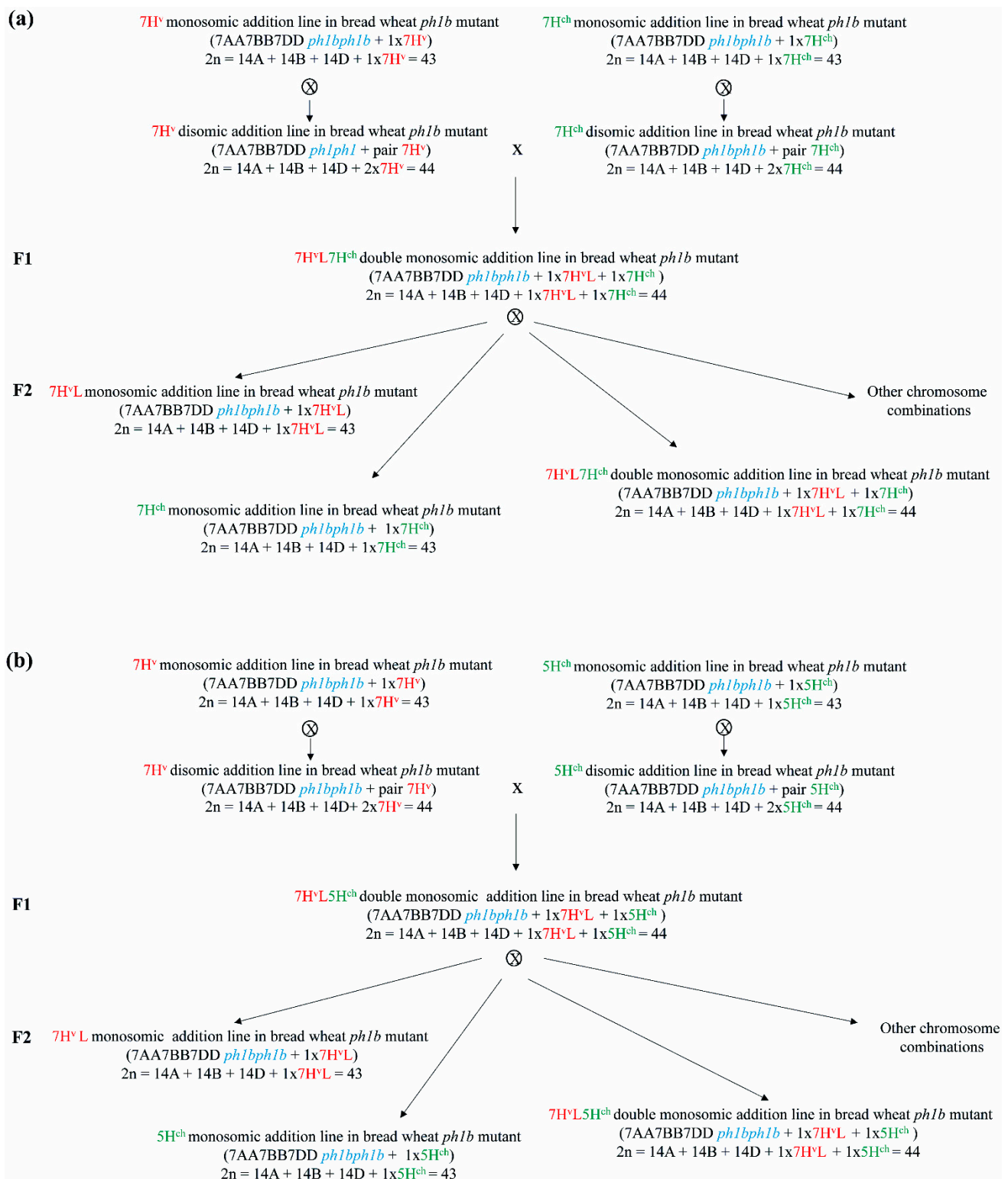


Figure 1. Development of double monosomic *H. vulgare* and *H. chilense* addition lines in wheat in the *ph1b* mutant background. (a) Diagram of genetic crosses developed to obtain the 7H^vL7H^{ch} double monosomic addition line in wheat in the *ph1b* mutant background. (b) Diagram of genetic crosses developed to obtain the 7H^vL5H^{ch} double monosomic addition line in wheat in the *ph1b* mutant background.

Only 20 grains were obtained from the genetic crosses between the 7H^v and the 7H^{ch} disomic addition lines in the absence of the *Ph1* locus. Similarly, only 26 grains were

obtained from the genetic crosses between the $7H^V$ and the $5H^{ch}$ disomic addition lines in wheat in the *ph1b* mutant background (Table 1). All these grains were germinated, and plants were screened by PCR using molecular markers to confirm simultaneously the presence of one *H. chilense* chromosome, one *H. vulgare* chromosome, and the *ph1b* deletion. Unfortunately, it was not possible to detect any *H. chilense* and *H. vulgare* double monosomic addition lines carrying the whole chromosome $7H^V$. Chromosome $7H^V$ was always identified as a telosomic $7H^VL$ chromosome. Thus, we only obtained *H. chilense* and *H. vulgare* double monosomic addition lines carrying the whole $5H^{ch}$ or $7H^{ch}$ *H. chilense* chromosome and the telosomic $7H^VL$ chromosome. This means that chromosome associations between *H. chilense* and *H. vulgare* chromosomes were only possible to study in the long chromosome arm of both *Hordeum* chromosomes in the absence of the *Ph1* locus. These double monosomic wild and barley addition lines in wheat in the *ph1b* mutant background were selected to study the effect of the *Ph1* locus on chromosome associations. Thus, plant lines were cultivated until meiosis to analyze *H. chilense* and *H. vulgare* chromosome associations for both the same homoeology group ($7H^{ch}7H^VL$) and for different homoeology groups ($5H^{ch}7H^VL$) in the wheat *ph1b* mutant background. Only the first spike was taken from each plant for meiosis scoring, leaving the rest of the spikes to develop until maturity to obtain F2 seeds. Spikes entering meiosis were isolated and chromosome spreads from anthers were used for GISH experiments to study chromosome associations between homoeologous chromosomes in the absence of the *Ph1* locus.

3.2. Non-Homologous Chromosome Associations between *H. vulgare* and *H. chilense* Are Allowed during Early Meiosis in Wheat in the *ph1b* Mutant Background

Chromosome associations between *H. vulgare* and *H. chilense* chromosomes for the same ($7H^VL$ and $7H^{ch}$) and for different ($7H^VL$ and $5H^{ch}$) homoeology groups were studied in pachytene in double *H. chilense*-*H. vulgare* monosomic addition lines in wheat in the absence of the *Ph1* locus. In addition, equivalent *H. chilense* and *H. vulgare* double monosomic addition lines, for the same and for different homoeology groups in the presence of the *Ph1* locus, were also grown. *Hordeum chilense* and *H. vulgare* chromosomes were visualized during meiosis by GISH to accurately assess the effect of the *Ph1* locus on chromosome associations. It is worth saying that *H. vulgare* chromosome $7H^V$ was complete in these wheat lines carrying the *Ph1* locus.

In situ hybridization experiments in meiosis were developed in more than 250 pollen mother cells (PMCs) of each genomic combination ($7H^VL7H^{ch}$ and $7H^VL5H^{ch}$) in the *ph1b* mutant and results were visualized in prophase I of meiosis (Figure 2). GISH experiments showed that *H. vulgare* and *H. chilense* homoeologous chromosomes ($7H^VL7H^{ch}$) were always fully-associated in pairs by the long chromosome arms during pachytene in both wheat backgrounds, in the presence and in the absence of the *Ph1* locus (Figure 2a,c). In contrast, non-homoeologous *Hordeum* chromosomes $7H^VL$ and $5H^{ch}$ were not observed associated at any time in the presence of the *Ph1* locus (Figure 2b), confirming previous results from our group [21]. However, we visualized some minor chromosome associations between $7H^VL$ and $5H^{ch}$ non-homoeologous chromosomes in 10.89% of the PMCs during pachytene in the *ph1b* mutant background (Figure 2d), involving a small chromosome portion of both non-homoeologues. Our observations showed that homoeologous barley chromosomes could associate both in the presence and in the absence of the *Ph1* locus. Chromosome pairing between non-homoeologous chromosomes from two different *Hordeum* species can also be allowed in the absence of the *Ph1* locus. Thus, homoeologous barley chromosomes can recognize each other by equivalent chromosome regions and trigger a chromatin conformational change independently of the *Ph1* locus (Figure 2a,c).

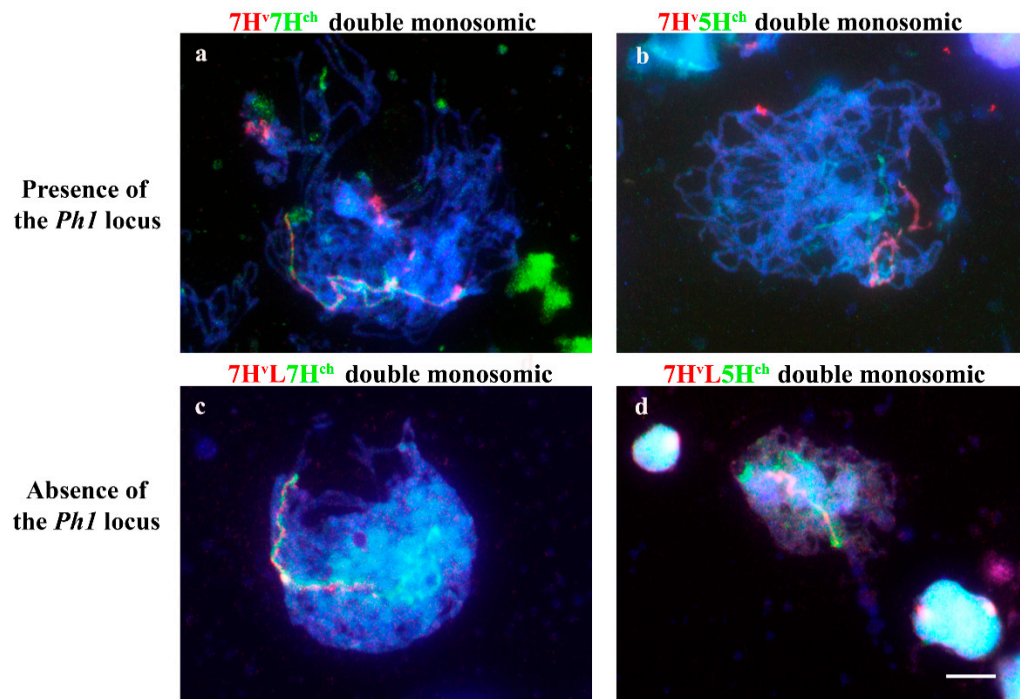


Figure 2. Analysis of pairing between homoeologous and non-homoeologous barley chromosomes during early meiosis in the presence and in the absence of the *Ph1* locus. *Hordeum vulgare* (red) and *H. chilense* (green) chromosomes. DNA was counterstained with DAPI (blue). (a) $7H^v7H^{ch}$ double monosomic addition line showing both barley chromosomes associated at early pachytene in the presence of the *Ph1* locus. (b) Non-homoeologous $7H^v$ and $5H^{ch}$ did not associate in the presence of the *Ph1* locus. (c) $7H^vL7H^{ch}$ double monosomic addition mutant line showing both barley chromosomes associated by the long chromosome arm at early pachytene in the *ph1b* mutant background. (d) $7H^vL5H^{ch}$ double monosomic addition mutant line showing both barley chromosomes partially associated at early pachytene in the *ph1b* mutant background. Bar: 10 μ m.

3.3. Recombination Can Occur Indistinctly between Wild Barley, Cultivated Barley, and Wheat Chromosomes in the Absence of the *Ph1* Locus

Taking into account that homoeologous and non-homoeologous chromosome associations between *H. chilense* and *H. vulgare* chromosomes did occur in wheat, we focused on studying whether or not these chromosome associations could allow overcrossing and recombination events in the absence of the *Ph1* locus. Thus, we analyzed chromosome associations in pollen mother cells (PMCs) in metaphase I from *H. vulgare* and *H. chilense* double monosomic addition lines for the same and different homoeologous group in wheat in the *ph1b* mutant background. $7H^v7H^{ch}$ and $7H^v5H^{ch}$ double monosomic additions in wheat in the presence of the *Ph1* locus were used as control. Cultivated and wild barley chromosomes remained always as univalent in GISH experiments in metaphase I in the presence of the *Ph1* locus, in both situations (same and different homoeologous group, Figure 3a,b), as was previously described [21]. In contrast, several types of associations between *H. vulgare* and *H. chilense* chromosomes were detected in the absence of the *Ph1* locus for both the same and different homoeologous groups (Figure 3c–l). Associations in metaphase I between *H. vulgare* and *H. chilense*, *H. chilense* and wheat, and *H. vulgare* and wheat chromosomes were observed (Figure 3c–h) in both $7H^vL7H^{ch}$ and $7H^vL5H^{ch}$ double monosomic addition lines in the *ph1b* mutant background, as well as double *H. chilense*-wheat and *H. vulgare*-wheat associations (Figure 3i,j) and tripartite *H. vulgare*-*H. chilense*-wheat associations (Figure 3k,l). The most frequent association in both wheat lines carrying homoeologous ($7H^vL7H^{ch}$) and non-homoeologous chromosomes ($7H^vL5H^{ch}$) occurred between *H. vulgare* and *H. chilense* chromosomes (20.85% and 7.26%, respectively,

Table 2). Our results showed that recombination was, as expected, more frequent between homoeologous chromosomes than non-homoeologous chromosomes, supporting chromosome associations observed during pachytene between 7H^vL and 7H^{ch} chromosomes, which were also more frequent than associations occurring between 7H^vL and 5H^{ch} chromosomes. The *H. vulgare*-*H. chilense*-wheat tripartite chromosomes association was the least frequent in both lines carrying 7H^vL7H^{ch} and 7H^vL5H^{ch} chromosomes in the *ph1b* background (0.47% and 0.33%, respectively) (Table 2). These observations suggested that, although homoeologous chromosomes 7H^vL and 7H^{ch} can associate in pairs during pachytene both in the presence and in the absence of the *Ph1* locus, recombination can only occur in the absence of the *Ph1* locus. In addition, although chromosome associations between non-homoeologues can occur in equivalent chromosome regions, recombination events were rare between non-homoeologues. Moreover, chromosome associations and recombination events between wild or cultivated barley chromosomes and wheat were less frequent, suggesting that chromosome associations might depend on genome homology, as *H. chilense* and *H. vulgare* species are phylogenetically closer between them than to wheat. The absence of homologues in the case of *Hordeum* chromosomes might also contribute to increase chromosome associations between both wild and cultivated barley chromosomes, which did not occur in the case of wheat chromosomes where the two homologues for all wheat chromosomes were always present. In summary, our observations suggested that the *Ph1* locus cannot suppress homoeologous chromosome associations in wheat but recombination, because no recombination events were detected between homoeologous *H. chilense* and *H. vulgare* chromosomes in any case in the presence of the *Ph1* locus.

Table 2. Frequency of pairing between *H. vulgare*, *H. chilense*, and wheat chromosomes in metaphase I in double monosomic addition lines in the presence and in the absence of the *Ph1* locus.

| Wheat Line | Number of Plants Analyzed in Metaphase | Number of PMCs Scored in Metaphase | Number (and Frequency) of PMCs Carrying Chromosome Associations Scored in Metaphase I | | | | | Total Pairing |
|--|--|------------------------------------|---|--------------------------------|-------------------------------|--|--|---------------|
| | | | H ^v -H ^{ch} Pairing | H ^{ch} -Wheat Pairing | H ^v -Wheat Pairing | H ^{ch} -Wheat and H ^v -Wheat Pairing | H ^v -H ^{ch} -Wheat Pairing | |
| 7H ^v 7H ^{ch} double monosomic <i>Ph1Ph1</i> | 2 | 107 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7H ^v 5H ^{ch} double monosomic <i>Ph1Ph1</i> | 2 | 159 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7H ^v L7H ^{ch} double monosomic <i>ph1bph1b</i> | 2 | 211 | 44 (20.85%) | 18 (8.53%) | 18 (8.53%) | 4 (1.89%) | 1 (0.47%) | 85 (40.27%) |
| 7H ^v L5H ^{ch} double monosomic <i>ph1bph1b</i> | 2 | 303 | 22 (7.26%) | 7 (2.31%) | 8 (2.64%) | 2 (0.66%) | 1 (0.33%) | 40 (13.20%) |

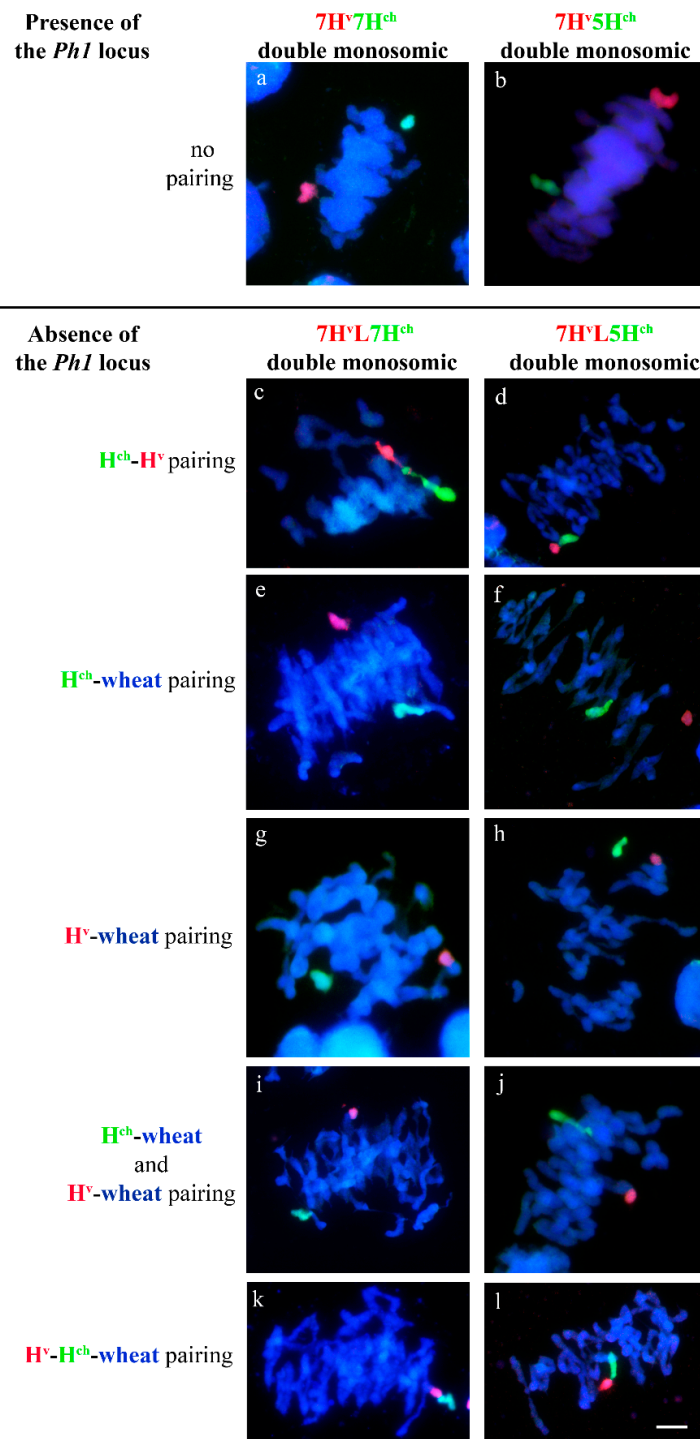


Figure 3. Interspecific chromosome associations during meiosis metaphase I between *H. vulgare*, *H. chilense*, and wheat in double monosomic addition lines in the presence and the absence of the *Ph1* locus. *Hordeum chilense* and *H. vulgare* were visualized in green and red, respectively. DNA was counterstained with DAPI (blue). (a,b) 7H^v7H^{ch} and 7H^v5H^{ch} barley chromosomes did not associate in the presence of *Ph1* locus. (c,d) H^{ch}-H^v associations in both wheat lines in the *ph1b* mutant background. (e,f) H^{ch}-wheat associations in the absence of the *Ph1* locus. (g,h) H^v-wheat pairing in the absence of *Ph1* locus. (i,j) Double H^{ch}-wheat and H^v-wheat pairing in wheat in the *ph1b* mutant background. (k,l) Tripartite chromosome associations in both lines in absence of the *Ph1* locus. Bar: 10 μm.

3.4. Segregation of the Inter-Specific Chromosome Associations Occurred Randomly in the Absence of the *Ph1* Locus

Chromosome reorganizations in PMCs as the result of inter-specific chromosome associations were studied in later stages of meiosis in wheat only in the *ph1b* mutant background, since no recombination events were detected in the presence of the *Ph1* locus. Almost 300 PMCs were screened in total in meiosis anaphase I using GISH. Several possible situations of inter-specific chromosome associations between *H. chilense*, *H. vulgare*, and wheat were identified in both lines carrying $7H^VL7H^{ch}$ and $7H^VL5H^{ch}$ chromosomes in wheat in the *ph1b* mutant background (Figure 4). *Hordeum chilense* and *H. vulgare* chromosomes were usually observed to be delayed in the metaphase plate during anaphase I/telophase I, independently of being associated or unpaired (Figure 4). *Hordeum chilense* and *H. vulgare* chromosomes remained associated during anaphase I in the $7H^VL7H^{ch}$ double monosomic addition line in the *ph1b* mutant background, but other chromosome associations between wheat and *H. chilense* or *H. vulgare* chromosomes were also visualized in anaphase I/telophase I, corresponding to previous chromosome associations observed in metaphase I (Figure 4a–h). When chromosomes $7H^VL$ and $7H^{ch}$ did not associate in metaphase I, both univalents segregated randomly, both in one cell, each one in each daughter cell or suffered chromosome misdivision (Figure 4i–k).

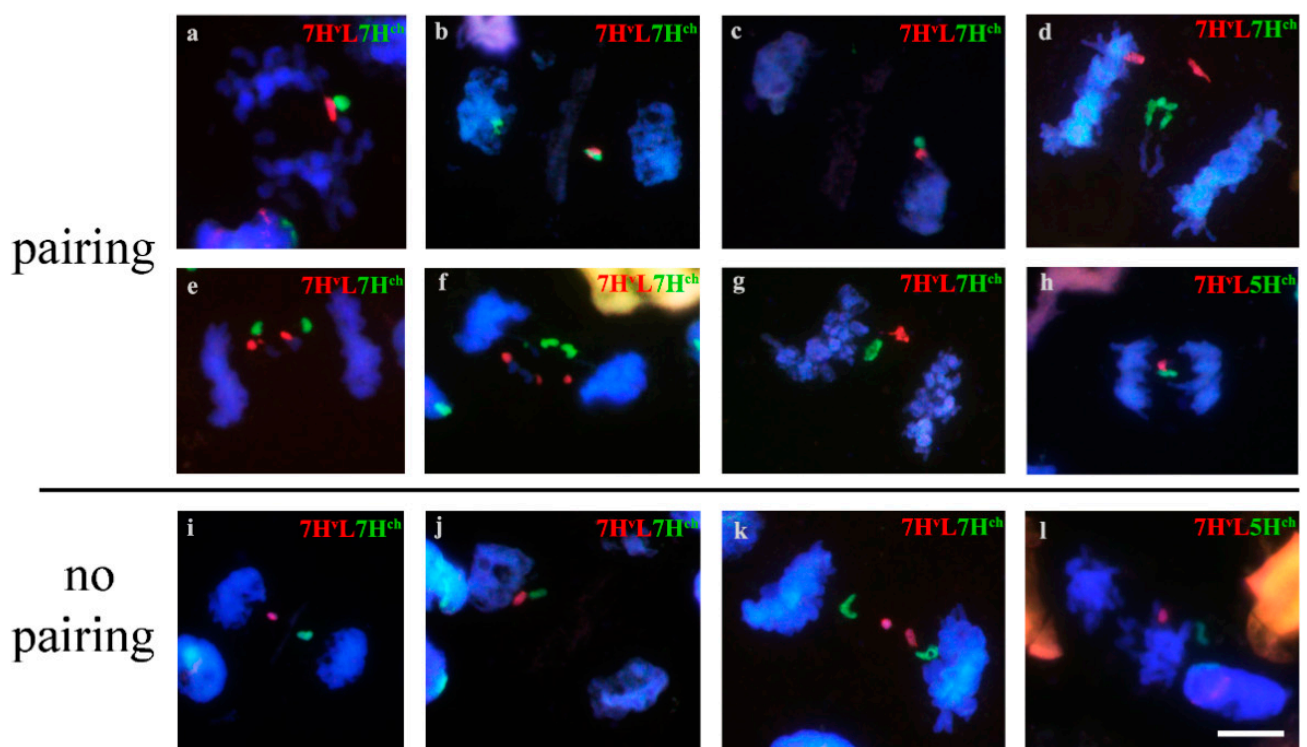


Figure 4. Chromosome segregation of barley chromosomes in double *H. vulgare* and *H. chilense* monosomic addition lines in wheat in the absence of the *Ph1* locus. Examples of barley chromosome segregation are shown in both situations, whether barley chromosomes did crossover or not. Both barley chromosomes remain delayed in all situations. *Hordeum vulgare* and *H. chilense* chromosomes were visualised in red and green, respectively. DNA was counterstained.

Similarly, we visualized in anaphase I/telophase I interspecific chromosome associations between *H. chilense* and *H. vulgare* in the non-homoeologous $7H^VL5H^{ch}$ double monosomic addition line in the *ph1b* mutant background. We scored 150 PMCs by GISH and we observed chromosome associations between *H. chilense* and *H. vulgare* chromosomes in few cells corresponding with the low frequency of pairing previously visualized in metaphase I (Figure 4h), but we did not detect other chromosome associations such as *H. vulgare*-wheat, double *H. chilense*-wheat and *H. vulgare*-wheat, or the tripartite *H. chilense*-*H. vulgare*-wheat. As expected, non-homoeologous chromosome associations between *H.*

chilense and *H. vulgare* were less frequent than *H. chilense* and *H. vulgare* associations for the same homoeologous group, corresponding to previous observations in pachytene and metaphase I (Table 2). *Hordeum chilense* and *H. vulgare* chromosomes segregated randomly when they did not associate previously in 7H^VL5H^{ch} double monosomic addition lines in the *ph1b* mutant background (Figure 4). Inter-specific chromosome associations were also visualized in tetrads, where several possibilities of *H. chilense* and *H. vulgare* segregation were detected, including the non-integration of the *Hordeum* chromosome, especially in the case of the *H. vulgare* 7H^VL chromosome (Figure 5).

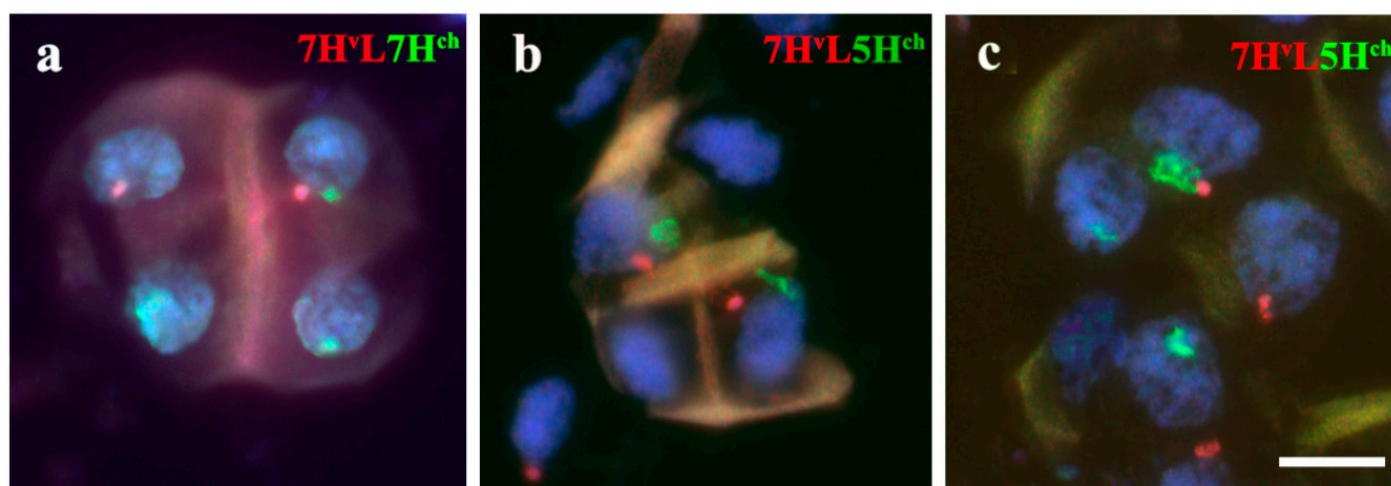


Figure 5. Visualization of barley chromosomes after meiosis in double *H. vulgare* and *H. chilense* monosomic addition lines in wheat in the absence of the *Ph1* locus. Examples of both *H. vulgare* (red) and *H. chilense* (green) chromosomes at the tetrads stage. DNA was counterstained with DAPI (blue). (a) *Hordeum vulgare* and *H. chilense* chromatin detected in two and three PMCs, respectively, in the double monosomic 7H^VL7H^{ch} in wheat in the *ph1b* mutant background. (b) 7H^VL5H^{ch} chromosomes detected simultaneously in two PMCs in wheat in the *ph1b* mutant background. (c) *Hordeum vulgare* and *H. chilense* chromatin detected in three and three PMCs, respectively, in the double monosomic 7H^VL5H^{ch} in wheat in the *ph1b* mutant background. Bar: 10 μ m.

Finally, it is worth mentioning that other chromosome rearrangements can occur during meiosis in the absence of the *Ph1* locus. We detected one mosaic plant carrying the 7H^VL5H^{ch} chromosomes in the *ph1b* mutant background. In situ hybridization in meiosis chromosome spreads of this plant revealed that both *H. chilense* and *H. vulgare* chromosomes were present only in some of the cell surrounding the meiocytes (Figure 6a,b). In fact, most of these cells and the meiocytes only carried the *H. chilense* chromosome, and just a few cells accompanying the meiocytes retained the *H. vulgare* chromosome. This observation suggested that a misdivision event occurred in an early mitosis event before meiosis, resulting in the loss of the *H. vulgare* chromosome in one cell, which later generated the meiocytes. This case of aneusomy was only detected in anthers, as in somatic root cells, both *H. chilense* and *H. vulgare* chromosome were present in all cells (Figure 6c). Whether or not the *Ph1* locus might be involved in this event remains to be elucidated.

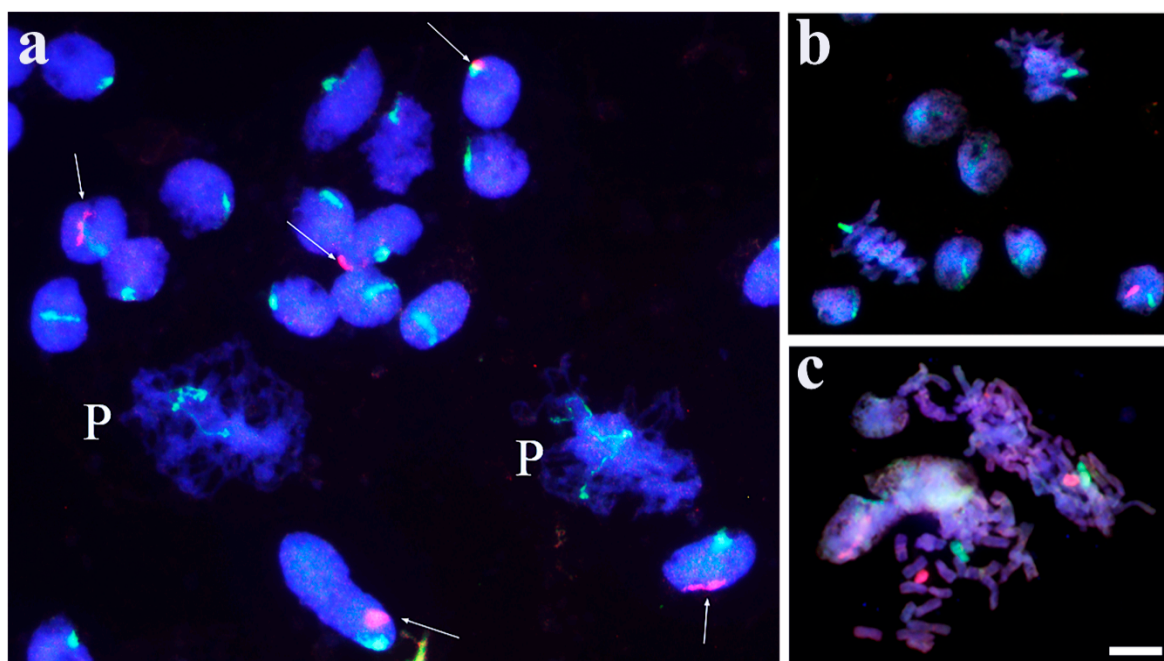


Figure 6. Mosaicism detected in the $7H^V L5H^{ch}$ double monosomic addition line in wheat in the absence of the *Ph1* locus. *Hordeum vulgare* (red) and *H. chilense* (green) were visualised in meiotic (a,b) and somatic cells (c). DNA was counterstained with DAPI (blue). (a) Meiocytes in pachytene (P) showed only the *H. chilense* chromosome although somatic surrounding cells within the anther contained both *H. vulgare* and *H. chilense* chromosomes (arrowed). (b) Meiocytes in metaphase only showed the *H. chilense* chromosome. (c) Somatic cells from root tips in interphase and metaphase showed both barley chromosomes in all cells. Bar: 10 μ m.

4. Discussion

Homologous chromosomes need to recognize each other to correctly associate, recombine and segregate. Plant breeders have always wished to transfer genetic variability from related species into a crop like wheat using genetic crosses, expecting recombination between chromosomes from the donor species and those from wheat. However, wheat genome has an enormous stability and recombination between non-homologous chromosomes is hampered. The genetic control of pairing and the physical divergence of the homoeologous genomes are considered the genetic systems responsible for the stable cytological diploid behavior of polyploid wheat [40,41]. The *Ph1* locus has been described controlling correct pairing and suppressing homoeologous recombination in wheat [19,24,42]. In this work, to go deeper into the knowledge of the *Ph1* locus function, we developed *H. chilense* and *H. vulgare* double monosomic addition lines in the wheat *ph1b* mutant background for the same and for different homoeology groups. For this, we used *H. chilense* and *H. vulgare* monosomic addition lines for chromosomes $7H^V$, $5H^{ch}$, and $7H^{ch}$ in the wheat *ph1b* mutant background developed previously [35] to be used as parental lines in genetic crosses. The wheat lines developed here in the absence of the *Ph1* locus are equivalent to double monosomic addition lines for the same *Hordeum* chromosomes previously developed in our laboratory in the presence of the *Ph1* locus [21]. However, we found difficulties in obtaining wheat lines carrying both *Hordeum* chromosomes intact at the same time in the *ph1b* mutant background. In fact, *Hordeum vulgare* chromosome 7 was always introgressed as a telochromosome $7H^V L$ and never the entire chromosome in the absence of the *Ph1* locus. Chromosome misdivision by the centromere was frequently observed when univalents were present in the wheat background [43–45]. Particularly, $7H^{ch}$ and $7H^V$ telosomic chromosomes were found in wheat in the *ph1b* mutant background in a higher frequency than the rest of the *H. chilense* and *H. vulgare* chromosomes [35]. This confirms the difficulties found in this work to retain the whole $7H^V$ chromosome in the *H. chilense* and *H. vulgare* double monosomic addition lines in the absence of the *Ph1* locus.

Thus, chromosome association and recombination in the *ph1b* mutant background was only possible to study between the long arm of chromosome 7H^V and the long arm of both 7H^{ch} (homoeologous) and 5H^{ch} (non-homoeologous) chromosomes.

Most of the studies performed during meiosis in wheat plants carrying alien chromosomes are focused on meiosis metaphase I or later stages [35,46,47]. Previously, we studied interspecific chromosome associations in early meiosis stages (pachytene) and we managed to track simultaneously extra wild and cultivated barley chromosomes (for the same and for different homoeology groups) in the wheat background using in situ hybridization [21]. Both wild and cultivated barley chromosomes were shown to be associated correctly in pairs in the presence of the *Ph1* locus, suggesting that the *Ph1* locus does not affect to specific chromosome associations [21]. In this work, our results confirmed these previous observations related to the effect of the *Ph1* locus on homoeologous chromosome associations. Wild and cultivated barley homoeologous chromosomes can associate intimately either in the presence or in the absence of the *Ph1* locus. In addition, we have clearly shown here that the *Ph1* locus hampers crossing-overs between wild and cultivated barley homoeologous chromosomes in the absence of homologous chromosomes. Thus, crossing-overs between *H. chilense* and *H. vulgare* homoeologous chromosomes are only observed in the absence of the *Ph1* locus. Crossovers between *H. chilense* and *H. vulgare* were previously detected in the *ph1b* mutant background [33]. Previously, an increment on the crossover frequency between wheat homoeologous chromosomes was described in the *ph1b* mutant background by targeting the MHL1 (a crucial component of the meiotic recombination machinery); sites that progressed to crossovers when environmental growing conditions were altered [48]. To the best of our knowledge, we have described here for the first time an increment of the crossovers and therefore recombination between both wild and cultivated barley species in the wheat background.

Other homoeologous chromosome associations have been previously described in the presence of *Ph* genes. For example, recombination between *H. vulgare* and *H. bulbosum* homoeologous chromosomes or between 5M^g chromosome from *Ae. geniculata* and 5D chromosome were also observed in the wheat background [49,50]. Robertsonian translocations between *H. chilense* and *H. vulgare* were described before in the presence of the *Ph1* locus [51]. However, different levels of allosyndesis between wheat chromosomes and *H. vulgare* and *H. chilense* or wheat and rye (*Secale cereale*) were detected in the *ph1b* mutant background [33,52]. Our results show that, although *H. vulgare* and *H. chilense* are phylogenetically quite distant [53], both species have a lot of similarities at the chromosomal level [54] allowing homoeologous chromosome associations and recombination between them in the absence of the *Ph1* locus. Thus, our results also showed that the *Ph1* locus suppresses recombination between homoeologous chromosomes as bivalents between homoeologous chromosomes were detected in metaphase I in the *ph1b* mutant background.

Alterations in chromosome content can also be frequently generated by mistakes during cell division, producing aneuploidy or polyploidy progeny cells. It is not strange that high polyploids show a variable chromosome number even for the same ploidy level. This phenomenon was labelled as aneusomaty, referring to an intra-individual aneuploidy [55]. This term can be equivalent to mosaicism and in wheat can be assumed to arise as the result of the meiotic instability characteristic of freshly created allopolyploids [56]. In our work, we detected this aneusomaty or mosaicism phenomenon in one plant developed in the *ph1b* mutant background carrying the *H. chilense* and *H. vulgare* non-homoeologous chromosomes. In our case, the *H. vulgare* chromosome was detected only in some somatic cells in the anther but in all somatic cells in the root tissue. Chromosome number variations in a population and at individual level can be frequent in high polyploids, especially in plants with an active vegetative reproduction [55,57–59]. This phenomenon has not been reported previously in the *ph1b* mutant background and more observations would be required to clarify whether or not the *Ph1* locus might have an effect on it.

Subtelomeres are high polymorphic DNA structures, located near telomeres at the end of the chromosome arms [60], and include recombination hot spots [61,62]. This extraordi-

nary polymorphism contributes to complicating the picture about the roles that subtelomeres play on chromosome associations and recombination during meiosis, although some studies in *Arabidopsis* and *Hordeum* species have shed some light on it [21,63–65]. In our work, crossovers were always observed at the distal region of the associated homoeologous chromosomes. Our results here also suggest that subtelomeres might play a function in the interspecific recombination between wild and barley chromosomes, as chiasmata are always located at the end of the chromosomes near the telomeres. The mechanism by which recombination at the subtelomeres between homoeologues is suppressed by the *Ph1* locus remains to be elucidated.

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