Programa de Doctorado

Biociencias y ciencias agroalimentarias

TESIS DOCTORAL

Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes.

Aplicación de herramientas citogenéticas en el estudio de las asociaciones cromosómicas durante la meiosis en trigo con fines de mejora genética.

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TITULO: *Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes*

AUTOR: *Maria del Carmen Calderon Perez*

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TÍTULO DE LA TESIS: Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes.

DOCTORANDA: María del Carmen Calderón Pérez

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La Dra. **Pilar Prieto Aranda**, científica titular del Instituto de Agricultura Sostenible, perteneciente al CSIC, como directora de la tesis doctoral con título: "Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes", realizada por **María del Carmen Calderón Pérez,**

INFORMA

Que dicha tesis ha sido realizada bajo mi dirección. Su principal objetivo ha sido profundizar en el conocimiento de las asociaciones cromosómicas al inicio de la meiosis en trigo.

Los resultados y conclusiones obtenidos son muy interesantes para programas de mejora genética en los que se pretende transferir caracteres de interés agronómico desde especies relacionadas al trigo.

La doctoranda, además, a lo largo del desarrollo de la tesis ha colaborado en varias líneas de investigación y asistido a diversos congresos nacionales e internacionales, de los que derivan las publicaciones siguientes:

Artículos en revistas SCI:

- Calderón MC, Rey MD, Prieto P (2014) The subtelomeric region is important for chromosome recognition and pairing during meiosis. **Scientific reports** 4: 6488. DOI: https://doi.org/10.1038/srep06488
  Impact factor 2014: 5.849
  ISI Journal Citation Reports Ranking 2014: 5/55 (Q1, D1 of Multidisciplinary Sciences)
- Calderón MC, Rey MD, Martín A, Prieto P (2018) Homoeologous chromosomes from two *Hordeum* species can recognize and associate during meiosis in wheat in the presence of the *Ph1* locus. **Frontiers in Plant Science** 9: 585.
  DOI: https://doi.org/10.3389/fpls.2018.00585
  Impact factor 2018: 4.106
  ISI Journal Citation Reports Ranking 2018: 20/228 (Q1, D1 of Plant Sciences)

Artículos en revistas SCI (no incluidas en la tesis doctoral)

- Rey MD, Calderón MC, Prieto P (2015) The use of the p*h1b* mutant to induce recombination between the chromosomes of wheat and barley. **Frontiers in Plant Science** 6:160.
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  Impact factor 2015: 4.495
  ISI Journal Citation Reports Ranking 2015: 15/209 (Q1, D1 of Plant Sciences)
- Rey MD, Calderón MC, Rodrigo MJ, Zacarías L, Alós E, Prieto P (2015) Novel bread wheat lines enriched in carotenoids carrying *Hordeum chilense* chromosome arms in the *ph1b* background. **PLoS One** 10(8): e0134598.
  DOI: https://doi.org/10.1371/journal.pone.0134598
  Impact factor 2015: 3.057

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- Calderón MC, Ramírez MC, Martín A, Prieto P (2012) Development of *Hordeum chilense* $4H^{ch}$ introgression lines in durum wheat: a tool for breeders and complex trait analysis. **Plant Breeding** 131, 733–738
  DOI: https://doi.org/10.1111/j.1439-0523.2012.02010.x
  Impact factor 2012: 1.175
  ISI Journal Citation Reports Ranking 2012: 29/78 (Q2 of Agronomy), 119/158 (Q3 of Biotechnology &Applied Microbiology Science), 106/196 (Q3 of Plant Sciences).

Otras aportaciones científicas que la autora ha realizado durante su tesis doctoral:

Aportaciones a Congresos internacionales:

- Calderón MC, Martín A, Prieto P. Wild and cultivated barley chromosomes can recognise and associate in pairs during early meiosis in the wheat background. 13th International Wheat Genetics Symposium. Wien, Austria. 23-28 abril 2017. Póster.

Aportaciones a Congresos nacionales:

- Calderón MC, Rey MD, Martín A, Prieto P. Unzipping how homologous chromosomes can recognise and associate in pairs in wheat. I Spanish Symposium on Cereal Physiology and Breeding. Zaragoza, España. 9-10 abril 2018. Póster.
- Calderón MC, Martín A, Prieto P. Estudio del apareamiento entre cromosomas de distintas especies de cebada en el fondo genético del trigo harinero. IX Seminario de Citogenética. Toledo, España. 8-10 junio 2016. Comunicación oral.
- Calderón MC, Cabrera A, Prieto P. Importancia de las regiones subteloméricas en el apareamiento cromosómico en *Hordeum chilense.*

VII Seminario de Citogenética. Pontevedra, España. 27-30 junio 2012. Comunicación oral.

Colaboraciones en congresos no incluidos en la tesis:

Congresos internacionales:

- Rey MD, Calderón MC, Prieto P**.** Wheat breeding using the *Ph1* mutant to transfer agronomic desirable traits from *Hordeum chilense* into wheat. 12th International Wheat Genetics Symposium. Yokohama, Japón. 08-14 septiembre 2013. Póster.
- Rey MD, Calderón MC, Prieto P. Uses of the *Ph1* mutants as a genetic tool for wheat breeding. 7th International Triticeae Symposium. Chengdu, China. 09-13 junio 2013. Comunicación oral.
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Congresos nacionales:

- Aguilar M, Garrido J, Calderón MC, Prieto P. Cytogenetic and molecular dissection of wheat subtelomeres to facilitate the transfer of desirable agronomic traits from related species. II Spanish Symposium on Cereal Physiology and Breeding. Córdoba, España. 6-7 marzo 2019. Póster.
- Osuna D, Calderón MC, Aguilar M, Prieto P. Identification and validation by FISH of subtelomeric sequences in long arms of ic sequences in long arms of wheat chromosomes 5 and 6. XL Congreso de la Sociedad Española de Genética. Córdoba, España. 16-18 septiembre 2015. Comunicación oral.
- Rey MD, Calderón MC, Prieto P. Obtención de líneas de interés entre *Hordeum chilense* y trigo harinero en el fondo genético del mutante

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- Prieto, P., Calderón, MC., Martín, A. Uso de mutantes de apareamiento cromosómico en la mejora genética de trigo. VI Seminario de citogenética. Córdoba, España. 27sep-2 oct 2010. Comunicación oral.
- Calderón MC, Martín A, Prieto P. Utilización del locus *Ph1* para la transferencia genética de caracteres de interés agronómico de cebada a trigo. V Congreso de Mejora Genética de Plantas. Madrid, España. 7-9 julio 2010. Póster.

Actividades formativas del programa de doctorado realizadas:

Actividades obligatorias:

- Introducción a legislación relativa al doctorado:
  - **Jornada Formativa Doctoral sobre EL DOCTORADO EN LA UNIVERSIDAD DE CÓRDOBA, MARCO NORMATIVO, PROCESOS Y PROCEDIMIENTOS**, 2019. Universidad de Córdoba. 13 diciembre 2019.
- Congreso Científico de Investigadores en Formación eidA3-ceiA3:
  - **I Congreso Científico de Investigadores en Formación en Agroalimentación del eidA3-ceiA3 (Córdoba, España)**, asistencia y presentación oral "Importancia de las regiones subteloméricas en el apareamiento cromosómico en *Hordeum chilense*". Universidad de Córdoba. 8-9 mayo 2012.
  - **II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba**, asistencia y comunicación oral "Importancia de las regiones subteloméricas en el apareamiento cromosómico en *Hordeum chilense*". Universidad de Córdoba. 8-9 mayo 2012.

- Congreso Nacional o Internacional, en el que el doctorando presente una comunicación dentro del campo científico de la Tesis Doctoral o derivada de ella:
    - Congreso internacional:
        - Calderón MC, Martín A, Prieto P. Wild and cultivated barley chromosomes can recognise and associate in pairs during early meiosis in the wheat background. 13th International Wheat Genetics Symposium. Wien, Austria. 23-28 abril 2017. Póster.
    - Congresos nacionales:
        - Calderón MC, Rey MD, Martín A, Prieto P. Unzipping how homologous chromosomes can recognise and associate in pairs in wheat. I Spanish Symposium on Cereal Physiology and Breeding. Zaragoza, España. 9-10 abril 2018. Póster.
        - Calderón MC, Martín A, Prieto P. Estudio del apareamiento entre cromosomas de distintas especies de cebada en el fondo genético del trigo harinero. IX Seminario de Citogenética. Toledo, España. 8-10 junio 2016. Comunicación oral.
        - Calderón MC, Cabrera A, Prieto P. Importancia de las regiones subteloméricas en el apareamiento cromosómico en *Hordeum chilense.* VII Seminario de Citogenética. Pontevedra, España. 27-30 junio 2012. Comunicación oral.

Actividades optativas:

- Curso de Aplicador de productos fitosanitarios (nivel cualificado). IFAPA Alameda del Obispo, Córdoba. Febrero-junio 2020.
- Jornada "UCO Divulga. Más allá de los papers", UCO, Córdoba. Marzo 2020.
- Actividad formativa "Agentes químicos y prevención de riesgos en laboratorios". IAS-CSIC, Córdoba. Febrero 2020.
- Actividad formativa “Formación en prevención de riesgos laborales para actividades de campo en el CSIC", IAS-CSIC, Córdoba. Febrero 2020.
- Actividad formativa “Riesgos y medidas preventivas en laboratorios de investigación”, Premap, Córdoba. Junio 2016.
- Curso teórico-práctico sobre Secuenciación automática y Genotipado de ADN. Universidad de Córdoba. Marzo 2013

  Curso de “Análisis de datos ecológicos en R”, organizado por Instituto de Agricultura Sostenible (CSIC), Córdoba. Abril 2012.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 27 de septiembre de 2020

Firma de la directora

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TÍTULO DE LA TESIS: Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes.

DOCTORANDO/A: María del Carmen Calderón Pérez

INFORME RAZONADO DEL TUTOR

Doña Adoración Cabrera Caballero, Catedrática de Universidad de la Universidad de Córdoba, como responsable de la línea de investigación con título: "Recursos Fitogenéticos y Mejora" informa que:

El trabajo titulado "Application of cytogenetic tools for studying chromosome associations in meiosis for plant breeding purposes.", realizado por la doctoranda María del Carmen Calderón Pérez, se considera finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en la Universidad de Córdoba.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 25 de septiembre de 2020

Firma del responsable de línea de investigación

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SUMMARY

Meiosis is one of the most important processes in eukaryotic cells with sexual reproduction, where homologous (equivalent) chromosomes associate, recognise, pair and recombine. Meiosis has been deeply studied because recombination between chromosomes from two species in a hybrid can introduce new genetic variability. In a crop such as wheat, the transfer of genes from related species to introduce new resistance to biotic or abiotic stresses is a target in breeding programs. Thus, going deeper into the knowledge of meiosis and the processes controlling chromosome associations at the initial stages might contribute to wheat breeding.

Chromosome associations at the beginning of meiosis initiate at the chromosome ends, where telomeres and subtelomeres are. Although the function of telomeres has been deeply studied, the subtelomere region continues being a meiosis black hole. In addition, the effect of the *Ph1* locus on recombination between interspecific chromosomes also hampers the incorporation of genetic variability from relative species wheat.

In this work we have shed light into the role of subtelomeres in initial chromosome recognition and pairing between homologous chromosomes in meiosis in the wheat background. Results revealed that the subtelomeric region is crucial in the processes of homologous chromosome recognition and pairing.

In addition, we have shown that homoeologous chromosomes from two barley species (*Hordeum vulgare* and *Hordeum chilense*) can associate correctly in pairs at the beginning of meiosis but do not recombine in the presence of the *Ph1* locus. Recombination between homoeologous chromosomes from these barley species was only allowed in wheat in the *ph1b* mutant background. The role of subtelomeres and the *Ph1* locus in early meiosis events in wheat is discussed.

RESUMEN

La meiosis es uno de los procesos más importantes en las células eucariotas con reproducción sexual, donde los cromosomas homólogos (equivalentes) se asocian, reconocen, aparean y recombinan. La meiosis ha sido estudiada en profundidad, ya que la recombinación entre cromosomas de dos especies dando lugar a un híbrido puede introducir una nueva variabilidad genética. En un cultivo como el trigo, la transferencia de genes de especies relacionadas para introducir una nueva resistencia al estrés biótico o abiótico es un objetivo principal en los programas de mejoramiento. Por lo tanto, profundizar en el conocimiento de la meiosis y los procesos que controlan las asociaciones de cromosomas en las etapas iniciales podría contribuir al mejoramiento del trigo.

Las asociaciones de cromosomas al comienzo de la meiosis se inician en los extremos de los cromosomas, donde se encuentran los telómeros y los subtelómeros. Aunque la función de los telómeros en meiosis se ha estudiado en profundidad, las regiones subteloméricas siguen siendo un agujero negro. Además, el efecto del locus *Ph1* en la recombinación entre asociaciones cromosómicas interespecíficas, también obstaculiza la incorporación de la variabilidad genética de las especies relativas de trigo.

En este trabajo hemos estudiado el papel de los subtelómeros en el reconocimiento cromosómico inicial y el apareamiento entre cromosomas homólogos en la meiosis en el fondo genético del trigo. Los resultados revelaron que la región subtelomérica es crucial en estos procesos. Además, hemos mostrado que los cromosomas homeólogos de dos especies de cebada (*H. vulgare* y *H. chilense*) pueden asociarse correctamente en pares al comienzo de la meiosis, pero no recombinan en presencia del locus *Ph1*. La recombinación entre cromosomas homeólogos de estas especies de cebada solo se produjo en trigo en el fondo mutante *ph1b*. Se discute el papel de los subtelómeros y el locus *Ph1* en los eventos de meiosis temprana en trigo.

Chapter I

General introduction and objectives

Cereals production

There are about 400.000 plant species in the word, but just 300.000 are eatable, being only 200 consumed or have contribution to the human food supply. The curiosity is that most of the proteins that we consume come just from three crops, all of them cereals: maize, rice and wheat. Wheat is the crop with the highest harvested area in the world (575.158.079 ha in 2018), but it is the third one in productivity (734.045.174 tonnes) (FAO-STAT 2020; http://faostat.org). In relation to consumption, more than two thirds of total cereals are used for animal feed and one third for human consumption.

Wheat has been successful as a global food crop and over others because it is adapted to a wide range of temperature environment and has unique dough properties, which allows a variety of breads, cakes, biscuits, pasta and other daily food products. Wheat is also an important nutritional source of carbohydrates, proteins, minerals and vitamins and few fats. The grains have low water what facilitates their conservation. Owing to those data, the 50% of daily dietary energy in industrialised and developing countries come from wheat-derived food (Tovoli, 2015). The success of production, storing and use has been relevant for the development of modern civilisation.

Spain is the 20th world country in wheat area harvested with more than 2 million hectares and the 19th wheat world producer with almost 8 million tonnes (Figure 1). In order to meet the market requirements, Spain still has to import more than 15 million tonnes of cereals every year (http://mapa.gob.es). Andalusia is the second Spanish region largest wheat producer (http://mapa.gob.es) (Figure 2).

Wheat production has to be increased every year to satisfy the growing world population (https://population.un.org/wpp/) and breeders have an essential role to improve yield, reduce stresses, increase disease resistant, crop harvestability and adaptation to climatic change using genetics tools.

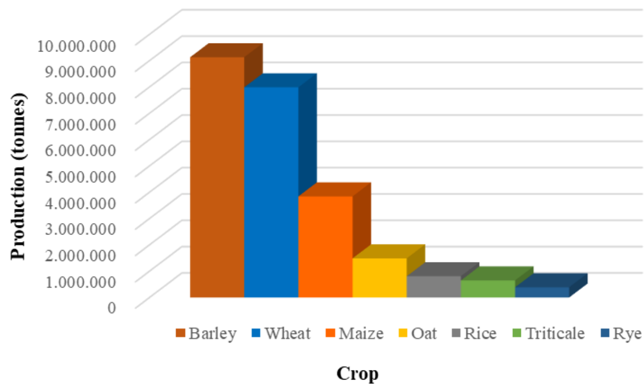

Figure 1. Main cereal crops in Spain (MAPA, 2020)

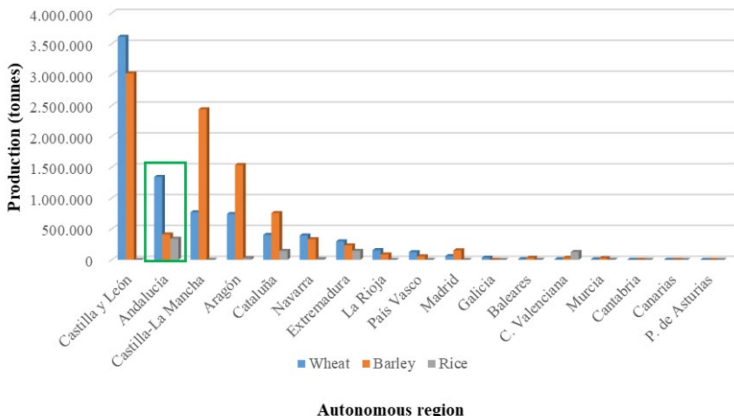

Figure 2. Wheat, barley and rice production in Spanish regions (MAPA)

Wheat origin, evolution and domestication

The first data of agriculture are from Holocene, 10.000 years ago, when human began to leave hunting and food gathering to settle agriculture (called the "Neolithic Revolution"). That change from nomadic life style provided new tools and techniques, commerce, communications, domestication of plants, culture, even religion promoting a civilisation evolution and cereals were part of this economic and cultural development (Shewry, 2009).

The term "cereal" comes from Ceres, the Roman Goddess of harvest and agriculture. Cereals are monocotyledons, have an annual vegetative cycle and are cultivated in temperate and subtropical zone. Cereals belong to the Poaceae or Gramineae family. The earliest cultivated vegetable forms, approximately 10.000 years ago, come from three cereals, two members of the *Triticeae* tribe: diploid (genome **AA**, 2n=2x=14, einkorn wheat, *Triticum monococcum* subsp. *monococcum*), tetraploid (genome **AABB**, 2n=4x=28, emmer wheat, *Triticum turgidum* subsp. *dicoccum*) and barley (*Hordeum vulgare*). Cereal grains found in the Middle East, indicate those cereals have provided the main source of calories for people from them (Heun, 1997; Dubcovsky and Dvorak, 2007; Zohary *et al*., 2012).

Einkorn and emmer wheat was domesticated from natural populations, but in contrast, bread wheat has only existed in cultivation, as the result of hybridisation between cultivated emmer (*Triticum turgidum*) with the unrelated wild grass *Triticum tauschii* (or *Aegilops tauschii* or *Ae. squarosa*). This hybridisation, through the time, resulted in the novel hexaploid wheat (genome **AABBDD**, 2n=6x=42, *Triticum aestivum*), which was selected by farmers for its superior properties (Shewry, 2009; (Marcussen *et al*., 2014) (Figure 3).

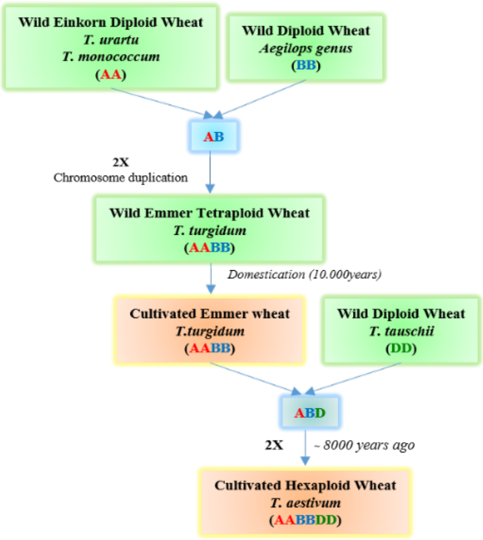

Figure 3. Natural evolution of hexaploid wheat

Bread wheat genome

Bread wheat is an allohexaploid with 21 pairs of chromosomes, organised in 3 homoeologous (partially homologous chromosomes of different genomes carrying the same gene loci order) sets (**A**, **B** and **D** genomes) having 7 pairs of homologous (identical chromosomes with the same genes in the same loci but different alleles) chromosomes each (Figure 4). Hexaploid genome size is 17Gb and contains more than 124.000 gene loci (Mayer *et al*., 2014; Appels *et al*., 2018) which is more than 5 times the human genome.

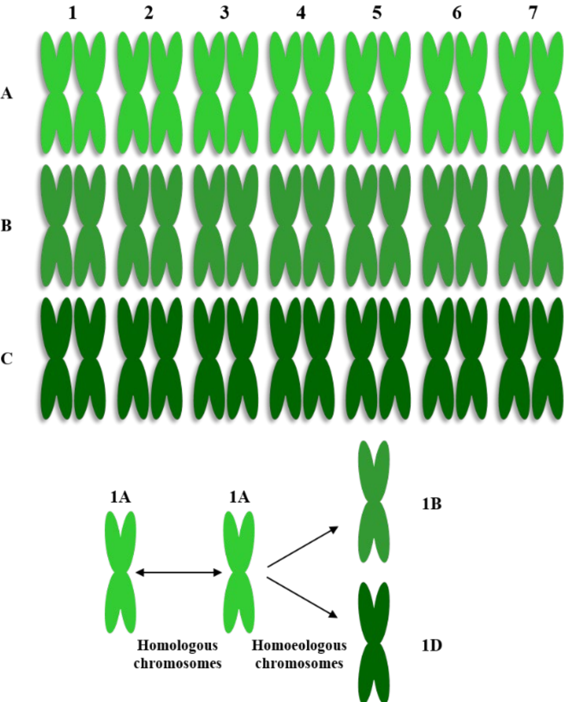

Figure 4. Diagram of bread wheat genomes (2n=6x=42) composed of three related genomes (**A**, **B** and **D**).

Meiosis in hexaploid wheat

Meiosis is the eukaryotic cell division where the number of chromosomes is reduced in the parent cell and produces 4 gametes. This process is needed for sexual reproduction. Meiosis begins with a diploid (2n) germ cell, and after a DNA replication, two separate cycles of nuclear division take place, resulting in 4 haploid daughter cells (n). Meiosis is divided in two phases: meiosis I and meiosis II. Meiosis I is split into prophase I (leptotene, zygotene, pachytene, diplotene and diakinesis), metaphase I, anaphase I, telophase I and cytokinesis. (Figure 6). Before meiosis I starts, premeiosis (A), includes the differentiation of meiocytes and the replication of the DNA (phase S). At the beginning of meiosis, DNA begins to condensate into chromosomes (leptotene). The homologous chromosomes are orientated (Rabl configuration) with centromeres and telomeres in opposite sides of the cell, occupying nuclear subdomain, known as chromosome territories (Rabl, 1885). The Rabl configuration facilitates homologous interactions at early meiosis (Figure 5) (Fussell, 1987).

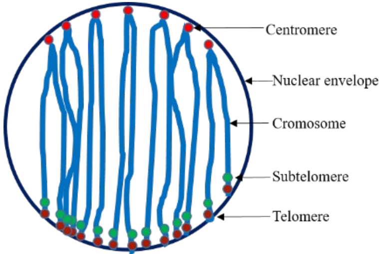

Figure 5. Diagram of the Rabl configuration in wheat

Later (zygotene), telomeres are congregated in a cluster or bouquet (Dawe, 1998; Harper, 2004), which facilitates initial chromosome interactions at the chromosome ends and the initiation of pairing and synapsis (Bass *et al.*, 2000; Trelles-Sticken *et al.*, 2000; Cowan, 2001; Scherthan, 2001, 2007; Bass, 2003; Harper, 2004). Chromosome pairing is initiated when both homologues are at the same conformational state (Prieto *et al.*, 2004b) and the synaptonemal

complex (SC) is accomplished and mediate recombination (Cowan, 2001). Pachytene stage (D) initiates when the homologues are paired and synapsed and recombination events take place (Prieto *et al.*, 2004b). The diplotene stage (E) begins with the separation of the homologues and recombination evens can be visualised by chiasmas, the cytological manifestation of a CO (crossover, a reciprocal exchange between two DNA molecules) (Mercier *et al.*, 2015). During diakinesis (F) chromosomes are more condensed and disjoin from the nuclear envelope which breaks down (G). The mechanism by chromosome dynamics are controlled is poorly understood (Ronceret and Pawlowski, 2010). During metaphase I (H) bivalents are aligned in the equatorial metaphase plate, and they separate toward each pole of the cell (anaphase I). During telophase I (J), the new nuclear envelopes are formed and, later, the diakinesis or cytoplasm division occurred and the original cell is divided into 2 daughter cells. The meiosis II (K, L, M and N) is a mitosis division when sister chromatids of each chromosome separate and haploid gametes are formed, resulting in 4 haploid gametes.

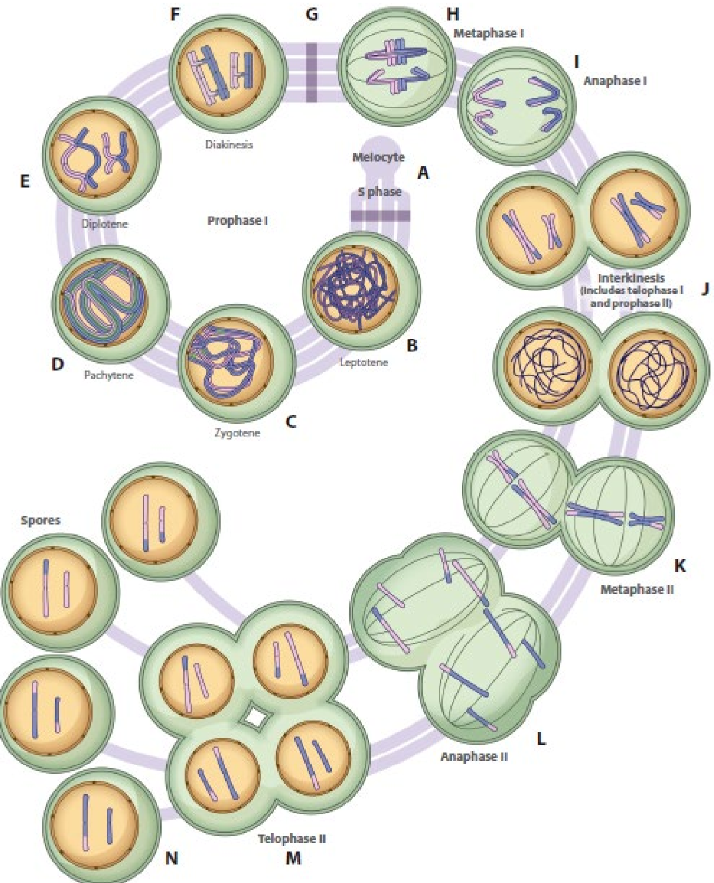

Figure 6. Diagram of the meiotic stages. Reproduced (with permission) from (Mercier *et al.*, 2015)

Chromosome regions involved in initial chromosome recognition and pairing

Eukaryotic chromosomes have specific regions playing essential functions to maintain the integrity of the genome and correct distribution of the DNA during cell division. One of these structures are the telomeres, which prevents the ends of linear chromosome degradation by exo-nucleases, end-to-end fusion with other chromosomes, prevention of homologous recombination between

telomeric regions, are implicated in DNA repairing and facilitate homologous chromosome associations at the beginning of meiosis (Chan and Blackburn, 2004). Telomeres consist in tandem repeats highly conserved during evolution (TTTAGGG repeats in most higher plants) (Richards and Ausubel, 1988). The stability and heredity of linear DNA is considered the most important fact of that evolution (Fuchs *et al.*, 1995).

During early meiosis, chromatin decondensation and chromosome movements allow homologues to find each other to associate in pairs (Scherthan, 2001; Prieto *et al.*, 2004a; Naranjo, 2014). In most organisms, and particularly in plants, chromosomes initiate a physical interaction at the bouquet stage (leptotene) of meiosis, while being all telomeres attached to the inner part of the nuclear envelope. DNA regions adjacent to telomeres (subtelomeres) might benefit of this telomere cluster as they are forced to occupy a restricted space where occur the instigation and temporal stabilisation of unstable chromosome interactions. The focus on subtelomeres is an exciting area of study, but the polymorphic nature of these regions represents a challenge from a technical perspective. Subtelomeres are less evolutionary conserved than telomeres and include recombination hot spots among other features that complicate the assessment of the potential conserved functions of these high-polymorphic regions (Linardopoulou *et al.*, 2005; Louis and Vershinin, 2005; Emden *et al.*, 2019). Together with their associated proteins, these DNA segments are essential for genome stability (Riethman *et al.*, 2005; Emden *et al.*, 2019).

Centromeres are also playing an important role in meiosis. Centromere is a chromosomal region containing highly repetitive sequences with a complex structure where the kinetochore, the protein complex assembled at each centromere, is formed. The kinetochores are the spindle microtubules fibres attachments during cell division. Thus, these together with the telomeres ensure the proper segregation of chromosomes during meiosis and mitosis. Centromeres are a main control mechanism of cell cycle and alterations centromeres might cause aneuploidies (loss or gain of a chromosome). As the

centromeres functions are essential, they might have evolved high flexibility. In addition, mechanisms to tolerate and preserve their functionality have been also required during evolution (Cleveland *et al.*, 2003; Achrem *et al.*, 2020; Leo *et al.*, 2020).

Pairing homologous locus

Although the polyploid bread wheat has 3 sets of related chromosomes, it behaves as a diploid at meiosis. The control of homoeologous pairing is prevented by *Ph* (*Pairing homologous*) locus (Riley and Chapman, 1958; Sears and Okamoto, 1958). Several studies during decades have been carried out to unzip the mechanism of the *Ph1* locus during meiosis. Initially, it was suggested that during premeiotic interphase nuclei, *Ph1* is the responsible of spatial organisation of chromosomes (Feldman, 1966). The *Ph1* locus was mapped in the long arm of 5B chromosome and its mutant version obtained by a deletion of the locus, is named *ph1b* (Sears, 1977). The *Ph1* locus was later characterised as a cluster of defective cyclin-dependent kinase, Cdk-like gene cluster related to Cdk-2 in humans. These protein kinases seem to be a main check-point gene implicated in the premeiotic replication, chromatin condensation, homologue pairing and repairing errors (Al-Kaff *et al.*, 2008; Greer *et al.*, 2012). The lack of the *Ph1* locus generates multivalents and not bivalents in more than 50% PMCs (Riley and Chapman, 1958; Riley, 1960), aneuploidy and genomic rearrangements (Sanchez-Moran *et al.*, 2001). More recently, other functions on chromosome recombination have raised (Martín *et al.*, 2017).

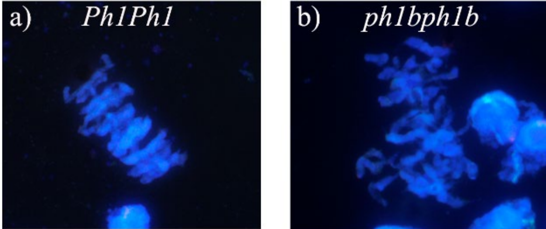

Figure 7. The effects of the *Ph1* locus during metaphase I in bread wheat. a) Stable metaphase I with 21 bivalents of *T. aestivum Ph1Ph1*. b) Metaphase I showing multivalents in the *ph1bph1b* background.

The introgression of alien chromosomes of relative species in wheat has been used in many studies to induce more genetic diversity and transfer desirable traits into wheat (O'Mara, 1953; Schlegel, 1996; Martín *et al.*, 1999). The *ph1b* mutation has been used to induce homoeologous chromosome recombinations between related chromosomes and those from wheat in hybrids or alien introgression lines the wheat background (Marais *et al.*, 2010; Niu *et al.*, 2011; Ayala-Navarrete *et al.*, 2013; Rey *et al.*, 2015a, 2015b; King *et al.*, 2019). Once an alien chromosome or chromosome segment has been introgressed in wheat in the absence of the *Ph1* locus, the wild phenotype of the *Ph1* locus might be restored by backcrosses with wheat lines carrying the *Ph1* locus in order to maintain chromosome However, some mutants with homoeologous chromosome pairing can be fairly steady (Svačina *et al.*, 2020). In this work, we have used wheat lines carrying *H. vulgare* and *H. chilense* chromosome introgressions in the presence and in the absence of the *Ph1* locus to go deeper into the knowledge of homologous chromosome associations during meiosis in wheat and the function of the *Ph1* locus in these specific associations. In addition, we have also tried to shed more light on the role of subtelomeres in the processes of chromosome recognition and pairing.

Objectives

General objective

The aim of this work is to evaluate the role of terminal chromosome regions (telomeres and subtelomeres) in chromosome pairing and whether there is an effect of the *Ph1* locus during the recognition and association processes at the beginning of meiosis.

Specific objectives

- Study of the implication of subtelomeric sequences in the processes of recognition/pairing between homologous chromosomes (Chapter II).
- Study of the effect of the *Ph1* locus in homoeologous chromosome association and pairing during meiosis (Chapters III and IV).

The thesis is written in chapters following the structure of peer-reviewed publications. Chapter II was published in Scientific Report (doi: 10.1038/srep06488). Chapter III has been published in Frontiers in Plant Science (doi: 10.3389/fpls.2018.00585). Chapter IV is in preparation for publication in Agronomy.

Chapter II

The subtelomeric region is important for chromosome recognition and pairing during meiosis

Published in:

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Abstract

The process of meiosis results in the formation of haploid daughter cells, each of which inherit a half of the diploid parental cells' genetic material. The ordered association of homologues (identical chromosomes) is a critical prerequisite for a successful outcome of meiosis. Homologue recognition and pairing are initiated at the chromosome ends, which comprise the telomere dominated by generic repetitive sequences, and the adjacent subtelomeric region, which harbours chromosome-specific sequences. In many organisms, telomeres are responsible for bringing the ends of the chromosomes close together during early meiosis, but little is known regarding the role of the subtelomeric region sequence during meiosis. Here, the observation of homologue pairing between a pair of *Hordeum chilense* chromosomes lacking the subtelomeric region on one chromosome arm indicates that the subtelomeric region is important for the process of homologous chromosome recognition and pairing.

Introduction

The outcome of meiosis is the generation of balanced gametes each carrying a full haploid complement. Proper homologue recognition is required in order to ensure ordered pairing and legitimate recombination. In a polyploid such as bread wheat, (hexaploid, 2n=6x=42), which has three related genomes (A, B and D), the presence of homoeologous (related) chromosomes complicates the picture, since homologues also need to be distinguished from homoeologues before the chromosomes can pair in an ordered way. The mechanism by which homologues identify one another is the most poorly understood aspect of meiosis (Ronceret and Pawlowski, 2010). It is accepted that the distal region of the chromosomes, which encompasses the telomere and the subtelomeric region, is critical to the process of homologue recognition and pairing in many organisms, but the specific role of these two structures is still unclear (Page and Hawley, 2003). Telomeric sequence is highly conserved across the eukaryotes, underlining the importance of the telomeres in cell division. In many organisms, at an early stage of meiosis, the telomeres appear to cluster at the nuclear envelope to form a ‘‘bouquet’’; the effect of this clustering is to bring the ends of the chromosomes close together, thereby facilitating the initiation of homologue recognition and pairing (Zickler and Kleckner, 1998; Bass *et al.*, 2000). Once initiated, pairing triggers a conformational change in the chromatin which advances in a proximal direction along the length of the chromosome arm, inducing the necessary intimate contact between the two homologues along their entire chromosome length (Prieto *et al.*, 2004b). How chromosomes identify their homologous partners to pair, however, remains unknown, since the DNA sequence of the telomeres is largely generic and not at all chromosome-specific. The polymorphic nature of subtelomeres is an exciting area for study, but also presents a difficult challenge from the technical perspective. Subtelomeres are the transition between chromosome-specific sequences and the arrays of telomeric repeats, gene-rich, less evolutionary

conserved than telomeres, and represents hot spots of recombination (Linardopoulou *et al.*, 2005; Louis and Vershinin, 2005). These features have contributed to the difficulty in assessing the potential conserved functions of these high-polymorphic regions, which are one of the most exciting frontiers left in genomics.

The addition of a pair of 'alien' chromosomes to the full genome complement of a crop species is a commonly used first step for accessing genetic variation from the secondary gene pool (Gale and Miller, 1987). Such addition lines have a long history of use for locating genes and markers, characterising the regulation of alien genes, isolating individual chromosomes and understanding meiotic pairing behaviour and chromosome structure (Cho *et al.*, 2006; Suchánková *et al.*, 2006; Naranjo and Corredor, 2008). Sets of both cultivated (*H. vulgare*) and wild (*H. chilense*) barley addition lines in a hexaploid wheat background have been established for some time (Islam *et al.*, 1978; Miller *et al.*, 1982). *Hordeum chilense* is highly polymorphic both morphologically and biochemically (Bothmer *et al.*, 1995), and has been used as a donor of various traits of relevance to wheat improvement (Martín and Cabrera, 2005). In this study, chromosome pairing in wheat was analysed at the onset of meiosis by following an extra pair of chromosomes from this wild barley. One of the added *H. chilense* chromosomes appears to have suffered a terminal chromosome deletion on its short arm, which has removed the subtelomeric region but retained the telomere, while a sister line carries a deletion on the long arm, but has retained both the long arm telomere and subtelomeric region (Said *et al.*, 2012). Since non-wheat chromosomes present in a wheat line can be readily tracked via *in situ* hybridisation (Prieto *et al.*, 2001), these materials provide an excellent opportunity to analyse the influence of the subtelomeric region on chromosome pairing and conformational changes during meiosis.

Methods

Plant material

Root tips and anthers were harvested from two addition lines constructed in the experimental bread wheat variety 'Chinese Spring' and involved two versions of *H. chilense* chromosome $3H^{ch}$. One of these carried a terminal deletion of the short arm (44% of the chromosome arm deleted, including the subtelomeric region) and the other a terminal deletion within the long arm (58% of the chromosome arm deleted, but retaining the subtelomeric region) (Said *et al.*, 2012).

Fluorescence *in situ* hybridisation

Three *in situ* hybridisation probes were fluorescently labelled following standard procedures: these comprised (1) the barley subtelomeric sequence HvT01 (Belostotsky and Ananiev, 1990), (2) the highly conserved telomeric sequence pAt74 (Richards and Ausubel, 1988), originally isolated from *A. thaliana*, and (3) total genomic *H. chilense* DNA (Prieto *et al.*, 2001). Methods for preparing meiotic and mitotic chromosomes spreads, *in situ* hybridisation and subsequent scoring have been described elsewhere (Prieto *et al.*, 2001, 2004a).

Immunocytological detection of CENH3, ZYP1 and MLH1 during wheat meiosis

To visualise centromeres, samples were rinsed in PBS buffer following the *in situ* hybridisation procedure, and then incubated with the α-CENH3 antibody (Nagaki *et al.*, 2004), kindly supply by Dr. J.M. Vega from the Complutense University of Madrid (Spain). The hybridisation signal was detected following incubation with a secondary incubation with the α-rabbit-ROD (Millipore, MA, USA). After rinsing once more in PBS buffer, a second immunolocalisation was carried out in the same meiocytes to detect the ZYP1 and MLH1 proteins

following the protocols previously described (Chelysheva *et al.*, 2010; Khoo *et al.*, 2012). The anti-ZYP1 and the anti-MLH1 were kindly supplied by Dr. J.A. Able from the University of Adelaide (Australia) and Dr. Chelysheva from the INRA (France), respectively.

Fluorescence microscopy and image processing

The fluorescence optical images were collected using the fluorescence microscope Eclipse 80i, Nikon UK. The images were processed by the Photoshop version 11.0.2 software.

Results

Detection of *H. chilense* subtelomeric regions in the wheat background

The presence/absence of the *H. chilense* subtelomeric regions in the wheat background was visualised using fluorescence *in situ* hybridisation. Mitotic chromosome spreads obtained from root tips were made from both the addition line carrying the terminal *H. chilense* chromosome short arm deletion (lacking the subtelomeric region) and the one carrying the deleted version of the long arm (subtelomeric region retained) (Figure 1). When probed with the *Arabidopsis thaliana* telomeric sequence pAt74, all of the wheat chromosomes and the *H. chilense* pair generated positive hybridisation signals. In contrast, only the barley subtelomeric regions were labelled when the barley-specific subtelomeric repetitive sequence HvT01 was used as the probe. Both the telomere and the subtelomeric region were present on both arms of the $3H^{ch}$ chromosome carrying the long arm deletion (Figure 1a), while the telomere but not the subtelomeric region were present on $3H^{ch}$ chromosome carrying the short arm deletion (Figure 1b). These wheat lines and the visualisation of the satellite regions in only one pair of chromosomes were used as a tool to help in

understanding the role of the subtelomeric regions in chromosome recognition/pairing at early meiosis in wheat.

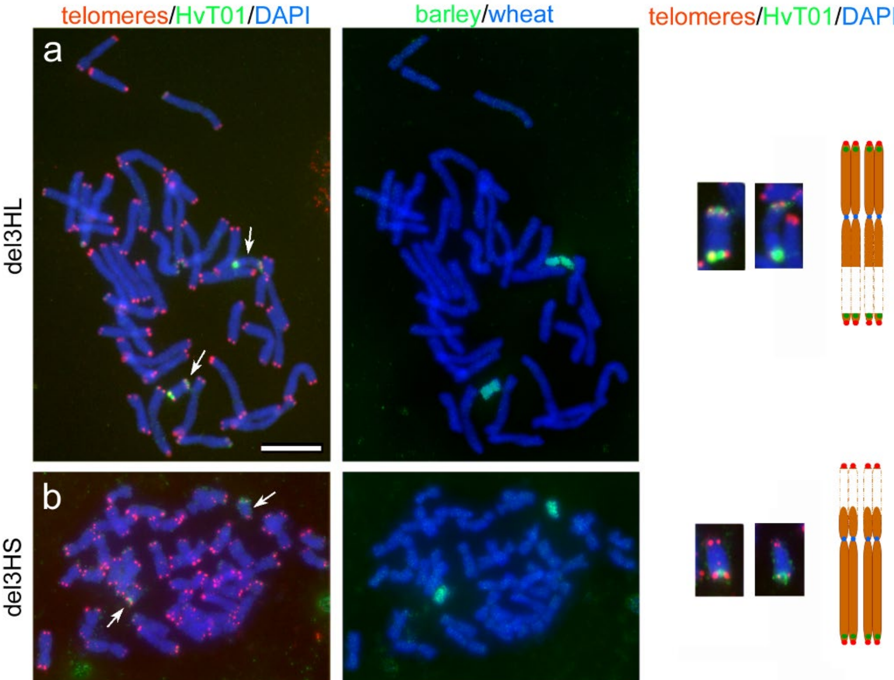

Figure 1. ***In situ* hybridisation-based detection of telomeres and the *H. chilense* chromosome 3H^{ch} subtelomeric regions in addition line root tip metaphase spreads.** (**a**) The line carrying the version of chromosome 3H^{ch} in which a deletion on the long arm has removed neither the long arm telomere nor the subtelomeric region, (**b**) the line carrying the version of chromosome 3H^{ch} in which a deletion on the short arm has removed the subtelomeric region. The 3H^{ch} chromosomes (indicated by a white arrow) can be distinguished by the presence of signal following hybridisation with HvT01, as confirmed by probing with labelled *H. chilense* genomic DNA. Bar: 10 µm.

Homologous chromosomes failed to recognise and pair with one another in the absence of the subtelomeric region

Chromosome pairing was analysed during early meiosis by fluorescence *in situ* hybridisation, visualising telomeres and subtelomeres on the wild barley chromosomes in the wheat background. The *in situ* hybridisation analysis of more than 500 pachytene meiocytes of each of the two addition lines showed that the *H. chilense* homologues were associated with one another along the whole of their length provided that the sequences recognised by HvT01 were

present on both chromosome arms (Figure 2a). In the absence of the *H. chilense* subtelomeric region on the $3H^{ch}$ short arm, homologous chromosomes were only associated by the distal region of long arm where the HvT01 sequence was present (Figure 2b). No chromosome remodelling was observed where the homologous arms were not associated with one another. A result, the short arms without the subtelomeric region were unable to initiate pairing. However, as meiosis progressed, the pairing signal initiated at the other end of the chromosome was propagated along the chromosome, even crossing the centromere into the short arm (Figure 2c), so that the homologues became fully associated by late pachytene (Figure 2d). No other chromosome patterns were visualised during pachytene. The implication was that DNA sequence(s) within the subtelomeric region must be important for the process of homologue recognition and pairing.

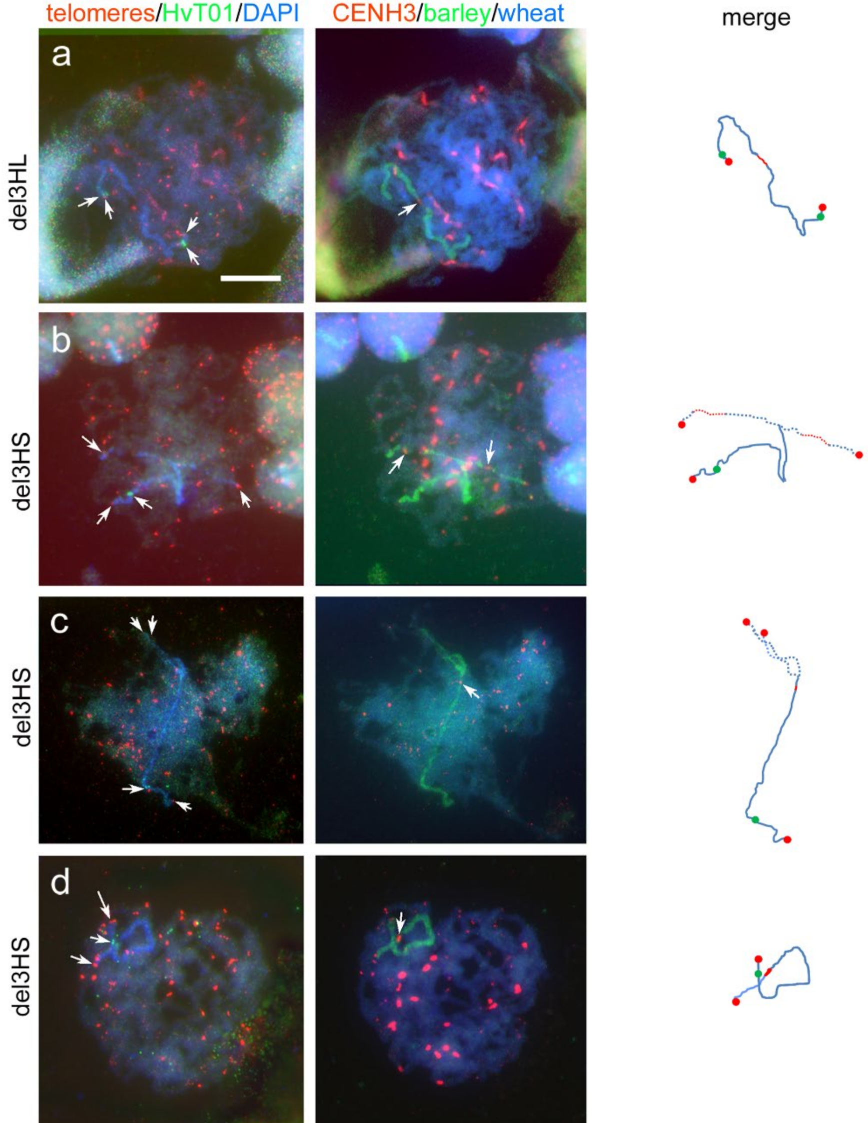

Figure 2. The behaviour of the chromosome 3H^{ch} homologues during pachytene. Telomeres, subtelomeric regions (first line) and centromeres (second line) of the two homologues are indicated by a white arrow. Solid and broken lines in the diagrams represent the paired and unpaired regions of the homologous chromosomes, respectively. (**a**) In the line carrying the version of chromosome 3H^{ch} in which a deletion on the long arm has removed neither the long arm telomere nor the subtelomeric region, the two homologues are fully paired, while (**b**) in the line carrying the version of chromosome 3H^{ch} in which a deletion on the short arm has removed the subtelomeric region, they are only associated along the distal region of the long arm, leaving the centromeric region and the short arm unpaired. (**c**) At a later stage during

meiosis, pairing is extended in the line carrying the version of chromosome $3H^{ch}$ in which a deletion on the short arm has removed the subtelomeric region. The homologues are fully associated along their long arm and the pairing signal has been transmitted through the centromeres, although the short arms remain largely unpaired. (**d**) At an even later stage, the homologues become fully associated. Bar: 10 µm.

Recombination does not occur in the absence of the subtelomeric region

Chromosome pairing was also analysed during meiotic metaphase I. Meanwhile homologous chromosomes remained associated correctly in ring bivalents by two chiasmata (the cytological equivalent of genetic crossing-over) at metaphase I when the subtelomeric region was present on both chromosome arms (Figure 3a), rod bivalents were always observed in all the cells analysed when the short arm subtelomeric region was absent, keeping homologues associated only by the long arm having subtelomeres (Figure 3b; Table 1). These observations implied that, even though in the absence of the subtelomeric regions the short arms were still able to associate with one another late in pachytene (Figure 2d), chiasma formation (and hence recombination) was not possible. The absence of the subtelomeric region therefore had an indirect effect on recombination.

Table 1. Chromosome $3H^{ch}$ pairing at metaphase I in the first ear sampled from four independent plants per addition line. Applying a *Chi-Square* test to test whether chromosome pairing was similar in the two lines suggested that the hypothesis should be rejected ($p<0.001$). del$3H^{ch}$L: the line carrying the version of chromosome $3H^{ch}$ in which a deletion on the long arm has removed neither the long arm telomere nor the subtelomeric region, del$3H^{ch}$S: the line carrying the version of chromosome $3H^{ch}$ in which a deletion on the short arm has removed the subtelomeric region. n: number of cells analysed

Line	Number of samples	Total n	Ring bivalents n (%)	Open bivalents n (%)	Univalents n (%)	*P*
del3**H**^{ch}L	4	275	273 (99.27)	2 (0.73)	0	2.2×10^{-16}
del3**H**^{ch}S	4	511	0	503 (98.43)	8 (1.57)	

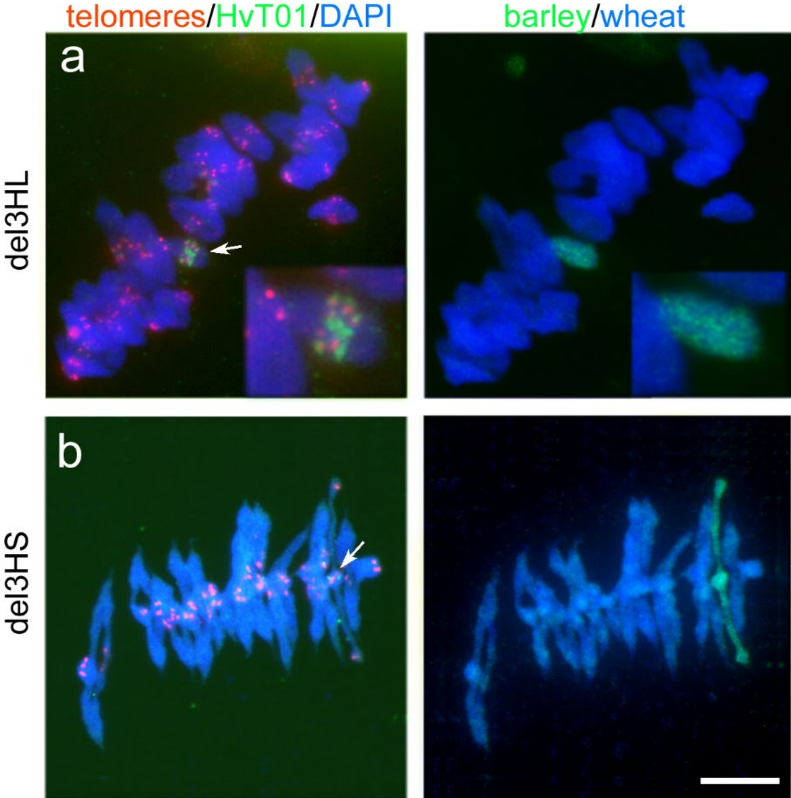

Figure 3. The behaviour of the chromosome 3H^{ch} homologues (labelled by genomic *in situ* hybridisation) during metaphase I. (**a**) In the line carrying the version of chromosome 3H^{ch} in which a deletion on the long arm has removed neither the long arm telomere nor the subtelomeric region, the homologues form a ring bivalent (see inset), reflecting chiasma formation on both arms. (**b**) In the line carrying the version of chromosome 3H^{ch} in which a deletion on the short arm has removed the subtelomeric region, a rod bivalent forms, reflecting the lack of chiasma formation on the short arm (arrowed). Scale bar 10 µm.

The ZYP1 and MLH1 proteins are known to be deposited in, respectively, the synaptonemal complex and the chiasma. Immunolocalisation experiments based on antibodies recognising each of these proteins were performed on the pachytene meiocytes of the addition line carrying the pair of 3H^{ch} chromosomes with the short arm deletion (the subtelomeric region is absent). ZYP1 was associated with both arms, so appeared not to be affected by the absence of the subtelomeric region (Figure 4a). However, MLH1 was only deposited on the

long arm of the alien chromosome (Figure 4b); consistent with the conclusion that recombination depended on the presence of the subtelomeric region at pachytene, the stage during which chromosome remodelling occurred and pairing is initiated.

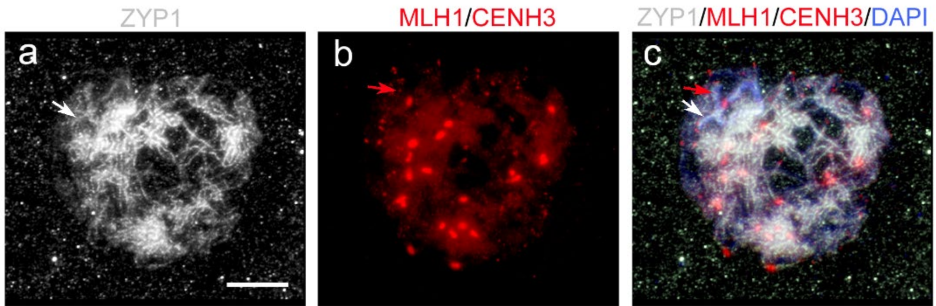

Figure 4. Immunolocalisation of ZYP1, MLH1 and CENH3 in the pachytene meiocytes of the line carrying the version of chromosome 3H^{ch} in which a deletion on the short arm has removed the subtelomeric region (the same preparations were assayed as described in Figure 2d). (**a**) ZYP1 is deposited along the synapsed homologues chromosomes, even in the short arm of the barley chromosome (white arrow) were the subtelomeric region is absent. (**b**) Foci of MLH1 deposition only show along the long arms having the subtelomeric region (red arrow). The large red signals relate to sites of CENH3 deposition and mark the centromere. (**c**) A merged image of (a) and (b). Bar: 10 µm.

Discussion

In silico modelling has suggested that telomere interactions alone may not be sufficient to assure the pairing of large chromosomes, so that interactions at additional chromosome sites are likely required (Penfold *et al.*, 2012). In addition, even when the telomere bouquet fails to form homologous pairs, recombination, synapsis and the formation of balanced gametes can still occur (Cooper *et al.*, 1998; Wu and Burgess, 2006), suggesting that other mechanisms involving subtelomeres, which also form a bouquet in rye (Mikhailova *et al.*, 2001), can determine homologue recognition and pairing. Because telomeres are highly conserved structures, it is logical that a less well conserved structure, such as the subtelomeric region, probably controls homologue recognition and pairing once the clustering of the telomeres has

ensured that the chromosome ends lie close to one another. Homologue recognition may depend not only on the molecular DNA-DNA interactions but also on chromatin structure, which is determined by the DNA sequence within the subtelomeric region. The formation of a homologue-specific structure close to its telomere could serve as a chromosome identifier, which would facilitate homologue recognition and the initiation of pairing at its distal ends; thereafter, the trigger to pair can pass along the chromosome arm (Prieto *et al.*, 2004b). In addition, it cannot be discarded the ability that the subtelomeric region might have in physical interaction between homologous chromosomes. The subtelomeric region can be able to bind with a specific set of proteins, so that recognition and pairing could be driven by protein-protein or DNA-protein, rather than DNA-DNA interactions (Page and Hawley, 2003).

Although chromosomes associate via centromeres into seven groups of homoeologues at the onset of meiosis in wheat (Martinez-Perez *et al.*, 2003), association of homologous barley centromeres have not been visualised at this stage until the pairing signal initiated at the chromosome ends was transmitted along the chromosome arms up to the centromeric regions. Sister chromatid cohesion and full synapsis among the arm of a pair of homologues are known to be a prerequisite for homologous centromere pairing at early prophase I to occur in both wheat and maize (Corredor and Naranjo, 2007; Zhang *et al.*, 2013). The present data suggest that centromeres are not sufficient to trigger chromosome pairing along the chromosome arms. Centromere associations might act to stabilise the centromere pole, to maintain a reference point for the oriented telomere migration, providing a physical structure for chromosome movements that facilitate pairing interactions along chromosome arms, as suggested for maize or wheat (Naranjo and Corredor, 2008; Zhang *et al.*, 2013). Our observations of the behaviour of the wheat addition line which carries the $3H^{ch}$ chromosome lacking its short arm subtelomeric region have illustrated that, although the centromeres do not trigger a signal inducing the homologues to pair, the signal which has been initiated at one end of the chromosome can

be transmitted through the centromere, thereby allowing homologues to become fully associated by the end of prophase I.

It has recently become clear that rather than location on the chromosome *per se*, it is either the DNA sequence or chromatin organisation which governs the site of chiasma formation (Valenzuela *et al.*, 2012). Regions which frequently feature crossing-over, such as the subtelomeric region, are more intimately involved in homologue recognition and synapsis than are cross-over poor regions (Linardopoulou *et al.*, 2005; Louis and Vershinin, 2005; Valenzuela *et al.*, 2012). Thus, the absence of a subtelomeric region, as in the short arm deletion version of chromosome $3H^{ch}$, would be expected to suppress chiasma formation, and consequently this arm would be less likely to be associated during meiotic metaphase I. During the telomere bouquet stage, the chromosome distal ends undergo remodelling, following which the homologue pairs associate with one another (Prieto *et al.*, 2004b). Chromatin remodelling has been shown to be a prerequisite for chromosome pairing and recombination in wheat (Prieto *et al.*, 2004b; Colas *et al.*, 2008). The requirement of chromatin remodelling for chromosome pairing and recombination has been also shown in other species such as *Caenorhabditis elegans*, which does not even display a telomere bouquet during early meiosis (Nabeshima *et al.*, 2011). The present observations of the behaviour of the version of chromosome $3H^{ch}$ which lacks the short arm subtelomeric region suggest that it fails to undergo remodelling at the bouquet stage, and the homologous arms do not pair until the pairing signal has been transmitted along the chromosome from the long arm. The lack of chromatin remodelling in the short arm and the consequent delay in pairing together can explain the localised absence of recombination, a process which needs to be completed before the meiocytes progresses beyond pachytene. The implication is that it is not so much the lack of the subtelomeric sequences that is important, but rather the consequent delay in chromatin remodelling and pairing which is responsible for the absence of recombination.

The present observations have shown that the subtelomeric region plays a key role in the processes of homologue recognition and subsequent pairing during early meiosis in wheat. It was also confirmed that it acts to trigger a conformational change to the chromatin, an event which allows the homologue pairs to associate intimately with one another, thereby permitting recombination later in meiosis. Unravelling the underlying molecular mechanisms involved will shed light to our understanding of how each chromosome associates with 'the right partner' during meiosis in wheat.

Ethics statement

The authors declare that the experiments comply with the current laws of the country (Spain) in which they were performed.

Acknowledgments

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Author contributions

M.C.C., M.D.R., A.C. and P.P. conceived the project and planned the experiments. M.C.C., M.D.R. and P.P. conducted the experiments. M.C.C.,

M.D.R., A.C. and P.P. wrote the paper (all authors provided constructive feedback).

Chapter III

Homoeologous chromosomes from two *Hordeum* species can recognise and associate during meiosis in wheat in the presence of the *Ph1* locus

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Abstract

Understanding the system of a basic eukaryotic cellular mechanism like meiosis is of fundamental importance in plant biology. Moreover, it is also of great strategic interested in plant breeding since unzipping the mechanism of chromosome specificity/pairing during meiosis will allow its manipulation to introduce genetic variability from related species into a crop. The success of meiosis in a polyploid like wheat strongly depends on regular pairing of homologous (identical) chromosomes and recombination, processes mainly controlled by the *Ph1* locus. This means that pairing and recombination of related chromosomes rarely occur in the presence of this locus, making difficult wheat breeding trough the incorporation of genetic variability from related species. In this work, we show that wild and cultivated barley chromosomes associate in the wheat background even in the presence of the *Ph1* locus. We have developed double monosomic wheat lines carrying two chromosomes from two barley species for the same and different homoeology chromosome group, respectively. Genetic *in situ* hybridisation revealed that homoeologous *Hordeum* chromosomes recognise each other and pair during early meiosis in wheat. However, crossing over does not occur at any time and they remained always as univalents during meiosis metaphase I. Our results suggest that the *Ph1* locus does not prevent chromosome recognition and pairing but crossing over between homoeologous. The role of subtelomeres in chromosome recognition is also discussed.

Keywords: wheat, barley, homoeologous pairing, introgressions, meiosis, chromosome recognition

Introduction

More than two-thirds of global cropland features annual grain crops, which represent roughly 70% of humanity's food energy needs and typically grown in monoculture. Annual grain production, at its current scale, is fundamentally unsustainable. Thus, the growing human population demands greater crops, more productive and better adapted to specific agro-climatic conditions (Godfray *et al.*, 2010). Plant breeders are playing a major role in worldwide efforts to understand gene functions and interactions with the aim of increasing quality and productivity of major crops. Wide-crossing in plant breeding is an important tool and sometimes the results are the starting point for new crops (O'Mara, 1953). For example, wide-crossing has been carried out in the Triticeae tribe, which includes wheat, to develop new plant species such as × *Triticosecale*, obtained after crossing wheat and rye, or ×*Tritordeum*, an amphyploid between the wild barley *Hordeum chilense* Roem. *et* Schult. and wheat (O'Mara, 1953; Martín and Sanchez-Mongelaguna, 1982). Breeders have also used related species as genetic donors for widening the genetic basis of wheat to get for example wheat cultivars better adapted to specific agro-climatic conditions, improving the quality or carrying resistance to diseases (Lukaszewski, 2000; Calderón *et al.*, 2012, 2014; Rey *et al.*, 2015a). In fact, there are many wild species carrying interesting traits that would be useful to be exploited in wheat breeding programmes, but unfortunately, hybridisation between wheat and a wild related species produces only a low level of chromosome pairing and recombination. So understanding wheat genetics and genome organisation is essential for plant breeding purposes.

Bread wheat is an hexaploid, which possesses three sets of related chromosomes because of doubling of chromosomes following sexual hybridisation between closely related species. However, chromosomes associate regularly in pairs in wheat during meiosis, the cellular process to produce gametes in sexually reproducing organisms. Thus, at meiosis each

chromosome only recognises and associate with its homologous and not with the related (homoeologous) chromosomes, which have a similar gene content and order but differ in repetitive DNA sequences. Several pairing homologous (*Ph*) genes control chromosome associations in wheat, although the major effect is due to the *Ph1* locus (Sears, 1976). The efficiency of chromosome associations during meiosis have a big influence on the fertility of wheat plants, being crucial for success in breeding, but has a negative effect preventing pairing and recombination between wheat chromosomes and those from related species. Therefore, it seems reasonable to go deeper into the knowledge of the biology of chromosome associations during meiosis in wheat, which will be valuable for wheat breeding.

Chromosome dynamics during meiosis have been extensively studied in a polyploidy such as hexaploid wheat (Moore, 2002; Corredor *et al.*, 2007; Colas *et al.*, 2008; Naranjo and Corredor, 2008). It is now well established that both interactions during recombination at the DNA level and assembly of a meiosis specific proteinaceous structure known as the synaptonemal complex (SC) play roles in stabilising associations between homologous chromosomes. However, how homologs became colocalised and how initial recognition is accomplished to establish chromosome associations remains poorly understood. When a chromosome recognises its homolog (and not another chromosome) in wheat, a localised conformational change in adjacent chromatin is triggered in both partners. This process facilitates recognition and association of homologous versus homoeologous chromosomes and is affected by the *Ph1* locus (Prieto *et al.*, 2005; Greer *et al.*, 2012). Thus, *Ph1* stabilises wheat during meiosis by both, promoting homolog synapsis during early meiosis and preventing homoeologous recombination later in meiosis (Martín *et al.*, 2014, 2017). The effect on synapsis occurs during the telomere bouquet *Ph1* stage, when promotes more efficient homologous synapsis, thereby reducing the chance of homoeologous synapsis (Martín *et al.*, 2017). The effect on CO formation occurs later in meiosis, when *Ph1* prevents MLH1 sites (Double Holliday

Junctions marked to become COs) on synapsed homoeologues from becoming COs. In addition, it has been also described that the level of a ZIP4 paralog included within the *Ph1* locus alters the number of CO between homoeologous chromosomes (Rey *et al.*, 2017).

Efforts focused on centromeres and telomeres behaviour during meiosis have been also made (Martinez-Perez *et al.*, 2000, 2001, 2003; Naranjo *et al.*, 2005). Telomeres, which are highly conserved structures among plants, including wheat (Simpson *et al.*, 1990; Ganal *et al.*, 1991; Schwarzacher and Heslop-Harrison, 1991), play an important role on initial chromosome associations at the onset of meiosis. In this stage, the association of telomeres in a bouquet facilitates the search and recognition of homologous chromosomes by bringing chromosomes closer (Corredor and Naranjo, 2007; Koszul *et al.*, 2008; Moore and Shaw, 2009) and its formation is affected by the *Ph1* locus (Richards *et al.*, 2012). Subtelomeres, which are the telomere associated sequences (TAS), are highly polymorphic and extraordinarily dynamic sequences (Eichler and Sankoff, 2003). The complex and variable nature of subtelomeres has made difficult to assess the possible functions(s) of these regions so far, but studies on *Arabidopsis* and *Hordeum* subtelomeres might suggest a possible role on chromosome specificity between homolog chromosomes at the onset of meiosis (Kotani, 1999; Heacock *et al.*, 2004; Calderón *et al.*, 2014). In fact, subtelomeres in *Hordeum* showed high variability not only from different chromosomes but also among chromosome arms within the same chromosome (Schubert *et al.*, 1998; Prieto *et al.*, 2004a). Thus, the copy number of the subtelomeric HvT01 sequence was variable among chromosomes in both *H. chilense* and *H. vulgare*. Since chromosome associations are initiated at the distal regions of the chromosomes and homologous chromosomes are zipping up from those to the centromeres (Prieto *et al.*, 2004b; Corredor *et al.*, 2007), it seems reasonable to go deeper into the role of the subtelomeric regions on homolog chromosome associations, rather than focusing on features that are common to all chromosomes like telomeres.

The addition of a pair of ‘alien’ chromosomes to the full genome complement of a crop species is commonly used as a first step for accessing genetic variation from the secondary gene pool, but addition lines are also relevant for understanding meiotic pairing behaviour and chromosome structure (Friebe *et al.*, 2005; Lukaszewski, 2010). Sets of both cultivated (*H. vulgare*) and wild (*H. chilense*) barley addition lines in a hexaploid wheat background were developed (Islam *et al.*, 1978; Miller *et al.*, 1982) and have potential in plant meiosis studies. Certainly, it allows tracking one specific pair of chromosomes or chromosome segments within the wheat background using genomic *in situ* hybridisation (GISH) and study chromosome rearrangements and associations exclusively in a pair of homologs (Naranjo *et al.*, 2010; Rey *et al.*, 2015b).

In this study, we have developed double monosomic addition lines of wild and cultivated barley in wheat for the same and for different homoeology group to go deeper into the knowledge of chromosome associations during meiosis. These double monosomic addition lines enabled to distinguish chromosomes from two different barley species in the wheat background, observe conformational changes during meiosis and analyse whether subtelomeres might play a role on chromosome recognition/pairing at early meiosis in the absence of homologs. Results showed that homoeologous chromosomes can recognise each other to associate correctly in pairs, even in the presence of the *Ph1* locus, although crossing over does not occur as they remained as univalents during metaphase I.

Materials and methods

Plant material

Crosses between *H. chilense* and *H. vulgare* addition lines in bread wheat (*Triticum aestivum* cv. Chinese Spring; AABBDD + pair of $H^{ch}H^{ch}$ and AABBDD + pair of $H^{v}H^{v}$, respectively) for the same and for different homoeology group were made to obtain double monosomic wheat lines

carrying one *H. chilense* and one *H. vulgare* chromosome for the same and for different homoeology group. *H. chilense* and *H. vulgare* addition lines were kindly provided by Steve Reader, JIC, Norwich, UK. The presence of each *Hordeum* sp. chromosome in parental and F1 wheat lines used in this work was confirmed by both PCR assays previously described (Liu *et al.*, 1996; Hagras *et al.*, 2005) and *in situ* hybridisation.
Seeds obtained from genetic crosses were germinated on wet filter paper in the dark for 5 days at 4◦C, followed by a period of 24h at 25ºC. Plants were then growth in the greenhouse at 26ºC during the day and 18ºC during the night (16 h photoperiod).

Fluorescence *in situ* hybridisation

Fluorescence genomic *in situ* hybridisation (GISH) was used to study chromosome associations between *H. chilense* and *H. vulgare* chromosomes in the wheat background as described previously (Prieto *et al.*, 2004a). Root tips were collected from germinating seeds and were pre-treated for 4 h in a 0.05% colchicine solution at 25ºC and fixed in 100% ethanol-acetic acid, 3:1 (v/v), for at least a week at room temperature. Spikes in meiosis were collected from mature plants and preserved in 100% ethanol-acetic acid, 3:1 (v/v) until were used to characterises chromosome associations. Chromosome spreads were prepared from both root tips cells and pollen mother cells (PMCs) at meiosis. Root tips and anthers were macerated in a drop of 45% glacial acetic acid on ethanol-cleaned slides, squashed under a cover slip and dipped in liquid nitrogen in order to fix the plant material on the slide. The cover slip was removed and the slides were air-dried and stored at 4ºC until used.
Both total genomic *H. vulgare* and *H. chilense* DNA were labelled by nick translation with biotin-11- (Boehringer Mannheim Biochemicals, Germany) and digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN, USA), respectively, and used as probes. Both probes were mixed to a final concentration of 5 ng/µl in the hybridisation mixture. The hybridisation mixture

consisted of 50% formamide, 2 × SCC, 5 ng of biotin-labelled or digoxigenin-labelled probe, 10% dextran sulphate, 0.14 μg of yeast tRNA, 0.1 μg of sonicated salmon sperm DNA and 0.005 μg of glycogen. Biotin-labelled *H. vulgare* DNA and digoxigenin-labelled *H. chilense* DNA were detected with a streptavidin- Cy3 conjugate (Sigma, St. Louis, MO, USA) and antidigoxigenin-FITC (Roche Diagnostics, Meylan, France), respectively. Chromosomes were counterstained with DAPI (4′, 6-diamidino-2-phenylindole) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Chromosome spreads from somatic cells and anthers of the F1 wheat lines were reprobed with the barley subtelomeric tandem repeat HvT01, which was obtained by amplification by the polymerase chain reaction (PCR) from genomic DNA from the barley cv. Betzes using primers made according to the published sequence (Belostotsky and Ananiev, 1990). PCR conditions were previously described by Prieto *et al*., 2004a. The PCR product corresponding to this barley satellite HvT01 probe was labelled with digoxigenin-11-dUTP, (Roche Applied Science, Indianapolis, IN, USA) by nick translation and detected with antidigoxigenin-FITC (Roche Diagnostics, Meylan, France). Meiosis metaphase samples were also reprobed to label centromeres using the RT sequence included in the barley centromeric BAC7 (Hudakova *et al.*, 2001), amplified by PCR following the same conditions as the amplification of the CCS1 centromeric repeat (Aragon-Alcaide *et al.*, 1996), labelled with biotin-11-(Boehringer Mannheim Biochemicals, Germany) and detected with the streptavidin- Cy3 conjugate (Sigma, St. Louis, MO, USA).

Fluorescence microscopy and image processing

Hybridisation signals were visualised 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, California, USA).

Statistical analysis

Statistical analyses were performed using STATISTIX 10.0 software (Analytical Software, Tallahassee, FL, USA). Anaphase I combinations were evaluated by an analysis of variance (ANOVA) as a completely randomised design. This analysis included a tangent transformation in the anaphase I combination where only one pole of the meiocytes showed *H. chilense* and *H. vulgare* signals. Tetrad combinations were analysed by the Kruskal–Wallis test (non-parametric one-way analysis of variance).

Results

Development of double monosomic *H. vulgare-H. chilense* addition lines in wheat

Crosses between disomic *H. chilense* and *H. vulgare* addition lines in bread wheat carrying chromosomes $7H^{ch}$ and $7H^{v}$, respectively, were made to obtain double monosomic barley additions in wheat carrying homoeologous *H. chilense* and *H. vulgare* chromosome 7. Similarly, genetic crosses between disomic *H. chilense* and *H. vulgare* addition lines in bread wheat for chromosomes $7H^{ch}$ and $5H^{v}$, respectively, were made to obtain double monosomic wheat lines carrying non-homoeologous chromosomes $7H^{ch}$ and $5H^{v}$. Finally, to corroborate observations on chromosome associations in a different homoeology group, we also developed genetic crosses between disomic *H. chilense* and *H. vulgare* addition lines in bread wheat for chromosomes $5H^{ch}$ and $5H^{v}$, respectively, to obtain double monosomic barley additions in wheat lines carrying homoeologous *H. chilense* and *H. vulgare* chromosomes for group 5. The F1 hybrid progeny from each genetic cross was analysed by GISH to ensure that they retained the expected both *H. chilense* and *H. vulgare* chromosomes (Figure 1). All the plants from all the genetic

crosses carried both barley chromosomes. In addition, fluorescence *in situ* hybridisation (FISH) was also performed in these wheat lines using the barley subtelomeric HvT01 repeat as a probe to label polymorphisms between the subtelomeric regions from the *H. chilense* and *H. vulgare* chromosomes added to the wheat background (Figure 1). Chromosome $7H^v$ had two strong signals for the barley subtelomeric HvT01 sequence on both chromosome arms meanwhile there was only a weaker signal on the short arm of chromosome $7H^{ch}$ Both $5H^{ch}$ and $5H^v$ chromosomes had a signal on the short arm for the HvT01 probe, which was stronger in the case of $5H^v$ chromosome, and only a weak signal on the subtelomeric region of the long arm of chromosome $5H^v$ was detected, which sometimes cannot be clearly seen and depended on the FISH experiment (Figure 1). No HvT01 subtelomeric signals were detected on the wheat chromosomes. The F1 progeny from each genetic cross was also growth until meiosis with the aim of studying the meiotic behaviour of both *Hordeum* chromosomes by *in situ* hybridisation in PMCs in the wheat background.

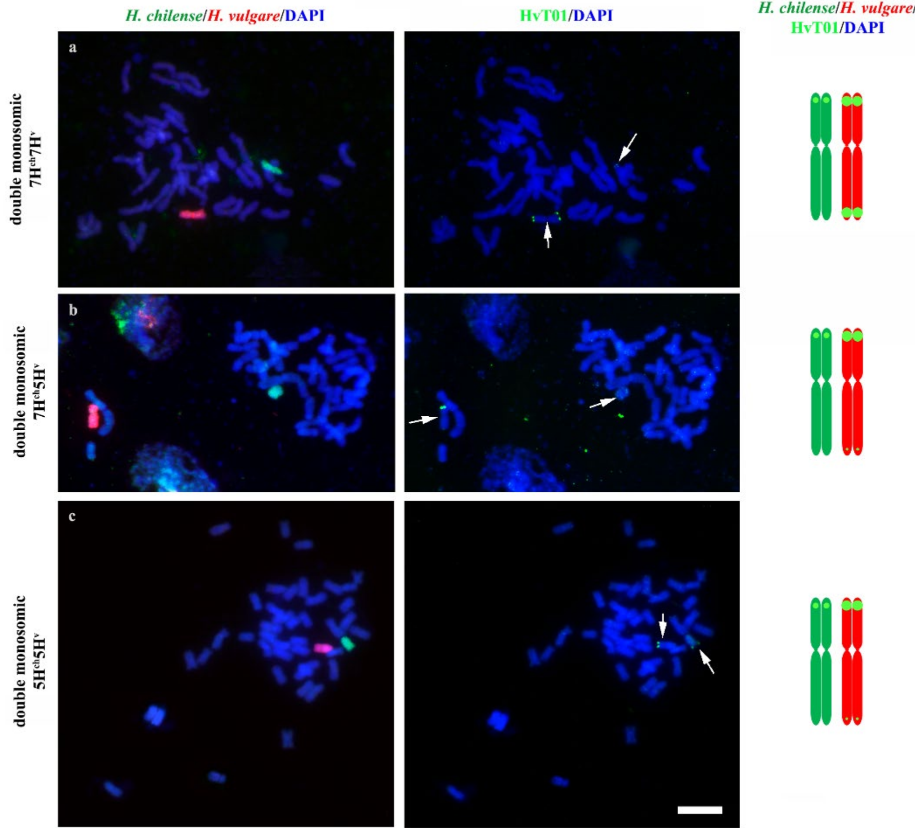


Figure 1. *Hordeum chilense* and *H. vulgare* double monosomic addition lines in wheat and physical location of the HvT01 subtelomeric repeat on barley chromosomes. *Hordeum chilense* (green) and *H. vulgare* (red) chromosomes were detected in GISH experiments in somatic chromosome spreads. In addition, the HvT01 subtelomeric sequence from barley was also detected (green). Total genomic DNA was counterstained with DAPI (blue). (**a**) Double monosomic $7H^{ch}7H^{v}$ addition line, including a diagram showing the HvT01subtelomeric signals in all barley chromosome arms, except in $7H^{ch}L$ arm. (**b**) Double monosomic $7H^{ch}5H^{v}$ addition line, including a diagram showing the subtelomeric barley sequence in all barley chromosome arms except in $7H^{ch}L$ arm. (**c**) Double monosomic $5H^{ch}5H^{v}$ addition line, including a diagram showing the subtelomeric barley sequence in all barley chromosome arms, except in $5H^{ch}L$ arm. Bar represents 10µm.

Homoeologous wild and cultivated barley chromosomes can fully associate in pairs in wheat in the presence of the *Ph1* locus

Chromosome pairing was analysed during early meiosis by GISH in F1 plants carrying one copy of *H. chilense* and one copy of *H. vulgare* homoeologous

chromosomes (7H^{ch} and 7H^{v}) and it was compared to those carrying non-homoeologous *H. chilense* and *H. vulgare* chromosomes (7H^{ch} and 5H^{v}, respectively). Experiments were developed in around a 100 cells of each genomic combination in prophase I of meiosis. Both wild and cultivated barley chromosomes were visualised simultaneously in the wheat background (Figure 2). In both cases, *H. chilense* and *H. vulgare* chromosomes were in proximity in the nucleus in early prophase (Figures 2a, c). As meiosis progressed, GISH experiments showed homoeologous *H. chilense* and *H. vulgare* chromosomes always fully-associated in pairs along the whole chromosome (Figure 2b). In contrast, non-homoeologous *Hordeum* chromosomes 7H^{ch} and 5H^{v} were not observed associated at this meiotic stage at any time, remaining always un-associated (Figure 2d).

GISH experiments were also carried out in cells in prophase I of meiosis in F1 plants carrying homoeologous chromosomes from *H. chilense* and *H. vulgare* for another homoeology group (group 5), with the aim of confirming the observations on chromosome associations between homoeologous chromosomes 7H^{ch} and 7H^{v} in the wheat background. Results showed that homoeologous *Hordeum* chromosomes 5H^{ch} and 5H^{v} did also associate in pairs during early meiosis in the wheat background, even in the presence of the *Ph1* locus (Figures 2e, f), suggesting that chromosome pairing between homoeologous chromosomes from two different *Hordeum* species is not hampered by the *Ph1* locus. In addition, results suggested that homoeologous barley chromosomes shared enough similar DNA sequences to recognise each other, a conformational chromatin change is observed in both homoeologues and chromosomes are finally associated completely in pairs.

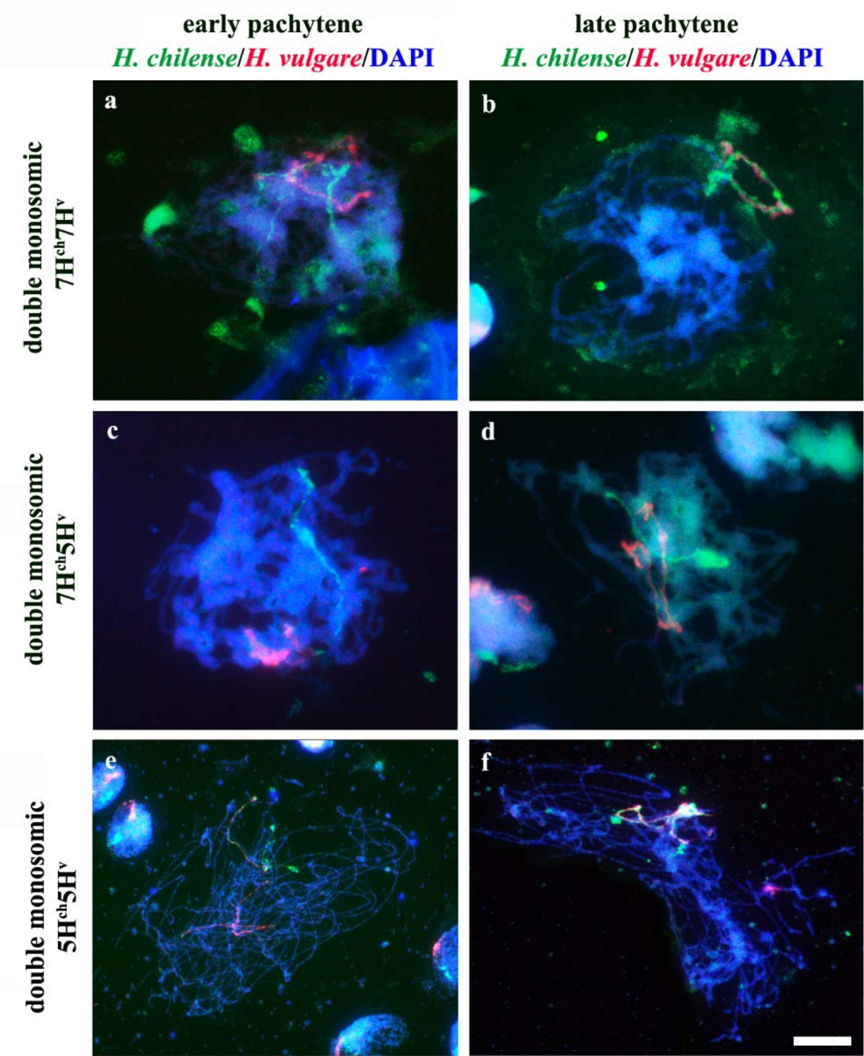


Figure 2. Behaviour of homoeologous and non-homoeologous barley chromosomes during early meiosis in wheat. *Hordeum chilense* chromosomes are visualised in green and *H. vulgare* chromosomes are visualised in red. (**a**) Double monosomic $7H^{ch}7H^{v}$ addition line showing both barley chromosomes un-associated at early pachytene. (**b**) Homoeologous barley chromosomes are fully associated at late pachytene in the double monosomic $7H^{ch}7H^{v}$ addition line. (**c**) Double monosomic $7H^{ch}5H^{v}$ addition line showing both barley chromosomes un-associated at early pachytene. (**d**) Non-homoeologous $7H^{ch}$ and $5H^{v}$ chromosomes remained un-associated at late pachytene. (**e**) Double monosomic $5H^{ch}5H^{v}$ addition line showing homoeologous wild and cultivated barley chromosomes half-paired at early pachytene. (**f**) Double monosomic $5H^{ch}5H^{v}$ addition line showing both homoeologous *Hordeum* chromosomes fully associated. Bar represents 10µm.

Subtelomeres might hamper chromosome associations between non-homologous *Hordeum* chromosomes in the wheat background

We have described that in the absence of homologous chromosomes, wild and cultivated barley homoeologous chromosomes can still recognise each other and associate completely in pairs during early meiosis. In addition, we have observed that, in these cases, initial chromosome recognition occurred by the chromosome ends where none or little copy number of the subtelomeric HvT01 repeat were detected, i.e., the long arm of homoeologous chromosomes $5H^{ch}$ and $5H^{v}$ (Figure 3). Thus, homoeologous *Hordeum* chromosomes did recognise and associate in pairs by these chromosome ends, which made chromosome recognition less restrictive and allowed homoeologous to recognise and associate in pairs. These observations were similar in the cells detected at the same stage from the double monosomic $7H^{ch}7H^{v}$ addition line in wheat (data not shown). Moreover, the observations were consistent in all the cells detected at the initiation of pairing (26 and 18 cells from the double monosomic $5H^{ch}5H^{v}$ and $7H^{ch}7H^{v}$ addition lines in wheat, respectively). Once homoeologous chromosomes had associated by these chromosome ends, a conformational change was observed along both homoeologous *Hordeum* chromosomes and pairing progressed along both chromosomes allowing a complete chromosome association between them (Figure 3).

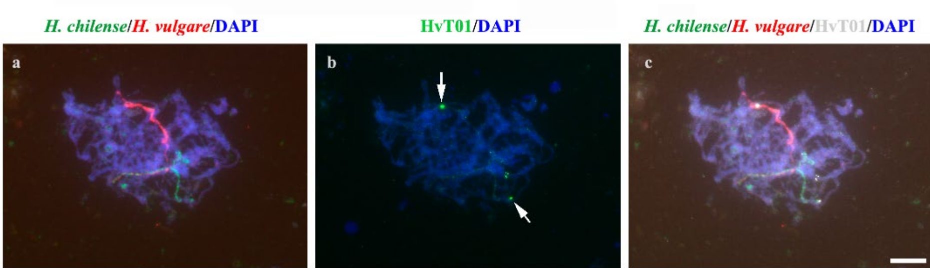


Figure 3. Detection of the subtelomeric HvT01 sequence in the wild and cultivated barley chromosomes in the $5H^{ch}5H^{v}$ double monosomic addition line. DNA was counterstained with DAPI (blue). (**a**) GISH of both *H. chilense* (green) and *H. vulgare* (red) homoeologous chromosomes initiating pairing during early pachytene. (**b**) Detection of the subtelomeric HvT01 probe (green) on the same cell. (**c**) Merge image showing the subtelomeric HvT01 signal (overlay in white) on the terminal region of the unpaired homoeologous wild and cultivated barley chromosome arms (arrowed). Bar represents 10µm.

Crossing over does not occur between wild and cultivated barley chromosomes in wheat although they previously associated in early meiosis

Once we observed full chromosome associations between homoeologous *Hordeum* chromosomes during early meiosis in the wheat background, we also analysed chromosome behaviour of both wild and cultivated barley chromosomes during metaphase I of meiosis in PMCs from double monosomic *H. chilense* and *H. vulgare* additions in wheat lines, both for the same and different homoeologous chromosomes. Meiosis metaphase I was also checked in the disomic *H. chilense* and *H. vulgare* addition lines in wheat used as parental lines for the genetic crosses developed in this work, to obtain the double *H. chilense* and *H. vulgare* double monosomic addition lines. Chromosome stability of the parental lines was confirmed as the homologous barley chromosomes carried in the *H. chilense* and *H. vulgare* disomic addition lines were always observed associated in pairs, indicating that crossing over occurred between homologous barley chromosomes (Figure 4).

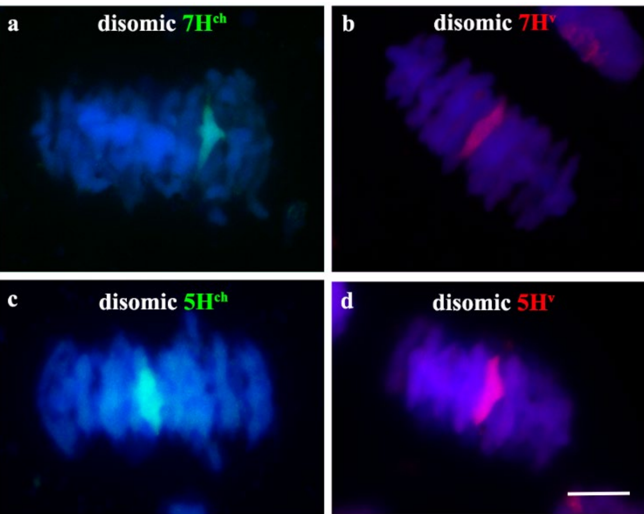


Figure 4. *Hordeum chilense* and *H. vulgare* chromosome behaviour during metaphase I in parental *H. chilense* and *H. vulgare* disomic addition lines in wheat. *Hordeum chilense* (green) and *H. vulgare* (red) chromosomes were observed always associated in pairs in all the cells in metaphase I in each *H. chilense* and *H. vulgare* disomic addition line. DNA was counterstained with DAPI (blue). (**a**) *H. chilense* chromosome 7H^{ch} disomic addition line. (**b**) *H. vulgare* chromosome 7H^{v} disomic addition line. (**c**) *H. chilense* chromosome 5H^{ch} disomic addition line. (**d**) *H. vulgare* chromosome 5H^{v} disomic addition line. Bar represents 10µm.

Similarly, wheat chromosomes associated correctly in bivalents at meiosis metaphase I and orientated by centromeres properly in double monosomic *H. chilense* and *H. vulgare* additions in wheat lines, both for the same and different homoeologous chromosomes (Figure 5). In contrast, *H. chilense* and *H. vulgare* chromosomes remained always un-associated in all the cells analysed for the three different genetic combinations analysed (Figure 5), despite the fact that homoeologous *H. chilense* and *H. vulgare* chromosomes (7H^{ch}7H^{v} and 5H^{ch}5H^{v}, respectively) did completely associate in pairs in early meiosis. These observations suggested that, although wild and cultivated barley homoeologous chromosomes can fully associate during pachytene, crossing over did not occur later between these chromosomes. Consequently, *Hordeum* homoeologous chromosomes were never observed associated by chiasmata during metaphase I and always remained as univalent (Figure 5), suggesting

other requirements for crossing over rather than full previous chromosome associations or similarities in the DNA sequence.

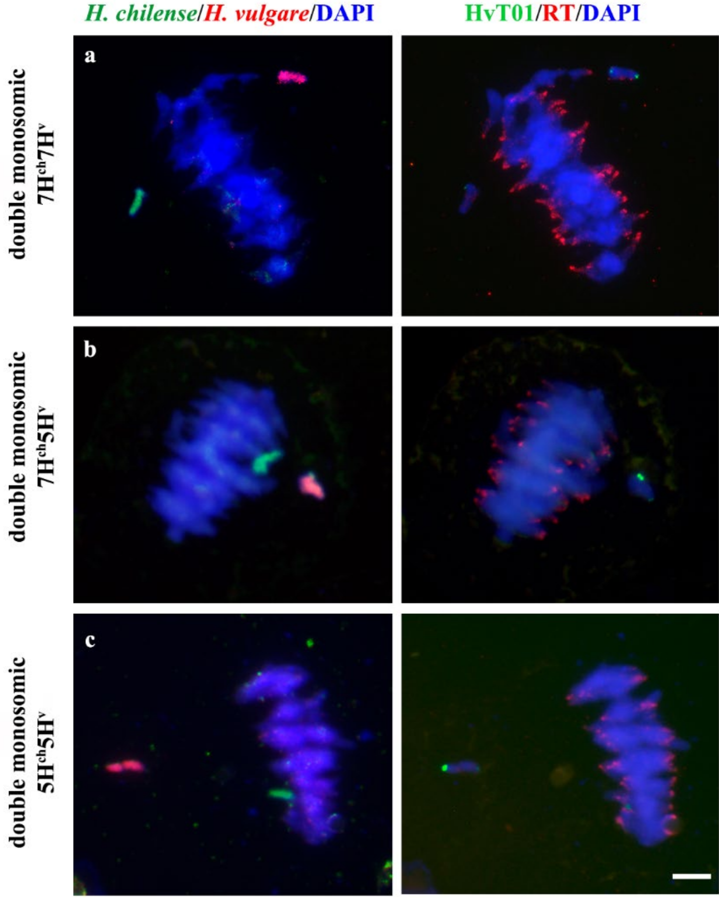


Figure 5. *Hordeum chilense* and *H. vulgare* chromosome behaviour in double monosomic barley addition lines in wheat during metaphase I. *Hordeum chilense* (green) and *H. vulgare* (red) chromosomes remained unassociated in all the cases. DNA was counterstained with DAPI (blue). Centromeres (red) were labelled with RT sequence to show the correct orientation of wheat chromosomes during metaphase I. Subtelomeres on barley chromosomes were labelled in green. (**a**) Double monosomic $7H^{ch}7H^{v}$ addition line. (**b**) Double monosomic $7H^{ch}5H^{v}$ addition line. (**c**) Double monosomic $5H^{ch}5H^{v}$ addition line. Bar represents 10µm.

Chromosome segregation does not depend on previous chromosome associations during early meiosis

Each PMC analysed at the MI stage was characterised by the presence of two barley univalents in double monosomic *H. chilense*-*H. vulgare* addition lines. Around 300 cells were observed in meiosis anaphase I. GISH analysis showed that both wild and cultivated barley univalents segregated simultaneously with wheat bivalents at stage anaphase I (Figure 6).

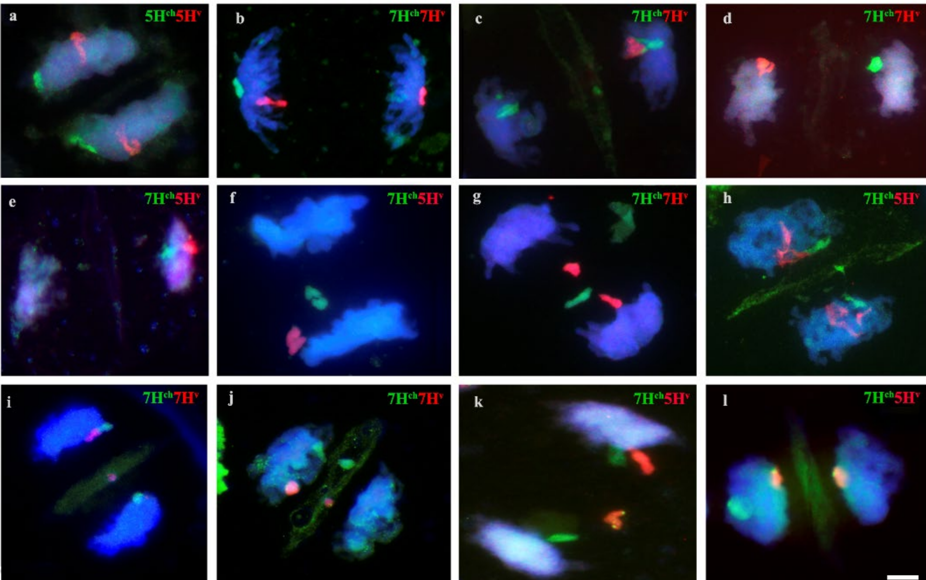


Figure 6. Behaviour of *Hordeum chilense* and *H. vulgare* chromosomes in double monosomic barley addition lines in wheat during anaphase I of meiosis. Examples of barley chromosome segregation after metaphase I. *Hordeum chilense* and *H. vulgare* were visualised in green and red, respectively. DNA was counterstained with DAPI (blue). (**a**) Double monosomic $5H^{ch}5H^{v}$ addition line, (**b**) Double monosomic $7H^{ch}7H^{v}$ addition line, (**c**) Double monosomic $7H^{ch}7H^{v}$ addition line; (**d**) Double monosomic $7H^{ch}7H^{v}$ addition line. (**e**) Double monosomic $7H^{ch}5H^{v}$ addition line. (**f**) Both $7H^{ch}5H^{v}$ barley chromosomes remained delayed. (**g**) Both $7H^{ch}7H^{v}$ barley chromosomes remained delayed and a misdivision of chromosome $7H^{v}$ was also observed. One or both barley micronuclei were positioned in the equatorial region on telophase I in (**h**) Double monosomic $7H^{ch}5H^{v}$ addition line, (**i**) Double monosomic $7H^{ch}7H^{v}$ addition line, and (**j**) Double monosomic $7H^{ch}7H^{v}$ addition line. The subtelomeric HvT01 probe was used in GISH experiments performed in cells in telophase I from the double monosomic $7H^{ch}5H^{v}$ addition line to visualise (**k**) $5H^{v}$ chromosome misdivision or (**l**) $5H^{v}$ chromosome segregation. Bar represents 10μm.

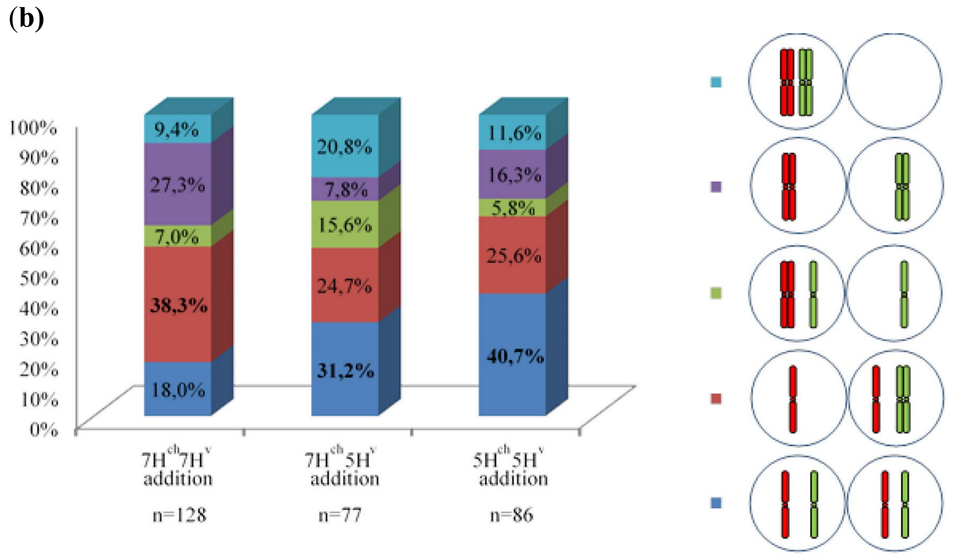
All the different possible situations for chromosome segregation of the unpaired *H. chilense* and *H. vulgare* chromosomes were identified (Figures 6a–e, Table 1): (i) both barley chromosome were detected in both nuclei; (ii) only *H. vulgare* chromosome was detected in both nuclei; (iii) only *H. chilense* chromosome was detected in both nuclei; (iv) each barley chromosome was detected in each daughter nucleus; and (v) both barley chromosomes were detected in the same anaphase/telophase pole. These different situations for chromosome segregation of both barley chromosomes were found in all the genetic combinations ($7H^{ch}7H^{v}$, $5H^{ch}5H^{v}$, and $7H^{ch}5H^{v}$ double monosomic addition lines) in the wheat background, although the ratio between them varied depending on the genetic stock (Table 1). Nevertheless, no significant differences were found for *H. chilense* and *H. vulgare* chromosome segregation between the different genetic combinations (Table 1), despite the fact that the most frequent observation for the segregation of the wild and cultivated barley chromosomes was different in $7H^{ch}7H^{v}$ addition line compared to $5H^{ch}5H^{v}$, and $7H^{ch}5H^{v}$ addition lines (Table 1). Moreover, no significant differences were found on the behaviour of the *Hordeum* chromosomes within the same F1 line. These results suggest that chromosome segregation in double *H. chilense* and *H. vulgare* monosomic addition lines in wheat occurred randomly, regardless the barley chromosomes added to the wheat background and whether or not chromosome associations took place previously in early meiosis.

Table 1. (a) Total number of PMCs scored at anaphase I showing the different combinations observed for both *Hordeum* chromosomes added to the wheat background. The most frequent observation per line and the total number of meiocytes examined are shown in bold. **(b)** Quantification of meiocytes (%) for each observation.

(a)

Wheat lines	**Anaphase I combinations scored in meiocytes**					
						Total no. of meiocytes
$7H^{ch}7H^{v}$ addition	23	**49**	9	35	12	128
$7H^{ch}5H^{v}$ addition	**24**	19	12	6	16	77
$5H^{ch}5H^{v}$ addition	**35**	22	5	14	10	86
Total no. of meiocytes	82	90	26	55	38	**291**

(b)



Chromosomes delay was usually observed in double monosomic *H. chilense* and *H. vulgare* addition lines in late anaphase I/telophase I (Figures 6f, g), and the presence of chromatin across the equator during phragmoplast formation either from *H. chilense*, *H. vulgare* or both species was also observed (Figures 6h–j). Missegregation or chromosome breaks that occurred in anaphase I in the double monosomic *H. chilense* and *H. vulgare* addition lines cannot be distinguished from sister chromatids segregation unless using, among others, the HvT01 subtelomeric probe (Figures 6k, l).

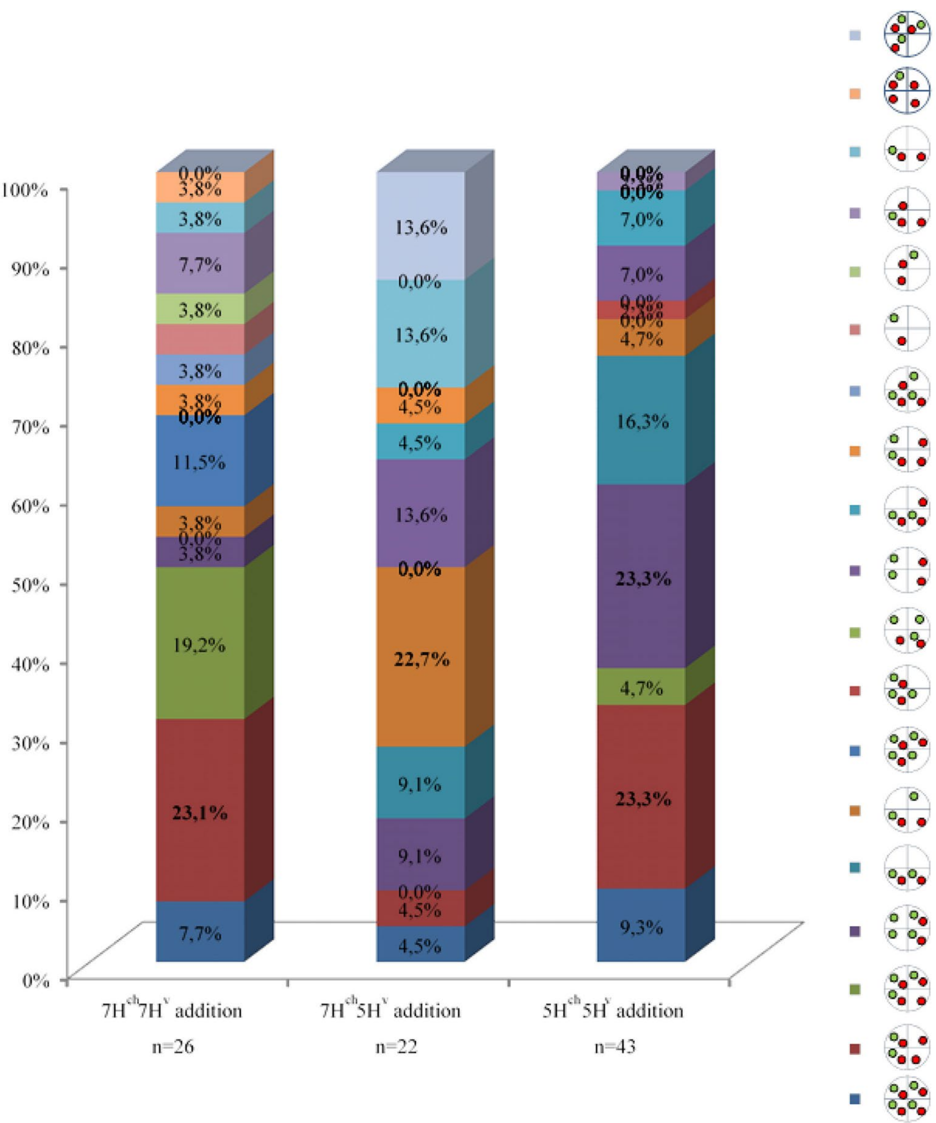
Depending on chromosome segregation of both wild and cultivated barley univalents during meiosis I, the number of different genetic combinations in the PMC increased during MII, resulting in a wide range of meiotic phenotypes observed (nineteen different cases; Table 2). The most frequent *H. chilense* and *H. vulgare* chromosome combination observed for each genetic combination during telophase II was different depending on whether *H. chilense* and *H. vulgare* chromosomes were included in the same or in different homoeology group (Figure 7; Table 2), but no significant differences were found. Results suggested that both sister chromatids separation and misdivision did occur randomly independently of the barley chromosome combination.

Table 2. (a) Total number of meiocytes showing different tetrad combinations of *Hordeum* sp. addition lines in bread wheat. **(b)** Quantification of meiocytes (%) displaying distinct chromosomal behaviour in the different lines during tetrads in meiosis. "n" signifies (or indicates) number of meiocytes examined in every addition line and the predominant combinations are shown in bold.

(a)

Wheat lines	**Tetrad combinations scored in meiocytes**																		
																			Total no. of meiocytes
$7H^{ch}7H^{v}$ addition	2	**6**	5	1	0	1	3	0	0	0	0	1	1	1	1	2	1	1	0	26
$7H^{ch}5H^{v}$ addition	1	1	0	2	2	**5**	0	0	0	3	1	1	0	0	0	0	3	0	3	22
$5H^{ch}5H^{v}$ addition	4	**10**	2	**10**	7	2	0	1	0	3	3	0	0	0	0	1	0	0	0	43
Total no. of meiocytes	7	17	7	13	9	8	3	1	0	6	4	2	1	1	1	3	4	1	3	**91**

(b)



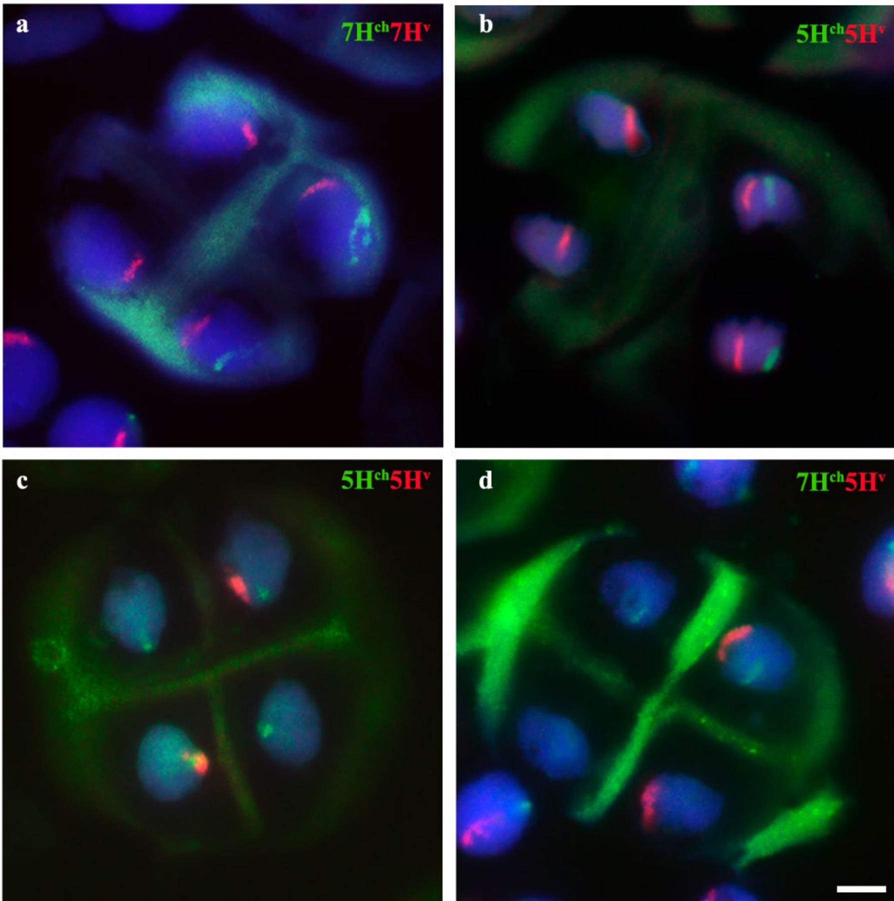


Figure 7. Behaviour of *H. chilense* and *H. vulgare* chromosomes in double monosomic *Hordeum* addition lines in wheat after the second meiosis division. Examples of the most frequent observations for both *H. chilense* (green) and *H. vulgare* (red) chromosomes are shown. DNA was counterstained with DAPI (blue). (**a**) *Hordeum vulgare* and *H. chilense* chromatin detected in four and two PMCs, respectively, in the double monosomic $7H^{ch}7H^{v}$ wheat line. (**b**) *Hordeum vulgare* and *H. chilense* chromatin detected in four and in two PMCs, respectively, in the double monosomic $5H^{ch}5H^{v}$ wheat line. (**c**) Four *H. chilense* signals and two *H. vulgare* signals were observed in the double monosomic $5H^{ch}5H^{v}$ wheat line at the same frequency as reported in b. (**d**) Wild and cultivated barley signals are present in the same nucleus and only one each in another two different nucleuses. Bar represents 10µm.

Discussion

Little is known about how chromosomes recognise each other to correctly associate in pairs at early meiosis and recombine. This is a key question for plant breeders to transfer genetic variability from related species into a crop like wheat. The lack of recombination between cultivated wheat and alien chromosomes limits the transfer of novel traits from relatives to wheat because *Ph1* suppresses homoeologous recombination between wheat and related species (Riley and Chapman, 1958; Sears, 1976). Different meiosis studies on chromosome pairing have been developed using wheat lines carrying an addition of one pair of homologous chromosomes or chromosome segments from one related species into wheat (Mikhailova *et al.*, 1998; Maestra *et al.*, 2002; Prieto *et al.*, 2004b) or hybrids between wheat and relatives (Molnár-Láng *et al.*, 2014; Rey *et al.*, 2017). In this work, we have developed wheat lines carrying double monosomic chromosome additions for wild and cultivated barley for the same and for different homoeology group, respectively, which allowed us to track simultaneously by GISH a couple of extra homoeologous and non-homoeologous chromosomes from two different *Hordeum* species during early meiosis. These double monosomic addition lines can contribute to go deeper into the knowledge of how chromosomes recognise and associate in pairs in the wheat background in the presence of the *Ph1* locus. In addition, most of the works carrying alien chromosomes in the wheat background are focused in meiosis metaphase I or later stages (Molnar-Lang *et al.*, 2000; Silkova *et al.*, 2014). The analysis of chromosome pairing focused only in metaphase I can result in an underestimation of homoeologous associations that might occur earlier in meiosis, as chromosomes might remain mostly as univalents due to the lack of homoeologous recombination. Few works analysed the behaviour of an extra pair of chromosomes at early stages of meiosis (Aragón-Alcaide *et al.*, 1997; Prieto *et al.*, 2004b; Valenzuela *et al.*, 2012, 2013; Koo *et al.*, 2016). Homologous barley chromosomes have been

previously observed associated during early meiosis and metaphase I in disomic addition lines in wheat (Aragón-Alcaide *et al.*, 1997; Calderón *et al.*, 2014). In this study we have reported by GISH analysis that homoeologous wild and cultivated barley chromosomes can also fully associate in pairs in the wheat background during early meiosis and that such chromosome pairing occurred even in the presence of the *Ph1* locus, although homoeologous barley chromosomes did not cross over and were always observed as univalent in metaphase I. The role of the *Ph1* locus was recently narrowed down preventing recombination between related chromosomes in interspecific hybrids (Moore, 2014; Martín *et al.*, 2017). Our results clearly showed that the *Ph1* locus does not hamper homoeologous chromosome associations but crossing over. Nevertheless, homoeologous recombination between related *Aegilops geniculata* and *Ae. searsii* has been detected in the wheat background in the presence of the *Ph1* locus due to the presence of chromosome 5Mg of *Ae. geniculata*, which harbours a homoeologous recombination promoter factor (Koo *et al.*, 2016). Recombination frequencies between *H. vulgare* and *H. bulbosum* homoeologues have been previously detected but are lower than association frequencies (Zhang *et al.*, 1999), probably because of non-chiasmate associations (Orellana, 1985). Homologous pairing has been also described in the absence of synapsis and meiotic recombination in *Caenorhabditis elegans* (Dernburg *et al.*, 1998). Our results showed that homoeologous *H. chilense* and *H. vulgare* chromosomes associated in pairs in wheat in the absence of crossing over and that the *Ph1* locus does not prevent such chromosome recognition and association between homoeologues. It is also worthy to mention that, although *H. chilense* and *H. vulgare* are phylogenetically quite distant, even included in two different sections among the *Hordeum* genus (Blattner, 2009), both species share a high degree of similarities at the chromosomal level, as it has been reported between them and other species within this genus (Hernández *et al.*, 2001; Aliyeva-Schnorr *et al.*, 2016). Thus, other elements such as cohesins or the DNA sequence itself might

play a major role on chromosome recognition and pairing at the onset of meiosis in a polyploidy like wheat.

So far, it is unclear whether initial recognition is mediated through protein-protein interactions, DNA base-pairing, or other chromosomal features. For example, a non-coding RNA (meiRNA-L) is responsible for the recombination-independent pairing of homologous loci in *Schizosaccharomyces pombe* (Ding *et al.*, 2012). Other chromosomal features different from DNA/DNA recombinational interactions or RNA-mediated pairing have been proposed to be involved in the homologous recognition such as the pattern of cohesins distribution in the axial elements of unmatched meiotic chromosomes in mice and *S. pombe* (Ishiguro *et al.*, 2011; Ding *et al.*, 2016). Subtelomeres have been also reported as crucial to promote chromosome recognition and pairing between homologous chromosomes (Gonzalez-Garcia *et al.*, 2006; Calderón *et al.*, 2014). In our study, variability for the HvT01 subtelomeric sequence was found between *H. chilense* and *H. vulgare* chromosomes 5 and 7, particularly for the long arm of both chromosomes. We observed that although homoeologous chromosomes can potentially associate by the telomeres, subtelomeric DNA blocks might hamper homoeologous chromosome to correctly associate in pairs and thus, in the absence of homologs, chromosome recognition and association between homoeologues can occurred by the chromosome end where the subtelomeric repeats are shorter or absent. However, as meiosis progressed, the pairing signal initiated at these chromosome ends can be propagated along the whole chromosome, so that the homoeologues became fully associated by late pachytene. Thus, our results might suggest that subtelomeres can play a key role in the specificity of chromosome recognition, restricting chromosome recognition to true homologs and therefore hampering homoeologous chromosomes to recognise each other and associate. The implication was that DNA sequence(s) within the subtelomeric region must be important for the process of initial homolog

recognition and pairing, although further studies are required to reveal how subtelomeres take part in such important meiosis processes.

The peculiarities of univalent behaviour in meiosis have been extensively studied in wheat aneuploids, particularly for the relation between the means of a chromosome segregation and its inclusion into a microspore (Sears, 1952; Marais and Marais, 1994; Friebe *et al.*, 2005; Lukaszewski, 2010). The knowledge about univalent behaviour in meiosis is necessary for the directed development of wheat lines carrying alien introgressions since univalents are subjected of incorrect division and segregation. Thus, abnormities in meiosis result in various modifications and/or in the loss of a transferred chromosome (Silkova *et al.*, 2014). Univalents in meiosis have a tendency to misdivide (break) across their centromeres producing telocentric. This process has been deeply described in wheat (Sears, 1952; Steinitz-Sears, 1966; Friebe *et al.*, 2005), and used to generate different cytogenetic stocks (Sears, E. R., and Sears, 1978; Lukaszewski, 1993, 1997). The most common alien introgression in wheat, chromosome translocations, is the result of centric misdivision and fusion of misdivision products. Translocations between *H. chilense* and *H. vulgare* have been detected previously when the genomes of these species are in the same background (Prieto *et al.*, 2001). Our results overview the univalent behaviour of two homoeologous and non-homoeologous barley chromosomes in the wheat background. We observed that chromosome misdivisions and sister chromatids segregated randomly at anaphase I similarly to previous works (Friebe *et al.*, 2005), and independently of whether or not related chromosomes associate in pairs in early meiosis.

In summary, homoeologous wild and cultivated barley chromosomes were observed fully associated in pairs in early meiosis in the presence of the *Ph1* although crossing over did not occur at any time, as both chromosomes were always visualised as univalents during metaphase I. Whether or not homoeologous *Hordeum* chromosomes can crossover in the absence of the *Ph1* locus remains to be elucidated. In addition, the role of the terminal

chromosome regions in chromosome recognition and paring and the proteins interacting with these chromosomes ends will be key questions to shed light in future works.

Human and animal rights and informed consent

This article does not contain any studies with human participants or animals.

Authors contributions

All authors contributed to this manuscript. MC and PP designed the research and performed the experiments. MC, MR, AM, and PP analysed, discussed the results. PP and MC wrote the manuscript. All authors read and approved the manuscript.

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Chapter IV

Cultivated and wild homoeologous barley chromosomes can associate and recombine in the wheat background in the absence of the *Ph1* locus

In preparation (for Agronomy Journal):

Calderón MC, Prieto P. Cultivated and wild homoeologous barley chromosomes can associate and recombine in the wheat background in the absence of the *Ph1* locus.

Abstract

Bread wheat is an allohexaploid that behaves as a diploid during meiosis, the cell division process occurring in organisms with sexual reproduction to generate the gametes. The knowledge of the mechanisms implicated in meiosis, is an essential tool that can help in breeding programs to transfer desirable traits from related species into a crop like wheat. It is particularly interesting to shed light into the mechanisms controlling correct pairing between homologous (equivalent) chromosomes and recombination, even more in polyploid species like wheat. The locus *Ph1* is implicated in these processes. In this work, we aimed to study the behaviour during meiosis of *Hordeum chilense* and *H. vulgare* chromosomes 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 in wheat in the *ph1b* mutant background. These wheat lines carry one *H. chilense* and one *H. vulgare* chromosomes for the same and for different homoeology group in the *ph1b* mutant background. Using genomic *in situ* hybridisation we have visualised the two (wild and cultivated) barley chromosomes during meiosis and we have 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 recombination. Results are discussed here.

Introduction

Wheat is one the oldest crops in the world. Its cultivation dates back 10,000 years old during the 'Neolithic Revolution' and it is considered one of the main reasons for which humans transformed from hunter-gatherer nomad to settled agriculturalist (Shewry, 2009). Due to easy seed storage for a long period of time, wheat promoted the development of cultural achievement since it was not necessary to seek food every day (Shewry, 2009). Nowadays, wheat is the third

most produced cereal in the world (734 million tons), behind maize (1.1 million tons) and rice (782 million tons) (data taken from 2018; http://faostat3.fao.org/). Every year, this production has to increase on account of the growing human population, therefore wheat crops have to be more productive and better adapted to specific agro-climatic conditions (Godfray *et al.*, 2010). Plant breeders are playing an essential role to exploit the existing genetic variability in the development of new varieties better adapted to the environment, as well as trying to understand gene functions and interactions with the aim of increasing quality and productivity of wheat crops. In plant breeding, wide-crossing is an important tool in and sometimes the results are the starting point for new crops. Thus, examples of new plant species carried out in the Triticeae tribe including wheat are ×*Triticosecale*, obtained after crossing wheat and rye, or ×*Tritordeum*, an amphyploid between the wild barley *H. chilense* Roem. et Schult. and wheat (O'mara, 1953; Martín and Chapman, 1977). Hence, these 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, improving the quality or carrying resistance to diseases (Lukaszewski, 2000; Friebe *et al.*, 1996; Calderón *et al.*, 2012; Rey *et al.*, 2015a).

The search for advantageous genes to improve the nutritional quality and increase the total yield potential of cereals and other crops using related species is based on interspecific hybridisation of many cultivars with their wild relatives (Bates and Deyoe, 1973). But in wheat, hybridisation between wheat chromosomes and those from the wild related species produces only a low level of chromosome pairing and recombination, increasing the difficulty of transferring desirable traits from the related species into wheat. Understanding wheat genetics and its genome organisation seems to be essential for plant breeding purposes.

Bread wheat is an allopolyploid with three homoeologous (related) sets of seven pairs of chromosomes in each subgenome (A, B and D). Nevertheless,

hexaploid wheat behaves as a diploid and chromosomes associate regularly in pairs during meiosis, being homoeologous pairing is prevented through the action of *Ph* (*Pairing homologous*) genes (Martinez-Perez *et al.*, 2001), although the major effect is due to the *Ph1* locus, located on the short arm of chromosome 5B (Sears, 1976). Deletion of the *Ph1* locus allows homoeologues (chromosomes from related genomes but not completely homologous) to pair relatively freely with one another (Moore, 2014). In fact, the *Ph1* locus has been studied for decades and was initially described controlling homologous pairing in bread wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Sears, 1977) although the molecular mechanisms behind these observations are still unclear. More recently, other functions on recombination have raised (Martín *et al.*, 2017). Other loci are implicated in chromosome associations during meiosis, such as the *Ph2* locus, which is located on the short arm of chromosome 3D and has a weaker effect than the *Ph1* locus (Mello-Sampayo, 1971), and the *Ph3* locus, which is located on the short arm of chromosome 3A and is the weaker regulator (Driscoll, 1972).

Precise control of chromosome pairing is crucial for conferring meiotic regularity, and hence reproductive stability in allopolyploids (Jenczewski and Alix, 2004). This big influence on the fertility is crucial for success in breeding, but in contrast, the negative effect in plant breeding since it avoids pairing and recombination between wheat chromosomes and those from related species, need to be unzipped. Therefore, it seems reasonable to go deeper into the knowledge of chromosome associations during meiosis in wheat, contributing to develop valuable tools for wheat breeding (Calderón *et al.*, 2018).

Several studies in plants 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 shed light on chromosome dynamics in a small-genome plant *A. thaliana* as well as in plants with large and complex genomes of polyploidy such as wheat and maize (Pawlowski, 2010).

In wheat, and the onset of meiosis, homologous chromosomes (equivalent chromosomes from the same subgenome) are sorted in pairs and are intimately aligned, or synapsed, along their lengths while a proteinaceous structure, the synaptonemal complex (SC), is assembled between them (Greer *et al.*, 2012). 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 (Prieto *et al.*, 2004b; Naranjo and Corredor, 2008).

When a chromosome recognises its homolog and not another chromosome in wheat, a localised conformational change in adjacent chromatin is triggered in both partners, facilitating recognition and pairing of homologous versus homoeologous chromosomes, being this process also under the effect of the *Ph1* locus (Prieto *et al.*, 2005; Greer *et al.*, 2012). In wheat, the *Ph1* gene also suppresses recombination between homoeologous chromosomes (Okamoto, 1957; Riley and Chapman, 1958, 1964; Sears and Okamoto, 1958; Riley and Kempanna, 1963; Martín *et al.*, 2017; Rey *et al.*, 2017) although it does not prevent chromosome associations between homoeologues (Calderón *et al.*, 2018).

The development of alien chromosome additions has an important utility not only for breeding but also in plant genetic studies. The specific genetic and cytogenetic properties of chromosome introgressions into a crop make these lines powerful tools for fundamental research, contributing to elucidate the processes of homoeologous recombination, distribution of chromosome-specific markers and repetitive DNA sequences, and regulation of heterologous gene expression (Chang and de Jong, 2005). Sets of both cultivated (*H. vulgare*) and wild (*H. chilense*) barley addition lines in a hexaploid wheat background were developed (Islam *et al.*, 1978, 1981; Miller *et al.*, 1982) and have an enormous potential in plant meiosis studies. These lines let a search of one specific pair of chromosomes or just a chromosome section within the wheat background using genomic *in situ* hybridisation (GISH), thus

rearrangements and associations in a pair of homologs can be studied uniquely (Naranjo *et al*., 2010; Rey *et al*., 2015b).

In this study, we have 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 to go deeper into the knowledge of chromosome associations during meiosis. These double monosomic addition lines allow the visualisation of chromosomes from two different barley species in wheat in the *ph1b* mutant background and analyse whether *Ph1* locus might play a role on chromosome recognition/pairing at early meiosis in the absence of homologues. Results showed that the *Ph1* locus does not impede homoeologous recognition and pairing but recombination during meiosis, which only occur in the absence of the *Ph1* locus. Results are discussed in this work.

Materials and methods

Plant material

In this work we used *H. vulgare* and *H. chilense* monosomic addition lines in bread *ph1b* mutant wheat background (*T. aestivum* cv. Chinese Spring *ph1b* mutant; AABBDD*ph1bph1b* + H^vH^v and AABBDD *ph1bph1b* + $H^{ch}H^{ch}$, respectively), developed previously in our lab. These lines were allowed to self-pollinate to obtain *H. chilense* and *H. vulgare* disomic addition lines in the wheat *ph1b* mutant background and 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 locus *Ph1* (Calderón *et al.*, 2018) were also used.

Seeds were germinated on wet filter paper in the dark for 5 days at 4°C, followed by a period of 24h at 25°C. Emerging seedling roots were cut,

incubated for 4h in 0.05% w/v colchicine at 25ºC, fixed in Carnoy´s solution (three parts 100% ethanol plus, one-part glacial acetic acid) and stored at 4ºC until their use. Plants were then growth in the greenhouse at 26°C during the day and 18°C during the night (16 h photoperiod). Immature spikes were collected when plants were at the reproductive stage and were also preserved in Carnoy´s solution and used to characterise chromosome pairing at meiosis I.

DNA characterisation

Genomic DNA was extracted from frozen seedling leaves (Murray and Thompson, 1980) with some modifications (Hernández *et al.*, 2001). The absence of *Ph1* locus in the lines used in this work was verified using the ABC920 SCAR marker (Wang *et al.*, 2002). The presence of each *Hordeum sp.* chromosome in original, self-pollinated F1 plants (*ph1b* and *Ph1* background) was confirmed by both PCR assays previously described (Liu *et al.*, 1996; Hagras *et al.*, 2005) and *in situ* hybridisation.

Fluorescence *in situ* hybridisation

Associations between *H. vulgare* and *H. chilense* chromosomes in the *ph1b* mutant wheat background were study using fluorescence genomic *in situ* hybridisation (GISH) (Prieto *et al.*, 2004a). Root tips and spikes in meiosis were collected and pre-treated as described previously (Calderón *et al.*, 2018). Chromosomes spreads were arranged from both root tips cells and pollen mother cells (PMCs) at meiosis. Root tips and anthers were macerated as described (Calderón *et al.*, 2018). The genomic DNA extracted from *H. vulgare* and *H. chilense* seedling leaves was the probe used for GISH. The DNA was labelled by nick translation with either biotin-11 for *H. vulgare* (Boehringer Mannheim Biochemicals, Germany) or digoxigenin-11-dUTP for *H. chilense* (Roche Applied Science, Indianapolis, IN, USA). The GISH protocol was performed according to Calderón *et al*. (2018).

Fluorescence microscopy and image processing

Hybridisation signals were visualised 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, California, USA).

Results

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 $7H^{v}$ previously developed in our lab in the absence of the *Ph1* locus (Rey *et al.*, 2015b). 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* mutation (Liu *et al*., 1996; Hagras *et al*., 2005; Wang *et al*., 2002). Those plants carrying either the *H. chilense* or *H. vulgare* chromosomes in the absence of the *Ph1* locus were selected and analysed by GISH, as described previously (Prieto *et al.*, 2004b), to identify disomic addition lines for each $5H^{ch}$, $7H^{ch}$ and $7H^{v}$ chromosomes. *In situ* hybridisation 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 GISH

experiments, these $5H^{ch}$, $7H^{ch}$ and $7H^{v}$ disomic addition lines in the *ph1b* mutant background were cultivated to obtain grain. 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.

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 the wheat *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* mutation (Liu *et al.*, 1996; Hagras *et al.*, 2005; Wang *et al.*, 2002). 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 $7H^{v}L$ chromosome. Thus, we only obtain *H. chilense* and *H. vulgare* double monosomic addition lines carrying the whole $5H^{ch}$ or $7H^{ch}$ *H. chilense* chromosome and the telosomic $7H^{v}L$ chromosome. Thus 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. These double monosomic wild and barley addition lines in wheat in the *ph1b* mutant background were selected to study chromosome associations in the absence of the *Ph1* locus. Thus, plant lines were cultivated until meiosis to analyse *H. chilense* and *H. vulgare* chromosome associations for both the same homoeology group ($7H^{v}L7H^{ch}$) and for different homoeology group ($7H^{v}L5H^{ch}$) 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 prepared as previously described (Calderón *et al.*, 2018; Prieto and Naranjo, 2020).

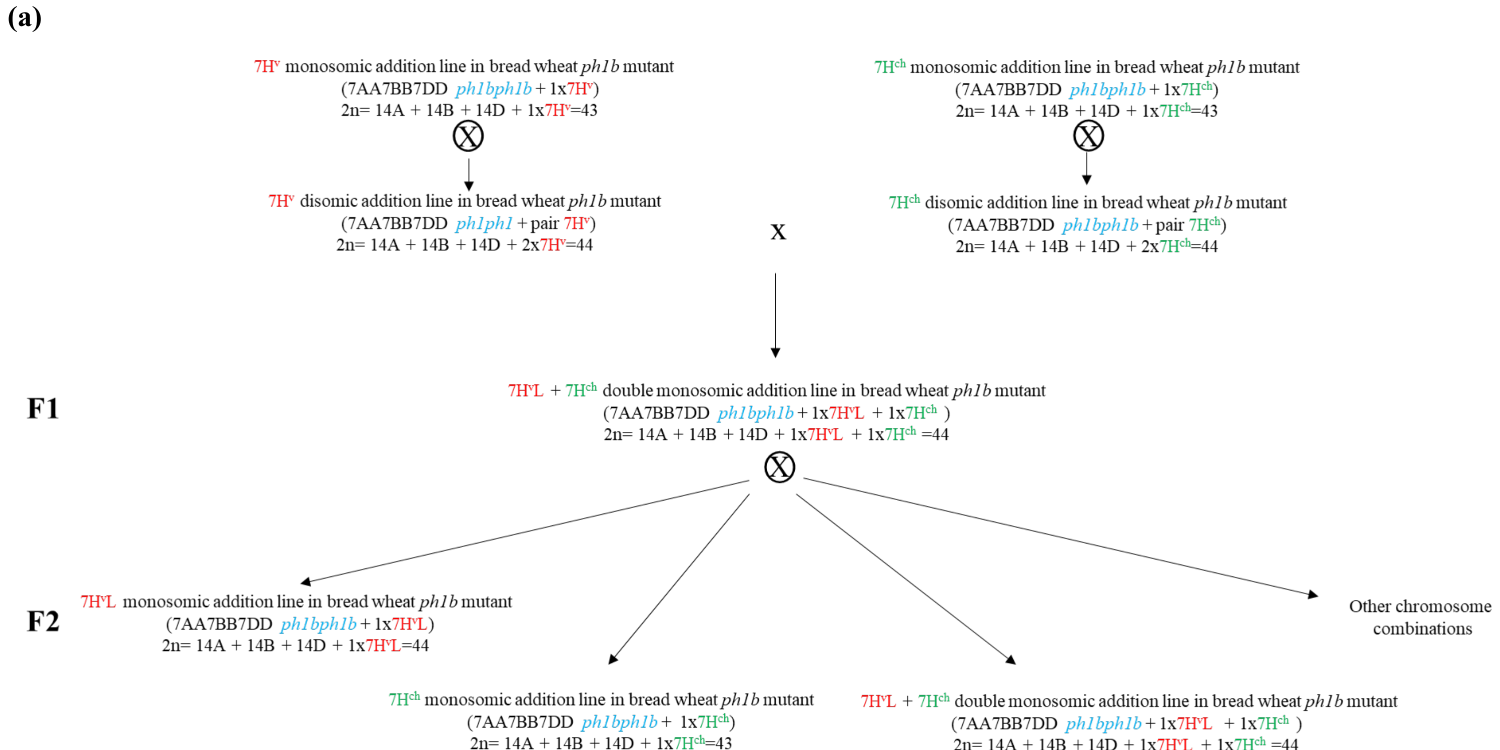
Table 1. (a) Plants used for crosses to engineer lines carrying a *Hordeum* disomic addition lines in a *ph1b* mutant background. **(b)** Plants obtained after the crosses of disomic addition lines.

(a)

parental lines *ph1bphb1*			**self-pollinated progeny *ph1bph1b***		
$7H^{v}$ 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 *ph1bph1b*		**F2 progeny *ph1bph1b***			
$7H^{v}L5H^{ch}$ 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



(a)
7H^v monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^v)
2n= 14A + 14B + 14D + 1x7H^v=43
7H^v disomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1ph1 + pair 7H^v)
2n= 14A + 14B + 14D + 2x7H^v=44
X
7H^ch monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^ch)
2n= 14A + 14B + 14D + 1x7H^ch=43
7H^ch disomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + pair 7H^ch)
2n= 14A + 14B + 14D + 2x7H^ch=44
F1
7H^vL + 7H^ch double monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^vL + 1x7H^ch)
2n= 14A + 14B + 14D + 1x7H^vL + 1x7H^ch =44
F2
7H^vL monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^vL)
2n= 14A + 14B + 14D + 1x7H^vL=44
7H^ch monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^ch)
2n= 14A + 14B + 14D + 1x7H^ch=43
7H^vL + 7H^ch double monosomic addition line in bread wheat ph1b mutant
(7AA7BB7DD ph1bph1b + 1x7H^vL + 1x7H^ch)
2n= 14A + 14B + 14D + 1x7H^vL + 1x7H^ch =44
Other chromosome combinations

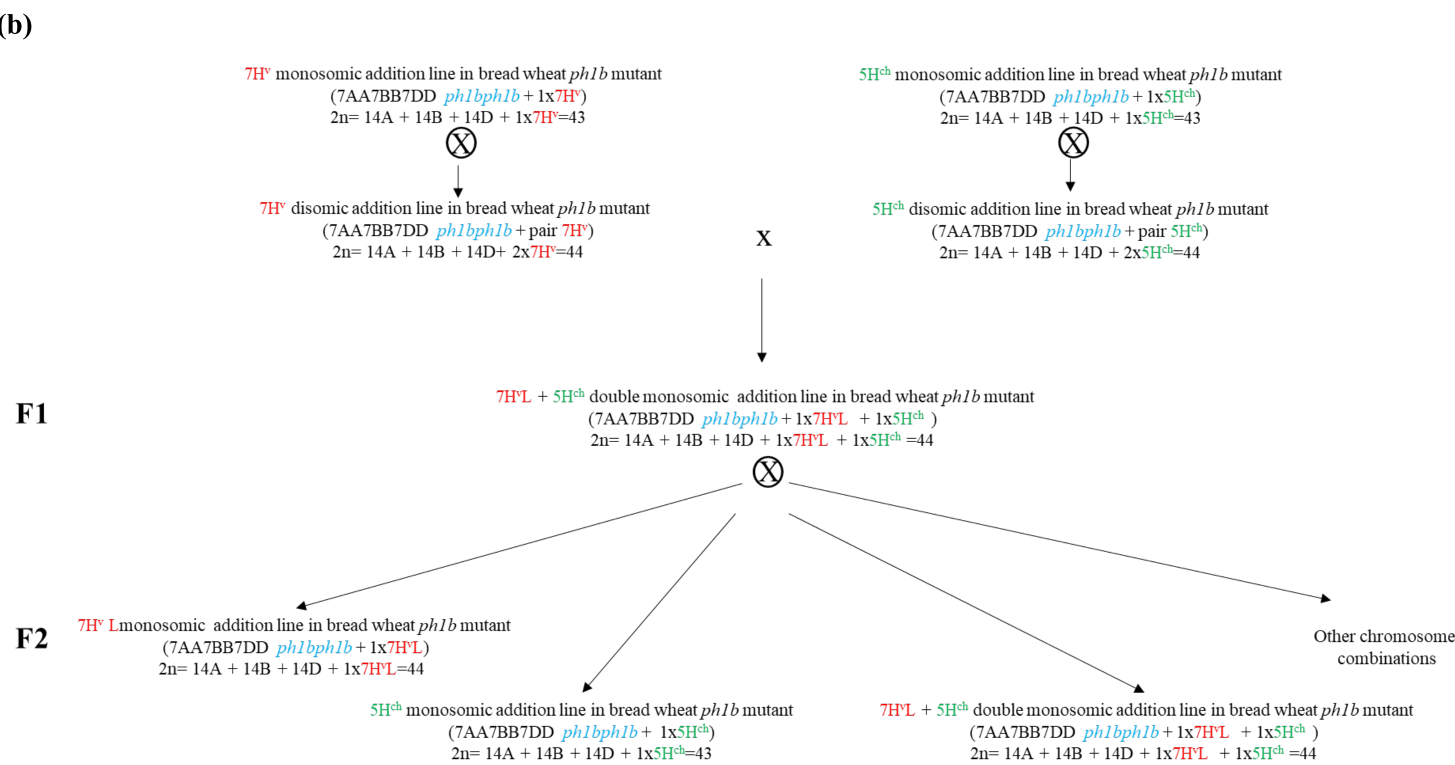


Figure 1. Development of double monosomic addition lines in wheat *ph1b* mutant background. **(a)** After backcrosses of $7H^{v}$ and $7H^{ch}$ *ph1b* monosomic addition lines of to obtain the disomic addition lines, crosses between both lines were developed to obtain the $7H^{v}L7H^{ch}$ double monosomic lines. **(b)** Process developed to obtain the $7H^{v}L5H^{ch}$ double monosomic lines in the *ph1b* background.

Homoeologous and non-homoeologous chromosome associations between *H. vulgare* and *H. chilense* are allowed during early meiosis in wheat in the *ph1b* mutant background

Chromosome associations in double monosomic addition lines carrying one copy of *H. vulgare* and one copy of *H. chilense* chromosomes for the same ($7H^vL$ and $7H^{ch}$) and for different ($7H^vL$ and $5H^{ch}$) homoeology group were studied in pachytene 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 group in the presence of the *Ph1* locus, were also growth and analysed by *in situ* hybridisation as control plants to study the effect of the *Ph1* locus on chromosome associations in meiosis. In these lines in the presence of the *Ph1* locus, *H. vulgare* chromosome $7H^v$ was complete.

In situ hybridisation experiments in meiosis were developed in more than 250 PMCs of each genomic combination ($7H^vL7H^{ch}$ and $7H^vL5H^{ch}$) in the *ph1b* mutant wheat background and results were visualised 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 phase in both wheat backgrounds, in the presence and in the absence of the *Ph1* locus (Figure 2a, c). In contrast, non-homoeologous *Hordeum* chromosomes $7H^v$ 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 (Calderón *et al.*, 2018). However, we visualised some minor and punctual chromosome associations between $7H^vL$ and $5H^{ch}$ homoeologous chromosomes in 10.89% of the PMCs during pachytene in the *ph1b* mutant background (Figure 2d), involving a small chromosome portion of both homoeologues. These observations suggested that chromosome pairing between non-homoeologous chromosomes from two different *Hordeum* species could be also allowed in the absence of the *Ph1* locus. Thus, these results suggested that non-homoeologous barley chromosomes can recognise each other by specific equivalent chromosome

regions and trigger a conformational change in the chromatin to associate in pairs independently of the *Ph1* locus.

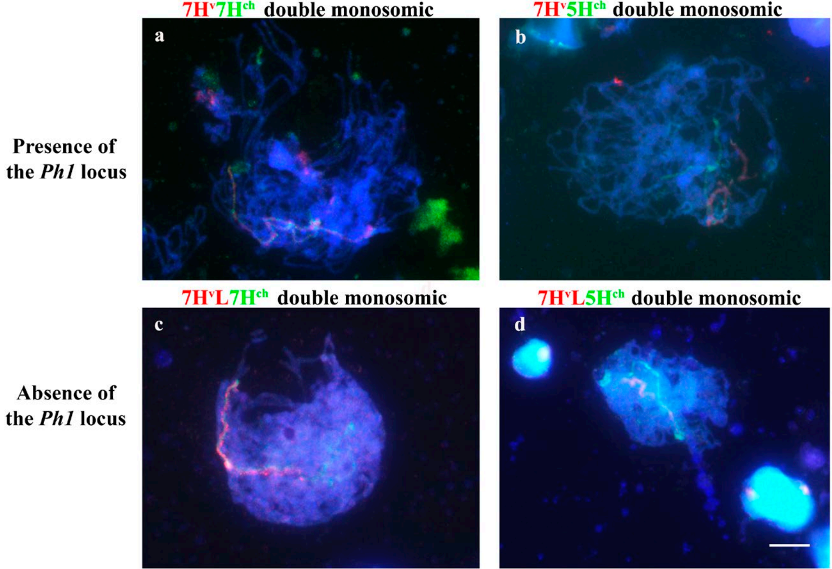


Figure 2. Behaviour of homoeologous and non-homoeologous barley chromosomes during early meiosis in the *Ph1* and *ph1b* wheat background. *H. vulgare* (red) and *H. chilense* (green). DNA was counterstained with DAPI (blue). **(a)** 7H^{v}7H^{ch} double monosomic addition line showing both barley chromosomes associated at early pachytene. **(b)** 7H^{v}5H^{ch} double monosomic addition line showing non-homoeologous chromosomes associated. **(c)** 7H^{v}L7H^{ch} double monosomic addition mutant line showing both barley chromosomes associated at early pachytene. **(d)** 7H^{v}L5H^{ch} double monosomic addition mutant line showing both barley chromosomes associated at early pachytene. Bar: 10µm.

Recombination can occur indistinctly between wild barley, cultivated barley and wheat 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, we focused to study whether or not these chromosome associations could allow overcrossing and recombination events. Thus, we analysed chromosome associations in metaphase I in the *ph1b* mutant background in PMCs from *H. vulgare* and *H. chilense* double monosomic addition lines in wheat for the same and different homoeologous group. 7H^{v}L7H^{ch} and 7H^{v}L5H^{ch} double

monosomic addition lines in the presence and in the absence 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 it was previously described (Calderón *et al.*, 2018). 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 group (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 (Fig 3c-h), as well as double *H. chilense*-wheat and *H. vulgare*-wheat associations (Fig 3i, j) and tripartite *H. vulgare-H. chilense*-wheat associations (Fig 3k, l) in both, $7H^{v}L7H^{ch}$ and $7H^{v}L5H^{ch}$ double monosomic addition lines in the *ph1b* mutant background. The most frequent association in both wheat lines carrying homoeologous ($7H^{v}L7H^{ch}$) and non-homoeologous chromosomes ($7H^{v}L5H^{ch}$) 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 $7H^{v}L$ and $7H^{ch}$ chromosomes, which were more frequent than associations occurring between $7H^{v}L$ and $5H^{ch}$ chromosomes. The *H. vulgare-H. chilense*-wheat tripartite chromosomes association was the least frequent in both lines carrying $7H^{v}7LH^{ch}$ and $7H^{v}L5H^{ch}$ chromosomes in the *ph1b* background (0.47% and 0.33%, respectively) (Table 2). These observations suggested that, although homoeologous chromosomes $7H^{v}L$ 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 metaphase I in the absence of the *Ph1* locus. In addition, chromosome associations can occur between non-homoeologues in some similar chromosome regions, but 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 and non-homoeologous chromosome associations in wheat but recombination, because no recombination events were detected between *H. chilense* and *H. vulgare* in any case in the presence of the *Ph1* locus.

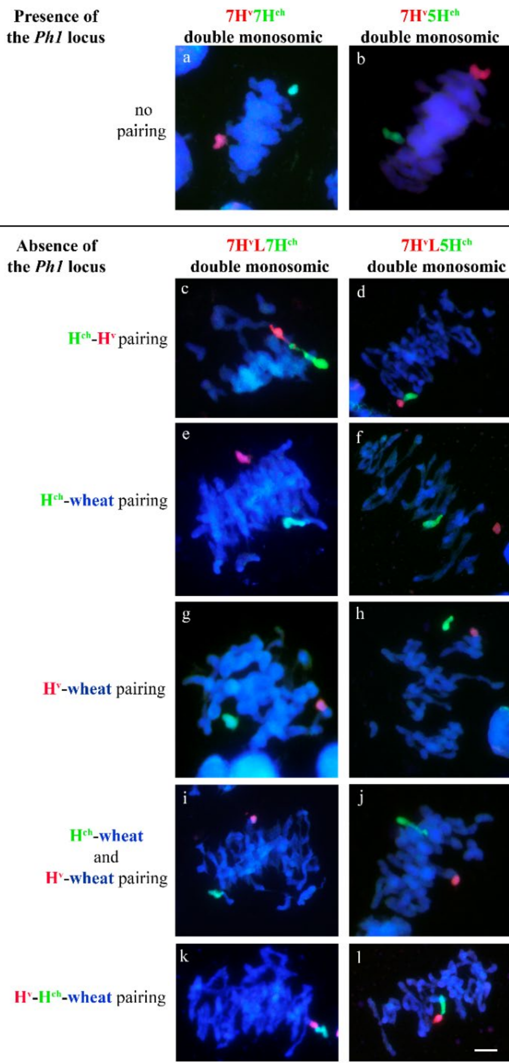


Figure 3. Behaviour of *H. vulgare* and *H. chilense* chromosomes in double monosomic addition lines in presence and absence of *Ph1* locus during metaphase I of meiosis. Examples of different pairing observed. *Hordeum chilense* and *H. vulgare* were visualised in green and red, respectively. DNA was counterstained with DAPI (blue). **(a, b)** $7H^{v}7H^{ch}$ and $7H^{v}5H^{ch}$ double monosomic addition line showing non-association in presence of *Ph1* locus. **(c, d)** H^{ch}-H^{v} pairing in both *ph1b* mutant lines. **(e, f)** H^{ch}-wheat pairing in the mutant lines. **(g, h)** H^{v}-wheat pairing in absence of *Ph1* locus. **(i, j)** Double H^{ch}-wheat and H^{v}-wheat pairing. **(k, l)** Tripartite pairing in both lines in absence of *Ph1* locus. Bar: 10µm.

Table 2. The frequency of pairing between *H. vulgare* and *H. chilense* and wheat chromosomes in the double monosomic addition lines in *Ph1* and *ph1b* background at metaphase I.

wheat Line	Number of plants analysed in metaphase	Number of PMCs scored in metaphase	Number (and frequency) of PMCs carrying chromosome associations scored in metaphase I					
			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	total pairing
$7H^v7H^{ch}$ double monosomic *Ph1Ph1*	2	107	0	0	0	0	0	0
$7H^v5H^{ch}$ double monosomic *Ph1Ph1*	2	159	0	0	0	0	0	0
$7H^vL7H^{ch}$ double monosomic *ph1bph1b*	2	211	**44(20.85%)**	18(8.53%)	18(8.53%)	4(1.89%)	**1(0.47%)**	85(40.27%)
$7H^vL5H^{ch}$ double monosomic *ph1bph1b*	2	303	**22(7.26%)**	7(2.31%)	8(2.64%)	2(0.66%)	**1(0.33%)**	40(13.20%)

Segregation of the inter-specific chromosome associations occurred randomly in the absence of the *Ph1* locus

Chromosome reorganisations 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 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 visualised in anaphase I/telophase I, corresponding to the previous observations in metaphase I (Figure 4a-h). When chromosomes $7H^vL$ and $7H^{ch}$ did not associate in metaphase I, both univalent segregated randomly, both in one cell, each one in each daughter cell or suffered chromosome misdivision (Figure 4i-k).

Similarly, we visualised 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 using GISH and we observed chromosome associations between *H. chilense* and *H. vulgare* chromosomes in few cells corresponding with the low frequency of pairing previously visualised in metaphase I (Figure 4h). 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. *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 4l). 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 in anaphase I in the $7H^vL5H^{ch}$ double monosomic addition lines in the absence of the *Ph1* locus.

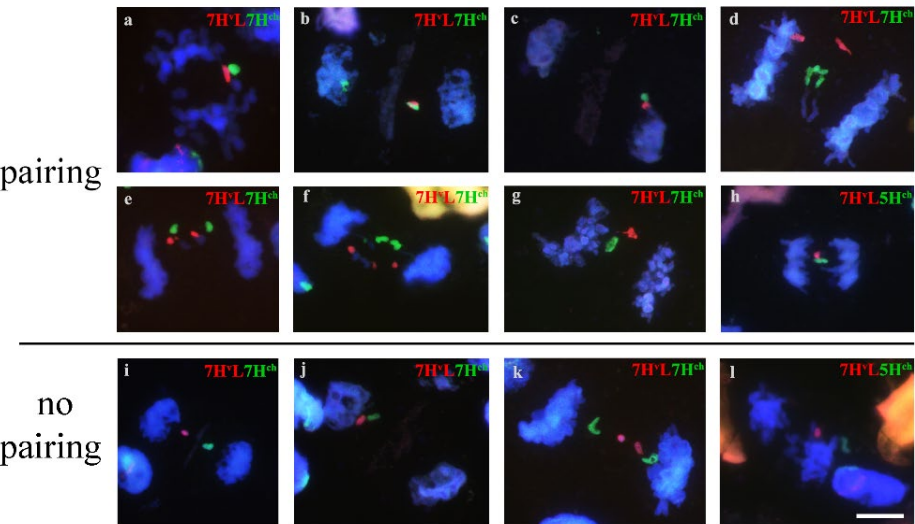


Figure 4. Behaviour of *Hordeum vulgare* and *H. chilense* chromosomes in the double monosomic *ph1b* mutant lines during anaphase I of meiosis. Examples of barley chromosome segregation after metaphase I. *Hordeum vulgare* (red) and *H. chilense* (green) were visualised in green and red, respectively. DNA was counterstained with DAPI (blue). Bar: 10µm.

Inter-specific chromosome associations were also visualised 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).

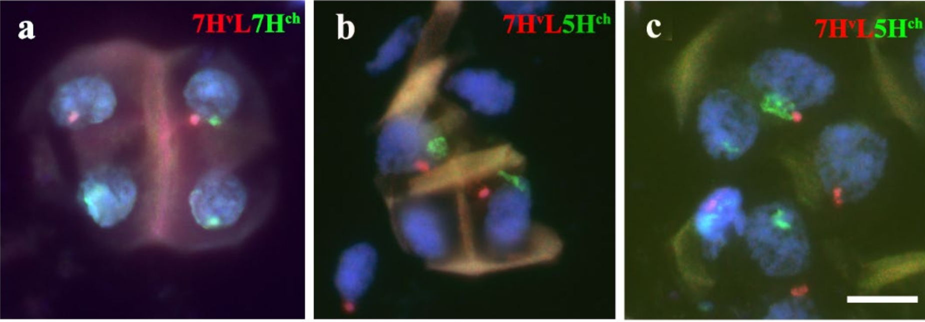


Figure 5. Behaviour of *H. vulgare* and *H. chilense* chromosomes in double monosomic *Hordeum* addition lines in wheat after the second meiosis division. Examples of some observation for both *H. vulgare* (red) and *H. chilense* (green) chromosomes are shown. DNA was counterstained with DAPI (blue). **(a)** *Hordeum vulgare* and *H. chilense* chromatin detected in two and three PMCs, respectively, in the double monosomic $7H^{v}L7H^{ch}$ wheat *ph1b* mutant line. **(b)** *Hordeum vulgare* and *H. chilense* chromatin detected in two and two PMCs, respectively, in the double monosomic $7H^{v}L5H^{ch}$ wheat *ph1b* mutant line. **(c)** *Hordeum vulgare* and *H. chilense* chromatin detected in three and three PMCs, respectively, in the double monosomic $7H^{v}L5H^{ch}$ wheat *ph1b* mutant line. Bar: 10µm.

Finally, it is worthy to mention that other chromosome rearrangements can occur during meiosis in the absence of the *Ph1* locus. We detected one mosaic plant carrying the $7H^{v}L5H^{ch}$ double monosomic addition line in the *ph1b* mutant background. *In situ* hybridisation 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 during premeiosis, resulting in the loss of the *H. vulgare* chromosome in one of descendence cell, which generate the meiocytes and result in a mosaic individual. This case of aneusomaty 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 remains to be elucidate.

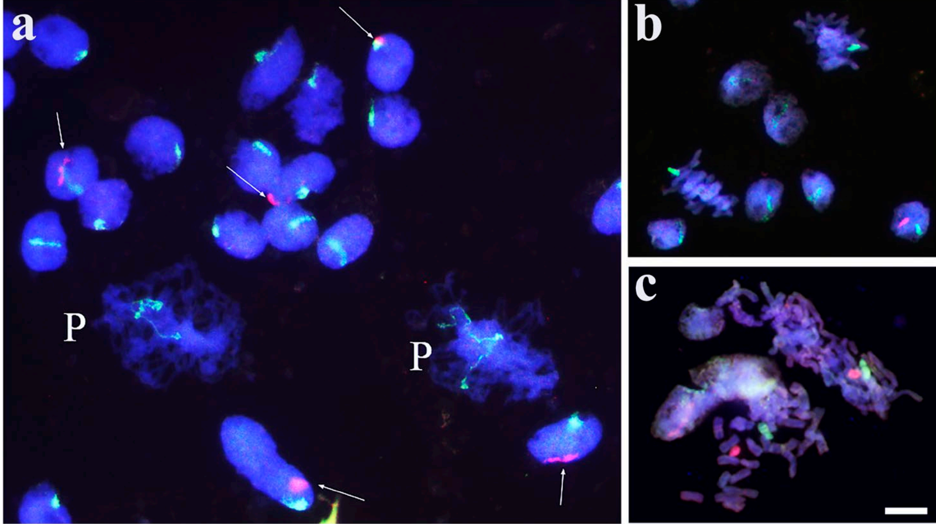


Figure 6. Behaviour of aneusomaty line at pachytene stage and somatic metaphase. *Hordeum vulgare* and *H. chilense* were visualised in green and red, respectively. DNA was counterstained with DAPI (blue). **(a)** The meiocytes at pachytene (indicated by P letter) show only the *H. chilense* chromosome. The interphase cells show *H. vulgare* (indicated by a white arrow) and *H. chilense*. **(b)** The meiocytes at metaphase show only the *H. chilense* chromosome. **(c)** The line in somatic metaphase showing both barleys chromosomes. Bar: 10µm.

Discussion

The mechanisms through chromosomes recognise each other to correctly associate in pairs at early meiosis to recombine and properly segregate are largely unknown. Plant breeders have always wished to transfer genetic variability from related species into a crop like wheat using genetic crosses, expecting that recombination events between chromosomes from the donor species and those from wheat should occur in the resulting interspecific hybrids. However, wheat genome has an enormous stability, behaving as a diploid in meiosis what means that homologous chromosomes correctly associate in pairs and do not associate with the related homoeologous chromosomes. The genetic control of pairing and the physical divergence of the homoeologous genomes are considered the genetic systems responsible for the stable cytological diploid behaviour of polyploid wheat (Feldman *et al*., 2012;

Leitch and Bennett, 1997). The *Ph1* locus has been described controlling correct pairing and suppressing homoeologous recombination in wheat (Martín *et al.*, 2017), but little is known about the mechanism through which this is achieved. In this work, to go deeper into the knowledge of the *Ph1* locus function, we have developed *H. chilense* and *H. vulgare* double monosomic addition lines in the wheat *ph1b* mutant background for the same and for different homoeology group. For this, we have used *H. chilense* and *H. vulgare* monosomic addition lines for chromosomes $7H^{v}$, $5H^{ch}$ and $7H^{ch}$ in the wheat *ph1b* mutant background as parental lines in genetic crosses developed previously (Rey *et al.*, 2015b). The lines developed here are equivalent to double monosomic addition lines for the same *Hordeum* chromosomes previously developed in our laboratory in the presence of the *Ph1* locus (Calderón *et al.*, 2018). However, we found difficulties to obtain 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 (Sears, 1952; Steinitz-Sears, 1966; Friebe *et al.*, 2005). Particularly, telosomic chromosomes for $7H^{ch}$ and $7H^{v}$ were found in wheat in the *ph1b* mutant background in a higher frequency than for the rest of the *H. chilense* and *H. vulgare* chromosomes (Rey *et al.*, 2015b), supporting 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 be studied 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 (Molnar-Lang

et al., 2000, Silkova *et al*., 2014; Rey *et al*., 2015b). Previously, we have 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 group) in the wheat background using *in situ* hybridisation (Calderón *et al*., 2018). In fact, both wild and cultivated barley chromosomes were shown associated correctly in pairs in the presence of the *Ph1* locus, suggesting that the *Ph1* locus does not affect to specific chromosome associations. In this work, our results confirmed that the *Ph1* locus does not impede homoeologous chromosome associations, but crossing-overs between homoeologous chromosomes. Crossovers between *H. chilense* and *H. vulgare* have been frequently detected among other interspecific chromosome recombination events in the *ph1b* mutant background. Previously, an increment on the crossover frequency between homoeologous chromosomes was described in the *ph1b* mutant background by targeting the MHL1 sites that progressed to crossovers when environmental growing conditions were altered (Martín *et al*., 2017). 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 was described but in a much lower frequency than observations on chromosome association (Zhang *et al*., 1999). Recombination between 5M^{g} and 5D chromosomes in wheat substitution lines containing 5M^{g} chromosome from *Ae. Geniculata* was also observed (Liu *et al*., 2011). Robertsonian translocations between *H. chilense* and *H. vulgare* were described before in the presence of the *Ph1* locus (Prieto *et al*., 2001). However, different levels of allosyndesis between wheat chromosomes and *H. vulgare* and *H. chilense* or wheat and rye (*Secale cereale*) (Miller *et al*., 1994) were detected in the *ph1b* mutant background (Miller *et*

al., 1994; Rey *et al.*, 2015b). Our results show that, although *H. vulgare* and *H. chilense* are phylogenetically quite distant (Blattner, 2009), both species has a lot of similarities at the chromosomal level (Aliyeva-Schnorr *et al.*, 2016) to allow homoeologous chromosome associations and recombination between them in the absence of the *Ph1* locus. Thus, our results also showed that the *Ph1* locus supresses recombination between homoeologous chromosomes as bivalents between homoeologous chromosomes were detected in metaphase I in the *ph1b* mutant background.

Alterations in chromosome content can be also 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 (Duncan, 1945). 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 (Luo *et al.*, 2020). In our work, we have 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 (Duncan, 1945; Lewis, 1970; Persson, 1974; Couderc *et al.*, 1980). 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 (Eichler and Sankoff, 2003), and include recombination hot spots (Linardopoulou *et al.*, 2005; Aguilar and Prieto, 2020). This extraordinary polymorphism contributes to complicate 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 (Kotani, 1999; Heacock *et al.*, 2004; Calderón *et al.*, 2014, 2018) 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 supressed by the *Ph1* locus remains to be elucidated.

Chapter V

Conclusions

1. The use of bread wheat lines carrying *H. chilense* chromosome additions without the subtelomeric region has revealed the relevance of subtelomeres in the processes of recognition and pairing between homologous chromosomes.

2. The development of wheat lines carrying double monosomic additions for wild and cultivated barley chromosomes (*H. chilense* and *H. vulgare*, respectively) for the same and different homoeology group has enabled the study of pairing and recombination between homoeologous chromosomes during meiosis.

3. The effect of the *Ph1* locus on chromosome associations during meiosis in wheat has been assessed using *H. chilense* and *H. vulgare* double monosomic addition lines in the presence and in the absence of the *Ph1* locus.

4. *In situ* hybridisation is an essential technique for the study of chromosome associations during meiosis in the wheat background.

Chapter VI

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